bHLH003, bHLH013 and bHLH017 Are New Targets of JAZ Repressors Negatively Regulating JA Responses

Sandra Fonseca1, Patricia Fernández-Calvo1, Guillermo M. Fernández1, Monica Díez-Díaz1, Selena Gimenez-Ibanez1, Irene López-Vidriero2, Marta Godoy2, Gemma Fernández-Barbero1, Jelle Van Leene3,4, Geert De Jaeger3,4, José Manuel Franco-Zorrilla2, Roberto Solano1

1 Departamento de Genetica Molecular de Plantas, Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma, Madrid, Spain, 2 Genomics Unit, Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma, Madrid, Spain, 3 Department of Plant Systems Biology, VIB, Gent, Belgium, 4 Department of Plant Biotechnology and Bioinformatics, Ghent University, Gent, Belgium

Abstract

Cell reprogramming in response to jasmonates requires a tight control of transcription that is achieved by the activity of JA-related transcription factors (TFs). Among them, MYC2, MYC3 and MYC4 have been described as activators of JA responses. Here we characterized the function of bHLH003, bHLH013 and bHLH017 that conform a phylogenetic clade closely related to MYC2, MYC3 and MYC4. We found that these bHLHs form homo- and heterodimers and also interact with JAZ repressors in vitro and in vivo. Phenotypic analysis of JA-regulated processes, including root and rosette growth, anthocyanin accumulation, chlorophyll loss and resistance to Pseudomonas syringae, on mutants and overexpression lines, suggested that these bHLHs are repressors of JA responses. bHLH003, bHLH013 and bHLH017 are mainly nuclear proteins and bind DNA with similar specificity to that of MYC2, MYC3 and MYC4, but lack a conserved activation domain, suggesting that repression is achieved by competition for the same cis-regulatory elements. Moreover, expression of bHLH017 is induced by JA and depends on MYC2, suggesting a negative feedback regulation of the activity of positive JA-related TFs. Our results suggest that the competition between positive and negative TFs determines the output of JA-dependent transcriptional activation.

Introduction

Jasmonates (JAs) are fatty acid derived molecules, ubiquitous in the plant kingdom and structurally similar to animal prostaglandins. They regulate many plant cellular and developmental processes such as cell cycle, plant growth, fertility, root elongation, gamete development, trichome initiation, and senescence [1–7]. JAs are also potent alert signals that trigger the activation of JA-responsive genes [24–26]. Phenotypic analysis of JA-regulated processes, including root and rosette growth, anthocyanin accumulation, chlorophyll loss and resistance to Pseudomonas syringae, on mutants and overexpression lines, suggested that these bHLHs are repressors of JA responses. bHLH003, bHLH013 and bHLH017 are mainly nuclear proteins and bind DNA with similar specificity to that of MYC2, MYC3 and MYC4, but lack a conserved activation domain, suggesting that repression is achieved by competition for the same cis-regulatory elements. Moreover, expression of bHLH017 is induced by JA and depends on MYC2, suggesting a negative feedback regulation of the activity of positive JA-related TFs. Our results suggest that the competition between positive and negative TFs determines the output of JA-dependent transcriptional activation.

Upon hormone recognition, JAZ are ubiquitinated and degraded by the proteasome [10,15]. TFs are then released and activate transcription. The bHLH TF MYC2 plays a central role in JA signaling and was the first TF identified regulating a subset of JA-responsive genes [24–26]. jin1-2/myc2 mutant was only partially impaired in JA responses, which suggested that other TFs should act additively or redundantly to it [26]. In fact, its closest protein homologs MYC3 and MYC4 were found to share redundant functions with MYC2 in the regulation of JA-regulated gene expression, root growth and pathogen and insect resistance [27–29]. Remarkably, the triple mutant myc2myc3myc4 is completely depleted of glucosinolates and therefore, fully susceptible to insects [26]. MYC2, MYC3 and MYC4 act cooperatively with MYB TFs to activate glucosinolate biosynthesis in response to JA adding another level of complexity to the regulation of JA responses [30].

Additional targets of JAZ from distinct TF families have been described in Arabidopsis. This is the case of the bHLH, Ghabra3 (GL3), Enhancer of Ghabra3 (EGL3) and Transparent Testa8 (TT8), involved in trichome formation and anthocyanin accumulation, and Inducer of CBF Expression1 (ICE1) and ICE2 involved in cold signaling [31–33]; the R2R3 MYBs, Ghabra 1 (GL1) and PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1)
and MYB21 that are also involved in trichome development, anthocyanin biosynthesis and male fertility [33,34]; and the ethylene related TFs, EIN3 and EIL1 which are involved in ET signaling and defense against necrotrophs, where the JA/ET crosstalk plays a relevant regulatory role [26,35,36].

Several mechanisms contributing to repress the JA pathway have been recently reported. Most JAZ genes are transcriptionally induced by JA, thus providing a negative feed-back mechanism for repression. Moreover, alternative splicing of some JAZ genes give rise to truncated forms of JAZ without the Jas domain. This domain is responsible for the interaction with COI1 and, therefore, such truncated forms are resistant to degradation but still repress the TFs [17,37–39]. Similarly, JAZ8 lacks the canonical Jas degron and is unable to interact with COI1, being therefore a stable protein that behaves as a constitutive repressor [23]. The catabolism of the bioactive hormone also contributes to shut-down the JA signal. The fatty acid ω-hydroxylase CYP94B3 is induced in response to JA and converts the bioactive JA-Ile in the inactive 12-hydroxy-JA-Ile ([2OH]-JA-Ile) [40–42]. The activation of parallel metabolic pathways that converts JA in inactive molecules as JA-glucose esters [43], 12-hydroxy-JA (12-OH-JA) and its sulfated and glycosylated derivatives [44,45], volatile methyl-JA (MeJA), and JA-amino conjugates other than JA-Ile [46] might play a complementary role on the process of active hormone depletion.

The existence of all these mechanisms of repression underscores the importance of a tight and timely regulation to prevent a harmful activation of the JA-pathway. Here we describe another repression mechanism based on competition of positive and harmful activation of the JA-pathway. Here we describe another repression mechanism based on competition of positive and inhibitory transcription factors (TFs). We recently showed that the JA-induced gene expression of the Arabidopsis bHLH (basic helix-loop-helix) TFs bHLH003, bHLH013 and bHLH017 is dependent on the interaction of these TFs with JAZ repressors. The interaction of these TFs with JAZ repressors in turn represses the expression of the JA-regulated genes. We found that a group of bHLH TFs including MYC2, MYC3 and MYC4, homo- and heterodimerize and interact with JAZ proteins in vivo. In contrast to MYCs, the three bHLHs described here behave as transcriptional repressors of all JA-related phenotypes tested. Our results are in line with very recent works on these TFs [47–49], showing that they bind efficiently to the G-box and that competition for DNA-binding motifs. We generated transgenic Arabidopsis plants expressing bHLH-HA fusions and selected homozygous lines. Consistent with yeast results, bHLH017-HA was pulled-down from the transgenic extracts by all JAZ proteins, though with different efficiencies (Figure 1B). Similarly, bHLH003-HA was pulled-down by JAZ1, JAZ2, JAZ3, JAZ4, JAZ9 and JAZ11. Most MBP-JAZ proteins with the exception of JAZ7, JAZ8, JAZ10 and JAZ12 were able to pull-down bHLH013-HA. Thus, in general, PD analyses are consistent with Y2H assays and demonstrate that these bHLH proteins can interact with JAZ repressors in vivo.

To test if the interaction between JAZ proteins and bHLHs occur also in vivo, we performed Tandem Affinity Purification (TAP) of protein complexes in cultured PSB-D Arabidopsis cells using N- and C-terminal fusions of bHLH003, bHLH013 and bHLH017 with the TAP epitope as baits. As shown in Table 1 and Table S2, bHLH017-TAP allowed the co-purification of protein complexes that included several JAZ proteins [JAZ2, JAZ3, JAZ11 and JAZ12] and NINJA (although JAZ12 and NINJA were found only in one independent experiment; Table 1 and Table S2). Interestingly, these complexes also included bHLH003. Purification of bHLH003-TAP complexes identified bHLH013 and, conversely, TAP on bHLH013 rendered the isolation of bHLH003 (Table 1). Therefore, TAP tagging experiments revealed that these three bHLH proteins form heterodimers in vivo and interact with core components of JA signaling modules in planta.

To further analyze the spectrum of heterodimeric interactions among the three bHLHs, we expressed and purified MBP-bHLH003, MBP-bHLH013 and MBP-bHLH017 proteins from E.coli, and tested them in PD experiments using extracts of the transgenic lines expressing HA fusions of the three bHLHs. Results of PD experiments (Figure 1C) confirmed the TAP-tagging data showing that all three proteins can form heterodimers and bHLH013 and bHLH017 can also form homodimers.

Expression patterns of bHLH003, bHLH013 and bHLH017

To get an insight into the activity of these TFs in the plant we generated transgenic plants that express the β-glucuronidase (GUS) reporter under the control of the bHLH003, bHLH013 or bHLH017 promoters, and analyzed their tissue expression patterns in seedlings and adult plants. Consistent with microarray data at BAR (http://bar.utoronto.ca) all three genes were expressed quite ubiquitously. In 6 day-old seedlings, all three bHLHs were expressed in leaves, cotyledons and roots, predominantly in the vasculature and root tips (Figure 2). bHLH017 expression was widespread over all aerial organs and bHLH003 expression could be detected in all root tissues. Among the genes analyzed, bHLH003 had the lowest expression levels in all tissues, but a strong GUS signal was detected in young emerging leaves.

In adult leaves, all three bHLHs showed the same expression patterns, however signal from bHLH003 was weaker (Figure 2). GUS expression driven by all three promoters could be detected in all floral organs. In sepalis, bHLH017 showed a widespread expression while expression of bHLH003 and bHLH013 was confined to vasculature. In petals, expression of the three bHLHs was similar, and in reproductive organs bHLH003 and bHLH013 were more abundant in stamen filaments, while bHLH017 is highly expressed in the anther. In all cases, GUS staining was detected in some pollen grains. In the pistil, all three bHLH genes are expressed across the ovary tissues while bHLH003 is the only being expressed in stigma and ovules.
Previous results suggest that bHLH003, bHLH013 and bHLH017 might play a redundant role in the regulation of JA-mediated responses. To test this hypothesis we obtained homozygous mutants from insertion lines available in NASC. RT-PCR analysis of gene expression confirmed that the homozygous T-DNA insertion lines in bHLH003 (GK-301G05) and bHLH017 (SAIL_536_F09) did not express full-length mRNAs (Figure S2), indicating that these lines should be Knock-Out mutants. However, T-DNA insertion in line GK-696A04 did not alter the expression of bHLH013 (Figure S2). We also generated transgenic Arabidopsis lines constitutively and ectopically expressing bHLH003, bHLH013 and bHLH017 under the control of the CaMV 35S promoter. Thus, we analyzed the insertion lines of bHLH003 and bHLH017 and the OE lines of all three bHLHs for defects in JA-related phenotypes such as root- and aerial-growth inhibition, anthocyanin accumulation, chlorophyll loss and pathogen resistance.

JA-Ile treatment inhibits root and aerial plant growth. Root-length analysis of seedlings germinated and grown for 8 days in vertical plates containing 10 μM JA showed that root-growth inhibition by JA was significantly higher in bhlh003 mutants than in WT plants (Figure 3A). In contrast, root growth of bHLH013 over-expressing plants was less sensitive to JA.

Analysis of the aerial part of seedlings grown for 13 days in MS plates containing 50 μM JA revealed that bhlh003 and bhlh017 mutants were smaller and reddish (a symptom of higher accumulation of anthocyanins) than WT plants, suggesting that mutation of bHLH003 or bHLH017 might promote hypersensitivity to JA (Figure 3B). Consistent with this hypothesis, the 35S:bHLH017 and 35S:bHLH013 OE lines were bigger than WT plants and more similar to the JA-insensitive jin1-2 mutant, indicating that constitutive activation of these two genes promote JA-insensitivity (Figure 3B). 35S:bHLH003 also had a weak effect increasing aerial plant size.

Next, we analyzed anthocyanin accumulation in the mutants and OE lines. Exogenous JA treatment promotes accumulation of the pigments in WT plants. This increase is highly attenuated in jin1-2 mutants and almost impaired in coi1-1 (Figure 3B, 3C and Figure S3). Consistent with previous results, bhlh003 and bhlh017
mutants showed higher levels of anthocyanins than WT, whereas transgenic plants overexpressing any of the three bHLH TFs accumulated lower levels than WT, similar to jin1-2 (Figure 3C). JA treatment also produces a reduction of chlorophylls in plant leaves, which depends on jin1-2 and coi1-1 (Figure 3D). In contrast, transgenic plants overexpressing bHLH013 or bHLH017 accumulated higher levels of chlorophylls than WT plants, further suggesting that these two TFs antagonize this JA effect. Consistent with this, levels of chlorophylls in bhlh003 plants are reduced. Levels in bhlh017 and 35S:bHLH013 were comparable to WT (Figure 3D and Figure S3).

Some strains of Pseudomonas, such as Pseudomonas syringae pv. tomato (Pto) DC3000, produce Coronatine (COR), a bacterial phytotoxin that functionally mimics JA-Ile [13,50]. COR activates the JA-pathway, which counteracts SA-dependent defenses against the bacteria [51,52]. Thus, JA-insensitive mutants such as jin1-2 or coi1-1 are more resistant to Pto DC3000 than WT plants ([51] and Figure 4). The bhlh017 mutant showed a strong susceptibility, increasing bacterial growth over one log compared to WT. Consistently, OE of bHLH017 or bHLH013 increased resistance reducing bacterial growth by one or half a log, respectively. The mutant or OE lines of bHLH003 did not show significant differences with WT plants.

Altogether, results from all phenotypic analyses are consistent with a role of bHLH003, bHLH013 and bHLH017 as repressors of different aspects of the JA function in seedling development and in plant defense.

DNA-binding specificity of bHLH003, bHLH013 and bHLH017

Results described above suggest that bHLH003, bHLH013 and bHLH017 have an antagonistic effect on the JA pathway to that of MYC2, MYC3 and MYC4 [28]. TAP tagging results obtained in this work for bHLH003, bHLH013 and bHLH017, and by Fernández-Calvo et al. [28], for MYC2, MYC3 and MYC4, suggest that these two groups of TFs do not interact with each other in vivo. Direct testing of bHLH017 interaction with MYC2, MYC3 and MYC4 in co-immunoprecipitation assays supports this conclusion [28]. This has been also recently confirmed by Song et al. [49]. Therefore, we hypothesize that the antagonistic activity of these two groups of TFs might occur by competition for their binding sites. To test this hypothesis we determined the subcellular localization and the DNA-binding specificity of bHLH003, bHLH013 and bHLH017.

To assess their subcellular localization we generated transgenic Arabidopsis plants expressing GFP fusions of these three TFs under the control of the 35S CaMV promoter. As shown in Figure 5, all three TFs showed a clear nuclear fluorescent signal in plant leaves, which depends on jin1-2 and coi1-1 (Figure 3D). In contrast, transgenic plants overexpressing bHLH013 or bHLH017 accumulated lower levels of chlorophylls than WT plants, further suggesting that these two TFs antagonize this JA effect. Consistent with this, levels of chlorophylls in bhlh003 plants are reduced. Levels in bhlh017 and 35S:bHLH013 were comparable to WT (Figure 3D and Figure S3).

Some strains of Pseudomonas, such as Pseudomonas syringae pv. tomato (Pto) DC3000, produce Coronatine (COR), a bacterial phytotoxin that functionally mimics JA-Ile [13,50]. COR activates the JA-pathway, which counteracts SA-dependent defenses against the bacteria [51,52]. Thus, JA-insensitive mutants such as jin1-2 or coi1-1 are more resistant to Pto DC3000 than WT plants ([51] and Figure 4). The bhlh017 mutant showed a strong susceptibility, increasing bacterial growth over one log compared to WT. Consistently, OE of bHLH017 or bHLH013 increased resistance reducing bacterial growth by one or half a log, respectively. The mutant or OE lines of bHLH003 did not show significant differences with WT plants.

Altogether, results from all phenotypic analyses are consistent with a role of bHLH003, bHLH013 and bHLH017 as repressors of different aspects of the JA function in seedling development and in plant defense.

### Table 1. Proteins interacting with bHLH003, bHLH013 and bHLH017 in TAP tagging assays.

| AT number | Protein name | bHLH003 (4) | bHLH013 (4) | bHLH017 (6) |
|-----------|--------------|-------------|-------------|-------------|
| AT4G16430 | bHLH003      | 4           | 4           | 5           |
| AT1G01260 | bHLH013      | 4           | 4           |             |
| AT2G46510 | bHLH017      |             |             | 6           |
| AT1G74950 | JAZ2         |             |             | 2           |
| AT3G17860 | JAZ3         |             | 2           |             |
| AT3G43440 | JAZ211       |             |             | 2           |
| AT5G20900 | JAZ12        | 1           |             |             |
| AT4G28910 | NINJA        |             |             | 1           |

The prey proteins identified in the TAP tagging assays using bHLH003-TAP, bHLH013-TAP and bHLH017-TAP as baits are listed in the left column. Numbers within parenthesis indicate the total number of TAP assays performed for each protein. The numbers in the table correspond to the number of positive results in the independent TAP assays. Half of the assays were performed with an N-terminal TAP fusion and the other half with a C-terminal TAP fusions expressed in Arabidopsis cells suspension cultures (PSB-D).

doi:10.1371/journal.pone.0086182.t001
We next determined the DNA-binding specificity of bHLH003, bHLH013, and bHLH017 in vitro using a protein binding microarray (PBM) assay [53]. The three proteins recognized with the highest affinity a perfect G-box (CACGTG; Figure 6A), similarly to that observed for MYC2, MYC3 and MYC4 using the same strategy [28,53]. Analysis of the affinity for all possible 8-mer containing the G-box showed that the three proteins have a preference for purine at 5'-end and pyrimidine at 3'-end flanking the G-box (Figure 6B). However, whereas bHLH013 and bHLH017 (as well as MYC2) recognized with high affinity all the G-box-containing 8mers, binding of bHLH003 was almost exclusive for DNA sequences flanked by purine and pyrimidine at 5' and 3' ends, respectively (Figure 6B).

In previous works, we determined that MYC2, MYC3 and MYC4 proteins recognize with high affinity other DNA-elements related to the G-box, referred to as T/G, G-related, G/A and G/C elements [28,53]. The analysis of binding to the DNA probes containing these variants revealed a strikingly similar pattern of affinities to the different variants for bHLH017 and MYC2 (Figure 6C), suggesting that they might bind similar cis-elements in vivo. bHLH013 showed also a clear binding to the MYC2-recognized elements, although with lower affinity than MYC2. bHLH003, however, seems to have a strict requirement for a perfect G-box flanked by purine and pyrimidine as mentioned above. Altogether, these results support that the antagonistic activity of these two groups of TFs might be achieved by competition for their binding sites.

bHLH003, bHLH013 and bHLH017 are transcriptional repressors
To test the repressor activity of bHLH003, bHLH013 and bHLH017 we performed transient expression assays in Nicotiana benthamiana leaves. As effectors, we obtained 35S:HA-fusions of all three bHLHs and also MYC2 as a control for transcriptional activator. The promoter region (2 kb) of JAZ2 fused to luciferase was used as reporter (Figure 7A). As shown in Figure 7B, co-expression of pJAZ2:Luc with 35S:MYC2-HA promoted an increase of the reporter activity, consistent with the function of MYC2 as a transcriptional activator. In contrast, co-expression of pJAZ2:Luc with 35S:bHLH003-HA or 35S:bHLH017-HA had an additive effect reducing the transactivation capacity of MYC2 (Figure 7C). These results support that these bHLH TFs compete with MYC2 for their DNA-binding site in vivo.

Expression of bHLH017 is induced by JA and depends on MYC2
According to public databases (http://bar.utoronto.ca), bHLH017 expression is induced by JA. To confirm it, we analyzed its mRNA accumulation by qRT-PCR in JA-treated WT plants and jin1-2 mutants. As shown in Figure 8, bHLH017 mRNA is induced by JA in WT plants, but this induction is greatly reduced

Figure 3. Responses to JA are affected in bHLH mutants and over-expression (OE) lines. (A) Root growth inhibition by JA (10 μM) of 8 days-old wild-type (WT), bhlh003 and bhlh017 mutant seedlings and OE lines of bHLH003, bHLH013 and bHLH017 grown in vertical MS plates. Values correspond to the average root growth between day 2 and day 8. Error bars represent standard deviations. Asterisks represents values that are significantly different statistically from WT applying a Student’s t-test (p<0.01). (B) Phenotype of rosette leaves of 13 days-old seedlings grown in 50 μM JA-containing MS medium. (C) Anthocyanin accumulation and (D) Chlorophyll content in WT, jin1-2, coi1-1, bhlh003 and bhlh017 mutants and OE lines for bHLH003, bHLH013 and bHLH017, grown in 50 μM JA for 13 days. Bars represent the average value of 3 pools of seedlings and error bars the standard deviation. Asterisks indicate statistically significant values compared to WT applying a Student’s t-test (p<0.05). doi:10.1371/journal.pone.0086182.g003
in *jin1-2* mutants. This suggests that *bHLH017* expression is regulated by MYC2, and therefore, that activation of MYC2-dependent transcription triggers a negative feed-back regulation by *bHLH017*.

**Discussion**

The current view of how JA responses are activated involves the de-repression of positive activators (TFs) of gene expression by protein degradation of JAZ repressors upon hormone binding to its receptor [5,32]. In this work, we found that JAZ proteins also interact with negative regulators of gene expression, which contribute to repress hormone responses. Therefore, our results support that the JA output depends on the equilibrium between activator and repressor TFs.

**New JA-signaling modules**

Identification of the JAZ-Interaction Domain (JID) of MYC2, MYC3 and MYC4 and BLAST searches with this sequence suggested new JAZ targets [28]. A combination of protein-protein interaction techniques used in this work (Y2H, PD and TAP tagging) has confirmed that JAZ proteins interact with *bHLH003*, *bHLH013* and *bHLH017* in *vivo* and in *vitro*, uncovering new JA-signaling modules. These results are in line with those very recently described by Song et al., [49], which also demonstrated that JAZ proteins repress these bHLHs. TAP tagging and PD results also showed that these three TFs can form homo- and hetero-dimers, thus indicating that a variety of combinations among these three TFs and JAZ proteins can be expected, broadening the regulatory possibilities of these signaling modules. This resembles the situation described for MYC2, MYC3 and MYC4, which can also homo- and hetero-dimerize to form distinct regulatory complexes [28]. Interestingly, based on Y2H, PD and TAP tagging results (this work and [28,49]) *bHLH003*, *bHLH013* and *bHLH017* do not seem to hetero-dimerize with MYC2, MYC3 or MYC4, suggesting that these two groups of TFs represent two separate clades that do not interact with each other and form distinct JA-signaling modules.

**Figure 4. Resistance of bHLH mutants and OE lines to the bacterial pathogen Pto DC3000.** (A) Growth of *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 on WT Arabidopsis plants, *jin1-2*, *coi1-1*, *bhlh003* and *bhlh017* mutants and OE lines for *bHLH003*, *bHLH0013* and *bHLH017* 3 days after spray inoculation. Bacterial counts are expressed as log (cfu cm\(^{-2}\)). Error bars indicate SE. The results are representative of two independent experiments. Asterisks indicate statistically significant differences compared with WT (Student’s t test: *, P<0.05; **, P<0.005; and ***, P<0.001). (B) Disease symptoms in leaves of WT, mutants and OE lines. Pictures of detached leaves were taken 4 days after inoculation. doi:10.1371/journal.pone.0086182.g004

**Figure 5. Sub-cellular localization of bHLH003, bHLH013 and bHLH017.** Microscopy images of GFP signal detected in root cells of transgenic Arabidopsis transgenic plants over-expressing *bHLH003-GFP*, *bHLH013-GFP* or *bHLH017-GFP* and grown for ten days in media supplemented (A) or not (B) with 50 μM JA for 3 h. doi:10.1371/journal.pone.0086182.g005
A new mechanism of negative regulation of JA responses

bHLH003, bHLH013 and bHLH017 are nuclear proteins with a DNA-binding specificity strikingly similar to that of MYC2, MYC3 and MYC4, and with similar tissue expression patterns, suggesting that they might regulate similar, or at least overlapping, sets of genes. However, sequence analysis of bHLH003, bHLH013 and bHLH017 fail to identify an activation domain conserved with MYC2, MYC3 and MYC4, which suggested that they might behave as repressors rather than activators [28,47]. Consistently, transient expression experiments in Nicotiana benthamiana and phenotypic analyses of mutants and transgenic OE lines demonstrated that these three bHLHs are transcriptional repressors, regulating JA responses in the opposite way to MYC2, MYC3 and MYC4 (this work and [28,47–49]).

The fact that these two clades do not seem to form heterodimers suggests that competition for DNA-binding might mechanistically explain their functional antagonism. Supporting this hypothesis, transcriptional activity of MYC2 can be repressed by bHLH003 or bHLH017 in transient experiments in Nicotiana benthamiana and Arabidopsis protoplasts (this work and [47,49]).

The lack of a repressor domain, such as the EAR present in many TFs [21] suggests that bHLH003, bHLH013 and bHLH017 may exert their repressive role through two independent mechanisms: i) by competition for the DNA-binding sites and interference with the transcriptional activators, and ii) by their interaction with JAZ repressors and recruitment of TPL and TPR co-repressors through the adaptor protein NINJA [22]. Thus, in our current view (Figure 9), bHLH003, bHLH013 and bHLH017 may form repression complexes with JAZ-NINJA-TPL, similar to those described for MYC2, MYC3 and MYC4. It should be noted that in the absence of the activation signal [JA-Ile] MYC2, MYC3 and MYC4 complexes with JAZ-NINJA-TPL are also repressor complexes because their DNA-binding specificity determines the genes to be repressed by TPL/TPRs. In the presence of JA-Ile, degradation of JAZ separates the TFs from the NINJA-TPL/TPRs co-repressors. In the case of MYC2, MYC3 and MYC4, which have an activation domain, the release of the TFs will allow the activation of their target genes. However, in the case of bHLH003, bHLH013 and bHLH017, the lack of an activation domain would prevent any transcriptional activation and would reduce the efficiency of MYC2, MYC3 and MYC4 due to competition for binding to their cis-regulatory elements. Moreover, since expression of bHLH017 is dependent on MYC2, activation of MYC2 will increase the concentration of bHLH017, therefore increasing competition for their DNA-binding targets and reducing the activity of the positive transcriptional activators.

Figure 6. DNA-binding specificity of bHLH003, bHLH013 and bHLH017.

(A) Position weight matrix (PWM) representation of the three top scoring 8-mers obtained in “seed-and-wobble” algorithm corresponding to bHLH003, bHLH013 and bHLH017. All three proteins showed highest binding affinity to a canonical G-box (CACGTG). (B) Median enrichment scores (E-scores) of the all the possible G-containing 8mers flanked by purine (R) and/or pyrimidine (Y) nucleotides recognized by the three bHLH proteins tested. bHLH003 showed a strong dependence for purine at 5'-end and pyrimidine at 3'-end. We included for comparison data corresponding to MYC2 previously described [53]. (C) Box-plot of E-scores of G-related variants. Boxes represent quartiles 25% to 75%, and black line represents the median of the distribution (quartile 50%). Bars indicate quartiles 1 to 25% (above) and 75 to 100% (below), and dots denote outliers of the distribution. Data corresponding to MYC2 were previously described [53]. doi:10.1371/journal.pone.0086182.g006
It is becoming evident that repression of the JA pathway is extremely important for the cell to prevent harmful responses and to fine-tune activation spatio-temporally. Several repression mechanisms have been described recently. Thus, transcriptional activation of the JAZ genes contributes to re-establish the repressor complexes [10,15,22]. Repression is also potentiated by the expression of JAZ repressor forms resistant to degradation, i.e. truncated forms of JAZ (JAZΔjas [17,37–39]) and JAZ8 [23]. The identification of these new repressor TFs adds a new example to these mechanisms of repression operating by TF competition for their cis-regulatory elements in the promoters of JA-regulated genes.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col-0) is the genetic background of wild-type and transgenic lines used throughout the work. Seedlings were grown in Murashige and Skoog medium (Sigma-Aldrich) at 21°C under a 16-h-light/8-h-dark cycle. The T-DNA insertion lines bhlh003 (GK-301G05), bhlh013 (GK-696A04) and bhlh017 (SAIL_536_F09) were obtained from the Nottingham Arabidopsis Stock Centre (NASC), myc2/jin1-2 was previously described [26] and coi1-1 was kindly provided by J. Turner.

To generate transgenic plants expressing bHLH003, bHLH013 or bHLH017 in Col-0 background, full-length coding sequences carrying or not the stop codon were amplified with Expand High Fidelity polymerase (Roche) using Gateway-compatible primers (Sup Table S1). PCR products were cloned into pDONR207 using the Gateway system (Invitrogen), and those without stop codon transferred to pGWB5 and pGWB14 and sequence verified. Agrobacterium strain GV3101, containing these constructs, was used to transform Col-0 plants by floral dipping [54]. Homozygous and independent lines of 35S:bHLH003-GFP, 35S:bHLH003-HA, 35S:bHLH013-GFP, 35S:bHLH013-HA, 35S:bHLH017-GFP and 35S:bHLH017-HA were selected and used for further analysis.

Figure 7. bHLH003, bHLH013 and bHLH017 are transcriptional repressors. (A) Schematic representation of reporter and effector constructs used in transient expression experiments in Nicotiana benthamiana. The reporter is the fusion of the JAZ2 promoter to firefly LUC coding sequence. MYC2, bHLH003, bHLH013 and bHLH017 genes expressed under the CaMV 35S promoter were used as effectors. (B) Induction or repression of pJAZ2:LUC reporter by MYC2, bHLH003, bHLH013 and bHLH017. Error bars indicate the SE from 16 replicates. (C) Effect of bHLH003 or bHLH017 expression on the MYC2 transactivation activity of pJAZ2:LUC. Error bars indicate the SE of results of 16 replicates. Asterisks represent p<0.05 in Students t-test. doi:10.1371/journal.pone.0086182.g007

Figure 8. bHLH0017 induction by JA is reduced in myc2 mutants. Quantitative real-time PCR of bHLH17 expression in WT plants or the myc2 mutant allele jin1-2 treated (or not) with 50 μM JA for 6 h. The measurements correspond to the average of three technical replicates and are relative to untreated WT. ACTIN8 expression was used as internal control. Error bars represent standard deviation. Asterisks indicate statistically significant differences compared to WT (Student’s t test: * P<0.05 and ** P<0.005). doi:10.1371/journal.pone.0086182.g008
**Sub-cellular localization**

Fluorescence of Arabidopsis 35S:bHLH003-GFP, 35S:bHLH013-GFP and 35S:bHLH017-GFP transgenic seedlings treated or untreated with 50 μM JA during 3 h was visualized by a Leica DMR UV/VIS microscope under UV light.

**Root measurements**

Root growth from day 2 to day 8 after germination was measured on 10 to 15 seedlings grown in vertical MS plates, in the presence or absence of 10 μM JA during 3 h was visualized by a Leica DMR UV/VIS microscope under UV light.

**Anthocyanin and chlorophyll quantification**

Seedlings were grown for 10 days in MS medium or 13 days in media supplemented with 50 μM JA. The aerial part of 6 to 12 seedlings from the same plates were pooled for DNA extraction and protein quantification. DNA was quantified in a spectrophotometer. Acetone 80% (V/V) was used for protein extraction and A663 was measured in a spectrophotometer. Values represent mean ± s.d. Student’s t-test was applied. Six to 10 seedlings from the same plates were pooled for chlorophyll measurements. Acetone 80% (V/V) was used for extraction and A645 and A665 was measured in a spectrophotometer. Data analysis was done according to Arnon, [56]. Values represent mean ± s.d. The experiment was repeated three times.

**Yeast two-hybrid assays**

Full-length bHLH003, bHLH013 or bHLH017 coding sequences carrying a stop codon were recombined from pDONR207 into pGADT7 (Gal4 AD). JAZ sequences cloned into pGBKTK7 (Gal4 BD) were previously described in Chini et al., [57]. To assess protein interactions, the corresponding plasmids were co-transformed into yeast AH109 cells following standard heat shock protocols. The method used for Y2H assays was previously described [28, 57]. Colonies from the co-transformed plates were collected and resuspended in minimal medium. A drop of each experiment was plated in medium lacking Leu and Trp to select for co-transformation and in medium lacking Ade, His, Leu, and Trp to select for interaction. Photos were taken after 5 days incubation at 30 °C. Empty gateway vectors pGADT7 and pGBKTK7 were used for co-transformation as negative controls.

**Protein extracts and pull-down assays**

MBP-bHLH fusion proteins were generated as previously described [11, 57] and MBP-bHLH fusions were transferred from the pDONR207 into the pDEST-TH1 [58] by recombination (Gateway, Invitrogen). Recombiant protein purification from E. coli was performed according to Fonseca and Solano [59]. Ten days-old Arabidopsis wild-type seedlings and transgenic lines expressing 35S:bHLH003-HA, 35S:bHLH013-HA and 35S:bHLH017-HA were ground in liquid nitrogen and homogenized in extraction buffer containing 30 mM Tris-HCl, pH 7.4, 80 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride, 50 μM MG132 (Sigma-Aldrich) and complete protease inhibitor (Roche) and used in pull-down assays [59].

**TAP-tagging purification**

Cloning of transgenes encoding tag fusions under control of the constitutive cauliflower mosaic virus 35S promoter and transformation of Arabidopsis cell suspension cultures were performed as previously described using the oligonucleotides listed in Sup Table S1 [60]. TAP of protein complexes was done using the GS tag [61] followed by protein precipitation and separation, according to Van Leene et al. [62]. The protocols of proteolysis and peptide isolation, acquisition of mass spectra by a 4000 Proteomics Analyzer (Applied Biosystems) and MS-based protein homology identification based on the TAIR genomic database are described in Van Leene et al. [63]. Experimental background proteins were subtracted based on ~40 TAP experiments on wild-type cultures and cultures expressing TAP-tagged mock proteins GUS, red fluorescent protein, and GFP [69].

**Determination of DNA-binding motifs**

DNA-binding specificities of bHLH003, bHLH013 and bHLH017 were determined using protein binding microarrays (PBM) as described by Godoy et al. [53], from soluble protein extracts. Extracts were obtained from 25 mL induced E. coli cultures containing the translational fusions to MBP. Pelleted cells were resuspended in 1 mL of 1x binding buffer, sonicated (2x30 s) and centrifuged twice to obtain cleared extracts of soluble proteins as in Godoy et al., [53]. The extract was adjusted to 175 μl containing 2% milk and 0.89 μg of denatured salmon sperm DNA. Synthesis of double-stranded microarrays, protein incubations and immunological detections of DNA-protein complexes were as described [53, 64].

**Promoter fusions and GUS staining**

bHLH003, bHLH013 and bHLH017 promoter regions of 1512 bp, 2121 bp and 2574 bp from the ATG, respectively, were amplified by combining the oligonucleotides listed in Table S1, cloned in pDONR207 (bHLH003 or bHLH013) or in pENTR/D-TOPO (Invitrogen, bHLH017) and transferred to pGBW3, to

---

**Figure 9. Schematic model of the role of bHLHs within the JA signaling pathway.** In the absence of JA-Ile, JAZ repressors are stable and interact with MYC2, MYC3 and MYC4 as well as bHLH003, bHLH013 and bHLH017. JAZs are part of a repression complex that comprises NINJA and TOPLESS (not shown). Upon a stimulus, hormone is perceived by the COI1/JAZ co-receptor and JAZ proteins are targeted for degradation by the proteasome. Once released from JAZ, MYC2, MYC3 and MYC4 will activate transcription, whereas bHLH003, bHLH013 and bHLH017 will repress it. Both sets of proteins (MYCs and bHLHs) compete for the G-box and, therefore, the output response will depend on the balance of activity between these two sets of TFs. Moreover, bHLH017 expression is activated by MYC2, therefore increasing the effective repressor over time and further contributing to reduce transcriptional activation. doi:10.1371/journal.pone.0086182.g009
drive GUS expression. Agrobacterium GV3101 strain was transformed with these constructs and used to transform Arabidopsis by floral dipping [54].

Six days-old seedlings or adult plant tissues from several transgenic lines were stained for GUS activity. Samples were placed in staining solution containing 50 mM phosphate buffer, pH 7, 0.1% (v/v) Triton X-100 (Sigma-Aldrich), 2 mM 5-bromo-4-chloro-3-indolyl b-D glucuronic acid (X-Gluc, Glycosynth), 1 mM potassium-ferrocyanide (Sigma-Aldrich), and 1 mM potassium-ferrocyanide (Sigma-Aldrich) and incubated at 37°C overnight. After staining, the tissue was soaked several times in 75% ethanol and kept in 5% glycerol until being photographed with a Leica DMR UV/VIS microscope (anthers, sepals, petals and pistils) or with a digital NIKON D1-x camera (seedlings and adult leaves).

Luciferase assay
Transcriptional activity of bHLH003, bHLH013 or bHLH017 and competition with MYC2 for their binding sites in the JAZ promoters was measured using the promoter of JAZ2 (2 Kb) fused to a Luciferase reporter gene cloned into pGWB435. Leaves of N. benthamiana were transiently infiltrated with Agrobacterium strains bearing the pGZ22-LUC and 35S:MYC2-HA, 35S:bHLH0003-HA; 35S:bHLH013-HA or 35S:bHLH017-HA constructs. All combinations included the silencing suppressor p19. 24 hours after agroinfiltration, 1 cm discs were collected from the leaves with the aid of a cylindrical borer and carefully transferred, the abaxial side upwards, to 96 well microplates filled with 175 μl of H2O and 25 μl of D-Luciferin substrate (0.1 mg/ml; Sigma #L9504). One disc was used per well and at least 16 disc replicates per sample. Levels of Luciferase activity were measured every hour, for a total of 48 hours, using the LB 960 Microplate Luminometer (Berthold) which operates through the Windows® PC MikroWin 2000 software.

Quantitative real time PCR (QPCR)
Seedlings untreated or treated with 50 μM JA for 6 h were harvested for RNA extraction with the Trizol reagent (Invitrogen). After DNase I digestion and cleanup by RNeasy mini kit (Qiagen) harvested for RNA extraction with the Trizol reagent (Invitrogen).

Real Time PCR was performed with 50 ng of cDNA in a 7500 Real Time PCR system (Applied Biosystems). Data analysis was performed using the 2-ΔΔCt method (using ACTIN8, L9504). One pair of primers was used per gene (Table S1) and competition with MYC2 for their binding sites in the JAZ promoters was measured using the promoter of JAZ2 (2 Kb) fused to a Luciferase reporter gene cloned into pGWB435. Leaves of N. benthamiana were transiently infiltrated with Agrobacterium strains bearing the pGZ22-LUC and 35S:MYC2-HA, 35S:bHLH0003-HA; 35S:bHLH013-HA or 35S:bHLH017-HA constructs. All combinations included the silencing suppressor p19.

Infection assays with Pseudomonas syringeae
Pseudomonas syringae pv tomato (Pto) DC3000 growth assays in Arabidopsis were performed by spray inoculation as previously described in Fernandez-Calvo et al, [28]. Briefly, overnight bacterial cultures were pelleted and resuspended in sterile 10 mM MgCl2. Three- to four-week-old plants were sprayed with a bacterial suspension containing 108 (colony-forming units)/mL bacteria (OD600 = 0.2) with 0.04% Silwet L-77. Leaf discs were harvested after 2 days and ground in 10 mM MgCl2. Population counts were performed at 2 days after infiltration. In both cases, serial dilutions of leaf extracts were plated on LB agar with appropriate antibiotics. Data points represent the average of four replicates, each containing two leaf discs from different plants. Error bars indicate SE. These experiments were repeated with similar results, and representative results are shown. Pictures of disease symptoms 4 days after inoculation on analyzed genotypes were taken with a digital NIKON D1-x.

Supporting Information
Figure S1 Co-transformation controls of Y2H assays. Yeast growth control of experiment shown in Figure 1A. Yeast cells co-transformed with the indicated combinations of JAZ-BD (pGBK7) and bHLH-AD (pGADT7), grown for 3 days in medium lacking Leu and Trp to select for co-transformation. Numbers represent the number of JAZ protein (JAZ1 to JAZ12) and C represents the empty pGBK7 vector containing only the BD.

Figure S2 T-DNA insertion lines for bHLH003, bHLH013 and bHLH017. (A) Representation of T-DNA insertions (black triangles) in bHLH003, bHLH013 and bHLH017 genes. The white bar represents the coding sequence where the bHLH domain is shown in grey. Pairs of oligonucleotides used for cDNA amplification are shown as arrows. (B) PCR products generated with the oligonucleotide pairs indicated above, in WT and mutant T-DNA insertion homozygous lines. Oligonucleotide sequence is listed in Table S1.

Figure S3 Anthocyanin and chlorophyll quantification in bHLH003, bHLH013 and bHLH017 mutants and OE lines in basal conditions. Anthocyanin accumulation (A) and chlorophyll content (B) in 10-days-old WT seedlings, jin1-2, coi1-1, bhlh003 and bhlh017 mutants and OE lines for bHLH003, bHLH0013 and bHLH017. Bars represent the average of three pools of seedlings and error bars the standard deviation. Differences are not statistically significant (Student’s T-test).

Table S1 Oligonucleotides used for PCR reactions described in Material and Methods section.

Table S2 Protein Identification details obtained with the 480 MALDI TOF/TOF Proteomics analyzer (AB SCIEX) and the GPS explorer v3.6 (AB SCIEX) software package combined with search engine Mascot version 2.2 (Matrix Science) and database TAIR. Column headers for Protein and Peptide data are explained below.

Acknowledgments
We thank Roberto Solano’s lab members for critical reading of the manuscript.

Author Contributions
Conceived and designed the experiments: SF PF-C GF MD-D SG-I GF-B JF-Z RS. Performed the experiments: SF PF-C GF MD-D SG-I GF-B IL-V MG JVL. Analyzed the data: SF PF-C GF MD-D SG-I GF-B JF-Z RS. Wrote the paper: SF RS.
References

1. Blecher S, Bockelmann C, Füßlein M, von Schrader T, Steinhach B, et al. (1999) structure-activity analyses reveal the existence of two separate groups of active octadecanoids in elicitation of the tendril-coiling response of bryonia dioica jaq. PLoS ONE 4: e60.

2. Fonseca S, Chico JM, Solano R (2009) the jasmonate pathway: the ligand, the receptor and the core signalling module. Curr opin plant biol 12: 339–347.

3. Pauwels L, Moreel K, De Witte E, Lammertyn V, Van Montagu M, et al. (2008) mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in Arabidopsis cells. Proc natl acad sci u s a 105: 1300–1305.

4. Reineboe C, Springer A, Samol I, Reineboe S (2009) plant oxylipins: role of jasmonic acid during programmed cell death, defence and leaf senescence. Festschr 267: 4666–4681.

5. Wasternack C, Haune B (2013) jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in annals of botony. Ann bot 111: 1021–1058.

6. Yoshida Y, Sano R, Wada T, Takahayashi J, Okada K (2009) jasmonic acid control of glabra3 links inducible defence and trichome patterning in Arabidopsis. Development 136: 1039–1048.

7. Zhang Y, Turner JG (2008) wound-induced endogenous jasmonates stunt plant growth by inhibiting mitosis. Plos one 3: e609.

8. Browse J, Howe GA (2008) new weapons and a rapid response against insect attack. Plant physiol 146: 832–838.

9. Devoe A, Ellis C, Magunin A, Chang HS, Chlrotch C, et al. (2005) expression profiling reveals coi1 to be a key regulator of genes involved in wound and methyl jasmonate-induced secondary metabolism, defence, and hormone interconnections. Planta 220: 58–71.

10. Chini a, fonseca s, fernandez g, adie b, chico jm, et al. (2007) the jaz family of repressors is the missing link in jasmonate signalling. Nature 448: 666–671.

11. Fonseca S, Chini A, Hamberg M, Ade B, Porzel A, et al. (2009) jasmonoyl-l-isoleucine is the endogenous biologically active jasmonate. Nat chem biol 5: 344–350.

12. Katsir L, Schmidler AM, Stawick PE, He SY, Howe GA (2008) coi1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc natl acad sci u s a 105: 7100–7105.

13. Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, et al. (2010) jasmonate perception by inositol-phosphatase-potentiated coi1-cjaz-receptor. Nature 468: 400–405.

14. Stuckey PE, Tsyaki I (2004) the oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant cell 16: 2117–2127.

15. Thines B, Katsir L, Melotto M, Niu Y, Mandolaar A, et al. (2007) jasmonate repressor proteins are targets of the scf(coi1) complex during jasmonate signalling. Nature 446: 661–665.

16. Xie DX, Frys BF, James S, Nieto-Rostro M, Turner JG (1998) coi1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280: 1091–1094.

17. Yan Y, Stolz S, Santuari L, Tartu S, Reymond P, et al. (2007) a downstream target of jaz10 encodes a myc transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant cell 19: 2225–2245.

18. Acosta IF, Gasperini D, Chatelat A, Stolz S, Santuari L, et al. (2013) role of ninja in jasmonate-regulated defense and fertility. Plant cell 25: 1641–1656.

19. Alonso-Blanco C, Ecker JR (2006) characterization of ein3/eil1, acts as a repressor to negatively regulate jasmonate signaling in Arabidopsis. Plant cell 18: 1577–1591.

20. Consortium AIM (2011) evidence for network evolution in an Arabidopsis interactome map. Science 333: 601–607.

21. Koo AJ, Cooke TF, Depew CL, Patel LC, Chuang HS, et al. (2011) negative feedback control of jasmonate signaling by an alternative splice variant of jaz10. Plant physiol 162: 1006–1017.

22. Heitz T, Widermann E, Lugar R, Miesch L, Ullmann P, et al. (2012) cytochromes p450 cyp94c1 and cyp94h3 catalyze two successive oxidation steps of plant hormone jasmonoyl-isoleucine for catabolic turnover. J biol chem 287: 6296–6306.

23. Kitaoka N, Matusbara T, Saito M, Takahashi K, Wakana S, et al. (2011) Arabidopsis cyp94h3 encodes jasmonoyl-isoleucine 12-hydroxylase, a key enzyme in the oxidative catabolism of jasmonate. Plant cell physiol 52: 1757–1765.

24. Koo AJ, Cooke TF, Howe GA (2011) cytochrome p450 cyp94h3 mediates catabolism and inactivation of the plant hormone jasmonoyl-isoleucine. Proc natl acad sci u s a 108: 12539–12544.

25. Schwender S, Ntsebeza P, Wasternack C (2013) jasmonate signaling synergy in Arabidopsis thaliana. J biol chem 288: 17895–17900.

26. Miersch O, Neumerkel J, Dippe M, Stenzel I, Wasternack C (2008) hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. New phytolet 177: 114–127.

27. Wang L, Halinschi R, Kang JH, Berg A, Harmich F, et al. (2007) independently silencing two jar family members impairs levels of tryptic protease inhibitors but not nicotine. Planta 226: 159–167.

28. Nakata M, Mitsuwa N, Herde M, Koo AJ, Moreno JE, et al. (2013) a bhlh-type transcription factor, ab3-inducible bhlh-type transcription factor/ja-associated myc2-like-1, acts as a repressor to negatively regulate jasmonate signaling in Arabidopsis. Plant cell 25: 1641–1656.

29. Sauki-Sekimoto Y, Ikimaru Y, Ohayashi T, Saito H, Masaoka S, et al. (2013) bhlh transcription factors ja-associated myc2-like-1, ja2 and ja3 are negative regulators of jasmonate responses in Arabidopsis thaliana. Plant physiol. doi:10.1104/pp.113.220129.

30. Song S, Qi T, Fan M, Zhang X, Gao H, et al. (2013) the bhlh subgroup iiid factors negatively regulate jasmonate-mediated plant defense and development. PloS genet 9: e1003653.

31. Benders CL, Alarcón-Chávez F, Gross DG (1999) pseudomonas syringae phytophthora: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol mol biol rev 63: 266–292.

32. Luinevin BH, Kunkel NL (2006) the arabidopsis thaliana jasmonate insensitive 1 gene is required for suppression of salicylic acid-dependent defense responses during infection by pseudomonas syringae. Mol plant microbe interact 19: 789–800.

33. Upadhyra SR, Ishiga Y, Komatsub T, Kunkel BN, Anand A, et al. (2007) the phytoxotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with pseudomonas syringae pv. Tomato dc3000. Mol plant microbe interact 20: 955–965.
53. Godoy M, Franco-Zorrilla JM, Perez-Perez J, Oliveros JC, Lorenzo O, et al. (2011) improved protein-binding microarrays for the identification of dna-binding specificities of transcription factors. Plant j 66: 700–711.
54. Clough SJ, Bent AF (1998) floral dip: a simplified method for agrobacterium-mediated transformation of arabidopsis thaliana. Plant j 16: 735–743.
55. Swain T, Hillis WE (1959) the phenolic constituents of prunus domestica. I.—the quantitative analysis of phenolic constituents. Journal of the science of food and agriculture 10: 63–68.
56. Arnon DI (1949) copper enzymes in isolated chloplasts. Polyphenoloxidase in beta vulgaris.plant physiol 24: 1–15.
57. Chini A, Fonseca S, Chico JM, Fernandez-Calvo P, Solano R (2009) the zim domain mediates homo- and heteromeric interactions between arabidopsis jaz proteins. Plant j 59: 77–87.
58. Hammarstrom M, Hellgren N, Van Den Berg S, Bergh H, Hard T (2002) rapid screening for improved solubility of small human proteins produced as fusion proteins in escherichia coli. . Protein sci 11: 313–321.
59. Fonseca S, Solano R (2013) pull-down analysis of interactions among jasmonic acid core signaling proteins. Methods mol biol 1011: 159–171.
60. Van Leene J, Stals H, Ecclethout D, Persiau G, Van De Slijke E, et al. (2007) a tandem affinity purification-based technology platform to study the cell cycle interactome in arabidopsis thaliana. Molecular & cellular proteomics 6: 1226–1238.
61. Burckstummer T, Bennett KL, Preradovic A, Schutze G, Hantschel O, et al. (2006) an efficient tandem affinity purification procedure for interaction proteomics in mammalian cells. Nat meth 3: 1013–1019.
62. Van Leene J, Wittens E, Inzè D, De Jaeger G (2008) boosting tandem affinity purification of plant protein complexes. Trends in plant science 13: 517–520.
63. Van Leene J, Hollandier J, Ecclethout D, Persiau G, Van De Slijke E, et al. (2010) targeted interactomics reveals a complex core cell cycle machinery in arabidopsis thaliana. Mol syst biol 6.
64. Berger MF, Bulyk ML (2009) universal protein-binding microarrays for the comprehensive characterization of the dna-binding specificities of transcription factors. Nat protocols 4: 395–411.