INTRODUCTION

Jasmonic acid and its derivatives, collectively referred to as jasmonates (JAs), act as important regulators in plant biotic and abiotic stress responses (Howe and Jander, 2008; Browse, 2009; Koo and Howe, 2009). JA also plays important roles in physiological and developmental processes, including root growth, senescence, trichome formation, cell cycle progression, and flower development (Wasternack, 2007; Pauwels et al., 2008)

The molecular mechanisms by which JA regulates gene expression were illuminated by the discovery of JAZ proteins and the finding that the SCF^{COI1} complex-mediated 26S proteasome degrades JAZs (Chini et al., 2007; Thines et al., 2007). In the absence of JA, JAZ proteins bind to transcription factors and prevent their activity by recruiting the general co-repressor TOPLESS, through interaction with the adaptor protein NINJA (Pauwels et al., 2010), or by directly recruiting histone-modifying proteins, such as histone deacetylases (Zhu et al., 2011). In the presence of the signal, JA is converted into jasmonoyl-isoleucine (JA-Ile) by JAR1 in *Arabidopsis* (Staswick et al., 2002; Staswick and Tiryaki, 2004). JA-Ile then promotes the interaction between JAZ proteins and the F-box protein, COI1, in the SCF complex, resulting in proteolytic degradation of JAZ proteins by the 26S proteasome (Chini et al., 2007; Thines et al., 2007). Degradation of JAZ proteins liberates TFs from NINJA, TPL or HDACs, and initiates transcriptional reprogramming in response to JA (Krogan and Long, 2009; Howe, 2010).

Recent reports on JA-responsive TFs have further improved our understanding of JA-responsive regulatory mechanisms. AtMYC2, a bHLH TF, is a primary target in the JA signaling pathway and interacts with some members of the AtJAZ family to regulate various JA-responsive target genes (Boter et al., 2004; Lorenzo et al.; 2004; Dombrecht et al., 2007). Other TFs also have been shown to interact with specific JAZ proteins (Fernández-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011). It has been speculated that the specific interactions between TFs and JAZs could be largely responsible for the specificity and diversity of JA responses to different stimuli (Pauwels and Goossens, 2011; Kazan and Manners, 2012).

These TFs bind to specific promoter elements of downstream genes and propagate JA signaling. One well-defined JA-responsive element, which is bound by MYC2, is the G-box (CACGTG) or G-box like motif (core ACGT) (Dombrecht et al., 2007). The G-box has been found in the promoters of many JA-responsive genes, such as *VSP1* in *Arabidopsis* (Kim et al., 1992), *PIN2* in potato (Mason et al., 1993), *VSPB* in soybean (Guerineau et al., 2003), and *ORCA3* in *Catharanthus* (Vom Endt et al., 2007). Another JA-responsive element is the GCC-motif in *PDF1.2* in *Arabidopsis* (Brown et al., 2003), *PMT* in tobacco (Xu et al., 2004), and *STR* in *Catharanthus* (Menke et al., 1999). Other JA-responsive sequence motifs have also been reported (Rouster et al., 1997; He and Gan, 2001). Transcriptome shifts of gene clusters responding to hormonal signals closely corresponded with the set of *cis*-elements in the genes' promoters (Adie *et al*., 2007). Some elements are

involved in signal transduction in response to a specific hormone; others respond to two or more hormonal signals (Yamamoto *et al*., 2011). Therefore JA-responsive *cis*-elements are key to understanding both JA-specific signal transduction and inter-hormonal cross-talk.

Histone acetyltransferases and histone deacetylases play key roles in regulating gene expression through histone modification. The addition of acetyl groups to conserved lysine residues neutralizes the positive charge of histone tails and decreases their affinity for DNA (Strahl and Allis, 2001; Chen and Tian, 2007). Hypoacetylation mediated by HDACs has the opposite effect on chromatin, enabling the histones to bind more tightly to the negatively-charged DNA, and is associated with the repression of gene expression (Hebbes et al., 1988; Chua et al., 2003). HATs and HDACs interact with co-activator and co-repressor complexes, respectively, to regulate expression of target genes (Utley et al., 1998; Kagale and Rozwadowski, 2011). There are reports that transcription levels of some JA-responsive genes are altered in *Arabidopsis HDA6* or *HDA19* knockout mutants and overexpression plants (Zhou et al., 2005; Wu et al., 2008).

One key aspect of JA signaling is feedback regulation of JA synthesis. In *Arabidopsis*, the expression of *AtJMT*, which encodes a jasmonic acid carboxyl methyltransferase responsible for MeJA formation, is developmentally regulated and induced upon wounding or JA application (Seo et al., 2001a). *BcNTR1* encodes the orthologous JA carboxyl methyltransferase in *Brassica campestris* (Seo et al., 2001b) and its expression pattern is similar to the pattern of *AtJMT* expression. In

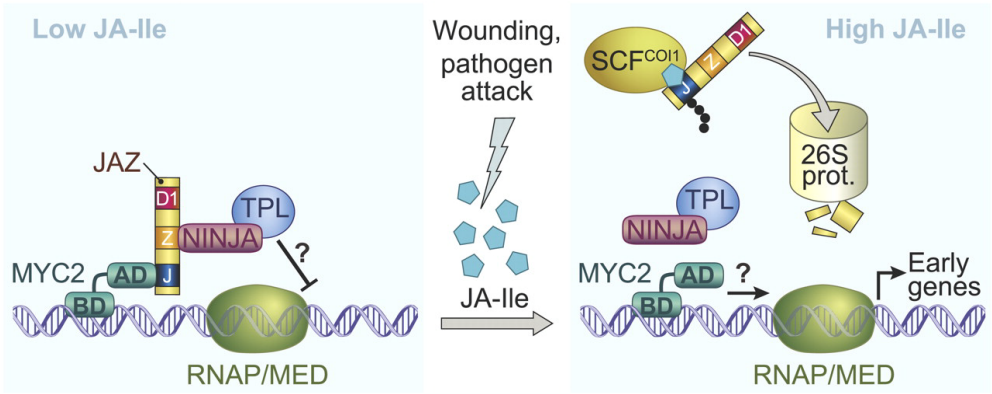
this study, it was identified a novel JA-responsive *cis*-element (JARE) in the *AtJMT* and *BcNTR1* promoters and isolated a *trans*-acting factor, AtBBD1, which binds to the JARE and interacts with JAZ1 and JAZ4. It was also showed that AtBBD1 regulates transcription of *AtJMT* and another JA-regulated gene.

LITERATURE REVIEW

Plant growth and development is exquisitely tuned to the environment. The capacity for plants to adapt to various environmental stimuli is mediated by hormones that regulate virtually all aspects of development, physiology, and metabolism. The fatty acid-derived hormone jasmonate is a prime example of a small molecule that orchestrates phenotypic plasticity in rapidly changing environments (Howe and Jander, 2008; Browse, 2009).

Recently, remarkable progresses on understanding JA signal pathway was made and it has opened up new opportunities to study mechanisms of hormone sensing, transcriptional regulation, and interconnectivity between hormone response pathways. COI1, a F-box protein, directly binds to JA-Ile and coronatine and serves as a receptor for jasmonate in *Arabidopsis* (Yan *et al.,* 2009). JASMONATE ZIM-domain (JAZ) proteins as substrates of the E3 ubiquitin ligase, interacts with COI1 protein in coronatine or JA-Ile dependent manner (Chini *et al*., 2007; Thines *et al*., 2007). JAZ proteins discovery led directly to the recognition of JA-Ile as the active form of the hormone, the identification of the receptor as the COI1 F-box protein that directs ubiquitination of JAZ proteins by the Skp/Cullin/F-box $(SCF)^{COI1}$ complex, and the recognition of MYC2 as one of the transcription factors regulated by the JAZ proteins (Figure 1).

Figure 1. Current model of JA signal transduction. Low JA-Ile levels (left section) permit the accumulation of JAZ proteins (denoted with their Jas [J], ZIM/TIFY [Z], and Domain 1 [D1] regions) that bind to the bHLH-type transcription factor MYC2 (BD, DNA-binding domain; AD, activation domain). Repression of JA response genes involves binding of JAZ to NINJA, which contains an EAR motif that recruits the corepressor TPL. The mechanism by which TPL silences gene expression is unknown (?). In response to stress-related cues that activate JA-Ile synthesis, high levels of the hormone (right section) promote SCF^{COI1}-mediated ubiquitination and subsequent degradation of JAZs by the 26S proteasome (26S prot.). JAZ degradation relieves TPL-mediated repression of gene expression, and may also alleviate passive repression by allowing MYC2 to engage RNA polymerase II (RNAP) and/or the Mediator complex that links RNAP to MYC2.



Howe, 2010, *Plant Physiol*..154:471-474.

1. Mechanism of jasmonate signal transduction in plant.

1.1. The COI1-JAZ Receptor Complex

When the *coi1-1* mutant was discorverd and it encodes an F-box protein (Xie et al., 1998), it was predicted to be part of an SCF complex (SCF^{COI1}) acting as an E3 ubiquitin ligase. Subsequent protein interaction studies identified CULLIN1 (CUL1; At4g02570), RING-BOX1 (At5g20570), the ARABIDOPSIS SKP1 HOMOLOG1 (ASK1; At1g75950), ASK2 (At5g42190), and the COP9 signalosome as interactors and confirmed this hypothesis (Devoto et al., 2002; Xu et al., 2002; Feng et al., 2003). These results and the phenotypes of mutants in the loci of members of the SCF^{COI1} complex (Feys et al., 1994; Xie et al., 1998; Xu et al., 2002; Feng et al., 2003; Ren et al., 2005) indicated that protein ubiquitination by SCF^{COI1} was essential for the JA response.

Recently, JAZ proteins, substrate of COI, were found and it interact with COI1 in a manner dependent on the presence of an Ile conjugate of JA, JA-Ile (Thines et al., 2007; Katsir et al., 2008), later pinpointed as (+)-7-iso-jasmonoyl-L-Ile, which is now accepted as the endogenous bioactive JA (Fonseca et al., 2009). Interestingly, the phytotoxin coronatine that is produced by *Pseudomonas syringae* is a structural mimic of JA-Ile and can substitute for JA-Ile action (Katsir et al., 2008; Fonseca et al., 2009).

JAZ proteins were degraded with a rapid and COI1-dependent manner

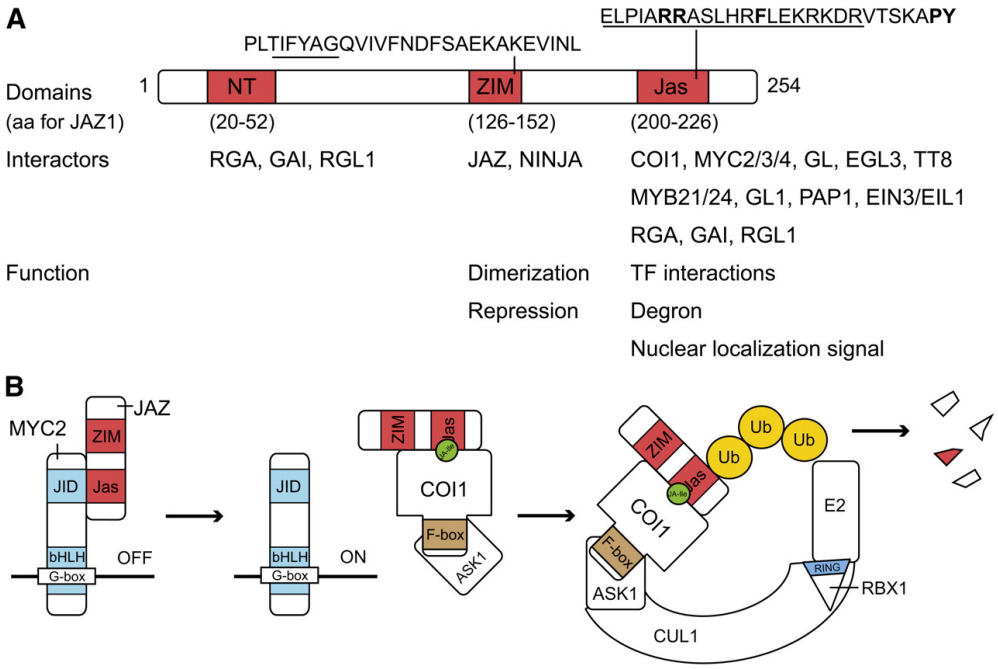
upon JA perception (Chini et al., 2007; Thines et al., 2007). The C-terminal region containing the JA-associated (Jas) domain was necessary and sufficient for interaction with COI1 and binding of COR to the complex (Katsir et al., 2008). The Jas domain, first described by Yan et al. (2007), is characterized by an S-L-X(2)-F-X(2)-K-R-X(2)-R core, delimited by a conserved N-terminal Pro and a C-terminal PY sequence (Figure 2A). Two extra N-terminal conserved basic residues (205R and 206R in JAZ1; Figure 1A) within the Jas domain were shown to be essential for COI1 interaction (Melotto et al., 2008). Direct interaction with COI1 has been shown for at least JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17860), JAZ6 (At1g72450), JAZ9 (At1g70700), and JAZ10 (At5g13220) (Thines et al., 2007; Melotto et al., 2008; Chini et al., 2009; Chung and Howe, 2009; Yan et al., 2009; Sheard et al., 2010). The COI1-interacting degron of JAZ1 includes a six-amino acid (LPIARR) loop region that encloses JA-Ile in its binding poket (Sheard et al., 2010). This sequence is largely conserved in other JAZs that strongly interact with COI1 in the presence of JA-Ile. But JAZ7 (At2g34600) and JAZ8 (At1g30135) lack the conserved these degrons (Shyu et al., 2012) (Figure 2A). (Chung et al., 2010). Also, it was reported that overexpressing AtJAZ8 transgenic plants showed insensitive phenotype respond to JA and it repressed gene expression through interaction with TPL directly (Shyu et al., 2012). Therefore, conserved degron is important to JA dependent JAZ degradation.

The hormone is probably perceived by a co-receptor complex consisting of a JAZ protein and COI1 (Figure 2B). Both the structural data and the observation

that degron binding to COI1-JAZ was more than 50-fold higher than to COI1 alone support this hypothesis (Sheard et al., 2010). Nonetheless, it appears that COI1 alone can bind JA-Ile to some extent (Yan et al., 2009; Sheard et al., 2010), suggesting a model in which COI1 might initially bind to JA-Ile and subsequently recruit JAZ proteins, which would in turn enhance the interaction significantly (Yan et al., 2009).

F-box proteins may form substrate adaptors together with the Skp1 proteins that, only when bound to their target, are recruited by cullins to complete the SCF E3 ubiquitin ligase complex. It is assumed that this maintains an uncommitted pool of cullin-RING ligases in the cell (Hua and Vierstra, 2011). It is widely accepted that binding with SCF^{COI1} is followed by JAZ polyubiquitination and degradation by the proteasome (Figure 2B). The dependence of JA-mediated JAZ degradation by the 26S proteasome has been confirmed (Chini et al., 2007; Thines et al., 2007). Nevertheless, information on JAZ (poly-)ubiquitination is lacking and only JAZ6 has been reported to be modified by ubiquitin (Saracco et al., 2009).

Figure 2. (A) Schematic representation of the JAZ1 protein and its conserved domains. Known interacting proteins and functions are depicted. The TIFY motif for the ZIM domain and the JAZ degron for the Jas domain are underlined. Important amino acids mentioned in the text are in bold. (B) In the absence of JA-Ile, JAZ binds MYC2 and represses gene expression. COI1-SKP may form a substrate adaptor that, with JA-Ile and InsP5 as a cofactor, binds the Jas domain of JAZ proteins. Upon formation of the SCF^{COI1} E3 ligase complex, JAZ1 is presumably polyubiquitinated, which marks it for degradation by the 26S proteasome.(aa, amino acid; NT, N-terminal domain).



Pauwels, 2011, *Plant Cell,* 23:3089-3100.

1.2. Inositol Pentakisphosphate Is a Cofactor of the JA Receptor

The similarity between the JA and auxin signaling pathways in regulation of JAZ and auxin/indole-3-acetic acid (Aux/IAA) protein stability, respectively, is striking. COI1 is a close homolog ofthe auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1), and the binding of the latter to the Aux/IAA proteins is mediated by the auxin IAA. Because COI1 and TIR1 share the core cullin-RING ligase subunits, such as CUL1, and the CUL1 regulation by rubylation, many mutants in these common components are compromised in both auxin and JA signaling (Hoffmann et al., 2011).

In the structure of the TIR1-Aux/IAA coreceptor, inositol hexakisphosphate (InsP6) was identified as a cofactor (Tan et al., 2007). Intriguingly, it is not InsP6, but presumably inositol pentakisphosphate (InsP5) that acts as a cofactor for the COI1-JAZ coreceptor (Sheard et al., 2010). This corresponds with the hypersensitivity to JA of the Arabidopsis ipk1-1 mutant (Mosblech et al., 2011), which is defective in the conversion of InsP5 to InsP6 by the INOSITOL POLYPHOSPHATE KINASE1 (IPK1) and accumulates InsP4 and InsP5 (Stevenson-Paulik et al., 2005).

Accordingly, in Y2H assays, deletion of the yeast IPK1 gene enhanced the COR-mediated COI1 interaction with JAZ proteins. This interaction depended on the amino acid residues in COI1 that coordinate the inclusion of InsP5 near the JA-Ile binding pocket (Mosblech et al., 2011). Finally, InsP5 is essential for COR

binding to the COI1-JAZ coreceptor in vitro (Sheard et al., 2010). Whether and how InsP5 levels are controlled by environmental stimuli, such as wounding, remains to be determined, but these results suggest that InsP5 has the potential to play a role as a signaling molecule in plants by fine-tuning of the COI1-JAZ coreceptor formation.

1.3. Alternative Splicing Leads to Dominant JAZ Repressors

The involvement of JAZ proteins in JA signaling has been proven by the expression of dominant JAZ variants resulting in JA-insensitive plants. These dominant variants all lacked (part of) the C-terminal Jas domain, either through a mutation in a splice acceptor site in JAZ3 (Chini et al., 2007), overexpression of a splice variant of JAZ10 (Yan et al., 2007), or overexpression of a truncated JAZ1 sequence lacking the Jas domain (Thines et al., 2007).

Several JAZ pre-mRNAs are subject to alternative splicing, which, at least for JAZ10, results in truncated JAZ proteins with a reduced COI1 binding potential, thereby causing dominantJA-insensitive phenotypes (Yan et al., 2007; Chung et al., 2010). The alternative splicing event provokes retention of an intron with the loss of the C-terminal X5PY (PY) motif in the Jas domain and the expression of DPY JAZ proteins as a consequence. Notably, the conserved PY sequence itself is not part of the JAZ degron (Sheard et al., 2010), and its replacement by two Ala residues does not affect COI1 binding (Chung et al., 2010).

Stable DPY JAZ proteins might be produced to terminate the activated JA

pathway (Chung et al., 2010). This hypothesis raises a new question as to which mechanism is responsible for the clearing of the DPY JAZ proteins that would be necessary to reset the JA pathway when required. A possible role for the conserved PY sequence might reside in the subcellular localization of JAZ proteins. Whereas full-length JAZ1 is exclusively located in the nucleus, deletion of the Jas domain leads to the accumulation of the mutant JAZ1 proteins in the cytoplasm, at least in transformed tobacco (Nicotiana tabacum) suspension cells. A green fluorescent protein–tagged version of the Jas domain alone was localized specifically to the nucleus only when the conserved PY sequence was included (Grunewald et al., 2009). Hence, JAZ proteins might contain a type of nuclear localization signal (PY-NLS) that is recognized by karyopherin b in yeast and humans (Lee et al., 2006; Bai et al., 2011). Nevertheless, the splice variants JAZ10.3 and JAZ10.4 that lack the PY motif and the entire Jas domain, respectively, are still localized in the nucleus, arguing for an alternative mode of nuclear localization, at least for the JAZ10 proteins (Chung and Howe, 2009).

1.4. JAZ-CONTROLLED TFs

The first TF described to be regulated by the JAZ proteins was MYC2 (At1g32640) (Chini et al., 2007). The importance of this basic helix-loop-helix (bHLH) protein for JA signaling initially was discovered through a forward genetics approach (Lorenzo et al., 2004). MYC2 acts both as an activator and repressor of downstream

JA responses. For example, it is a positive regulator of JA-mediated inhibition of primary root growth, anthocyanin biosynthesis, and oxidative stress tolerance but a negative regulator of JA-mediated resistance to necrotrophic fungi and biosynthesis of Trp and indole-glucosinolates (Lorenzo et al., 2004; Dombrecht et al., 2007). Nevertheless, MYC2 functions as a transcriptional activator when targeted to a heterologous promoter (Pauwels and Goossens, 2008). After its discovery by forward genetics, JAZ3 was found to interact with MYC2, indicating that JAZ proteins might function as regulators of JA responses by binding and inhibiting the function of TFs, such as MYC2 (Figure 3A) (Chini et al., 2007). MYC2 binds most JAZ proteins, and, conversely, most JAZ proteins interact with its closest homologs, MYC3 (At4g17880) and MYC4 (At5g46760) (Table 1) (Cheng et al., 2011; Fernandez-Calvo et al., 2011; Niu et al., 2011). Although these three bHLH proteins have similar DNA binding preferences, they seem to regulate specific subsets of JA responses (Figure 3A). For example, in contrast with MYC2, MYC3 and MYC4 play only a weak role in regulating JA-mediated inhibition of primary root growth, whereas MYC3 and MYC4 are important for JA-mediated resistance to the herbivore Spodoptora littoralis. These differences between MYC2 and MYC3/MYC4 are postulated to correlate with their preferential production in root and shoot tissues, respectively (Cheng et al., 2011; Ferna´ ndez-Calvo et al., 2011; Niu et al., 2011).

All three MYC proteins belong to group III of the bHLH family (Heim et al., 2003), in which at least five different protein domains have been identified

(Figure 2B). Besides the DNA binding bHLH domain, MYC2/MYC3/MYC4 and other bHLH proteins contain a conserved ACT-like domain at their C terminus that is necessary and essential for homo- and heterodimerization of the related bHLH proteins GLABRA3 (GL3; At5g41315) and ENHANCER OF GL3 (EGL3; At1g63650) (Zhang et al., 2003). Therefore, the ACTlike domain in MYC2/MYC3/MYC4 might have a similar dimerization function. At their N terminus, they contain three adjacent conserved domains of which the second domain is essential for interaction with JAZ proteins and designated the JID (Fernandez-Calvo et al., 2011). A mutation in a conserved Asp (Asp94Asn) in the JID of MYC3 (Asp100 in MYC2; Figure 3B) resulted in a dominant phenotype (Smolen et al., 2002), which might correspond to a loss of interaction with and repression by JAZ proteins (Fernandez-Calvo et al., 2011).

The JID domain is also found in several other bHLH proteins, suggesting that JAZ proteins might target more bHLH proteins than MYC2/MYC3/MYC4 (Fernandez-Calvo et al., 2011). For instance, the JID domain is present in GL3, EGL3, and TRANSPARENT TESTA8 (TT8; At4g09820), all of which belong to group IIIf of the bHLH family (Heim et al., 2003) and interact with eight different JAZ proteins (Qi et al., 2011). Remarkably, however, this interaction has been observed only with the C-terminal part of TT8 and EGL3, which lacks the JID domain. Nonetheless, the Jas domain of JAZ1 and JAZ8 is as essential for interaction with these bHLH proteins as for the MYC2/MYC3/MYC4 proteins (Figure 3A) (Qi et al., 2011). GL3, EGL3, and TT8 function in complexes in which

they interconnect directly with the WD40 protein TRANSPARANT TESTA GLABRA1 (At5g24520), on the one hand, and different R2R3 MYB proteins, on the other hand. The GL3/EGL3/TT8 complexes regulate multiple processes, including development of trichomes and root hairs, biosynthesis of flavonoids (anthocyanins and proanthocyanidins), stomata patterning on hypocotyls, and seed coat mucilage production (Zhang et al., 2003; Dubos et al., 2010).

Anthocyanin biosynthesis and trichome initiation are both inducible by JAs (Feys et al., 1994). This induction requires both the JA receptor component COI1 and the GL3/EGL3/TT8-type bHLH proteins (Maes et al., 2008; Yoshida et al., 2009; Qi et al., 2011). Interestingly, the MYC2/MYC3/MYC4 complex is also involved in the JA-mediated induction of anthocyanin biosynthesis (Lorenzo et al., 2004; Dombrecht et al., 2007; Niu et al., 2011), but trichome induction seems to be independent of the MYC2 presence (Yoshida et al., 2009). How and whether MYC2/3/4 on the one hand and GL3/EGL3/TT8 on the other hand interact to regulate anthocyanin biosynthesis remains to be resolved.

Within the GL3/EGL3/TT8 complexes, the R2R3 MYB proteins provide the specificity for the downstream effects (Zhang et al., 2003; Dubos et al., 2010). Several JAZ proteins interact directly with the R2R3 MYB proteins PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1; At1g56650) and GL1 (At3g27920), which have specific roles in anthocyanin synthesis and trichome initiation, respectively. These connections are mediated by the Jas domain of the JAZ proteins and the C-terminal domain of PAP1/GL1, respectively (Figure 2A). Moreover,

overexpression of JAZ1 could disturb the bHLH–MYB interaction between PAP1-TT8 and GL1-GL3 (Qi et al., 2011). Correspondingly, in plants producing stabilized JAZ proteins, such as the coi1-2 knockout mutant plant, the levels of anthocyanin production and trichome initiation are lower than those of wild-type plants, either in the absence or presence of JA elicitation. Overexpression of PAP1, GL3, and EGL3 suppresses the inhibition of these processes by the JAZ proteins (Qi et al., 2011). To date, no R2R3 MYB proteins have been identified that interact with MYC2/MYC3/MYC4. More bHLH-type interactors of JAZ proteins may exist; hence, additional cellular processes under yet unrevealed control of JA signaling might be discovered. For instance, JID occurs in bHLH013 (At1g01260), which was found to interact with JAZ1, JAZ8, and JAZ10 in systematic Y2H screens (Arabidopsis Interactome Mapping Consortium, 2011).

On the other hand, JA signaling is essential for male fertility in Arabidopsis. Mutants defective in JA biosynthesis or JA-insensitive mutants, such as coi1-1, are male sterile because of a combination of defective anther dehiscence, insufficient filament elongation, and severely reduced pollen viability (Browse, 2009b). Transcriptome analysis of JA-treated stamens identified two R2R3 MYB proteins, MYB21 (At3g27810) and MYB24 (At5g40350), as key regulators of the stamen maturation processes triggered by JA (Mandaokar et al., 2006). Overexpression of MYB21 in the coi1-1 or oxophytodienoate reductase3 background could partially restore male fertility (Song et al., 2011), whereas the myb21-1 knockout mutant had strongly reduced fertility that could not be rescued

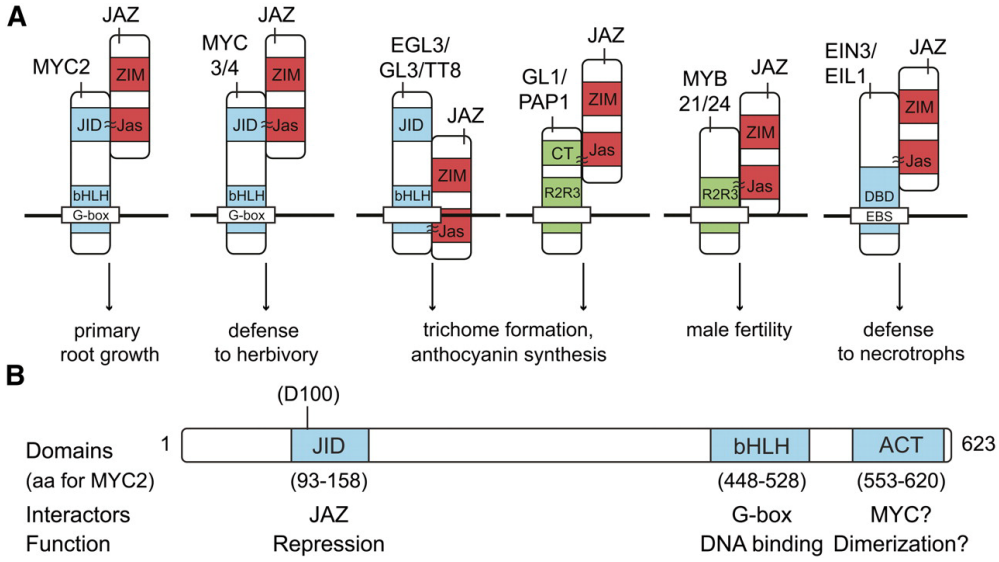
by exogenous JA (Mandaokar et al., 2006).

A select set of JAZ proteins (i.e., JAZ1, JAZ8, and JAZ11 [At3g43440]) interact directly with MYB21 and MYB24 (Figure 3A) (Song et al., 2011), revealing a model in which developmentally regulated JA biosynthesis triggers COI1-dependent JAZ degradation to control MYB21 and MYB24 levels and thereby stamen development. Like PAP1 and GL1, MYB21 and MYB24 bind the Jas domain of the JAZ proteins, but in contrast with PAP1 and GL1, the connection between JAZ proteins and MYB21/MYB24 is mediated through the N-terminal R2R3 domain (Songet al., 2011). Ectopic expression of JAZ1DJas (Thines et al., 2007) and JAZ10.4 (Chung and Howe, 2009), both of which lack the full Jas domain, results in male sterility, whereas the JAZ3 splice acceptor mutant jai3-1, which expresses JAZ3 without the Jas domain, is still fertile (Chini et al., 2007). Overall, this suggests that production of truncated JAZ proteins under the control of their own promoter might help reveal the specific roles of the different JAZ proteins in plants. In a related approach, cell type–specific production of a nondegradable DELLA protein in the endodermis established that GA-mediated regulation of cell proliferation occurs in a subset of root cells rather than in all root cells (Ubeda-Tomas et al., 2009).

Part of this crosstalk apparently is mediated through the connection of JAZ proteins with ETHYLENE INSENSITIVE3 (EIN3; At3g20770) and EIN3-LIKE1 (EIL1; At2g27050), two TFs that are the central positive regulators of the ET response (Zhu et al., 2011). At least JAZ1, JAZ3, and JAZ9 can bind EIN3 and

EIL1. The Jas domain–harboring C terminus of JAZ1 is necessary and sufficient for interaction with a fragment of EIN3 (comprising amino acids 200 to 500) that overlapped with its DNA binding domain (amino acids 59 to 359) (Figure 3A). Thereby, the JAZ proteins can repress the function of EIN3/EIL1, possibly by suppressing the DNA binding of EIN3 (Zhu et al., 2011). The emerging model, in which ET is needed for EIN3/EIL1 stabilization and JA for EIN3/EIL1 release from the JAZ protein repression, might provide a plausible explanation for the synergy in many ET/JA-regulated processes (Zhu et al., 2011).

Figure 3. (A) Model depicting different JAZ-TF combinations and the protein domains involved. The most important known JA responses controlled by the TFs are shown. Approximation symbols connect domains or approximate regions involved in physical interactions. (B) Schematic representation of the MYC2 protein and its conserved interaction domains. Known protein interactors and functions are depicted. The location of the Asp-100 mutation in the JID domain is shown. (aa, amino acid; CT, C-terminal domain; DBD, DNA binding domain; EBS, EIN3 binding sites.



Pauwels, 2011, *Plant Cell,* 23:3089-3100

2. JA-responsive *cis*-elements in plants

The expression of a gene is determined by the *cis*-acting DNA elements located in the vicinity of the gene and the trans-acting protein factors that interact with them. In general, these *cis*-acting elements are concentrated in a relatively small promoter region of a few hundred nucleotides upstream of the transcriptional start site, although there are examples of regulatory sequences located at a distance of several thousands of nucleotides from the gene they control. Several *cis*-acting elements in various gene promoters that mediate jasmonate responsiveness have been identified. The most common jasmonate-responsive promoter sequences are the GCC motif and the G-box. In addition several other jasmonate-responsive promoter elements have been reported.

2.1. JA-responsive promoter elements: the GCC motif

In the promoter of the terpenoid indole alkaloid biosynthesis gene strictosidine synthase (*STR*) from Catharanthus roseus a jasmonate- and elicitor-responsive element (JERE) has been identified (Menke et al., 1999). Mutation or deletion of this JERE results in an inactive and unresponsive *STR* promoter derivative. A tetramer of the JERE fused to a minimal promoter confers MeJA-responsive gene expression on a reporter gene, showing that the JERE is an autonomous MeJA-responsive sequence (Menke et al., 1999).

Within this JERE a GCC-box-like sequence is present. In Arabidopsis, a GCC motif (GCCGCC) plays a role in conferring JA responsiveness to the *PDF1.2* promoter (Brown et al., 2003). The GCC motif has also been shown to function autonomously as an ethylene-responsive element (Ohme-Takagi and Shinshi, 1995; Fujimoto et al., 2000). The *PDF1.2* gene is synergistically induced by a combination of JA and ET (Penninckx et al., 1998), which is likely caused by a convergent action of both signals on the GCC motif. However, not all GCC motifs confer JA- and ET-responsive gene expression, since the STR gene does not respond to ET (Memelink, unpublished results). This may be due to the sequence of the STR GCC motif (GACCGCC), which differs slightly from the consensus sequence.

2.2. JA-responsive promoter elements: the G-box

The G-box (CACGTG) or G-box-like sequences (e.g. AACGTG) that are essential for the jasmonate response were found in thepromoters of the potato proteinase inhibitor 2 gene (*PIN2*; Kim et al., 1992), the soybean vegetative storage protein B gene (*VSPB*; Mason et al., 1993), the Arabidopsis *VSP1* gene (Guerineau et al., 2003), the tomato leucine aminopeptidase gene (*LAP*; Boter et al., 2004), the tobacco putrescine N-methyltransferase 1a gene (*PMT1a*; Xu and Timko, 2004) and the Octadecanoid-derivative Responsive Catharanthus AP2-domain gene (*ORCA3*; Vom Endt et al., 2007). Also, analysis of the promoters of JA-responsive

Arabidopsis genes showed that the G-box element was statistically significantly over-represented (Mahalingam et al., 2003).

In the tomato *LAP* promoter, the G-box-like sequence is flanked by another sequence characterized by a GAGTA repeat, which is also essential for JA-responsive expression (Boter et al., 2004). In the *NtPMT1a* promoter, the G-box is flanked by a GCC motif, and both sequences are essential for MeJA-responsive promoter activity (Xu and Timko, 2004). In the *ORCA3* promoter the G-box-like sequence is flanked by an A/T-rich sequence which is important for the expression level (Vom Endt et al., 2007).

2.3. JA-responsive promoter elements: other motifs

TGACG (as-1-type) sequences were found to be essential for JA inducibility of the promoter of the Agrobacterium tumefaciens T-DNA nopaline synthase (nos) gene in tobacco (Kim et al., 1993, 1994) and of the barley lipoxygenase 1 gene promoter (*LOX1*; Rouster et al., 1997). A monomer or a tetramer of the as-1 sequence from the Cauliflower Mosaic Virus (CaMV) 35S promoter also conferred JA-responsive expression to a reporter gene in transgenic tobacco (Xiang et al., 1996). Two jasmonate-responsive elements, JASE1 (50-CGTCAATGAA-30) and JASE2 (50-CATACGTCGTCAA-30), were identified in the promoter of the *OPR1* gene in Arabidopsis (He and Gan, 2001). JASE1 is a new motif without any signature sequence so far reported, whereas JASE2 possesses an ACGT core which is also

found in the G-box and in as-1-type elements. In the Long Terminal Repeat (LTR) promoter of the tobacco retrotransposon Tto1 a 13 bp element, which contains a box L/AC-I or H-box-like motif, is involved in responsiveness to MeJA (Takeda et al., 1998).

In conclusion, a variety of jasmonate-responsive elements appear to exist. The best characterized elements are the G-box and closely related variants, which are commonly found in promoters that respond to jasmonates and are negatively affected by ET, and the GCC motif, which is commonly present in promoters that respond in a synergistic manner to JAs combined with ET. It has been well established that the JAs-responsive activity of promoters containing the GCC motif (e.g. PDF1.2; Lorenzo et al., 2003) or the G-box (e.g. VSP; Benedetti et al., 1995) is dependent on COI1. For promoters containing other elements COI1 dependency has not been established. The *OPR1* gene for example, containing the JASE1/2 motifs in its promoter, has been shown to be woundinducible in a coi1 mutant background (Reymond et al., 2000), and is inducible by OPDA but not by JA in an *opr3* mutant background (Stintzi et al., 2001). Therefore it remains to be established whether so-called JA-responsive elements other than the GCC motif and the G-box confer responses to bioactive JAs via COI1.

3. Histone deacetylation in Arabidopsis

Chromatin, consisting of both DNA and proteins, is responsible for storing heritable and instructional information in a cell. Chromatin is highly organized and consists of nucleosomes. In each nucleosome, four core histone proteins, H2A, H2B, H3, and H4, are organized into octameric protein complexes containing two molecules of each of the four core histones. Approximately 146 base pairs (bp) of DNA wrap around each nucleosome, and approximately 80 bp of DNA link adjacent nucleosomes with the help of histone H1, forming the so-called "beads-on-a-string" organization. This basic level of chromatin packaging is further arranged into higher order conformations (Alberts et al., 2002). Protruding from the nucleosome are the positively charged amino-terminal tails of the core histone proteins that tightly associate with DNA's negatively-charged phosphate backbone. Reversible post-translational modifications of histone H3 and H4 amino-terminal tails, such as methylation, phosphorylation, ubiquitination, adenosine diphosphate (ADP)-ribosylation and acetylation, alter interactions between the DNA and core histones, resulting in changes in chromatin conformation. It was discovered that specific histone modifications at certain residues of the amino-terminal tails of H3 and H4 constitute the "histone code" that instructs the chromatin to adopt either "open" or "closed" configurations, thereby regulating the availability of *cis*-regulatory elements of genes to transcriptional machinery (Jenuwein and Allis, 2001). Some of the histone modifications, such as methylation, are heritable. Therefore, the histone code not only expands the information-storing capacity of DNA but also offers rapid and reversible changes in chromatin accessibility when organisms are

challenged with internal or external stresses. As plants are sessile, the ability to rapidly change their gene expression programs in response to internal or external stresses underlies the very plastic growth and developmental programs in plants.

Histone acetylation is a reversible process that plays vital roles in the epigenetic regulation described above. Therefore, histone acetylation and deacetylation are of particular importance to plant growth, development, defense and adaptation. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes required to perform histone acetylation and deacetylation, respectively, acting on the ε-amino group of lysine residues located near the amino-termini of core histone proteins. The prime acetylation targets are H3, lysine (K) residues 9, 14, 18 and 23, and H4 lysine (K) residues 8, 12, 16 and 20 (Fuchs et al. 2006). Although the lysine residues can only accommodate one acetyl group at a time, each nucleosome has over 20 possible targets for acetylation. The addition of acetyl groups, mediated by HATs, neutralizes the positive charge of histone tails and decreases their affinity for DNA. Growing evidence also indicates that acetylation may help shape the binding surface for activators and repressors (Kurdistani and Grunstein, 2003). Thus, acetylation allows the chromatin to open up and provides transcription factors and RNA polymerases access to the DNA (Mutskov et al., 1998). This is supported by much experimental data indicating that hyperacetylation of histone H3 and H4 is associated with transcriptionally active euchromatic regions. Hypoacetylation mediated by HDACs has an opposite effect on the chromatin, enabling the histones to bind more tightly to the negatively-

charged DNA. As a result, hypoacetylation is associated with the repression of gene expression (Hebbes et al. 1988; Chua et al., 2003). In order to carry out their intended functions, HDACs and HATS interact with co-repressor or co-activator complexes, respectively, to regulate the expression of target genes (Utley et al. 1998; Gonzalez et al. 2007).

MATERIALS AND METHODS

1. Bacterial strains and culture media

Bacterial strains used in this experiment were *E. coli* DH10B [F-, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ-, *rps*L(str^r), *nup*G] for subcloning and *E. coli* BL21 (DE3) *pLys*S [F$^-$, *omp*T, *gal*, *dcm*, *lon*, *hsd*S$_B$(r$_B^-$ m$_B^-$) λ(DE3) *pLys*S(cm^R)] for protein expression. *E. coli* DH10B and BL21 (DE3) *pLys*S were gown in LB (Luria-Bertani) medium (1% tryptone; 0.5% yeast extract; 0.5% NaCl). Transformed strains were cultured in LB broth media with ampicillin (50 μg/ml). Solid medium was made with 1.5% (w/v) Bacto-agar.

2. Enzymes and chemicals

Restriction enzymes, Klenow fragment and T4 DNA ligase were purchased from Takara. Taq DNA polymerase was purchased from Takara and Bioneer. Reverse transcriptase was purchased from Invitrogen. Abscisic acid, methyl jasmonate, ethylene, salicylic acid and agarose were purchased from Sigma and Duchefa. Amylose resin was purchased from New England Biolab (NEB). dNTPs and [α-^{32}P]dATP were purchased from Izotope. Acrylamide and N, N'-bisacrylamide were purchased from Elpis Biotech. Nylon membrane was purchased from PerkinElmer. Nitrocellulose membrane was purchased from Whatman. MS salt was purchased from Duchefa. Other chemicals were purchased from Aldrich, Merck, Sigma,

Duchefa and Duksan.

3. Yeast strain

The yeast strain used for one hybrid assay was *S. cerevisiae* Y187 [*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*]. The yeast strain used for two hybrid assay was *S. cerevisiae* AH109 [*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ, MEL1*] (Clontech).

4. Plant materials and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type for all experiments. Variety and source of *Brassica campestris* was described in Song et al. (2000) [44]. Plants were grown on soil or one-half-strength Murashige and Skoog agar medium (Duchefa) in a growth chamber maintained at 22 ℃ and 60% relative humidity under long-day conditions (16-h-light/8-h-dark cycle). *Arabidopsis* was transformed with *Agrobacterium tumefaciens* (strain C58C1) using the floral dip method [45]. A construct list of transgenic plants used in this study is provided in Supplemental Table 1 online. Transformed lines (T1 generation) were selected on MS plates containing kanamycin (30 μg/ml) or hygromycin (20 μg/ml). At least 40 independent T1 plants per genotype were transferred to soil for subsequent analysis. Homozygous lines were identified by testing T3 progeny for resistance to

antibiotics. The basal level and MeJA responsive induction of reporter gene were variable among transformants. A line showing medium level of expression was selected from each construct by RT-PCR analysis. Several lines showing extremely high or low level of basal expression were excluded. For chemical treatment, solutions of 100 μM MeJA (Aldrich), 100 μM (±)-JA (Duchefa), 100 μM (±)-ABA (Duchefa), 50 μM SA (Sigma), or 5 mM ethephon were applied to soil-grown 4-week-old plants by spraying.

5. Genomic Southern blot analysis

For genomic Southern blots, 5 μg of genomic DNA was digested with restriction enzymes, separated on 0.8% agarose gels, and transferred to GeneScreen Plus hybridization transfer membranes (PerkinElmer). The probe used in Southern blotting was obtained from pBI121 vector by PCR. Primer pair is listed in Supplementary Table 1. The synthesis of ^{32}P-labelled nucleic acid probes, and Southern blot analysis was followed standard procedures (Sambrook *et al*., 2001).

6. RNA gel blot and RT-PCR analyses

RNA gel blot analysis was performed with total RNA extracted from frozen and ground samples using the phenol–SDS–LiCl method (Carpenter *et al*., 1998). Ten micrograms of total RNA was separated on 1.2% agarose formaldehyde gels and transferred onto GeneScreen Plus hybridization transfer membranes (PerkinElmer). cDNA probes were obtained by RT-PCR of RNA isolated from wild-type

Arabidopsis leaves, cloned into a pGEM-T Easy Vector (Promega), and verified by DNA sequencing. These clones were used as templates for PCR reactions with gene-specific primer pairs to generate cDNA fragments that were used as probes in an RNA gel blot analysis. cDNA probes were labeled by random primer extension with [α-^{32}P] dATP (Izotope). Primer pairs used to amplify cDNA probes are listed in Supplementary Table 1. For RT-PCR analysis, the first-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) at 50°C for 1 h from 2 μg of total RNA with oligo $(dT)_{15}$ according to the manufacturer's instructions. PCR was conducted from the first-strand cDNA with sequence-specific primers described in Supplementary Table 1 by 24 cycles.

7. Chromatin Immunoprecipitation

ChIP-PCR was carried out as described by Saleh et al. (2008). Anti-acetylated H4K12 and anti-acetylated H3K14 antibodies were purchased from Upstate. After immobilization using protein A agarose (Pierce), bound DNA was eluted and amplified by primers corresponding to sequences neighboring the AtBBD1 binding sites in the promoter of *AtJMT*. PCR products were separated on ethidium bromide-stained agarose gel or real-time PCR was used to quantify the amplification. For real-time PCR, amplification of P_{JMT} (near the JARE) was normalized to that of Actin (Johnson et al., 2002). Chromatin precipitated without antibody was used as negative control, and the chromatin before precipitation was used as input control. ChIP assays were independently repeated twice with the same result. Real-time

qRT-PCR was performed using a LightCycler 480 II machine (Roche Diagnostics) with a SYBR Premix EX Taq Kit (TaKaRa). Primers used for qRT-PCR are listed in Table S1. Each qRT-PCR was independently repeated three times with the same expression pattern.

8. Subcellular Localization of AtBBD1 Protein

For subcellular localization, the full length CDS of the *AtBBD1* without the stop codon was amplified by RT-PCR (Table S1). The PCR product was then inserted under the CaMV 35S promoter and in frame with the 3' terminal GFP gene in the pBI121 vector. Young roots of 2-week-old transgenic plants were examined for GFP fluorescence with a confocal laser-scanning microscope LSM510 (Carl Zeiss). T3 transgenic lines were used for the subcellular localization experiment. Histochemical staining for GUS activity in transgenic plants was carried out as described by Weigel and Glazebrook (2002).

9. Yeast one-hybrid (Y1H) screening and assay

The yeast one-hybrid screening was performed using MATCHMAKER One-Hybrid Library Construction and Screening Kit (Clontech). To isolate JARE-binding proteins, a cDNA library was prepared by RT-PCR from MeJA-treated seedlings of *Arabidopsis* Col-0 into pGADT7-Rec2. Bait DNA (-3518 to -3390 bp) containing JARE was cloned into the pHIS2 reporter vector. Positive clones were identified by nucleotide sequencing with AD sequencing primers. To identify the AtBBD1

binding sequence, various promoter fragments were cloned into the pHIS2 vector. The full length CDS or specific domains of AtBBD1 were cloned into pGADT7-Rec2.

10. Yeast two-hybrid (Y2H) assay

To test the protein–protein interactions, full-length cDNA of *AtJAZ* genes was amplified by RT-PCR from 14-day-old seedlings of wild-type rice plants. Primer pairs of each *AtJAZ* are listed in Supplementary Table 1. These genes were cloned into the Y2H prey vector pGADT7 (Clontech) to obtain prey gene constructs. Full-length cDNA of a AtBBD1 by RT-PCR using primer pairs listed in Supplementary Table 1. These genes were cloned into the Y2H bait vector pGBKT7 (Clontech) to obtain bait gene constructs. Bait and prey gene constructs were co-transformed into yeast *Saccharomyces cerevisiae* strain AH109. Co-transformed colonies were selected on yeast synthetic dropout (SD) glucose medium lacking Leu and Trp (–2). For AtBBD1 and AtJAZs interactions, a co-transformed single colony (2 mm diameter) grown on selection medium for 3 days was re-suspended in 100 μl of autoclaved distilled H_2O and 10 μl of re-suspended cells were dropped on SD-glucose medium lacking Ade, His, Leu, and Trp (–4) with (±) JA, MeJA, or coronatine (Sigma) at a concentration of 30 μM and 120 μM coronatine for an OsCOI1–OsJAZ interaction.

11. *In vitro* pull-down assay

The procedure for *in vitro* pull-down experiments was as described previously (Thines *et al.,* 2007). Total protein was extracted from 14-day-old seedlings (for *35S:AtBBD1-MYC* plants) in a buffer containing 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.05% (v/v) Tween 20, 20 mM 2-mercaptoethanol, the EDTA-free complete miniprotease inhibitor cocktail (Roche) and 10% (v/v) glycerol. To obtain recombinant MBP-AtJAZ1, full-length cDNA of *AtJAZ1* was introduced into a pMALc2E, which encodes a MBP fusion protein and includes a cleavage site for the Tobacco Etch Virus protease, expression vector. MBP-AtJAZ1 was expressed in BL21 (DE3) pLysS (Novagen) and purified by amylose resin as per manufacturer's instructions (Invitrogen). To test AtJAZ1-AtBBD1 interaction, 100 μg of MBP-AtJAZ1 was incubated with 1 mg of total plant extract in total volume of 300 μl with gentle rocking for 1 h at 4°C. Reaction complex was purified using amylose resin as per the manufacturer's instructions (NEB). Pulled-down mixtures were separated on SDS-PAGE, transferred nitrocellulose membrane (Whatman), and detected with anti-MYC antibody (Santa Cruz Biotechnology) and goat anti-mouse IgG-horseradish peroxidase (HRP) as the secondary antibody (Thermo Scientific) or anti-MBP antibody (Santa Cruz Biotechnology) and donkey anti-rabbit IgG-HRP as the secondary antibody (Thermo Scientific). ECL analysis was carried out with HRP substrate kit (Pierce)

12. Electrophoretic Mobility Shift Assay

Full length CDS or DNA binding domain (a.a. residue 257 to 325) of AtBBD1 were

fused in frame with the maltose-binding protein (MBP) at the C-terminus and expressed in *Escherichia coli.* A soluble crude extract of recombinant protein was used for EMSA. DNA fragments labeled with [γ-^{32}P]dCTP were incubated with MBP-AtBBD1 or MBP-AtBBD1DB in the binding buffer [20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM DTT, 1 mM EDTA, 10 % glycerol, 5 mM MgCl2, 0.01% Triton X-100, and 100 ng poly(dI-dC)] for 1 hour. For competition analysis, unlabeled DNA fragments were included in the binding reactions as competitors in 30-fold molar excess relative to the labeled probes. The reaction mixture was analyzed by 10% polyacrylamide gel electrophoresis and the wet gel was exposed and detected by BAS reader (BAS-2010, Fujifilm).

13. Agroinfiltration and Transient Protein Expression

Agroinfiltration for transient protein expression in tobacco leaves was performed according to the method described by Voinnet et al., with minor modifications. Briefly, *Agrobacterium tumefaciens* strains C58C1 carrying either the 6xMYC-BBD1 or 3xHA-AtJAZ1 construct under the control of the 35S promoter were grown at 28°C in Luria-Bertani medium and resuspended in infiltration media. For cotransfections, *Agrobacterium* cultures carrying each construct were mixed in equal proportion. Leaves of 4-week-old *Nicotiana benthamiana* plants were infiltrated with a needleless syringe carrying bacterial cultures through the abaxial air spaces.

Table S1. List of constructs and primers used in this study

Assays	Clone Name	Forward Primers (5'→3')	Reverse Primers (5'→3')	Vector	Cloning Site
Promoter	NP4.0	CCGCTGCAGCTCGAGGAGGGGTAATTAAG	CGGGATCCTTCTCTCTCTCTCTATGTCT	pBI121	PstI-BamHI
	NP3.0	CCGCTGCAGAATCGATTCTTGAAATAGTA	CGGGATCCTTCTCTCTCTCTATGTCT	pBI121	PstI-BamHI
	NP2.0	CCGCTGCAGCCACGAATCATATGCCATGT	CGGGATCCTTCTCTCTCTCTATGTCT	pBI121	PstI-BamHI
	NPfr1	CGCTCGAGGGTTAAAATTTTATAAAAGAT	CAGTCGACTGATTGTGGTTGAAAAATGT	pBI121M	XhoI-SalI XbaI-BamHI
		CGTCTAGACCAATTTTCTTCCAACCAGG	AAGGATCCTTCTCTCTCTCTCTATGTCTC		
	NP4-A	CCTCGAGCTCGAGGAGGGGTAATTAAG	CAGTCGACCAATTACAGAATCCAAAGT	pBI121M	XhoI-SalI XbaI-BamHI
		CGTCTAGAATGTTAGAAAAAGGGATGC	AAGGATCCTTCTCTCTCTCTCTATGTCT		
	JP4.5	CCCAAGCTTATTGGATTCTGTATTGGAGG	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP4.0	CCCAAGCTTGACTGTCTTTTTCAACCACA	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP3.5	CCCAAGCTTTTAAATTTCAAAGCATACCC	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP3.0	CCCAAGCTTATCAAACAGGGTAGACAAAC	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2.5	CCCAAGCTTCAAAATGAAGTCTGTATACA	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2.0	CCCAAGCTTATTCGGTCTATATTCTTTAG	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP1.5	CCCAAGCTTGATTACGACAAATCCTTTGA	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP1.0	CCCAAGCTTTTGTGTAGACCTAACGAGAG	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP0.5	CCCAAGTTGGACATATATAATGGTAC	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2400	CCCAAGCTTCTACTTCTACATTTGGCAAT	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2294	CCCAAGCTTACTCCTGAAAATGAAAATG	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2280	CCCAAGCTTTGAAAATGCATTGCCTATCT	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2200	CCCAAGCTTGTTGATAGATTGAACTATAC	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
	JP2100	CCCAAGCTTCTTCGGATATAGTTCGAATA	CGGGATCCCTCTCTCCCTCTCTCGCTCT	pBI121	HindIII-BamHI
Y1H	pHIS2-128	GGAATTCTGAGTTGTGACATGATTTG	CGGAGCTCGGTGAACAAACTGTACCTGA	pHIS2	EcoRI-SacI
	pHIS2-a	GGAATTCAAGTGAGTTGTGACATGATT	CGGAGCTCTTTTCTAACATCAGGACCAT	pHIS2	EcoRI-SacI
	pHIS2-b	GGAATTCGGGATGCATCGTCGATCGCC	CGGAGCTCATCCTTTGTTTGGTCATATA	pHIS2	EcoRI-SacI
	pHIS2-c	GGAATTCTTATATGACCAAACAAAGGA	CGGAGCTCGGTGAACAAACTGTACCTGA	pHIS2	EcoRI-SacI
	pHIS2-a1	GGAATTCAAAAAAGTTGTGACATGATTTGTATA	CGGAGCTCTTTTCTAACATCAGGACCAT	pHIS2	EcoRI-SacI
	pHIS2-a2	GGAATTCAAGTGAAAAAAAACATGATTTGTATAATG	CGGAGCTCTTTTCTAACATCAGGACCAT	pHIS2	EcoRI-SacI
	pHIS2-a3	GGAATTCAAGTGAGTTGTGAAAAAATTTGTATAATG	CGGAGCTCTTTTCTAACATCAGGACCAT	pHIS2	EcoRI-SacI
	pHIS2-a4	GGAATTCAAGTGAGTTGTGACATGAAAAAAATAATGTATGG	CGGAGCTCTTTTCTAACATCAGGACCAT	pHIS2	EcoRI-SacI
	pHIS2-a5	GGAATTCAAGTGAGTTGTGACATGATT	CGGAGCTCCTAACATCAGGACCATTTTTTTTTACAAATCATG	pHIS2	EcoRI-SacI
	pHIS2-a6	GGAATTCAAGTGAGTTGTGACATGATT	CGGAGCTCTTCTAACATCAGTTTTTTACATTATACA	pHIS2	EcoRI-SacI
	pHIS2-a7	GGAATTCAAGTGAGTTGTGACATGATT	CGGAGCTCTTCTAATTTTTTGACCATACATTATACA	pHIS2	EcoRI-SacI
	pHIS2-a8	GGAATTCAAGTGAGTTGTGACATGATT	CGGAGCTCTTTTTTTCATCAGGACCATACATTATAC	pHIS2	EcoRI-SacI
	pHIS2-M0	GGAATTCTAAAAAAAAGTCCTGATGTTAGAAAAAGGG	CGGAGCTCCCCTTTTTCTAACATCAGGACTTTTTTTTT	pHIS2	EcoRI-SacI
	pHIS2-M1	GGAATTCTAATGTAAAAACTGATGTTAGAAAAAGGG	CGGAGCTCCCCTTTTTCTAACATCAGTTTTTTACATTA	pHIS2	EcoRI-SacI
	pHIS2-M2	GGAATTCTAATGTATGAAAAAAATGTTAGAAAAAGGG	CGGAGCTCCCCTTTTTCTAACATTTTTTTCATACATTA	pHIS2	EcoRI-SacI
	pHIS2-M3	GGAATTCTAATGTATGGTCAAAAAATTAGAAAAAGGG	CGGAGCTCCCCTTTTTCTAATTTTTTGACCATACATTA	pHIS2	EcoRI-SacI
	pHIS2-M4	GGAATTCTAATGTATGGTCCTGAAAAAAGAAAAAGGG	CGGAGCTCCCCTTTTTCTTTTTTCAGGACCATACATTA	pHIS2	EcoRI-SacI
	pHIS2-M5	GGAATTCTAATGTATGGTCCTGATGAAAAAAAAAGGG	CGGAGCTCCCCTTTTTTTTTCATCAGGACCATACATTA	pHIS2	EcoRI-SacI
	pHIS2-P_{NTR1}	GGAATTCTAATGTATGGTCCTGATG	CGGAGCTCCCCTTTTTCTAACATCAG	pHIS2	EcoRI-SacI
	pHIS2-P_{JMT}	GGAATTCTTATTAGTATACTGAAA	CGGAGCTCGGATTTTCATTTTCAGGAG	pHIS2	EcoRI-SacI
	pHIS2-P_{GLBOX}	GGAATTCTTATTAGTATACACGTGAAAATGAA	CGGAGCTCGCATTTTCATTTTCACGTGTATACTAA	pHIS2	EcoRI-SacI
	pHIS2-CM1	GGAATTCATTAGTATAATCCTGAAAA	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CM2	GGAATTCTTAGTATACGCCTGAAAAT	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CM3	GGAATTCTAGTATACTACTGAAAATG	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CM4	GGAATTCTAGTATACTACTGAAAATG	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CM5	GGAATTCTAGTATACTACTGAAAATG	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CM6	GGAATTCTAGTATACTACTGAAAATG	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CM7	GGAATTCTAGTATACTACTGAAAATG	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
	pHIS2-CMR	GGAATTCTCAGGAGAAAATGAAAATGCAT	GTGAGCTCTCTATCAACTTTTAAAAC	pHIS2	EcoRI-SacI
Y2H assay	AD-BBD1F	CGAATTCATGAGATCGGTTCAAGCACC	CGGGATCCTCATGTGTATTTCCTCAAGT	pGADT-7	EcoRI-BamHI
	AD-BBD1A	CGAATTCATGAGATCGGTTCAAGCACC	CGGGATCCTAGTTGCCTACCATCCCTCA	pGADT-7	EcoRI-BamHI
	AD-BBD1B	CGAATTCCGATGTGTCCATAACAATCC	CGGGATCCTCATGTGTATTTCCTCAAGT	pGADT-7	EcoRI-BamHI
	AD-BBD1C	CGAATTCCGATGTGTCCATAACAATCC	CGGGATCCCTCTCTCCTCATCGACTCC	pGADT-7	EcoRI-BamHI
	AD-BBD1D	CGAATTCATGAGATCGGTTCAAGCACC	CGGGATCCCTTCCCTGACTCAATGACTC	pGADT-7	EcoRI-BamHI
	AD-JAZ1	GGAATTCATGTCGAGTTCTATGGAATG	CGGGATCCTCATATTTCAGCTGCTAAAC	pGADT-7	EcoRI-BamHI
	AD-JAZ2	GGAATTCATGTCGAGTTTTTCTGCCGA	CGGGATCCTTACCGTGAACTGAGCCAAG	pGADT-7	EcoRI-BamHI
	AD-JAZ3	GGAATTCATGGAGAGAGATTTTCTCGG	CCGCTCGAGTTAGGTTGCAGAGCTGAGA	pGADT-7	EcoRI-XhoI
	AD-JAZ4	GGAATTCATGGAGAGAGATTTTCTCGG	CCGCTCGAGTTAGTGCAGATGATGAGCTG	pGADT-7	EcoRI-XhoI
	AD-JAZ5	GGAATTCATGTCGTCGAGTCATGAAA	CGGGATCCTCATCTAGATCGAGAT	pGADT-7	EcoRI-BamHI
	AD-JAZ6	GGAATTCATGTCAACGGGACAAGCGCC	CGGGATCCCTAAAGCTTGAGTTCAAGGT	pGADT-7	EcoRI-BamHI
	AD-JAZ7	GGAATTCATGATCATCATCATCAAAAA	CCATCGATCTATCGGTAACGGTGGTAAG	pGADT-7	EcoRI-ClaI
	AD-JAZ8	GGAATTCATGAAGCTACAGCAAAATTG	CGGGATCCTTATCGTCGTGAATGGTACG	pGADT-7	EcoRI-BamHI
	AD-JAZ9	GGAATTCATGGAAAGAGATTTTCTGGG	CGGGATCCTTATGTAGGAGAAGTAGAAG	pGADT-7	EcoRI-BamHI
	AD-JAZ10	GGAATTCATGTCGAAAGCTACCATAGA	CGGGATCCTTAGGCCGATGTCGGATAGT	pGADT-7	EcoRI-BamHI
	AD-JAZ11	GGAATTCATATGATGGCTGAGGTAAGGGAGA	CGGGATCCTGTCACAATGGGCTGGTTT	pGADT-7	EcoRI-BamHI
	AD-JAZ12	GGAATTTCATATGATGACTAAGGTGAAAGATGA	CGGGATCCAGCAGTTGGAAATTCCTCCT	pGADT-7	EcoRI-BamHI
	AD-JAZ1F	GGAATTCATGTCGAGTTCTATGGAATG	CGGGATCCTCATATTTCAGCTGCTAAAC	pGADT-7	EcoRI-BamHI
	AD-JAZ1A	GGAATTCATGTCGAGTTCTATGGAATG	CGGGATCCAGCAATAGGAAGTTCTGTCA	pGADT-7	EcoRI-BamHI
	AD-JAZ1B	GGAATTCTCCTCTCTTCCAAAGGAAGA	CGGGATCCGCTTCTGATATCGGTTTGAT	pGADT-7	EcoRI-BamHI
	AD-JAZ1C	GGAATTCGCTAGAAGAGCTTCACTTCA	CGGGATCCTCATATTTCAGCTGCTAAAC	pGADT-7	EcoRI-BamHI
	AD-JAZ1D	GGAATTCCATCCAAGAAAAACCACAAC	CGGGATCCTCATATTTCAGCTGCTAAAC	pGADT-7	EcoRI-BamHI
	BD-BBD1	CGAATTCATGAGATCGGTTCAAGCACC	CGGGATCCTCATGTGTATTTCCTCAAGT	pGADT-7	EcoRI-BamHI
	BD-JAZ1	GGAATTCATGTCGAGTTCTATGGAATG	CGGGATCCTCATATTTCAGCTGCTAAAC	pGADT-7	EcoRI-BamHI
Protein	MBP-BBD1	CGGAATTCATGAGATCGGTTCAAGCACC	CGGGATCCTCAATGTGTATTTCCTCAAGT	pMALc2E	EcoRI-BamHI
	MBP-BBD1E	CGGAATTCCCGGCACCAGCTTCAGATGG	CGGGATCCTCAATGTGTATTTCCTCAAGT	pMALc2E	EcoRI-BamHI
	MBP-JAZ1	CGGAATTCATGTCGAGTTCTATGGAATG	CGGGATCCTCATATTTCAGCTGCTAAAC	pMALc2E	EcoRI-BamHI
Transgenic	35S:AtBBD1	GCTCTAGAGATCTCTCAAGTGAAGAATC	CCCTCGAGGCACGAAAACAAATACGACG	pBI111L	XbaI-XhoI
	AtBBD1-GFP	GCTCTAGAATGAGATCGGTTCAAGCACC	GCTCTAGCCTGTGTATTTCCTCAAGTTAC	pBI-sGFP	XbaI-XbaI
	MYC-AtBBD1	CGGGATCCTAATGAGATCGGTTCAAGCA	CGGGATCCTGTGTATTTCCTCAAGTTAC	myc-pBA	BamHI-BamHI
	HA-AtJAZ1	TCCCCCGGGATGTCGAGTTCTATGGAATG	TCCCCCGGGTCATATTTCAGCTGCTAAAC	HA-pBA	SmaI
	4xJRE-GUS 4xmJRE-GUS	TGATTACGCCAAGCTTATCTACTAGTTTATTAG	ACCTGCAGCCAAGCTTGGATCCGATTGCATTTTC	pCambia1391Z pCambia1391Z	HindIII HindIII
	TATA-GUS	GGATCCCCGGGAATTCTCGCAAGACCCTTCCTCT	CGACGGCCAGTGAATTCCAGCGTGTCCTCTCCAA	pCambia1391Z	EcoRI
RT-PCR	gusA expression	TATGCCGGAATCCATCGCAG	CCAGTCGAGCATCTCTTCAG		
	AtJMT expression	CCACCGCTCAGAGTAACATA	TTGGCCATGGACATAAGAGC		
	TUB2 expression	ATCCGTGAAGAGTACCCAGAT	AAGAACCATGCACTCATCAGC		
Genotyping	Salk_131906	TGCAATTCGACCAAGACAAG	CACATCGTAGTTGCCTACCA		
	GABI_641C01	GGAGGCAACTTCGATGTGTC	ACGAAGATCGAAGCTGACAC		
Blot probe	gusA	TATGCCGGAATCCATCGCAG	CCAGTCGAGCATCTCTTCAG		
	AtBBD1	AACAATCACTCTTAGATTGT	ATCTGAAGCTGGTGCCGGTG		
	AtBBD2	GAATCTTCGTCTGCATCTTC	ATCAGAAGCAGGTGTTTGCT		
	JR2	ATGGCAACCCTTAAGTGCAT	CTGTTTGAGATGAGCCTCGG		
ChIP PCR	JMTP-ChIP-PCR	GGAGTGCATGGCTTAGTATCTTCAT	TATGAGAAGACATTATTTTGTCACG		

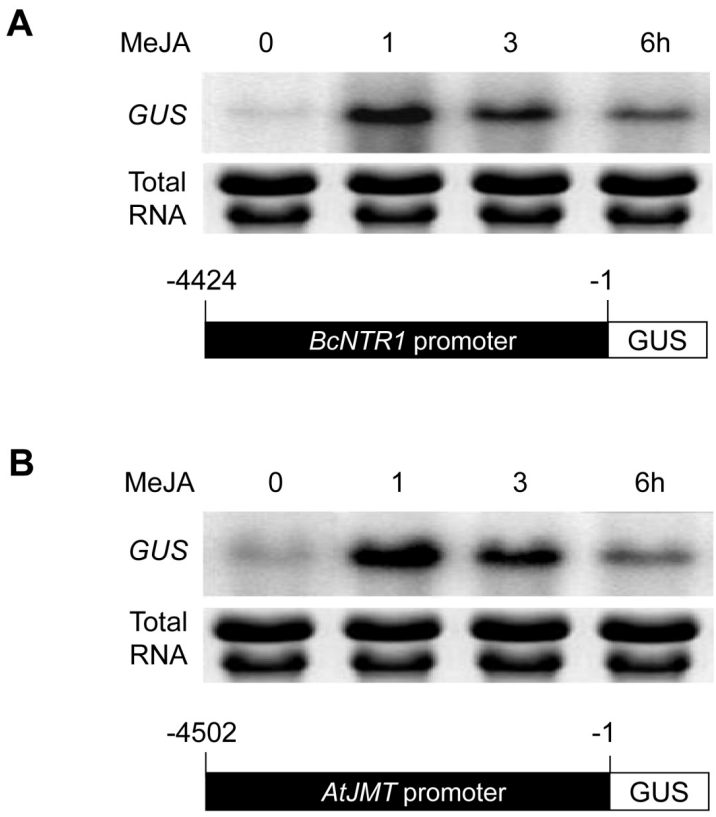
BcNTR1 promoter for NP series was described in Koo et al (2004) Agric. Chem. Biotechnol. 47:119-124
4xJRE and 4xmJRE constructs was cloned by using "In Fusion HD Cloning Kit (Clontech)'. Multimerized JRE and mJRE was synthesized and cloned by using TA-cloning(Takara).

RESULTS

1. The promoter regions of *BcNTR1* and *AtJMT* contain a JA-responsive element

AtJMT and *BcNTR1* are orthologues, which are both induced by MeJA treatment (Song et al., 2000; Seo et al., 2001a and 2001b). To understand the regulatory mechanism of *BcNTR1* and *AtJMT* JA-responsive expression, it was first defined the promoter regions that mediate induction by JA. Promoter regions 4.4 kb upstream of *BcNTR1* and 4.5 kb upstream of *AtJMT,* respectively, were combined with the *GUS* coding sequence and transformed into *Arabidopsis*. Transcription of *GUS* was induced within 1 hour after MeJA treatment in both sets of transgenic plants (Fig. 4), showing that these promoter regions could recapitulate JA-responsive induction. These results indicate that both fragments contain putative JA-responsive *cis*-acting elements (JARE). Also it showed that the promoter of *BcNTR1* works in *Arabidopsis* although it originated from *Brassica.*

Figure 4. Northern blot analysis of the recombinant *GUS* gene after MeJA treatment of transgenic *Arabidopsis*. Promoter structures of the *BcNTR1* (4.4 kb length, A) and *AtJMT* (4.5 kb length, B) fused to *GUS* gene, respectively, are shown.


A
MeJA
0
1
3
6h
GUS
Total
RNA
-4424
-1
BcNTR1 promoter
GUS
B
MeJA
0
1
3
6h
GUS
Total
RNA
-4502
-1
AtJMT promoter
GUS

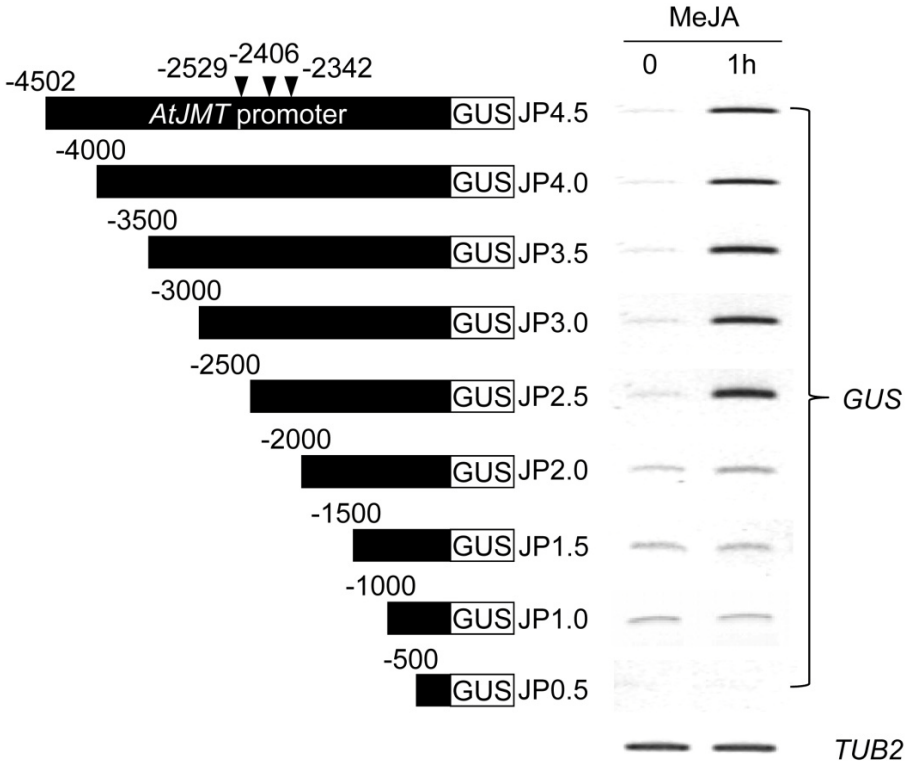
2. Identification of a JARE in the *AtJMT* promoter

Next promoter-deletion analysis was used to locate the JARE(s) present in the *AtJMT* promoter. A series of 5'-deleted promoters was fused to the *GUS* coding region and transformed into *Arabidopsis*. Each construct contains from 4.5 kb to 500 bp of the promoter region (Fig. 5A). GUS expression in response to MeJA treatment was examined by RT-PCR. When MeJA was applied, transgenic plants containing promoter fragments longer than 2.0 kb (JP4.5~JP2.5) showed *GUS* induction within 1 hour; however, those containing promoter fragments shorter than 2.0 kb (JP2.0, ~JP0.5) did not show *GUS* induction (Fig. 5A). These results indicate that a putative JARE is located in the 500 bp region between position -2,500 and -2,000 bp in the *AtJMT* promoter

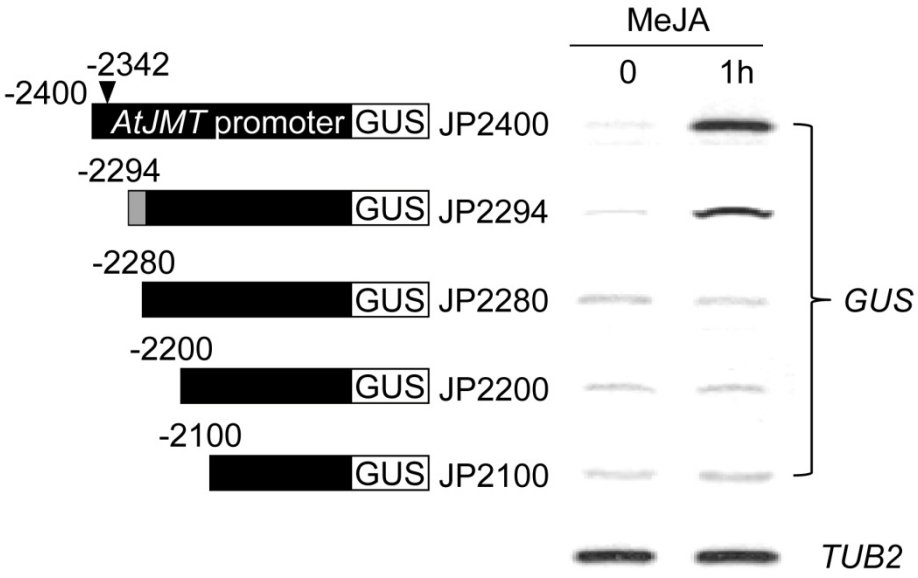
To narrow down the position of the JARE, additional 5' deletion constructs subdividing the -2,400 to -2,000 bp region of the *AtJMT* promoter were generated. As before, promoter regions were fused to *GUS* and transformed into *Arabidopsis*. When *GUS* mRNA levels were analyzed by RT-PCR after MeJA treatment, constructs containing 2,400 and 2,294 bp of the *AtJMT* promoter were responsive to MeJA, but constructs containing regions shorter than 2,294 bp were not responsive to MeJA (Fig 5B). Therefore, the putative JARE is located in the 15 bp region between the positions -2,294 and -2,280 bp of the *AtJMT* promoter.

Figure 5. (A) A series of 5' deleted promoters (closed bar) was cloned upstream of *GUS* coding region (open bar) and transformed into *Arabidopsis*. RT-PCR analysis of each transgenic plant was carried out after 1 hour of MeJA treatment. The JARE is located in the region between -2,500 and -2,000. (B) Additional promoter deletion constructs between -2,500 and -2,000 are shown and their *GUS* gene expression in response to MeJA treatment is shown. The putative JARE is located in the region between -2,294 and -2,280 (gray bar). The positions of the G-boxes are shown at -2,529, -2,406 and -2,342 (▼)

A


MeJA
0
1h
-4502
-2529
-2406
-2342
AtJMT promoter
GUS
JP4.5
-4000
JP4.0
-3500
JP3.5
-3000
JP3.0
-2500
JP2.5
-2000
JP2.0
-1500
JP1.5
-1000
JP1.0
-500
JP0.5
GUS
TUB2

B


MeJA
0
1h
-2342
-2400
AtJMT promoter
GUS
JP2400
-2294
GUS
JP2294
-2280
GUS
JP2280
-2200
GUS
JP2200
-2100
GUS
JP2100
GUS
TUB2

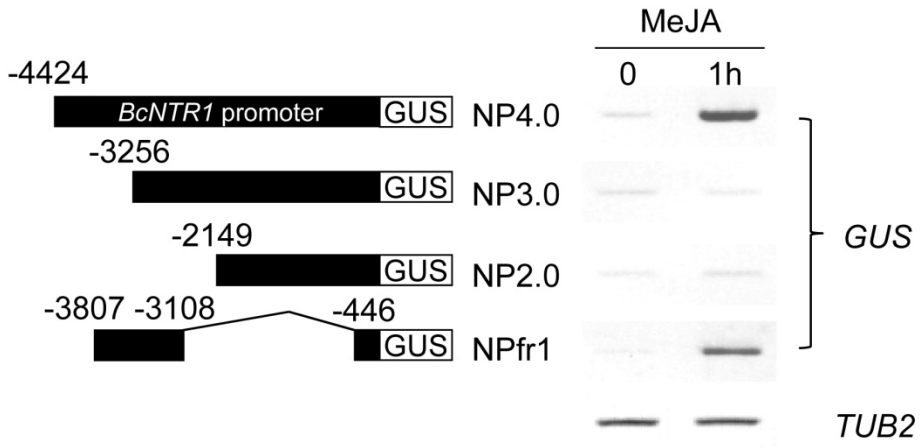
3. Identification of a JARE in the *BcNTR1* promoter

In parallel, a series of *BcNTR1* promoter deletion constructs was made and transformed into *Arabidopsis* to identify the JARE in *BcNTR1*. *GUS* mRNA levels were examined by Northern blot and were induced rapidly, within 1 hour after MeJA treatment, in NP4.0 but not in NP3.0 and NP2.0 lines. Another construct, NPfr1, containing a 3,807 bp fragment of the *BcNTR1* promoter with a deletion between -3,108 and -446, showed a similar response to NP4.0 (Fig. 6A). These results showed that the region between -3,807 and -3,256 in the *BcNTR1* promoter also contains a JARE. To test this hypothesis, an additional deletion construct, NP4-A, was made, in which the -3,518 to -3,480 region was deleted. When transgenic *Arabidopsis* plants containing the NP4-A construct were treated with MeJA, these lines showed no induction of *GUS* (Fig. 6B). These results show that the JARE is localized in the 39 bp region, between -3,518 and -3,480 bp of the *BcNTR1* promoter.

Sequence alignment between the JARE-containing regions of the *AtJMT* promoter (15 bp) and the *BcNTR1* promoter (39 bp) showed a highly conserved sequence motif, TCCTGA (Fig. 7). It was hypothesized that this conserved sequence element is a putative JARE (TCCTGA) that could play a critical role in the JA responsiveness of *AtJMT* and *BcNTR1* expression.

Figure 6. (A) Structures of promoter deletion constructs of *BcNTR1* for JA response tests (left) and RT-PCR analysis of transgenic *Arabidopsis* after MeJA treatment (right). The JARE is located in the region between -3,807 and -3,256. (B) Additional promoter deletion constructs (left) and RT-PCR analysis of transgenic *Arabidopsis* after MeJA treatment (right). The JARE is located in the region between -3,518 and -3,480

A


MeJA
0
1h
-4424
BcNTR1 promoter
GUS
NP4.0
-3256
GUS
NP3.0
-2149
GUS
NP2.0
-3807 -3108
-446
GUS
NPfr1
GUS
TUB2

B

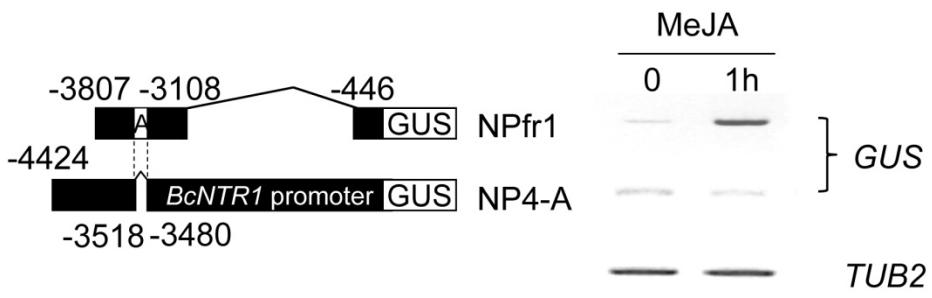
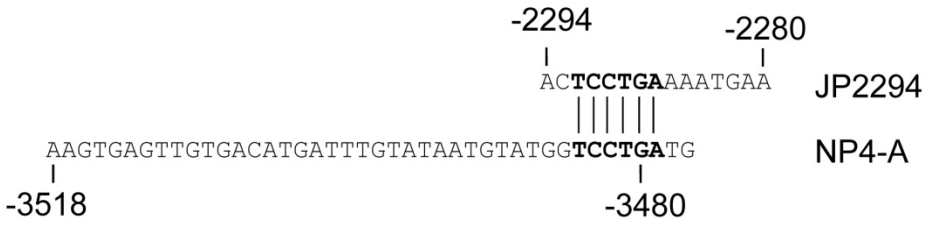

-3807 -3108
-446
GUS
NPfr1
-4424
BcNTR1 promoter
GUS
NP4-A
-3518 -3480
MeJA
0
1h
GUS
TUB2

Figure 7. Sequence alignment between putative JARE-containing regions in JP2294 of Fig 2B and A region in NPfr1. Identical sequence elements (putative JARE) between JP2294 and NP4-A are shown in bold


-2294
-2280
ACTCCTGAAAATGAA
JP2294
AAGTGAGTTGTGACATGATTTGTATAATGTATGGTCCTGATG
NP4-A
-3518
-3480

4. A multimerized JARE-containing construct responds to MeJA

To show regulation of JA responses by the JARE, next a construct containing multimers of the JARE-containing promoter region linked to a minimal promoter was made and tested whether it could mediate JA-responsive induction of transcription. A region containing the putative JARE, between -2,305 and -2,278 of the *AtJMT* promoter, was duplicated 4 times and fused with the TATA-box sequence (-46 to +8) of the CaMV 35S promoter and a GUS coding sequence. Also a mutant version, in which the core 6 nucleotides, TCCTGA, were mutated to TTTTTT, was constructed in the same manner to determine the role of this core element in response to JA (Fig 8).

All the constructs were transformed into *Arabidopsis* (Col-0) and transgenic lines were treated with MeJA. RT-PCR analysis showed that GUS transcript was induced within 1 hour in 4xJARE-GUS lines in response to MeJA, but was not induced in 4xmJARE lines (Fig. 9).

Histochemical staining of transgenic plants also showed that JA-responsive GUS activity was present only in 4xJARE-GUS lines, but not in 4xmJARE-GUS lines (Fig 10). Taking these data together, it is concluded that the conserved 6-nucleotide element (TCCTGA) in the *AtJMT* and *BcNTR1* promoters is indeed a JARE.

Figure 8. Schematic representation of multimerized JARE- containing construct (4xJARE-GUS), and its mutant version (4xmJARE-GUS). The DNA fragment from the *AtJMT* promoter (-2,305 to -2,278) containing the JARE was repeated 4 times and combined with the GUS reporter containing a minimal promoter (TATA) from CaMV 35S. JARE (TCCTGA) and its mutant version (mJARE) are shown in bold.


JARE
JARE
JARE
JARE
TATA
GUS
4xJARE-GUS
mJARE
mJARE
mJARE
mJARE
TATA
GUS
4xmJARE-GUS
-2305
-2278
JARE
ATTATTAGTATACTCCTGAAAATGAAAA
mJARE
ATTATTAGTATACTTTTTTAAATGAAAA

Figure 9. RT-PCR analysis of transgenic plants containing multimerized JARE constructs was carried out after MeJA treatment. *GUS* gene expression was detected by RT-PCR with time course. Two independent transgenic lines for each consctruct were tested.

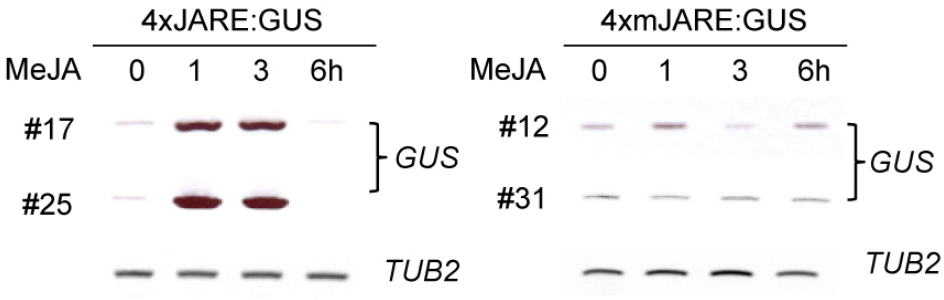
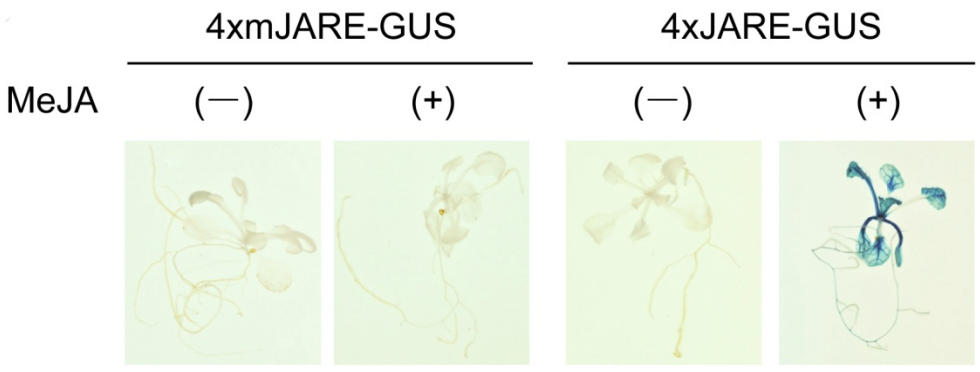

4xJARE:GUS
MeJA
0
1
3
6h
#17
#25
GUS
TUB2
4xmJARE:GUS
MeJA
0
1
3
6h
#12
#31
GUS
TUB2

Figure 10. Histochemical staining was carried out with 3-week old seedlings of 4xJARE-GUS and 4xmJARE-GUS transgenic plants. Each transgenic plant was treated with (+) or without (-) MeJA for 4h.


4xmJARE-GUS
4xJARE-GUS
MeJA
(—)
(+)
(—)
(+)

5. Identification of a JARE-binding protein

Next yeast one-hybrid (Y1H) screening was carried out to isolate protein factors that bind to the JARE. A segment (-3,518 to -3,390) of the *BcNTR1* promoter containing the JARE was employed as bait in the reporter construct. The yeast cells were co-transformed with activator constructs incorporating cDNA libraries prepared from MeJA treated *Arabidopsis*. Clones were sequenced from positive colonies and sequence analysis showed that multiple positive clones corresponded to AtBBD1 (Fig. 11A). *AtBBD1* is an *Arabidopsis* homologue of the *Oryza minuta bifunctional nuclease in basal defense response* (*OmBBD1*), which acts in abscisic-acid (ABA)-dependent callose deposition (You et al., 2010). To confirm the DNA binding ability of AtBBD1, EMSA assay was carried out with MBP-AtBBD1 expressed in *E. coli.* The result showed that AtBBD1 is DNA binding protein (Fig. 11B)

The *Arabidopsis thaliana* genome also contains an *AtBBD1* homologue, *AtBBD2,* with 81% amino acid sequence identity to *AtBBD1* (Fig 12A). To understand these homologuous proteins, gene expression pattern respond to various hormones was investigated (Fig. 12B). AtBBD2 expression was lower than AtBBD1 responding to the hormones, but the induction pattern was similar with each other. To verify the cellular localization of AtBBD1, 35S:AtBBD1-GFP transgenic plant was observed with confocal microscope. And it was found that AtBBD1 was localized in nucleus (Fig. 12C).

To determine whether AtBBD1 binds to the putative JARE core sequence of GTCCTGA in the *BcNTR1* promoter fragment, or to another *cis*-element, the bait region (-3518 to -3390) of the *BcNTR1* promoter was divided into three segments; a (-3,518 to -3,471), b (-3,471 to -3,430) and c (-3,430 to -3,390) (Fig. 13A). Each segment was tested for interaction with AtBBD1 by Y1H assay. AtBBD1 was fused with the activation domain of GAL4 (AD) in the activator construct. These assays showed that AtBBD1 bound only to the segment (-3,518 to -3,471) that contains the GTCCTGA core sequence.

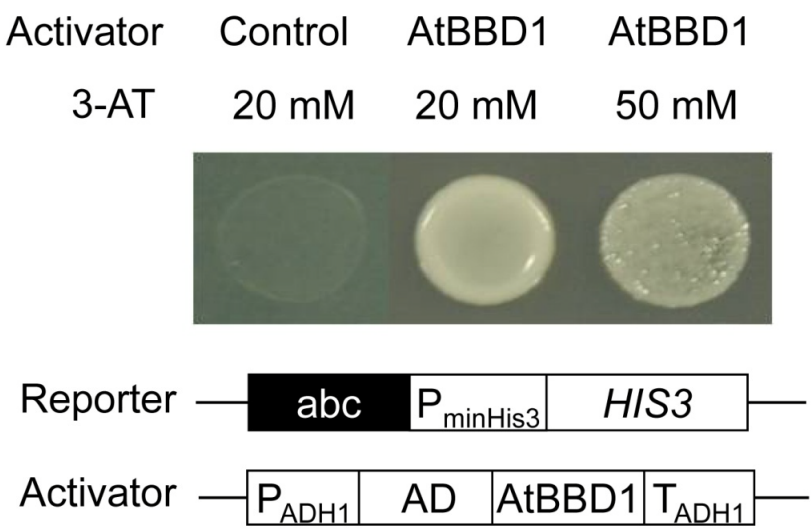
To further narrow down the binding sequences within this segment (-3,518 to -3,471), a series of mutated bait segments (a1 to a8) was designed by changing 6 nucleotides of each subsegment into 6 adenines (Fig. 13B) and testing by Y1H whether these changes affected AtBBD1 binding. AtBBD1 did not bind mutant segments a6 and a7 in yeast; therefore, those 12 nucleotides include sequences necessary for AtBBD1 binding (Fig. 13C). Another series of six overlapping mutant constructs, M0-M5, in which 6 nucleotides were mutated into 6 adenines, revealed that the nucleotide element, GTCCTGA, is necessary for AtBBD1 interaction (Fig. 13C).

Additional point mutation experiments showed that adenine and thymine in the first nucleotide eliminated AtBBD1 binding (Fig 13D). However, cytosine was acceptable as in P_{JMT} (see Fig 14). The 7th adenine, which was not tested in the M0-M5 constructs above, was also necessary. The orientation of the heptameric element was also important in the Y1H assay, as the CMR construct, which has the

JARE sequence in reverse orientation, did not show AtBBD1 binding in yeast (Figure 13D). In conclusion, the heptameric nucleotide element, G(C)TCCTGA, is critical for AtBBD1 to interact with these DNA sequences (JARE). This result is consistent with the promoter deletion experiments and the multimerized JARE analysis (Fig. 9-10).

Figure 11. (A) AtBBD1 binding intensity was tested by increasing 3-aminotriazole (3-AT). 20 mM 3-AT was completely suppress background growth of empty vector control. AtBBD1 showed binding ability to abc segment (Fig 5A) containing JARE in high 3-AT concentration. (B) Electrophoretic mobility shift assays were performed using MBP-AtBBD1 fusion protein and a segment of *BcNTR1* promoter (Fig 5A) was used as a probe.

A


Activator
Control
AtBBD1
AtBBD1
3-AT
20 mM
20 mM
50 mM
Reporter
abc
P minHis3
HIS3
Activator
P ADH1
AD
AtBBD1
T ADH1

B

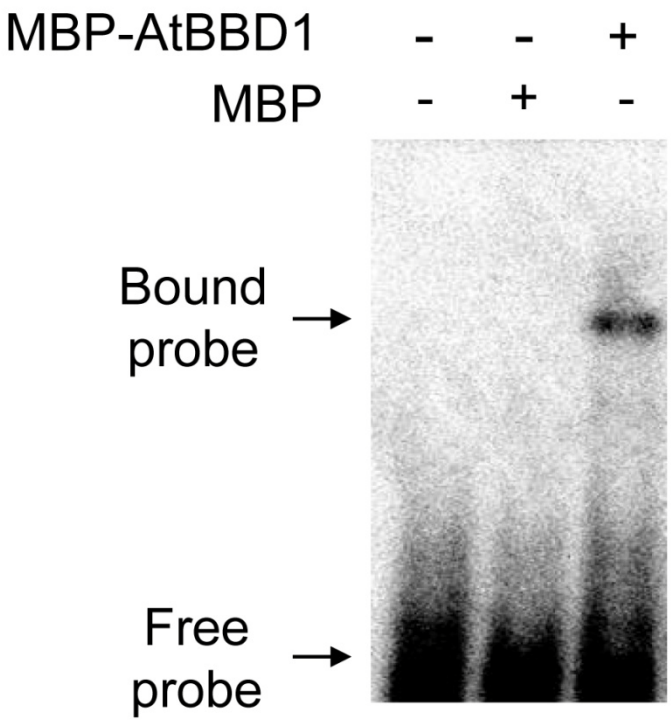
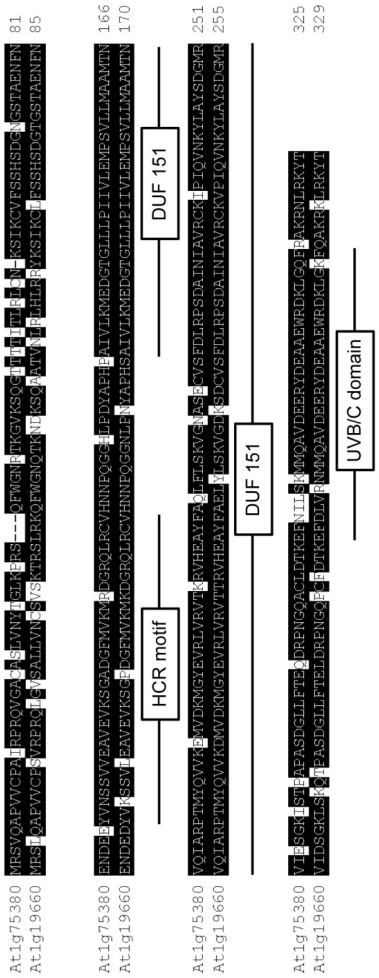
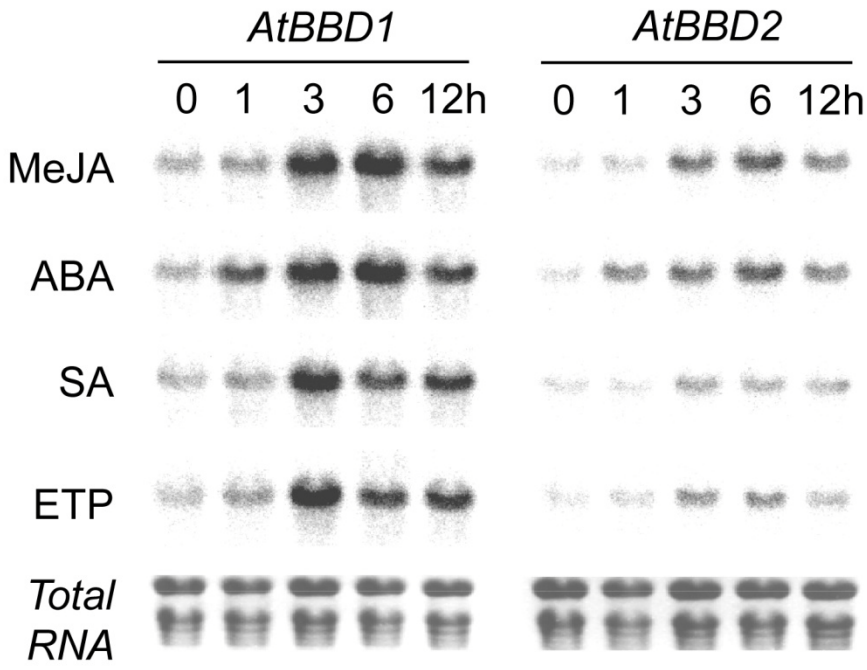


Figure 12. (A) Amino acid sequence alignment between AtBBD1 and AtBBD2 using the CLUSTALW. Conserved regions are shown on dark background. The result shows 81% similarity between AtBBD1 and AtBBD2. (B) Gene expression pattern of AtBBD1 and AtBBD2. 4-week old *Arabidopsis* were treated with JA (100 μM), ABA (100 μM), SA (100 μM), or ETP (5 mM). Northern blot analysis was carried out with *AtBBD1* as a probe. (C) Cellular localization of AtBBD1. *AtBBD1* cDNA was fused to GFP under the control of the CaMV 35S promoter and the construct was stably transformed into *Arabidopsis*. GFP fluorescence patterns were confirmed by observing at least five different transgenic lines under a confocal laser-scanning microscope (detailed procedure was described in Supplementary experimental procedure).

A



B



C

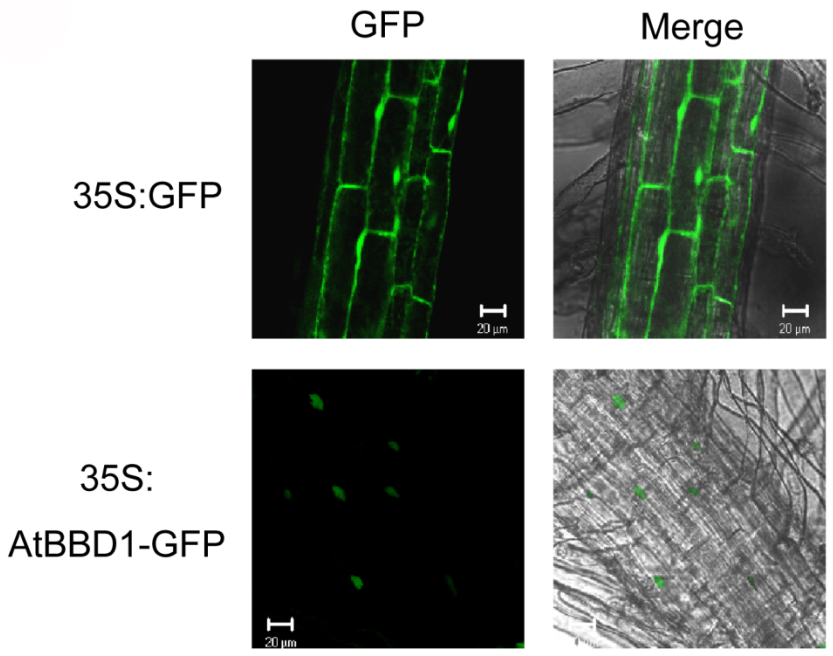
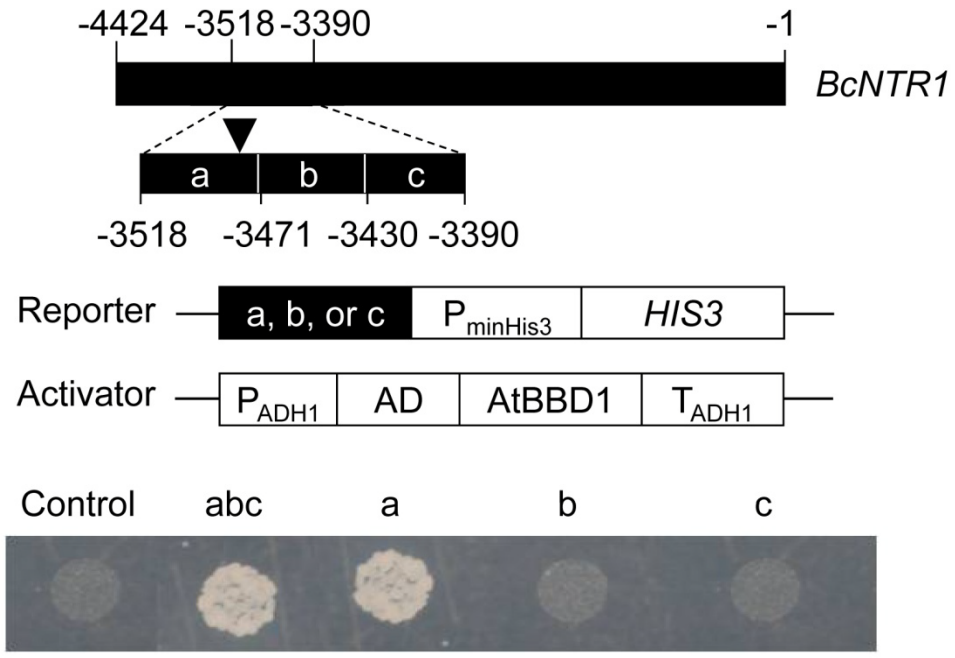
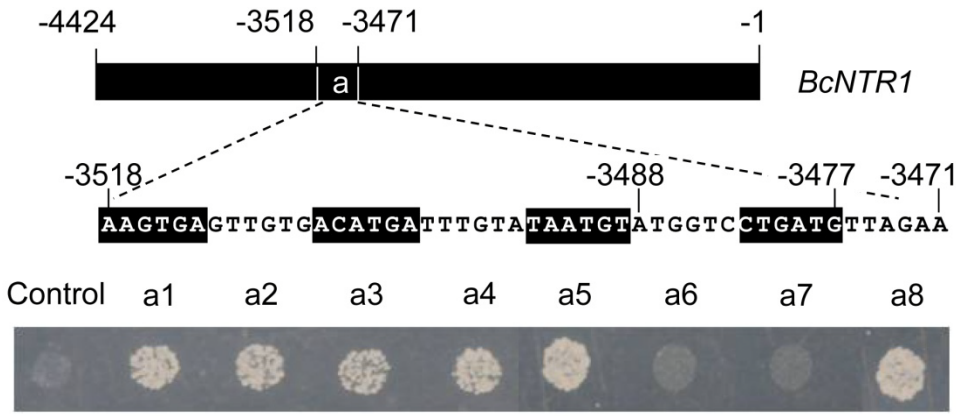


Figure 13. (A) Structures of reporter and activator genes used in Y1H assays. The promoter region of *BcNTR1*, -3,518 to -3,390, was divided into 3 segments and each segment was used as bait for Y1H assays. The control does not contain any of those segments. AtBBD1 was fused with the GAL4 activating domain (AD) as an activator. The position of the putative JARE is shown (▼). (B) The segment a was divided further into 8 subsegments (6 nt each) and each subsegment, a1 to a8, was mutated into 6 adenines. Each mutant segment was tested as bait in Y1H assays.
(C) Subsegments a6 and a7 to which AtBBD1 bound to, were dissected further by mutation in overlapping frames. In each mutant, 6 nucleotides were mutated into 6 adenines. Each mutant subsegment, M0-M5, was tested by Y1H assays. The sequence motif to which AtBBD1 binds is shown in bold. (D) Mutation analysis of the AtBBD1 binding element. Mutant series (CM1 to CMR) of JARE was created by changing a single nucleotide from purine to pyrimidine, or *vice versa*, in the fragment -2,305 to -2,278 as shown in Fig 4A as a bait and Y1H assays were carried out with AD-AtBBD1. CMR is a JARE in reverse orientation

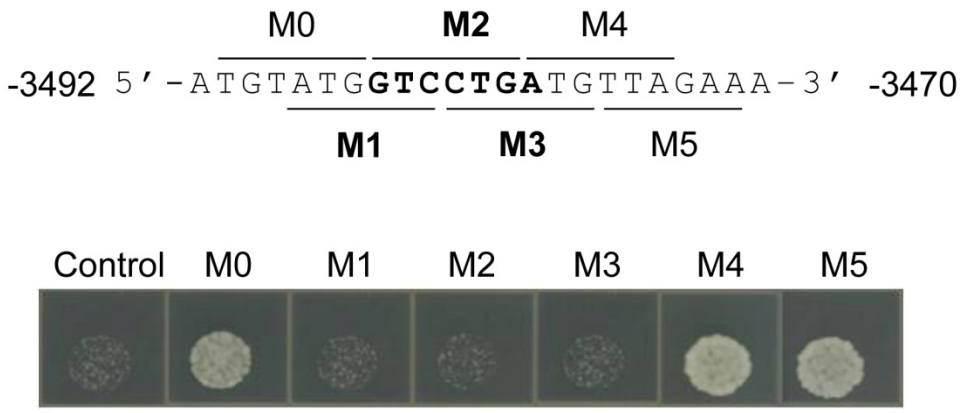
A


-4424 -3518 -3390 -1
BcNTR1
a b c
-3518 -3471 -3430 -3390
Reporter
a, b, or c
P minHis3
HIS3
Activator
P ADH1
AD
AtBBD1
T ADH1
Control
abc
a
b
c

B

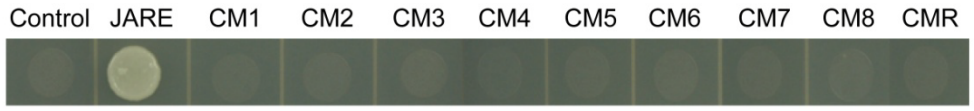

-4424
-3518
-3471
-1
a
BcNTR1
-3518
-3488
-3477
-3471
AAGTGAGTTGTGACATGATTTGTATAATGTATGGTCCTGATGTTAGAA
Control
a1
a2
a3
a4
a5
a6
a7
a8

C


M0
M2
M4
-3492 5′-ATGTATGGTCCTGATGTTAGAAA-3′ -3470
M1
M3
M5
Control
M0
M1
M2
M3
M4
M5

D

GTCCTGA	JARE	CTCC**G**GA	CM5
ATCCTGA	CM1	CTCCT**A**A	CM6
C**G**CCTGA	CM2	CTCCTG**C**	CM7
CT**A**CTGA	CM3	**T**TCCTGA	CM8
CTC**A**TGA	CM4	**TCAGGAC**	CMR



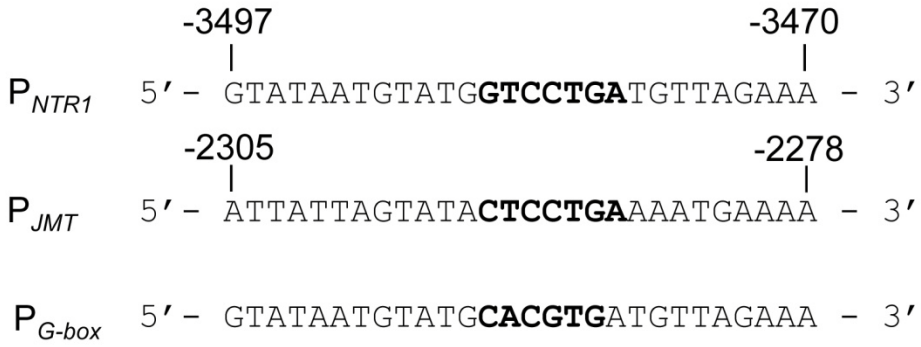
6. JARE is distinct from the G-box

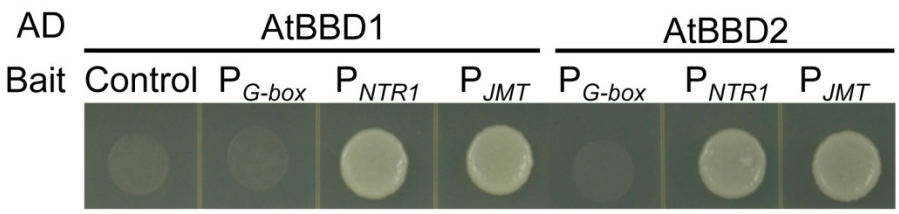
To test the specificity of AtBBD1 binding, Y1H assays were carried out with promoter segments containing different JA-responsive *cis*-elements, including the G-box, placed into the same sequence context as the JARE. For JARE-containing constructs, $P_{NTR1,}$ (-3,497 to -3,470 of the *BcNTR1* promoter) or P_{JMT} (-2,305 to -2,278 of the *AtJMT* promoter), which contain the JARE, were used as bait in the Y1H assay. For the G-box, $P_{G\text{-}box}$ is a mutant version of P_{NTR1} in which the JARE was replaced with a G-box. The G-box is a typical JA-responsive element and is bound by AtMYC2 (Boter et al., 2004). Y1H results showed that AtBBD1 interacted with P_{JMT} and P_{NTR1} but did not interact with $P_{G\text{-}box}$ (Fig. 14A). Therefore, the JARE ((G/C)TCCTGA) of P_{NTR1} and P_{JMT}, is a distinct *cis*-element in the *AtJMT* and *BcNTR1* promoters for JA-responsive gene expression. AtBBD1 could regulate expression of *BcNTR1* and *AtJMT* in response to JA through binding to the JARE. AtBBD2, a homologue of AtBBD1, also bound to the same DNA sequences as JARE in Y1H assays (Fig 14A).

On the other hand, *myc2* knockout mutant analysis was carried out to understand *AtJMT* gene expression in response to jasmonate. Two T-DNA insertion knockout mutants of MYC2, *jin1-7* and *jin1-8* ,were analysed by RT-PCR. Gene expression pattern of *AtJMT* in the *myc2* mutants after MeJA application was the same as that in wild type Col-0 (Fig 14B). It means that MYC2 TF is not a critical factor for *AtJMT* induction in response to jasmonate.

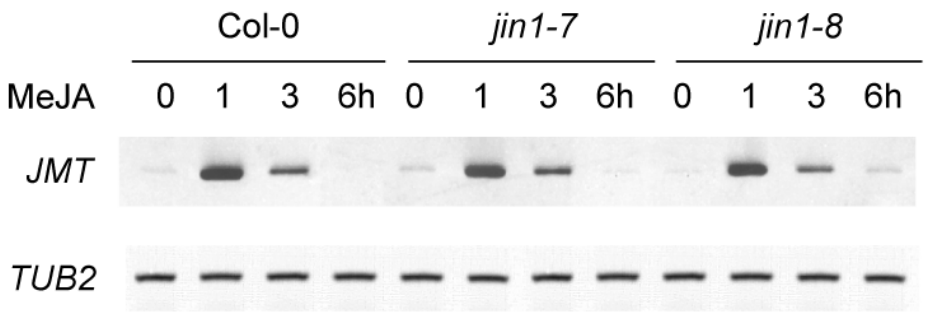
Figure 14. (A) Promoter segments, $P_{NTR1,}$ (-3,497 to -3,470 of *BcNTR1* promoter) or P_{JMT} (-2,305 to -2,278 of *AtJMT* promoter) were used as bait in Y1H assays. A mutated segment, $P_{G\text{-}box}$, which contains G-box sequence in P_{NTR1} was used as a bait and an empty vector (pHIS2) was used as a control. AtBBD1 and AtBBD2 were fused with AD. G-box is AtMYC2 binding element (CACGTG) (Boter et al., 2004). (B) Gene expression pattern in *myc2* knockout mutatnt. MeJA response of *AtJMT* gene in Col-0, *jin1-7* (salk_040500), and *jin1-8* (salk_061267) after MeJA treatment . *AtJMT* and *TUB2* was analyzed by RT-PCR.

A


-3497
-3470
P NTR1 5′- GTATAATGTATGGTCCTGATGTTAGAAA - 3′
-2305
-2278
P JMT 5′- ATTATTAGTATACTCCTGAAAATGAAAA - 3′
P G-box 5′- GTATAATGTATGCACGTGATGTTAGAAA - 3′


AD
AtBBD1
AtBBD2
Bait
Control
P G-box
P NTR1
P JMT
P G-box
P NTR1
P JMT

B



7. The C-terminal region of AtBBD1 has DNA binding activity

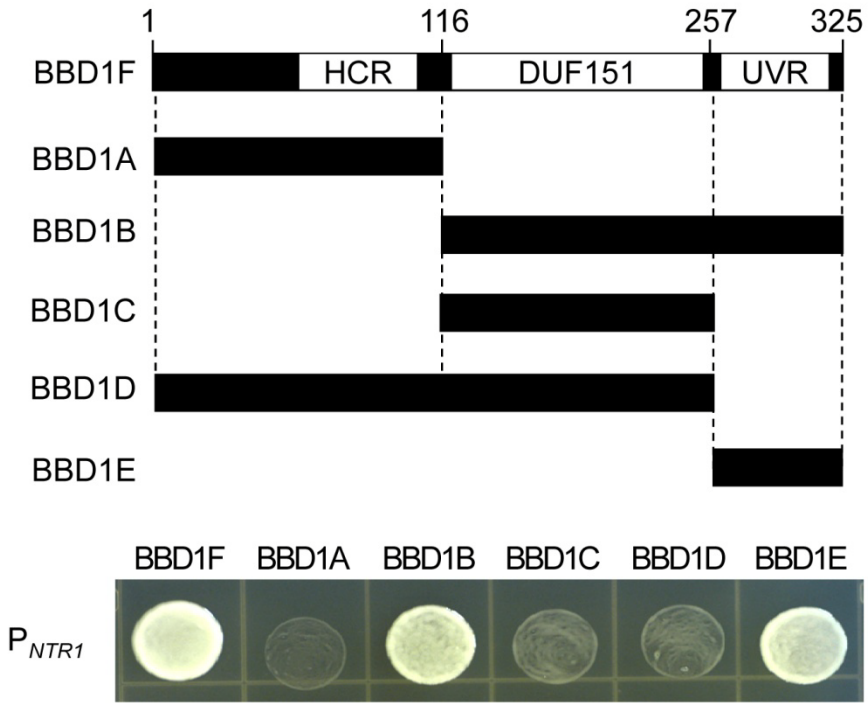
Sequence analysis of the BBD1 protein family had previously shown that the BBD1 proteins contain several conserved domains, including a highly conserved region (HCR), a domain of unknown function 151 (DUF151), and a UV responsive (UVR) domain at the C-terminus (You et al., 2010). However, this analysis did not identify a known DNA-binding motif; therefore, it was tested whether different domains of AtBBD1 had DNA-binding activity. Five truncated protein constructs (BBD1A-E) each containing one or two domains of AtBBD1 were made and fused these with AD for Y1H assays to determine their DNA binding activity (Fig. 15A). Each construct was co-transformed into yeast with a bait DNA sequence (P_{NTR1}) that is known to interact with full-length AtBBD1. Constructs BBD1B (116-325) and BBD1E (257-325), which both contain the C-terminal predicted UVR domain, showed DNA binding activity in yeast, but the other constructs showed no DNA binding activity (Fig. 15A). This result suggests that the AtBBD1 DNA binding domain resides in the C-terminal region.

To confirm the DNA binding activity of BBD1E (257-325) by electrophoretic mobility shift assays (EMSA), MBP-BBD1E, which fused amino acids 257-325 with MBP was made and expressed in this fusion protein in *E. coli*. Crude extracts containing MBP-BBD1E bound to the 70 bp DNA fragment containing the JARE of the *BcNTR1* promoter. Competition assays with unlabeled probe showed the specificity of binding (Fig. 15B). These results show that amino

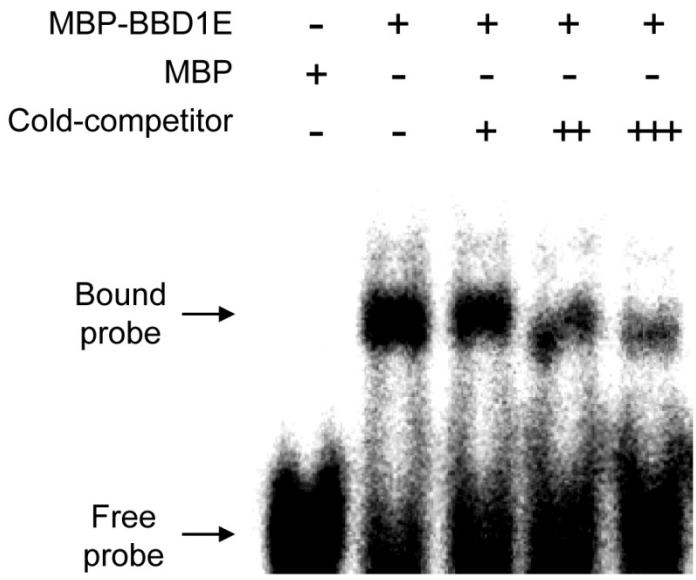
acid residues from 257 to 325 at the C-terminus are involved in DNA binding by AtBBD1.

Figure 15. (A) A schematic representation of truncation mutants of AtBBD1. Numbers indicates amino acid residues, and putative domains are represented (HCR, Highly Conserved Region; DUF151, Domain Unknown Function 151; UVR , putative UV-Response domain) (You et al., 2010). Each truncated protein was fused with AD as shown in Fig 5A. P_{NTR1} (Fig.6) was used as a bait DNA sequence (bottom). (B) Electrophoretic mobility shift assays were carried out using fusion protein (MBP-BBD1) and a 70 bp fragment containing JARE was used as a probe.

A


1
116
257
325
BBD1F
HCR
DUF151
UVR
BBD1A
BBD1B
BBD1C
BBD1D
BBD1E
BBD1F
BBD1A
BBD1B
BBD1C
BBD1D
BBD1E
P NTR1

B



8. AtBBD1 interacts with the ZIM/TIFY domain of AtJAZ1 through its HCR domain

Because JAZ proteins interact with various transcription factors involved in JA-responsive gene expression, yeast-two-hybrid (Y2H) assays was used to test whether AtBBD1 interacts with *Arabidopsis* JAZ proteins. Full-length AtBBD1 was fused to the GAL4 DNA binding domain (BD) and the full-length protein for each of of 12 AtJAZs was fused to the AD. AtBBD1 showed strong interactions with AtJAZ1 and AtJAZ4 in Y2H assays (Fig. 16A).

To confirm the results of the Y2H assay, *in vitro* pull-down assays were carried out. Recombinant MBP-AtJAZ1 bound to amylose resin was incubated with plant extracts prepared from a 35S::6xMYC-AtBBD1 transgenic plant and pulled-down proteins were analyzed by Western blotting with anti-MYC antibody. Recombinant 6xMYC-AtBBD1 was pulled down by recombinant MBP-AtJAZ1 (Fig. 16B). To confirm the interaction between AtBBD1 and AtJAZ1 *in vivo*, 35S:6xMYC-AtBBD1 and 35S:3xHA-AtJAZ1 constructs were transiently coexpressed in tobacco leaves by agroinfiltration. Leaf extract was immunoprecipitated with anti-HA antibody and then immunoblotted with anti-MYC antibody (Figure 16C). These results showed that AtBBD1 directly interacts with AtJAZ1.

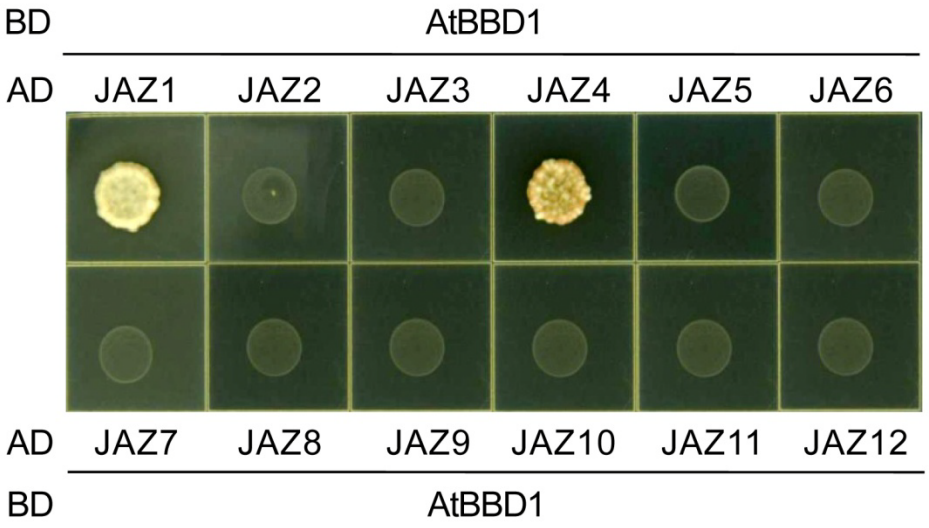
To identify the domain of AtBBD1 that mediates interaction with AtJAZs, truncated AtBBD1 proteins were fused with the AD and full length AtJAZ1 was

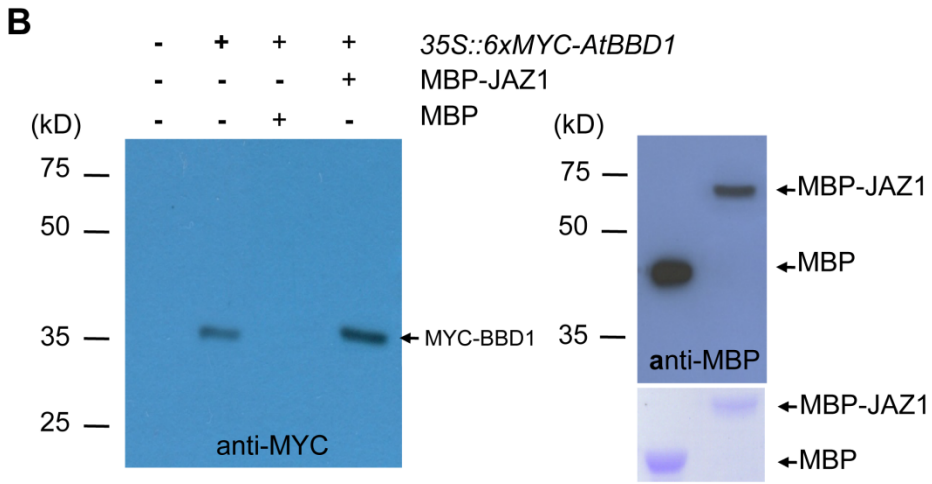
fused with BD for Y2H assays. Y2H results showed that BBD1A (1-116), and D (1-257) interacted with AtJAZ1 in yeast, indicating that the N-terminal domain between amino acid residues 1 and 116 of AtBBD1, which includes the HCR domain, interacts with AtJAZ1 (Figure 17).

Reciprocally, to identify the domain of AtJAZ1 that mediates interaction with AtBBD1, 5 truncated protein constructs containing the ZIM/TIFY or Jas domains of AtJAZ1 were designed for Y2H assays. JAZ1A (1-204) and JAZ1B (100-181) fragments as well as JAZ1F (full length JAZ1) interact with AtBBD1. These results indicate that the amino acid sequence from 100 to 181 of AtJAZ1, which contains the ZIM/TIFY domain, is responsible for interaction with AtBBD1 (Figure 18). Therefore these results lead us to conclude that the N-terminal region containing the HCR domain of AtBBD1 interacts with ZIM/TIFY domain of AtJAZ1 in *Arabidopsis*.

Figure 16. (A) Y2H assay between AtBBD1 and each of 12 AtJAZs. Full length CDS of AtBBD1 was fused to GAL4 DNA binding domain (BD) and each full length CDSs of 12 AtJAZs was fused to AD. (B) The pull-down assay between AtBBD1 and AtJAZ1. *35S:6xmyc-AtBBD1* plant extract (input) was incubated with amylose resin bound recombinant MBP-AtJAZ1 protein. Pulled-down protein complex was detected by immunoblotting using anti-MYC antibody (left). MBP protein was used as a pull-down control. The panel on the right shows input recombinant MBP and MBP-AtJAZ1 proteins in the pull-down assay.
(C) Immunodetection of the AtBBD1 and AtJAZ1 complex *in vivo.* 35S:6xMYC-AtBBD1 and 35S:3xHA-AtJAZ1 constructs were transiently coexpressed in tobacco leaves by agroinfiltration. The expressed proteins were immunoprecipitated (IP) using anti-HA antibody (+/+) and immunoblotting was carried out with anti-myc antibody. Left lane (-/-) is control leaf extract that was not agroinfiltrated. MYC-AtBBD1 and HA-AtJAZ1 proteins were detected in input coexpressed leaf extracts by each antibody (right).

A


BD
AtBBD1
AD
JAZ1
JAZ2
JAZ3
JAZ4
JAZ5
JAZ6
AD
JAZ7
JAZ8
JAZ9
JAZ10
JAZ11
JAZ12
BD
AtBBD1


B
- + + + 35S::6xMYC-AtBBD1
- - - + MBP-JAZ1
(kD) - - + - MBP
75
50
35
25
anti-MYC
MYC-BBD1
(kD)
75
50
35
anti-MBP
MBP-JAZ1
MBP
MBP-JAZ1
MBP

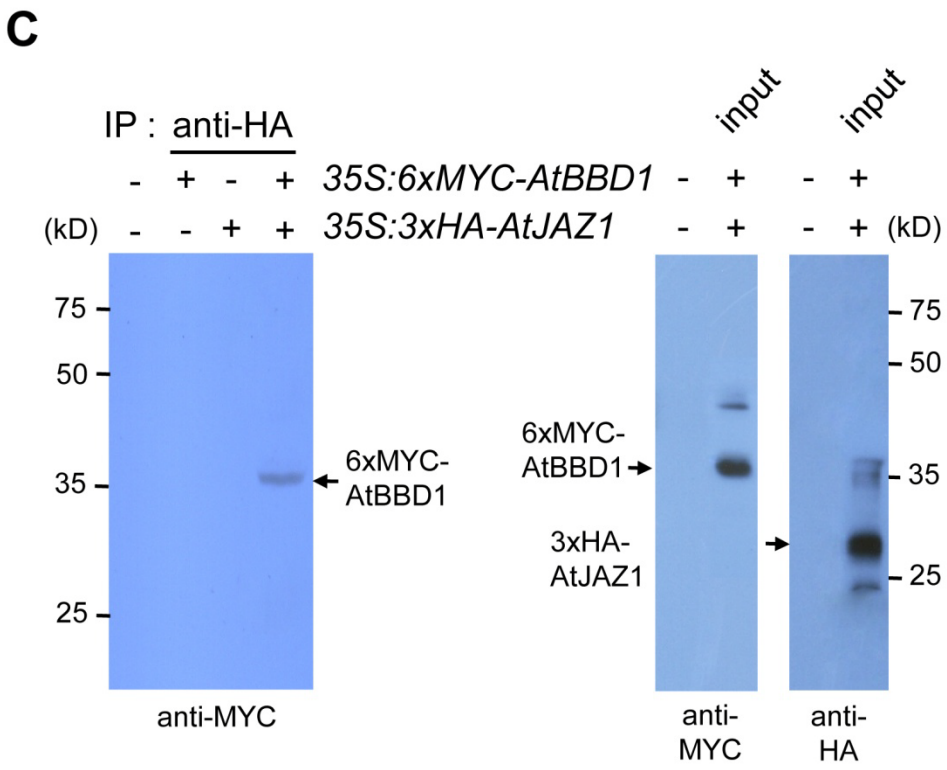

C
IP : anti-HA
input
input
- + - + 35S:6xMYC-AtBBD1 - + - +
(kD) - - + + 35S:3xHA-AtJAZ1 - + - + (kD)
75
50
35
25
6xMYC-
AtBBD1
anti-MYC
6xMYC-
AtBBD1
3xHA-
AtJAZ1
75
50
35
25
anti-
MYC
anti-
HA

Figure 17. A schematic representation of truncation mutants of AtBBD1 (top) and Y2H results between JAZ1 and each truncated construct (bottom). Each truncated AtBBD1 protein was fused to AD as a prey for Y2H assay with AtJAZ1. AtJAZ1 was fused to BD as a bait. Numbers indicates amino acid residues, and putative domains were represented.

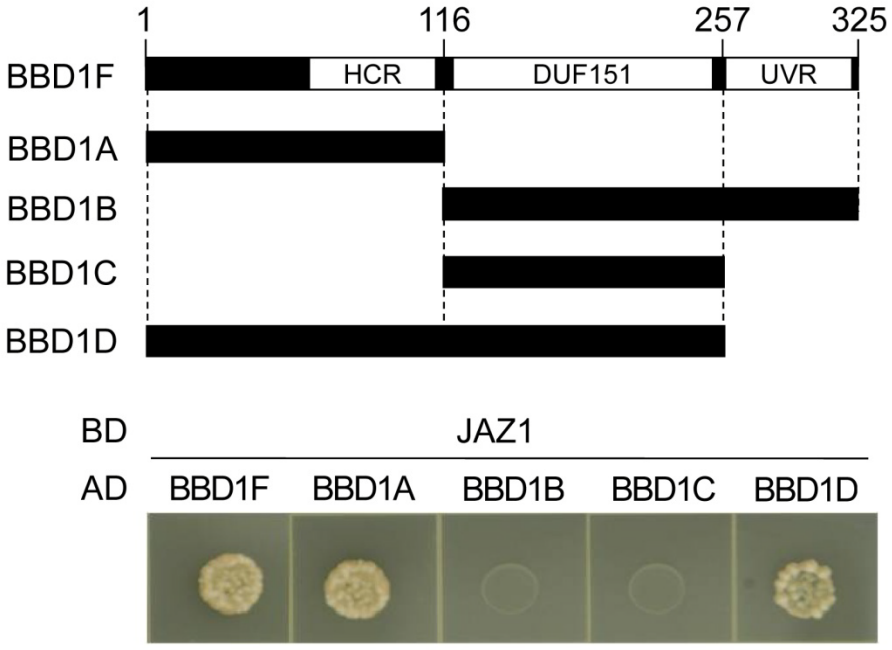
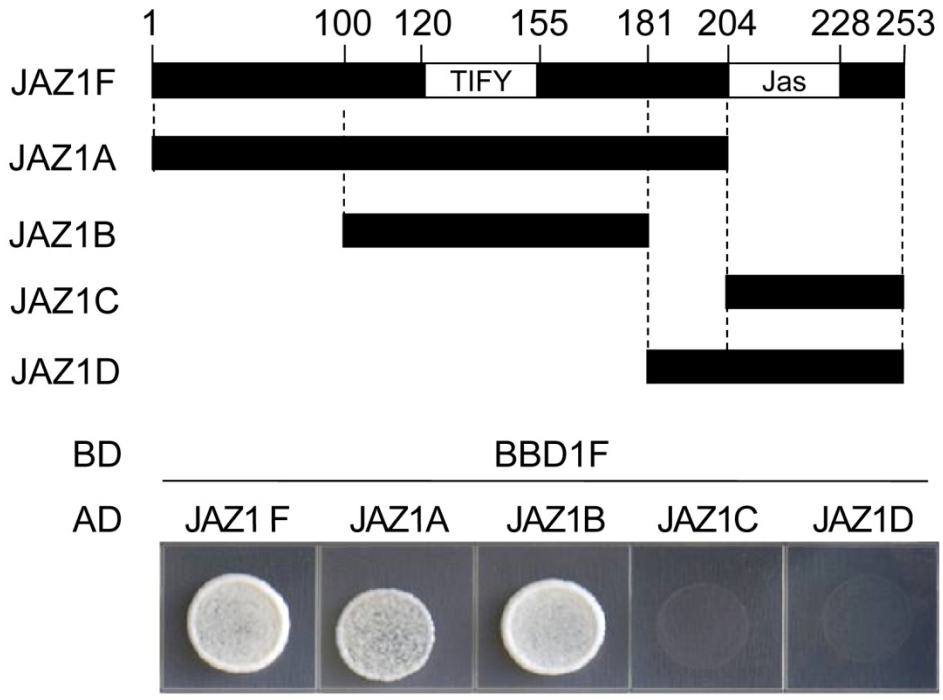

1
116
257
325
BBD1F
HCR
DUF151
UVR
BBD1A
BBD1B
BBD1C
BBD1D
BD
JAZ1
AD
BBD1F
BBD1A
BBD1B
BBD1C
BBD1D

Figure 18. A schematic representation of truncation mutants of AtJAZ1 (top) and Y2H results between BBD1 and each truncated construct (bottom). Each truncated AtJAZ1 protein was fused to AD as a prey for Y2H assay with AtBBD1 protein. AtBBD1 was fused to BD as a bait.. Numbers indicates amino acid residues, and putative domains were represented.

.


1
100
120
155
181
204
228
253
JAZ1F
TIFY
Jas
JAZ1A
JAZ1B
JAZ1C
JAZ1D
BD
BBD1F
AD
JAZ1 F
JAZ1A
JAZ1B
JAZ1C
JAZ1D

9. AtBBD1 negatively regulates *AtJMT*

To investigate the *in vivo* function of AtBBD1 in regulating *AtJMT* gene expression, a T-DNA insertion knockout mutant, *atbbd1,* was examined. When treated with MeJA, the *atbbd1* mutant showed no difference from wild type plants (Col-0) in *AtJMT* gene expression (Fig 20A). However, *AtBBD2,* which has 81% amino acid sequence identity to *AtBBD1* (Fig 12), may have overlapping functions. But *atbbd2* mutant also showed no difference from wild type plants (Col-0) in *AtJMT* and *JR2* gene expression (Fig 20B). To test whether these two genes act redundantly, the double knockout mutant, *atbbd1 atbbd2,* was made by crossing the *atbbd1* and *atbbd2* single mutant plants (Fig. 19A, B and Fig 20B). When the double knockout plants were treated with MeJA, *AtJMT* expression was induced to a higher level and the induction lasted longer than in wild type. In wild type, induction of *AtJMT* transcription by JA was short-lived and transcript levels began to decline after 3 hours of MeJA induction, but in double knockout plants, *AtJMT* transcript levels continued to increase, even 6 hours after MeJA treatment (Fig. 20A). The *atbbd1 atbbd2* plants however, showed reduced levels of the JA-regulated gene *JR2* in response to MeJA treatment. These results showed that AtBBD1 and AtBBD2 have redundant functions as negative regulators of *AtJMT* gene expression in response to MeJA, but may act as positive regulators of *JR2*.

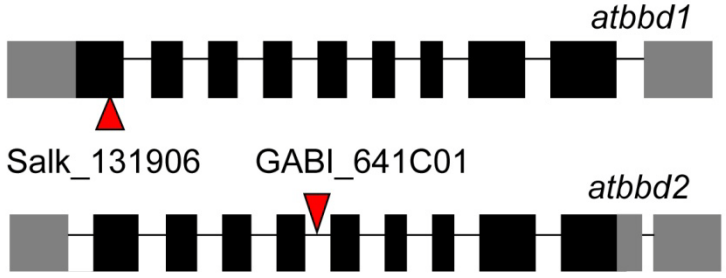
To understand *AtBBD1* function further, *AtBBD1* was overexpressed under the control of the CaMV 35S promoter in transformed lines (Fig. 21A and B).

Transgenic lines OX4 and OX13, single copy transformants, were selected for further analysis. Consistent with the *atbbd1 atbbd2* mutant phenotype, the basal and JA-induced levels of *AtJMT* expression were lower in OX4 and OX13 compared to wild type (Col-0) (Fig. 22A and B). Also, JA-regulated *JR2* gene expression was enhanced in overexpression plants. These results further support the hypothesis that AtBBD1 functions as a repressor of *AtJMT* gene expression *in vivo* by binding to JARE but acts as a positive regulator of *JR2*.

Figure 19. (A) Gene structure of *AtBBD1* and *AtBBD2*. Closed boxes and solid lines denote exons and introns, gray boxes represent UTRs and red triangles show T-DNA insertion site, respectively. (B) RT-PCR anaylsis demonstrated that single and double knockout plant showed no transcripts.

.

A



B

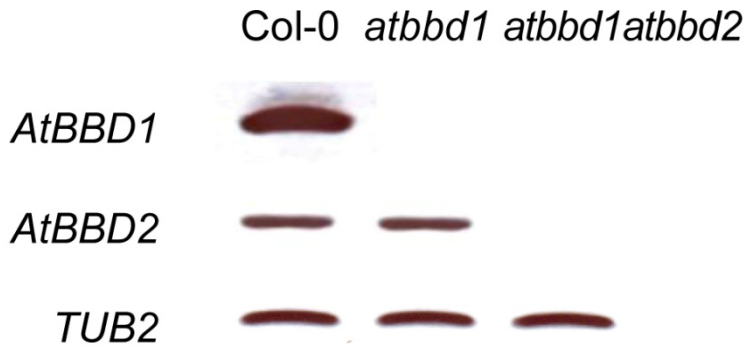
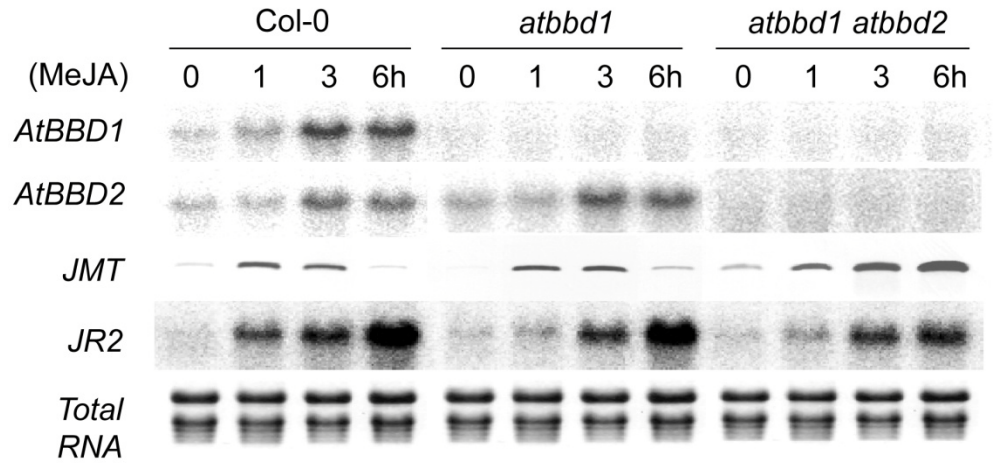


Figure 20. (A) MeJA response of *AtJMT* in Col-0, *atbbd1,* and *atbbd1 atbbd2* mutants after MeJA treatment. *AtBBD1, AtBBD2* and *JR2* were analyzed by Northern blot, and *AtJMT* was analyzed with RT-PCR. (B) MeJA response of *AtJMT* gene in Col-0 and *atbbd2* after MeJA treatment. *AtBBD2* and *JR2* were analyzed by Northern blot, and *AtJMT* was analyzed with semi-quantitative RT-PCR.

A


Col-0
atbbd1
atbbd1 atbbd2
(MeJA)
0 1 3 6h 0 1 3 6h 0 1 3 6h
AtBBD1
AtBBD2
JMT
JR2
Total RNA

B

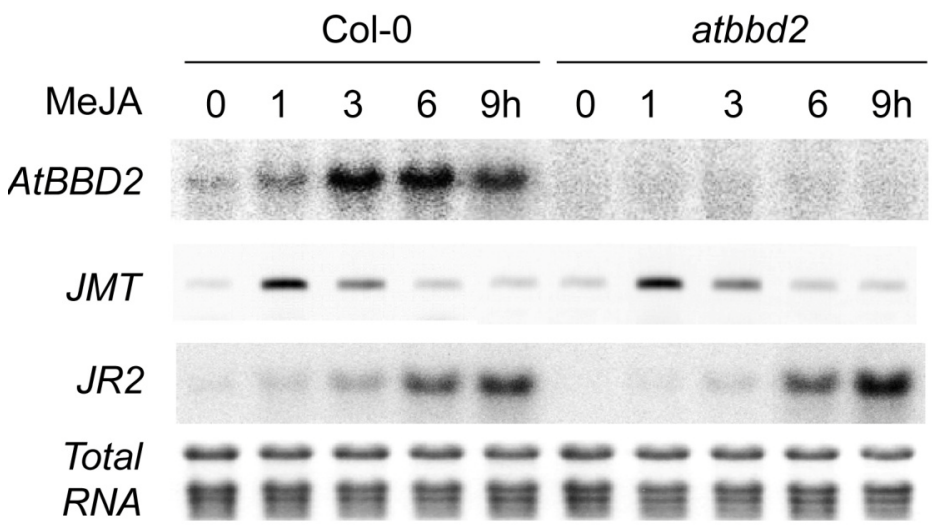
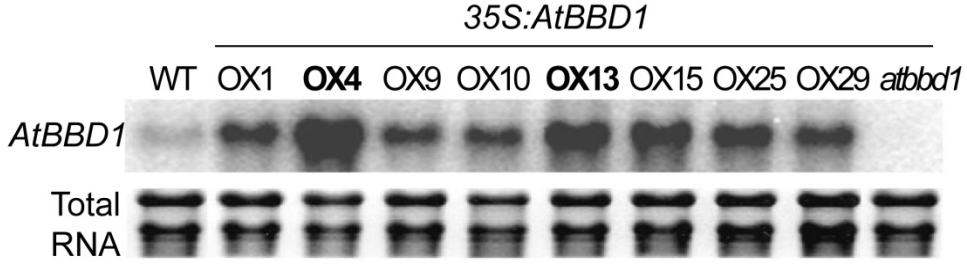


Figure 21. (A) Northern blot shows the overexpression of *AtBBD1* in transgenic plants. (B) Genomic Southern blot indicates copy numbers of the inserted T-DNA . Genomic DNA was digested with *Pst*I and *Eco*RI, and the blot was hybridized with a *NPTII* probe. The probe position is shown by solid line on the recombinant construct.

A


35S:AtBBD1
WT
OX1
OX4
OX9
OX10
OX13
OX15
OX25
OX29
atbbd1
AtBBD1
Total
RNA

B

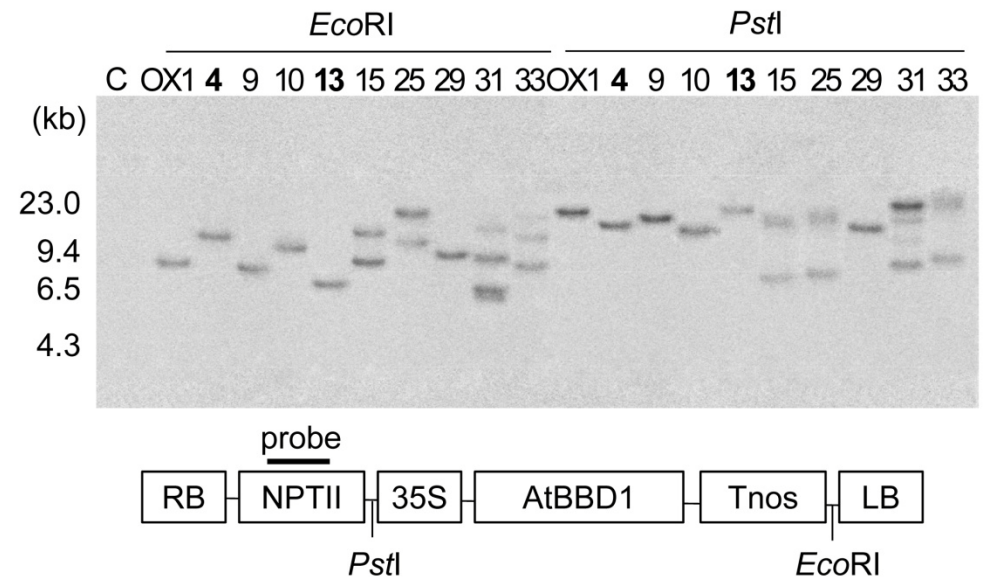
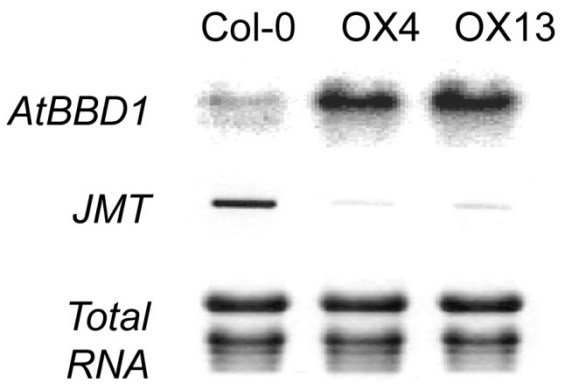
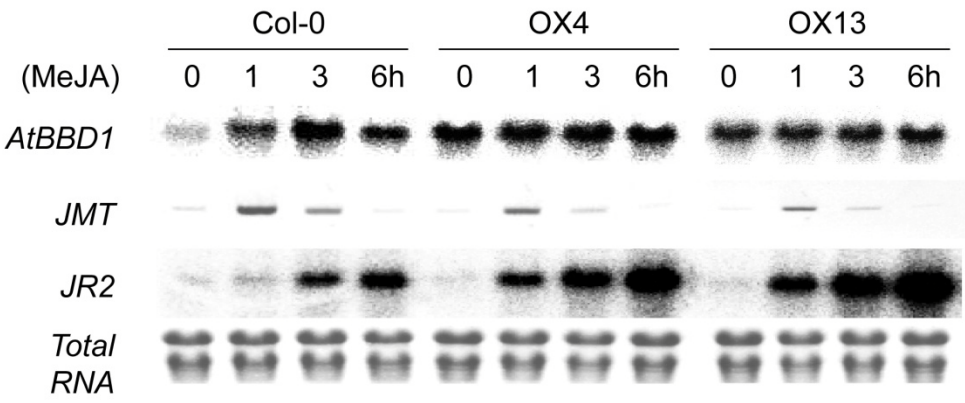

EcoRI
PstI
C OX1 4 9 10 13 15 25 29 31 33 OX1 4 9 10 13 15 25 29 31 33
(kb)
23.0
9.4
6.5
4.3
probe
RB
NPTII
35S
AtBBD1
Tnos
LB
PstI
EcoRI

Figure 22. (A) Basal levels of *AtJMT* expression in Col-0, OX-4, and OX-13. (B) MeJA response of *AtJMT* expression between Col-0, OX-4, and OX-13. *AtBBD1* and *JR2* was analyzed by Northern blot and *AtJMT* was analyzed by RT-PCR

A



B

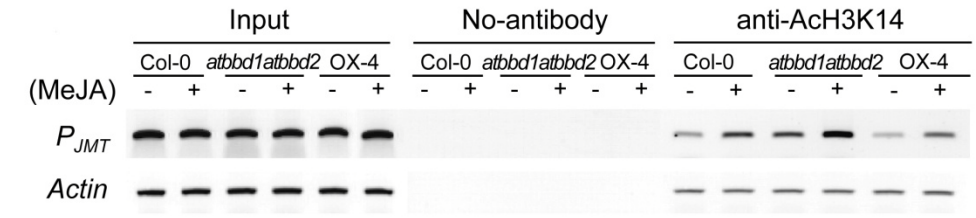

Col-0
OX4
OX13
(MeJA)
0 1 3 6h 0 1 3 6h 0 1 3 6h
AtBBD1
JMT
JR2
Total RNA

10. Chromatin immunoprecipitation reveals that AtBBD1 repression of *AtJMT* is associated with histone deacetylation

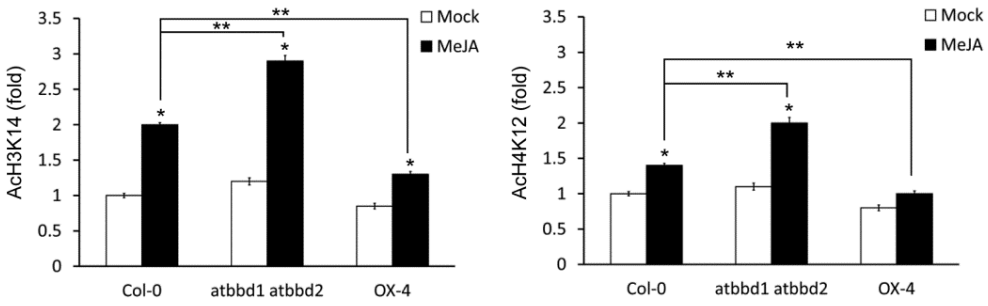
To understand the repression mechanism by which AtBBD1 affects *AtJMT* gene expression, the level of histone acetylation in the promoter region of *AtJMT* was examined. It has been reported that JAZ1 interacts with HDA6 directly and contributes to histone deacetylation (Zhu et al., 2011). Chromatin immunoprecipitation was carried out with antibodies against modified histones. Fragmented chromatin DNA was incubated with anti-AcH3K14 or anti-AcH4K12 antiserum and isolated DNA was amplified with sets of primers specific to the *AtJMT* promoter region containing the JARE. Upon MeJA treatment, the levels of histone H3 and H4 acetylation in the promoter region of *AtJMT* were enhanced in *atbbd1 atbbd2* mutant plants but reduced in OX-4 compared to wild type plants (Fig 23A). The basal level of histone acetylation in the promoter region of *AtJMT* was also higher in *atbbd1 atbbd2* double knockout plants and lower in OX-4 compared to wild type. qPCR data showed that the histone acetylation level of *AtJMT* was significantly different from wild type, double knockout and OX-4 plants at a confidence level of $P<0.05$ (Figure 23B). These results showed that AtBBD1 repression of *AtJMT* is associated with histone deacetylation; this deacetylation may occur through the AtBBD1 interaction with JAZ1, which was reported to interact with HDAC6 (Zhu et al., 2011).

Figure 23. (A) Chromatin immunoprecipitation was carried out with antibodies recognizing acetylated histone H3 (AcH3K14) or H4 (AcH4K12). Precipitated DNA was amplified by primers corresponding to sequences adjacent to the AtBBD1 binding sites in the *AtJMT* promoter (P_{JMT}). PCR product was analyzed by agarose gel electrophoresis. Actin was used as a control. Input indicates samples before immunoprecipitation. (B) qPCR analysis of ChIP assay with Col-0, *atbbd1 atbbd2*, and OX-4. Open bar is without MeJA treatment and closed bar is with MeJA treatment for 3 hours. Relative fold difference is represented. Mean ±SD, $n = 3$ (t test: $^{*}P \leq 0.05$: comparison with the value of Col-0, $^{**}P \leq 0.05$: comparison between indicated values). Statistical significance of the measurements was determined using a t-test ($P \leq 0.05$) by comparison with the value of Col -0 (*). and by Comparison between indicated values is also shown by **. Data represent the mean values of 3 independent experiments and error bars represent standard deviation.

A


Input
No-antibody
anti-AcH3K14
Col-0
atbbd1atbbd2
OX-4
(MeJA)
- + - + - + - + - + - + - + - + - + - +
P_{JMT}
Actin

B


AcH3K14 (fold)
3.5
3
2.5
2
1.5
1
0.5
0
Mock
MeJA
**
**
*
*
*
Col-0
atbbd1 atbbd2
OX-4
AcH4K12 (fold)
3.5
3
2.5
2
1.5
1
0.5
0
Mock
MeJA
**
**
*
*
Col-0
atbbd1 atbbd2
OX-4

DISCUSSION

Identification of a JARE in the *AtJMT* and *BcNTR1* promoters

Here we have identified a novel *cis*-element, the JARE, which regulates JA-responsive gene expression and contains a heptanucleotide sequence motif (G/C)TCCTGA. The JARE was identified in two orthologous genes encoding an enzyme involved in JA biosynthesis, -3480 bp upstream of *BcNTR1* and –2290 bp upstream of *AtJMT*, respectively. The same JARE sequence is present in both genes, although at slightly different positions in each promoter. Considering that the two genes are orthologous, encoding the same enzymatic activities in the same plant family, the two loci could have conserved mechanisms of transcriptional regulation in which they share homologous *cis*-acting elements and *trans*-acting factors. The JARE is also found in the promoters of other Arabidopsis JA-responsive genes, including *LOX2, COI1, JAZs (6, 7, 8), WRKY70, PDF1.2, VSP1,* and *MYBs (24, 44)*, and in other plants, including the promoter of the rice JA-responsive gene *OsbHLH148*. Therefore, JA-responsive regulation through the JARE may affect many Arabidopsis genes and may also be conserved beyond the *Brassicaceae*.

The JARE is distinct from other JA-responsive elements previously reported. For example, G-box (CACGTG) and GCC motifs (GCCGCC) are known JA-responsive elements in plants (Dombrecht et al., 2007; Memelink, 2009). There are several G-boxes or G-box like elements in the *BcNTR1* and *AtJMT* promoters,

but tests of promoter deletions and multimerized JARE constructs showed that these G-box elements are not necessary for the JA response of *AtJMT* and *BcNTR1* expression (Fig. 9). Moreover, MYC2 is a TF that interacts with the G-box to regulate JA-responsive genes (Boter et al., 2004) and *AtJMT* gene expression in response to MeJA treatment was not affected in *myc2* knockout plants (*jin1-7 and jin1-8*) (Fig. 14B). These results therefore indicate that JA-responsive regulation of *AtJMT* and *BcNTR1* occurs through the JARE and not through G-box elements and suggest that the transcription factors binding to JARE could be different from bHLH transcription factors like MYC2.

The JARE is also distinct from other reported JA-responsive *cis*-elements. For example, the GCC motif was initially defined as an ethylene (ET)-responsive element in EREBPs (Ohme-Takagi and Shinshi, 1995), but it also plays a role in conferring JA- and ET-responsive expression of the *PDF1.2* gene (Brown et al., 2003). Also, TGACG sequences were found to be essential for the JA response in promoters of tobacco *nopaline synthase* (*nos*) and barley *lipoxygenase 1* (*LOX1*) genes (Kim et al., 1993, 1994; Rouster et al., 1997). JASE1 (CGTCAATGAA) and JASE2 (CATACGTCGTCAA) of *Arabidopsis OPR1* are also reported to be JA-responsive motifs (He and Gan, 2001). All of these motifs reported as JA-responsive elements are different from the JARE, which is therefore a novel *cis*-element controlling JA-responsive gene expression.

AtBBD1 binds to JARE

To understand the mechanism of *AtJMT* gene expression regulation by the JARE *cis*-acting element, we identified a *trans*-acting factor, AtBBD1, which binds to the JARE. *AtBBD1* is an *Arabidopsis* homologue of *OmBBD1*, which is involved in ABA dependent callose deposition (You et al., 2010). *AtBBD1* expression was induced by various plant hormones such as MeJA, SA, ABA, and ETP (Fig. 12B). The complex regulation of *AtBBD1* indicates that it may act in additional hormonal responses, or in cross-talk among hormonal signaling pathways.

AtBBD1 specifically binds to the JARE sequence, which we defined by mutational analysis as (G/C)TCCTGA, and does not bind to other sequences such as the G-box (Fig. 14A). Also *AtJMT* expression in MYC2 knockout mutant showed that MYC2 is not critical for JA-responsive *AtJMT* gene exression (Fig. 14B).

Sequence analysis of AtBBD1 did not identify a known DNA binding motif, but Y1H assays revealed that that the AtBBD1 C-terminal domain, containing the UVR domain, binds to JARE. The DNA binding motif of AtBBD1 is also similar to the C-terminal region of DELLA proteins (GAI, and RGA/RGLs) although the DNA binding domain of DELLA proteins has not yet been clearly defined. Consistent with the ability of AtBBD1 to bind DNA, AtBBD1-sGFP fusion proteins were localized in the nuclei of the transformed *Arabidopsis* plants (Fig. 12C). Therefore, the characteristics of AtBBD1 are consistent with a role as a nuclear transcription factor.

AtBBD1 interacts with AtJAZ1

The finding that AtBBD1 interacts with JAZ1 and JAZ4 (Fig. 16) suggests a possible mechanism for JA-responsive regulation through the JARE, as many JA-regulated TFs are controlled by interaction with JAZ proteins. For example, various responses to JA including root growth, stress response, anthocyanin accumulation, trichome initiation, and stamen development are regulated by the interaction between JAZs and transcription factors such as DELLAs, MYCs, MYBs, and bHLHs (Hou et al., 2010; Fernandez-Calvo et al., 2011; Song et al., 2011; Qi et al., 2011). JAZ proteins bind to transcription factors and recruit the co-repressor TPL directly or through NINJA (Howe et al., 2010; Pauwels et al., 2010).

There are reports that JAZ proteins also interact with HDA6 directly and contribute to histone deacetylation (Pauwels et al., 2010; Zhu et al., 2011). Transcriptional repression is released when JAZs are degraded by SCF^{COI1} complex-mediated 26S proteasome in a JA-dependent manner and the TFs can then activate target gene transcription. These TFs therefore activate early responses when JAZ proteins are degraded by the 26S proteasome in initiation of JA signaling.

Considering their narrow spectrum of interaction with JAZs, AtBBD1 and 2 are expected to be involved in a specific subset of JA-related defense signaling, rather than global JA-responses. AtMYC2 interacts with most of the JAZ proteins and is involved in most JA-related phenotypes (Pauwels and Goossen, 2011). By contrast, other TFs involved in specific JA-responses, including TFs such as

MYB21/24 and EGL3/GL3/TT8, interact with a small set of JAZ proteins (Qi et al., 2011; Song et al., 2011).

AtBBD1 and AtBBD2 repress *AtJMT* gene expression

Unlike other JA-dependent transcription factors, which act as transcriptional activators after release from JAZ interaction, AtBBD1 acts to repress expression of the JARE-regulated target gene *AtJMT*. This repression is shown by induction of *AtJMT* expression in mutants lacking both AtBBD1 and its close homologue AtBBD2 (Fig. 20). These results also suggest that AtBBD2 is functionally redundant with AtBBD1. Similar to other JAZ-interacting proteins, the JAZ-AtBBD1 complex could repress *AtJMT* gene expression by recruitment of co-repressors, and by histone deacetylation through interaction between JAZ proteins and HDACs. For example, EIN3/EIL1 directly interact with JAZ1 and HDA6 to repress *ERF1* in JA- and ethylene (ET)-responses through histone deacetylation (Zhu et al., 2011).

AtBBD1/2 can also act, directly or indirectly, as positive activator, as shown by increased *JR2* expression in the *atbbd1 atbbd2* mutant (Fig. 20A). Also, Arabidopsis overexpressing *OmBBD1* showed enhanced expression of the JA-related gene *PDF 1.2* and the ABA-related genes *ABA1, RD29*. Also the *atbbd1* mutant showed susceptibilty to *B. cinerea*. There is no study reporting the double

mutant phenotype yet. According to our results as described in this manuscript, it is expected that the double mutant would be more susceptible to JA-responsive necrotrophic pathogens. AtBBD1 might be involved in blocking of a MeJA metabolic sink and thus may contribute to increasing the local concentration of JA-Ile, an active form of JA (Stitz et al., 2011). AtBBD1 could function as a positive regulator responding to JA by post-translational modification or interaction with other proteins (Eferl and Wagner, 2003). For example, bifunctional TFs, APETALA2 and WUSCHEL, act as activators or repressors on different target genes in plant flower development (Ikeda et al., 2009; Yant et al., 2010)

Moreover, in addition to, or instead of, acting in initial JA responses, AtBBD1 may act during the recovery after JA induction. Slow induction of *AtBBD1* by JA could reflect its role in the recovery phase. Indeed, the double knockout plant *atbbd1 atbbd2* shows a higher level of *AtJMT* transcript at a later time (Fig. 20), indicating a failure of recovery from JA stimulation.

Our results also suggest the existence of a positive regulator or activator responding to JA. For example, induced expression of the multimerized JARE reporter construct (Fig. 9) and increased expression of *AtJMT* in the *atbbd1 atbbd2* mutant are consistent with the presence of an activator that also binds the JARE. Although we did not find such an activator by Y1H hybrid screening, it is possible that the specific activator could compete with AtBBD1 for binding to the JARE. The postulated positive regulator may also be subject to JAZ-dependent regulation, as *AtJMT* expression is still strongly induced by JA in the *atbbd1 atbbd2* mutant

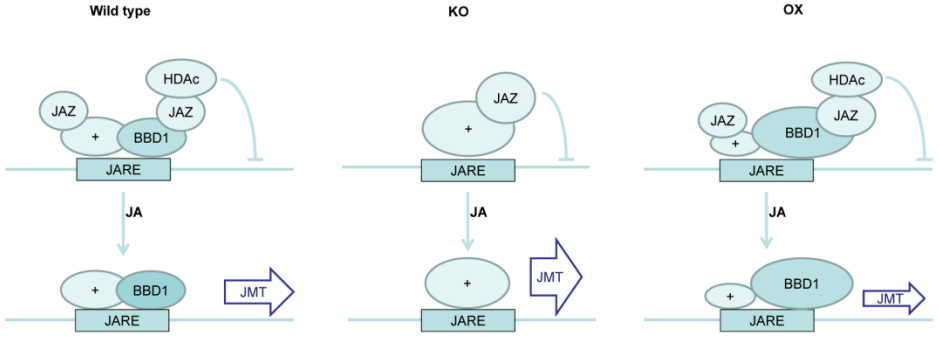
plants.

Regulation by competing positive and negative transcription factors has substantial precedent. In plants, a family of transcription factors, such as the auxin-responsive element binding factors (ARFs) or ET-responsive element binding factors (ERFs), can share the same DNA binding domain and the same *cis*-element, but many of the members have opposite functions in target gene regulation (Fujimoto et al., 2000; Guilfoyle and Hagen, 2007; Tiwari et al., 2003). For example, ERFs bind to the same *cis*-element (GCC-box) but regulate target gene expression in the opposite manner. ERF1, 2, and 5 function as activators and ERF3, 4, and 7-12 function as repressors (Fujimoto et al., 2000; Yang et al., 2005). Their repression activity is conferred by the EAR motif in ERF3 and 4 (Ohta *et al*., 2001; Yang *et al*., 2005). The EAR motif interacts with the corepressor TPL. ERF-TPL complexes repress target genes by modification of chromatin structure through histone deacetylase (Kagale and Rozwadowski, 2011). In the case of ARF family TFs, ARF5-8 and 19 activate target gene expression and the others repress target gene expression although they bind to the same auxin-responsive *cis*-element (Guilfoyle and Hagen, 2007). ARFs require the association with Aux/IAA repressors for an auxin response (Guilfoyle and Hagen, 2007; Tiwari et al., 2003). Many Aux/IAA proteins are degraded by the SCF^{TIR1} complex in an auxin-dependent manner (Chapman and Estelle, 2009). These examples are reminiscent of the mechanism of JA signal transduction through JARE. Therefore, it is possible that AtBBD1 could bind to the JARE as a repressor and that other TFs with a similar DNA binding

domain could compete with AtBBD1 and act as activators.

In conclusion, the mechanism of AtBBD1 negative regulation of *AtJMT* could be postulated to function as follows (Fig. 24); AtBBD1 recognizes the JARE in the *AtJMT* promoter and interacts with AtJAZs. When the JA signal is absent, the AtBBD1-AtJAZ complex recruits co-repressors or HDACs. An activator also competes with AtBBD1 for binding to the JARE; in the absence of JA, this activator may be bound by JAZs. When the JA signal comes in, JAZs are degraded by the 26S proteasome pathway through SCF^{COI}, and AtBBD1 is then released from JAZs. At the same time, the activator is also released from JAZs and activates *AtJMT*. The *AtJMT* expression level is regulated by the balance between activator and AtBBD1. In knockout plants, the activator occupies the JARE and *AtJMT* gene expression is activated to higher levels than wild type because the AtBBD1 repressor is absent. In AtBBD1-overexpressing plants, AtBBD1 occupies the JARE dominantly over the activator and *AtJMT* gene expression is repressed more than in wild type. Identification of the activator protein interacting with JARE would fill out the model more precisely.

Figure 24. In the absence of signal, AtBBD1 represses *AtJMT* gene expression by recruiting corepressor or HDAc through AtJAZ. In the presence of signal, the JA-Ile is released and JAZ proteins are degraded by SCF^{AtCOI1} complex. Putative activator (+) which binds to JARE competes with AtBBD1 (repressor). In knockout plant, putative activator occupies JARE dominantly and *AtJMT* gene expression is activated higher than wild type. In overexpressed plant, AtBBD1(repressor) compete with putative activator and occupies JARE dominantly and *AtJMT* gene expression is repressed more than in wild type. Size of each circle represents relative abundance


Wild type
HDAc
JAZ
JAZ
+
BBD1
JARE
JA
+
BBD1
JARE
JMT
KO
JAZ
+
JARE
JA
+
JARE
JMT
OX
HDAc
JAZ
JAZ
+
BBD1
JARE
JA
+
BBD1
JARE
JMT

REFERENCES

Adie, B.A., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A., and Solano, R. (2007) ABA is an essential signal for plant resistance to pathogens sffecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* **19**: 1665-1681.

Alberts B., Johnson A., Lewis J., Raff M., Roberts K., Walter P. (2002) *Molecular Biology of the Cell*. 4th Edition. Garland Science, NewYork.

Arabidopsis Interactome Mapping Consortium (2011) Evidence for network evolution in an Arabidopsis interactome map. *Science* **333**: 601–607.

Bai, Y., Meng, Y., Huang, D., Qi, Y., and Chen, M. (2011). Origin andevolutionary analysis of the plant-specific TIFY transcription factor family. *Genomics* **98**: 128–136.

Benedetti, C.E., Xie, D., Turner, J.G. (1995) COI1-dependent expression of an Arabidopsis vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. *Plant Physiol.* **109**: 567–572.

Boter, M., Ruı´z-Rivero, O., Abdeen, A., and Prat, S. (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. *Genes Dev.* **18**: 1577–1591.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M. (2003) A role for the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of *Arabidopsis*. *Plant Physiol.* **132**: 1020–1032.

Browse, J. (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annu. Rev. Plant Biol*. **60**: 183-205.

Carpenter, C.D. and Simon, A.E. (1998) Preparation of RNA. *Methods Mol. Biol*. 82, 85-89.

Chapman, E.J., and Estelle, M. (2009) Mechanism of auxin-regulated gene expression in plants. *Annu. Rev. Genet*. **43:** 265-285.

Chen, Z.J., and Tian, L. (2007) Roles of dynamic and reversible histone acetylation in plant development and polyploidy. *Biochim. Biophys. Acta* **1769**: 295-307.

Chini, A., Fonseca, S, Ferna´ndez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcı´a-Casado, G., Lo´pez-Vidriero, I., Lozano, F.M., and Ponce, M.R. (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**: 666–671.

Chini, A., Fonseca, S., Chico, J.M., Ferna´ ndez-Calvo, P., and Solano, R. (2009) The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. *Plant J.* **59**: 77–87.

Chua, Y.L., Watson, L.A., Gray, J.C. (2003) The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. *Plant Cell* **15**: 1468-1479

Chung, H.S., and Howe, G.A. (2009) A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. *Plant Cell* **21**: 131–

145.

Chung, H.S., Cooke, T.F., Depew, C.L., Patel, L.C., Ogawa, N., Kobayashi, Y., and Howe, G.A. (2010) Alternative splicing expands the repertoire of dominant JAZ repressors of jasmonate signaling. *Plant J.* 63: 613–622.

Clough, S.J., and Bent, A.F. (1998) Floral dip: A simpli fied method for Agrobacterium-mediated transformation of *Arabidopsis* thaliana. *Plant J.* **16**: 735–743.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G. (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. *Plant J.* **32**: 457–466.

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., Fitt, G.P., Sewelam, N., Schenk, P.M., Manners, J.M., and Kazan, K. (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* **19**: 2225–2245.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., and Lepiniec, L. (2010) MYB transcription factors in Arabidopsis. *Trends Plant Sci.* **15**: 573–581.

Eferl, R., and Wagner, E.F. (2003) AP-1: a double-edged sword in tumorigenesis. *Nat. Rev. Cancer* **3**: 859–868.

Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (2003) The COP9 signalosome interacts physically with SCF COI1 and

modulates jasmonate responses. *Plant Cell* **15**: 1083–1094.

Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J.M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., Pauwels, L., Witters, E., Puga, M.I., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G., and Solano, R. (2011) The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant Cell* **23**: 701–715

Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6: 751–759.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., and Solano, R. (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nat. Chem. Biol.* 5: 344–350.

Fuchs J, Demidov D, Houben A, Schubert I (2006). Chromosomal histone modification patterns – from conservation to diversity. *Trends Plant Sci.* **11**, 199–208.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000) *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* **12**: 393-404.

Guilfoyle, T.J., and Hagen, G. (2007) Auxin response factors. Curr. Opin. Plant Biol. **10**:453-460.

Grunewald, W., Vanholme, B., Pauwels, L., Plovie, E., Inze´, D., Gheysen, G., and Goossens, A. (2009) Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. *EMBO Rep*. **10**: 923–928.

Gonzalez D, Bowen AJ, Carroll TS, Conlan RS (2007) The transcription corepressor LEUNIG interacts with the histone deacetylase HDA19 and mediator components MED14 (SWP) and CDK8 (HEN3) to repress transcription. *Mol. Cell. Biol.* **27:** 5306–5315.

Guerineau, F., Benjdia, M., and Zhou, D.X., (2003) A jasmonate-responsive element within the A. thaliana *vsp1* promoter. *J. Exp. Bot*. **54**: 1153–1162.

He, Y., and Gan, S. (2001) Identical promoter elements are involved in regulation of the *OPR1* gene by senescence and jasmonic acid in *Arabidopsis*. *Plant Mol. Biol*. **47**: 595–605.

Hebbes, T.R., Thorne, A.W., and Crane-Robinson, C. (1988) Adirect link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* **7**:1395-1402.

Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., and Bailey, P.C. (2003) The basic helix-loop-helix transcription factor family in plants: A genome-wide study of protein structure and functional diversity. *Mol. Biol. Evol*. **20**: 735–747.

Hou, X., Lee, L.Y., Xia, K., Yan, Y., and Yu, H. (2010) DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Dev Cell*. **19**: 884-94

Howe, G.A. (2010) Ubiquitin Ligase-Coupled Receptors Extend Their Reach to Jasmonate. *Plant Physiol.* **154:** 471-474.

Howe, G.A., and Jander, G. (2008) Plant immunity to insect herbivores. *Annu. Rev. Plant Biol*. **59:** 41–66.

Hua, Z., and Vierstra, R.D. (2011) The cullin-RING ubiquitin-protein ligases. *Annu. Rev. Plant Biol*. **62**: 299–334.

Ikeda, M., Mitsuda, N., and Ohme-Takagi, M. (2009) Arabidopsis WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning. *Plant Cell* **21**:3493-505.

Jenuwein T, Allis CD (2001) Translating the histone code. *Science* **293**: 1074–80.

Johnson, L., Cao, X., and Jacobsen, S. (2002) Interplay between two epigenetic marks. DNA methylation and hitone H3 lysine 9 methylation. *Curr. Biol*. **12**: 1360-1367.

Kagale, S., and Rozwadowski, K. (2011) EAR motif-mediated transcriptional repression in plants. *Epigenetics* **6**: 141-146.

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y., and Howe, G.A. (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. USA* **105**: 7100–7105.

Kazan, K. and Manners, J.M. (2012) JAZ repressors and the orchestration of phytohormone crosstalk. *Trends Plant Sci*. **17**:22-31.

Kim, S.R., Kim, and Y., An, G. (1993) Identification of methyl jasmonate and salicylic acid response elements from the *nopaline synthase (nos)* promoter. *Plant Physiol.* **103**: 97-103.

Kim, S.R., Choi, J.L., Costa, M.A., and An, G., (1992) Identification of G-box sequence as an essential element for methyl jasmonate response of potato proteinase inhibitor II promoter. *Plant Physiol.* **99:** 627–631.

Kim, Y., Buckley, K., Costa, M.A., and An, G. (1994) A 20 nucleotide upstream element is essential for the nopaline synthase (nos) promoter activity. *Plant Mol. Biol*. **24**: 105-117.

Koo, A.J., and Howe, G.A. (2009) The wound hormone jasmonate. *Phytochemistry* **70**: 1571-1580.

Koo, Y.J, Song, J.T., and Choi, Y.D. (2004) Promoter analysis of the *NTR1* gene of *Brassica campestris* L. ssp. *Pekinensis* in transgenic tobaccos. *Agric. Chem. Biotechnol*. **47**: 119-124.

Krogan, N.T., and Long, J.A. (2009) Why so repressed? Turning off transcription during plant growth and development *Curr. Opin. Plant Biol.* **12**: 628.

Kurdistani SK, Grunstein M (2003). *In vivo* protein-protein and protein-DNA crosslinking for genomewide binding microarray. *Methods* **31**: 90–95.

Lee, B.J., Cansizoglu, A.E., Su¨ el, K.E., Louis, T.H., Zhang, Z., and Chook, Y.M. (2006). Rules for nuclear localization sequence recognition by karyopherin beta 2. *Cell* **126**: 543–558.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004)

JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *Plant Cell* **16**: 1938–1950.

Mahalingam, R., Gomez-Buitrago, A., Eckardt, N., Shah, N., Guevara-Garcia, A., Day, P., Raina, R., Fedoroff, N.V. (2003) Characterizing the stress/defense transcriptome of Arabidopsis. *Genome Biol.* **4**: R20.

Mandaokar, A., Thines, B., Shin, B., Lange, B.M., Choi, G., Koo, Y.J., Yoo, Y.J., Choi, Y.D., Choi, G., and Browse, J. (2006) Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. *Plant J.* **46**: 984–1008.

Mason, H.S., Dewald, D.B., and Mullet, J.E., (1993) Identification of a methyl jasmonateresponsive domain in the soybean vspB promoter. *Plant Cell* **5**: 241–251.

Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A., and He, S.Y. (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. *Plant J*. **55**: 979–988.

Memelink, J., (2009) Regulation of gene expression by jasmonate hormones. *Phytochemistry* **70**: 1560-1570.

Menke, F.L.H., Champion, A., Kijne, J.W., and Memelink, J. (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary

metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO J.* **18**: 4455–4463.

Mosblech, A., Thurow, C., Gatz, C., Feussner, I., and Heilmann, I. (2011) Jasmonic acid perception by COI1 involves inositol polyphosphates in Arabidopsis thaliana. *Plant J.* **65**: 949–957.

Mutskov V, Gerber D, Angelov D, Ausio J, Workman J, Dimitrov S (1998) Persistent interactions of core histone tails with nucleosomal DNA following acetylation and transcription factor binding. *Mol. Cell Biol.* **18**: 6293–6304.

Niu, Y., Figueroa, P., and Browse, J. (2011) Characterization of JAZinteracting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. *J. Exp. Bot*. **62**: 2143–2154.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* **13**: 1959-1968.

Ohme-Takagi, M., and Shinshi, H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* **7**: 173-182.

Pauwels, L., Barbero, G.F., Geerinck, J., Tilleman, S., Grunewald, W., Pérez, A.C., Chico, J.M., Bossche, R.V., Sewell, J., Gil, E., García-Casado, G., Witters, E., Inzé, D., Long, J.A., De Jaeger, G., Solano, R., and Goossens, A. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* **464**: 788–791.

Pauwels, L., and Goossens, A. (2011) The JAZ Proteins: A Crucial Interface in the Jasmonate Signaling Cascade. *Plant Cell* **23**: 3089-3100.

Pauwels, L., Morreel, K., De Witte, E., Lammertyn, F., Van Montagu, M., Boerjan, W., Inze´, D., and Goossens, A. (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. *Proc. Natl.Acad. Sci. USA* **105**: 1380–1385.

Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., Fan, M., Peng, W., Ren, C., and Xie, D. (2011) The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis* thaliana. *Plant Cell* **23**: 1795–1814.

Ren, C., Pan, J., Peng, W., Genschik, P., Hobbie, L., Hellmann, H., Estelle, M., Gao, B., Peng, J., Sun, C., and Xie, D. (2005). Point mutations in Arabidopsis Cullin1 reveal its essential role in jasmonate response. *Plant J*. **42**: 514–524.

Reymond, P., Weber, H., Damond, M., Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. *Plant Cell* **12:** 707–720.

Rouster, J., Leah, R., Mundy, J., and Cameron-Mills, V. (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. *Plant J*. **11**: 513–523.

Sambrook, J. and Russell, D.W. (2001) Molecular cloning: a laboratory manual, 3rd ed, Cold Spring Laboratory Press, Cold Spring Harbor, New York

Saleh, A., Alvarez-Venegas, R., and Avramova, Z. (2008) An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. *Nature Protocols* **3**: 1018-1025.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D. (2001a) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonateregulated plant responses. *Proc Natl Acad Sci USA* **98**: 4788–4793.

Seo, H.S., Song, J.T., Koo, Y.J., Jung, C., Yeu, S.Y., Kim, M.K., Song, S.I., Lee, J.S., Hwang, I., Cheong, J.J., and Choi, Y.D. (2001b) Floral nectarty-specific gene NTR1 encodes a jasmonic acid carboxyl methyltransferase, *Agric. Chem. Biotechnol*. **44**: 119-124.

Seo, J.S., Joo, J., Kim, M.J., Kim, Y.K., Nahm, B.H., Song, S.I., Cheong, J.J., Lee, J.S., Kim, J.K., and Choi, Y.D. (2011) OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. Plant J. **65**:907-921.

Sheard, L.B., Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, He SY, Rizo J, Howe GA, Zheng N. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature* **468**: 400–405.

Shyu C., Figueroa P., Depew C.L., Cooke T.F., Sheard L.B., Moreno J.E., Katsir L., Zheng N., Browse J., Howe G.A. (2012) JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in Arabidopsis. *Plant Cell* **24**: 536–550.

Smolen, G.A., Pawlowski, L., Wilensky, S.E., and Bender, J. (2002) Dominant alleles of the basic helix-loop-helix transcription factor ATR2 activate stress-responsive genes in Arabidopsis. *Genetics* **161**:1235–1246.

Song, J.T., Seo, H.S., Song, S.I., Lee, J.S., and Choi, Y.D. (2000) NTR1 encodes a floral nectary-specific gene in Brassica campestris L. ssp. pekinensis. *Plant Mol. Biol.* **42**: 647-655.

Song, S., Qi, T., Huang, H., Ren, Q., Wu, D., Chang, C., Peng, W., Liu, Y., Peng, J., and Xie, D. (2011) The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect jasmonate-regulated stamen development in *Arabidopsis*. *Plant Cell* **23**: 1000–1013.

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature* **403**: 41–45.

Staswick, P.E., and Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. *Plant Cell* **16**: 2117–2127.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002) Jasmonate response locus JAR1 and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. *Plant Cell* **14**: 1405–1415.

Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.-T., Frye, R.A., and York, J.D. (2005). Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. *Proc. Natl. Acad. Sci. USA* **102**: 12612–12617.

Stitz, M., Gase, K., Baldwin, I.T., and Gaquerel, E. (2011) Ectopic expression of *AtJMT* in Nicotiana attenuata: creating a metabolic sink has tissue-specific consequences for the jasmonate metabolic network and silences downstream gene expression. *Plant Physiol*. **157**: 341-354.

Takeda, S., Sugimoto, K., Otsuki, H., Hirochika, H. (1998) A 13-bp cis-regulatory element in the LTR promoter of the tobacco retrotransposon Tto1 is involved in responsiveness to tissue culture, wounding, methyl jasmonate and fungal elicitors. *Plant J.* **18**: 383–393.

Tan, X., Calderon-Villalobos, L.I.A., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M., and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**: 640–645.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G.H., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* **448**: 661–665.

Tiwari, S.B., Hagen, G., and Guilfoyle, T. (2003) The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* **15**:533-543.

Ubeda-Toma´ s, S., Federici, F., Casimiro, I., Beemster, G.T.S., Bhalerao, R., Swarup, R., Doerner, P., Haseloff, J., and Bennett, M.J. (2009). Gibberellin signaling in the endodermis controls Arabidopsis root meristem size. *Curr. Biol*. **19**: 1194–1199.

Utley, R.T., Ikeda, K., **Grant P.A., Cote J., Steger D.J., Eberharter A., John S.,**

and Workman J.L. (1998) Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. *Nature* **394**:498-502.

Vom Endt, D., Soares e Silva, M., Kijne, J.W., Pasquali, G., and Memelink, J. (2007) Identification of a bipartite jasmonate-responsive promoter element in the Catharanthus roseus ORCA3 transcription factor gene that interacts speci fically with AT-hook DNA-binding proteins. *Plant Physiol*. **144**:1680–1689.

Wasternack, C. (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany* **100**: 681-697.

Weigel, D and Glazebrook, J. (2002) Arabidopsis: A Laboratory Manual. CSHL Press 241-245; 176-180.

Wu, K., Zhang, L., Zhou, C., Yu, C.W., and Chaikam, V. (2008) HDA6 is required for jasmonate response, senescence and flowering in *Arabidopsis*. *J. Exp. Bot*. **59**:225-234.

Xiang, C., Miao, Z.H., Lam, E. **(**1996**)** Coordinated activation of as-1-type elements and a tobacco glutathione S-transferase gene by auxins, salicylic acid, methyljasmonate and hydrogen peroxide. *Plant Mol. Biol*. **32**: 415–426.

Xie, D.-X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998) COI1: An Arabidopsis gene required for jasmonate-regulateddefense and fertility. Science **280**: 1091–1094.

Xu, B., and Timko, M. (2004) Methyl jasmonate induced expression of the tobacco putrescine N-methyltransferase genes requires both G-box and GCC-motif

elements. *Plant Mol. Biol.* **55**: 743–761.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002) The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell* **14**: 1919–1935.

Yamamoto, Y.Y, Yoshioka, Y., Hyakumachi, M., Maruyama, K., Yamaguchi-Shinozaki, K., Tokizawa, M., and Koyama, H. (2011) Prediction of transcriptional regulatory elements for plant hormone responses based on microarray data. *BMC Plant Biol.* **11**:39.

Yang, Z., Tian, L., Latoszek-Green, M., Brown, D., and Wu, K. (2005) *Arabidopsis* ERF4 is a transcriptional repressor capable of modulating ethylene and abscisic acid responses. *Plant Mol. Biol.* **58**: 585-596.

Yan, Y., Stolz, S., Che´ telat, A., Reymond, P., Pagni, M., Dubugnon, L., and Farmer, E.E. (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. *Plant Cell* **19**: 2470–2483.

Yan J.B., Zhang C., Gu M., Bai Z.Y., Zhang W.G., Cheng Z.W., Peng W., Luo H.B., Nan F.J., Wang Z., and Xie D. (2009) The *Arabidopsis* CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell* **21**: 2220–2236.

Yant, L., Mathieu, J., Dinh, T.T., Ott, F., Lanz, C., Wollmann, H., Chen, X., and Schmid, M. (2010) Orchestration of the floral transition and floral development in Arabidopsis by the bifunctional transcription factor APETALA2. *Plant Cell* **22**:2156-2170.

Yoshida, Y., Sano, R., Wada, T., Takabayashi, J., and Okada, K. (2009)

Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in Arabidopsis. *Development* **136**: 1039–1048.

You, M.K., Shin, H.Y., Kim, Y.J., Ok, S.H., Cho, S.K., Jeung, J.U., Yoo, S.D., Kim, J.K., and Shin, J.S. (2010) Novel bifunctional nucleases, OmBBD and AtBBD1, are involved in abscisic acid-mediated callose deposition in *Arabidopsis. Plant Physiol.* **152**: 1015-29

Zhang, F., Gonzalez, A., Zhao, M., Payne, C.T., and Lloyd, A. (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. *Development* **130**: 4859–4869.

Zhou, C., **Zhang, L., Duan, J., Miki, B., and Wu, K.** (2005) HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. *Plant Cell* **17**:1196-1204.

Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., A, M., Jiang, Z., Kim, J.M., To, T.K., Li, W., Zhang, X., Yu, Q., Dong, Z., Chen, W.Q., Seki, M., Zhou, J,M., and Guo, H. (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **108**: 12539–12544.

국문 요약

자스모네이트 (jasmonates) 는 병해충에 대한 식물의 방어 기작, 한발 (drought), 냉해 (cold stress), 염해 (salinity) 와 같은 외부 환경 스트레스 및 다양한 식물의 발달 과정에 관여하는 중요한 식물 호르몬이다. 애기장대 유전자인 *AtJMT*는 자스몬산에 메틸기를 전이하여 메틸화된 자스몬산 합성에 관여하는 유전자로 밝혀져 있으며, 배추의 *BcNTR1* 유전자는 *AtJMT*와 상동 유전자인 것으로 밝혀져 있다. 두 유전자들은 자스몬산의 메틸화뿐만 아니라 자스모네이트 신호전달 과정에도 영향을 미치는 것을 알려져 있다. 따라서 이들 유전자의 신호전달 과정에서의 역할을 알아내기 위하여 이들 유전자의 프로모터를 분석하였다. 두 유전자의 4.5kb에 해당하는 프로모터 부분을 리포터 유전자인 *GU*S 유전자와 재조합하여 형질전환 애기장대 식물을 만들었으며, 이들 형질전환 식물에 자스모네이트를 처리한 결과 이들 프로모터에 자스모네이트에 반응하는 *cis*-element 가 존재하고 있음을 알 수 있었다. 이를 찾기 위해 다양한 종류의 프로모터 결실 재조합체를 제작하였으며, 이를 애기장대에 형질전환하여 자스모네이트에 대한 반응성을 살펴본 결과 *AtJMT* (-2,294~-2,280)와 *BcNTR1* (-3,518~-3,480) 두 유전자의 프로모터에 공통적으로 존재하는 염기서열이 있는 것을 확인할 수 있었으며, 이는 이전에 알려진 자스모네이트에 반응하는 *cis*-element 들과는 다른 새로운 염기서열임을 알 수 있었다. 따라서 이 염기서열 (TCCTGA)를 JARE로 새롭게 명명하였다. 추가적으로 JARE를 반복한 재조합체를 만들어 형질전환체를 RT-PCR 및 GUS staining으로 분석한 결과 JARE가 자스모네이트에 특이적으로 반응하는 *cis*-element 임을 다시 한번 확인할 수 있었다.

다른 한편으로 새롭게 발견한 JARE *cis*-element 와 상호작용하는 전사 인자를 찾기 위해서 애기장대 cDNA를 가지고 Y1H screening을 실시한 결과 애기장대 *BBD1* 유전자를 선별하였고, 추가적인 Y1H 실험과 EMSA 실험을 통하여 BBD1 단백질이 JARE와 상호작용하고 있음을 알 수 있었다. 또한 여러 종류의 BBD1 단백질의 결실체를 만들어 Y1H 실험을 실시한 결과 이 단백질의 C-말단 부위가 DNA와 상호작용하는 역할을 하고 있음을 알 수 있었다. 그리고 목표 유전자인 *JMT* 유전자 발현 조절 및 자스모네이트 신호전달 작용기작에서 BBD1의 역할을 알아내기 위해서 BBD1과 상호작용하는 단백질을 찾기 위해 애기장대 JAZ 단백질들과의 상호작용 여부를 Y2H 시스템에서 조사한 결과 BBD1이 JAZ1과 JAZ4와 특이적으로 상호하고 있음을 알 수 있었다. 또한 in vitro pulldown assay 및 agroinfiltration 실험을 통해서도 BBD1과 JAZ1 단백질이 상호작용하고 있음을 확인하였다. 그리고 JAZ1과 BBD1 단백질 결실체들의 상화작용을 Y2H로 확인한 결과 JAZ1의 tify motif와 BBD1의 HCR motif가 서로 상호작용하고 있음을 밝혀내었다. JAZ 단백질은 자스모네이트 신호전달에 있어 핵심 역할을 하는 것으로 알려져 있으며, 작용 기작은 자스모네이트 신호가 없을 때에는 전사인자와 결합하여 전사 개시를 저해하고 있다가 신호가 발생할 경우 F-box 단백질인 COI1 단백질에 의해 특이적으로 ubiquitination 된 후 26S proteosome 에 의해 분해되고 전사인자에 의해 자스모네이트에 반응하는 여러 유전자들의 전사가 활성화 되는 것으로 알려져 있다. BBD1의 목표 유전자인 *JMT* 유전자의 발현에 미치는 영향을 알아내기 위하여, *bbd1* 결실돌연변이체와 BBD1과 높은 상동성을 가지는 BBD2가 함께 결실된 돌연변이체인 *bbd1bbd2*를 분석한 결과 자스모네이트 처리시 야생형에 비해 목표유전자인 *JMT* 유전자의 발현이 현저히 증가 되어 있음

을 알 수 있었다. 다른 한편으로 *BBD*1의 과다발현 형질전환체 식물을 만들어 분석한 결과 반대로 자스모네이트 처리시 목표유전자인 *JMT* 유전자의 발현이 감소하고 있음을 알 수 있었다. 따라서 BBD1은 자스모네이트에 반응하여 *JMT* 유전자 발현 억제에 관여하고 있음을 알 수 있었다. 이 억제 작용 기작을 알아내기 위하여 염색체의 아세틸화 상태를 ChIP 실험을 통해 조사한 결과 *bbd1bbd2* 돌연변이체에서 아세틸화가 더 높게 되어 있음을 알 수 있었으며, 반대로 *BBD1* 과다발현체에서는 낮게 되어 있음을 알 수 있었다. 이는 JAZ 단백질이 궁극적으로 co-repressor인 TOPLESS 단백질 또는 histone deacetylase (HDAC)과 상호작용하고 있다는 연구 결과와 TOPLESS는 HDAC을 통해 염색체 아세틸화를 조절하는 것으로 알려진 사실을 볼 때, BBD1이 JAZ 단백질과 상호작용하면서 궁극적으로 HDAC을 통해 *JMT* 유전자의 프로모터의 아세틸화 상태를 조절하면서 유전자 발현 수준을 조절할 것으로 생각되며, 이는 자스모네이트의 신호전달 작용 기작에 의해 이루어 질 것이라는 사실을 알 수 있었다.

주요어; AtJMT, 자스모네이트 신호 전달 체계, AtJAZs, AtBBD1, JARE, 자스모네이트 반응 시스인자.

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