Plant Physiology Preview. Published on October 19, 2011, as DOI:10.1104/pp.111.187302

Running Head: ***spy*** **mutants are more tolerant to salt and drought**

Corresponding Author:

Kazuko Yamaguchi-Shinozaki

Biological Resources and Post-harvest Division, Japan International Research Center for Agricultural Sciences

Tel: +81-29-838-6641

Fax: +81-29-838-6643

E-mail: kazukoys@jircas.affrc.go.jp

Journal Research Area: Environmental Stress and Adaptation – Associate Editor Julia Bailey-Serres

Copyright 2011 by the American Society of Plant Biologists

SPINDLY, a Negative Regulator of GA Signaling, Is Involved in the Plant Abiotic Stress Response[1]

Feng Qin[2], Kensuke Kodaira, Kyonoshin Maruyama, Junya Mizoi, Lam-Son Phan Tran, Yasunari Fujita, Kyoko Morimoto, Kazuo Shinozaki and Kazuko Yamaguchi-Shinozaki

Biological Resources and Post-harvest Division, Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki 305-8686, Japan (F.Q., K.K., K.M., Y.F., K.M., K.Y.-S.); Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657 (J.M., K.K., K.M., K.Y.-S.); Plant Science Center, RIKEN, Yokohama, Kanagawa 230-0045, Japan (L.-S.P.T, K.S.)

Footnotes

[1] This work was supported by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (in part by Genomics for Agricultural Innovation, Development of Abiotic Stress Tolerant Crops by DREB Genes), the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

[2] Feng Qin was supported by the JIRCAS Visiting Research Fellowship Program and by a JSPS Postdoctoral Fellowship for Foreign Researchers (P07618). Current address: Key Laboratory for Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences (IB-CAS), Xiangshan, Beijing 100093, China.

The author responsible for the distribution of materials integral to the findings presented in this article, in accordance with the policy described in the Instructions for Authors, (www.plantphysiol.org) is Kazuko Yamaguchi-Shinozaki (kazukoys@jircas.affrc.go.jp)

Abstract

The *SPINDLY* (*SPY*) gene was first identified as a negative regulator of plant gibberellic acid (GA) signaling because mutation of this gene phenocopies plants treated with an overdose of bioactive GA and results in insensitivity to a GA inhibitor during seed germination. The *SPY* gene encodes an *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferase (OGT) that can modify the target protein and modulate the protein activity in cells. In this study, we describe the strong salt and drought tolerance phenotypes of *spy-1* and *spy-3* mutants, in addition to their GA-related phenotypes. *SPY* gene expression was found to be drought stress-inducible and slightly responsive to salt stress. Transcriptome analysis of *spy-3* revealed that many GA-responsive genes were upregulated, which could explain the GA-overdosed phenotype of *spy-3*. Some stress-inducible genes were found to be upregulated in *spy-3*, such as genes encoding LEA proteins, RD20 and AREB1-like transcription factor, which may confer stress tolerance on *spy-3*. *CKX3*, a cytokinin (CK) catabolism gene, was upregulated in *spy-3*; this upregulation indicates that the mutant possesses a reduced CK signaling, which is consistent with a positive role for SPY in CK signaling. Moreover, overexpression of *SPY* in transgenics (SPY-OX) impaired plant drought stress tolerance, opposite to the phenotype of *spy*. The expression levels of several genes, such as *DREB1E/DDF1* and *SNH1/WIN1*, were decreased in SPY-OX but increased in *spy*-3. Taken together, these data indicate that *SPY* plays a negative role in plant abiotic stress tolerance, probably by integrating environmental stress signals via GA and CK crosstalk.

Introduction

The *SPINDLY* (*SPY*) gene was first identified by ethyl methanesulfonate (EMS) mutagenesis screening for mutants resistant to paclobutrazol (PAC), an inhibitor of gibberellic acid (GA) synthesis in plants, and isolated by map-based cloning (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996). Unlike GA-insensitive mutants, most of the identified *spy* mutants phenocopy wild-type (WT) plants treated with GA. Relative to WT plants, *spy* mutants exhibit lighter green-colored leaves, early flowering, increased stem elongation and partial male sterility, suggesting that *SPY* functions as a negative regulator to repress GA signaling in plants, and mutation of the *SPY* gene gives rise to an elevated GA response.

GA is one of the most important plant hormones and has been known to play a major role in various aspects of plant biology, including seed germination, leaf expansion, stem and root elongation, flowering time and fruit development. A major breakthrough in our understanding of GA perception and signal transduction was achieved in the last ten years when several components of the GA signaling pathway were identified. Initially, a GA signal is perceived by a soluble GA receptor, GID1 (GA-Insensitive Dwarf 1) (Ueguchi-Tanaka et al., 2005). A null mutation in the single *GID1* gene in rice leads to an extremely short and GA-insensitive plant. In Arabidopsis, when three homologs of *GID1* (*GID1a*, *GID1b* and *GID1c*) were disrupted, plants also displayed a similar dwarf phenotype (Griffiths et al., 2006; Iuchi et al., 2007), indicating that GID1 has an essential role in GA signaling. Following GID1 binding to bioactive GA, the signal is transduced to the DELLA proteins, leading to their degradation (Hirano et

al., 2008). The DELLA proteins are conserved repressors of GA signaling that modulate all aspects of GA-induced growth and development in plants. DELLA proteins were named for an N-terminal conserved DELLA domain, which is essential for GA-dependent proteasomal degradation (Willige et al., 2007). In Arabidopsis, there are five DELLA proteins: GA Insensitive (GAI), Repressor of ga1-3 (RGA), RGA-like 1 (RGL1), GRL2, and GRL3, and they form a subfamily of the GRAS family of putative transcription regulators (Pysh et al., 1999; Richards et al., 2001). The N-terminal DELLA and VHYNP domains are necessary for protein interactions with GID1 (Griffiths et al., 2006). Loss-of-function mutations of DELLA proteins can rescue the dwarf phenotype of the GA biosynthesis mutant *ga1-3* (Dill and Sun, 2001; King et al., 2001), indicating that DELLA proteins function as negative regulators in GA signaling. Another important component of GA signal transduction is an F-box protein, SLY1, that functions as a subunit of the E3 ubiquitin ligase complex SCF^{SLY1}. SLY1 interacts with GAI and RGA in a yeast two-hybrid system and mediates their ubiquitination and degradation (Dill et al., 2004; Fu et al., 2004; Tyler et al., 2004). Mutations in the GA1 locus block GA biosynthesis prior to the formation of ent-kaurene. The phenotypes of *ga1* mutants, including dwarfism, germination failure, male sterility and incomplete petal development, can be reversed by exogenous GA application (reviewed in Richards et al., 2001).

The *SPY* gene is expressed throughout the plant and can be detected not only in all organs where the phenotypes of *spy* mutants have been observed but also in the roots, indicating a role for the gene in root development (Swain et al., 2002). The SPY protein is predominantly localized to the nucleus, where it modifies

components of the GA signaling pathway (Swain et al., 2002). Cytosolic SPY activity was reported to promote CK responses and repress GA signaling (Maymon et al., 2009). The SPY protein consists of 915 a.a., including multiple tetratricopeptide repeats (TPRs) at the N-terminus and a serine and threonine *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferase (OGT) domain at the C-terminus (Jacobsen et al., 1996). Each TPR motif consists of a highly degenerate sequence of 34 a.a., with eight loosely conserved residues. The TPR motifs and OGT domain are important for mediating protein-protein interactions and correct assembly for the enzyme activity, respectively (Kreppel and Hart, 1999; Lubas and Hanover, 2000; Tseng et al., 2001, 2004). Similar to animal OGTs, *in vitro*-expressed SPY protein possesses OGT activity (Thornton et al., 1999). OGTs transfer a GlcNAc monosaccharide to the *O*-linkage of Ser or Thr of cytosolic and nuclear proteins. In animals, more than 1000 *O*-GlcNAc-modified proteins have been identified, and this kind of protein modification is believed to regulate many basic cellular and disease processes. In some cases, *O*-GlcNAc modification and phosphorylation occur at the same site on the substrate protein, leading to the hypothesis that these two modification processes compete with each other to fine tune substrate protein activity under different circumstances (Comer and Hart, 2001; Wells et al., 2001; Slawson and Hart, 2003; Love and Hanover, 2005). In Arabidopsis, there are only two genes encoding *O*-GlcNAc transferases, *SPY* and *SEC* (SECRET AGENT) (Hartweck et al., 2002). Mutations in or knockout of the *SPY* gene leads to elevated GA responses, indicating that SPY functions as a negative regulator of GA signaling, whereas loss-of-function mutations of the SEC gene do not result in any obvious phenotypic changes in

plants. It has been suggested that *SPY* plays a primary role in GA signaling. Double mutants containing loss-of-function mutations in both the *SPY* and *SEC* genes are embryonically lethal (Hartweck et al., 2002, 2006). Although the *SPY* gene has been identified as a negative regulator of GA signaling, the molecular action of SPY in GA signaling and the functions of plant OGTs remain largely unknown. Although the role of *SPY* in GA-related processes is indisputable, it is also known that *SPY* is involved in other cellular processes. The *spy* mutants exhibit altered phyllotaxy, CK responses, light responses, and reduced hypocotyl and rosette growth, none of which are directly related to GA responses (Swain et al., 2001; Tseng et al., 2004; Greenboim-Wainberg et al., 2005).

Recent research has begun to elucidate the molecular events involved in plant hormone responses and environmental stress adaption. As an important plant phytohormone, GA has been reported to affect the plant abiotic stress response. The growth variability promoted by treatment with GA is able to reverse the inhibitory effects of salt, oxidative, and heat stresses on germination and seedling establishment. Additionally, GA promotes plant growth by inducing the degradation of the growth repressor DELLA in the nucleus, and a quadruple loss-of-function DELLA mutant is more sensitive to extremely high salinity stress than the WT plants (Achard et al., 2006). It has been proposed that SPY can alter DELLA protein activity or stability via *O*-GlcNAc modification. Moreover, *SPY* can suppress GA signaling and promote CK responses in Arabidopsis (Greenboim-Wainberg et al., 2005). Interestingly, reducing CK signaling by knocking out two CK receptors, AHK2 and AHK3, can enhance plant survival rates under severe salt stress conditions (Tran et al., 2007).

In this study, we describe the salt and drought stress tolerance phenotypes of *spy-1* and *spy-3* mutants, in addition to their GA-related phenotypes. *SPY* gene expression was found to be drought stress-inducible and slightly responsive to salt stress, indicating a role for this gene in stress response. Transcriptome analysis of the *spy-3* mutant revealed that many GA-responsive genes are upregulated, explaining the GA-overdosed phenotype observed in the *spy-3* mutant. Some stress-inducible genes were found to be upregulated in *spy-3*, such as genes encoding LEA proteins, RD20, ABC transporters and AREB1-like transcription factor under normal growing condition. Under dehydration stress, the expression of many LEA protein genes was higher in the mutant than that in wild type, which may confer stress tolerance on the *spy-3* mutants. Increased expression of the *CKX3* gene was detected in the *spy-3* mutant under both normal and stressed conditions, which may also contribute to plant stress tolerance. Transcriptomic comparison of *spy-3* versus WT and SPY-OX versus WT plants showed that the expression levels of *DREB1E/DDF1* and *SHN1/WIN1* were increased by the *spy-3* mutation but decreased by *SPY* overexpression, which may result in the reversed drought stress phenotypes. The function of the *SPY* gene in plant abiotic stress response and plant hormone crosstalk is also discussed.

Results

spy Mutants Are More Tolerant to High Salinity Stress

To examine whether the *SPY* gene also plays a role in the plant abiotic stress response, we obtained point mutation mutants of *spy-1* and *spy-3* (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996). Both the *spy-1* and *spy-3* mutants were

generated by EMS mutagenesis screening of *Arabidopsis* ecotype Columbia (*Col.*) for plants that were more tolerant to PAC during seed germination compared with WT plants. The seedlings of both mutants exhibited a GA-overdosed phenotype. The *spy-1* mutant exhibited a stronger phenotype than the *spy-3* mutant in terms of characteristics affected by GA responses, such as an elongated stem, pale leaves, slender seedlings and male sterility, whereas the *spy-3* mutant exhibits only a mild phenotype, and this is probably due to a more severe mutation in the *spy-1* mutant (Fig. 1A). The *spy-3* mutation caused an amino acid substitution in the OGT domain, while the *spy-1* mutation caused an in-frame deletion of 23 a.a. of the protein, which located in the 8^{th} and 9^{th} TPR (Jacobsen et al., 1996; Fig. 1B). We compared the ability of the *spy* mutants and wild-type *Col.* plants to survive severe high salinity stress conditions. If 7-day-old plants were transferred from normal growth conditions to 200 mM NaCl-containing plates after 7-10 days, less than 20% of the WT plants survived. In contrast, more than 80% of *spy-3* and 70% of *spy-1* plants continued to grow and retained a greenish color, which suggested that these two mutants were strongly salt tolerant (Fig. 1C). Statistical analysis of the data obtained in these experiments is presented in Fig. 1D. Because the *spy* mutants grew a little bit more quickly after germination than the WT plants, we compared the salt tolerance of 8-day old *Col.* and 7-day old *spy-3* mutants to eliminate any differences due to plant age and size. Similarly, we found that the survival rate of *spy-3* plants was much higher than that of the WT plants (Fig. 1E). In order to verify *spy* mutations really altered plant stress tolerance, we obtained two additional *spy* alleles, *spy-8* and *spy-12*, which were generated from EMS mutagenesis screen for suppressors of the *ga1-3* mutant (Silverstone et al., 2007).

An increased resistance to the inhibitory effects of salt on germination was clearly observed in these mutants (Supplemental Fig. S1). Taken together, it strongly suggested that mutations in the *SPY* locus could enhance plant resistance to salinity stress.

spy Mutants Consume Less Water and Survive Longer under Water Deficit Stress

Because salt and drought stresses usually cause similar water stress in plant cells, we extended our plant survival tests to water deficit conditions. First, we compared the plant survival rates in individual soil-pots. When the water supply for 4-week-old plants was terminated for 2 weeks, most of the Col. plants died, whereas almost all of the *spy-3* and *spy-1* mutants survived (Fig. 2A). Since the rosette leaves of the 4-week-old mutants were smaller than those of WT plants, which could bring about the differences in transpiration and water content in the soil in different pots after withholding water, we exerted out efforts to dehydrate the plants similarly by ensuring that the soil water content dropped to the same level in the soil-pots. The water supply to 4-week-old plants growing in different pots was terminated. The soil water content was recorded daily from the twelfth day after watering was cut off. Lower water content was observed in the soil in which WT plants were growing compared with soil containing *spy-3* plants, indicating a higher water usage or transpiration of the WT plants. On the sixteenth day, when the total water amount had fallen to approximately 10% of the original in the pots containing WT plants, watering was resumed. Approximately 28% percent of the plants recovered from severe wilt. Whereas, watering was

reinitiated for the *spy-3* plants after 19 days of dehydration, when the soil-water content in these pots had dropped to a similar level as it is in the pots of the wild type (Fig. 2B). Although the plants were dehydrated for 3 days longer than the WT plants, 88.0% of the *spy-3* plants survived (Fig. 2C). We also compared plant survival in same tray. In this experiment, the WT plants and *spy-3* mutants were planted side by side in one soil plot in which the soil water content was more comparable between plants than that in different pots. The earlier bolting and flowering phenotypes of the *spy-3* mutant as compared with the WT plants were typical GA-elevated phenotypes (Fig. 2D). In our drought experiments, water was usually withheld when the *spy-3* mutant was flowering. Water consumption at this stage is considered to be the most crucial for the plant. However, we again observed a much higher survival rate of the *spy-3* mutants compared with the WT plants (Fig. 2D). Additionally, the water loss from detached leaves of *spy-3* and WT plants was evaluated using an assay that is considered to be less affected by the size of the plants. The aerial parts of 15-day-old plants were placed in empty Petri dishes and periodically weighed. As expected, both the *spy-1* and *spy-3* plants were better able to retain water than the WT plants (Fig. 2E). The leaf electrolyte leakage of the mutants and WT was compared after dehydrating samples for 2.5 hours in Petri dishes. Significantly lower ion leakage was observed in the *spy* mutants as compared with the WT plants (Fig. 2F). From all these observations, we concluded that loss-of-function mutation of *SPY* gene led to increased water stress tolerance in comparison with the WT, which was probably due to a less leaf water loss and a better membrane integrity under drought stress. Because *spy* mutants exhibit elevated GA signaling and a GA-overdosed

phenotype, we wondered whether the increased GA response caused the drought tolerance. We treated WT plants with either water or a bioactive GA_3 solution. This treatment led, as expected, to the typical GA phenotype, characterized by earlier flowering and light green coloring, which is similar with *spy-3* mutants. The plants were then subjected to drought stress. Interestingly, we found that unlike the *spy-3* mutants, the GA-treated plants were much more sensitive to water stress, with a much lower survival rate compared with the water-treated plants (Fig. 2G). This result indicated that although *SPY* is involved in the GA response, the salt and drought stress tolerance observed in the *SPY* mutation was probably not due to the increased GA signaling.

SPY Gene Expression Is Stress Inducible

Because the analysis of salt and drought tolerance indicated a role for the *SPY* gene in the plant stress response, we extended our study to determine whether *SPY* expression is stress inducible. We found that *SPY* expression was responsive to dehydration stress. After a 10 min dehydration treatment, there was a clear increase in *SPY* gene expression, and this remained high for up to 24 hours. Gene expression of *SPY* was slightly responsive to NaCl treatment but not to treatment with ABA. The responsiveness of the gene to cold was not clear (Fig. 3A). From the eFPBrowser database, which is a digital resource that indicates gene expression information based on collective microarray analyses, we found that *SPY* is expressed at higher levels in guard cells relative to epidermal cells, but this expression does not respond to ABA treatment (Fig. 3B).

Drought-inducible Expression of Some Genes Is Enhanced in the *spy-3* Mutant

Because the *spy* mutants exhibited increased tolerance to both salinity and water deficit stresses, we compared genome-wide gene expression changes between the *spy* mutants and WT plants (Columbia ecotype). For this analysis, gene expression was compared between *spy-3* and WT plants because the *spy-3* mutant displayed a strongly tolerant phenotype with the fewest morphological changes. The expression of 506 genes was found to be enhanced in the *spy-3* mutant compared to WT, and 287 genes were downregulated in the mutant. The criteria for identifying up- or downregulated genes were a fold change absolute (FCA)≥2; p-value<0.05 (Supplemental Table S1). To obtain an overview of the genes with altered expression, the top 200 up- or downregulated genes were submitted to Genevestigator to determine their expression in response to various treatments, including chemical, biotic, abiotic, and hormone treatments. The 200 most strongly upregulated genes did not show any predominant response to the various treatments, except that many genes were found to be drought stress inducible (Supplemental Fig. S2A). However, among the 200 most strongly downregulated genes, many were found to be ABA or drought repressible (Supplemental Fig. S2B). Furthermore, microarray analysis of *Col*. plants growing in soil that were dehydrated for 3 days, which identified drought-inducible genes genome-wide (Maruyama et al., 2009), showed that of the 506 genes upregulated in *spy-3* mutant, 59 were drought stress inducible, suggesting that these genes may contribute to enhanced drought tolerance of *spy* mutant. (Supplemental Table S2, Fig. 4A).

Expression of GA-Responsive Genes Is Increased in the *spy-3* Mutant

Because the *spy-3* mutant displays many of the same phenotypes as GA-treated plants, we were interested to determine whether the expression of GA-responsive genes was altered in the *spy-3* mutant. Microarray analysis of *ga1-3* mutants versus WT plants using flower buds identified 826 GA-responsive genes (Cao et al., 2006). When we compared our data with these results, we found that of the 506 genes upregulated in the *spy-3* mutant, 114 were GA-responsive, including *RGL1* (Fig. 4B, Supplemental Table S3). Such a large overlap in gene expression may provide a good explanation for the GA-overdosed phenotype of the *spy-3* mutant. In addition to these 114 genes, there were at least another 3 GA-responsive genes found to be upregulated in the *spy-3* mutant: *RGL2*, *GA 2-oxidase* and *GA-responsive gene* (*At5g59845*). Although DELLA proteins are degraded by treatment with GA, the expression of these genes was found to be slightly upregulated in the *spy-3* mutant.

We also examined the correlation between gene expression in the *spy-3* mutant and ABA-treated *Col.* plants. According to the microarray analysis of the *Col.* plants treated with ABA for 6 h, there were 2,119 and 2,427 genes that were up- or downregulated, respectively (Fujita et al., 2009). Of the 2119 ABA-upregulated genes, the expression of 79 was found to be increased, and of the 2427 ABA-downregulated genes, the expression of 46 was decreased in the *spy-3* mutant (Fig. 4C). Compared with the overlap observed between the genes upregulated in both GA-treated and *spy*-3 mutant plants, those upregulated in ABA-treated plants overlapped with those upregulated in the *spy* mutants much

less, indicating that the *spy-3* mutation has strong effects on GA-dependent gene expression, whereas it does not considerably affect ABA-responsive gene expression.

Transcriptome Alterations in Dehydrated *spy-3* Mutants Compared with WT Plants

We also compared the gene expression changes in *spy-3* and WT plants that were dehydrated for 2 h. Overall, the expression of 612 genes was found to be enhanced in the *spy-3* mutant compared with *Col.*, and 720 genes were downregulated in the mutant, as determined by selecting genes with FCA≥2; p-value<0.05 (Supplemental Table S4). The top 200 up- or downregulated genes were analyzed using Genevestigator. Similarly, among the top 200 upregulated genes, many were found to be drought stress inducible (Supplemental Fig. S3A). However, among the top 200 downregulated genes, there were many genes that are ABA- or drought stress repressible, which was similar to the results of the microarray analysis of the untreated *spy-3* mutants. Unexpectedly, many biotic stress-inducible genes were revealed to be downregulated in the *spy-3* mutant (Supplemental Fig. S3B). Compared with the results of the microarray analysis of *Col*. plants growing soil that were dehydrated for 3 days (Maruyama et al., 2009), we found that of the 612 upregulated genes, 98 were drought stress inducible (Supplemental Table S5, Fig. 4D). Importantly, 16 LEA protein genes were found to be upregulated in the *spy-3* plants that were dehydrated for 2 h, and all of them were drought stress inducible, which might contribute to the drought stress tolerance of plants. Similarly, the expression of five lipid transfer proteins and

seven transporter genes was found to be enhanced in the *spy-3* mutants dehydrated for 2 h. Some transcription factor and protein enzyme encoding genes were also revealed to be transcriptionally enhanced.

To verify the microarray results, the expression levels of several genes that are potentially related to water stress response in the *spy-3* mutant were monitored by quantitative RT-PCR analyses. Three late embryogenesis abundant (LEA) protein-encoding genes, *At2g03850*, *At2g36640* and *At3g17520*, were determined to be significantly upregulated under both normal growing conditions and stress conditions in the *spy-3* mutant as compared with WT plants (Fig. 5). These LEA proteins may confer the stress-tolerant phenotype of the *spy-3* mutant. Two genes encoding transporters, an ATP-binding cassette (ABC) transporter gene *ABCG29* and an outward K+ channel gene (GORK) that may be involved in leaf water transpiration or ABA response, were revealed to be upregulated in the *spy-3* mutant (Fig. 5). Moreover, the *CKX3* gene encoding a CK oxidase/dehydrogenase was found to be 15.6-fold and 3.4-fold upregulated in the *spy-3* mutant under normal and dehydration conditions, respectively, according to the qRT-PCR analyses (Fig. 5). Recently, it was reported that overexpression of the *CKX3* gene, which leads to a reduction in CK content, enhanced the drought and salt tolerance of *Arabidopsis* and tobacco transgenic plants (Werner et al., 2010; Nishiyama et al., 2011). Increased expression levels of two other stress-related genes, *RD20* and an *AREB1-like* gene (*At2g36270*), were also found in the mutant under both normal and stress conditions (Fig. 5). Taken together, we concluded that the microarray data are reliable and reproducible. The enhanced expression of LEA genes, genes encoding transporters, stress-related genes and *CKX3* might

contribute to the salt- and drought-tolerant phenotypes of the *spy-3* mutant. It is worth mentioning that among the 720 genes downregulated in the dehydrated *spy-3* mutants, the expression of 84 was found to be drought-repressible. Many genes encoding disease resistance proteins, leucine-rich repeat (LRR) protein kinases, and receptor-like protein kinases were transcriptionally downregulated, which was consistent with the results of Genevestigator analysis of the gene expression responsiveness of the top 200 downregulated genes (Supplemental Table S4).

Overexpression of the *SPY* Gene Reduces Plant Drought Stress Tolerance

To further elucidate the function of the *SPY* gene in the plant water deficit stress response, we generated transgenic *Arabidopsis* plants overexpressing the *SPY* gene using the 35S constitutive promoter (35S:SPY OX). Several lines of transgenic plants were obtained, and transgene expression was checked by RNA gel blot analysis (Fig. 6A). In contrast with *spy* mutants, the *SPY* overexpressors did not exhibit dramatic morphological changes (Fig. 6B). Three lines of 35S:SPY OX plants, a, b and c, with comparably high levels of transgene expression, were tested for plant drought stress tolerance. Water was withheld from 4-week-old plants for approximately 2 weeks, and the plants were then watered again. The survival rate was recorded and compared between the vector-transformed control and 35S:SPY OX lines. While 78.9% of the control plants survived, 19.4, 15.6 and 13.9% of the 35S:SPY-OXa, b and c lines, respectively, survived after the drought stress treatment. The statistical analysis of the plant survival rates from three replicate experiments is shown in Fig. 6C and Supplemental Table S6. It suggested

that drought stress tolerance was decreased in these three types of 35S:SPY-OX transgenic plants.

We were further interested in exploring alterations of gene expression in SPY-OX plants because the expression levels of hundreds of genes were changed in the *spy-3* mutants. Mutations in *spy-3* affected GA and CK signaling and led to a variety of morphological changes, whereas *SPY* overexpression only negatively altered plant stress tolerance without any obvious morphological effects. Analyzing SPY-OX plants may facilitate the identification of a direct relationship between *SPY* function and plant stress tolerance. Two additional microarray experiments were constructed comparing two independent 35S:SPY OX-a and OX-c lines to vector-transformed transgenic plants. Transcriptomic changes were compared under both normal and 2-hour-dehydrated conditions. In total, 48 genes were upregulated, and 156 genes were downregulated (FCA≥2; p-value<0.05) in SPY-OX plants during normal growth. Among them, the expression of the *SPY* gene was 12.1-fold higher in the transgenic plants as compared with the WT plants (Supplemental Table S7). According to the same criteria, 66 and 184 genes were found to be up- and downregulated in 2-hour-dehydrated SPY-OX plants (Supplemental Table S8). From both microarray experiments, more genes were found to be downregulated than upregulated in the SPY-OX plants, indicating a negative, genome-wide effect of SPY on gene expression regulation. Based on the drought tolerant phenotype of the *spy-3* mutants and sensitive of SPY-OX plants, we were particularly interested in the genes that were upregulated in *spy-3* and downregulated in the SPY-OX plants, which might be the genes most affected by the SPY protein. Out of 156 downregulated genes in the SPY-OX plants, 19 genes

were found to be upregulated in the *spy-3* mutant under normal growing conditions (FCA≥2; p-value<0.05). Six of these genes are GA-upregulated genes in Arabidopsis buds, but none of these genes is GA repressive; this result is consistent with a negative role for SPY in GA signaling (Supplemental Table S9). Indeed, there is no overlap of genes upregulated in the SPY OX plants that are also downregulated in the *spy-3* plants.

Results from the 2-hour-dehydrated samples showed that among the 184 genes downregulated in the SPY-OX plants, 3 were upregulated in the *spy-3* mutant (FCA≥2; p-value<0.05, Supplemental Table S10). These genes are *SHN1/WIN1 (SHINE/WAX INDUCER1)*, *ABCG26* and *AP3 (APETALA3)*. Two additional genes, *DREB1E/DDF1 (Dehydration Responsive Element Binding protein 1E/ Dwarf and Delayed Flowering 1)* and *At5g62080*, were verified to be inversely regulated by *SPY* overexpression and *spy-3* mutation based on further qRT-PCR analyses. The expression of all these genes was found to be drought stress inducible (Fig. 6D). *SHN1/WIN1* encodes an AP2/ERF transcription factor (TF) belonging to the A-6 subgroup. Overexpression of *SHN1/WIN1* increased the leaf cuticular wax content, leading to reduced water loss and enhanced tolerance to drought stress (Aharoni et al., 2004; Brounet al., 2004; Kannangara et al., 2007). DREB1E/DDF1 is also an AP2/ERF TF, classified as a member of the A-1 subgroup, that can bind the DRE sequence in the promoter of dehydration-responsive genes (Sakuma et al., 2002). Transgenic plants overexpressing the *DREB1E/DDF1* gene exhibited improved tolerance to drought, cold and heat stresses (Kang et al., 2011). The upregulated expression of *SHN1/WIN1* and *DREB1E/DDF1* may contribute to the maintenance of higher

water content, resulting in stronger drought stress tolerance of the *spy-3* mutant (Fig. 2). Although expression of the *ABCG26* and *ABCG29* genes was increased in the *spy-3* mutant, only *ABCG26* transcription was repressed by overexpression of SPY under both normal and stress conditions (Fig. 5 and 6D). The function of *ABCG26* was reported to be associated with male fertility and pollen maturation (Quilichini et al., 2010). Together with the *AP3* gene, *ABCG26* might cause the flowering-related phenotypes of the *spy* mutant.

Discussion

To properly respond to environmental signals, plants need to integrate external and internal signals and a complex network of signal transduction pathways. In recent years, different components involved in these signal cascades have been identified, including genes involved in stress-induced transcriptional regulation and complex cross-talk among different hormones at either the biosynthesis or action levels (Yamaguchi-Shinozaki and Shinozaki et al., 2007; Fujita et al., 2006). GA is an important plant phytohormone involved in several processes, including seed germination, vegetative growth, flowering induction, and fruit development (Sun and Gubler, 2004). *SPY* gene was firstly identified as a negative regulator in GA signaling, because the mutant displayed a set of GA-treated phenotypes, such as in sensitive to PAC during germination, early-flowering, partial male sterility, pale green etc. (Jacobsen and Olszewski, 1993, Jacobsen et al., 1996). Although the involvement of *SPY* in GA signaling is indisputable, it has also been recognized that *SPY* is involved in other cellular processes. *spy* mutants also display phenotypes, such as altered leaf phyllotaxy,

CK responses, light responses, and reduced hypocotyls and rosette growth, that are not directly related to GA responses (Swain et al., 2001; Tseng et al., 2004; Greenboim-Wainberg et al., 2005). Additionally, double mutation of the *SPY* and *SEC* genes, the only two OGTs in plants, leads to lethality, which may not be caused by GA treatment. These phenomena support the hypothesis that *SPY* has an unidentified function in one or more processes unrelated to GA signaling. Here, we demonstrated mutation of *spy* led to a strong salt and drought tolerance in plant whose mechanism is independent of GA-DELLA signaling. *SPY* gene expression was found to be drought stress inducible and slightly responsive to salinity stress. Furthermore, based on microarray analysis, we revealed that under normal growing condition, in addition to the upregulation of many GA-responsive genes, the expression of a number of drought-inducible genes was increased; these genes encode LEA proteins, RD20, ABC transporters and AREB1-like transcription factor (Fig. 5). Under drought conditions, the expression of 16 LEA protein genes was higher in *spy-3* mutants compared with that in the WT plants (Supplemental Table S5). LEA proteins function in an unfolded state as ion sinks or water replacement molecules and that water deficit stress can induce folding and conformational shifts in LEA proteins, so that they act as molecular chaperons to protect important proteins (Goyal et al., 2003; Wise and Tunnacliffe, 2004). Overexpression of DREB1A, constitutively active DREB2A or an activated form of AREB1 (Maruyama et al., 2004; Sakuma et al., 2006; Fujita et al., 2005) in *Arabidopsis* resulted in the upregulation of the expression of some LEA genes, and all of these transgenic plants displayed improved drought stress tolerance, further supporting the hypothesis that LEA proteins play a role in plant water deficit

tolerance. Direct evidence has shown that overexpression of a rice *OsLea3* gene was able to enhance plant water-deficit stress tolerance (Xiao et al., 2007). The elevated stress-responsive gene expression may confer the stress tolerance on *spy* mutant.

In addition to the negative regulation of GA responses, the *SPY* gene has been shown to play a positive regulatory role in CK signaling. *spy* mutants were found to be more resistant to exogenously applied CK and exhibited inhibition of the induction of *Arabidopsis* type-A response regulator 5 (ARR5) after CK treatment (Greenboin-Wainberg et al., 2005). Interestingly, according to the results of our microarray analysis, we found that the expression of *CKX3* is increased 15.6- and 3.4-fold relative to WT in both normal and dehydrated conditions, which may cause a decrease in CK level in the *spy-3* mutant. (Fig. 5), which may cause a decrease in CK level in the *spy-3* mutant (Werner et al., 2003; 2010). Overexpression of the *CKX3* gene resulted in reduced *ARR5* and *ARR7* promoter activity, a decrease in the total surface area of rosette leaves and plant growth retardation (Werner et al., 2003; Nishiyama et al., 2011). Similarly, *spy-3* mutant rosette leaves were smaller than those of WT, despite the fact that GA treatment usually increases the size of these leaves. This morphological phenotype is consistent with the increased expression of *CKX3* in the *spy-3* mutant. Interestingly, recent research has shown that reduction of the CK level via overexpression of the *CKX3* gene in roots can improve plant drought tolerance and leaf mineral enrichment (Werner et al., 2010). Functional analyses of CK-deficient *AtCKX* overexpressors and *atipt* mutants provided direct evidence that CKs negatively regulate salt and drought stress responses in plants (Nishiyama et al.,

2011). Moreover, when the CK receptors AHK2 and/or AHK3 were disrupted, both the single and double mutants were more tolerant of high salt and drought stresses (Tran et al., 2007). Taken together, the up-regulation of *CKX3* gene expression might reduce CK signaling in the *spy-3* mutant, contributing to plant salt and drought stress tolerance. Although *CKX3* gene expression was significantly upregulated in the *spy-3* mutant, it was not downregulated in the SPY-OX plants. This lack of downregulation probably explains why the stress-tolerant phenotype of SPY-OX was not as evident as that of the mutant. No remarkable tolerance to high salinity stress was observed in the SPY-OX plants. The molecular mechanism by which SPY mediates CK signaling must be investigated further.

Because our knowledge concerning the molecular actions of the SPY protein have been limited by the inability to reliably detect modified proteins that are direct targets of the SPY protein (Olszewski et al., 2010), we generated SPY-OX plants and compared transcriptome alterations in *spy-3* versus WT and SPY-OX versus WT plants to identify the direct effects of the *SPY* gene on the transcriptome, especially in relation to plant stress response. The SPY-OX transgenic plants were morphologically similar to the WT plants but were more sensitive to drought stress than the WT plants (Fig. 5). Comparative transcriptomic analyses uncovered genes that showed inverse transcriptional regulation by *SPY* overexpression and *spy-3* mutation under both normal and drought stress conditions (Fig. 6D, Supplemental Tables S9 and S10). The expression of *DREB1E/DDF1* and *SHN1/WIN1* was upregulated in the *spy-3* mutant but downregulated in SPY-OX plants. Both genes positively contribute to plant drought or salt tolerance. The *DREB1E/DDF1* gene not only activated

stress-responsive gene expression but also enhanced plant tolerance to drought, cold and heat stresses (Kang et al., 2011). Moreover, the *DREB1E/DDF1* gene can activate *GA2ox7*, a gene encoding a GA-deactivation enzyme, under salinity stress. The expression of this gene may reduce the active GA content in the plant, thus reducing plant growth under stress (Magome et al., 2008). *GA2ox7* gene expression was also consistently decreased 1.68-fold as the *DREB1E/DDF1* gene was reduced in SPY-OX plants. Our results provide evidence that overexpression of the *SPY* gene can suppress the GA response, supporting the hypothesis that *SPY* functions as a negative regulator of GA signaling. The *SPY* gene may not directly regulate the gene activity involved in GA metabolism but may instead regulate transcription factors that control metabolic gene expression. SHN1/WIN1 is also an AP2/ERF transcription factor, which can activate cuticular wax synthesis, alter cuticle properties and increase plant drought stress tolerance. SHN1/WIN1 may act by controlling the expression of *LACS2*, a long-chain acyl-CoA synthetase gene (Aharoni et al., 2004; Kannangara et al., 2007). Recently, plant cuticle composition was found to be essential for osmotic stress response and tolerance. The cuticle not only functions as a physical barrier to minimize water loss but also mediates abiotic stress signaling (Wang et al., 2011). The expression of *SHN1/WIN1* was significantly increased in the *spy-3* mutant and downregulated in SPY-OX plants, which may result in the different drought-related phenotypes of these two kinds of plants. The expression of *CKX3* and some LEA protein genes was upregulated by the *spy-3* mutation but not downregulated by *SPY* overexpression, which probably explains why the SPY-OX plants were not only sensitive to drought but also to salt stress in comparison to WT plants (data not

shown).

In recent decades, great progress has been made regarding the characterization of GA signal reception and transduction, due largely to the identification of the upstream GID1 GA receptors and the downstream GA-targeted DELLA proteins (Ueguchi-Tanaka et al., 2005; Harberd et al., 2009). GA promotes plant growth by destroying a class of transcription factors, the DELLA proteins, which function as repressors to restrain plant growth. DELLA proteins are potential candidates target of SPY (Olszewske et al., 2002, Silverstone et al., 1998). The *O*-GlcNAc modification, mediated by SPY, was proposed to compete with phosphorylation to modulate DELLA protein abundance and activity in response to GA or environmental signals (Hussain et al., 2007; Itoh et al., 2005). It has been proposed that salt stress inhibits plant growth by reducing endogenous bioactive GA levels, leading to the accumulation of DELLAs (Achard et al., 2006). Studies have demonstrated that the growth restraint conferred by DELLA proteins is beneficial and promotes plant survival under severe salinity stress conditions. A quadruple-DELLA mutant lacking GAI, RGA, RGL1 and RGL2 has been found to be less tolerant to severe salt stress and exhibit suppression of the salt tolerance conferred by *ga1-3* (Achard et al., 2006). Unexpectedly, in various *spy* mutants, the level of RGA protein was consistently higher than in WT plants although *spy* mutants displayed GA-treated phenotypes (Silverstone et al., 2007). Higher levels of DELLA proteins in the *spy* mutants indicate that the absence of the *O*-GlcNAc modification might enhance the stability of the proteins but it likely reduces their activity in terms of GA signaling. It is also possible that abundant DELLA proteins might confer plant stress tolerance on *spy-3* mutant. Additionally, many

biotic-responsive genes were downregulated in the 2-hour-dehydrated *spy-3* mutant (Supplemental Fig. S3B, Supplemental Table S4). Whether the mutant is more susceptible to biotic attack is unknown.

Research on *O*-GlcNAc modification is more advanced in mammalian cells than in plants. *O*-GlcNAc modification modulates signaling by influencing gene expression, protein degradation and trafficking in cells and also plays roles in nutrient sensing, cell cycle progression and stress response (Hart et al., 2007). In mammalian cells, a rapid and global increase in *O*-GlcNAcylation on many proteins upon exposure to stress stimuli was found, indicating a positive role for OGTs (Hart et al., 2007). Here, we characterized a negative role for SPY, a plant OGT that plays a negative role in plant survival under severe salinity and drought stress because the *spy* mutants were more tolerant to these stresses. Unlike mammals and insects, plants have two distinct genes encoding OGTs, *SPY* and *SEC*, and both genes have unique and overlapping roles (Olszewski et al., 2010). The activity of a single OGT gene is essential for cell viability in mammals (Hart et al., 2007). In plants, the simultaneous deletion of the SPY and SEC genes is embryo lethal, whereas single mutations of each gene produce different phenotypes. A phylogenetic analysis of all eukaryotic *OGT* genes found that mammalian OGTs are SEC-like but not SPY-like proteins (Olszewski et al., 2010). The gene function of *SEC* in plant stress and/or hormone response and the relationship between these two gene functions merit further investigation.

MATERIALS AND METHODS

Plant Materials and Transgenic Plant Construction

Arabidopsis plants were grown, transformed, and treated as previously described (Qin et al., 2008). *spy-1* and *spy-3* mutant seeds were obtained from the ABRC (Arabidopsis Biological Resource Center) (catalog numbers CS6266 and CS6268, respectively). *spy-8* and *spy-12* mutant seeds were kindly provided by Dr. Tai-ping Sun. The full-length coding sequence of the *SPY* gene was constructed downstream of the 35S promoter with an Omega enhancer sequence in the pGKX vector (Qin et al., 2008) and transformed into *Col.* plants for the purpose of overexpressing this gene.

RNA Gel Blot and Real-time RT-PCR Analysis

Total RNA was isolated with the RNAiso (Takara) reagent from 3-week-old plants. RNA gel blot analysis was performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). A *SPY*-specific probe was prepared by PCR amplification of the C-terminal end of the *SPY* gene from nucleotide 2003 to the final nucleotide. For real-time RT-PCR analysis, 1 μg of total RNA was used for the first-strand cDNA synthesis using Superscript III transcriptase (Invitrogen). The resultant cDNA solution was diluted five times, and a 1 μl solution was used as the template in subsequent real-time PCRs. PCR was performed in a 10-μl volume using the ABI7500 system and SYBR green I mixture (Takara). Each reaction was performed in triplicate to obtain the average and standard deviation for the expression level of each gene.

Microarray Analysis and Statistical Analysis

Genome-wide expression studies with Arabidopsis 44K Oligo Microarrays

(Agilent Technologies) were performed using 3-week-old WT and spy-3 plants. Gene expression was compared between WT and spy-3 plants under both unstressed and 2 h dehydration stress conditions. For each sample (pooled from six plants), 200 ng of total RNA was isolated using the RNAiso reagent (Takara) and used for the analysis. Biological replication was performed by analyzing the samples obtained from two independent treatments (for both the control and 2 h dehydration stress conditions). For each experiment, two slides were analyzed to perform a cy3 and cy5 dye-swap, and a total of eight slides were hybridized for the four experiments. Statistical analysis of the microarray data to integrate and normalize each spot's signal intensity was performed using the Lowess method with the Feature Extraction 9.5 program (Agilent Technologies). The statistical analysis was carried out using ArrayAssist software (Stratagene). The Welch t test was used as a parametric test, and the Benjamini and Hochberg False Discovery Rate (FDR) for multiple testing corrections were used with a threshold set at a $P < 0.05$ to identify reliable genes. Following this statistical analysis, we generated lists of the genes showing significant differences in expression between WT and spy-3 plants under normal condition and after exposure to a 2 h dehydration stress. Of the genes that were considered to show significant expression differences based on the previous tests, those that exhibited an Absolute Fold Change (AFC) greater than 2.0 were selected. Such genes were considered to be upregulated in spy-3 plants relative to WT plants. All of the microarray data are available at the website http://www.ebi.ac.uk/arrayexpress/ under the accession number E-MEXP-3171 and E-MEXP-3362.

Salt Stress Tolerance Test

For the plant seedling tolerance test, seeds were sown on GM agar plates and post-stratified at 4℃ for 4 days. After growing on GM plates for 7 days, the plants were transferred onto 0.5 × MS agar plates containing 200 mM NaCl. The plates were maintained at 22℃ under a 16 h light/8 h dark cycle until visual symptoms could be observed and photographed. For the plant salt tolerance test at the germination stage, the seeds were sown on MS agar plates with 150 mM NaCl and post-stratified at 4℃ for 4 days. Then, they were transferred to 22℃ under a 16 h light/8 h dark cycle until visual symptoms could be observed and photographed.

Drought Stress Tolerance Test

Briefly, 3-week-old plants grown on GM agar plates were transferred to soil. After one week of conditioning, the plants were subjected to a water-withholding treatment for approximately 14 days, after which the most obvious differences were observed between the WT and mutant plants. The plant survival rate was recorded after one week of reinitiated watering and recovery. For the GA treatment, *Col*. plants were transferred to soil after 3 days of germination on GM agar plates and sprayed with a 50 μM GA_3 solution or water every other day for 3 weeks. The water supply was then cut off for approximately 2 weeks for the dehydration treatment. To ensure that the pots in which *Col*. and *spy*-3 plants were growing contained equal water levels during the dehydration stress treatment, the plants were transferred into pots containing the same amount of soil saturated by water. The initial weight of each pot was controlled, and the total weight of each pot was

measured daily after 12 days of dehydration stress, so as to calculate and record the water content of each pot daily. Watering was resumed when the average total weights of the pots containing *Col.* and *spy-3* were comparable. For the plant water loss assay, the aerial parts of 2-week-old *spy-1, spy-3* and *Col.* plants were placed in empty Petri dishes, and the plant weights were periodically recorded. For each assay, the average and standard deviation were calculated from four plants. The leaf electrolyte leakage was determined as described in Qin et al. (2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *spy-8* and *spy-12* mutants were more tolerant to salt stress during seed germination.

Supplemental Figure S2. Heat map of transcriptomic analysis of normal growing *spy-3* versus Col. Plants.

Supplemental Figure S3. Heat map of transcriptomic analysis of 2h dehydrated *spy-3* versus Col. Plants.

Supplemental Table S1. 506 upregulated and 287 downregulated genes in 0h spy-3 mutant.

Supplemental Table S2. 59 Drought-stress-inducible genes upregulated in 0h dehydrated spy-3 plants.

Supplemental Table S3. 114 GA-upregulated genes upregulated in spy-3 plants.

Supplemental Table S4. 612 upregulated and 720 downregulated genes in 2h dehydrated spy-3 mutant.

Supplemental Table S5. 98 Drought inducible genes upgrulated in 2h-dehydrated

spy-3 plants.

Supplemental Table S6. Drought tolerance test of SPY OX plants.

Supplemental Table S7. Up- or downregulated genes in SPY-OX plants under normal growing conditions.

Supplemental Table S8. Up- or downregulated genes in SPY-OX plants after 2 h of dehydration stress.

Supplemental Table S9. Genes upregulated in the spy-3 mutant but downregulated in SPY OX plants under normal growing conditions.

Supplemental Table S10. Genes upregulated in the spy-3 mutant but downregulated in SPY OX plants under 2-h dehydration conditions.

Supplemental Table S11. Primer list for qRT-PCR.

ACKNOWLEDGEMENTS

We thank E. Murai, K. Amano, K. Yoshiwara and H. Kishi for their excellent technical support and M. Toyoshima for skillful editorial assistance.

REFERENCES

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science. **311**: 91-94

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis,

alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. Plant Cell **16**: 2463-2480

Cao D, Cheng H, Wu W, Soo HM, Peng J (2006) Gibberellin mobilizes distinct DELLA-dependent transcriptomes to regulate seed germination and floral development in Arabidopsis. Plant Physiol **142**: 509-525

Comer FI, Hart GW (2001) Reciprocity between *O*-GlcNAc and *O*-phosphate on the carboxyl terminal domain of RNA polymerase II. Biochemistry **40**: 7845-7852

Dill A, Sun T (2001) Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana. Genetics **159**: 777-785

Dill A, Thomas SG, Hu J, Steber CM, Sun TP (2004) The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell **16**: 1392-1405

Fu X, Richards DE, Fleck B, Xie D, Burton N, Harberd NP (2004) The Arabidopsis mutant sleepy1$^{gar2\text{-}1}$ protein promotes plant growth by increasing the affinity of the SCFSLY1 E3 ubiquitin ligase for DELLA protein substrates. Plant Cell **16**: 1406-1418

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol **9**: 436-442

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K., and Yamaguchi-Shinozaki,

K. (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. Plant Cell **17**: 3470-3488

Goyal K, Tisi L, Basran A, Browne J, Burnell A, Zurdo J, Tunnacliffe A (2003) Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. J Biol Chem **278**: 12977-12984

Greenboim-Wainberg Y, Maymon I, Borochov R, Alvarez J, Olszewski N, Ori N, Eshed Y, Weiss D (2005) Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. Plant Cell **17**: 92-102

Griffiths J, Murase K, Rieu I, Zentella R, Zhang ZL, Powers SJ, Gong F, Phillips AL, Hedden P, Sun TP, Thomas SG (2006) Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis. Plant Cell **18**: 3399-3414

Harberd NP, Belfield E, Yasumura Y (2009) The angiosperm gibberellin-GID1-DELLA growth regulatory mechanism: how an "inhibitor of an inhibitor" enables flexible response to fluctuating environments. Plant Cell **21**: 1328-1339

Hartweck LM, Genger RK, Grey WM, Olszewski NE (2006) SECRET AGENT and SPINDLY have overlapping roles in the development of Arabidopsis thaliana L. Heyn. J Exp Bot. **57**: 865-875

Hartweck LM, Scott CL, Olszewski NE (2002) Two *O*-linked N-acetylglucosamine transferase genes of Arabidopsis thaliana L. Heynh. have

overlapping functions necessary for gamete and seed development. Genetics **161**: 1279-1291

Hirano K, Ueguchi-Tanaka M, Matsuoka M (2008) GID1-mediated gibberellin signaling in plants. Trends Plant Sci **13**: 192-199

Hussain A, Cao D, Peng J (2007) Identification of conserved serine/threonine residues important for gibberellin sensitivity of Arabidopsis RGL2 protein. Planta **226**: 475-483

Itoh H, Sasaki A, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Hasegawa Y, Minami E, Ashikari M, Matsuoka M (2005) Dissection of the phosphorylation of rice DELLA protein, SLENDER RICE1. Plant Cell Physiol. **46**: 1392-1399

Iuchi S, Suzuki H, Kim YC, Iuchi A, Kuromori T, Ueguchi-Tanaka M, Asami T, Yamaguchi I, Matsuoka M, Kobayashi M, Nakajima M (2007) Multiple loss-of-function of Arabidopsis gibberellin receptor AtGID1s completely shuts down a gibberellin signal. Plant J **50**: 958-966

Jacobsen SE, Binkowski KA, Olszewski NE (1996) SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in Arabidopsis. Proc Natl Acad Sci USA **93**: 9292-9296

Jacobsen SE, Olszewski NE (1993) Mutations at the SPINDLY locus of Arabidopsis alter gibberellin signal transduction. Plant Cell **5**: 887-896

Kang HG, Kim J, Kim B, Jeong H, Choi SH, Kim EK, Lee HY, Lim PO (2011) Overexpression of FTL1/DDF1, an AP2 transcription factor, enhances tolerance to cold, drought, and heat stresses in Arabidopsis thaliana. Plant Sci **180**: 634-641

Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Hofte H, Pauly M, Riechmann JL, Broun P (2007) The transcription factor WIN1/SHN1 regulates Cutin biosynthesis in Arabidopsis thaliana. Plant Cell **19**: 1278-1294

King KE, Moritz T, Harberd NP (2001) Gibberellins are not required for normal stem growth in Arabidopsis thaliana in the absence of GAI and RGA. Genetics **159**: 767-776

Love DC, Hanover JA (2005) The hexosamine signaling pathway: deciphering the "*O*-GlcNAc code". Sci STKE **29**: re13

Hart GW, Housley MP, Slawson C (2007) Cycling of *O*-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. Nature. 446:1017-22.

Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K (2008) The DDF1 transcriptional activator upregulates expression of a gibberellin-deactivating gene, GA2ox7, under high-salinity stress in Arabidopsis. Plant J **56**: 613-626

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J **38**: 982-993

Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, Sakakibara H, Schmülling T, Tran LS (2011) Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in

drought, salt and abscisic Acid responses, and abscisic Acid biosynthesis. Plant Cell **23**: 2169-2183

Olszewski NE, West CM, Sassi SO, Hartweck LM (2010) *O*-GlcNAc protein modification in plants: Evolution and function. Biochim Biophys Acta **1800**: 49-56

Pysh LD, Wysocka-Diller JW, Camilleri C, Bouchez D, Benfey PN (1999) The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the SCARECROW-LIKE genes. Plant J **18**: 111-119

Richards DE, King KE, Ait-Ali T, Harberd NP (2001) HOW GIBBERELLIN REGULATES PLANT GROWTH AND DEVELOPMENT: A Molecular Genetic Analysis of Gibberellin Signaling. Annu Rev Plant Physiol Plant Mol Biol **52**: 67-88

Sakuma, Y., Maruyama, K., Osakabe, K., Qin, F., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell **18**: 1292-1309

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot **58**: 221-227

Silverstone AL, Tseng TS, Swain SM, Dill A, Jeong SY, Olszewski NE, Sun TP (2007) Functional analysis of SPINDLY in gibberellin signaling in Arabidopsis. Plant Physiol **143:** 987-1000

Slawson C, Hart GW (2003) Dynamic interplay between *O*-GlcNAc and *O*-phosphate: the sweet side of protein regulation. Curr Opin Struct Biol **13**: 631-636

Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol **55**: 197-223

Swain SM, Tseng TS, Thornton TM, Gopalraj M, Olszewski NE (2002) SPINDLY is a nuclear-localized repressor of gibberellin signal transduction expressed throughout the plant. Plant Physiol **129**: 605-615

Thornton TM, Swain SM, Olszewski NE (1999) Gibberellin signal transduction presents ellipsisthe SPY who *O*-GlcNAc'd me. Trends Plant Sci **4**: 424-428

Tran LS, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. Proc Natl Acad Sci USA **104**: 20623-20628

Tseng TS, Swain SM, Olszewski NE (2001) Ectopic expression of the tetratricopeptide repeat domain of SPINDLY causes defects in gibberellin response. Plant Physiol **126**: 1250-1258

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun TP (2004) Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. Plant Physiol **135**: 1008-1019

Ueguchi-Tanaka M, Ashikari M, Nakajima M, Itoh H, Katoh E, Kobayashi M, Chow TY, Hsing YI, Kitano H, Yamaguchi I, Matsuoka M (2005) GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. Nature **437**: 693-698

Wells L, Vosseller K, Hart GW (2001) Glycosylation of nucleocytoplasmic proteins: signal transduction and *O*-GlcNAc. Science **291**: 2376-2378

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15**: 2532-2550

Willige BC, Ghosh S, Nill C, Zourelidou M, Dohmann EMN, Maier A, Schwechheimer C (2007) The DELLA domain of GA INSENSITIVE mediates the interaction with the GA INSENSITIVE DWARF1A gibberellin receptor of *Arabidopsis*. Plant Cell **19**: 1209-1220

Wise MJ, Tunnacliffe A (2004) **POPP the question: what do LEA proteins do?** Trends Plant Sci **9**: 13-17

Xiao B , Huang Y , Tang N , Xiong L (2007) Over-expression of a *LEA* gene in rice improves drought resistance under the field conditions. Theor Appl Genet **115**: 35-46

FIGURE LEGENDS

Figure 1. *spy* mutants were more tolerant of high salinity stress compared with wildtype plants. (A) Morphological phenotype of *spy* mutants. Three-week-old *Col*., *spy-1* and *spy-3* plants growing on GM agar plates were photographed. (B) Schematic structure of the SPY protein and the locations of the mutations in the mutants. (C) *spy-1*, *spy-3* and WT plants grown on GM plates for 7 days and subsequently transferred onto 0.5 × Murashige and Skoog (MS) medium plates with or without (w/o) 200 mM NaCl. (D) The survival rate was calculated from independent experiments. ($n \geq 3$; ** $p < 0.01$, * $p < 0.05$, student *t*-test). (E) Salt stress tolerance was compared between 8-day-old *Col.* and 7-day-old *spy-3*

mutants, as described in panel A.

Figure 2. *spy-1* and *spy-3* mutants were more tolerant of drought stress. (A) The plant survival rate under drought stress was compared in individual soil-pots. (B) Four-week old *Col*. and *spy-3* plants were subjected to dehydration in pots containing the same amounts of soil. After 12 days of water withholding, the weight of each pot was recorded daily. Watering of the plants was reinitiated once they were equally dehydrated, which occurred on the 16^{th} and 19^{th} days for WT and *spy-3* plants, respectively. (C) Representative photographs of panel B are shown. (D) Plant drought tolerance was compared in a large tray containing soil in which the plants were cultivated side-by-side. Water was withheld from the plants for approximately 14 days, after which the significant difference was observed between the *spy-3* and wildtype plants. (E) Water loss rates of detached *spy-1*, *spy-3* and WT shoots. WT Columbia (Col.) is shown as the control. The mean and standard deviation (SD) were obtained from four plants in each assay. (F) Leaf electrolyte leakage was compared among *spy-1*, *spy-3* and *Col.* plants after 2.5 h of dehydration stress. (G) Three-week-old *Col.* plants treated with 50 μM GA_3 or H_2O after germination were subjected to drought stress, and their survival rates were compared. For all drought tolerance experiments, photographs were taken after one week of reinitiated watering. The survival rates were calculated from at least three independent experiments.

Figure 3. (A) Expression of the *SPY* gene under various stress treatments. Twenty micrograms of total RNA from 3-week-old plants that had been treated as

indicated were used for RNA blot hybridization with a gene-specific probe. (B) *SPY* was preferentially expressed in guard cells in leaf tissue based on data from the Arabidopsis eFP Browser.

Figure 4. Venn diagrams of gene expression alterations in *spy-3* plants under normal or dehydration stress conditions based on microarray analysis. (A) Overlap between *Col.* plants dehydrated for 3 days and non-dehydrated *spy-3* plants. (B) Overlap between genes up or downregulated by GA with those of untreated *spy-3* plants. (C) Overlap between *Col.* plants treated for 6 h with ABA and untreated *spy-3* plants. (D) Overlap between *Col.* plants dehydrated for 3 days and *spy-3* plants dehydrated for 2 h.

Figure 5. qRT-PCR analysis of the expression of genes that were identified as being upregulated in the *spy-3* mutant under normal growing and 2-hour-dehydrated conditions.

Figure 6. Overexpression of *SPY* reduced plant drought stress tolerance. (A) RNA gel blot analysis of transgene expression in vector and *35S:SPY*-transformed plants; 10 µg total RNA was loaded. (B) Morphological phenotype of 3-week-old SPY-OX plants as compared with wild type. (C) Survival rate of vector-transformed and SPY OX lines. The averaged data were obtained from three independent experiments; ** $p < 0.01$, * $p < 0.05$, Student's t-test. (D) qRT-PCR analysis of the expression of genes that were upregulated in *spy-3*

mutants but downregulated in SPY-OX plants. Empty bars indicate the relative expression level in the untreated plants, and filled bars indicate the relative expression level in the 2-h dehydrated plants.

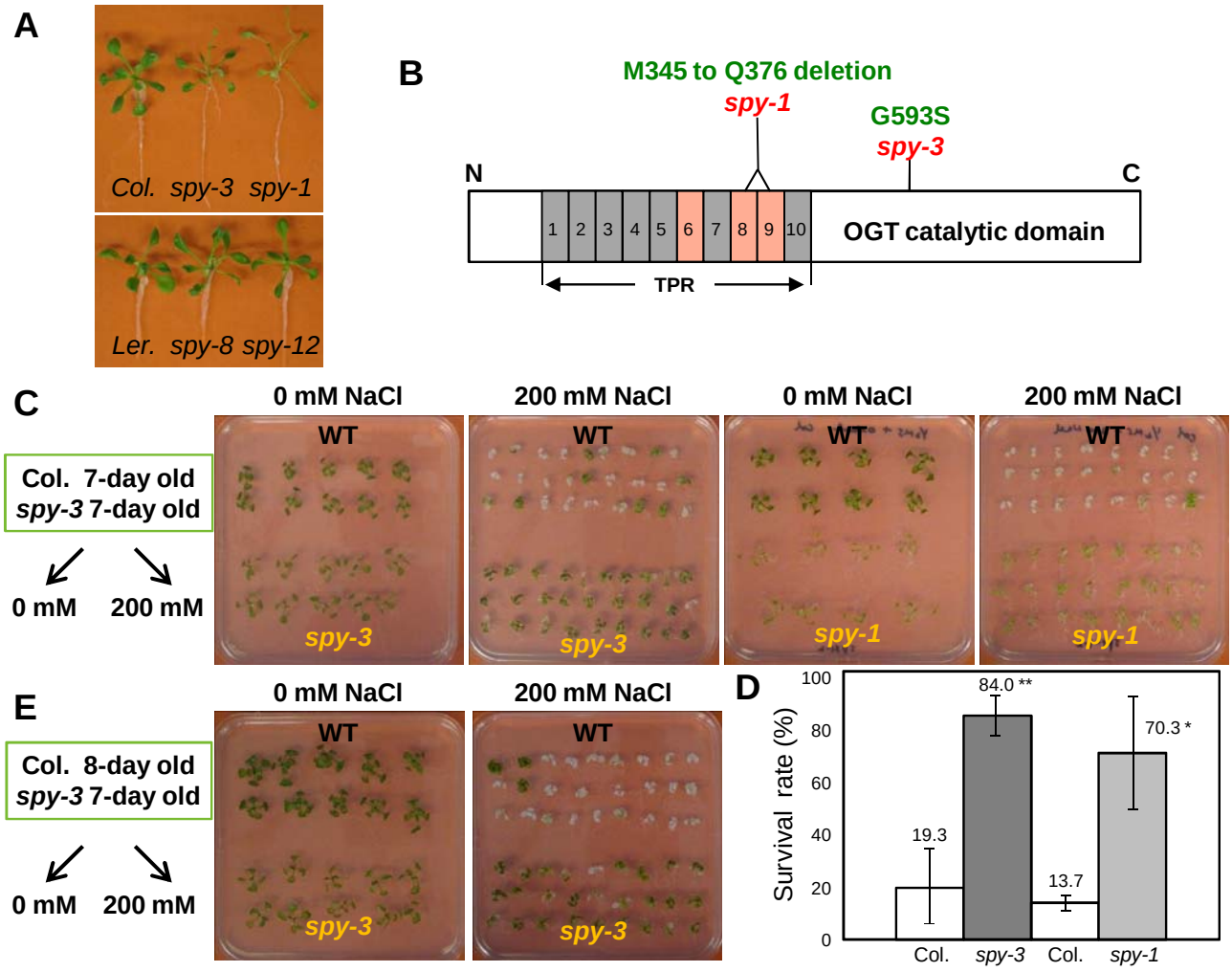

Figure 1. *spy* mutants were more tolerant of high salinity stress compared with wildtype plants. (A) Morphological phenotype of *spy* mutants. Three-week-old *Col.*, *spy-1* and *spy-3* plants growing on GM agar plates were photographed. (B) Schematic structure of the SPY protein and the locations of the mutations in the mutants. (C) *spy-1, spy-3* and WT plants grown on GM plates for 7 days and subsequently transferred onto 0.5 × Murashige and Skoog (MS) medium plates with or without (w/o) 200 mM NaCl. (D) The survival rate was calculated from independent experiments. (n ≥ 3; ** $p < 0.01$, * $p < 0.05$, student *t*-test). (E) Salt stress tolerance was compared between 8-day-old *Col.* and 7-day-old *spy-3* mutants, as described in panel A.

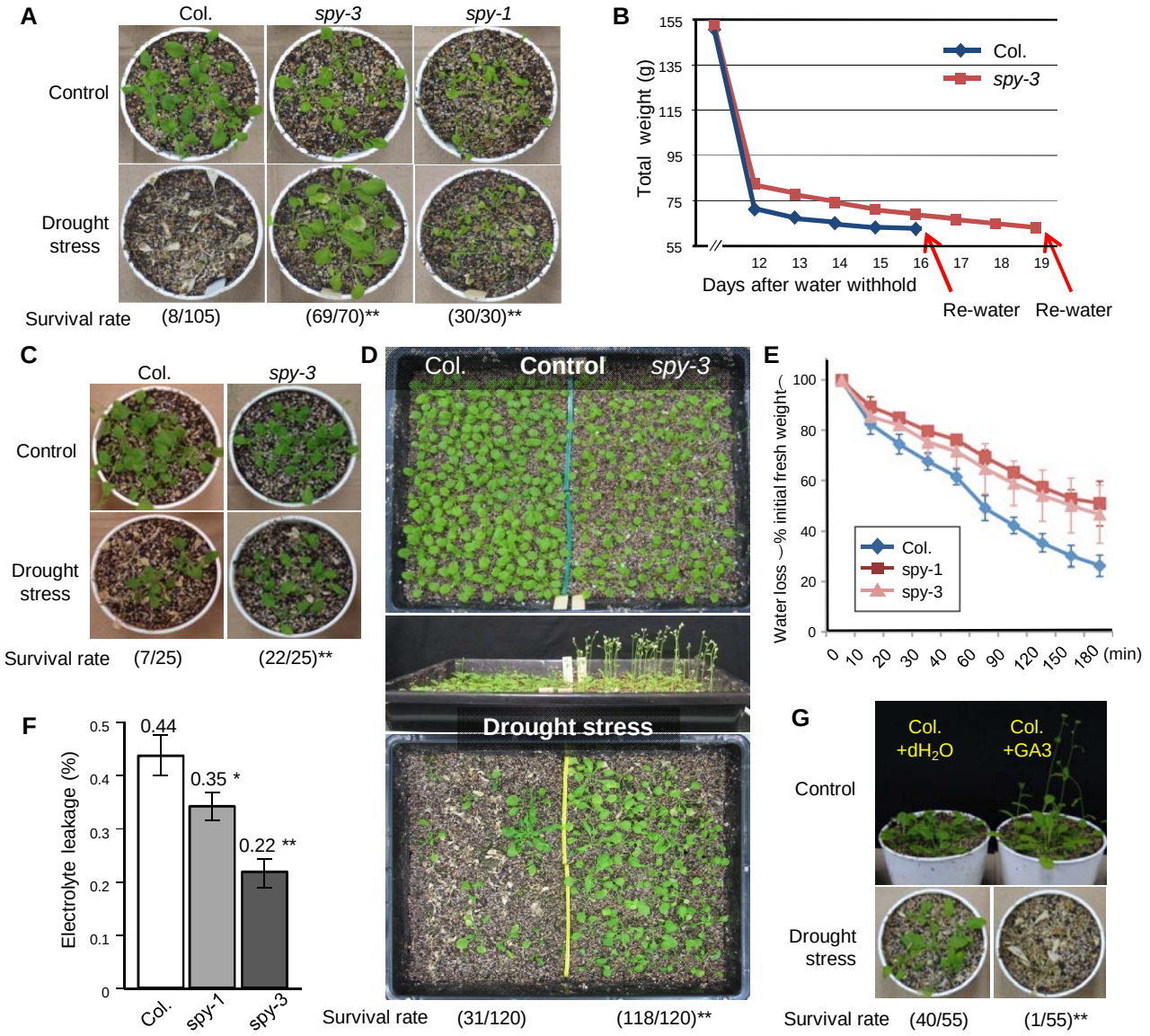

Figure 2. *spy-1* and *spy-3* mutants were more tolerant of drought stress. (A) The plant survival rate under drought stress was compared in individual soil-pots. (B) Four-week old *Col.* and *spy-3* plants were subjected to dehydration in pots containing the same amounts of soil. After 12 days of water withholding, the weight of each pot was recorded daily. Watering of the plants was reinitiated once they were equally dehydrated, which occurred on the 16th and 19th days for WT and *spy-3* plants, respectively. (C) Representative photographs of panel B are shown. (D) Plant drought tolerance was compared in a large tray containing soil in which the plants were cultivated side-by-side. Water was withheld from the plants for approximately 14 days, after which the significant difference was observed between the *spy-3* and wildtype plants. (E) Water loss rates of detached *spy-1, spy-3* and WT shoots. WT Columbia (Col.) is shown as the control. The mean and standard deviation (SD) were obtained from four plants in each assay. (F) Leaf electrolyte leakage was compared among *spy-1*, *spy-3* and *Col.* plants after 2.5 h of dehydration stress. (G) Three-week-old *Col.* plants treated with 50 µM GA_3 or H_2O after germination were subjected to drought stress, and their survival rates were compared. For all drought tolerance experiments, photographs were taken after one week of reinitiated watering. The survival rates were calculated from at least three independent experiments.

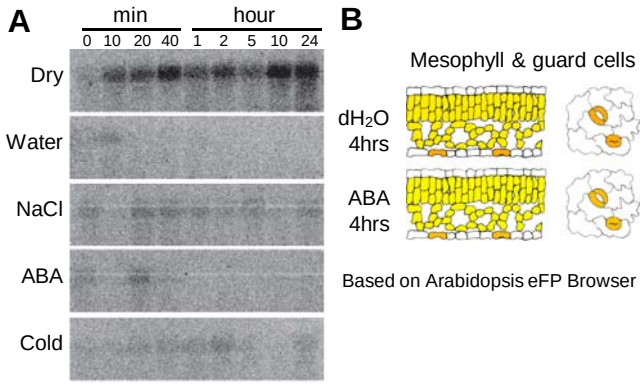

Figure 3. (A) Expression of the *SPY* gene under various stress treatments. Twenty micrograms of total RNA from 3-week-old plants that had been treated as indicated were used for RNA blot hybridization with a gene-specific probe. (B) *SPY* was preferentially expressed in guard cells in leaf tissue based on data from the Arabidopsis eFP Browser.

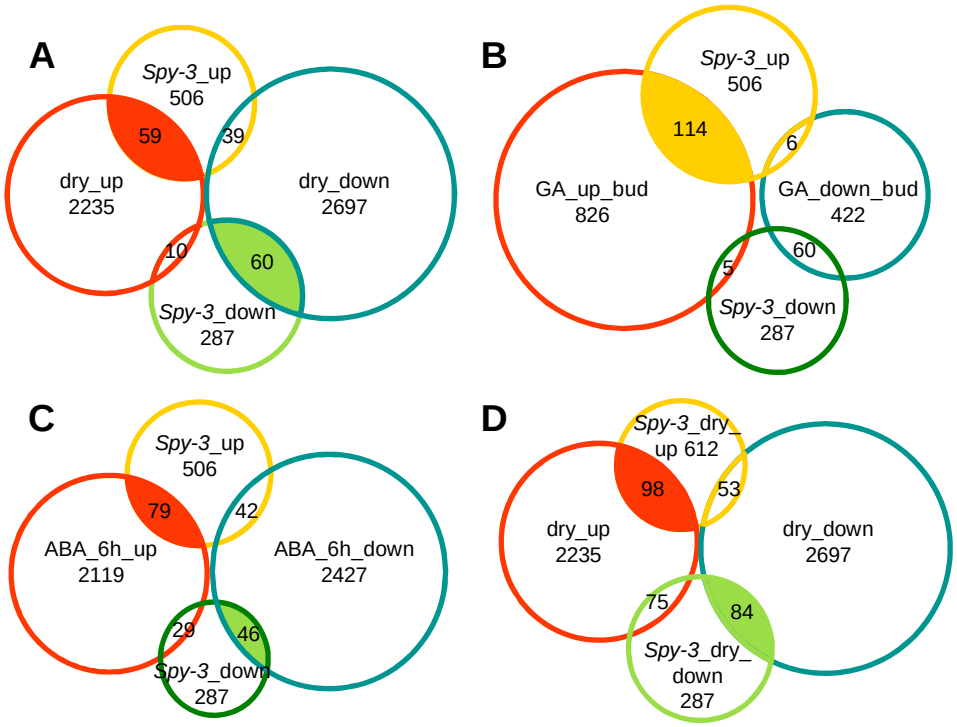

Figure 4. Venn diagrams of gene expression alterations in *spy-3* plants under normal or dehydration stress conditions based on microarray analysis. (A) Overlap between *Col.* plants dehydrated for 3 days and non-dehydrated *spy-3* plants. (B) Overlap between genes up or downregulated by GA with those of untreated *spy-3* plants. (C) Overlap between *Col.* plants treated for 6 h with ABA and untreated *spy-3* plants. (D) Overlap between *Col.* plants dehydrated for 3 days and *spy-3* plants dehydrated for 2 h.

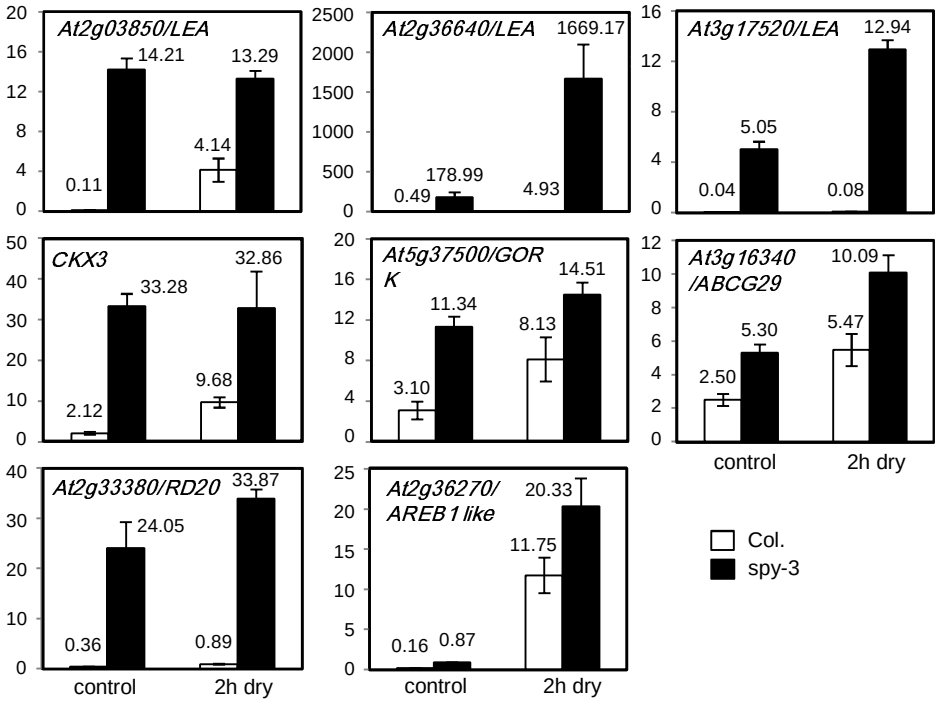

Figure 5. qRT-PCR analysis of the expression of genes that were identified as being upregulated in the *spy-3* mutant under normal growing and 2-hour-dehydrated conditions.

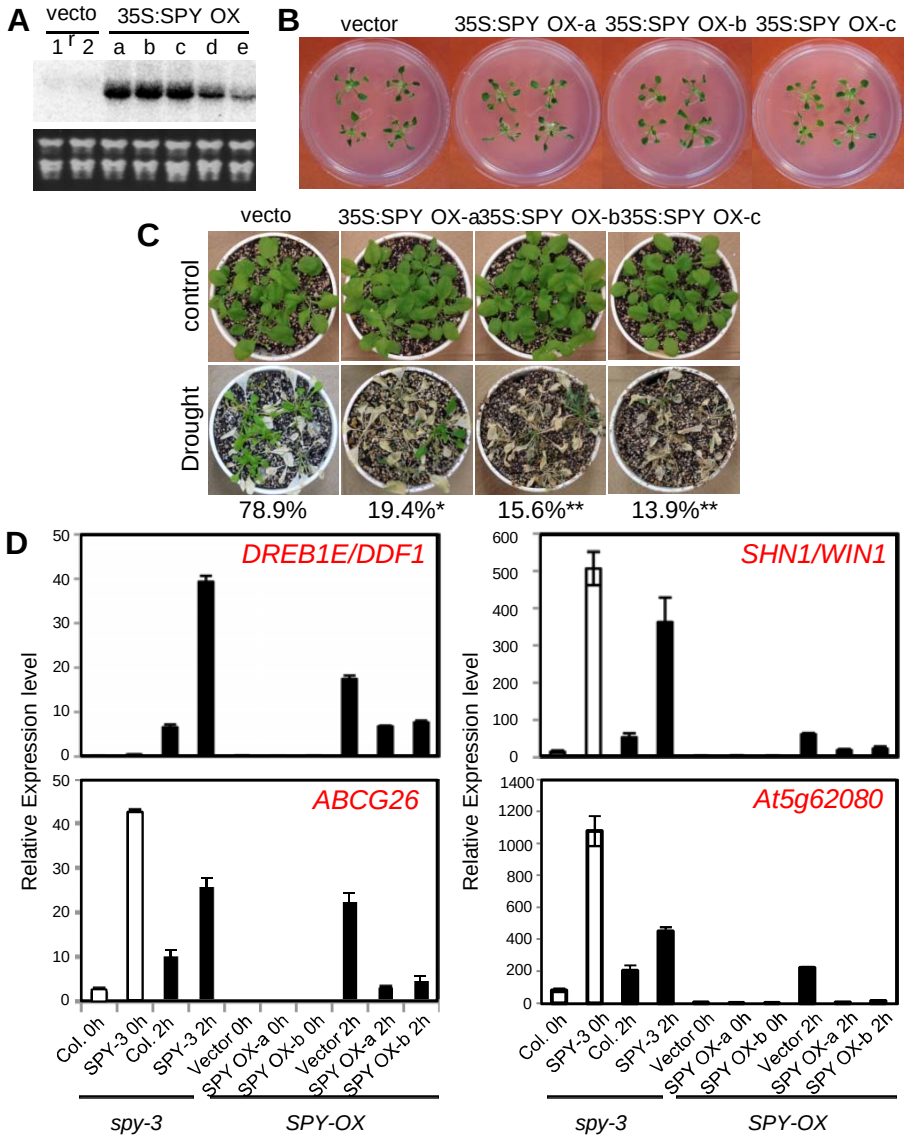

Figure 6. Overexpression of *SPY* reduced plant drought stress tolerance. (A) RNA gel blot analysis of transgene expression in vector and *35S:SPY*-transformed plants; 10 µg total RNA was loaded. (B) Morphological phenotype of 3-week-old SPY-OX plants as compared with wild type. (C) Survival rate of vector-transformed and SPY OX lines. The averaged data were obtained from three independent experiments; ** $p < 0.01$, * $p < 0.05$, Student's t-test. (D) qRT-PCR analysis of the expression of genes that were upregulated in *spy-3* mutants but downregulated in SPY-OX plants. Empty bars indicate the relative expression level in the untreated plants, and filled bars indicate the relative expression level in the 2-h dehydrated plants.