

# Integration of Wounding and Osmotic Stress Signals Determines the Expression of the *AtMYB102* Transcription Factor Gene<sup>1</sup>

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Transcript levels of the Arabidopsis R2R3-*AtMYB102* transcription factor gene, previously named *AtM4*, are rapidly induced by osmotic stress or abscisic acid (ABA) treatment. Reporter gene expression studies revealed that in addition, wounding is required for full induction of the gene. Histochemical analysis showed a local  $\beta$ -glucuronidase induction around the wounding site, especially in veins. In ABA-treated plants, wounding-induced  $\beta$ -glucuronidase activity could be mimicked by the wound signaling compound methyl jasmonate. In silico studies of the *AtMYB102* promoter sequence and its close homolog *AtMYB74* demonstrated several conserved putative stress regulatory elements such as an ABA-responsive element, its coupling element 1 (CE1), and a W box. Interestingly, further studies showed that the 5'-untranslated region is essential for the osmotic stress and wounding induced expression of the *AtMYB102* gene. This 5'-untranslated region contains putative conserved regulatory elements such as a second W box and an overlapping MYB-binding element. These studies suggest that *AtMYB102* expression depends on and integrates signals derived from both wounding and osmotic stress.

Abscisic acid (ABA) is the major plant hormone in water stress signaling. ABA regulates plant water balance and osmotic stress tolerance. The role of ABA in the osmotic stress responses has been studied extensively, and the availability of ABA-deficient and -insensitive mutants especially has been most helpful in these studies (Koornneef et al., 1998). Several of these mutants show severe wilting even upon a mild water stress treatments, supporting the role of ABA under stress conditions. ABA levels increase in wilting leaves due to the de novo synthesis of the carotenoid cleavage enzyme 9-cis-epoxycarotenoid dioxygenase (NCED), VP14 in maize (*Zea mays*). This enzyme catalyzes what appears to be the rate-limiting step in ABA biosynthesis (Tan et al., 1997). A clear correlation in *Phaseolus vulgaris* was found between NCED (*PvNCED1*) mRNA expression, NCED protein levels, and ABA levels in dehydrated leaves and roots, indicating the important role of NCED in stress ABA biosynthesis. Moreover, overexpression of the Arabidopsis *AtNCED3* results in the accumulation of ABA (Iuchi et al., 2001).

Two separate groups of stress-responsive genes are induced during osmotic stress: the "early response genes" and the "delayed-response genes" (Kiyosue et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The early response genes can be induced within min-

utes. Expression is often transient and is independent of protein synthesis. These genes typically encode transcription factors that activate downstream delayed-response genes. The latter constitute the vast majority of the stress-responsive genes, which are activated by stress more slowly but often show sustained expression. In osmotically stressed Arabidopsis plants, ABA accumulation starts after approximately 2 h after the treatment (Kiyosue et al., 1994). Several genes respond already within this time period to dehydration stress via a rapid ABA-independent signaling pathway (Yamaguchi-Shinozaki and Shinozaki, 1994; Gosti et al., 1995; Hong et al., 1997). The *RPK1* gene of Arabidopsis is induced by dehydration stress and by ABA treatment. The dehydration-induced expression is not impaired in the *aba1* ABA-deficient mutant or in the ABA-insensitive mutants *abi1*, *abi2*, and *abi3*, suggesting that this ABA-induced gene also is regulated in an ABA-independent way (Hong et al., 1997). Expression of the stress-responsive *RD29A* gene is rapidly induced in an ABA-independent way followed by a strong ABA-dependent induction phase. These observations can be explained by separate cis-acting elements in the promoter, such as a dehydration-responsive element (DRE) and an ABA-responsive element (ABRE; Yamaguchi-Shinozaki and Shinozaki, 1993, 1994). The DRE element was necessary and sufficient for the first rapid response to osmotic stress induction without the involvement of ABA through the DREB2A and related transcription factors. Full activation of *RD29A* transcription depends upon the synergy between DRE and ABRE elements in its promoter. Transcription factors with ERF/AP2

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domains have been shown to bind to DRE elements, whereas several ABRE-interacting proteins are classified as basic/Leu zipper transcription factors (Carles et al., 2002; Sakuma et al., 2002).

Wounding is another stress factor that often is closely related to osmotic stress. Tissue damage is usually associated with decompartmentalization, release of cellular contents, and a loss of water. Such damage induces local osmotic stress responses similar to those occurring in water-stressed intact plants (Reymond et al., 2000). The central role of jasmonic acid (JA) in plant responses to wounding is well established, but other signaling molecules have been proposed to play important roles in wound signaling as well. These include ABA, ethylene, oligosaccharides, and the oligopeptide systemin. Studies on the wound signal transduction cascades showed involvement of  $Ca^{2+}$  ions (Knight et al., 1993), fatty acids (Ryu and Wang, 1998), systemin (Ryan, 2000), hydrogen peroxide (Orozco-Cardenas and Ryan, 1999), and poly-GalUA in triggering wound signaling. Many of the wound-responsive genes are induced through jasmonate-dependent signal transduction cascades, but jasmonate-independent cascades also exist. For example, in Arabidopsis, oligogalacturonides activate gene expression via a JA-independent pathway (Titarenko et al., 1997).

In several Solanaceous plants, both JA and ABA can activate the expression of wound-responsive genes through the systemin-activated signaling system (Hildmann et al., 1992; Peña-Cortés et al., 1995). This system seems to be absent in species such as barley (*Hordeum vulgare*), potato (*Solanum tuberosum*), and Arabidopsis (Leon et al., 2001). In tomato (*Lycopersicon esculentum*), ABA is necessary for the wound-induced expression of proteinase inhibitor genes (Carrera and Prat, 1998), but ABA does not appear to be a primary signal in wound signaling (Birkenmeier and Ryan, 1998). In Arabidopsis, ethylene acts as a cross talk regulator between the JA-dependent and -independent oligosaccharide-mediated wound signaling pathways (Rojo et al., 1999). The oligosaccharide-mediated repression of the JA-dependent signaling pathway is exerted through the local production and perception of ethylene in damaged tissue.

It is of interest to unravel the regulatory connections between wound-activated signaling pathways and signal transduction pathways triggered by other stress factors such as osmotic stress. Here, the expression analysis of a putative Arabidopsis transcription factor of the R2R3 MYB class is presented. This *AtMYB102* gene is responsive to osmotic stress and wounding. Dehydration, osmotic or salinity stress, and ABA application enhance *AtMYB102* transcript levels. Also, methyl jasmonate (MeJa) enhances expression synergistically in combination with ABA, whereas MeJa alone has a limited effect. It is proposed that *AtMYB102* is a regulatory component,

which integrates osmotic stress and wounding signaling pathways in Arabidopsis.

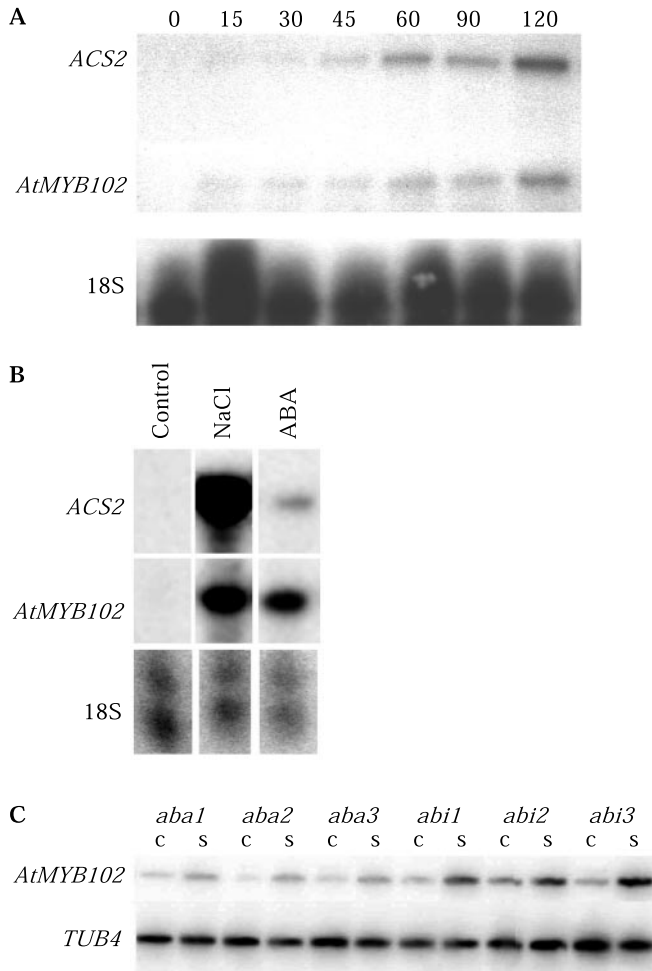
## RESULTS

### *AtMYB102* mRNA Levels Respond to Osmotic Stress and ABA

Previously, it was reported that Arabidopsis ecotype Columbia-0 (Col-0) *AtMYB102* mRNA levels are induced by dehydration stress in leaves (Kranz et al., 1998). This response of *AtMYB102* was further investigated in a time course dehydration experiment. Rosettes were detached from the roots at different time points, and the RNA transcript levels were determined by RNase protection assays. *AtMYB102* transcript levels in leaves increase within 30 min after detachment (Fig. 1A). The stress-responsive *ACS2* gene was used as an osmotic stress induction control. *ACS2* encodes an enzyme involved in the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. Salinity stress and ABA effectively induce *AtMYB102* mRNA levels (Fig. 1B) as do mannitol- or polyethylene glycol-induced osmotic stress (data not shown). In mutant lines deficient in ABA biosynthesis (*aba1-1*, *aba2-1*, and *aba3-1*) or ABA signal transduction (*abi1-1*, *abi2-1*, and *abi3-*), *AtMYB102* mRNA levels are induced (Fig. 1C). Thus, *AtMYB102* induction probably does not depend on ABA or ABA-induced signaling per se. *AtMYB102* expression and ABA-dependent induction were studied in whole plants. After treatment, plants were dissected and *AtMYB102* transcript levels determined by real-time PCR. The gene is prominently expressed in young flowers, leaves, cauline leaves, and roots and is ABA responsive in these tissues (Fig. 2).

### Osmotic Stress and Wounding Are Both Required for *AtMYB102* Expression

The Arabidopsis Col-0 ecotype was transformed with a construct containing a 2-kb *AtMYB102* promoter fragment including the mRNA 5' leader sequence and the first three amino acids of the mature protein fused in frame to the  $\beta$ -glucuronidase (GUS) reporter gene (see "Materials and Methods"). Transformants were generated using the *Agrobacterium tumefaciens*-mediated floral transformation method and homozygous lines obtained. Surprisingly, neither salt stress nor ABA enhanced GUS reporter enzyme activity in leaf tissues when analyzed histochemically (Fig. 3) or enzymatically (Fig. 4). This was observed in all independent transgenic Col-0 lines tested. Such treatments efficiently induce endogenous *AtMYB102* transcript levels (Figs. 1 and 2). These observations were confirmed in all independent transgenic lines tested in the C24 ecotype. These C24 lines were generated with the same construct as the Col-0 ecotype except that a 1-kb promoter element was used. Also, in this ecotype, no induction of



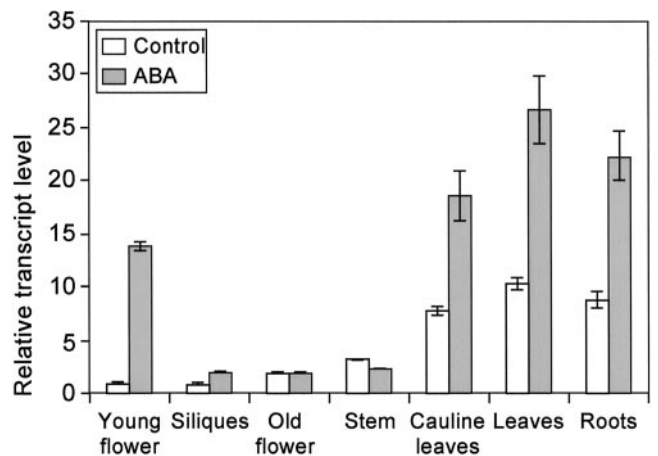
**Figure 1.** A, Time course dehydration stress experiment. Rosettes were detached and left on a filter paper in a climate room at 70% humidity. RNA was extracted at the time points indicated (in minutes), and RNA protection analyses were performed. The *ACS2* gene was used as a control for dehydration stress conditions. B, RNAse protection analyses of the *AtMYB102* and the *ACS2* control transcripts. Rosettes stage plants were subjected to salinity stress by soil saturation with 200 mM NaCl or were sprayed with 100  $\mu$ M ABA. The plants were left in a climate room for 4 h before harvest. C, Reverse transcriptase (RT)-PCR analyses of *AtMYB102* gene expression. ABA mutant lines were exposed to salinity stress. S, Salinity stress (2 h, 200 mM NaCl soil saturation); C, control.

GUS activity as determined by histochemistry was found when treated with ABA or salinity stress, whereas in C24 *AtMYB102*, mRNA levels are induced also (data not shown). With the more sensitive enzymatic GUS assay, an approximately 2-fold induction was observed (Fig. 3B). Interestingly, GUS activity was highly induced in both ecotypes at wounding sites (Fig. 3, A and B; data not shown). This effect of wounding was tested via incisions in leaves and exposure of plants to salt stress by saturating the soil with 200 mM NaCl. Histochemical analysis revealed that GUS activity was prominently induced specifically at the wounding sites in stressed and wounded

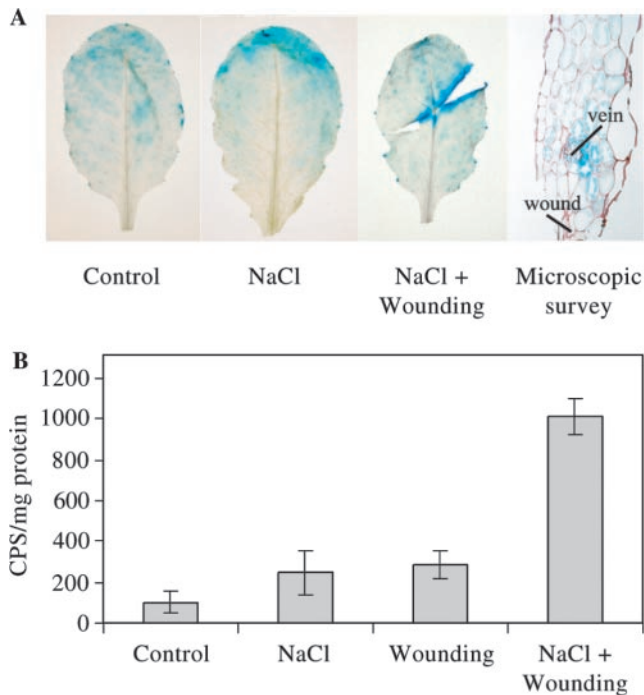
plants. GUS activity is especially prominent in the vicinity of damaged veins, but a diffuse activity in the adjoining parenchymal tissues was also observed. Staining was absent in the epidermal layer (Fig. 3A).

The combination of osmotic stress and wounding and its effect on mRNA levels and GUS enzyme activity was further investigated in the Col-0 ecotype. Pots with 3-week-old transgenic plants were treated with hormones known to be important in osmotic stress and wound signaling. Endogenous *AtMYB102* and GUS transcript levels were determined by real-time PCR with *Actin2* as the endogenous reference. ABA treatment induces transcript levels as expected. However, GUS mRNA level seems less responsive compared with the endogenous transcript (2- over 10-fold, respectively), as was found for all other treatments (Fig. 4, A and B). Possibly, GUS transcript stability differs from that of the endogenous *AtMYB102* transcripts. Alternatively, this might be due to a less efficient PCR with the GUS primers. However, the expression patterns of the GUS and the *AtMYB102* transcripts are nearly identical over the treatments as was observed in a number of experiments with similar treatments (data not shown).

The wound signaling hormone jasmonate enhances *AtMYB102* transcript levels approximately 2-fold compared with a 10-fold induction by ABA (Fig. 4A). Combined application of these compounds induced the transcript abundance 13-fold. Interestingly, GUS activity was not altered compared with the control treatment for both separate treatments, but the activity increased when both ABA and jasmonate were applied (Fig. 4C). Wounding in combination with ABA further enhances GUS enzymatic activity (Fig. 4C). Wounding disrupts tissues and is accompanied by osmotic stress. This combination boosts transcript levels and GUS activity. These results suggest that *AtMYB102* functions in a wound response pathway



**Figure 2.** Real-time PCR analyses of the *AtMYB102* transcript levels in several plant organs. The plants were sprayed with 100  $\mu$ M ABA and left for 3 h in a climate room before harvest. Adult plants were dissected in the organs indicated.



**Figure 3.** A, Histochemical GUS analyses in Col-0 line 2.6 harboring the 2-kb promoter fragment from *AtMYB102* translationally fused to the GUS reporter gene. The soil was saturated with 200 mM NaCl, and the plants were left for 4 h in a climate room. Wounding was applied by incision. B, GUS activity assays (line 2.6). The rosettes were exposed for 4 h to 200 mM NaCl via soil saturation. Wounding was applied through cutting of leaf tips. Three leaf slices around the wounding site of each plant were harvested ( $n = 5$ ).

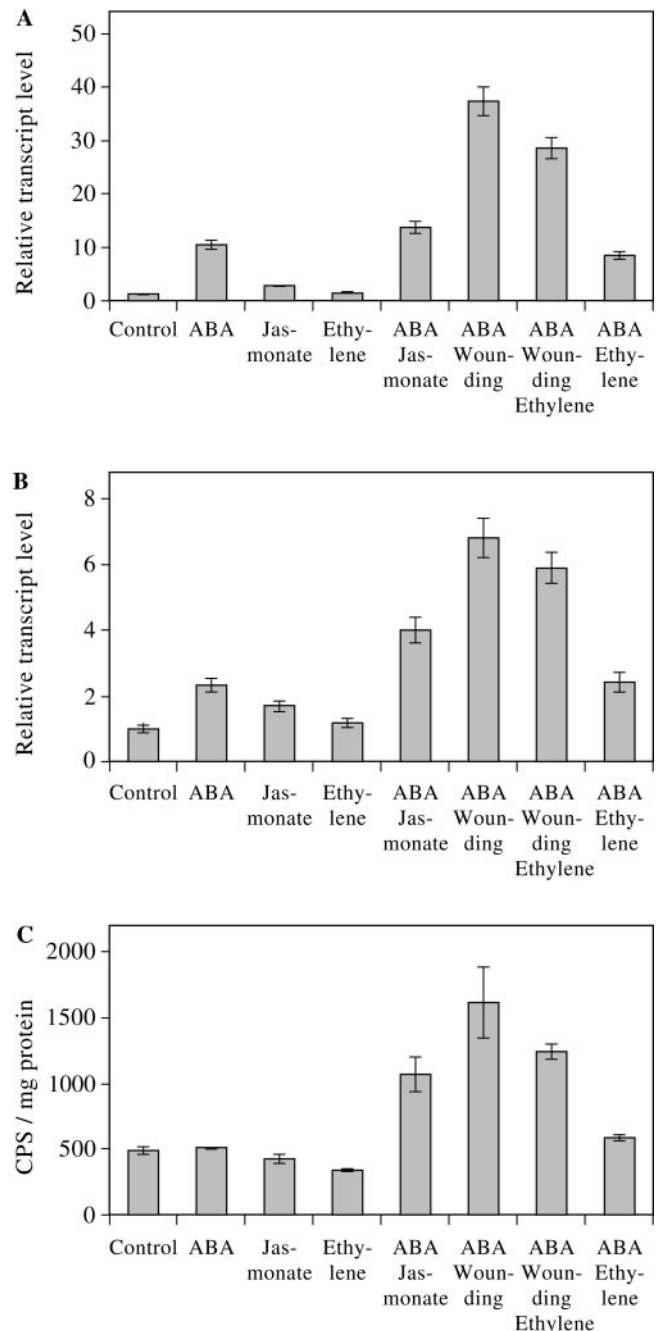
that may involve jasmonate and that is integrated with an osmotic stress and ABA response pathway.

Ethylene is a plant hormone that is induced locally upon wounding. Therefore, the effect of this hormone on the wounding response was tested. ACC, a precursor of ethylene, was applied to 3-week-old wounded and ABA-treated plants. Ethylene in combination with wounding has a small but consistent inhibitory effect on transcript levels and GUS activity (Fig. 4). No effect was found in combination with ABA alone. Ethylene has been proposed as an inhibitory compound of the local jasmonate-dependent wound response pathway (Rojo et al., 1999), and our results suggest a similar but small inhibitory effect on *AtMYB102* expression at the wound site.

#### The 5'-Untranslated Region (UTR) Is Essential for Gene Expression

Experiments with the GUS reporter construct revealed that ABA induces GUS transcript levels, but GUS activity is not enhanced (Fig. 4, B and C). This observation suggests a posttranscriptional regulatory mechanism of the *AtMYB102* gene. The *AtMYB102* promoter-GUS construct includes the 5'-UTR of 108 nucleotides (see "Materials and Methods"). Such 5'-UTR regions are often involved in translation regu-

lation, and this possibility was investigated using an *AtMYB102* promoter-GUS construct lacking the 108-bp 5'-UTR. Six independent hygromycin-resistant Col-0 lines showing different expression levels were analyzed for GUS mRNA levels and GUS enzymatic activity. Surprisingly, the endogenous *AtMYB102* transcript levels and the GUS transcript



**Figure 4.** Real-time PCR analyses of *AtMYB102* (A) and GUS fusion transcript levels (B). Ten rosettes of 3-week-old plants were used per treatment and sprayed with 100  $\mu$ M ABA, 10  $\mu$ M MeJA, or 10  $\mu$ M ACC (ethylene precursor). Wounding was applied over the leaf with forceps. Plants were incubated for 4 h in a climate room before processing. C, Representation of the GUS activity in the same samples.

levels were differentially regulated by salt stress (Fig. 5A). This indicates that the 5'-UTR is essential for the stress response. The GUS transcript levels basically are in agreement with GUS enzymatic activity as observed in the independent lines (Fig. 5B). Moreover, in the lines transformed with the construct lacking the 5'-UTR, wounding does not enhance GUS activity as it does in lines with the intact constructs.

## DISCUSSION

### AtMYB102 Is Responsive to Osmotic Stress and ABA

The *AtMYB102* was identified previously as a light-regulated gene (*AtM4*) expressed in seedlings (Quaedvlieg et al., 1996). Further analysis revealed that *AtMYB102* is responsive to dehydration stress as examined by the reverse northern technique (Kranz et al., 1998). A time course dehydration experiment showed a rapid increase of *AtMYB102* mRNA levels in leaves within 30 min after stress application (Fig. 1A). Experiments with salinity stress (Fig. 1B, lane 2) and osmotic stress induced by mannitol or polyethylene glycol (data not shown) showed the same increase in *AtMYB102* transcript levels. Similar to many other dehydration-responsive genes, *AtMYB102* is ABA responsive but probably does not depend on ABA or ABA signaling. This is suggested by the salinity re-

sponse of *AtMYB102* in several *aba* and *abi* mutants (Fig. 1, B, lane 3, and C). Moreover, the dehydration stress response occurs within 30 min, whereas significant stress ABA levels accumulate after a 2-h lag period (Kiyosue et al., 1994; Zhu et al., 1997).

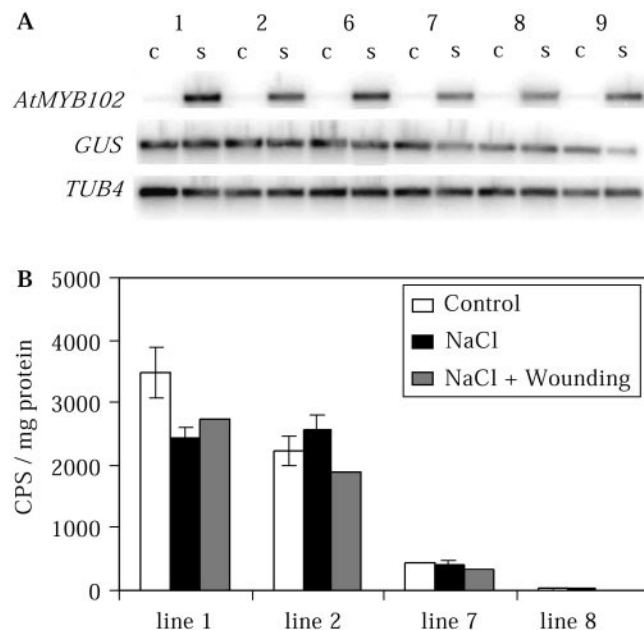
### AtMYB102 Is Wound Responsive

Analyzing GUS reporter gene constructs in the Col-0 and C24 ecotypes provided additional information on regulation and location of *AtMYB102* gene expression. Some enhancement of GUS activity was observed in plants of both ecotypes exposed to salinity stress or ABA. This enhancement was only observed with sensitive enzymatic assays but not in histochemical experiments. Surprisingly, GUS activity was strongly induced by wounding in combination with either osmotic stress or ABA treatment (Figs. 3 and 4). Thus, wounding is important for full expression of the GUS reporter gene. Jasmonate is a well-known hormone involved in wounding responses. Applying MeJa in combination with ABA to 3-week-old Col-0 transgenic plants tested its possible involvement. ABA, and to a lesser extent MeJa, enhance *AtMYB102* transcript levels but fail to induce GUS activity (Fig. 4), suggesting a posttranscriptional regulatory mechanism. Only the combined application of MeJa and ABA significantly induced GUS activity. These results indicate that jasmonate signaling is required for expression of *AtMYB102*, but this needs further investigation. Wounding is more effective in combination with ABA than with jasmonate in inducing transcript levels and GUS activity (Fig. 4). Perhaps wounding is more effective than jasmonate due to the locally induced osmotic stress at the wounding site.

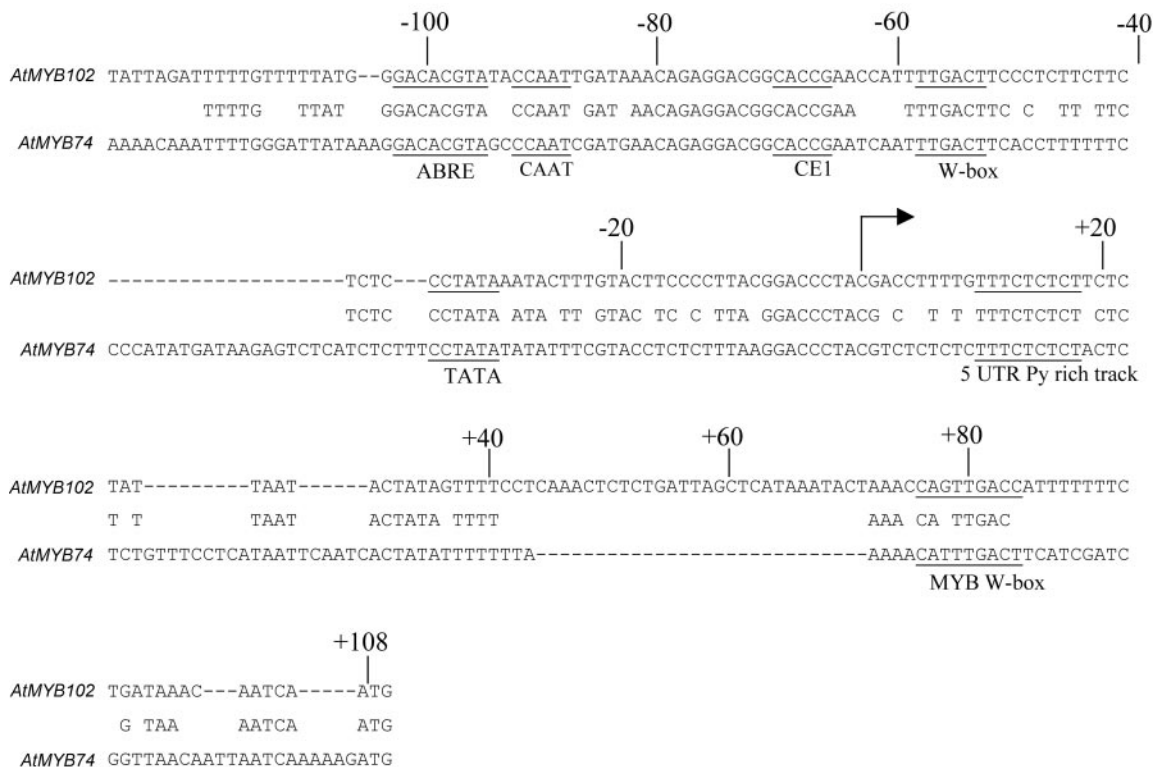
Ethylene might be another factor involved in the observed expression because it is synthesized locally after wounding. Studies on jasmonate-responsive genes like *JR1* and *JR2* suggest that ethylene inhibits the local jasmonate-dependent signaling pathway (Rojo et al., 1999). Plants treated with ABA and wounding showed a small inhibitory effect of ethylene on both transcript levels and GUS activity. The inhibitory effect of ethylene is restricted to the wound response because the ABA response was not affected (Fig. 4). Several independent experiments confirmed this small inhibitory effect of ethylene (data not shown).

### AtMYB102 Integrates Osmotic Stress and Wound Signaling

Full expression of *AtMYB102* requires both osmotic stress- and wounding-induced signaling pathways. Most likely, in Arabidopsis the *AtMYB102* protein is produced after local wounding with its associated osmotic stress. This allows as of yet unknown target genes to be regulated accordingly in a wounding-



**Figure 5.** Analysis 5'-UTR-deleted transgenic GUS reporter lines. A, RT-PCR analyses of GUS transcript levels of six independent lines. C, Control; S, salinity stress (4 h, 200 mM NaCl soil saturation). B, Four lines varying in GUS activity were subjected to 4 h of salt treatment by soil saturation with 200 mM NaCl. In a parallel experiment, wounding was applied to salt-stressed plants by cutting (three leaves of five plants per line). The leaf material at the vicinity of the wounding site was harvested and pooled per line. The error bars are based on two independent experiments.



**Figure 6.** Promoter alignment using the ClustalW program of the homologous *AtMYB102* and *AtMYB74* genes. The *AtMYB102* coding sequence starts at position +108, and the transcript starts at position 0. Transcript initiation site was determined by 5'-RACE (see "Materials and Methods"). Putative conserved boxes are underlined. General gene regulatory elements like the TATA box and CCAAT box are present at positions -30 and -92, respectively. The latter is located between the ABRE motif (GACACGTA) and its Coupling Element (CE1) with the consensus CACCG (Niu et al., 2002). A W box is present with the consensus sequence TTGACT, just 8 bp downstream of the ABRE-CE1 elements (Eulgem et al., 1999). A second W-box consensus sequence is found in the 5'-UTR and overlaps with a MYB-binding consensus sequence, CAGTT (Urao et al., 1993). A 5'-UTR pyrimidine-rich track is present just after the transcription initiation site (Sickinger and Schweizer, 1999).

dependent manner. Prolonged osmotic stress induces ABA accumulation and a further enhancement of the response in time. In tomato, ABA and jasmonate can activate proteinase inhibitor genes through a common signal transduction pathway (Hildmann et al., 1992; Peña-Cortés et al., 1995). Moreover, the presence of an early common wounding and ABA-mediated signaling pathway was suggested. The dominant Arabidopsis *abi1-1* allele reduced the wound-induced expression of the proteinase inhibitor 2 and *LAP* transcripts (Carrera and Prat, 1998), but ABA does not appear to be a primary signal in wound signaling (Birkenmeier and Ryan, 1998). In these studies, it was observed that the increased ABA levels due to osmotic stress do not induce wound-responsive genes. Similarly, wounding does not enhance osmotic stress-responsive genes except when wounding causes local osmotic stress (Hildmann et al., 1992; Peña-Cortés et al., 1995; Reymond et al., 2000). The Arabidopsis *AtMYB102* gene might be a candidate signaling component for such a rapid response to wounding.

The 5'-UTR sequence could be important for a posttranscriptional regulatory mechanism as sug-

gested by experiments where ABA induces GUS mRNA levels but not enzymatic activity in the promoter-GUS transgenic lines. This notion was tested using a leaderless *AtMYB102* promoter-GUS construct, but, surprisingly, these experiments showed the presence of essential stress-responsive elements in the 5'-UTR. Steady-state GUS transcript levels in the lines lacking the 5'-UTR remain unchanged during exposure to salt stress alone or combined with wounding (Fig. 5). The GUS transcripts levels were representative for the observed GUS activity, and wounding did not enhance GUS activity in these lines (Fig. 5).

#### In Silico Promoter Analyses Reveal Conserved Stress-Responsive Elements

The closest homolog of *AtMYB102* in the genome of Arabidopsis is *AtMYB74*. This *AtMYB74* revealed considerable sequence conservation with *AtMYB102* and has a similar expression pattern (Kranz et al., 1998). Most likely, these genes arose from a single ancestor. The promoter sequences of both genes

show conserved cis-elements with homology to previously described sequence motifs (Fig. 6). These include elements like ABRE and its coupling element (CE1), which are spaced as found in other ABA- and dehydration-responsive promoters (Niu et al., 2002). These elements are known to be involved in abiotic stress responses and interact with putative trans-acting factors. The ABRE can interact with basic domain/Leu zipper-type transcription factors such as ABI5. The CE1 element might interact with AP2-like transcription factors such as ABI4 (Niu et al., 2002). Another conserved element, located 8 bp downstream of the CE1 element, is a W box (Maeo et al., 2001). The W-box is a target of WRKY zinc finger-type transcription factors that are associated with processes like wounding, pathogen defense, and senescence (Hara et al., 2000; Robatzek and Somssich, 2002). The involvement of *AtMYB102* in pathogen defense was tested using compatible strains of *Peronospora parasitica* and *Pseudomonas syringae*. Neither pathogen was able to induce GUS activity in the *AtMYB102* promoter-GUS lines after inoculation (data not shown). In Arabidopsis, wounding- or jasmonate-responsive WRKY proteins might be involved in transcriptional regulation of *AtMYB102*, possibly interacting with other trans-acting factors mentioned above. The conserved putative promoter elements, like ABRE-CE1 and W box and their interacting factors, likely determine transcription activity of *AtMYB102*.

The 5'-UTR is essential for stress-induced expression of *AtMYB102*. This region contains overlapping putative MYB and W-box elements that are conserved in the 5'-UTRs of *AtMYB102* and *AtMYB74* (Fig. 6). Another conserved element in the 5'-UTR is a pyrimidine-rich tract (Fig. 6). The RNA-binding protein, polypyrimidine tract-binding protein, is known to interact with such tracts and regulates the utilization of premessenger and mRNA in association with other proteins involved in splicing and translation initiation (Shav-Tal and Zipori, 2002). Such a protein might affect translation efficiency of the *AtMYB102* mRNA.

In conclusion, osmotic stress in combination with wounding is needed for full expression of *AtMYB102*. Thus, *AtMYB102* seems to integrate signals from these separate signal transduction pathways. It will be interesting to identify the expression mechanisms involved and the downstream processes controlled by *AtMYB102*. Moreover, overexpression of the gene in combination with phenotypic and metabolic analyses are underway and hopefully will shed light on its function.

## MATERIALS AND METHODS

### Plant Material

Arabidopsis ecotype Col-0 seeds were sown on soil in 5-cm pots, moistened, and placed for 3 to 5 d in a cold room at 4°C in the dark to synchronize

germination. The pots were transferred to a climate room to allow growth to rosette stage with an 8-h photoperiod at 22°C. The plants were sprayed with solutions containing 100  $\mu$ M ABA (mixed isomers, Sigma-Aldrich Chemie, Steinheim, Germany), 10  $\mu$ M MeJa (Duchefa, Haarlem, The Netherlands), or 10  $\mu$ M ACC (Sigma-Aldrich Chemie) and water was used as control. All solutions contained 0.01% (v/v) Silwet L77 (Van Meeuwen Chemicals, Weesp, The Netherlands). About 5 mL of the solution was sprayed on five rosette stage plants. The plants were returned to the climate room at 22°C, 70% relative humidity until harvesting. For salinity stress, the soil was saturated with 200 mM NaCl solution, and about 1 L of the solution was poured on the soil surface of 10 plants and allowed to drain. Wounding of the plants was performed using scalpel or forceps.

### RNA Analyses

#### RNAse Protection and RT-PCR Analyses

Total RNA was prepared from leaves of rosette stage plants using the PUREscript RNA isolation Kit (Gentra Systems, Minneapolis) as described by the manufacturer. RNAse protection analyses were performed on 10  $\mu$ g of total RNA using the RPAIII Kit (Ambion, Austin, TX) as described by the manufacturer. The 18S endogenous control probe and the RNA ladder were obtained from Ambion and used according to the protocols provided. The *AtMYB102* probe was prepared as described before (Quaedvlieg et al., 1996). For preparing an *ACS2* probe, a PCR fragment was generated using primers F1213ACS2 (5'-CGAGATCTACGCCGACAGTC-3') and R1719ACS2 (5'-CAACGAAGGAAGAGCCAGGAGA-3'), cloned in a pGEM-T vector (Promega, Madison, WI), and linearized with *Xho*I. For generating the RNA probe, the Riboprobe Combination system T7/T6 (Promega) was used according to the protocol provided.

The first strand cDNA for RT-PCR analysis was synthesized with oligo(dT) primers on 1 to 2.5  $\mu$ g of total RNA using SUPERSCRIPT II (Invitrogen, Carlsbad, CA) as described by the manufacturer. All RNA was pretreated with DNase I using the DNA free kit (Ambion) according to the protocol. Amplification reactions contained 2  $\mu$ L of the 2-fold diluted cDNA pool, 1 $\times$  polymerase buffer (Roche Diagnostics), 2.5 mM of each dNTP, 10 pmol of each primer, and 2.5 units of *Taq* DNA polymerase. PCR was performed with denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A final extension at 72°C for 10 min was performed to complete the reaction.

The primers used were: *AtMYB102*, FS102cDNA 5'-TAAACAA-TCAATGGCAAGGTAC-3' and 5RACE102C 5'-CTGTGAGTCACTGGATCAATCC-3'; and *Tubuline 4*, F-B-TUBULIN4 5'-GCTTACGAATCCGAGGGTGCC-3' and R-B-TUBULIN4 5'-GTCCAGTGTCTGTGATATTGCA-CC-3'.

PCR products (5  $\mu$ L) were fractionated on a 1% (w/v) agarose gel, transferred onto Hybond N<sup>+</sup> membranes (Amersham, Buckinghamshire, UK), and hybridized with <sup>32</sup>P-labeled fragments generated with the PCR primers specified. Hybridization signals were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

#### Real-Time PCR

RNA was extracted from 100 mg of plant material with the RNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD), subsequently pretreated with DNase I using the DNA free kit (Ambion), and cDNA was synthesized with Moloney murine leukemia virus RNase H<sup>-</sup> (Promega) using 2  $\mu$ g of total RNA. The cDNA was diluted to 100  $\mu$ L with Tris-EDTA buffer, and 5  $\mu$ L was used in a 25- $\mu$ L PCR reaction. The mixture was set up with 12.5  $\mu$ L of Taq-Man 2 $\times$  Universal PCR Master Mix (Applied Biosystems, Warrington, UK), 5  $\mu$ L of cDNA, 2.5  $\mu$ L of probe (250 nM final), and 2.5  $\mu$ L of each primer (900 nM final). PCR was performed with denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 1 min. The comparative threshold cycle (Ct) method was used to determine relative RNA levels (User Bulletin no. 2, Applied Biosystems). *Actin2* is used as the internal reference, and expression levels are relative to the control treatment.

The primer probe combination of *AtMYB102* was: probe, FAM 5'-AGAAGCTCCAATCTTCCATGCCACCATTC-3' Tamra; forward primer, 5'-AACCAATATTTCGAGAACACGATTACT-3'; and reverse primer, 5'-TGTTGTTAAACTGACGAGCTTCATT-3'. The primer probe combination of

the Promoter::GUS fusion construct was: probe, FAM 5'-CGGCCG-CCTGCAGCAGCCAA'-3' Tamra; forward primer, 5'-CAATGGCAAGGT-CACAATCACT'-3; and reverse primer, 5'-TAAACTAGTCAGATCTACAG-CGCTAAG'-3. The primer probe combination of *Actin2* was: probe, FAM 5'-AAGTCTTGTTCAGCCCTCGTTTGTGG'-3 Tamra; forward primer, 5'-GCTGAGAGATTCAGATGCCCA'-3; and reverse primer, 5'-GTGGATT-CACAGCCTCCAT'-3.

### Determination of the Transcription Initiation Site of *AtMYB102* Using 5'-RACE

Total RNA was prepared as mentioned for RT-PCR analyses from leaves of rosette stage plants exposed to dehydration stress for 4 h and reverse transcribed as above using 2.5 µg of total RNA and 1.5 pmol gene-specific primer 5'RACE102A (5'-GCTGATGACGACGATTAGTATCC-3') in a 12-µL reaction mix. The reaction was stopped by heating at 70°C for 15 min and treated with 1 µL of RNase H (Promega) at 37°C for 30 min. The gene-specific cDNA was purified over a spin column Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and eluted in 19 µL of 10 mM Tris buffer (pH 8.0). The cDNA was C tailed by adding 2.5 µL of One-Phor-All buffer (Amersham-Pharmacia Biotech, Uppsala) and 2.5 µL of 2 mM dCTP. The mixture was heated at 94°C for 3 min, transferred to ice for 5 min, 25 units of terminal deoxynucleotidyl transferase (Amersham-Pharmacia Biotech) was added, and C tailing was performed at 37°C for 10 min and terminated at 70°C for 5 min. PCR was performed on 5 µL of the C-tailed gene-specific cDNA using 20 pmol 5RACE-AP (5'-GGCCACGCGTCTGACTAGTACGG-GIIGGGIIGGGIIG-3') and 20 pmol 5RACE102B (5'-GACATGTTTCATGT-GATGTGAAG-3') under RT-PCR conditions as described before with a melting temperature of 55°C and for 35 cycles. The PCR products were separated on a 1% (w/v) agarose gel along a size marker. Visible bands were isolated from the gel using Qiaex II gel extraction kit (Qiagen) and eluted in 20 µL of 10 mM Tris buffer (pH 8.0). A second identical PCR was performed on 5 µL of 1:250 (v/v) diluted gel-isolated fragments with 20 pmol 5RACE-AUAP (5'-GGC-CACGCGTCTGACTAGTAC-3') and 20 pmol 5RACE102C (5'-CTGT-GAGTCACTGGATCAATCC-3'). The obtained fragment was cloned in a pGEM-T vector (Promega) according to the protocol and sequenced. The transcription initiation site could be located 108 bp upstream of the ATG codon.

### Histochemistry, Microscopy, and Quantitative GUS Assay

For GUS histochemistry, plant material was stained overnight at 37°C in a 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Biosynth AG, Staad, Switzerland) solution in 50 mM sodium phosphate (pH 7.0) buffer, 0.1% (v/v) Triton X-100, 10 mM EDTA, 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>. The plant material was cleared in 70% and 95% (v/v) ethanol.

Plant material for microscopy was infiltrated and embedded in a Technovit 7100 (Kulzer, Heraeus, Germany) as instructed by the manufacturer. Sections of 4 µm were made on a Reichert-Jung 1140 rotary microtome (Hanau, Germany) carrying a disposable Adams steel knife. Sections were stained with 0.1% (w/v) Ruthenium red (Sigma-Aldrich Chemie) in distilled water for 2 to 5 min at room temperature and fixed under a glass slide cover.

Relative GUS-activity was measured using the GUS-Light, Chemiluminescent Reporter Gene Assay (Tropix, Inc., Bedford, MA) as described by the manufacturer. Proteins were extracted from samples containing at most 3 cm<sup>2</sup> leaf tissue in a buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100, and 10 mM β-mercaptoethanol. Equal amounts of protein as determined by the Bradford assay were used in the reactions.

### GUS Reporter Constructs

For the *AtMYB102* promoter-GUS construct in C24, the plasmid pM4.1 harboring the promoter was digested with *Sall* and *BstEII* and made blunt with Klenow (Amersham-Pharmacia Biotech). The fragment was cloned in the binary vector pBi101.2 using the *Sall*/*SmaI* sites, obtaining a translational fusion including the first three gene-specific amino acids. The construct was transformed to C24 ecotype via a root transformation procedure mediated by *Agrobacterium tumefaciens* (Valvekens et al., 1988). Transfor-

mants were selected on Murashige and Skoog medium containing 50 mg L<sup>-1</sup> kanamycin.

Using a PCR-based cloning strategy, a 2-kb promoter fragment was fused to the GUS reporter gene in pCAMBIA3381Xa. The primers F102LDR (5'-AATGCGCTCCCTTTCTC-3') and R102LDR (5'-GTGACCTTGCCAT-TGATTGTTTA-3') were used to amplify the 2-kb promoter region of the *AtMYB102* gene on Col-0 ecotype DNA. The proofreading DNA polymerase *Pfu* was used with *Taq* DNA polymerase in a 1:80 (v/v) unit ratio. The fragment was cloned in a pGEM-T vector (Promega) and sequenced. A construct with the 5'-end of the *AtMYB102* promoter at the vector T7 promoter site was digested with *SphI* and made blunt with T<sub>4</sub> DNA polymerase (Amersham-Pharmacia Biotech) and with *PstI*. The fragment was cloned into the *SmaI* and *PstI* sites of pCAMBIA3381Xa. The translational fusion with the GUS coding region includes three amino acids of the *AtMYB102* protein.

The constructs were transformed into Col-0 ecotype by an *A. tumefaciens* (C58C1)-mediated floral dip method (Clough and Bent, 1998). The putative Basta-resistant transformants (pCAMBIA3381Xa) were sown on pot soil and sprayed once with 75 mg L<sup>-1</sup> DL-phosphinothricin after germination (Basta, Duchefa). Hygromycin-containing Murashige and Skoog plates (30 mg L<sup>-1</sup>) were used for selection of putative transformants containing the binary vector pCAMBIA1381.

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