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*Rapid report*

# Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity

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## Summary

- The bHLH transcription factor MYC2, together with its paralogues MYC3 and MYC4, is a master regulator of the response to the jasmonate (JA) hormone in Arabidopsis (*Arabidopsis thaliana*). In the absence of JA, JASMONATE ZIM (JAZ) proteins interact with the MYC proteins to block their activity. Understanding of the mechanism and specificity of this interaction is key to unravel JA signalling.
- We generated mutant MYC proteins and assessed their activity and the specificity of their interaction with the 12 Arabidopsis JAZ proteins.
- We show that the D94N mutation present in the *atr2D* allele of *MYC3* abolishes the interaction between MYC3 and most JAZ proteins. The same effect is observed when the corresponding conserved Asp (D105) was mutated in MYC2. Accordingly, $MYC2^{D105N}$ activated target genes in the presence of JAZ proteins, in contrast to wild-type MYC2. JAZ1 and JAZ10 were the only JAZ proteins still showing interaction with the mutant MYC proteins, due to a second MYC interaction domain, besides the classical Jas domain.
- Our results visualize the divergence among JAZ proteins in their interaction with MYC proteins. Ultimately, the transferability of the Asp-to-Asn amino acid change might facilitate the design of hyperactive transcription factors for plant engineering.

## Introduction

Jasmonates (JAs) are phytohormones involved in abiotic and biotic stress responses, and several developmental processes (Kazan & Manners, 2008, 2011; Pauwels *et al.*, 2008, 2009; Browse, 2009; Pauwels & Goossens, 2011; Wasternack & Hause, 2013). Although the perception of the bioactive hormone jasmonoyl-isoleucine (JA-Ile) and the core JA signalling complex are very conserved for different JA-controlled processes, specificity in downstream responses is mediated by (combinations of) transcription factors (TFs) (De Geyter *et al.*, 2012), among which the best studied and arguably the most important are the basic Helix-Loop-Helix (bHLH) TFs MYC2, MYC3 and MYC4 from Arabidopsis (*Arabidopsis thaliana*) (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011; Kazan & Manners, 2013).

In the absence of bioactive JAs, the switch to downstream processes is blocked due to repression of the MYC TFs by the JASMONATE ZIM domain (JAZ) proteins (Chini *et al.*, 2007, 2009; Chung & Howe, 2009). When JA-Ile is present, a co-receptor complex is assembled, consisting of CORONATINE INSENSITIVE 1 (COI1), JA-Ile and a JAZ protein. COI1 is an F-box protein, functioning as a substrate-specific adaptor of an Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex, that mediates ubiquitin-dependent degradation of the JAZ protein by the 26S proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008; Fonseca *et al.*, 2009; Sheard *et al.*, 2010), which releases the MYC TFs from repression. The three MYC TFs show very high sequence similarity, which is manifested in their functional redundancy (Cheng *et al.*, 2011; Fernández-Calvo

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*et al.*, 2011; Niu *et al.*, 2011; Frerigmann *et al.*, 2014). Nonetheless, they each have a certain specific input in the JA response, which is likely constituted by their tissue-specific expression patterns (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011).

The MYC TFs have a nearly identical C-terminal bHLH domain responsible for DNA binding and a transcriptional activation domain responsible for the interaction with MED25, a subunit of the Mediator complex connecting them to the RNA polymerase (Çevik *et al.*, 2012; Chen *et al.*, 2012). The JAZ-Interacting Domain (JID) is essential for the interaction with the JAZ repressors (Fernández-Calvo *et al.*, 2011) and R2R3-MYB proteins involved in the biosynthesis of glucosinolates (Schweizer *et al.*, 2013).

The JAZ family consists of 12 members, all with a similar modular build-up (Pauwels & Goossens, 2011). JAZ proteins are capable of repressing gene expression due to the interaction of their central ZINC-FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM (ZIM) domain with the NOVEL INTERACTOR OF JAZ (NINJA), an adaptor protein bridging JAZ with the co-repressors TOPLESS (TPL) and TPL-related proteins (TPRs) through its ERF-associated amphiphilic repression (EAR) domain (Pauwels *et al.*, 2010). This ZIM domain is also responsible for homo- and heterodimerization among the JAZ proteins. A Jasmonate-associated (Jas) domain, localized at the C-terminus, is responsible for their interaction with COI1, the MYC TFs and several other bHLH and R2R3-MYB TFs (Pauwels & Goossens, 2011).

Here, we show that a single amino acid change in the *atr2D* allele of *MYC3* abolishes binding with most, but not all, Arabidopsis JAZ proteins and leads to activation of JA responses. This amino acid residue is conserved in JAZ-interacting MYC TFs in Arabidopsis and other plant species. We show that the corresponding amino acid change in the paralogue MYC2 also leads to loss of JAZ repression and an increased MYC2 activity. Furthermore, we demonstrate that different JAZ proteins have multiple ways and capacities of binding MYC proteins; in particular, two JAZ proteins have evolved to have two MYC interaction domains.

## Materials and Methods

### Gene cloning

All cloning was carried out by Gateway® recombination (Life Technologies). The point mutation in $MYC2^{D105N}$ was generated with the GeneTailor™ Site-Directed Mutagenesis system (Life Technologies, Carlsbad, CA, USA).

### Plant material and growth conditions

The *Arabidopsis thaliana* (L.) Heynh ecotype Col-0 was used as wild-type for all experiments. For overexpression, the full-length open reading frame (ORF) of *MYC2* or $MYC2^{D105N}$ was put under control of the CaMV35S promoter in the binary vector pK8m34GW-FAST (Vanholme *et al.*, 2013), which was introduced into the *Agrobacterium tumefaciens* strain C58C1 (pMP90) for transformation of Arabidopsis by the floral dip method (Clough & Bent, 1998). Plants were propagated to the $T_3$ generation for selection of homozygous plants for analysis. The *atr2D* mutant line is described in Smolen *et al.* (2002). Arabidopsis seeds were sterilized by the chlorine gas method, sown on plates containing MS medium (10 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agarose, pH 5.7), supplemented or not with dimethylsulfoxide (DMSO), methyl jasmonate (MeJA; Duchefa-Biochemie, Haarlem, the Netherlands), ethanol or abscisic acid (ABA; Sigma-Aldrich), incubated in the dark at 4°C for 3 d, and transferred to a growth room with controlled conditions (21°C, 16 h : 8 h, light : dark regime).

### Quantitative real-time PCR (qRT-PCR)

For each biological repeat, 20–80 seedlings were grown as described above for 8–10 d, pooled and snap-frozen. The material was ground in a Retsch MM300 mixer and total RNA was extracted using the Qiagen RNeasy kit (Qiagen). One microgram of RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed in the LightCycler 480 system (Roche) using the Fast Start SYBR Green I PCR mix (Roche) via the following program: pre-incubation (95°C, 10 s), 45 amplification cycles (incubation 95°C, 10 s; annealing 65°C, 15 s; elongation 72°C, 15 s). Transcript levels were normalized relative to the housekeeping genes *UBC10* (*AT5G53300*) and *PP2A* (*AT1G13320*). Primer sequences are presented in Supporting Information Table S1.

### Yeast two-hybrid (Y2H) analysis

Y2H analysis was performed as described (Cuéllar Pérez *et al.*, 2013). Bait and prey were fused to the GAL4-AD or GAL4-BD via cloning into pGAL424gate or pGBT9gate, respectively (Cuéllar Pérez *et al.*, 2013). The *Saccharomyces cerevisiae* PJ69-4A yeast strain (James *et al.*, 1996) was co-transformed with bait and prey using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) media lacking Leu and Trp (−2) (Clontech, Saint-Germain-en-Laye, France). Three individual colonies were grown overnight in liquid cultures (−2) at 30°C and 10- or 100-fold dilutions were dropped on control media (−2) and selective media lacking Leu, Trp and His (−3) (Clontech).

In order to allow quantitative Y2H analysis, we adapted the above classical Y2H set-up as follows. Three individual colonies were grown overnight in liquid cultures (−2) at 30°C. A five-fold dilution in yeast extract peptone dextrose adenine (YPDA) medium (Clontech) was grown for another 2–3 h until an $OD_{600}$ of 1.0–1.5 was reached. Lysis of pelleted cells was performed in Z buffer (0.06 M $Na_2HPO_4$; 0.04 M $NaH_2PO_4$; 0.01 M KCl; 0.001 M $MgSO_4$; pH 7) containing 0.15% 2-mercaptoethanol, 18% chloroform and 0.1% SDS. β-Galactosidase activity was quantified via spectrophotometry using the substrate o-nitrophenyl B-D-galactopyranoside (ONPG) and presented in Miller units.

In order to assess the protein stability of the different MYC2 versions in the transformed yeasts, immunoblot analysis was carried out. To this end, a pool of three individual transformant colonies was grown overnight in liquid selective medium at 30°C. The

culture was diluted to an $OD_{600}$ of 0.1 in 15 ml of YPD medium (Clontech) and grown for another 4–5 h. Total protein was extracted and separated by SDS-PAGE (4–15% Mini-PROTEAN® TGX™ Precast Gel, Bio-Rad) and blotted on a polyvinylidene fluoride (PVDF) membrane (Trans-Blot® Turbo™ Mini PVDF Transfer; Bio-Rad). After incubation with anti-GAL4-AD (Sigma-Aldrich) and anti-rabbit-horseradish peroxidase (GE Healthcare, Freiburg, Germany), the signal was captured using detection substrate (Western Lightning® Plus-ECL; Perkin Elmer) and X-ray films (Amersham Hyperfilm ECL; GE Healthcare). Total protein loading was visualized using Coomassie Brilliant Blue (Thermo Scientific, Waltham, MA, USA) staining of the PVDF membrane.

### Transient expression assays

Transient expression assays in protoplast cells prepared from Bright Yellow-2 (BY-2) *Nicotiana tabacum* suspension cultured cells were performed as described (Vanden Bossche *et al.*, 2013). The reporter plasmid contained a *firefly luciferase* (*fLUC*) gene under control of the MYC2-inducible *LOX3* promoter. *GUS*, *MYC2*, *MYC2*$^{D105N}$ and *JAZ* genes were expressed under control of the pCaMV35S promoter in effector plasmids. Plasmids with the *Renilla luciferase* (*rLUC*) expressed under control of the pCaMV35S promoter serve for normalization for transfection efficiency. Protoplast cells were transfected with 2 μg of each plasmid using the PEG/$Ca^{2+}$ method and grown overnight in the dark at room temperature with gentle agitation. After lysis of the cells, the luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega).

In order to assess the protein stability of the different MYC2 versions in the tobacco protoplasts, *MYC2* and *MYC2*$^{D105N}$ ORFs were fused to a 3xFlag-6xHis-tag and expressed under control of the pCaMV35S promoter in the pm43GW7 plasmid (Pauwels *et al.*, 2010). The resulting vectors were used for protoplast transfection as described above. Transfected protoplasts were pooled and total protein was extracted. Immunoblot analysis was performed as described above but with anti-Flag (Sigma-Aldrich) and anti-mouse-horseradish peroxidase (GE Healthcare) antibodies.

## Results

### The *atr2D* mutation releases MYC3 from JAZ interaction

The *atr2D* allele of *MYC3* has been described by Smolen *et al.* (2002) as a dominant mutant salvaging plants from the toxic effect of 5-methyl-Trp, an inhibitor of Trp biosynthesis, by increased expression of *Trp* genes. Furthermore, altered expression of stress-related genes has been observed in the *atr2D* mutant plants. The phenotype of *atr2D* is caused by a point mutation of a conserved Asp (D94N) in the JID of MYC3 (Smolen *et al.*, 2002; Fernández-Calvo *et al.*, 2011). Therefore, we performed a Y2H analysis between ATR2D, MYC3 and JAZ proteins to investigate the possibility that this mutation abolishes the interaction with the JAZ repressors. Indeed, ATR2D lost the capacity to interact with the majority of the JAZ repressors; only interaction with JAZ1, JAZ2, JAZ5 and JAZ10 remained (Fig. 1a). Immunoblot analysis demonstrated that MYC3 and ATR2D were present at similar levels in transformed yeast cells (Fig. S1), indicating that MYC3 protein stability was not affected by the D94N mutation. Quantitative Y2H revealed that the interaction of ATR2D was significantly diminished with all JAZ proteins (Fig. 1b), even with those that still showed interaction with MYC3 under selective pressure in the 'classical' Y2H (Fig. 1a). In the quantitative assay, only interaction of ATR2D with JAZ1 and JAZ10 was significantly different from the negative control. Together, our results indicate that the *atr2D* mutation abolishes or attenuates the interaction between MYC3 and the JAZ repressors.

### The D105N mutation increases MYC2 activity

MYC3 belongs to the clade of bHLH IIIe TFs, all containing a conserved N-terminal JID responsible for the interaction with the family of JAZ repressors (Heim *et al.*, 2003). Nearly all members of the subgroups bHLH IIId and bHLH IIIf also have this JID (Fig. S2) containing the conserved Asp residue of MYC3. Only TRANSPARANT TESTA 8 and MYC1 possess instead an Asn and a Glu residue, respectively. We hypothesized that the conserved Asp is essential for JAZ interaction in each homologue containing a JID. To test this hypothesis, we generated the MYC2$^{D105N}$ mutant by introducing a point mutation in MYC2. 'Classical' Y2H analysis indicated that MYC2$^{D105N}$ lost interaction with most of the JAZ proteins that were able to interact with MYC2 in this assay, except JAZ1 and JAZ10 (Fig. S3), which is similar to the Y2H analysis with ATR2D. Nevertheless, quantitative Y2H revealed that the strength of interaction between MYC2$^{D105N}$ and JAZ1 or JAZ10 was also severely reduced compared to their interaction with MYC2, as was also observed for ATR2D (Fig. 2a).

JAZ proteins exert their function as repressors by interaction with the adaptor NINJA that recruits co-repressors like TPL and TPRs (Pauwels *et al.*, 2010). Therefore, we would expect MYC2$^{D105N}$ to have a greater activity than MYC2 because of the loss of interaction with most of the JAZ proteins, hence with NINJA and the co-repressors. To assess this, we compared the transactivation potential of MYC2$^{D105N}$ and MYC2 by a transient expression assay in tobacco protoplasts with the MYC2-inducible *pLOX3::fLUC* construct as read-out (Pauwels *et al.*, 2008). In the absence of co-transfected JAZ, both MYC2 and MYC2$^{D105N}$ were able to transactivate the *LOX3* promoter to a similar extent (Fig. 2b). When co-expressed with JAZ proteins, complete inhibition of MYC2 transactivation activity was observed with all JAZ members tested (Fig. 2b). By contrast, depending on the JAZ member, a reduced to nearly no attenuation of transactivation activity was observed with MYC2$^{D105N}$ (Fig. 2b). To exclude the possibility that the D105N mutation increases the stability of MYC2, we transfected tobacco protoplasts with tagged versions of MYC2 and MYC2$^{D105N}$. Immunoblot analysis of the protein extracts revealed that MYC2 and MYC2$^{D105N}$ were present at similar levels in transfected tobacco protoplasts (Fig. S4), proving that the hyperactivity of MYC2$^{D105N}$ is not caused by increased protein stability. Hence, our data demonstrate that loss of binding to the JAZ repressors results in an increased transactivation potential of MYC2.

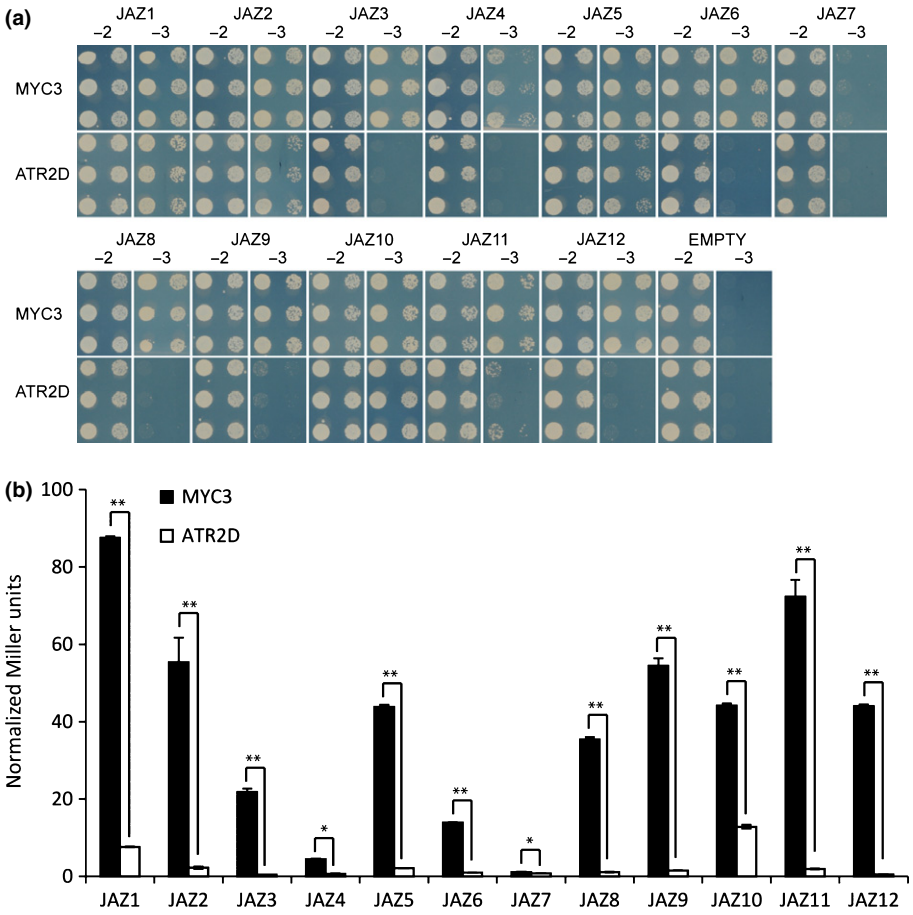


**Fig. 1** The *atr2D* mutation abolishes interaction with most JAZ repressors. Through Y2H, MYC3 and the mutant version ATR2D fused to GAL4-AD were tested for interaction with the 12 JAZ proteins fused to GAL4-BD. Empty vectors were used as negative control. (a) Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium, respectively, control and selective medium. (b) A liquid culture β-galactosidase assay was performed on the transformed yeasts. The activity of β-galactosidase was measured in Miller units and normalized to the negative control. Values are the mean (± SE) of three biological repeats. Significant differences (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$.

## An N-terminal Jas-like domain is responsible for the interaction of JAZ1 and JAZ10 with MYC2$^{D105N}$

The Y2H analysis indicated that JAZ1 and JAZ10 are the main JAZ proteins able to maintain proper interaction with the mutated MYC constructs. Previously, Moreno *et al.* (2013) reported that JAZ10 contains an additional, N-terminal, Jas-like domain that was able to interact with MYC2. This cryptic MYC2-interacting domain (CMID) lacks conserved residues that have been shown to be essential for COI1 interaction and can thus be considered more specific for interaction with MYC TFs (Fig. 3a) (Melotto *et al.*, 2008; Chung & Howe, 2009; Chung *et al.*, 2010; Sheard *et al.*, 2010; Withers *et al.*, 2012). To assess which domain is responsible for the remaining interaction with MYC2$^{D105N}$, truncated versions of JAZ10, that is JAZ10-N and JAZ10-C containing the CMID and the Jas domain, respectively, were generated and tested via Y2H. Both JAZ10 fragments were able to bind MYC2, but whereas the JAZ10-C fragment lost interaction with MYC2$^{D105N}$, the JAZ10-N fragment containing the CMID did not (Fig. 3b).

Because JAZ1 was also able to interact with MYC2$^{D105N}$ (Figs 2a, S3), we reasoned that a similar CMID motif might be present at the N-terminus of JAZ1. Alignment of JAZ1 with Jas domains of the JAZ proteins revealed a Jas-like motif in the N-terminus (Fig. 3a). Considering this motif to be a true 'Jas motif', we postulate that the conserved core sequence of the Jas motif responsible for MYC2 interaction would be Ba-F-X-X-Ba-Ba, in which Ba refers to a basic residue (Arg or Lys). To test this hypothesis, we performed Y2H with truncated versions of JAZ1, which demonstrated that the N-terminal part of JAZ1 could indeed bind both MYC2 and MYC2$^{D105N}$, whereas the C-terminal Jas domain of JAZ1 could bind MYC2, but not MYC2$^{D105N}$ (Fig. 3b). Hence, both JAZ1 and JAZ10 have two MYC interaction domains. Shorter constructs of the N-terminal domain or deletion constructs missing the N-terminal part of JAZ1 were not able to bind MYC2, indicating that amino acids residing somewhere in the first 66 amino acids of the protein sequence are essential for interaction of JAZ1ΔJas with MYC2 (Fig. S5).

## Hyperactive MYC transcription factors cause a JA-related phenotype in Arabidopsis

It has been reported that the *atr2D* mutant shows an increased expression of *Trp* and stress-related genes (Smolen *et al.*, 2002). However, because the role of MYC TFs in JA signalling was still

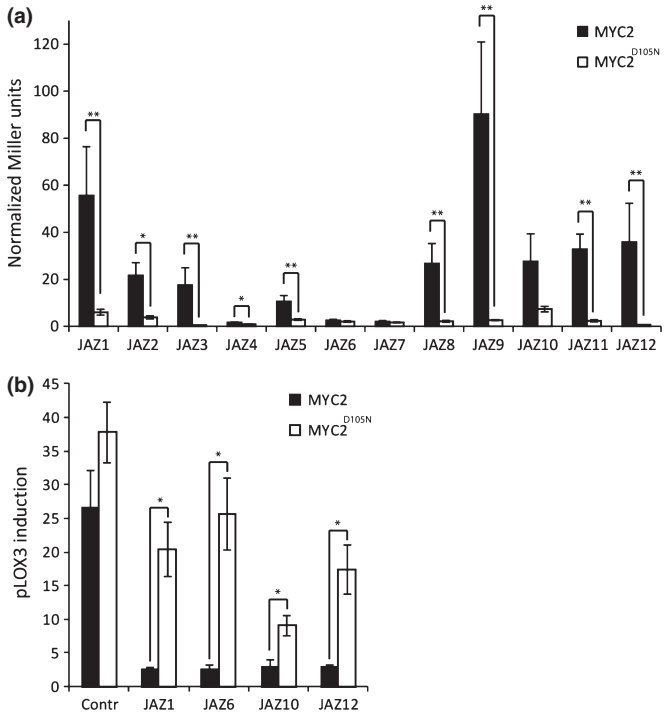


**Fig. 2** MYC2$^{D105N}$ loses interaction with most of the JAZ proteins, resulting in the preservation of the transactivation potential in the presence of JAZ proteins. (a) Quantitative Y2H analysis. MYC2 and MYC2$^{D105N}$ fused to GAL4-AD were tested for interaction with the 12 JAZ proteins fused to GAL4-BD. Empty vectors were used as negative control. A liquid culture β-galactosidase assay was performed on the transformed yeasts shown in Supporting Information Fig. S3. The activity of β-galactosidase was measured in Miller units and normalized to the negative control. Values are the mean (± SE) of three biological repeats. Significant differences (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$. (b) Transactivation assay by transient expression in tobacco protoplasts. The MYC2-inducible *pLOX3::fLUC* reporter construct was co-transfected with effector constructs overexpressing *MYC2*, *MYC2$^{D105N}$*, and/or *JAZ* genes and an *rLUC* construct for normalization. 'Contr' indicates a control assay with *MYC2* effector constructs co-transfected with a *GUS* expression construct instead of a *JAZ* effector construct. Values are fold-changes relative to protoplasts transfected only with a *GUS* expression construct instead of *MYC2* effector constructs and are the mean (± SE) of eight biological repeats. Significant differences (Student's *t*-test): *, $P < 0.05$.

unsuspected at that time, a direct correlation with the JA signalling response was not investigated. To explore the effect of the Asp-to-Asn mutation in the MYC TFs on JA signalling *in planta*, we analysed the expression of JA response genes in the *atr2D* mutant and in *MYC2$^{D105N}$* overexpressing plants. As expected, qRT-PCR analysis of the *atr2D* mutant revealed increased transcript levels of *OPR3, LOX2* and *AOS*, involved in JA biosynthesis, and of the JA marker genes *JAZ10*, *TAT3*, *VSP2* and *PDF1.2*, compared to those in wild-type plants (Fig. 4a). We also confirmed the increased expression of genes described by Smolen *et al.* (2002), such as *TSB1*. Notably, the *PDF1.2* gene showed a markedly higher induction (> 20-fold) in *atr2D* plants, as compared to the other tested genes that showed a rather moderate increase of *c.* two-fold only.

Induction of most JA marker genes was also observed in plants overexpressing the hyperactive *MYC2$^{D105N}$*, as compared to plants overexpressing *MYC2* or wild-type plants (Fig. 4b), which showed no altered expression of these target genes. Contrasting with the *atr2D* plants was the absence of *PDF1.2* induction in the *MYC2$^{D105N}$* overexpressing plants. Conversely, an extreme increase in *TAT3* transcript levels (by > 250-fold) was observed in plants overexpressing *MYC2$^{D105N}$*, whereas this was not the case in the *atr2D* plants. It should be noted here that we selected overexpression lines with comparable transcript levels of *MYC2* and *MYC2$^{D105N}$*, to allow comparative analysis of their activity *in planta*. The fact that *MYC2* overexpression was even slightly higher in this line compared to that of *MYC2$^{D105N}$* in the presented lines supports the proposition that MYC2$^{D105N}$ contains a higher transactivation activity than MYC2 *in planta*. Nonetheless, as illustrated in Fig. 4(d), altered expression of, for example, the *TAT3* gene could also be observed in the extreme *MYC2* overexpressing line that we generated.

Finally, we investigated whether the activation of JA-related transcription affected JA sensitivity by assessing inhibition of root growth, a typical JA response (Staswick *et al.*, 1992). In comparison to wild-type plants, root growth was significantly lower both for *MYC2* and *MYC2$^{D105N}$* overexpressing plants in mock conditions, although this effect was more pronounced in the *MYC2$^{D105N}$* plants (Fig. S6). The same trend was observed in plants grown in the presence of MeJA; however, no JA-hypersensitivity in *MYC2* and *MYC2$^{D105N}$* overexpressing plants could be distinguished (Fig. S6).

## Hyperactive MYC transcription factors cause an ABA-related phenotype in *Arabidopsis*

MYC2 is also a known positive regulator of abscisic acid (ABA) signalling and has been shown to be correlated with ABA-mediated inhibition of germination. Overexpression of *MYC2* results in hypersensitivity to ABA (Abe *et al.*, 2003; Lorenzo *et al.*, 2004) and *myc2* knock-outs show a decreased sensitivity to ABA (Abe *et al.*, 2003; Yadav *et al.*, 2005; Gangappa *et al.*, 2010). Accordingly, it was interesting to observe inhibition of germination without ABA treatment in *MYC2$^{D105N}$* overexpressing plant lines but not in plant lines overexpressing *MYC2* at a similar level (*c.* five-fold; OE *MYC2*-B; Figs 4c,d, S7). However, in a line overexpressing *MYC2* by *c.* 40-fold (OE *MYC2*-A), a similar phenotype as for the *MYC2$^{D105N}$* overexpressing plants was observed (Figs 4c,d, S7). Using *TAT3* as a marker of MYC2 activity (Fig. 4d) (Chini *et al.*, 2007; Dombrecht *et al.*, 2007; Niu *et al.*, 2011), we can conclude that either through higher expression or through loss of JAZ binding and repression, MYC2 activity is correlated with a decreased germination efficiency and smaller germinated seedlings.

Lastly, we investigated the sensitivity to ABA by comparing germination efficiency between plants grown in the absence or presence of ABA. We used a small dose of ABA (0.1 μM) that did not inhibit the germination of wild-type plants (Fig. 4e). Nonetheless, this low dose of ABA led to decreased germination efficiency in plants overexpressing *MYC2* or *MYC2$^{D105N}$*, with plants overexpressing *MYC2$^{D105N}$* showing a higher sensitivity than those overexpressing *MYC2* at a similar level (OE *MYC2*-B). Comparable ABA-hypersensitivity was only achieved in the plants

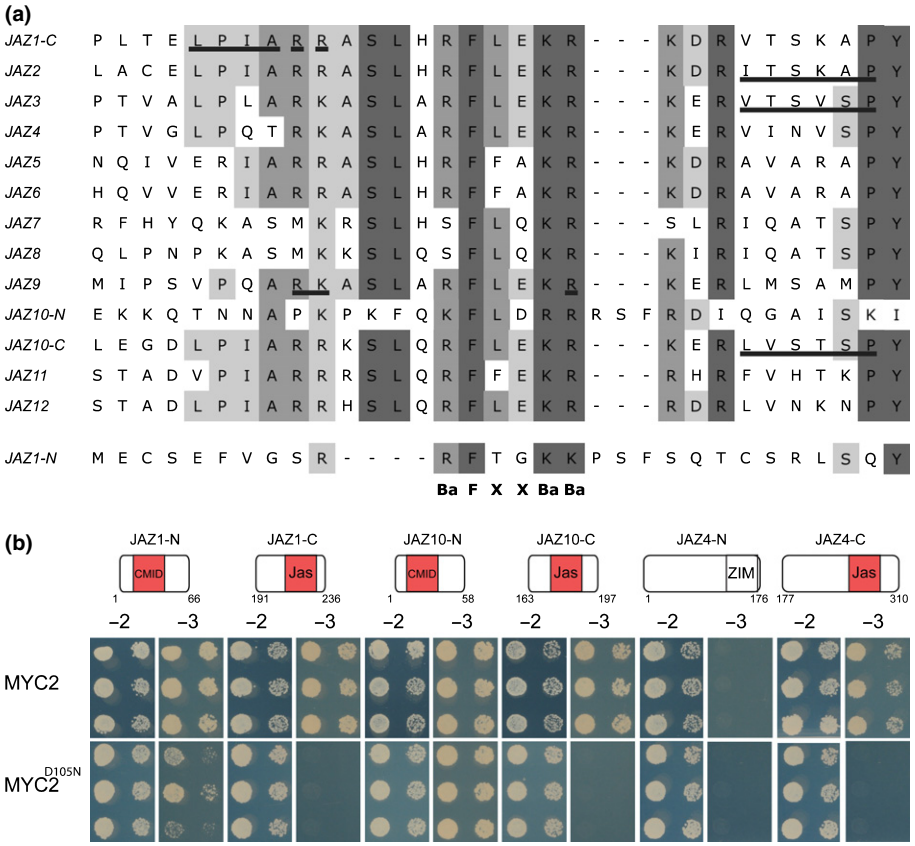


**Fig. 3** An N-terminal Jas-like domain enables JAZ1 and JAZ10 to interact with MYC2$^{D105N}$. (a) Sequence alignment of the cryptic MYC2-interacting domain (CMID) and Jas domain of the JAZ proteins. The putative N-terminal CMID of JAZ1 and JAZ10 are aligned to the Jas domains of all JAZ proteins. Shading of the residues represents conservation among the different proteins. The sequences were aligned by MUSCLE and edited with UGene. Underlined residues are proven to be essential for interaction with COI1. The putative core sequence of the Jas motif is represented in bold underneath the alignment. Ba, basic amino acid residue; X, any amino acid residue; F, phenylalanine. (b) MYC2 and MYC2$^{D105N}$ fused to GAL4-AD were tested for interaction with N-terminal and C-terminal constructs of JAZ1 and JAZ10 fused to GAL4-BD. Yeasts transformed with both plasmids were selected on SD-Leu-Trp (-2) or SD-Leu-Trp-His (-3) medium. N-terminal and C-terminal parts of JAZ4 were included as negative and positive controls, respectively.

overexpressing *MYC2* by *c.* 40-fold (OE *MYC2*-B). Together, these results indicate that the presence of the Asp-to-Asn mutation in MYC2 leads to an increased inhibition of germination associated with ABA-hypersensitivity.

## Discussion

Interaction between JAZ repressors and the MYC TFs is key in the JA signalling cascade. The Arabidopsis JAZ family consists of 12 members and the expansion of this protein family might point to functional specialization of individual JAZ members. In 2002, Smolen *et al.* described an allele of *MYC3* – that is, *ATR2D* – which caused a dominant stress-responsive phenotype. It has been noted before that the mutation in this allele caused an amino acid change (D94N) in the JID, possibly leading to loss of JAZ interaction (Fernández-Calvo *et al.*, 2011; Frerigmann *et al.*, 2014). Here, we show that this single amino acid change was sufficient to cause loss of interaction with most of the 12 JAZ proteins. Interaction with ATR2D was detected only with JAZ1 and JAZ10, although the strength of this interaction was also diminished compared to the interaction with MYC3. Similar results were obtained for the artificial allele *MYC2*$^{D105N}$, containing the same conserved residue change (D105N), which confirms the importance of the Asp residue for JAZ interaction and the transferability of the *atr2D* mutation to paralogues and presumably orthologues that have a conserved JID.

Previously, interaction analysis with truncated JAZ proteins has established the presence of an extra MYC interaction domain in the N-terminus of JAZ10, described as CMID by Moreno *et al.* (2013). Here, we reveal the presence of an additional MYC interaction domain in JAZ1 as well. Importantly, these CMIDs were essential for the remaining interaction of JAZ1 and JAZ10 with ATR2D and MYC2$^{D105N}$. We can infer that JAZ1 and JAZ10 proteins have a similar domain organization, which is different from those found in other JAZ proteins. A rationale for the functional relevance of this extra MYC interaction domain in JAZ10 has been forwarded by Moreno *et al.* (2013) and Chung & Howe (2009). For *JAZ10*, an alternative splice variant, *jaz10.4*, has been described, which lacks the Jas domain, making it resistant to COI1 interaction and JA-Ile-mediated degradation. However, the CMID still allows interaction with MYC2 and repression of JA responses, possibly attenuating JA-signalling. Our results might point to a similar specialization of JAZ1. Correspondingly, overexpression of *JAZ1ΔJas* but not of *JAZ9ΔJas* led to

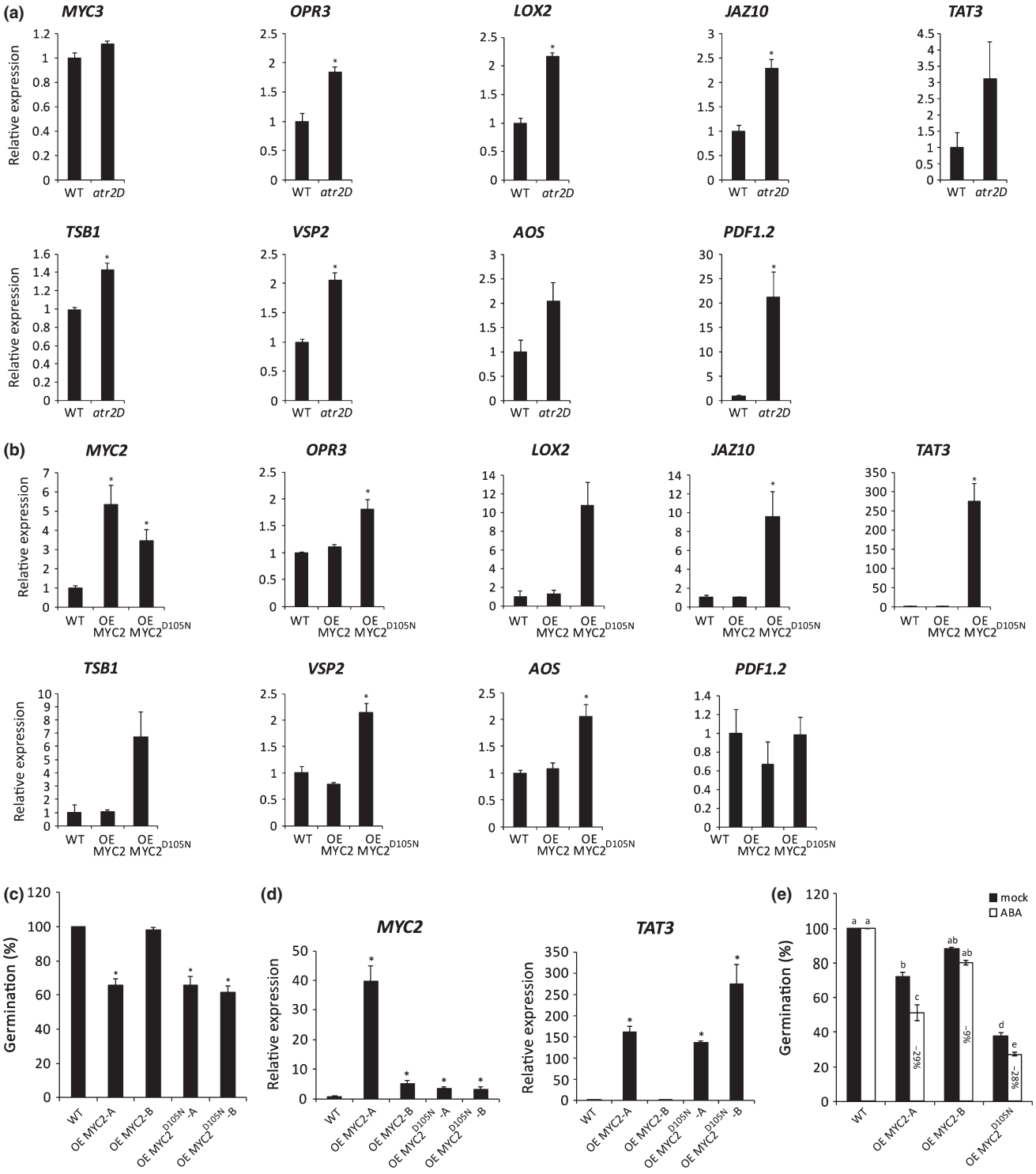


**Fig. 4** The *atr2D* mutant and *MYC2*$^{D105N}$ overexpressing plants show activation of the jasmonate (JA) and abscisic acid (ABA) response. (a, b) qRT-PCR analysis of Arabidopsis seedlings from (a) *atr2D* plants and (b) plants overexpressing (OE) *MYC2* or *MYC2*$^{D105N}$. Values represent the expression ratios plotted relative to those in control wild-type (WT) plants and are the mean (± SE) of three biological repeats. Significant differences with respect to the WT (Student's *t*-test): *, $P < 0.05$. (c) Germination rate of 7-d-old Arabidopsis seedlings from WT plants and two different *MYC2* or *MYC2*$^{D105N}$ OE lines (A and B). Values represent the mean (± SE) of three biological repeats. Significant differences with respect to the WT (Student's *t*-test): *, $P < 0.05$. The experiment was performed twice, obtaining similar results. (d) *MYC2* and *TAT3* transcript levels in the germinating seedlings from (c). Values represent the expression ratios plotted relative to those in control WT plants and are the mean (± SE) of three biological repeats. Significant differences with respect to the WT (Student's *t*-test): *, $P < 0.05$. (e) Germination rate of WT plants, two different *MYC2* OE lines (A and B) and one *MYC2*$^{D105N}$ OE line grown for 7 d in ethanol (mock) or 0.1 μM ABA. The experiment was performed twice, obtaining similar results. Values represent the mean (± SE) of three biological repeats. Letters represent significant differences according to a Tukey's Honestly Significant Difference (HSD) test (*, $P < 0.05$). Percentages in white bars depict the difference in germination rate between the mock and ABA condition of each line.

JA-insensitivity (Withers *et al.*, 2012) and phenotypic changes were observed only for loss-of-function lines for *JAZ1* and *JAZ10*, but not for other *JAZ* genes (Grunewald *et al.*, 2009; Demianski *et al.*, 2012; Moreno *et al.*, 2013). However, *JAZ1* lacks the Jas intron responsible for alternative splicing present in most *JAZ* genes (Chung *et al.*, 2010), hence the biological relevance of the JAZ1 CMID needs further investigation.

Our results demonstrate that different JAZ domains (CMID and Jas), and hence different JAZ proteins, have different ways and capacities of binding the MYC proteins. This is consistent with the fact that JAZ proteins show several subtle but important differences in their modular domain sequence and architecture. For instance, JAZ8 lacks a COI1-interacting degron (Shyu *et al.*, 2012) and contains an EAR domain that is responsible for direct interaction with TPL without the need for the adaptor protein NINJA. The latter property is also observed for JAZ5 and JAZ6 (Causier *et al.*, 2012; Shyu *et al.*, 2012). Combining this with tissue-, cell- and organelle-specific expression of the different *JAZ* genes, this provides the plant with a robust and pleiotropic control machinery that allows appropriate fine-tuning of the JA response across the plant body.

MYC2$^{D105N}$ was still capable of promoter transactivation in the presence of JAZ repressors, in contrast to MYC2. The consequence for JA signalling was further analysed *in planta* by analysis of JA marker gene expression in the *atr2D* mutant, and in *MYC2* and *MYC2*$^{D105N}$ overexpressing Arabidopsis lines. All tested genes have previously been linked to MYC2 and/or MYC3 activity (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007; Hou *et al.*, 2010; Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011; Sasaki-Sekimoto *et al.*, 2013; Schweizer *et al.*, 2013). Induction of these genes in *atr2D* mutant plants with respect to wild-type plants, establishes the direct correlation of the Asp-to-Asn amino acid change with JA signalling. Correspondingly, MYC2$^{D105N}$, but not MYC2 at comparable expression levels, was able to trigger expression of a common set of target genes. In agreement with previous reports (Niu *et al.*, 2011), some specificity was observed when comparing the *ATR2D* and *MYC2*$^{D105N}$ effects on the degree of induction of some genes, with a marked increased level of *TAT3* transcripts in the *MYC2*$^{D105N}$ overexpressing plants, and of *PDF1.2* in the *atr2D* mutant.

In conclusion, a change of the conserved Asp to Asn in the JID results in hyperactive TFs due to loss of interaction with JAZ repressors, which is translated into activation of (part of) the JA response and other MYC-controlled processes. Our results suggest that the Asp-to-Asn mutation is transferable to paralogues and orthologues of the MYC TFs in other plant species. Thereby, this mutation might ultimately become a useful concept for the design of hyperactive TFs for plant engineering.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** The D94N mutation does not affect MYC3 protein stability in transformed yeasts.

**Fig. S2** Comparison of the JID sequence of different Arabidopsis bHLH proteins.

**Fig. S3** $MYC2^{D105N}$ loses interaction with most of the JAZ proteins.

**Fig. S4** The D105N mutation does not affect MYC2 protein stability in transfected tobacco protoplasts.

**Fig. S5** Y2H analysis with JAZ1 fragments.

**Fig. S6** *MYC2* or $MYC2^{D105N}$ overexpressing plants are not hypersensitive to exogenous JA.

**Fig. S7** $MYC2^{D105N}$ overexpression decreases germination efficiency.

**Table S1** Primers used for qRT-PCR analysis

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