LebZIP2 induced by salt and drought stress and transient overexpression by Agrobacterium

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INTRODUCTION

Basic leucine zipper (bZIP) proteins are one of the largest transcription factor families in plants (1). The bZIP domains form two parallel \( \alpha \)-helices arranged as coils, which enable dimerization of two proteins (2). More than 80 bZIP proteins have been found in the Arabidopsis genome project (3). Interactions of each bZIP factor have been determined from the distribution of the bZIP region and the identities of the available partners in a given tissue (4).

The functional role of bZIP transcription factors has been linked to responses to light (5). The bZIP transcription factors are involved in plant defense, plant senescence, responses to various environmental stresses, and developmental processes (6). One of the bZIP proteins related to stress responses is comprised of TGA protein, which regulates the expression of some stress-responsive genes (7). Some bZIP factors have been associated with auxin, gibberellin, or abscisic acid (ABA) , and one group of bZIP factors mediates ABA-regulated gene expression (8). bZIP transcription factors are also involved in plant development and morphogenesis (9). For example, tobacco BZI-1 is associated with flower development (10).

Nitric oxide synthase (NOS)-like activity has been identified in soybean and pathogen interactions (11). Nitric oxide (NO)-associated 1 (NOA1) is detected as a factor related to NO production in Arabidopsis thaliana (At) (12). However, AtNOA1 has no sequence similarity with NOS of animals, and does not show any NOS-like activity itself. It has been suggested that NOA1 can be expected to be one of the genes involved in a nitrate reductase (NR)-independent NO-generation system (12).

In the present study, we employed reverse transcriptase-polymerase chain reaction (RT-PCR) to characterize a tomato bZIP transcription factor expressed in tomato leaves in response to elicitors related to environmental stresses. To better understand tomato bZIP gene functions, we also analyzed the tissue-specific expression of LebZIP2. The results suggest that the LebZIP2 gene might play a role in abiotic stress responses in tomatoes. Finally, we show that LebZIP2 can be a transcriptional regulator of NbNOA1 or NbNR genes by Agrobacterium-mediated transient overexpression.

RESULTS AND DISCUSSION

Sequence analysis of tomato LebZIP2

The sequence homologies among six bZIP transcription factors including LebZIP2 were analyzed (Fig. 1A). LebZIP2 was most similar in sequence to pepper CcbZIP (85% identity, GenBank Accession No. AAD21199) and tobacco NtbZIP (85% identity, GenBank Accession No. AAK92213). LebZIP2 was found to also share 66% sequence identity with soybean GmbZIP (GenBank Accession No. ABI34666) and 64% sequence identity with Vitis VdNtZIP (GenBank Accession No. CAN73127). Heterodimerization of the BZI transcription factor, which in-
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LebZIP2 includes a bZIP domain, may regulate several genes involved in plant development (10). Similar to bZIP transcription factors, expression of LebZIP1, which encodes a DNA-binding protein, is up-regulated by cold and dark treatment (13). Moreover, ABA prevents the rapid wound-induced increase in LebZIP1 transcription, and the basal level of LebZIP1 transcription is higher in ABA mutants (13); however, the functions of other genes with homology to LebZIP1 have not been reported in detail. The LebZIP2 polypeptide is composed of 164 amino acids. The deduced amino acids of LebZIP2 cDNA are indicative of a bZIP transcription factor with a basic region leucine zipper (BRLZ) domain (26-90; Fig. 1). The LebZIP2 protein is highly homologous to other previously cloned plant bZIP DNA-binding proteins including those from tobacco (14), snapdragon (15), rice (16), maize (17), and Arabidopsis (18). Comparison of the deduced DNA-binding domains of LebZIP2 and LebZIP1 (GenBank Accession No. AF176641) revealed only a 45% identity at the amino acid level.

LebZIP2 transcription was highly expressed in flower tissues (Fig. 1B), consistent with observations that another homologous group of bZIPs are also transcriptionally activated after stress treatment or are specifically expressed in certain parts of the flower (10, 14, 15).

LebZIP2 induction by NaCl and mannitol

Fig. 2 shows the pattern of LebZIP2 mRNA accumulation over...
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Expression of LebZIP2 in response to oxidative and osmotic stresses

To simulate oxidative stress, tomato plants were exposed to 50 μM methyl viologen. LebZIP2 gene expression was induced after 3 h and 6 h (Fig. 3A). The responses of LebZIP2 to hydrogen peroxide and ABA were the same as the results of MV treatment (Figs. 3B and 3C). The transgenic lines of overexpressed CabZIP1 are resistant to oxidative stress (25), and CabZIP1 overexpression can enhance the tolerance to stress by regulating the scavenging enzymes (26, 27). Survival of plants involved in efficient anti-oxidizing systems under serious environmental conditions has been studied (28).

Two bZIP transcription factors have been cloned and demonstrated capable of interaction with the ABA responsive element (ABRE) (29). The fab2B promoter is important to the induction of expression for ABA and stress (30, 31). The other transcription factor, ZmBZ-1, is involved in rice OsBZ8 (32). The ABRE binding factor and the ABRE binding proteins can bind to different ABRE-containing promoters in vitro and in yeast (33, 34). AbZP39/ABI5, AbZIP36/ABF2/AREB1, AtbZIP38/ABF4/AREB2, AtbZIP66/AREB3, AtbZIP40/GFB4, AtbZIP35/ABF1, and AtbZIP37/ABF3 have been studied for their roles in ABA response or signaling in response to stresses such as cold, drought, and high salinity (8, 33-35). Induction of ABA after various stimuli can evoke the expression of defense proteins such as Pin2 as well as specific transcription factors such as bZIP (8).

Transient expression by Agrobacterium

We investigated the role of LebZIP2 in the response to abiotic and biotic stress. To demonstrate whether this transcription factor could contribute to the expression of genes related to defense and nitric accumulation, Agrobacterium tumefaciens strain LBA4404 harboring pMBP1 binary vectors with a coding region of LebZIP2 under the control of the CaMV 35S promoter, or vector only, was infiltrated into leaves of N. benthamiana. After 1 day, the total RNA from the N. benthamiana leaves was extracted for RT-PCR analysis. When LebZIP2 was overexpressed in tobacco leaves, NbHSR203J, NbPR1a, NbPAL and NbSIP gene transcripts did not vary as compared with control plants harboring only the vector (Fig. 4). However, LebZIP2 transiently increased the expression of NOA1 and NR genes in the overexpressed leaves (Fig. 4). The nitrate-nitrite pathway catalyzed by NR and NOS inhibitors affect NO generation systems in plants (36). NO triggers defense-related gene expression and hypersensitive response (37, 38). Silencing of NbNOA1 af-
fecteds PR1a gene expression, whereas expression is not affected in HSR203J (39). By contrast, NO production is responsible for the expression of hsr genes (40). NbhNOA1 is necessary for defense responses and is involved in NO production (39). HY5 and HYH as well as the bZIP transcription factors are involved in photomorphogenesis signaling, with the genes being essential for induction of NR, a key enzyme in nitrogen assimilation (41). HY5 and HYH are also essential for strong expression of NR in seedlings as well as rosette-stage plants (42). A more detailed analysis of LebZIP2 is necessary to any extensive understanding of the overexpressed transgenic plant in the transcriptional regulatory network.

We investigated several both abiotic and biotic stress signaling-related genes in N. benthamiana. The genes have been previously shown to have antioxidant effects and to play roles in defense and nitrate accumulation. We screened for genes that regulate LebZIP2 by means of the transient expression of the gene. LebZIP2 expression was induced by ROS production, but did not differ based on the expression of the ROS scavengers APX and SOD. Two genes known to be involved in nitrate accumulation (NR and NOA) were up-regulated in the Agrobacterium-infiltrated N. benthamiana leaves. Interestingly, these genes are involved in plant defense (36).

In this study, we provide new evidence concerning the function of LebZIP2 during plant stress. The role of LebZIP2 in salt and drought stress signaling was confirmed and its involvement in stress tolerance is indicated.

MATERIALS AND METHODS

Isolation of LebZIP2 from tomato

We searched for and analyzed full-length nucleotide and deduced amino acid sequences to obtain the tomato bZIP2 gene clone (http://www.sgn.cornell.edu/). Full-length primers were designed to amplify the LebZIP2 of tomato. The primer sequences were 5’-TCAGTACTGCAAGACATC-3’ (forward) and 5’-ATGGCTTCCGTCAAGTGGT-3’ (reverse). The band detected by PCR amplification was cloned into the pGEM T Easy vector (Promega, Madison, WI). We confirmed that the obtained sequences matched those at http://www.sgn.cornell.edu/.

Chemical treatments of tomato

‘MicroTom’ tomato seeds were inoculated on MS medium (MS salts, 3% sucrose, 0.8% agar, pH 5.8). After 2 weeks, germinated plants were transferred to pots and kept in a growth chamber at 28°C for 4 weeks. Various abiotic elicitors were applied to the leaves of the tomato plants. The plants were placed in a solution containing 400 mM NaCl, 400 mM mannitol and 50 μM methyl viologen. The leaves of some whole plants were sprayed with 100 μM ABA and 10 mM hydrogen peroxide. For cold treatment, they were placed in distilled water and kept in a 4°C cold room under dim lighting for 8 h or 24 h. The treated leaves were frozen in liquid nitrogen and stored at −70°C until used for RNA extraction.

RNA preparation

Total RNA was extracted from various tomato tissues (young leaves, old leaves, stems, flowers) as previously described (43). Five ml of Trizol reagent (Invitrogen, Carlsbad, CA) were then added to 1 g of plant materials that had been frozen in liquid nitrogen and ground to a powder. The mixture was incubated for 5 min at room temperature and chloroform was added. The samples were centrifuged and the supernatant was transferred to a new tube. This process was repeated. Precipitation was performed with isopropanol at −20°C for 1 h. After centrifugation, the pellet was washed in 70% ethanol and resuspended in diethylpyrocarbonate water.

RNA gel blot hybridization and RT-PCR

For Northern-blot analyses, total RNA was separated on formaldehyde containing agarose gels and blotted onto nylon membranes following standard procedures (44). Equal loading of RNA
was verified by visualizing of rRNA following staining with ethidium bromide. Blots were hybridized with [α-32P]dCTP-labeled probes (LebZIP2). The amplified DNA fragments were cloned into a pGEM-T easy vector (Promega) and the determination of the correct gene was confirmed. First-strand DNA was synthesized from 1 μg samples of DNase-treated total RNA (M-MLV RT, Invitrogen). The primers used for RT-PCR were as follows: (5'-ATGGCTCTGCAAGTTGT-3' and 5'-TCAGTACTGCAAGCATC-3') for LebZIP2. Other gene-specific primers for each sequence were: NbActin forward primer, 5'-CAGCTCATCGGTTAGAAGA-3', NbActin reverse primer, 5'-AGGATACGGGGAGGAGAGAG-3', NbBABA1 reverse primer, 5'-GTCTGCTTCCGCCAC-3', NbHSR203J forward primer, 5'-TTAGTATGGACCCGCACGAAAC-3', NbHSR203J reverse primer, 5'-ATGGCTTCGTCAAGTGGT-3' and 5'-TCAGTACTGCAAGAAGA-3'. Other gene-specific primers for each sequence were: NbActin forward primer, 5'-CAGCTCATCGGTTAGAAGA-3', NbActin reverse primer, 5'-AGGATACGGGGAGGAGAGAG-3', NbBABA1 reverse primer, 5'-GTCTGCTTCCGCCAC-3', NbHSR203J forward primer, 5'-TTAGTATGGACCCGCACGAAAC-3', NbHSR203J reverse primer, 5'-ATGGCTTCGTCAAGTGGT-3' and 5'-TCAGTACTGCAAGAAGA-3'.

**Transient expression of LebZIP2 in N. benthamiana**

For agroinfiltration experiments, LebZIP2 were inserted into Agrobacterium tumefaciens strain LBA4404 (45). Other gene-specific primers for each sequence were: NbActin forward primer, 5'-CAGCTCATCGGTTAGAAGA-3', NbActin reverse primer, 5'-AGGATACGGGGAGGAGAGAG-3', NbBABA1 reverse primer, 5'-GTCTGCTTCCGCCAC-3', NbHSR203J forward primer, 5'-TTAGTATGGACCCGCACGAAAC-3', NbHSR203J reverse primer, 5'-ATGGCTTCGTCAAGTGGT-3' and 5'-TCAGTACTGCAAGAAGA-3'.

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