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StMYB1R-1 enhances drought tolerance in potato

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Research area: Environmental Stress and Adaptation
Expression of \textit{StMYB1R-1}, a Novel Potato Single MYB-like domain Transcription Factor, Increases Drought Tolerance

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Abstract

Potato (*Solanum tuberosum* L.) is relatively vulnerable to abiotic stress conditions such as drought, but the tolerance mechanisms for such stresses in potato are largely unknown. To identify stress-related factors in potato, we previously carried out a genetic screen of potato plants exposed to abiotic environmental stress conditions using reverse Northern blot analysis. A cDNA encoding a putative R1-type MYB-like transcription factor was identified (*StMYB1R-1*) as a putative stress-response gene. Here, the transcript levels of *StMYB1R-1* were enhanced in response to several environmental stresses in addition to drought, but were unaffected by biotic stresses. The results of intracellular targeting and Q9-protein binding microarray analysis indicated that StMYB1R-1 localizes to the nucleus and binds to the DNA sequence $^{G/A}GATAA$. Overexpression of a *StMYB1R-1* transgene in potato plants improved plant tolerance to drought stress while having no significant effects on other agricultural traits. Transgenic plants exhibited reduced rates of water loss and more rapid stomatal closing than wild-type plants under drought stress conditions. In addition, overexpression of *StMYB1R-1* enhanced the expression of drought regulated genes such as *AtHB-7*, *RD28*, *ALDH22a1*, and *ERD1-like*. Thus, the expression of *StMYB1R-1* in potato enhanced drought tolerance via regulation of water loss. These results indicated that StMYB1R-1 functions as a transcription factor involved in the activation of drought-related genes.
Introduction

As plants are sessile organisms, environmental stresses such as drought and high salinity conditions can compromise economic output and the overall human food supply (Barnabas et al., 2008). Drought stress gives rise to biochemical, molecular, physiological, and morphological changes that adversely affect plant growth, development, and productivity (Reynolds and Tuberosa, 2008; Moore et al., 2009). Under drought stress conditions, the phytohormone abscisic acid (ABA) is synthesized. ABA modulates the expression of stress-related genes and activates signal transduction pathways that lead to a variety of physiological responses, including changes in stomatal aperture (McCourt and Creelman, 2008). ABA accumulation in guard cells triggers an increase in cytosolic Ca\(^{2+}\), resulting in the activation of calcium-dependent protein kinases (CDPKs) (Pei et al., 1996). These CDPKs, especially CPK23, in turn, regulate the activity of anion channels such as slow anion channel 1 (SLAC1) (Mori et al., 2006; Geiger et al., 2010). The consequent efflux of anions and depolarization of the membrane induce stomatal closure to prevent transpirational water loss (Israelsson et al., 2006). In Arabidopsis, \textit{slac1} mutants exhibit reduced stomatal closure in response to ABA, Ca\(^{2+}\), and H\(_2\)O\(_2\) (Vahisalu et al., 2008), and SLAC1 anion channel activity is directly controlled by the ABA-activated protein kinase OPEN STOMATA 1 (OST1/SRK2E/SnRK2.6) and ABI1/PP2C phosphatase complexes (Geiger et al., 2009; Lee et al., 2009; Vahisalu et al., 2010). Recently, it was reported that RCAR/PYR1/PYL family of START proteins can bind ABA through a gate-latch-lock mechanism and interacts with ABI1/PP2C as co-receptors in Arabidopsis (Melcher et al., 2009; Nishimura et al., 2009; Santiago et al., 2009). ABA perception by the RCAR/PYR1/PYL proteins suppresses PP2C-mediated dephosphorylation of the SnRKs and allows their activation (Umezawa et al., 2009). The \textit{pyr1/pyl1/pyl2/pyl4} quadruple mutant plants show insensitivity in ABA-induced stomatal closure and ABA-inhibition of stomatal opening (Nishimura et al., 2010).

The expression of various stress-response genes by ABA is mediated by a number of transcriptions factors (TF) such as the MYB family of TFs and ABA response element-
binding factors (AREB) (Fujii et al., 2009). MYB TFs are composed of one, two or three imperfect helix-turn-helix repeats that recognize the major groove of DNA (Yanhui et al., 2006). MYB TFs are grouped into three subfamilies according to their MYB domain arrangement: R1R2R3, R2R3, and MYB-related (containing a single MYB-like domain) (Du et al., 2009). In animals, MYB TFs are typically of the R1R2R3 type, which contain three repeats of the MYB domain (Ramsay and Gonda, 2008). In contrast, most plant MYB TFs are of the R2R3 type, which contain two repeats of the MYB domain (Du et al., 2009). Over the past decade, R2R3-type MYB TFs have been implicated in a variety of plant-specific processes, including cell morphogenesis, secondary metabolism, cell differentiation, and stress responses (Nesi et al., 2001; Baumann et al., 2007; Ishida et al., 2007; Zhao et al., 2008; Lippold et al., 2009; Ma et al., 2009). In Arabidopsis, MYB60 and MYB61 are specifically expressed in guard cells. The \textit{atmyb60} mutant exhibits reduced light-induced stomatal opening; whereas, the \textit{atmyb61} mutant exhibits reduced ABA-induced stomatal closing (Liang et al., 2005).

In Arabidopsis and rice, 49 and 84 R1-type MYB genes, respectively, have been described (Yanhui et al., 2006). Only one contains the typical two or three tryptophan repeat in the MYB domain (Yanhui et al., 2006). To date, 66 MYB-like TFs have been described in potato plants (http://planttfdb.cbi.pku.edu.cn/), but the potato genome project is ongoing and will likely yield a more precise number upon completion. Compared with R2R3-type MYB TFs, there are few reports of functional studies of single MYB-like domain TFs in plants. Potato MybSt1, the first reported single MYB-like domain TF in plants, recognizes a target sequence located between nucleotides -73 and -48 of the cauliflower mosaic virus (CaMV) 35S promoter and functions as a transcriptional activator (Baranowskij et al., 1994). In Arabidopsis, the single MYB-like domain TF \textit{CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)} has been shown to bind to two imperfect repeats in the light-harvesting chlorophyll a/b (Lhcb) promoter and acts as a specific activator of phytochrome signal transduction (Wang et al., 1997). Constitutive expression of \textit{CCA1} results in longer hypocotyls and substantially delayed flowering (Wang and Tobin, 1998). In rice, \textit{anther indehiscence1 (AID1)} is closely related to other single MYB-like domain TFs in plants. A \textit{Ds}-tagged recessive \textit{aid1} mutant, in which the transposon is inserted in the coding region of \textit{AID1}, was identified in a genetic screen as playing a role in partial to complete spikelet sterility (Zhu et al., 2004). Three \textit{Oryza sativa} MYB proteins have been identified that interact with the promoter of \textit{α}-amylase, an
important component of the gibberellin (GA) and sugar responses (Lu et al., 2002). Interestingly, OsMYBS1 and 2 positively regulate the α-amylase promoter when sugar is present, whereas OsMYBS3 represses transcription from the same promoter in response to sugar starvation (Lu et al., 2002). Recently, it was reported that expression of an OsMYBS3 transgene in rice confers cold tolerance without a significant penalty in yield under normal field conditions (Su et al., 2010).

Potato (Solanum tuberosum L.) is an important food crop worldwide, with annual production approaching 300 million tons (Camire et al., 2009). Potato is relatively vulnerable to abiotic stresses such as drought and high salinity and is classified as an environmentally-sensitive crop along with paddy rice and sugarcane (Levy 1985; Bray et al., 2000; Vasquez-Robinet et al., 2008). Potato tuber initiation, bulking, and tuber growth stage especially are sensitive to drought stress (Evers et al., 2010). Therefore, water availability is an important factor in increasing tuber formation and potato crop yields. Several studies in potato were done to overcome crop yield loss under environmental stress conditions through genetic and molecular biological approaches (Jeong et al., 2001; Schafleitner et al., 2007; Stiller et al., 2008; Evers et al., 2010). And recently, drought-related elements in potato are profiled through a combined transcriptomic and targeted metabolite approach (Vasquez-Robinet et al., 2008; Evers et al., 2010). However, little is known about stress tolerance mechanisms in potato plants. In the current work, we identified an environmental stress-responsive R1-type MYB TF in potato and characterized its biological role in detail by examining its expression and physiological phenotype in potato plants.

Results
Isolation of a stress-responsive single MYB-like domain TF from Potato

To explore the molecular mechanisms of tolerance in potato (Solanum tuberosum L. cv Superior), we previously identified 17 genes by reverse Northern blot analysis that were upregulated by cold, drought, and salt stress (Lee et al., 2007). Here, we carried out a series of molecular experiments to characterize one of these abiotic-responsive genes, clone SOT171183 (accession no. AU279205). Clone SOT171183 was 434 basepairs (bp) in length and contained no ATG start codon. Using the sequence of SOT171183 as a probe in a colony hybridization screening assay, we identified and sequenced 6 putative clones of different lengths that were all derived from the same gene. The longest clone was 1,166 bp in length,
with an open reading frame that encoded a putative protein of 297 amino acids and a calculated molecular weight of 32.5 kDa (Supplemental Fig. S1). An in silico database search revealed that the sequence of this clone was similar to single MYB-like domain TFs from Arabidopsis, rice, pepper, and soybean belonging to the CCA1-like clade II subgroup (Yanhui et al., 2006). Outside of the MYB domain, the sequence similarity between our clone and other members of the CCA1-like clade II family was lower than the MYB domain itself, with the exception of GmMYB176 and AT1G74840, which showed a high degree of conservation within and outside of the MYB domain with our clone (Fig. 1A and B). The gene mapped to chromosome 6 and contained 3 exons and 2 introns. To begin to characterize the biological role of this putative stress-responsive gene in potato, we analyzed RNA transcript levels under various stress conditions. Expression was increased under environmental stress conditions such as drought, but not biotic stress conditions such as SA treatment (Fig. 2). These results indicated that this novel gene, which we designated StMYB1R-1, encodes a putative stress-responsive R1-type MYB transcription factor in potato.

**StMYB1R-1 Functions as a TF**

Most TFs, including MYB, localize to the nucleus and bind specific DNA sequences to regulate gene expression. Sequence analysis indicated that StMYB1R-1 belongs to the MYB family of TFs. To determine whether StMYB1R-1 functioned as a TF, we first examined the subcellular localization of StMYB1R-1 in Arabidopsis protoplasts and onion epidermal cells. Recombinant full-length StMYB1R-1, expressed as a transgene encoding full-length StMYB1R coupled to green fluorescent protein (StMYB1R-1:GFP), localized predominantly to the nucleus, and to a lesser extent the cytosol (Fig. 3B and Supplemental Figure S2). An in silico database search yielded no evidence of a nuclear localization signal (NLS) or nuclear export signal (NES) peptide in the StMYB1R-1 protein. We generated variants of StMYB1R-1 that contained only the N- or C-terminal region, and then examined the subcellular localization of each domain. Only the N-terminal domain of StMYB1R-1, which contained the MYB-like domain, localized to the nucleus, whereas the C-terminal segment localized to the cytosol (Fig. 3B). These results indicated that StMYB1R-1 localizes to the nucleus where it could function as a TF.

We took advantage of a recently developed protein binding microarray to determine the
putative DNA binding sequence of StMYB1R-1. The quadruple 9-mer (Q9)-protein binding array was developed as a screening tool for rapid identification of DNA binding sequences of TFs, and contains more than 100,000 unique double-stranded DNA oligomers synthesized as quadruples of all possible 9-mer combinations (Kim et al., 2009). Using a DsRed fusion protein of StMYB1R-1 (StMYB1R-1:DsRed) to monitor binding, Q9-protein binding microarray analysis yielded 1,028 putative DNA binding sequences (>3,500 relative signal intensity). The DNA sequences were segregated into 13 groups, and two sequences, GGATAA and AGATAA, emerged as the predominant DNA binding sequences (Fig. 4A; Supplemental Tables S1 and S2). The signal intensity of GGATAA was 21,860, while the signal intensities of the other groups, with the exception of group 8, were below 16,000. To test whether the GGATAA sequence was essential for DNA binding by StMYB1R-1, we analyzed the relative signal intensities of single nucleotide substitution variants of the putative binding sequence (with the exception of AGATAA). Individual substitutions at all position of the GGATAA sequence significantly reduced the signal intensity of binding, whereas substitutions outside this core sequence had no effect (Fig. 4B). To confirm these results, we performed an electrophoresis mobility shift assay (EMSA) using StMYB1R-1:DsRed as the probe. Recombinant StMYB1R-1 was able to bind to and cause a shift in the mobility of an oligonucleotide containing the GGATAA sequence, but not any of the single nucleotide substitution variant sequences (Supplemental Fig. S4). These results indicated that StMYB1R-1 selectively binds to DNA at $^\text{G/AGATAA}$ sequences and may function as a transcription factor.

**Expression of StMYB1R-1 Enhances Drought Tolerance**

Expression analysis of StMYB1R-1 indicated that it is positively regulated under drought stress conditions. We generated transgenic potato plant lines that overexpressed StMYB1R-1 to investigate whether overexpression of StMYB1R-1 increases drought tolerance. We first examined the effect of StMYB1R-1 expression on potato agricultural traits by morphological analysis. The expression of StMYB1R-1 did not significantly alter the growth characteristics of transgenic potato plants as compared to wild-type plants under greenhouse conditions (Supplemental Fig. S5). To assess the effect of StMYB1R-1 overexpression on drought tolerance, eight-week-old potato plants were deprived of water for 15 days, and then watering was resumed in the greenhouse. After 12 days without water, foliar tissue drooping was
evident in wild-type plants, but not transgenic plants. After 15 days without water, most of
the leaves of wild-type potato plants had wilted, while in transgenic plants, drooping of foliar
tissue was just beginning to become apparent. After watering was resumed, some of the
leaves of transgenic potatoes turned yellow-brown in color as a result of drought stress, but
the plants survived and eventually developed green leaves. By comparison, wild-type potato
plants did not go on to develop green leaves (Fig. 5). Seven days after the resumption of
watering and growth under normal conditions, survival rates were determined for wild-type
and transgenic potato plants. More than 50% of the transgenic potato plants survived after the
period of drought stress compared to 19% of wild-type potato plants (Fig. 6A). We also
measured the weight of fresh leaves before and after drought stress. Before drought stress
treatment, the leaf weights of wild-type and transgenic plants were similar. After 5 days of
water deprivation, the water content of wild-type leaves decreased by 20%, whereas that of
transgenic leaves decreased by only 5%. After 7 days without water, leaf water content in
wild-type and transgenic leaves decreased by 40% and 30%, respectively (Fig. 6B).

**Expression of StMYB1R-1 Prevents Water Loss and Enhances Hypersensitivity to ABA-
induced Stomatal Closure**

To determine whether *StMYB1R-1* was involved in regulating water loss under
conditions of drought stress, we performed a water loss assay of wild-type and transgenic
plants. The rate of water loss was determined by measuring the fresh weight of detached
leaves at 30-minute intervals. The fresh weight of transgenic and wild-type leaves was
decreased by approximately 22% and 27%, respectively, after drought stress (Fig. 6C). When
we examined transpiration rate and stomatal conductance of detached potato leaves, the time
to reach a 50% reduction in transpiration rate was approximately 9 minutes for wild-type
plants, and less than 7 minutes for transgenic plants. Transgenic plants also exhibited a more
rapid loss of stomatal conductance than wild-type plants (Supplemental Fig. S6). Transgenic
line 2 in particular had a more rapid physiological response under drought stress conditions.
These results indicated that *StMYB1R-1* overexpression results in a significant decrease in the
rate of water loss in potato plants.

Water loss in plants is regulated by guard cell density, guard cell size, and/or stomatal
opening and closing. To determine whether StMYBR-1 was involved in regulating these
parameters in potato plants, we first compared guard cell density and guard cell size in wild-
type and transgenic plants. Guard cell density and size were similar between wild-type and transgenic plants under normal growth conditions (data not shown). To determine the effect of \textit{StMYB1R-1} overexpression on stomatal opening, leaves from wild-type and transgenic potato plants were incubated in the light for 3 hours to allow stomata to open fully, and then the widths of the stomatal apertures were determined. In wild-type leaves, stomata measured 12.10 $\mu$m $\pm$ 0.017 in width as compared to 12.13 $\mu$m $\pm$ 0.024 (L1, 12.47 $\mu$m $\pm$ 0.027; L2, 12.07 $\mu$m $\pm$ 0.021; L8, 12.21 $\mu$m $\pm$ 0.025) in transgenic plants. Thus, overexpression of \textit{StMYB1R-1} did not appear to significantly affect stomatal opening. However, after treatment with ABA for 1 hour, there was a marked dose-dependent decrease in the size of stomatal pores (ratio of width to length) in leaves from transgenic plants as compared to wild-type (Fig. 7A).

As indicated above, in response to treatment with 1 $\mu$M ABA, there was a marked difference in stomatal aperture between wild-type and transgenic plants. We performed a kinetic analysis of stomatal aperture closing in wild-type and transgenic plants in the presence of 1 $\mu$M ABA. After 30 minutes of ABA treatment, stomatal aperture size in wild-type and transgenic plants decreased to 0.132 $\pm$ 0.005 and 0.113 $\pm$ 0.02 (L1, 0.115 $\pm$ 0.004; L2, 0.112 $\pm$ 0.005; L8, 0.118 $\pm$ 0.004), respectively. After an additional 60-minute incubation period in the presence of ABA, the stomatal aperture size of transgenic plants was close to that observed under dark conditions, while the stomatal aperture of wild-type leaves remained slightly open (Fig. 7B). These results suggested that the expression of \textit{StMYB1R-1} potentiates the ABA response of guard cells, leading to reduced water loss under drought conditions. This drought-tolerance phenotype of transgenic plants was consistent with the slow rate of water loss from leaves.

\textbf{Expression of \textit{StMYB1R-1} Enhances the Expression of Drought Stress-related Genes}

Given that overexpression of \textit{StMYB1R-1} led to drought tolerance in potato, we explored whether the phenotypic changes in the transgenic plants correlated with changes in the expression patterns of stress responsive genes. Microarray analysis using the TIGR 10K potato chip (Lee et al., 2007) revealed that 295 genes were up-regulated 2-fold or more in transgenic plants as compared to wild-type plants. Most of the annotations were hypothetical or unknown proteins (Supplemental Table S3), but 16 of the genes were abiotic stress-responsive genes. We analyzed these 16 genes by semi-quantitative reverse transcription
(RT)-PCR to determine whether they were regulated by drought stress, and to validate the results of the microarray analysis. Expression of the Na\(^+\)-dependent bile acid transporter (AtBAT5-like) was similar in the presence and absence of drought stress treatment, and trehalose-6-phosphate synthase (TPSI-like) and fumarylacetoacetate hydrolase (FAH1-like) were undetectable by RT-PCR under these experimental conditions. Notably, the transcript levels of several genes with high similarity to AtHB-7, RD28, ALDH22a1, and ERD1-like, which are involved in plant stress tolerance, were dramatically upregulated after drought stress treatment and were enhanced in transgenic plants (Fig. 8).

**Discussion**

Potato is one of the world’s major food crops (Gopal and Iwama, 2007). Despite this, there are a few reports in the literature of molecular engineering to improve or manipulate stress tolerance in potato plants (Stiller et al., 2008; Tang et al., 2008). A limited number of single MYB-like domain TFs have been identified in plants (Penfield and Hall, 2009). Here, we report the identification and characterization of a putative single MYB-like domain TF, StMYB1R-1, in potato.

Various TFs are rapidly induced during the early phases of environmental stress conditions such as drought and high salinity. AtMYB2, GmMYB92, and CpMYB10, which encode R2R3-type MYB transcription factors, are induced by ABA as well as drought (Urao et al., 1993; Villalobos et al., 2004; Liao et al., 2008), and AtMYB44 and AtMYB102 are induced after ABA and drought treatment. These genes are also upregulated by jasmonic acid (JA) and wounding stress (Denekamp and Smeekens, 2003; Jung et al., 2008). Although functional roles for single MYB-like domain TFs such as CCA1 have been identified in light-related and other developmental processes (Wang et al., 1997; Zhu et al., 2004), other MYB family TFs, such as GmMYB117, an R1-type MYB TF found in soybean, and OsMYBS3 in rice are enhanced by ABA and abiotic stresses (Liao et al., 2008) and cold stress conditions (Su et al., 2010), respectively. Here, we showed that the expression of StMYB1R-1 is induced after ABA treatment and by various abiotic stresses, but is unaffected by SA and *R. solanacearum* treatment (Fig. 2). These results suggest that single MYB-like domain TFs may function specifically in abiotic stress responses, and that StMYB1R-1, a novel R1-type member of the MYB family in potato, is involved in regulating these types of stress responses.

TFs interact with specific DNA sequences (cis-acting elements) in target genes to
modulate the transcription process (Priest et al., 2009). Protein-DNA binding properties are traditionally investigated by methods such as EMSAs and filter binding assays (Garner and Revzin, 1981; Soderman and Reichard, 1986). The development of the Q9-protein binding microarray along with the availability of whole-genome sequence information and advances in microarray technology have greatly facilitated our ability to characterize protein-DNA binding specificities in vitro (Kim et al., 2009). The results of Q9-protein binding microarray analysis and EMSA demonstrated that StMYB1R-1 binds DNA mainly at \( G/AGATAA \) sequences (Fig. 4 and Supplemental Fig. S4). Previously, it was shown StMYB1 binds specifically to the sequence GGATA within a region (nucleotides -73 to -48) of the CaMV 35S promoter to activate transcription in tobacco protoplasts (Baranowskij et al., 1994).

Three single MYB-like domain proteins in rice (OsMYBSs) interact with a TATCCA element in the promoter of \( \alpha \text{Amy}3 \), a rice \( \alpha \)-amylase gene that is strongly induced in response to sugar starvation (Lu et al., 2002). In addition, OsMYBS3 in rice regulates the expression of cold-related genes such as \( WRKY77 \) and \( glutamate de carboxylase \), both of which contain a TATCCA element in their promoters (Su et al., 2010). The fact that the DNA binding sequences of StMYB1 and the OsMYBSs are similar to the target binding sequence identified here for StMYB1R-1 supports the use of the Q9-protein binding microarray as a reliable technique for studying genome-wide protein-DNA interactions. These results also indicate that the DNA binding sequences of plant single MYB-like domain TFs may be similar, and that binding specificity may be regulated by interactions with other regulatory proteins.

Overexpression of \( StMYB1R-1 \) in potato plants induced the expression of several genes, including \( AtHB-7 \), \( RD28 \), \( ALDH \), and \( ERD15 \), and follow-up RT-PCR analysis confirmed that these genes are upregulated under drought stress conditions. \( AtHB-7 \) is up-regulated in response to ABA treatment and water deficit, and expression of \( AtHB-7 \) has been shown to increase ABA sensitivity (Olsson et al., 2004). RD28 is a member of the major intrinsic protein 2 family of water channels (Chaumont et al., 1997). The expression of water channels in Arabidopsis is up- or downregulated under conditions of abiotic stress such as drought (Jang et al., 2004). In particular, \( PIP2;2 \), which encodes a water channel protein, is activated predominantly in roots, and PIP2;2 functions in root water uptake under conditions of reduced transpiration (Javot et al., 2003). Glycine betaine is an important osmolyte source in plants, and \( ALDH \) genes, which encode betaine aldehyde dehydrogenases, are activated under saline or water deficit conditions (Kirch et al., 2005). \( ERD15 \) is rapidly expressed in response
to abiotic and biotic stresses, particularly dehydration (Kariola et al., 2006). Thus, the enhanced drought tolerance observed in StMYB1R-1 transgenic plants might depend in part on changes in the expression levels of these genes. That some of these upregulated genes such as RD28 contain an $^{\Lambda}_{\omega}$GATAAA box in their promoters (data not shown) supports a mechanism in which binding of StMYB1R-1 to cis-elements in the promoters of target genes results in transcriptional regulation of environmental stress-inducible genes.

The expression of stress inducible genes, including MYB TFs, affects stress tolerance in plants. AtMYB2 is induced by dehydration and ABA treatments, and overexpression of AtMYB2 results in increased sensitivity to ABA (Abe et al., 2003). AtMYB44 functions in leaf epidermal guard cells, and AtMYB44 transgenic plants exhibit a more rapid ABA-induced stomatal closure response, reduced rate of water loss, and markedly enhanced drought tolerance as compared to wild-type plants (Jung et al., 2008). Ectopic expression of GmMYB177 confers salt and freezing tolerance in Arabidopsis (Liao et al., 2008). OsMYBS3 transgenic rice plants acquire cold tolerance with no penalty in terms of yield under normal field conditions (Su et al., 2010). AtMYB41 is expressed in response to drought and salt treatment in an ABA-dependent manner, but transgenic plants that overexpress AtMYB41 lose water more rapidly than wild-type plants and are hypersensitive to desiccation (Cominelli et al., 2008). Furthermore, AtMYB41 has been shown to negatively regulate salt-induced genes such as AtDREB2a and AtNCED3 (Lippold et al., 2009). Here, overexpression of StMYB1R-1 greatly improved drought tolerance in potato plants, as demonstrated by increased survival rates, decreased water loss, and enhanced stomatal closure in transgenic plants as compared to wild-type (Figs. 6-8). StMYB1R-1 transgenic plants also exhibited salt tolerance, as observed by comparing root elongation in MS media under high saline conditions between wild-type and transgenic plants (Supplemental Fig. S4). These results indicate that StMYB1R-1 functions in drought and salt tolerance to regulate water content via transcriptional regulation of stress-responsive genes in potato plants.

Trehalose-6-phosphate synthase (TPS1) is involved in the synthesis of trehalose. Expression of Saccharomyces cerevisiae trehalose-6-phosphate synthase (TPS1) from the constitutive CaMV35S promoter resulted in drought tolerant in tobacco and potato plants (Goddijn et al., 1997; Romero et al., 1997). Transgenic potato plants expressing TPS1 have 30–40% fewer guard cells than the wild-type plants, and this correlates with their ability to retain significantly more water than wild-type plants (Stiller et al., 2008). Ectopic
expression of Arabidopsis *AtCBF* genes under the control of the constitutive CaMV 35S promoter in potato enhances freezing tolerance; however, plant growth is inhibited, as is development, particularly tuber formation and flowering time (Pino et al., 2007). Expression of *AtCBF* genes under the control of the rd29A promoter, on the other hand, ameliorated these agricultural traits without affecting freezing tolerance (Pino et al., 2007). Transgenic potato plants expressing *AtNDPK2* under the control of the stress-inducible SWPA2 promoter or CaMV 35S promoter exhibit enhanced tolerance to oxidative stress and salt stress. However, expression of *AtNDPK2* under the control of the SWPA2 promoter resulted in much less leaf damage than non-transgenic plants, whereas under the control of the CaMV 35S promoter, *AtNDPK2* expression resulted in an intermediate tolerance phenotype between *AtNDPK2*-SWPA2 transgenic and non-transgenic plants (Tang et al., 2008). Here, the overexpression of *StMYB1R-1* did not affect plant growth and development. In *StMYB1R-1* transgenic plants, foliar tissue size and biomass were similar to wild-type (Fig. 5). Importantly, tuber shape and size in *StMYB1R-1* transgenic lines were largely unaffected; tuber yield, on the other hand, was slightly reduced. These results suggest that stress-inducible promoters such as the rd29A and SWPA2 promoters are more efficient than the CaMV 35S promoter in terms of developing stress tolerant transgenic plants without negatively affecting other agricultural traits (Pino et al., 2007; Tang et al., 2008).

In summary, *StMYB1R-1* functions as a transcription factor to improve drought tolerance in potato. Although the detailed mechanism of *StMYB1R-1* function in response to abiotic stresses is not yet clear, this report provides valuable information for molecular breeding that could lead to improved stress tolerance in agricultural crops. Loss-of-function analysis of *StMYB1R-1* transgenic lines using RNA interference, currently underway in our laboratory, will further improve our understanding of the biological and molecular functions of *StMYB1R-1*.

**Materials and Methods**

**Plant Growth Conditions and Stress Treatments**

Potato (*Solanum tuberosum* L. cv Superior) was used throughout this study. Plants were grown in soil in a greenhouse or in hormone-free Murashige and Skoog (MS) agar medium in a growth chamber maintained at 21°C - 23°C and 60% relative humidity under long-day conditions (16 h light/8 h dark cycle). 3 µM of (±) ABA or 10 µM of SA were applied to the
surface of solid MS agar medium containing 3-week-old potato progenies. Abiotic stresses were applied to the 3-week-old progenies by either treating them with 100 mM NaCl, incubating them at 4°C under continuous light, or drying them on Whatman 3MM paper (Lee et al., 2007). *Ralstonia solanacearum* KACC10699 bacterial cells were grown on nutrient broth (NB) agar medium with phosphate for 24 h at 28°C, suspended in sterile distilled water, and adjusted to $10^8$ CFU/ml. The roots of the potato plants were immersed in 50 ml of bacterial suspension for 1 hour.

**Northern Blot Analyses, Microarray and Semi-quantitative RT-PCR**

Total RNA from 200 mg of tissue was extracted using the RNeasy Plant Mini kit (Qiagen) following the manufacturer’s instructions. Samples (10 μg lane$^{-1}$) were separated by 1.2% formaldehyde agarose gel electrophoresis, transferred to nylon membranes (Hybond N$^+$; Amersham, Piscataway, NJ) by capillary blotting and UV cross-linked. Hybridization was performed in Church buffer using a full-length *StMYB1R-1* probe, after which the membranes were washed with 2x, 1x and 0.5x SSC for 10 min each, and then rewashed with 0.1x SSC/0.1% SDS at 65°C for 10 min. The blots were then exposed to X-ray film (Eastman Kodak, Rochester, NY).

For microarray experiments, total RNAs were extracted from *StMYB1R-1* overexpression transgenic plants and wild type plants using TRIzol (invitrogen) as described by the manufacturer and were used for preparation of Cy5- and Cy3- labeled cDNA probes. Microarray was conducted with the TIGR cDNA potato microarray chip. Probe labeling and chip hybridization were carried out through the Korea TAKARA custom service (http://takara.co.kr/index.asp) by following the TIGR standard protocol (http://jcvi.org/potato/sol_ma_protocols.shtml). Normalization was performed according to the standard TIGR protocol to allow the comparison of the samples for each set of experiments. The Excel for significance analysis of microarrays was used to identify differentially expressed genes between *StMYB1R-1* overexpression transgenic plants and wild type plants.

For semi-quantitative RT-PCR, 2 μg of total RNA extracted from potato progenies were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen, Gaithersburg, MD, USA). The PCR program consisted of 25 to 30 cycles of amplification (20 sec, 95°C; 30 sec, 56°C; and 30 sec, 72°C) using ExTaq polymerase (TAKARA, Japan) with gene specific
primers. RT-PCR reactions were repeated three times. All of gene-specific primers are listed in Supplemental Table S4.

**Subcellular Localization of StMYB1R-1**

For subcellular localization, the cDNA fragments without the stop codon were amplified by PCR with specific primers containing appropriate restriction sites and subcloned directly upstream from the N-terminus of the GFP coding region in the p326GFP vector (Niwa et al., 1999). The plasmids were introduced into Arabidopsis protoplasts that had been prepared from leaf tissue by polyethylene glycol-mediated transformation (Jin et al., 2001). Expression of the fusion constructs was monitored at various time points after transformation and images were captured with a cooled CCD camera through a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Jena, Germany). Data were then processed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA) and presented in pseudo-color format.

**Determining of putative DNA binding sequence of StMYB1R-1**

To determine the DNA binding sequence of StMYB1R-1, a full-length cDNA of StMYB1R-1 was inserted into the pET32(a) vector (Novagen, San Diego, CA, USA) with an N-terminal fusion to a polyhistidine-tag and DsRed-monomer fluorescent protein (Novagen, San Diego, CA, USA). The StMYB1R-1 fusion protein was purified from E. coli strain BL21-CodonPlus (Stratagene, La Jolla, CA, USA). 200 nM of the StMYB1R-1 protein was incubated with quadruple 9-mer protein binding microarray (Q9-PBM), included 101,073 features from each 9-mers, in PBS-2% BSA, 50 ng/μl salmon testes DNA (Sigma) and 50 μM zinc acetate at 25°C for 1 h (Kim et al., 2009). Fluorescence images were obtained with a 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA). The signal intensities were ranged from 0 to 70,000. The significant probes were selected by statistical analysis modified (Kim et al., 2009). Probe sequence showing a $>3,500$ signal intensity from microarray fluorescence image were extracted and analyzed.

**Stomatal Aperture Bioassays**

To measure stomatal closure, detached leaves from 3-week-old potato progenies cultured in MS medium were floated in stomatal opening solution (15 mM KCl, 10 μM CaCl$_2$, and 10 mM MES-KOH, pH 6.15) in the light (200 μmol m$^{-2}$ sec$^{-1}$, 22°C). After 3 hours, the buffer
was replaced with fresh stomatal opening solution containing various concentrations of ABA. After an additional 1 hour incubation, the leaves were cut into 3 mm pieces, placed on a microscope slide, and covered with a cover slip. The abaxial epidermal layers of the leaves were observed using bright-field microscopy (Axioskop 2, Carl Zeiss, Jena, Germany) and images were captured using a CCD camera (Axio Cam, Carl Zeiss, Jena, Germany). Aperture size was measured from the photographs using the Interactive Measurement software package AxioVision 3.0.6 (Carl Zeiss, Germany).

Water loss

To determine water loss, nine leaves from 3-week-old potato progenies cultivated in the growth chamber were detached and placed in a weighing dish. The dishes were maintained in the growth chamber, and the loss of fresh weight was determined at 30-minute intervals during 6 hours (Bouchabke et al., 2008).

Generation of Transgenic Plants and Phenotype Observation

To generate StMYB1R-1 overexpression plants, the full-length cDNA of StMYB1R-1 was cloned into the pBIN121 vector (Clontech) under the control of the CaMV 35S promoter. The recombinant plasmid was introduced into Agrobacterium tumefaciens LBA4404, and transgenic potato plants were obtained by internode transformation (Lee et al., 2007). Twenty-four independent transgenic potato lines were generated and confirmed by hygromycin selection and Northern blot analysis. Among these transgenic potato lines, three independent transgenic lines were selected and grown in growth chamber before being used in later experiments.

To observe the phenotype under drought stress conditions, four-week-old potato plants cultured on MS medium and hydroponic chamber were transferred to soil (each 900 g of soil). To adapt the potato plants to the soil, potato progenies were grown for more 4 weeks in a greenhouse. Water was withheld from 8-week-old progenies for 15 days before resuming watering and everyday pot position was changed to minimize side effects. Photographs were taken, and the survival rate was determined on the 7th day after watering resumed. All stress assays were performed in three independent experiments, and mean values and standard errors are presented.
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FIGURE LEGENDS

Figure 1. Sequence comparison of single MYB-like domain TFs and StMYB1R-1. (A) Phylogenetic analysis of StMYB1R-1. The phylogenetic tree was constructed with the DNASTAR program (DNASTAR, Inc., Madison, WI, USA) using the deduced amino acid sequence of StMYB1R-1 and other single MYB-like domain protein sequences. (B) Amino acid sequence alignment of StMYB1R-1 and other single MYB-like domain proteins. Accession numbers are as follows: StMYB1R-1, ABB86258; At1g19000, BAH19529;
Figure 2. Transcript levels of StMYB1R-1 are upregulated in response to environmental stress, but not biotic stress conditions. (A) Transcript levels of StMYB1R-1 are enhanced in response to ABA, NaCl, and mannitol stress, but not SA or *Ralstonia solanacearum* treatment. Total RNA was extracted from 3-week-old potatoes treated with ABA (3 μM), NaCl (100 mM), mannitol (200 mM), SA (10 μM), and *Ralstonia solanacearum* for 1 hour. A gel pre-stained with ethidium bromide (lower) was used to confirm equal loading in all wells. (B) Transcript levels of StMYB1R-1 increase within 1 hour in response to environmental stress conditions. Total RNA was extracted from 3-week-old potato progenies treated for various lengths of time with ABA (3 μM), NaCl (100 mM), cold (4 °C), and drought (on 3MM paper). Total RNA (20 μg) was analyzed by Northern blot analysis using full-length StMYB1R-1 as the probe. Potato β-tubulin was used as a quantitative control.

Figure 3. StMYB1R-1 localizes to the nucleus. (A) Physical map of the StMYB1R-1 segment cloned into p326GFP. Black box indicates putative single MYB-like domain. (B) Protoplasts prepared from *Arabidopsis thaliana* leaves were transfected with StMYB1R-1:GFP and then observed by fluorescence microscopy 12 and 24 hours after transformation. Protoplasts expressing GFP were used as a control. Scale bar indicates 20 μm.

Figure 4. Identification of DNA binding sequences of StMYB1R-1. (A) Summary of putative StMYB1R-1 DNA binding sequences. Q9-protein binding microarray analysis was performed using a StMYB1R-1-DsRed fusion protein. A total of 1,028 probe sequences that produced high signal intensities were selected for further analysis. (B) Signal intensities of the GGATAA sequence and single nucleotide substitution derivatives using the Q9-protein binding microarray. W; wild-type sequence; 1-9, single nucleotide substitution variants (number corresponds to the position of the nucleotide substitution). One of probe sets which is irrelevant with the GGAAA sequences was used as negative control (N).
Figure 5. Transgenic potato plants overexpressing StMYB1R-1 are tolerant to drought stress conditions. Eight-week-old T0 plantlets were deprived of water for 15 days and then watering was resumed for 7 days. n = 3 independent experiments (20 plants per experiment). D, day; RW, rewatering.

Figure 6. Physiological traits of transgenic potato plants overexpressing StMYB1R-1 under drought stress conditions. (A) Survival rates of transgenic potato plants overexpressing StMYB1R-1 under drought stress conditions. Eight-week-old T0 plantlets were deprived of water for 15 days, watering was resumed for 7 days, and then the plants were scored for viability. Plants were considered dead if all the leaves were brown and there was no growth after 7 days of watering. n = 3 independent experiments (20 plants per experiment). (B) Fresh weight of leaves from transgenic potato plants overexpressing StMYB1R-1 under drought stress conditions. Detached leaves from transgenic and wild-type potato plants were measured. n = 3 independent experiments (20 detached leaves per data point). (C) The kinetics of water loss in detached leaves from wild-type and transgenic potato plants overexpressing StMYB1R-1. Water loss is presented as the percentage of weight loss versus initial fresh weight. Values represent the means ± SE of 3 independent experiments.

Figure 7. Stomatal closure of transgenic potato plants overexpressing StMYB1R-1 is more sensitive to ABA. (A) Stomatal apertures were measured following exposure to the indicated concentrations of ABA for 1 hour. ABA-induced stomatal closing was analyzed by measuring stomatal apertures. Detached leaves from 4-week-old T0 plantlets were floated on opening solution for 3 hours before the addition of ABA. (B) After the addition of 1 μM ABA, stomatal apertures were measured over time. n = 3 independent experiments (>100 stomatal apertures per data point). M, minutes.

Figure 8. Upregulation of drought stress-responsive genes in transgenic potato plants overexpressing StMYB1R-1. (A) Transcript levels of putative stress-responsive genes selected based on cDNA microarray analysis. Three-week-old wild-type potatoes were incubated on 3MM paper for 1 or 6 hours to simulate drought stress, and then total RNA was extracted. Transcript levels for each gene were analyzed using gene specific primers and semi-quantitative RT-PCR. (B) Transcript levels of drought stress-responsive genes are enhanced
in transgenic potato plants overexpressing StMYB1R-1. Potato β-tubulin was used as a quantitative control (Nicot et al., 2005).