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Dissection of the one-MegaDalton JAZ1 protein complex

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Jasmonates (JAs) comprise a class of plant-specific hormones that mediate a large variety of processes involved in plant growth, development and defense. Perception of jasmonoyl-isoleucine (JA-Ile), the bioactive amino acid conjugate of JA, initiates the expression of JA-responsive genes through the degradation of the jasmonate ZIM domain (JAZ) repressor proteins and the subsequent release of the transcriptional activator MYC2. By using a tandem affinity purification based approach, we demonstrated that the Groucho/Tup1-type co-repressor TOPLESS (TPL) and TPL-related proteins are connected to the JAZ proteins via an adaptor protein, designated Novel Interactor of JAZ (NINJA). Both NINJA and TPL were shown to function as negative regulators of JA signaling. Here, we provide additional data, demonstrating that JAZ1 incorporates into high-molecular weight (HMW) protein complexes of >1 MDa and speculate about their composition.

Key words: jasmonate, transcription, repressor, MYC2, JAZ1, NINJA, TOPLESS, tandem affinity purification, blue native page

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JA-Ile, the endogenous bioactive JA, is perceived by the F-box protein CORONATINE INSENSITIVE1 (COI1) that functions as the hormone receptor. In the presence of JA-Ile, the E3 ubiquitin ligase complex SCF^{COI1} recognizes its targets, the JAZ proteins that are subsequently ubiquitinylated and destroyed by the 26S proteasome.[1-5] In an unelicited state, the JAZ proteins bind and inactivate the transcription factor MYC2, thereby repressing the activation of early JA-responsive genes through a mechanism that remained elusive to date.

In our study,[6] we used a tandem affinity purification (TAP) technology platform that had been established in *Arabidopsis thaliana* cell suspension cultures,[7] to retrieve new interactors of JAZ1 and, thereby, unravel the core JA signaling module. JAZ1-TAP cultures were mock treated or elicited with JA for 1 min and subsequently harvested and analyzed. JAZ1 was found to interact with JAZ12 and MYC3, a close relative of MYC2. Also interaction between COI1 and JAZ1 was observed, but only in the presence of JA, in accordance with the proposed models.[1-5] We focused on a previously uncharacterized protein, designated NINJA (At4g28910) that was retrieved with JAZ1 independently of the JA elicitation. Studies with green fluorescent protein (GFP)-tagged proteins demonstrated that the stability of NINJA was not affected by JAs, in contrast to the JAZ proteins that were degraded within minutes after JA application.

The specificity of NINJA for JAZ proteins was confirmed by yeast-two hybrid (Y2H) analysis and pull-down experiments. Furthermore, these experiments revealed that NINJA interacted with most JAZ proteins as well as with other ZIM-domain proteins that contain the conserved TIF[F/Y]XG (TIFY) motif and belong to the group II TIFY proteins,[8] such as PEAPOD1 (PPD1), PPD2 and TIFY8. In a Y2H analysis with deletion series of JAZ1, a 39-amino-acid fragment with the TIFY motif was found to be necessary and sufficient for the interaction with NINJA. Conversely, a deletion series of NINJA that is characterized by three conserved protein motifs, designated A, B and C, showed that the C-domain was responsible and sufficient for interaction with JAZ proteins.

Analogously to the JAZ1-TAP, a TAP analysis with NINJA as bait revealed that NINJA was present in a complex with the

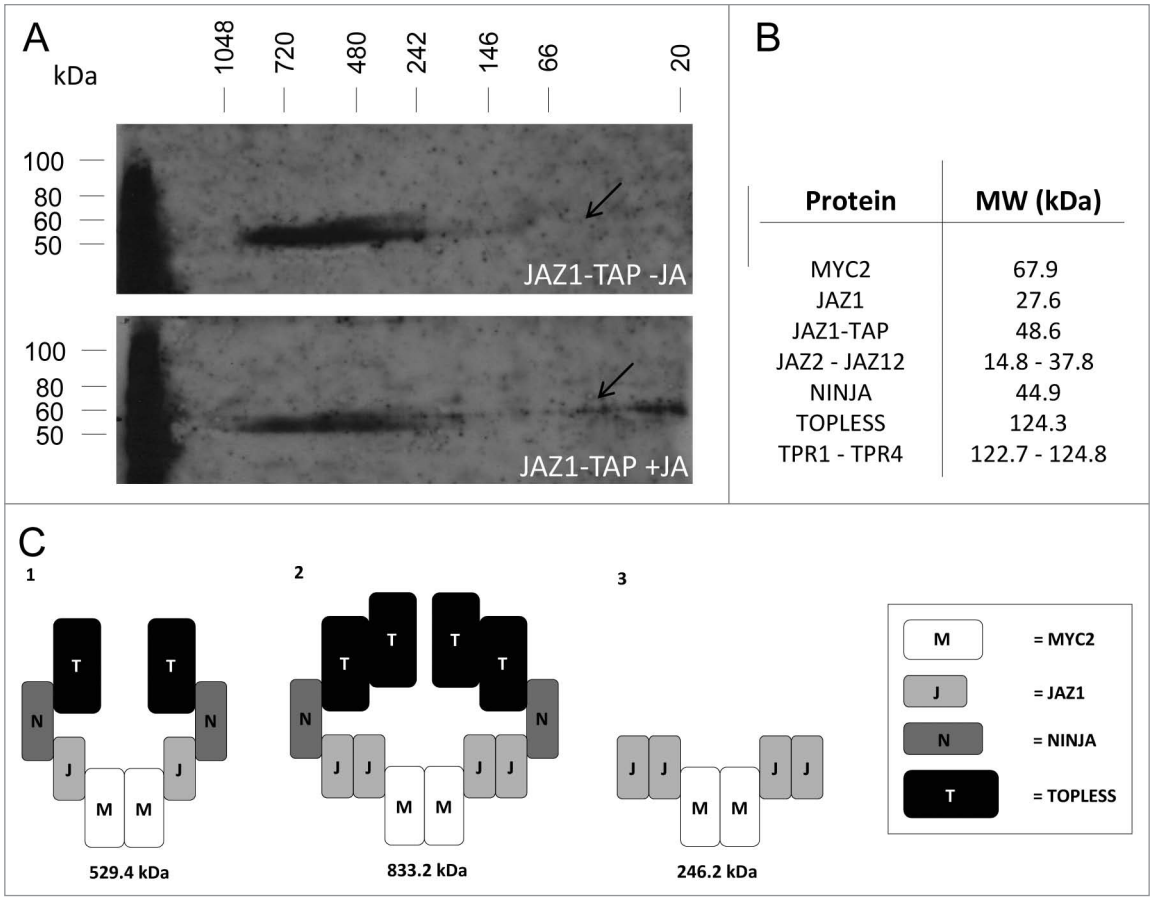

Figure 1. JAZ1 is incorporated in HMW protein complexes. (A) BN/SDS-PAGE analysis of the JAZ1-TAP complex. Total protein extracts of a JAZ1-TAP (48.6 kDa) overexpressing Arabidopsis cell culture, mock treated (-JA) or elicited with JA (+JA) for 1 min, separated over two dimensions by BN/SDS-PAGE. In the first (BN-PAGE) and second (SDS-PAGE) dimension, native protein complexes were separated according to their size and into their constituting components, respectively. The JAZ1-TAP fusion protein was visualized by immunoblot analysis with an antibody against the affinity tag. The molecular weight (MW) marker on top and on the left indicates the complex and protein sizes in the first and second dimension, respectively. Arrows mark the expected position of the monomeric JAZ1-TAP fusion protein, visible only in the JA-elicited sample. Degradation products of JAZ1-TAP can be observed in the 20-kDa region of the JA-elicited sample. Methods were as described.[19] (B) Overview of the MW (in kDa) of the proteins known to (in) directly interact with JAZ1. (C) Hypothetical HMW JAZ1 repressor complexes and their calculated sizes.

Groucho/Tup1-type co-repressor TPL and its homologs TPR2 and TPR3, both in the presence and the absence of JA. Via Y2H and bimolecular fluorescence complementation, TPL was demonstrated to interact with the ethylene-responsive element-binding factor associated amphiphilic repression (EAR)-motif present in the A-domain of NINJA. The EAR motif is a hallmark of transcriptional repressors[9] and accordingly, functional analysis established that both NINJA and TPL function as negative regulators of JA signaling. A model has been proposed in which JAZ proteins repress MYC2 activity by recruiting the TPL co-repressor through the adaptor protein NINJA and which illustrates that the transcriptional repression machinery of the JA signaling pathway has striking similarities with that that controls the auxin response pathway.

In this addendum, we provide additional data supporting the assembly of the JAZ-NINJA-TPL repressor module by demonstrating the incorporation of JAZ1 in HMW protein complexes of the megaDalton (MDa) range. Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a powerful technique that allows the isolation of native HMW protein complexes.[10,11] In combination with SDS-PAGE, the isolated protein complexes can be separated into their constituting components according to size. Via immunoblot analysis with specific antibodies, the incorporation of a protein of interest into one or more HMW complexes can be visualized. Applying this technique to the JAZ1-TAP-overexpressing Arabidopsis cell line, we were able to show that the JAZ1 protein assembles into protein complexes of up to 1 MDa (**Fig. 1A**). Furthermore, release of JAZ1 from these complexes and its subsequent degradation indicated by a smear in the 20-kDa region could be observed after treatment with JA for 1 min (**Fig. 1A**), which is in accordance with the reported rapid, JA-triggered, degradation of JAZ proteins.[6,12]

A number of pairwise interactions between several candidate elements of such a JAZ1 protein complex have already been established and may account for the observed HMW. First, interactions with MYC2 have been shown for almost all JAZ proteins and the JAZ1-TAP analysis supported interaction between JAZ1

and the closely related MYC3 as well.[6,13] Second, although dimerization of MYC2 or MYC3 has not been reported yet, it might occur, at least under certain conditions, because, for instance, heterodimerization of the basic-helix-loop-helix (bHLH) proteins GLABRA3 and ENHANCER OF GLABRA3, both involved in trichome development and closely related to MYC2, has already been described.[14,15] In addition, in maize (*Zea mays*) the homologous bHLH factor R is capable of forming homodimers.[16] Third, JAZ-JAZ dimerizations might facilitate the simultaneous binding of multiple JAZ proteins with MYC proteins.[13,17] Fourth, the JAZ-NINJA and NINJA-TPL interactions are necessary for recruiting TPL to the JA signaling module.[6] Finally, TPL dimerization has been reported and might be required for optimal transcriptional repression.[18] The TAP analysis already suggested the co-occurrence of multiple of these pairwise interactions between the MYC, JAZ, NINJA and TPL proteins.[6] The BN/SDS-PAGE analysis corroborates this hypothesis. Based on the possible, known, pairwise interactions listed above and the observed HMW complexes in the BN/SDS-PAGE analysis, a few tentative JA signaling repressor modules and their calculated sizes can be postulated (**Fig. 1B and C**). Obviously, these models merely represent a partial or static view because more or other, yet to be discovered, proteins could participate in the assembly of the JAZ HMW complexes either constitutively or transiently. The identification of such new interactors represents an exciting challenge for the research community and will undoubtedly benefit from the continuous technological advances in the proteomics field that will guarantee an increased sensitivity and resolution in time and space, essential parameters for the full elucidation of the JA signaling cascade.

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