A small molecule antagonizes jasmonic acid perception and auxin responses in vascular and nonvascular plants

Andrea Chini ^{1,*,#} Isabel Monte ^{1,†} Gemma Fernández-Barbero,¹ Marta Boter ^{1,‡}
Glenn Hicks ² Natasha Raikhel ² and Roberto Solano ^{1,#}

¹ Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología-CSIC, Campus Universidad Autónoma, Madrid, 28049, Spain

² Department of Botany and Plant Sciences, Center for Plant Cell Biology, University of California, Riverside, California, 92521, USA

*Author for correspondence: achini@cnb.csic.es

†Present address: Institute of Plant and Microbial Biology, Zurich-Basel Plant Science Center, University of Zurich, Zurich, 8008, Switzerland.

‡Present address: Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid –Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain.

#Senior authors

The authors have made the following declarations about their contributions: A.C., G.R.H., N.V.R., and R.S. conceived and designed the experiments; A.C., I.M., and G.F.B. performed the experiments; A.C., I.M., G.F.B., G.R.H., N.V.R., and R.S. analyzed the data; M.B. contributed reagents/materials; A.C. and R.S. wrote the paper. A.C. and R.S. agree to serve as authors responsible for contact and ensure communication.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is: Andrea Chini (achini@cnb.csic.es).

Abstract

The phytohormone jasmonoyl-L-isoleucine (JA-Ile) regulates many stress responses and developmental processes in plants. A co-receptor complex formed by the F-box protein Coronatine Insensitive 1 (COI1) and a Jasmonate (JA) ZIM-domain (JAZ) repressor perceives the hormone. JA-Ile antagonists are invaluable tools for exploring the role of JA-Ile in specific tissues and developmental stages, and for identifying regulatory processes of the signaling pathway. Using two complementary chemical screens, we identified three compounds that exhibit a robust inhibitory effect on both the hormone-mediated COI–JAZ interaction and degradation of JAZ1 and JAZ9 *in vivo*. One molecule, J4, also restrains specific JA-induced physiological responses in different angiosperm plants, including JA-mediated gene expression, growth inhibition, chlorophyll degradation, and anthocyanin accumulation. Interaction experiments with purified proteins indicate that J4 directly interferes with the formation of the Arabidopsis (*Arabidopsis thaliana*) COI1–JAZ complex otherwise induced by JA. The antagonistic effect of J4 on COI1–JAZ also occurs in the liverwort *Marchantia polymorpha*, suggesting the mode of action is conserved in land plants. Besides JA signaling, J4 works as an antagonist of the closely related auxin signaling pathway, preventing Transport Inhibitor Response1/Aux–indole-3-acetic acid interaction and auxin responses *in planta*, including hormone-mediated degradation of an auxin repressor, gene expression, and gravitropic response. However, J4 does not affect other hormonal pathways. Altogether, our results show that this dual antagonist competes with JA-Ile and auxin, preventing the formation of phylogenetically related receptor complexes. J4 may be a useful tool to dissect both the JA-Ile and auxin pathways in particular tissues and developmental stages since it reversibly inhibits these pathways.

Introduction

Plant hormones are bioactive small signaling molecules directly perceived by plant receptor complexes. Phytohormones regulate, alone or in combination, multiple aspects of plant development, responses to the environment, and to biotic challenges primarily through transcriptional reprogramming. Phytohormones regulate most known plant defenses, and different plant-interacting organisms have evolved the capability to produce phytohormones or phytohormone mimics to induce disease susceptibility and counteract plant defenses (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Fonseca et al., 2018). For example, the phytotoxin coronatine (COR) is a jasmonoyl-L-isoleucine (JA-Ile) functional analog produced by *Pseudomonas syringae* to increase its virulence hijacking the plant defense signaling network (Kloek et al., 2001; Brooks et al., 2004; Gimenez-Ibanez and Solano, 2013). In addition to the evolutionary relevance, natural mimic molecules are also important tools in research; for instance, the use of COR was determinant to the identification of several components of the jasmonate (JA) signaling cascade including the JA-Ile and COR co-receptor COR Insensitive 1 (COI1; Xie et al., 1998; Sheard et al., 2010). In addition, several plant hormone analogs are synthetically synthesized and exploited for agricultural and research purposes. For example, several synthetic auxin analogs and anti-auxins represent “classical” herbicides (Van Overbeek and Velez, 1946; Grossmann, 2010). Mutants completely impaired in auxin perception are not viable; however, auxin-related molecules, such as auxinole and α -phenylethyl-2-one (PEO)-indole-3-acetic acid (IAA), are commonly employed to reversibly block auxin perception and study auxin-regulated processes in several plant species (Hayashi et al., 2012; Leyser, 2018). Therefore, the identification of new molecules interfering with hormonal signaling cascades may be very useful in agriculture and research.

Chemical genomics aims to identify small molecules modifying different biological processes (Norambuena et al., 2009; Toth and van der Hoorn, 2010; Hicks and Robert, 2014). The structural diversity of small compounds is extraordinarily broad (Dobson, 2004). Large chemical libraries are screened to identify molecules affecting the activity of a protein, protein families, or a pathway in a spatial–temporal and reversible manner. An established advantage of this methodology is to overcome the common limitations of genetic mutant tools such as redundancy or lethality of essential genes (Toth and van der Hoorn, 2010; Hicks and Raikhel, 2012). Chemical genomic approaches successfully identified hormone analogs or previously unidentified functions of phytohormones (Hicks and Raikhel, 2012). A paradigmatic example is the agonist of abscisic acid (ABA) pyrabactin, originally identified as a synthetic inhibitor of growth and cell expansion (Zhao et al., 2007). Use of pyrabactin was instrumental in discovering the redundant ABA receptors PYR/PYL (Park et al., 2009). Furthermore, chemical genomics helped to uncover the function of strigolactone in light response (Tsuchiya et al., 2010).

JAs are lipid-derived phytohormones that mediate responses to abiotic and biotic stress such as drought, salinity, wounding, and pathogen attacks (Kazan, 2015; Ebel et al., 2018; Wasternack and Feussner, 2018). In addition, JAs regulate the biosynthesis of many secondary metabolites often involved in plant defense, such as anthocyanins or terpenoids (Howe et al., 2018; Wasternack and Feussner, 2018). JAs are also involved in many developmental processes including growth inhibition and senescence (Howe et al., 2018; Wasternack and Feussner, 2018). Endogenous developmental processes, as well as plant adaptation to environment, induce the accumulation of the bioactive form of the hormone, (+)-7-*iso*-JA-Ile (Fonseca et al., 2009; Sheard et al., 2010). The identification of the core JA-Ile pathway components evidenced the striking mechanistic parallelism between the JA-Ile and auxins pathways. Both pathways are composed of three elements: (1) redundant transcription factors regulating JA-Ile or auxins responses (e.g. MYCs or Auxin Responsive Factors, respectively); (2) a family of redundant repressors of the transcription factors (TF) (i.e. JA ZIM-domain [JAZs] or Aux/IAA), which directly or indirectly (through the NINJA adaptor; Pauwels et al., 2010) recruits the co-repressor TOPLESS; and (3) a co-receptor formed by an F-box protein (COI1 for JA-Ile and Transport Inhibitor Response1 [TIR1]/Auxin F-Box [AFBs] for auxin) and its repressors targets (JAZs for JA-Ile and Aux/IAA for auxins; Chini et al., 2016; Leyser, 2018). In accordance with the “molecular glue” model, in both cases the hormone binding to its co-receptor triggers ubiquitination and degradation of the repressor and, therefore, releases the corresponding TF to activate the pathway (Chini et al., 2007; Maor et al., 2007; Thines et al., 2007; Chini et al., 2009a; Saracco et al., 2009; Lumba et al., 2010; Chini et al., 2016; Howe et al., 2018; Leyser, 2018). It is remarkable that the receptors COI1 and TIR1/AFBs have a common evolutionary origin and, although strikingly similar, they are adapted to perceive structurally different ligands (Bowman et al., 2017).

The JAZ proteins are repressors that inhibit a large number of TFs belonging to several different families, including bHLH (basic helix-loop-helix), MYB, and Ethylene-Insensitive-3 (EIN3)/EIL (Chini et al., 2016; Howe et al., 2018; Zander et al., 2020). The identification of these components of the JA perception and signaling pathway were carried out using mutant screens or reverse genetics (Xie et al., 1998; Browse, 2009; Chini et al., 2016). However, several components of the JA-signaling pathway belong to large protein families with redundant functions, such as the 13 Arabidopsis JAZ repressors or the 4 MYC transcription activators, where genetic approaches are very laborious and their success may be more limited (Chini et al., 2007; Thines et al., 2007; Fernandez-Calvo et al., 2011; Qi et al., 2015; Guo et al., 2018; Zander et al., 2020). Therefore, specific antagonist molecules of JA perception would represent excellent tools to overcome redundancy and to explore the role of JA in spatio-temporal analyses. A chemical screen identified jirin1 as inhibitor of JA responses, impairing the synthesis of JA-Ile

(Meesters et al., 2014). In addition, COR-O-methyloxime (COR-MO) was rationally designed as specific antagonist of JA perception that shows strong inhibiting activity of COI1–JAZ interaction, JAZ degradation and several JA-mediated responses (Monte et al., 2014).

In this work, we searched for different and cheaper antagonists of JA-Ile perception by chemical screens. Here we describe the identification of three commercially available JA-Ile antagonists and their characterization. These molecules prevent the JA-Ile-mediated COI1–JAZ interaction and the degradation of JAZ1 and JAZ9 in vivo. Moreover, one molecule (J4) exhibited JA-antagonist effects in planta, preventing several JA-mediated responses such as gene expression, growth inhibition, chlorophyll degradation, and anthocyanin accumulation in *Arabidopsis*, tomato (*Solanum lycopersicum*), and *Nicotiana benthamiana*. In addition to JA signaling, J4 also inhibited the closely related auxin pathway but no other hormonal pathways. Furthermore, the mode of action of this molecule as direct antagonist of JA-Ile perception by COI1–JAZ complexes is conserved in land plants, indicating its potential use in any plant species. This commercially available compound is a potentially powerful tool for the pharmacological analysis and dissection of the JA and auxin signaling pathways.

Results

Chemical screens

The perception of the bioactive form of the JA-Ile hormone is mediated by the co-receptor COI1–JAZ (Sheard et al., 2010). In order to identify synthetic molecules promoting or interfering with the COI1–JAZ interaction, we carried out two independent chemical screens based on the interaction between COI1 and JAZ9 in yeast-two-hybrid (Y2H) assays (Chini et al., 2009b; Fonseca et al., 2009). Three chemical libraries containing approximately 22,500 molecules (see “Materials and methods”) were tested to identify agonists and antagonists of JA-Ile perception, inducing or preventing the hormone-triggered COI1–JAZ9 interaction, respectively. None of the agonist compounds found could reproducibly induce COI1–JAZ9 interaction. However, five antagonist molecules were identified and confirmed (Y10, Y11, Y17, Y18, and Y20; Y defines molecules identified in the Y2H screen [Figure 1, A and B; Supplemental Figure S1A and Supplemental Table S1]), with Y10 being weaker than the other four. We also confirmed that these compounds do not affect other interactions of these proteins (JAZ9 with itself or with NINJA) ruling out general unspecificity. We also tested whether these molecules could inhibit the formation of additional COI1–JAZ complexes, confirming that these five compounds prevent the COR-induced COI1–JAZ3 interaction (Figure 1B).

Two complementary chemical screens were also carried out in planta exploiting the rapid hormone-dependent JAZ degradation using the 35S::JAZ1- β -glucuronidase (GUS) reporter line (Thines et al., 2007). Approximately 15,000 compounds (see “Materials and methods”) were tested for

triggering degradation of JAZ1–GUS in the absence of JA-Ile/COR or preventing COR-induced degradation of JAZ1–GUS. Again, no agonist compounds were confirmed, whereas we validated seven antagonist molecules (J1, J2, J3, J4, J9, J10, and J11; J defines the compounds identified in the JAZ1–GUS screen), preventing hormone-induced degradation of JAZ1 (Figure 1, A and C; Supplemental Figure S2 and Supplemental Table S1). Since the original screen was carried out with COR, we tested the candidate molecules in response to JA to discard unspecific effects of COR or an effect of the compound on JA conversion into JA-Ile. All J1 to J11 compounds were confirmed (Supplemental Figure S2).

Among the seven compounds obtained in the screens in planta (J) only one was also active in yeast assays, whereas among the five compounds identified in Y2H screens (Y) only three were positive in planta (Supplemental Figure S3; despite Y10 was positive we discarded this compound because of its weak activity both in yeast and plant). Therefore, we confirmed three compounds (J4, Y11, and Y20; Figure 1A) with robust antagonistic activity in both Y2H and in vivo assays. These three compounds prevented the JA-triggered degradation of JAZ1–GUS and JAZ9–GUS in vivo, showing that their effect is not restricted to a particular JAZ (Figure 1B). Similarly, the three compounds inhibited the interaction of COI1–JAZ9 and COI1–JAZ3 in Y2H, without affecting other interactions of these proteins (JAZ9 with itself or with NINJA, Figure 1C). The effect of J4 and Y11 as antagonists of COR-induced COI1–JAZ9 interaction was confirmed in semi in vivo pull-down (PD) experiments between recombinant JAZ9 fused to maltose-binding protein (MBP) and COI1-flag expressed in planta (Figure 1D).

In summary, these results confirmed that two molecules, namely J4 and Y11, interfere with the COI1 interaction with more than one JAZ co-receptor both in the heterologous yeast system and in PD experiments. This suggests that these compounds might compete with COR or JA-Ile for the binding to COI1 in vivo. However, an indirect effect of these molecules acting through a regulator of COI1 or JAZ cannot be discarded yet.

Minimal active concentration of antagonist molecules of JA-Ile/COR perception

Next, we estimated the minimal active concentration at which these molecules could act as antagonist of the JA-Ile/COR perception. The J4 and Y20 compounds prevent the formation of the COI1–JAZ receptor complex in yeast at a concentration between 5 and 50 μ M, similar to the concentration of COR (5 μ M) used to induce COI1–JAZ interaction (Supplemental Figure S4). The compound Y11 inhibits the COI1–JAZ9 interaction at a concentration as low as 300 nM; however, Y11 is toxic for the yeast at a concentration of 1 μ M as shown by the lack of yeast growth in the control JAZ9–JAZ9 interaction assay (Supplemental Figure S4).

Similarly, the J4 and Y20 molecules prevent JAZ1-degradation in planta at minimal concentrations between 10 and 25 μ M, whereas Y11 had a weaker effect (Supplemental

Figure 1 Identification of molecules with JA-Ile antagonist activity in yeast two-hybrid, in planta and in the hormone-dependent formation of the receptor complex COI1–JAZ9. **A**, Chemical structure of the JA-antagonist compounds. **B**, Yeast cells co-transformed with pGAD-JAZ9 (preys) and pGBK-COI1, pGBK-NINJA, or pGBK-JAZ9 (baits) were selected and subsequently grown on yeast synthetic drop-out lacking Leu and Trp (-LW) as a transformation control, or on selective media lacking Ade, His, Leu, and Trp (-AHLW) to test protein interactions. COI1 interaction with JAZ9 or JAZ3 is detected only in presence 5 mM or 20 mM COR, respectively. Antagonist molecules inhibit COI1–JAZ interaction in presence of COR (J4 and Y20 were used at 15 μ M, and Y11 at 300 nM). Compounds were dissolved in DMSO; therefore, an equivalent volume of DMSO was used in the negative control (labeled as -). As control, we tested that the antagonist molecules did not interfere with the interaction between JAZ9 and NINJA or JAZ9 dimerization. **C**, Six-day-old JAZ1-GUS and JAZ9-GUS seedlings were concurrently treated with 2.5 μ M JA and the indicated molecules (J4 and Y20 were used at 25 μ M and Y11 at 50 μ M). Jasmonic acid triggers the degradation of JAZ-GUS protein, whereas the addition of antagonist molecules prevented JAZ degradation. Compounds were dissolved in DMSO; a volume of DMSO equivalent to that in the compounds was used in the negative control (identified as -). **D**, Immunoblot analysis of COI1–flag/JAZ9–MBP interaction performed with anti-flag antibodies to COI1-flag protein recovered from MBP–JAZ9 and extracts of transgenic COI1-flag plants. MBP was employed as negative control. The COI1-flag/JAZ9–MBP interaction is dependent on the presence of COR (0.5 μ M). Compounds were dissolved in DMSO; therefore, a volume of DMSO equal to that in 0.5 μ M of the compounds was used as control. The compounds Y11 and J4 reduce COI1-flag/JAZ9–MBP interaction (at 100 and 500 μ M). The lower panel shows Coomassie blue staining of the MBP and the JAZ9–MBP after cleavage with Factor Xa.

Figure S5). In summary, J4 and Y20 show an antagonistic activity on processes mediated by JA-Ile and COR at a concentration close to that usually employed for exogenous hormone treatments.

Structure–activity relationship

To define the active moiety of these molecules, we performed an analysis of structure–activity relationship (SAR) of the three identified compounds (Rosado et al., 2011). No structurally related compounds of Y11 were available. In contrast, we found three derivatives of Y20 (named Y20-L1, Y20-L2, and Y20-L3; Supplemental Figure S6A). Y20-L1 and Y20-L2 prevented COI1 interaction with both JAZ9 and JAZ3 at the same concentration as Y20 in Y2H (Supplemental Figure S6B). Similarly, they prevented the JA-

induced degradation of both JAZ1-GUS and JAZ9-GUS in planta (Supplemental Figure S6C), whereas Y20-L3 fails to show antagonistic activity in all bioassays (Supplemental Figure S6, B and C). These results suggest that the antagonistic activity of Y20 resides in the minimal 3-butyryl-4-hydroxy-2H-chromen-2-one structure. Moreover, we identified that either or both the hydroxy group and the butyryl lateral chain are essential for the activity of this molecule as JA antagonist, as simultaneous shortening of two carbons in the butyryl chain and loss of the hydroxy group in Y20-L3 abolish the activity (Supplemental Figure S6A).

The use of J4 derivatives (Supplemental Figure S6A) showed that the different substitutions of the benzene ring did not have any impact on the activity of these molecules, since all of them retained the activity as JA antagonists in

planta in the JAZ1 and JAZ9 degradation assays (Supplemental Figure S6C). We concluded that the 5-(benzylidene)-1,3-thiazolidine-2,4-dione moiety is important for the antagonist activity.

Molecules inhibiting JA-Ile perception affect JA-mediated transcription in vivo

We subsequently analyzed the effect of these molecules on JA-induced gene expression in planta. JAZ2 and JAZ9 are among the genes most rapidly and strongly induced by JA in a COI1-dependent manner (Chini et al., 2007; Thines et al., 2007). Therefore, we assessed the effect of these molecules on the JA-induced gene expression in the *pJAZ2:GUS* and *pJAZ9:GUS* reporter lines (Monte et al., 2014; Gimenez-Ibanez et al., 2017). In basal conditions, *pJAZ2:GUS* and *pJAZ9:GUS* are expressed at very low levels but they are quickly and strongly induced in response to JA and after wounding stress, which stimulates the synthesis of endogenous JA-Ile (Figure 2, A and B; Monte et al., 2014). Simultaneous treatment of exogenous JA or wounding with the identified molecules, and derivative compounds, strongly inhibited the JA-mediated activation of both JAZ2 and JAZ9 expression in planta (Figure 2, A–B; Supplemental Figure S7).

Interestingly, the JAZ9 transcript accumulates specifically in trichomes in basal conditions (Supplemental Figure S8A). This basal expression is abolished in the *coi1-30* background, which indicates that perception of endogenous JA-Ile is required for the specific JAZ9 expression in the trichomes (Supplemental Figure S8B). We assessed the inhibitory effect of the identified molecules on the trichome tissue-specific expression of JAZ9. Interestingly, only the compound J4 repressed the JA-regulated expression of JAZ9 in the trichomes (Supplemental Figure S8C). To further assess the inhibitory effect of J4 on additional JA-markers, we analyzed the expression of the JA-induced *OPR3*, *TAT3*, and *JAZ10* genes. Indeed, exogenous treatment with J4 partially prevents the transcriptional activation of these markers by JA (Supplemental Figure S9A).

To test if the JA inhibitory activity of J4 is conserved across angiosperm plants, we tested the effect of J4 on JA-regulated gene expression in two species of the Asterid clade, tomato, and *N. benthamiana*. Similar to the results in Arabidopsis, exogenous treatment with J4 significantly prevents the JA-induced transcriptional activation of several marker genes in both tomato and *N. benthamiana* plants (Figure 2, C and D).

These results show that these three compounds can prevent transcriptional activation induced by both endogenous and exogenous JA. Moreover, J4 can also inhibit the expression of a marker gene mediated by endogenous JA-Ile in a specific tissue as well as in different angiosperm plants.

Physiological effect of the identified antagonists in planta

JA induced several physiological responses, such as growth inhibition, anthocyanin accumulation, and reduction of

chlorophylls, among others (Wasternack and Feussner, 2018). In order to assess the effect of the identified molecules on JA-regulated physiological responses, we grew plants in presence of JA and the identified molecules. The concurrent treatment of the J4 compound at concentrations as low as 5 μ M could partially prevent the root growth-inhibition, chlorophyll degradation, and anthocyanin accumulation induced in planta by JA (10 μ M; Figure 3). In contrast, Y11 and Y20 failed to prevent JA-mediated responses in planta (Supplemental Figure S10). We also determined the half-maximal inhibitory concentration (IC₅₀) of J4. For a concentration of 15 μ M of JA in the JAZ1-GUS degradation assay, the IC₅₀ of J4 was 17.66 μ M (Supplemental Figure S11). To study if J4 conserved its JA inhibitory activity in different plants, we tested the effect of J4 on JA-induced chlorophyll and carotenoids degradation in *N. benthamiana*. Exogenous treatment of *N. benthamiana* with J4 significantly prevents the JA-induced degradation of both chlorophyll and carotenoids in planta (Supplemental Figure S12).

Altogether, these data confirm that J4 acts as an antagonist of the JA pathway and inhibits several hallmark JA-mediated responses in planta.

Specificity of J4

To study J4 specificity, we analyzed possible inhibitory side effects of J4 on the ubiquitin/proteasome process that would in turn stabilize the JAZ proteins. Therefore, we analyzed the effect of J4 on other hormonal pathways regulated by the ubiquitin/proteasome system, similarly to the JA pathway. First, we monitored the gibberellin-mediated proteasome degradation of Repressor of GA (RGA)-GFP and the constitutive proteasome-dependent degradation of EIN3-GFP, which is inhibited by ethylene or its precursor ACC (Silverstone et al., 2001; Guo and Ecker, 2003). As shown in Figure 4A, J4 did not prevent the GA-triggered degradation of RGA. Similarly, J4 did not substantially inhibit the constitutive degradation of EIN3 in the absence of ACC (Figure 4B). Second, we assessed the effects of J4 on the turnover of the auxin Aux/IAA repressors, whose degradation depends on the activity of the F-box TIR1, the receptor of auxins, and the closest homolog of COI1 (Chico et al., 2008). In contrast to GA and ethylene, J4 partially inhibited the auxin-mediated degradation of the dII-VENUS auxin-repressor marker and the auxin-repressor IAA1 (Figure 4, C and D). To further assess the effect of J4 on the auxin pathway, we tested the impact of this molecule on the auxin transcriptional marker *DR5:GUS*. Exogenous IAA treatment highly induced the expression of *DR5:GUS*, whereas the anti-auxin 2,3,5-triiodobenzoic acid (TIBA) and J4 could partially inhibit the IAA-mediated *DR5:GUS* expression (Figure 5A). PD analyses in Figure 5B show that IAA induces the interaction between TIR1 and IAA7 whereas J4 partially inhibited this interaction, similarly to the effect of the specific auxin perception inhibitor auxinole (Figure 5B). Finally, J4 also impairs the auxin-regulated gravitropic response in root (Figure 5, C–D).

Figure 2 Antagonistic molecules prevent the JA-induced expression of several JAZ genes in planta. A, Representative seedlings ($N > 30$) of the *pJAZ2:GUS* and *pJAZ9:GUS* line were concurrently treated with 5 μM JA for 75 min and the indicated molecules (J4 at 10 μM , Y20 at 25 μM , and Y11 at 100 μM) for 3 h. Jasmonic acid triggers the expression of JAZ, whereas the addition of antagonist molecules could prevent JAZ transcriptional activation. Compounds were dissolved in DMSO; an equivalent volume of DMSO was used in the negative control (identified as -) in (A) and (B). White bars are equal to 1 mm in (A) and (B). B, Seedlings of *pJAZ2:GUS* and *pJAZ9:GUS* were wounded several times and concurrently treated with the indicated compounds (J4 at 10 μM , Y20 at 25 μM , and Y11 at 100 μM) for 2 h. The mechanical wounding induces the JAZ expression whereas the JA-antagonists can prevent wound-induced JAZ expression. C and D, Gene expression analysis of JA-marker genes in *Solanum lycopersicum*, moneymaker cultivar, (C) and *N. benthamiana* (D) plants in response to JA treatment and/or the J4 molecule. Tomato (C) plants were pretreated with DMSO (-; untreated control), 50 or 100 μM J4 for 1 h, and then with 50 μM JA for 1 h. *SlATUB4* was used as housekeeping control gene. *Nicotiana benthamiana* plants were pretreated with DMSO (-; untreated control), 50 or 100 μM J4 for 1 h, and then with 25 μM JA for 1 h. *Nb β ACT* was used as housekeeping control gene. Experiments were repeated 3 times with similar results. One-way analysis of variance (ANOVA) with post-hoc Tukey's honestly significant difference (HSD) test ($P < 0.01$) analyses define the significant differences in gene expression. Each biological sample consisted of tissue pooled from 10 to 15 plants. Data show mean $\pm$ SD of 3–4 technical replicates.

Figure 3 Antagonistic effect of the compound J4 on JA-induced responses in planta. A, 13-d-old WT seedlings grown for 10 d on vertical plates in presence of 10 μ M JA with or without 5 μ M J4. White bar stands for 1 cm. B, Root growth inhibition by 10 μ M JA of 13-d-old seedlings in presence or absence of 5 μ M J4. J4 was dissolved in DMSO; therefore, an equivalent volume of DMSO was used in the negative control (identified as -) in (A)–(D). C, Anthocyanin accumulation, shown as Absorbance (530) per gram of plant fresh weigh, in 7-d-old WT seedlings grown for 2 d in presence of 10 μ M JA with or without 2.5 μ M J4. D, Chlorophyll a and b content of 13-d-old WT seedlings grown for 10 d on vertical plates in presence of 10 μ M JA with or without 5 μ M J4. B–D, Bars represent the average value and error bar the SD. Asterisks indicate statistically significant values of JA-treated plants according to a Student's *t* test ($P < 0.05$).

These results show that J4 does not generally affect ubiquitin/proteasome-regulated systems but is not a fully specific antagonist of JA-Ile perception, having also an inhibitory effect on the auxin pathway.

J4 mode of action: a direct antagonist of hormone-receptor complex

Previous results suggest a possible mode of action of J4 as a direct competitor with JA-Ile or COR for induction of

COI1–JAZ complex (or competition with auxin in the case of TIR1–IAA complex). To further study the J4 mode of action on the JA pathway, we checked if this molecule interferes directly with the assembly of the COI1–JAZ perception complex in vitro, in the absence of other plant proteins. For that, we used a heterologous baculovirus–insect cell expression system to express and purify the COI1-flag protein. Since purified COI1 is quite unstable and mostly inactive, we also expressed in insect cells a key component of the

Figure 4 The compound J4 does not affect the ubiquitin/proteasome system. A, Immunoblot analysis of the effect of antagonists on RGA-GFP stability (with anti GFP antibodies). pRGA:GFP-RGA seedlings were concurrently treated for 2 h with 10 μ M gibberellic acid (GA_3) and J4 at 10 μ M. GA_3 induces RGA degradation and J4 fails to prevent RGA destabilization. J4 was dissolved in DMSO; an equivalent volume of DMSO was used in the negative control (labeled as -) in (A)–(D). B, Immunoblot analysis of the effect of antagonists on EIN3-GFP stability (with anti GFP antibodies). Seedlings of the 35S:EIN3-GFP line were treated for 2 h with the J4 at 10 μ M or 10 μ M of the ethylene precursor ACC. EIN3 is continuously degraded and stabilized by ACC. The synthetic proteasome inhibitor MG132 (at 100 μ M) was included as positive control. C and D, Effect of J4 the stability of the on dII-VENUS (with anti GFP antibodies) auxin sensor and IAA1-HA (with anti HA antibodies) in planta. Immunoblot analysis of 35S:dII-VENUS and 35S:IAA1-HA seedlings of the dII-VENUS auxin repressor marker (C) or the auxin repressor IAA1 (D) treated for 1 h with 1 μ M IAA in absence or presence of J4 at 10 μ M. The first band shows constitutive expression of the dII-VENUS auxin marker (C) or IAA1-HA (D).

SCF^{COI1} complex, the adaptor Arabidopsis SKP-like protein 1 (ASK1), which stabilizes and maintains the bioactivity of COI1 (Li et al., 2017). In the presence of ASK1, COR promotes the interaction between the purified COI1 and several recombinant JAZ-MBP proteins (JAZ1, JAZ2, JAZ3, and JAZ9). J4 efficiently inhibits the COR-induced formation of all the tested COI1–JAZ complexes (Figure 6A). To test if the mode of action of J4 is conserved across land plants, we tested the effect of J4 on the MpCOI1–MpJAZ co-receptor from the bryophyte *Marchantia polymorpha* (Monte et al., 2018). This co-receptor is the most different COI1–JAZ complex known from that of Arabidopsis, which even perceives a different ligand (dinor-OPDA (dn-OPDA) instead of JA-Ile), and diverged evolutionarily more than 450 million years ago (Bowman et al., 2017; Monte et al., 2018). The MpCOI1 and MpASK1 proteins were expressed and purified in insect cells and their hormone-induced interaction with *Escherichia coli*-purified MBP-MpJAZ protein was tested in PD experiments. As shown in Figure 6B, dn-OPDA induces the interaction of the purified MpCOI1 and MpJAZ, similar to the effect of JA-Ile on Arabidopsis COI1–JAZs. In contrast, treatment with J4 substantially inhibited the dn-OPDA-dependent formation

of the MpCOI1–MpJAZ complex (Figure 6B). We subsequently analyzed the effect of J4 in planta by studying the OPDA-regulated transcriptional expression of several OPDA marker genes such as MpJAZ, MpDIR, MpPAT, and MpBHLH4 (Monte et al., 2018; Monte et al., 2019; Peñuelas et al., 2019). Indeed, exogenous pretreatment with J4 partially prevents the transcriptional activation of these marker genes by OPDA (Figure 6C; Supplemental Figure S13).

Altogether, these results show that J4 directly interferes with the hormone-triggered establishment of the COI1–JAZ receptor complexes of vascular and nonvascular plants in absence of any other plant proteins. In addition, J4 has an in vivo inhibitory effect on the JAs-mediated responses in both vascular and nonvascular plants. Finally, the absence of putative regulators of COI1 or JAZ proteins in these assays (Figure 6, A and B) indicates that J4 has a direct effect on the formation of the hormone-promoted COI1–JAZ complexes, likely competing with the ligands for the binding pocket.

Discussion

To date, the role of JA regulating plant development and adaptation to biotic and abiotic stresses has been primarily analyzed using classical genetic approaches relying on mutants impaired in the biosynthesis or response to JA (Browse, 2009; Chini et al., 2016). However, these loss-of-function genetic tools hold some intrinsic limitations such as functional redundancy, sterility, and lethality (Xie et al., 1998; Sanders et al., 2000; Browse, 2009; Fernandez-Calvo et al., 2011; Qi et al., 2015; Chini et al., 2018b). Thus, the identification of molecules that specifically prevent JA-Ile-perception represents excellent tools to explore the role of JA in specific plant stages or tissues, avoiding these limitations. For example, specific auxin inhibitors were critical tools to understand the role of auxins in various stages of plant development and different plant species (Hayashi et al., 2012; Leyser, 2018). Using the auxin inhibitor naxillin researchers defined a previously unknown function for root cap in root branching by specifically stimulating the accumulation of active endogenous auxin only in the root cap (De Rybel et al., 2012).

Molecules antagonizing JA-perception would allow the manipulation of the JA pathway in a specific tissue or developmental stage in a reversible manner. Ideally, the antagonists identified in Arabidopsis should be easily transferred to other plant species and might clarify the role of JA-Ile in different plants. The two-step approach reported here strongly minimizes the identification of false hits, one of the most problematic constraints in chemical screens (Hicks and Robert, 2014; Serrano et al., 2015). Thus, we identified only three molecules (among the more than 20,000 compounds screened) with robust JA-Ile antagonistic activity in both bioassays (Figure 1). Further biochemical and physiological analyses confirmed J4 as the only compound with robust JA-Ile antagonistic activity on several transcriptional and

Figure 5 Effect of the compound J4 on several auxin-regulated responses. A, Effect of J4 on auxin-induced *DR5:GUS* reporter gene expression. Six-day-old seedlings were treated with IAA (5 μ M) alone and in presence of the auxin inhibitor TIBA (100 μ M) or J4 (10 μ M) for 90 min. J4 was dissolved in DMSO, therefore an equivalent volume of DMSO was used as negative control (defined as -) in (A)–(D). B, Immunoblot analysis of TIR1-myc/IAA7-GST interaction performed with anti-myc antibodies to TIR1-myc protein recovered from IAA7-GST and extracts of transgenic *TIR1-myc* plants. IAA (5 μ M) induces the interaction between the auxin co-receptors TIR1-myc and IAA7-GST, whereas the specific auxin-perception inhibitor auxinole and J4 (at 100 μ M) partially inhibits the TIR1–IAA7 complex formation. C and D, Effect of J4 on auxin-mediated gravitropic response. Four-day-old seedlings grown on MS plates were transferred to control DMSO (-), NPA (1 μ M) or J4 (2.5 μ M) plates and then rotated 135° from the gravity vector (arrow 1 versus 2). Photographs were taken 24 h after re-orientation (C). Bar lengths represent the percentage of seedlings ($n = 28$ –32 seedlings for each treatment) growing at the indicated orientation 24 h after re-orientation (D).

physiological JA-responses in planta at concentrations similar to that of JA (Figure 3; Supplemental Figure S9).

The toxicity and secondary effects of current agrochemicals can seriously affect the environment and, potentially, animal and human health (Enserink et al., 2013; Lamberth et al., 2013). Multiple, unspecific or pleiotropic effects are rather common among compounds identified in chemical screens (Fonseca et al., 2014b; Serrano et al., 2015). Exceptionally, chemical screens also identified molecules with very specific targets and limited or none off-target effects. For example, Kynurenine, identified as inhibitor of the ethylene responses in root tissues (He et al., 2011), is the first specific inhibitor of auxin biosynthesis binding to the substrate pocket of the TAA1/TAR aminotransferases family but no other related aminotransferases.

Here, we showed that J4 acts as a specific and dual inhibitor of the closely related JA and auxin signaling pathways by antagonizing the hormone-induced formation of the co-receptor complexes. Moreover, this inhibitory activity is evolutionarily conserved in land plants (at least between liverworts and eudicots) since J4 also inhibits the formation of the *Marchantia* MpCOI1–dn-OPDA–MpJAZ complex. JA and auxin pathways share strikingly similar receptors (COI1

and TIR1), which have a common evolutionary origin and share many conserved residues (Bowman et al., 2017). Thus, we propose that J4 can partially mimic dn-OPDA, JA-Ile, and auxin to enter the COI1/TIR1 binding pocket, but cannot establish the complex due to a lack of interaction with the JAZ/Aux-IAA co-receptors.

Therefore, J4 may be considered a specific antagonist of two related receptors that, however, do not affect other hormonal pathways or general proteasome-related mechanisms. Our results support the use of J4 as a commercially available inhibitor of JA and auxin pathways in a particular tissue or developmental stage, useful in plant research and for agronomic purposes. J4 could also be useful in dissecting JA/auxin crosstalk. However, the concurrent inhibition of these two hormonal pathways may be undesirable in some circumstances. Therefore, additional structure–SARs analysis may be carried out to identify molecules structurally related J4 that potentially act as specific inhibitors of the JA or auxin pathway.

An alternative to chemical screen is the rational design of antagonist molecules specifically binding to the active pocket of key proteins. This approach, based on structural information of receptor–ligand complexes, is extensively

Figure 6 J4 interferes with the hormone-dependent formation of several COI1-JAZ receptor complexes. A, Immunoblot analysis of AtCOI1-flag/AtJAZ-MBP interaction performed with anti-flag antibodies to COI1-flag proteins recovered from MBP-AtJAZ columns. AtCOI1-flag and COI1-flag were expressed in Sf9 insect cells, whereas AtJAZ were expressed in *Escherichia coli*. The AtCOI1-flag/AtJAZ-MBP interaction is dependent on the presence of COR (0.5 μM); the compound J4 (at 100 and 200 μM) inhibits the hormone-dependent AtCOI1-flag/AtJAZ-MBP interaction. J4 was dissolved in DMSO; therefore, an equivalent volume of DMSO was used as negative control (labeled as -) in (A)–(C). The lower part shows Coomassie blue staining of the AtJAZ-MBP after cleavage with Factor Xa. Asterisks indicate the JAZ-MBP bands of the expected size in the Coomassie panel of each pull-down assay. B, Immunoblot (antiflag antibody) of recovered MpCOI1-flag after pull-down reactions using recombinant MpJAZ-MBP protein alone (-) or with dinor-OPDA. MpASK1-flag and MpCOI1-flag were expressed in Sf9 insect cells, and MpJAZ was expressed in *E. coli*. The MpCOI1-flag/MpJAZ-MBP interaction depends on the presence of dn-OPDA (50 μM). The J4 compound (at 100 and 200 μM) inhibits the hormone-dependent MpCOI1-flag/MpJAZ-MBP interaction. Bottom, Coomassie blue staining of MpJAZ-MBP after cleavage with Factor Xa. C, Gene expression analysis of MpDIR (Dirigent-like protein) and MpBHLH4 (basic helix-loop-helix 14) in Tak-1 Marchantia plants in response to 10 μM OPDA for 1 h; plants pretreated with DMSO (-, untreated control), 15 or 30 μM J4 (+) for 1 h. MpACT was used as house-keeping control gene. One-way ANOVA with post-hoc Tukey's HSD test ($P < 0.01$) analyses define the significant differences in gene expression. Each biological sample consisted of tissue pooled from 5 to 8 plants. Data show mean $\pm$ SD of four technical replicates.

exploited in medical research but just emerging in the agrochemical field (Lamberth et al., 2013). For instance, the rational design of auxin analogs successfully obtained receptor antagonists useful in evolutionary distant plants species and that overcome the redundancy of auxin receptors (Hayashi et al., 2008, 2012). Similarly, based on the crystal structure of the COI1-JAZ co-receptor, we designed a COR-derivative (COR-MO) with a very strong and specific activity preventing COI-JAZ interaction (Monte et al., 2014). Despite rational design being a very efficient and specific approach, it requires a deep knowledge of the structures of receptor-ligand complexes. In addition, the synthesis of rational-designed molecules is often very complex and expensive. A limited amount of compounds is therefore suitable only for

specific, small-scale laboratory assays and not for agronomic use. Moreover, their specificity may prevent their use in different species. For instance, bryophytes such as *M. polymorpha* do not synthesize JA-Ile, and the bioactive JA and ligand of the Marchantia COI1-JAZ co-receptor is dn-OPDA (Monte et al., 2018). The Marchantia COI1-JAZ fails to bind JA-Ile or COR, and therefore, COR-MO cannot be used as JA inhibitor in nonvascular plants (Monte et al., 2018). In contrast, J4 is able to block all COI1-JAZ complexes tested (from Marchantia and Arabidopsis), suggesting that J4 is potentially active in all plants, from bryophytes to angiosperm. This broad activity of J4 underscores the high agronomic potential of this compound and its derivatives because they can be active in all crops. Therefore, the “unbiased” chemical

screen using commercially available chemically diverse libraries is an informative approach.

Finally, the robustness of the reported screening system encourages to undertake additional screens using newly accessible “natural compound libraries” to identify natural molecules directly perturbing the COI1–JAZ co-receptor complex. Natural JA-Ile agonists could represent either novel activators of hormone biosynthesis or different active forms of the plant hormone, modulating specific COI1–JAZ complexes. Besides, the in planta optimized screen described here opens the way to identify natural molecules affecting previously unknown posttranscriptional regulatory mechanisms of the key components of the JA signaling pathway, the JAZ repressors.

Materials and methods

Plant materials, growth conditions, and transgenic plants

Arabidopsis thaliana Col-0 is the genetic background of wild type and transgenic lines used throughout the work. Seeds were surface sterilized and kept at 4°C in the dark for 48 h, then grown at 23°C with a 16-h d cycle for 6 d, as previously described (Chini et al., 2018b; Chico et al., 2020). *Nicotiana benthamiana* and tomato seeds were germinated and grown in the same conditions. Vertically grown seedlings were germinated in the same conditions. The transgenic line 35S:JAZ1-GUS was generated by (Thines et al., 2007). The generation of transgenic plants expressing 35S:JAZ9-GUS was previously described (Monte et al., 2014). The *pJAZ9:GUS* and *pJAZ2:GUS* reporter lines were also previously described (Monte et al., 2014; Gimenez-Ibanez et al., 2017). The homozygous *pJAZ9:GUS* line was crossed with the loss-of-function *coi1-30* mutant and the double homozygous *pJAZ9:GUS coi1-30* line was isolated.

Y2H assays

The growth, handling, and transformation of yeast were as previously described (Chini et al., 2009b; Chini, 2014). Briefly, the described plasmids were co-transformed into *Saccharomyces cerevisiae* AH109 cells following standard lithium acetate (LiAC) protocols to assess protein interactions. Successfully transformed colonies were identified on yeast synthetic dropout lacking Leu and Trp. Three days after transformation, yeast colonies were grown in selective—WL liquid media for 6/7 h, and the cell density was adjusted to 3×10^7 cells/mL (optical density (OD)₆₀₀ = 1). An aliquot of 4 µL of the cell suspensions was plated out on yeast synthetic dropout lacking Ade, His, Leu, and Trp to test protein interaction (supplemented with COR and antagonist compounds as indicated). Plates were incubated at 28°C for 2–4 d. As positive control, the yeast suspensions were also plated on—AHLW media containing COR and the appropriate volume of DMSO.

Chemical libraries and compounds

In the COI1–JAZ Y2H screen, we tested 22,680 compounds from three chemical libraries: the 2,320 molecules of MicroSource (from MDSI, <http://www.msdiscovery.com/spectrum.html>), the approximately 20,000 compounds of the 20 K DIVERSet (from ChemBridge, <http://www.chembridge.com>) and the 360 bioactive compounds described by (Drakakaki et al., 2011). In the JAZ1-GUS screen, compounds from the libraries MicroSource and 360 bioactive, and a subset of the 20 K DIVERSetTM were employed. All chemicals were stored at –20°C as 10 mg/mL stocks in 100% DMSO and supplemented to a final concentration of 50–100 µM based on the molecular mass of each compound.

GUS staining and visualization

The visualization of GUS in the four JAZ1-GUS, JAZ9-GUS, *pJAZ2:GUS*, and *pJAZ9:GUS* marker lines was carried out as previously described (Chini, 2014; Chini et al., 2018a). JAZ1-GUS and JAZ9-GUS seeds were germinated in MS plates congaing 1% agar, kept in vertical for 7 d, transferred to 2 mL Johnson's liquid media supplemented with the described chemicals and incubated in an orbital shaker at 100 rpm, as described in Chini (2014). Seedlings of the *pJAZ9:GUS* and *pJAZ2:GUS* lines were germinated in liquid MS media in an orbital shaker at 100 rpm. Chemical treatments were carried out 6 d after germination.

After the described treatments, samples were placed in staining solution 50 mM phosphate buffer (pH 7), 0.1% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA), 1 mM X-Gluc (Glycosynth, Wellington, New Zealand), 1 mM K-ferrocyanide (Sigma), 1 mM K-ferricyanide (Sigma), and incubated at 37°C overnight. After staining, the seedlings were washed in 75% ethanol to eliminate chlorophyll. JAZ1-GUS seedlings were otherwise treated as described above and roots were collected. Protein extract was employed for fluorometric quantification of GUS activity using a Spectra Max M2 fluorometer (Molecular Devices, San Jose, CA, USA; Monte et al., 2014).

Degradation of RGA-GFP and EIN3-GFP

pRGA:GFP-RGA, *35S:EIN3-GFP*, and *35S:HA-IAA1* (Silverstone et al., 2001; Guo and Ecker, 2003) seeds were germinated in 2 mL liquid MS media in an orbital shaker at 100 rpm and the described compounds were added directly to the media 6 d after germination. After 2 h, 20 plants of the same size were collected and proteins were extracted as previously described (Fonseca and Solano, 2013). Samples were denatured, loaded on 9% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and incubated with anti-GFP-HRP antibody (Milteny Biotec, Bergisch Gladbach, Germany). Blots were developed using ECL (Pierce, Waltham, MA, USA).

Protein extracts and pull-down assays

MBP–JAZ fusion proteins were generated as previously described (Fonseca et al., 2009). Pull-down experimental

procedure is extensively described by (Fonseca and Solano, 2013). Briefly, 35:COI1-flag seedlings were ground in liquid nitrogen and homogenized in extraction buffer. For PD experiments, 6 μ g of resin-bound MBP fusion protein was added to 1 mg of total protein extract and incubated for 1 h at 4°C with rotation in presence of COR and the indicated compounds or DMSO control. After washing, samples were denatured, loaded on 8% SDS–PAGE gels, transferred to nitrocellulose membranes, and incubated with anti-flag antibody (Sigma). To confirm equal protein loading, 7 μ L of MBP-fused protein of each sample was run into SDS–PAGE gels and stained with Coomassie blue.

Recombinant protein expression by baculovirus–insect cell expression systems

The expression of Arabidopsis and Marchantia ASK1 and COI1 in Sf9 insect cells was carried out as previously described (Sheard et al., 2010; Li et al., 2017; Takaoka et al., 2018). Briefly, pFast-HIS-flag-COI1 and pFastBac1-flag-ASK1 vectors were transformed into DH10Bac competent cells and further transposed into the bacmid. Blue/white selection was used to identify colonies containing the recombinant bacmid. The recombinant bacmid was transfected into Sf9 insect cells using Lipofectamine (Invitrogen, Waltham, MA, USA) according to the manufacturers' instructions. After the titter check, the isolated P1 recombinant baculovirus was further amplified to generate P2 and P3 stocks. P3 recombinant baculoviruses were added to large cultured Sf9 cells at a volume ratio of 4.5:1,000. An aliquot of 50 μ L of insect cell culture supernatant for ASK1 and 50 μ L for AtCOI1 or 50 μ L for MpCOI1 were employed, together with 100 μ L of PD buffer, for pull-down assays in presence of AtJAZ-MBP or MpJAZ proteins expressed in *E. coli* as reported above and extensively described by (Fonseca and Solano, 2013).

Root measurement, anthocyanin, and chlorophyll quantification

Root growth inhibition assay was carried as previously described (Fonseca et al., 2014a). Three-day-old Arabidopsis seedlings germinated in vertical plates were transferred onto vertical Johnson medium in presence of JA with or without 5 μ M J4 for 10 d and root length of 15–50 seedlings was measured. Roots were quantified using ImageJ software. Values represent mean and the error bars standard deviation (SD).

The same growing conditions were employed to measure chlorophylls using 13-d-old Arabidopsis seedlings grown for 10 d in presence 10 μ M JA with or with 5 μ M J4. *Nicotiana benthamiana* seeds were germinated and grown at 23°C in a 16-h d cycle. *Nicotiana benthamiana* seedlings were germinated and grown in vertical MS plates for 7 d; seedlings were then transferred in 5 mL liquid MS media supplemented with 25 μ M JA with or without 10 μ M J4. Three days later, the aerial part of 5–7 seedlings was collected for each measurement and 4–6 independent replicates were analyzed. Chlorophyll measurements were

performed as previously described (Fonseca et al., 2014a). Acetone 80% (v/v) was used for extraction and absorbance at 645 and 663 nm was measured in a spectrophotometer (Spectra Max M2 Molecular Devices). For carotenoids measurements, samples were extracted overnight in 95% ethanol and absorbance at 470, 645, and 663 nm was measured in a spectrophotometer. Values represent mean and the error bars correspond to SD. The experiment was repeated three times with similar results.

Anthocyanin quantification was carried as previously described (Fonseca et al., 2014a). Briefly, seedlings grown in vertical Johnson plates for 5 d and transferred to liquid Johnson media supplemented with 10 μ M JA with or without 2.5 μ M J4. Two days later, the aerial parts of 10–15 seedlings were pooled for each replicate and anthocyanin quantification was performed using a spectrophotometer. Four independent replicates (seedling pools) were measured for each sample. Values represent mean and the error bars SD. The experiment was repeated at least 3 times with similar results.

Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using biological samples of tissue pooled from 10 to 15 Arabidopsis seedlings. RNA was extracted and purified using a Plant Total RNA isolation kit (Favorgen, Ping-Tung, Taiwan), including on-column DNase digestion to remove genomic DNA contamination. cDNA was synthesized from 1 μ g total RNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Gene amplification was carried out using 4 μ L from a 1:10 cDNA dilution, 4 μ L of EvaGreen[®] qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and gene-specific primers (Supplemental Table S2). Quantitative PCR was performed in 96-well optical plates in a HT 7900 Real Time PCR system (Applied Biosystems) using standard thermocycler conditions. Relative expression values given as the means of three or four technical replicates (from 10 to 15 Arabidopsis seedlings), which is relative to the mock wild-type control using ACT8 as the housekeeping gene. Data were analyzed using one-way analysis of variance (ANOVA) with post-hoc Tukey's HSD test ($P < 0.01$).

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers_. AtJAZ1/AT1G19180 (gene ID 838501), AtJAZ3/AT3G17860 (821055), AtCOI1/AT2G39940 (818581), MpJAZ/Mapoly0097s0021/Mp6g06230 (PTQ32535), and MpCOI1/Mapoly0025s0025/Mp2g26590 (PTQ43322).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Inhibition of COR-induced COI1–JAZ interactions in Y2H assays.

Supplemental Figure S2. Molecules antagonizing the JA-mediated degradation of JAZ1-GUS protein *in planta*.

Supplemental Figure S3. Confirmation of the antagonistic effect of the 12 identified molecules on COI1–JAZ interaction in Y2H and JAZ1 degradation *in planta*.

Supplemental Figure S4. Minimal concentrations of antagonist molecules required to prevent COR-mediated COI1–JAZ interactions in Y2H.

Supplemental Figure S5. Quantification of the inhibition of JAZ1-GUS degradation by antagonist molecules *in vivo*.

Supplemental Figure S6. Inhibition of COI1–JAZ interactions in Y2H and of JAZ degradation in *planta* by derivate molecules of J4 and Y20.

Supplemental Figure S7. Inhibition of JAZ2 and JAZ9 expression of derivate molecules of J4 and Y20.

Supplemental Figure S8. Specific expression of JAZ9 in trichomes requires COI1 and it is inhibited by J4 treatment.

Supplemental Figure S9. J4 partially inhibits JA-mediated transcriptional activation in *Arabidopsis*.

Supplemental Figure S10. Y11 and Y20 do not prevent JA-mediated responses *in planta*.

Supplemental Figure S11. Half IC50 of J4 in JA-promoted JAZ1-GUS degradation.

Supplemental Figure S12. Antagonistic effect of the compound J4 on JA-induced responses in *Nicotiana benthamiana* plants.

Supplemental Figure S13. J4 partially inhibits OPDA-mediated transcriptional activation.

Supplemental Table S1. List of all identified molecules including their basic information.

Supplemental Table S2. List of primers sequences used for qPCR analyses.

Acknowledgments

We thank John Browse, Salome Prat, Joe Ecker, and Judy Callis for providing 35S:JAZ1-GUS, pRGA:GFP-RGA, 35S:EIN3-GFP, and 35S:HA-IAA1 seeds, respectively. We also thank Daoxin Xie for providing the pFast-HIS-FLAG-COI1 and pFastBac1-FLAG-ASK1 vectors for expression in insect cells. We are grateful to Monica Diez Diaz for the selection of JAZ9-GUS transgenic line, Abel Rosado, David Carter, and Michelle Brown for the technical help setting-up the chemical screen, and Guillermo Gimenez Aleman for the structure images using ChemDraw.

Funding

This work was financed by grants to R.S. and A.C. (BIO2013-44407-R and BIO2016-77216-R from MINECO/FEDER, and PID2019-107012RB-I00 from the Spanish Ministry of Science and Innovation AEI/FEDER) and the DE-FG02-11ER15295 grant to N.V.R. and G.R.H. from the US Department of Energy. A.C. was supported by the Ramon y Cajal (RYC-2010-05680) and HSPO Fellowships. I.M. was supported by a master fellowship from Fundación Ramón Areces/UAM and a predoctoral fellowship from the Ministerio de Educación

(AP2010-1410). M.B. was supported by a JAE-DOC fellowship (CSIC).

Conflict of interest statement. The authors declare that they have no conflict of interest.

References

- Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, Yamaoka S, Nishihama R, Nakamura Y, Berger F, et al. (2017) Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* **171**: 287–304.e15
- Brooks DM, Hernandez-Guzman G, Kloek AP, Alarcon-Chaidez F, Sreedharan A, Rangaswamy V, Penaloza-Vazquez A, Bender CL, Kunkel BN (2004) Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant-microbe Interactions: MPMI* **17**: 162–174
- Browse J (2009) The power of mutants for investigating jasmonate biosynthesis and signaling. *Phytochemistry* **70**: 1539–1546
- Chico JM, Chini A, Fonseca S, Solano R (2008) JAZ repressors set the rhythm in jasmonate signaling. *Curr Opin Plant Biol* **11**: 486–494
- Chico JM, Lechner E, Fernandez-Barbero G, Canibano E, Garcia-Casado G, Franco-Zorrilla JM, Hammann P, Zamarreno AM, Garcia-Mina JM, Rubio V, et al. (2020) CUL3(BPM) E3 ubiquitin ligases regulate MYC2, MYC3, and MYC4 stability and JA responses. *Proc Natl Acad Sci USA* **117**: 6205–6215
- Chini A (2014) Application of yeast-two hybrid assay to chemical genomic screens: a high-throughput system to identify novel molecules modulating plant hormone receptor complexes. *Methods Mol Biol* **1056**: 35–43
- Chini A, Boter M, Solano R (2009a) Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signalling module. *FEBS J* **276**: 4682–4692
- Chini A, Cimmino A, Masi M, Reveglia P, Nocera P, Solano R, Evidente A (2018a) The fungal phytotoxin lasiojasmonate A activates the plant jasmonic acid pathway. *J Exp Bot* **69**: 3095–3102
- Chini A, Fonseca S, Chico JM, Fernandez-Calvo P, Solano R (2009b) The ZIM domain mediates homo- and heteromeric interactions between *Arabidopsis* JAZ proteins. *Plant J Cell Mol Biol* **59**: 77–87
- Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, et al. (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**: 666–671
- Chini A, Gimenez-Ibanez S, Goossens A, Solano R (2016) Redundancy and specificity in jasmonate signalling. *Curr Opin Plant Biol* **33**: 147–156
- Chini A, Monte I, Zamarreno AM, Hamberg M, Lassueur S, Reymond P, Weiss S, Stintzi A, Schaller A, Porzel A, et al. (2018b) An OPR3-independent pathway uses 4,5-didehydrojasmonate for jasmonate synthesis. *Nat Chem Biol* **14**: 171–178
- De Rybel B, Audenaert D, Xuan W, Overvoorde P, Strader LC, Kepinski S, Hoye R, Brisbois R, Parizot B, Vanneste S, et al. (2012) A role for the root cap in root branching revealed by the non-auxin probe naxillin. *Nat Chem Biol* **8**: 798–805
- Dobson, C.M. (2004) Chemical space and biology. *Nature* **432**: 824–828
- Drakakaki G, Robert S, Szatmari AM, Brown MQ, Nagawa S, Van Damme D, Leonard M, Yang Z, Girke T, Schmid SL, et al. (2011) Clusters of bioactive compounds target dynamic endomembrane networks *in vivo*. *Proc Natl Acad Sci USA* **108**: 17850–17855
- Ebel C, BenFeki A, Hanin M, Solano R, Chini A (2018) Characterization of wheat (*Triticum aestivum*) TIFY family and role of *Triticum Durum* TdTIFY11a in salt stress tolerance. *PLoS One* **13**: e0200566

- Enserink M, Hines PJ, Vignieri SN, Wigginton NS, Yeston JS (2013) Smarter pest control. The pesticide paradox. Introduction. Science (New York, N.Y.) **341**: 728–729.
- Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, et al. (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
- Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-iso-jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol **5**: 344–350
- Fonseca S, Fernandez-Calvo P, Fernandez GM, Diez-Diaz M, Gimenez-Ibanez S, Lopez-Vidriero I, Godoy M, Fernandez-Barbero G, Van Leene J, De Jaeger G, et al. (2014a) bHLH003, bHLH013 and bHLH017 are new targets of JAZ repressors negatively regulating JA responses. PLoS One **9**: e86182
- Fonseca S, Radhakrishnan D, Prasad K, Chini A (2018) Fungal production and manipulation of plant hormones. Curr Med Chem **25**: 253–267
- Fonseca S, Rosado A, Vaughan-Hirsch J, Bishopp A, Chini A (2014b) Molecular locks and keys: the role of small molecules in phytohormone research. Front Plant Sci **5**: 709
- Fonseca S, Solano R (2013) Pull-down analysis of interactions among jasmonic acid core signaling proteins. Methods Mol Biol (Clifton, N.J.) **1011**: 159–171
- Gimenez-Ibanez S, Boter M, Ortigosa A, Garcia-Casado G, Chini A, Lewsey MG, Ecker JR, Ntoukakis V, Solano R (2017) JAZ2 controls stomata dynamics during bacterial invasion. New Phytol **213**: 1378–1392
- Gimenez-Ibanez S, Solano R (2013) Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens. Front Plant Sci **4**: 72
- Grossmann K (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manage Sci **66**: 113–120
- Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115**: 667–677
- Guo Q, Yoshida Y, Major IT, Wang K, Sugimoto K, Kapali G, Hayko NE, Benning C, Howe GA (2018) JAZ repressors of metabolic defense promote growth and reproductive fitness in Arabidopsis. Proc Natl Acad Sci USA **115**: E10768–E10777
- Hayashi K, Tan X, Zheng N, Hatate T, Kimura Y, Kepinski S, Nozaki H (2008) Small-molecule agonists and antagonists of F-box protein-substrate interactions in auxin perception and signaling. Proc Natl Acad Sci USA **105**: 5632–5637
- Hayashi K, Neve J, Hirose M, Kuboki A, Shimada Y, Kepinski S, Nozaki H (2012) Rational design of an auxin antagonist of the SCF(TIR1) auxin receptor complex. ACS Chem Biol **7**: 590–598
- He W, Brumos J, Li H, Ji Y, Ke M, Gong X, Zeng Q, Li W, Zhang X, An F, et al. (2011) A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in Arabidopsis. Plant Cell **23**: 3944–3960
- Hicks GR, Raikhel NV (2012) Small molecules present large opportunities in plant biology. Ann Rev Plant Biol **63**: 261–282
- Hicks GR, Robert S (2014) Plant Chemical Genomics, Ed. 1. Humana Press, New York, NY
- Howe GA, Major IT, Koo AJ (2018) Modularity in Jasmonate signaling for multistress resilience. Ann Rev Plant Biol **69**: 387–415
- Kazan K (2015) Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci **20**: 219–229
- Kloeck AP, Verbsky ML, Sharma SB, Schoelz JE, Vogel J, Klessig DF, Kunkel BN (2001) Resistance to *Pseudomonas syringae* conferred by an Arabidopsis thaliana coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. Plant J Cell Mol Biol **26**: 509–522
- Lamberth C, Jeanmart S, Luksch T, Plant A (2013) Current challenges and trends in the discovery of agrochemicals. Science (New York, N.Y.) **341**: 742–746
- Leyser O. (2018) Auxin signaling. Plant Physiol **176**: 465–479
- Li H, Yao RF, Ma S, Hu SH, Li SH, Wang YP, Yan C, Xie DX, Yan JB (2017) Efficient ASK-assisted system for expression and purification of plant F-box proteins. Plant J **92**: 736–743
- Lumba S, Cutler S, McCourt P (2010) Plant nuclear hormone receptors: a role for small molecules in protein-protein interactions. Ann Rev Cell Dev Biol **26**: 445–469
- Maor R, Jones A, Nuhse TS, Studholme DJ, Peck SC, Shirasu K (2007) Multidimensional protein identification technology (MudPIT) analysis of ubiquitinated proteins in plants. Mol Cell Proteom **6**: 601–610
- Meesters C, Monig T, Oeljeklaus J, Krahn D, Westfall CS, Hause B, Jez JM, Kaiser M, Kombrink E (2014) A chemical inhibitor of jasmonate signaling targets JAR1 in Arabidopsis thaliana. Nat Chem Biol **10**: 830–836
- Monte I, Hamberg M, Chini A, Gimenez-Ibanez S, Garcia-Casado G, Porzel A, Pazos F, Boter M, Solano R (2014) Rational design of a ligand-based antagonist of jasmonate perception. Nat Chem Biol **10**: 671–676
- Monte I, Franco-Zorrilla JM, García-Casado G, Zamarreño AM, García-Mina JM, Nishihama R, Kohchi T, Solano R, et al. (2019) A single JAZ repressor controls the jasmonate pathway in Marchantia polymorpha. Mol Plant **12**: 185–198
- Monte I, Ishida S, Zamarreño AM, Hamberg M, Franco-Zorrilla JM, Garcia-Casado G, Gouhier-Darimont C, Reymond P, Takahashi K, Garcia-Mina JM, et al. (2018) Ligand-receptor co-evolution shaped the jasmonate pathway in land plants. Nat Chem Biol **14**: 480–488
- Norambuena L, Raikhel NV, Hicks GR (2009) Chemical genomics approaches in plant biology. Methods Mol Biol **553**: 345–354
- Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, et al. (2009) Absciscic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science (New York, N.Y.) **324**: 1068–1071
- Pauwels L, Barbero GF, Geerinck J, Tillemans S, Grunewald W, Perez AC, Chico JM, Bossche RV, Sewell J, Gil E, et al. (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464**: 788–791
- Peñuelas M, Monte I, Schweizer F, Vallat A, Reymond P, García-Casado G, Franco-Zorrilla JM, Solano R, et al. (2019) Jasmonate-related MYC transcription factors are functionally conserved in Marchantia polymorpha. Plant Cell **31**: 2491–2509
- Pieterse CM, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SC (2012) Hormonal modulation of plant immunity. Ann Rev Cell Dev Biol **28**: 489–521
- Qi T, Huang H, Song S, Xie D (2015) Regulation of jasmonate-mediated stamen development and seed production by a bHLH-MYB complex in Arabidopsis. Plant Cell **27**: 1620–1633
- Robert-Seilanianz A, Grant M, Jones JD (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Ann Rev Phytopathol **49**: 317–343
- Rosado A, Hicks GR, Norambuena L, Rogachev I, Meir S, Pourcel L, Zouhar J, Brown MQ, Boersdore MP, Puckrin RS, et al. (2011) Sortin1-hypersensitive mutants link vacuolar-trafficking defects and flavonoid metabolism in Arabidopsis vegetative tissues. Chem Biol **18**: 187–197
- Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB (2000) The Arabidopsis DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12**: 1041–1061
- Saracco SA, Hansson M, Scalf M, Walker JM, Smith LM, Vierstra RD (2009) Tandem affinity purification and mass spectrometric analysis of ubiquitinated proteins in Arabidopsis. Plant J Cell Mol Biol **59**: 344–358

- Serrano M, Kombrink E, Meesters C** (2015) Considerations for designing chemical screening strategies in plant biology. *Front Plant Sci* **6**: 131
- Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, et al.** (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature* **468**: 400–405
- Silverstone AL, Jung HS, Dill A, Kawaide H, Kamiya Y, Sun TP** (2001) Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. *Plant Cell* **13**: 1555–1566
- Takaoka Y, Iwahashi M, Chini A, Saito H, Ishimaru Y, Egoshi S, Kato N, Tanaka M, Bashir K, Seki M, et al.** (2018) A rationally designed JAZ subtype-selective agonist of jasmonate perception. *Nat Commun* **9**:3654
- Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J** (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* **448**: 661–665
- Toth R, van der Hoorn RA** (2010) Emerging principles in plant chemical genetics. *Trends Plant Sci* **15**: 81–88
- Tsuchiya Y, Vidaurre D, Toh S, Hanada A, Nambara E, Kamiya Y, Yamaguchi S, McCourt P** (2010) A small-molecule screen identifies new functions for the plant hormone strigolactone. *Nat Chem Biol* **6**: 741–749
- Van Overbeek J, Velez I** (1946) Use of 2,4-dichlorophenoxyacetic acid as a selective herbicide in the tropics. *Science (New York, N.Y.)* **103**: 472
- Wasternack C, Feussner I** (2018) The oxylipin pathways: biochemistry and function. *Ann Rev Plant Biol* **69**: 363–386
- Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG** (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science (New York, N.Y.)* **280**: 1091–1094
- Zander M, Lewsey MG, Clark NM, Yin L, Bartlett A, Saldierna Guzman JP, Hann E, Langford AE, Jow B, Wise A, et al.** (2020) Integrated multi-omics framework of the plant response to jasmonic acid. *Nat Plants* **6**: 290–302
- Zhao Y, Chow TF, Puckrin RS, Alfred SE, Korir AK, Larive CK, Cutler SR** (2007) Chemical genetic interrogation of natural variation uncovers a molecule that is glycoactivated. *Nat Chem Biol* **3**: 716–721