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Comprehensive analysis of protein interactions between JAZ proteins and bHLH transcription factors that negatively regulate jasmonate signaling

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Abbreviations: bHLH, basic helix-loop-helix; JAM, JA-ASSOCIATED MYC2-LIKE; COI1, CORONATINE INSENSITIVE1; JA, Jasmonic acid; MJ, methyl jasmonate; qRT-PCR, quantitative reverse transcription PCR

Jasmonates have crucial roles in plant responses to biotic and abiotic stresses. Given the importance of transcriptional regulation in jasmonate-mediated stress responses, transcription factors are key regulators of jasmonate signaling. The transcription factors JASMONATE-ASSOCIATED MYC2-LIKE 1 (JAM1), JAM2, and JAM3 are negative regulators of jasmonate signaling, although the mechanisms that control the activities of these transcription factors remain unclear. To understand the regulatory mechanisms of JAM proteins, we used a yeast two-hybrid assay to screen for protein interaction partners of JAM1 and found that JAM1 interacted with JAZ proteins.

As JAZ proteins interact with MYC2 and suppresses its transcriptional activity, we performed comprehensive interaction analysis between JAM1 through JAM3 and JAZ1 through JAZ12. We showed that JAM1 and JAM2 interacted with several of the JAZ proteins analyzed here. In contrast, JAM3 interacted with none of the tested JAZ proteins. These results suggest that JAZ proteins regulate JAM1 and JAM2 through protein-protein interactions in a manner similar to the repressive effects of JAZ proteins on the transcriptional activity of MYC2.

Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, the so-called jasmonates, are growth and stress response regulators that are ubiquitous throughout the plant kingdom. Jasmonates are rapidly synthesized in response to certain external stimuli, such as wounding, pathogen infection, and insect attack.[1-3] Those stresses and JA treatment each induce similar sets of jasmonate-responsive genes, which are involved in defense-related metabolism.[4-9]

Jasmonoyl-L-isoleucine (JA-Ile), which is an active form of jasmonate,[10,11] promotes interaction between COI1 and JAZ proteins, and then JAZ proteins are presumably degraded via the 26S proteasome system.[12-14] MYC2, as well as its close homologs MYC3 and MYC4, is a central transcriptional regulator of jasmonate signaling and is negatively regulated by JAZ proteins through protein interactions. The degradation of JAZ proteins results in de-repression of the MYC transcription factors, which leads to transcriptional activation of downstream jasmonate-responsive genes.[15-20] The N-terminal regions of MYC2 and MYC3 each interact with JAZ proteins.[18] Moreover, the formation of homo- and heterodimers by the MYC transcription factors[18] suggests the importance of protein interactions among those factors that are required for jasmonate signaling.

JA-ASSOCIATED MYC2-LIKE 1 (JAM1), JAM2 and JAM3, which are homologs of MYC2, were recently shown to function redundantly as negative regulators of jasmonate signaling because

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the *jam1jam3jam3* triple mutants (*jam*×3) shows enhanced responsiveness to jasmonate, JAM1, JAM2 and JAM3 have repression activities, and they form homo-and hetero dimers.[21-24] Here, we screened for interaction partners of JAM1 and further showed that JAM1 and JAM2, but not JAM3, interacted with several JAZ proteins. We concluded that JAZ proteins are possible regulators of JAM transcription factors.

Results

Identification of interaction targets of JAM transcription factors

Yeast two-hybrid screens using JAM1 as bait identified 12 positive clones, which were sequenced to identify JAM1 targets (data not shown). Ten of the 12 were JAZ proteins such as JAZ1 (At1g19180, 2 clones), JAZ2 (At1g74950), JAZ3 (At3g17860, 4 clones), JAZ9 (At1g70700), JAZ10 (At5g13220) and JAZ12 (At5g20900), and the remaining two clones were JAM3 and HY1 (heme oxygenase, At2g26670). To test whether JAM1 interacts specifically with other JAZ proteins, we used yeast two-hybrid assays to check all possible combinations between full-length bHLH transcription factors (BD-JAM1 through BD-JAM3 and BD-MYC2 as bait) and full-length AD-JAZ1 through AD-JAZ12 proteins (as prey). We also tested the opposite combination (BD-JAZ proteins as bait and AD-bHLH proteins as prey) (**Fig. 1A and B**). JAM1 interacted with JAZ3 and JAZ12, and JAM2 interacted with JAZ12 in both sets of experiments. JAM3 did not interact with any JAZ proteins under the experimental conditions used. As previously reported, MYC2 interacted with several JAZ proteins, with the exception of JAZ4 (**Fig. 1**).[18,25] To quantify the possible interactions between JAM1 and the 12 JAZ proteins, we measured the β-galactosidase activity of cell cultures that expressed those proteins. Detection of β-galactosidase activity in cell cultures that expressed the combinations of BD-JAM1 and AD-JAZ3 (**Fig. 1C**) and BD-JAZ1, -JAZ3, or -JAZ9 and AD-JAM1 (**Fig. 1D**) confirmed the interactions of these proteins in yeast. We also tested the interaction between truncated derivatives of JAM1 and full-length JAZ3. The interaction of JAM1ΔC, but not JAM1ΔN, with JAZ3 indicated the necessity of the N-terminal region for the interaction of JAM1 with JAZ3 (**Fig. 1E**), which is also true for the interaction between N-terminal region of JAZ3 and MYC2 or MYC3.[18]

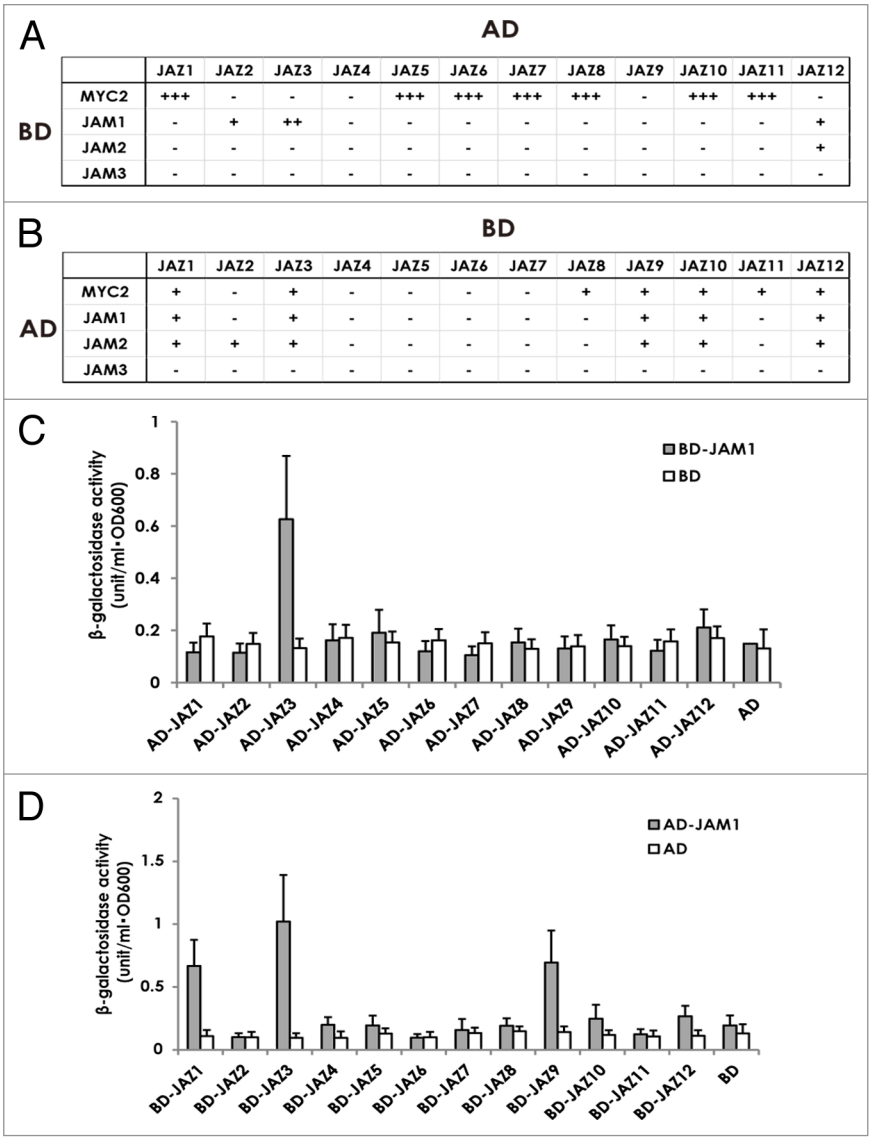

Figure 1A–D. (**A**) Protein-protein interaction between bHLH transcription factors and JAZ proteins. A summary of yeast two-hybrid results to assay for interactions between bHLH transcription factors as bait (BD) and JAZ repressors as prey (AD) (**A**), and the opposite combination (JAZ proteins as bait and bHLH proteins as prey) (**B**). AD, DNA activation domain; BD, DNA binding domain. Based on the number of colonies formed, the strength of each interaction was rated as strong (+++), medium (++), weak (+), or undetectable (–). (**C**), (**D**) Quantitative assays of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranose as a substrate to determine protein interactions between JAM1 and JAZ proteins. Results are shown as the mean ± SD for biologically independent experiments (n = 3).

Expression analysis of *JAZ* genes in *jam*×3 plants

Given that JAM proteins negatively regulate the expression of jasmonate-responsive genes,[21-23] we focused on the expression level of *JAZ* genes in *jam*×3 plants, which are loss of function mutant of JAM1, JAM2 and JAM3.[21] **Figure 2A** shows a summary of expression levels of *JAZ* genes obtained from GeneChip analyses.[21] The expression of all *JAZ* genes, except *JAZ4*, was induced by MJ treatment both in wild-type plants of the ecotype Columbia (Col) and in *jam*×3 plants. With the exception of *JAZ3*, the expression levels of all *JAZ* genes in mock-treated Col

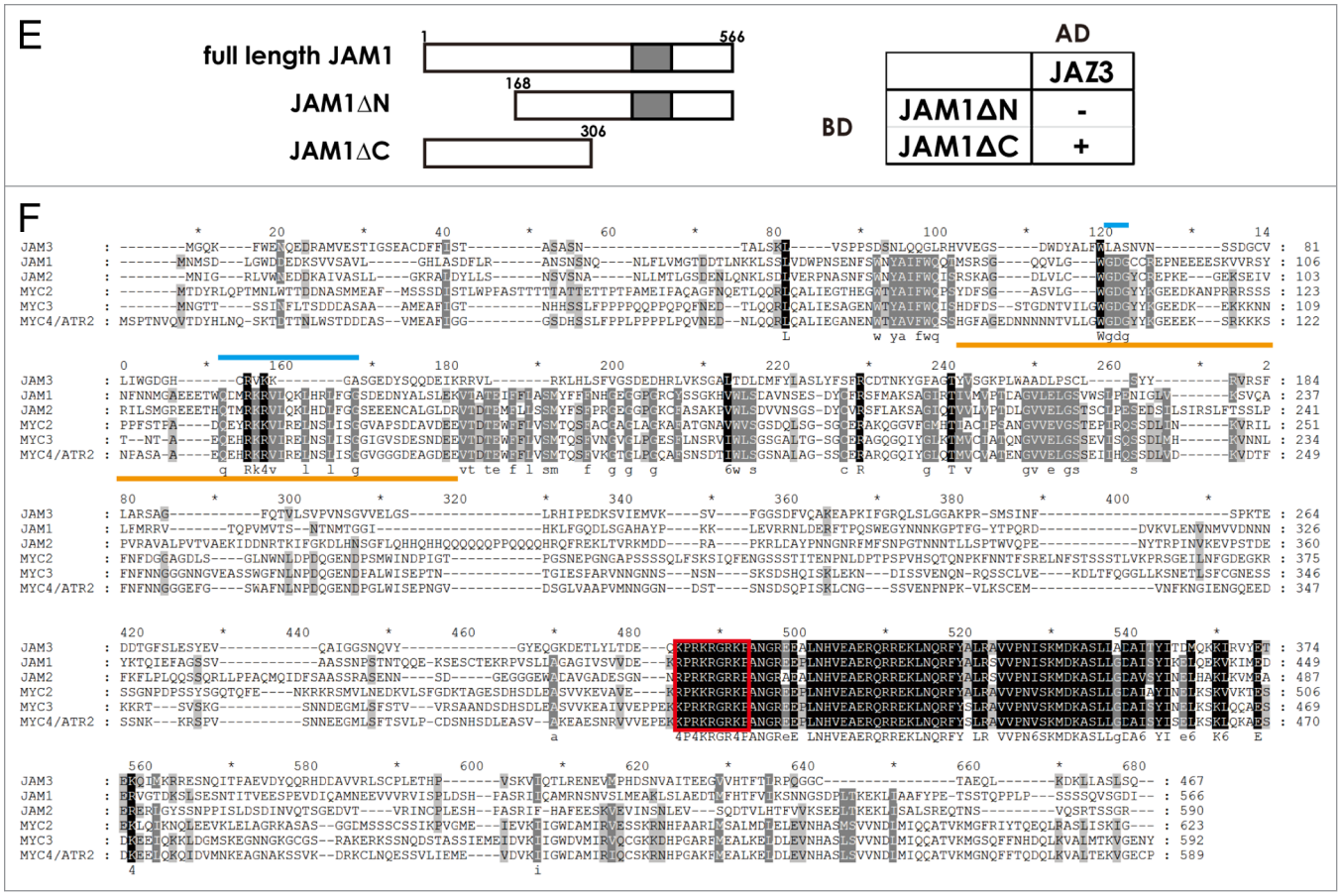

Figure 1E and F. (**E**) The interaction of truncated JAM1 derivatives with full-length JAZ3 was tested. (**F**) Alignment of the amino acid sequences of JAM1, JAM2, JAM3, MYC2, MYC3, and MYC4. Identical and similar amino acids are shaded black and gray, respectively. Red boxes indicate the nuclear localization signals. Orange lines under the sequences indicate the JAZ interaction domain of MYC2 and MYC3.[18] Light blue lines above the sequences indicate the conserved amino acid residues within a JAZ-interacting domain among JAM1, JAM2, MYC2, MYC3, and MYC4, but not JAM3.

and *jam*×3 plants were comparable as well as in MJ treated plants (**Fig. 2A**). Thus the expression levels of *JAZ* genes are almost comparable between Col and *jam*×3 in spite of the presence or absence of MJ. We further tested the expression levels of *JAZ1* in Col, *jam1jam2*, and *jam*×3 plants by qRT-PCR analysis in more detail (**Fig. 2B**). The expression level of *JAZ1* in *jam1jam2* and *jam*×3 plants was only 1.5-fold higher than that in Col plants ($P < 0.05$). The almost comparable expression levels of *JAZ1* in *jam1jam2* and *jam*×3 plants indicated that the negative effect on *JAZ1* expression derives from JAM1 and JAM2 but such effect is limited.

Discussion

Our yeast two-hybrid analysis indicated that JAM1 and JAM2 interact with JAZ proteins (**Fig. 1**). The interaction of the N-terminal region of JAM1 with JAZ3 (**Fig. 1E**) indicated the existence of a JAZ-interaction motif in that region. The N-terminal region of MYC2 (Y^{93} to E^{160}) is necessary for the interaction of MYC2 with JAZ proteins (**Fig. 1F**, orange lines).[18] This region contains several amino acids that are conserved in MYC2 through MYC4, as well as in JAM1 and JAM2, but not in JAM3 (**Fig. 1F**, pale blue lines). Given that JAM3 did not interact with any JAZ proteins in our experimental conditions, these results imply that these amino acids are involved in the interaction of JAZ proteins and JAM proteins.

JAZ proteins interact with bHLH transcription factors via a C-terminal region, which contains a Jas motif,[22,25] and also interact with COI1.[26,27] JA-Ile–dependent formation of protein complexes that contain COI1 and JAZ proteins is followed by the degradation of JAZ proteins. Thus, removal of the COI1-intereacting region of JAZ proteins might stabilize them and thereby compromise the expression of jasmonate-responsive genes and suppress jasmonate responses by blocking the transcriptional activity of transcription factors, such as MYC2.[12,13] Our demonstration here that some JAZ proteins interacted with JAM1 and JAM2 (**Fig. 1**) led us to speculate that physical interactions between JAZ and JAM proteins might inhibit the function of JAM proteins as transcriptional repressor. It is not clear whether the JAM and JAZ proteins could co-localize in the same cell. Therefore, further research is required to determine the protein interaction among those proteins in *vivo*. The expression of *JAZ* genes is induced by MYC2, MYC3, and MYC4,[12,18] and the expression of *JAM* genes is partially MYC2-dependent.[21]

A

		Fold-Change Information							
		Col (MJ/mock)		*jam* x3 (MJ/mock)		mock (*jam* x3/Col)		MJ (*jam* x3/Col)	
Locus ID	Gene name	ratio	significance	ratio	significance	ratio	significance	ratio	significance
At1g19180	*JAZ1*	30.8	**	11.0	**	3.2	ns	1.1	ns
At1g74950	*JAZ2*	20.8	**	22.1	**	1.1	ns	1.1	ns
At3g17860	*JAZ3*	8.3	**	4.0	**	2.5	**	1.2	ns
At1g48500	*JAZ4*	1.1	ns	1.1	ns	1.1	ns	1.0	ns
At1g17380	*JAZ5*	108.7	**	85.2	**	1.8	ns	1.4	ns
At1g72450	*JAZ6*	15.9	**	11.0	**	1.5	ns	1.1	ns
At2g34600	*JAZ7*	368.8	**	573.2	**	0.7	ns	1.2	ns
At1g30135	*JAZ8*	216.9	**	274.5	**	1.1	ns	1.4	ns
At1g70700	*JAZ9*	24.1	**	16.2	**	1.9	ns	1.3	ns
At5g13220	*JAZ10*	298.9	**	99.9	**	3.9	ns	1.3	ns
At5g20900	*JAZ12*	2.6	**	3.0	**	1.2	ns	1.4	ns

B

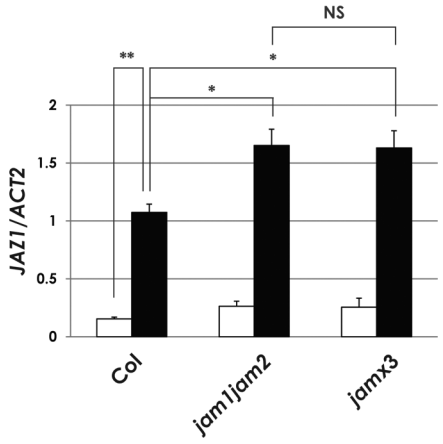

Figure 2. Expression analysis of *JAZ* genes in *jam*×3 plants. (**A**) A summary of fold-change information and statistical analyses of *JAZ* expression levels, based on the normalized intensity of *JAZ* genes obtained from GeneChip analyses.[21] Results are shown as the mean for two biologically independent RNA samples. ** $P < 0.01$, * $P < 0.05$, NS = not significant ($P \geq 0.05$); Tukey-Kramer multiple comparison test. (**B**) Total RNA was isolated from 7-d-old plants, which were grown in GM liquid medium with 1% sucrose and treated with mock (white bars) or 50 μM MJ (black bars) for 1 h. Results are shown as the mean ± SE for three biologically independent RNA samples.

Although JAM proteins negatively regulate the expression of jasmonate-responsive genes, such effects on the expression of *JAZ* genes were relatively weak (**Fig. 2**). Thus, MYC2 simultaneously induces the expression of both *JAZ* and *JAM* genes, with newly synthesized JAZ and JAM proteins negatively regulating jasmonate signaling. Although the three JAM transcription factors have redundant roles, JAM3 differs from JAM1 and JAM2 both in terms of its interactions with JAZ proteins and its expression profile after jasmonate treatment (**Fig. 1**).[21] Thus, the transcriptional activity of JAM3 to negatively regulate jasmonate-responsive genes might be regulated neither at the transcriptional level nor through interactions with JAZ proteins. In this study, we performed yeast two-hybrid screening and our results as well as the results of yeast two hybrid assay among JAM proteins[24] indicated that JAM1 interacted with JAM3. One possibility is the formation of a heterodimer between JAM3 and JAM1 or JAM2 to modulate the transcriptional activity of JAM3. Given that *JAM3* is constitutively expressed in almost all tissues (Genevestigator, https:// www.genevestigator.com/gv/),[28] JAM3 protein may be stable in the presence or absence of stressors that cause the accumulation of JA-Ile. Given that the expression of *JAM1* and *JAM2* is quickly induced after MJ treatment,[21] it is plausible that newly synthesized JAM1 and JAM2 form heterodimers with JAM3 and that such interactions might reinforce the repression activity of the JAM protein complex.

Our results suggest that JAZ proteins are possible regulators of JAM proteins. Unlike MYC2, MYC3, and MYC4, JAM proteins are transcriptional repressors.[23,24] Therefore, the effect of an interaction with JAZ proteins on the transcriptional repression activity of JAM proteins should be clarified. Further characterization of the protein interaction partners of JAM proteins is needed to understand the molecular mechanisms that regulate aspects of jasmonate signaling as controlled by JAM proteins.

Materials and Methods

Yeast two-hybrid screening

The *JAM1* coding sequence was PCR-amplified with PrimeSTAR HS DNA Polymerase (TaKaRa, R010A) and

Gateway-compatible primers by using a cDNA library from *Arabidopsis thaliana* seedlings (see **Supplemental Table 1**; primers 1 and 2). PCR products were cloned into pDONR/Zeo with a Gateway® BP Clonase® II enzyme mix (Invitrogen, 11789–020), and their sequences were verified. The JAM1 sequence was introduced into the destination low-copy yeast expression vector pDEST32 (with the GAL4 DNA binding domain [BD]) according to the manufacture's instruction of Gateway® LR Clonase® enzyme mix (Invitrogen, 11791–019), and the sequence of the resulting plasmid was verified.

The pDEST32-JAM1 plasmid was transformed into yeast strain AH109. The prey cDNA library, Mate and Plate Library - Universal *Arabidopsis* (Clontech, 630487), was prepared in the plasmid pGADT7-Rec and transformed in yeast mating strain Y187. The transformed AH109 and the Y187 strain-based library were mixed with 1 mL and 5 mL of cultures, respectively (1 × 108 cells mL–1), and were incubated overnight in 45 mL of 2 × YPDA medium at 30 °C. Yeast diploids were selected by plating at 30 °C for 4 d on minimal medium SD lacking histidine (His), leucine (Leu), tryptophan (Trp), and adenine. Putative interacting partners of JAM1 that were isolated from this screen and their sequences were verified.

Yeast two-hybrid assay

cDNA clones that encode full-length bHLH transcription factors (JAM1 through JAM3 and MYC2), truncated derivatives of JAM1, and 12 full-length JAZ proteins were amplified with PrimeSTAR HS DNA Polymerase using Gateway-compatible primers (see **Supplemental Table 1**; For BP Gateway cloning). Those cDNA clones were introduced into the destination low-copy yeast expression vectors pDEST22 (with the Gal4 activation domain [AD]) and pDEST32. Truncated derivatives of JAM1 were used in combination with the destination low-copy yeast expression vector pDEST32. All constructs were checked by sequencing.

To assess protein interactions, the corresponding plasmids were cotransformed into *Saccharomyces cerevisiae* AH109 cells with a standard heat shock protocol.[25] Successfully transformed colonies were identified on yeast synthetic dropout medium that lacked Leu and Trp. At 3 d after transformation, yeast colonies were grown on yeast synthetic dropout medium lacking His, Leu, and Trp and supplemented with 5 mM 3-aminotriazole to test for protein interactions. Plates were incubated at 30 °C for 4 d. Cells were transformed with the empty Gateway vectors pDEST22 or pDEST32 to provide negative controls.

Alignment of the amino acid sequences of JAM1, JAM2, JAM3, MYC2, MYC3, and MYC4

The amino acid sequences of JAM1 and its closely related bHLH proteins were aligned using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/).[29] Identical and similar amino acids are shaded black and gray, respectively, using the software GeneDoc ver 2.7 (http://www.nrbsc.org/gfx/genedoc/). Nuclear localization signals were predicted by cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).[30]

Statistical analysis of GeneChip data

We used GeneChip data to obtain normalized intensities, the MJ/mock ratio for wild-type (WT) and *jam*×3 plants, and the *jam*×3/WT ratio for mock- and MJ-treated plants for all *JAZ* genes except *JAZ11*.[21] Then we performed a Tukey-Kramer multiple comparison test using the normalized intensities of *JAZ* genes and calculated the p values.

Quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA was isolated from 7-d-old *Arabidopsis* seedlings as described in Sasaki-Sekimoto et al.[21] cDNA was generated using ReverTra Ace® qRT-PCR RT Master Mix (TOYOBO, FSQ-201) and was used as a template for q RT-PCR analyses. We used THUNDERBIRD SYBR q RT-PCR Mix (TOYOBO, QPS-201) according to the manufacturer's instructions. Gene-specific primers for *ACTIN2* and *JAZ1* are shown in **Supplemental Table 1** (primers for qRT-PCR analysis).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplementary material may be found here:
http://www.landesbioscience.com/journals/psb/article/27639/

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