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Date:
2021-11-01

Citation:
Cao, L., Tian, J., Liu, Y., Chen, X., Li, S., Persson, S., Lu, D., Chen, M., Luo, Z., Zhang, D. & Yuan, Z. (2021). Ectopic expression of OsJAZ6, which interacts with OsJAZ1, alters JA signaling and spikelet development in rice. Plant Journal, 108 (4), pp.1083-1096. https://doi.org/10.1111/tpj.15496.

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Article type : Original Article

Ectopic Expression of OsJAZ6, which Interacts with OsJAZ1, Alters JA Signaling and Spikelet Development in Rice

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RUNNING TITLE: OsJAZ6 regulates spikelet development

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Summary

Jasmonates (JA) are key phytohormones that regulate plant responses and development. JASMONATE-ZIM DOMAIN (JAZ) proteins safeguard JA signaling by repressing JA-responsive gene expression in the absence of JA. However, the interaction and cooperative roles of JAZ repressors remain unclear during plant development. Here, we found that OsJAZ6 interacts with OsJAZ1 depending on a single amino acid in the so-called ZIM domain of OsJAZ6 in rice JA signaling transduction and JA-regulated rice spikelet development. *In vivo* protein distribution analysis revealed that the OsJAZ6 content is efficiently regulated during spikelet development, and biochemical and genetic evidence showed that OsJAZ6 is more sensitive to JA-mediated degradation than OsJAZ1. Through over- and mis-expression experiments, we further showed that the protein stability and levels of OsJAZ6 orchestrate the output of JA signaling during rice spikelet development. A possible mechanism, which outlines how OsJAZ repressors interact and function synergistically in specifying JA signaling output through degradation titration, is also discussed.

Key words: Jasmonate, OsJAZ repressor complex, degradation rate, stability, spikelet, rice (*Oryza sativa* L.).

INTRODUCTION

Jasmonic acid (JA), its metabolic precursors and derivatives, are collectively referred to as jasmonates (JAs) (Wasternack and Feussner 2018). JAs orchestrate plant responses to biotic and abiotic stress (Campos *et al.* 2014, Machado *et al.* 2016), and regulate diverse growth and developmental processes, such as Arabidopsis flowering and fertility (Figueroa and Browse 2015, Guo *et al.* 2018, Qi *et al.* 2015, Song *et al.* 2011, Zhu *et al.*

2011), maize sex determination (Dellaporta and Calderon-Urrea 1994), sorghum pedicellate spikelet fertility (Jiao *et al.* 2018), tomato seed maturation (Li *et al.* 2004) and rice spikelet development (Cai *et al.* 2014, You *et al.* 2019a).

Forward genetic studies revealed a canonical JA signaling pathway in Arabidopsis, which appears conserved in other plants (Chini *et al.* 2016, Howe *et al.* 2018, Yuan and Zhang 2015). In response to external stress factors or internal developmental cues, plants rapidly generate JAs, especially (+)-7-*iso*-JA-Ile (JA-Ile), which is the bioactive form of JAs (Fonseca *et al.* 2009). In the nucleus, JA-Ile induces the formation of co-receptor complexes consisting of the F-box protein CORONATINE INSENSITIVE1 (COI1) and JASMONATE-ZIM domain (JAZ) proteins (Katsir *et al.* 2008, Sheard *et al.* 2010). COI1 is the subunit of a Skp-Cullin1-F-box $(SCF)^{COI1}$ E3 ubiquitin ligase complex (Xie *et al.* 1998), and the JA-Ile-COI1-JAZ interaction leads to polyubiquitination of JAZ proteins and subsequent proteasome-dependent degradation (Chini *et al.* 2007, Thines *et al.* 2007, Yan *et al.* 2007). JAZ proteins typically hold a TIF[F/Y] XG motif [also named as zinc-finger protein expressed in the inflorescence meristem (ZIM) motif] and a Jas (JA-associated motif) motif (Chini *et al.* 2009). The ZIM motif promotes the formation of JAZ dimers and mediates interactions between JAZs and NOVEL INTERACTOR OF JAZ (NINJA), which is an adaptor protein that recruits the transcriptional corepressors TOPLESS (TPL) and TPL-related proteins (TPRs) (Pauwels *et al.* 2010). The Jas motif is required for interactions among JAZs, COI1 and transcription factors (TFs), including MYB and MYC that confer specificity of JA signaling responses (Boter *et al.* 2015, Qi *et al.* 2015, Zhang *et al.* 2015).

When JA-Ile levels are low, JAZ proteins function as negative regulators and inhibit the expression of JA responsive genes by recruiting corepressors, including NINJA and TPL (Pauwels *et al.* 2010), chromatin modifier HISTON DEACETYLASE6 (HDA6) (Zhu *et al.* 2011), and impede the accession of the co-activators, such as Mediator subunit MED25 and LEUNIG HOMOLOG (LUH), to their genomic locations (An *et al.* 2017, Chen *et al.* 2012, You *et al.* 2019b, Zhang *et al.* 2015). In addition, some JAZ proteins, such as JAZ8, contain ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif that represses transcriptional activity. These may act

independently of JAZ-COI1 mediated protein degradation (Shyu *et al.* 2012). The noncanonical degron and sequence diversity of the Jas domain also affect the stability of JAZ proteins, which in turn adjust the sensitivity of JA signal transduction (Shyu *et al.* 2012, Thireault *et al.* 2015, Tian *et al.* 2019). Therefore, JAZ interactions occur dynamically to control off and on status of JA signaling. A better understanding of how JAZ proteins are degraded is crucial in unraveling how JAs modulate responses in diverse physiological processes.

Genetic studies revealed that most Arabidopsis JAZs work redundantly since *jaz* mutants, such as a null allele mutant *jaz1-1*, did not show obvious JA-hypersensitive phenotypes (Demianski *et al.* 2012). For example, a *jaz* quintuple (*jaz1*/*3*/*4*/*5*/*10*, also named as *jazQ*) mutant showed relatively mild JA-related phenotypes as compared to wild type (Campos *et al.* 2016, Major *et al.* 2017). Nevertheless, constitutive JA effects were observed in a *jaz* decuple (*jaz1-jaz7*/*9*/*10*/*13*, also named as *jazD*) mutant, and the undecuple mutant (*jazU* with mutation in *JAZ1–JAZ10* and *JAZ13*) showed more severe growth defects and increased sensitivity to JA (Guo *et al.* 2018), confirming the additive effects of JAZs in JA responses. A recent report also shows that Arabidopsis JAZ proteins (including *JAZ1-7*, *9-12*) play specific and redundant role in JA/COI1-regulated development and defense response (Liu *et al.* 2021). While most higher order *jaz* mutants show enhanced sensitivity to JA, there are notable exceptions, such as *jaz10* loss-of-function mutant that exhibited reduced sensitivity to JA due to a JA-sensitive alternative splice variant of JAZ10 (Chung *et al.* 2010, Chung and Howe 2009, Wu *et al.* 2020a). Here, the JAZ10.3 lacked a portion of the Jas motif and the JAZ10.4 lacked the entire Jas motif, which caused reduced ability of the JAZ10 to engage with COI1; however, these splice variants may still interact with MYC2 resulting in constitutively repressed JA responses (Chung and Howe 2009, Demianski *et al.* 2012, Thireault *et al.* 2015, Yan *et al.* 2007). Moreover, overexpression of *JAZ8* resulted in JA-insensitive phenotype because JAZ8 lacks a canonical Jas motif and therefore is resistant against JA-mediated degradation (Chini *et al.* 2007, Chung *et al.* 2010, Chung and Howe 2009, Shyu *et al.* 2012, Thines *et al.* 2007, Yan *et al.* 2007). Hence, JAZ8, together with JAZ10.3 and JAZ10.4, are proposed to be parts of a JA-triggered negative feedback loop to safeguard

against uncontrolled JA activation. This may constitute a route to desensitize plants to of JA signaling.

The JAZ family has expanded widely in angiosperms (Bai *et al.* 2011, Howe and Yoshida 2019), and the specificity of JAZs appears linked with their spatiotemporal expression patterns and/or protein accumulation during growth and development. As an example, *JAZ2* is expressed in stomatal guard cells and regulates stomata dynamics during bacterial invasion in Arabidopsis (Gimenez-Ibanez *et al.* 2017). In tobacco, *NaJAZi* is specifically expressed during early flower development to tailor defense response and promote reproductive development, thus balancing potential pathogen interactions and fitness (Li *et al.* 2017). While we have extensive evidence that JAZ proteins control JA signaling (Howe *et al.* 2018), the roles of the JAZ proteins, and consequently the corresponding repressor complexes, during developmental processes are less clear.

We have previously shown that *OsJAZ1* regulates rice spikelet development through OsMYC2 interactions, which in turn activates the expression of *OsMADS1*, one of the E-function TFs that specifies rice floral organ and meristem identity (Cai *et al.* 2014, Hu *et al.* 2015), and that the Jas domain degron in OsJAZ1 modulates JA signaling sensitivity (Tian *et al.* 2019). Here, we show that OsJAZ6 interacts with OsJAZ1 through the conserved ZIM domain. Furthermore, through multiple lines of evidence we outline how OsJAZ6 orchestrates JA-mediated rice spikelet development. We therefore propose that the protein levels of different OsJAZ repressors affect repressor complex stability and fine tune JA signaling output, exemplified in the degradation of OsJAZ6 to ensure successful rice reproductive development.

RESULTS

OsJAZ6 interacts with OsJAZ1

To identify OsJAZ1 interacting proteins that regulate rice spikelet development, we performed a yeast two-hybrid (Y2H) screen of a rice inflorescence cDNA library using OsJAZ1 as bait. All putative OsJAZ1 interacting protein colonies were sequenced, and included the rice gene *LOC_Os03g28940*, which encodes the TIFY family protein OsJAZ6. We first confirmed the interaction by pairwise Y2H assays. We, furthermore, conducted Y2H between truncated OsJAZ1 and OsJAZ6 and found that the ZIM domains of the respective proteins were sufficient and necessary for the interaction to occur (Figure 1a and b).

To further confirm the interaction, we employed a yellow fluorescence protein (YFP) bimolecular fluorescence complementation (BiFC) system to test interactions *in planta*. Rice contains three JA receptor orthologs, OsCOI1a, OsCOI1b and OsCOI2, and our previous study found that OsJAZ1 only interacts with OsCOI1b for JA signaling transduction (Cai et al., 2014). Here, OsJAZ6-cYFP and nYFP-OsJAZ1 were co-expressed in tobacco leaf epidermal cells, and OsCOI1a-cYFP and nYFP-OsJAZ1 were used as negative control. Co-expression of OsJAZ6-cYFP/nYFP-OsJAZ1, but not OsCOI1a-cYFP/nYFP-OsJAZ1, cYFP/nYFP-OsJAZ1 or OsJAZ6-cYFP/nYFP, resulted in strong fluorescence signals in tobacco cells (Figure 1c), supporting interaction between OsJAZ1 and OsJAZ6 *in vivo*. Furthermore, the interaction was nuclear-localized when co-expressed with OsMYC2 (Figure 1c), indicating that the nuclear localization of OsJAZ1 and OsJAZ6 is dependent on OsMYC2, consistent with the previous report on AtJAZ1 and AtJAZ9 localization (Withers *et al.* 2012).

Over-expression of *OsJAZ6* inhibits JA signaling and affects spikelet development

To determine a possible role for OsJAZ6 in the regulation of rice spikelet development, we generated *OsJAZ6* mutant alleles using CRISPR-Cas9 system (Figure S1a), and *OsJAZ6* over-expression lines using the *UBQ* promoter upstream of *OsJAZ6* cDNA, with a C-terminal 6HA tag (named as *OsJAZ6-6HA*, Figure S2a). Under normal growth conditions, *Osjaz6* mutants exhibited normal vegetative and reproductive growth, and no obvious abnormal spikelet phenotypes were observed compared with the wild type (Figure 2a). Interestingly, *OsJAZ6-6HA* over-expressing plants exhibited abnormal

spikelet development with extra glume-like organs outside of lemma or palea (Figure 2a). This phenotype is reminiscent of that in the JA-deficient mutant *coleoptile photomorphogenesis 2* (*cpm2*) and *extra glume 1* (*eg1*) (Cai *et al.* 2014, Riemann *et al.* 2013), and the *OsJAZ1* dominant mutant *Osjaz1-1D/extra glume 2* (*eg2*), which has an amino acid substitution in the degron of the Jas domain that affects OsCOI1b-dependent degradation (Cai *et al.* 2014). These results indicated that over-expression of *OsJAZ6* might block JA signaling by mimicking the function of OsJAZ1-1D.

To corroborate that *OsJAZ6* is involved in typical JA responses, we conducted root growth inhibition assays where JA signaling is prevalent (Chini *et al.* 2007, Thines *et al.* 2007). Root growth was strongly inhibited when wild type plants were grown on 10 μM MeJA, while root growth of both *OsJAZ6-6HA* line and *Osjaz1-1D/eg2* mutant were less sensitive to JA inhibition (Figure 2b and c), confirming that over-expression of *OsJAZ6* impairs JA-mediated root growth inhibition. Besides the weak root inhibition, both *eg2* mutant and *OsJAZ6-6HA* transgenic plants exhibited larger shoots and leaves in seedlings (Figure 2c), indicating that defense-growth balance might be changed in these plants.

To further characterize the role of OsJAZ6 in JA signaling, we applied Y2H assay to investigate if OsJAZ6 interacts with the rice COI1-like proteins, OsCOI1a, OsCOI1b and OsCOI2. Consistent with that of OsJAZ1, OsJAZ6 only interacted with OsCOI1b in the Y2H assay, and the interaction was dependent on a biologically active analog of JA-Ile, COR (Figure 3a). The JAZ-COI1 interaction can induce JAZ protein degradation by the 26S proteasome (Chini *et al.* 2007, Thines *et al.* 2007). To determine whether degradation of OsJAZ6 protein is mediated by 26S proteasome, we generated transgenic rice plants expressing *OsJAZ1pro:OsJAZ1gDNA-GFP* (named as OsJAZ1-GFP) and *OsJAZ6pro:OsJAZ6gDNA-GFP* (named as OsJAZ6-GFP), and treated them with MeJA and MG132 (a 26S proteasome-specific inhibitor), respectively. Both OsJAZ1 and OsJAZ6 localized in the nucleus in the OsJAZ1-GFP and OsJAZ6-GFP lines (Figure 3b, left panel). After 1 hour (h) treatment with 100 μM MeJA, GFP signals were greatly reduced in both OsJAZ1-GFP and OsJAZ6-GFP transgenic plants (Figure 3b, middle panel). However, the reduction in GFP signal was strongly inhibited by the application of 100μM MG132 (Figure 3b, right panel). Together, these results demonstrated *OsJAZ6*

functions in JA signaling pathway and might regulate rice spikelet development similar to that of OsJAZ1.

OsJAZ6 and OsJAZ1 synergistically regulate rice spikelet development

Previous studies found that JA signals promote 26S proteasome-mediated OsJAZ1 degradation to release OsMYC2, which in turn activates the expression of E-class MADS box TF*OsMADS1* to regulate rice spikelet development (Cai *et al.* 2014, You *et al.* 2019a). To assess whether OsJAZ6 employs canonical JA signaling elements, similar to OsJAZ1, to regulate rice spikelet development, we investigated whether OsJAZ6 interacts with OsMYC2. Both Y2H and BiFC assays demonstrated that OsJAZ6 interacted with OsMYC2 in yeast and *in planta* (Figure 3c and d). Consistently, *OsMADS1* expression was repressed in the *OsJAZ6-6HA* lines compared to wild-type plants (Figure 3e). These results suggested that OsJAZ6 also regulated rice spikelet development through activating expression of OsJAZ1-regulated E-class gene *OsMADS1*.

Following the above results, we investigated the relationship between *OsJAZ1* and *OsJAZ6* in rice reproductive development. We firstly constructed *Osjaz1* single mutant and *Osjaz1 Osjaz6* double mutant that showed normal spikelet development (Figure 4a; Figure S1b and c), confirming that plants with loss-of-function of some JAZ repressor proteins grow normally (Guo *et al.* 2018). We next over-expressed *OsJAZ6-6HA* in the *Osjaz1-1D/eg2* mutant to enhance OsJAZ activity (Figure S2b). The *OsJAZ6-6HA Osjaz1-1D/eg2* transgenic lines generated spikelets with severely abnormal phenotypes. These phenotypes were enhanced compared to that of *Osjaz1-1D*/*eg2* plants and displayed growth of reiterative glume-like organs on the spikelet surface. In addition, most spikelets (above 79% of spikelets, n=120) in the over-expressing lines had no stamens and pistil in the inner whorls of spikelet (Figure 4a), similar to that of *eg1 eg2* double mutant and *OsJAZ1-1D osmads1* transgenic lines (Cai *et al.* 2014). These data indicated that OsJAZ1 and OsJAZ6 work synergistically in modulating JA signal transduction during rice spikelet development.

An isoleucine residue in the ZIM domain of OsJAZ6 is critical for OsJAZ1-OsJAZ6 interaction and its repressive function

In Arabidopsis, the formation of homo- and hetero-dimer of JAZ proteins enhanced the repressive activity of JAZ complexes (Chung *et al.* 2010, Chung and Howe 2009, Yan *et al.* 2007). Our results indicated that the ZIM domain of OsJAZ6 may contribute to its interaction with OsJAZ1. Alignment of the ZIM domains across the JAZ family members revealed that the amino acids contributing to the TIFY motif are well conserved across the JAZ members (Figure 4b). We hypothesized that some of these amino acids are critical for OsJAZ6 and OsJAZ1 interaction, as well as for the repressive function of OsJAZ6 based on previous results in Arabidopsis (Chung and Howe 2009). We therefore mutated the two most well conserved amino acids, i.e. an isoleucine (I) and a glycine (G; amino acids 84 and 88, respectively), in OsJAZ6 to alanine (A; Figure 4b). We next tested how these substitutions impacted the interaction between OsJAZ1 and OsJAZ6 in Y2H assays. We found that the I, but not the G, was critical for the OsJAZ1-OsJAZ6 interaction (Figure 4c). To determine whether the I residue also was of importance for OsJAZ6 interaction with other OsJAZs, we conducted Y2H experiments with the mutated OsJAZ6 against other OsJAZ proteins. The results showed that wild-type OsJAZ6 could form a homo-dimer, and hetero-dimers with OsJAZ1, OsJAZ7, OsJAZ8, OsJAZ9, OsJAZ10, OsJAZ11 and OsJAZ12. The I to A substitution abolished almost all of these interactions, with the exception of OsJAZ8, OsJAZ9 and OsJAZ11 (Figure 4d). These data indicated that OsJAZ proteins can form multiple versions of hetero-dimeric repressor complexes, and that the interactions typically depend on few conserved amino acids in the TIFY motif of the ZIM domain in rice, similar to that of Arabidopsis (Chung and Howe 2009). To further test the effect of the I to A change in OsJAZ6 repression function, we generated *OsJAZ6*^{I84A}*-6HA* overexpression transgenic plants. These plants did not display defective spikelet growth such as those seen in the *OsJAZ6-HA* plants (Figure 4a), despite that the expression levels of *OsJAZ6-HA* and *OsJAZ6*^{I84A}*-6HA* transgenic plants were comparable (Figure S2c), suggesting that the isoleucine residue in the ZIM domain of OsJAZ6 is critical for its repression function.

Based on the fact that OsJAZ1 could interact with OsMYC2 to repress its role in activating *OsMADS1* expression (Cai *et al.* 2014), we wondered whether the I to A substitution in OsJAZ6 could abolish its interaction with OsMYC2. The Y2H assay found that both the OsJAZ6 and OsJAZ6^{I84A} interacted with OsMYC2 (Figure 3c, 3d and 4d), confirming the previous result that the ZIM domain doesn't mediate OsJAZ-OsMYC2 interaction (Cai *et al.* 2014). Therefore, these results suggested that the ZIM domain of OsJAZ6 determines its interaction with OsJAZs, and its repressive function in rice spikelet development.

OsJAZ6 protein levels is spatio-temporally regulated and more sensitive to JA-mediated degradation

In *Arabidopsis*, overexpression of full-length *JAZ1*, *JAZ2*, *JAZ3*, or *JAZ10* did not lead to visible developmental phenotypes different from wild type (Chini *et al.* 2007, Chung *et al.* 2010, Chung and Howe 2009, Thines *et al.* 2007, Yan *et al.* 2007). We therefore asked why over-expression of the *OsJAZ6* led to clear phenotypic variations in rice. It is possible that boosting the *OsJAZ6* expression levels and changing its expression patterns during rice early inflorescence meristem stages might increase overall JAZ activity. To assess this, we first analyzed the expression patterns of all the 15 *OsJAZ* genes at different inflorescence and spikelet developmental stages by applying RT-qPCR experiment. We found that the expression patterns of *OsJAZ1*, *OsJAZ2*, *OsJAZ6*, and *OsJAZ8* were similar to each other with high expression in young inflorescence that decreased during inflorescence development (Figure S3a). Although the *OsJAZ6* expression was lower than that of *OsJAZ1* in mature spikelet organs, the expression patterns followed similar trends (Figure S3a). By contrast to OsJAZ6, OsJAZ8 and OsJAZ2 contain EAR domains (Tian *et al.* 2019), presumably acting together with TPL and thus function differently than OsJAZ6. To see if overall JAZ levels changed flower development, we therefore selected to generate transgenic plants overexpressing *OsJAZ1-6HA*. These plants did not show any flower development defects (Figure S3b), indicating that simply increasing JAZ expression may not explain why we are seeing the phenotypes associated with *OsJAZ6* overexpression.

Another possibility for the abnormal spikelet phenotypes in *OsJAZ6-HA* transgenic lines could be that the OsJAZ6 might be a limiting factor in forming OsJAZ repressor complex during spikelet development. If so, only the accumulation of OsJAZ6, but not higher content of OsJAZ1, could stabilize OsJAZ1-OsJAZ6 repressor complex and block JA signaling transduction (Figure 5a). To investigate this, we first observed the native protein accumulation patterns of OsJAZ1 and OsJAZ6 during rice reproductive development using the OsJAZ1-GFP and OsJAZ6-GFP transgenic plants. Both OsJAZ1 and OsJAZ6 showed strong GFP signals in the inflorescence meristem, but the fluorescence of OsJAZ6-GFP dropped substantially in primordia of rudimentary glumes, sterile lemma, lemma and flower meristem, with GFP signals detected again during later developmental phases in the palea primordium (Figure 5b). OsJAZ1-GFP showed different fluorescent patterns from OsJAZ6-GFP, with signals decreasing but still detectable in sterile lemma, lemma and flower meristem (Figure 5b). These results indicate that the OsJAZ1 and OsJAZ6 protein accumulation is different during inflorescence development. It is possible that this reflects a JA gradient across the inflorescence that would efficiently degrade OsJAZ6 but retain OsJAZ1. However, *OsJAZ6* is expressed at lower levels than *OsJAZ1* during inflorescence development (Figure S3a) and this could also drive the differences in protein accumulation. To exclude the possibility that transcriptional regulation of *OsJAZ6* impacted the reduction in OsJAZ6 protein, we created *OsJAZ6gDNA-GFP* transgenic lines controlled by the *OsJAZ1* promoter instead of the native *OsJAZ6* promoter. Consistent with our hypothesis, the *OsJAZ1pro:OsJAZ6gDNA-GFP* lines followed similar GFP patterns as those of the *OsJAZ6* promoter driven construct (Figure 5b), indicating that the amount of OsJAZ6 is stringently regulated during rice spikelet development, which therefore might explain why *OsJAZ6-HA* transgenic lines blocked JA signaling output.

The differences in OsJAZ6-GFP and OsJAZ1-GFP levels at the same development stages, indicate that the OsJAZ6 stability has a different sensitivity to JA as compared to OsJAZ1. Research in both Arabidopsis and rice show that the interaction between JAZ and COI1 depends on the COR concentrations (Melotto *et al.* 2008, Tian *et al.* 2019). We therefore explored the COR-dependent OsJAZ-OsCOI1b interaction assays to test the JA

sensitivity of OsJAZ6 protein. Compared to OsJAZ1, OsJAZ2 and OsJAZ8, OsJAZ6 can interact with OsCOI1b at low COR concentration (Figure 5c). To test the degradation rate of OsJAZ6 *in vivo*, we used confocal laser scanning microscopy to compare JA-mediated protein degradation rates and patterns in both OsJAZ6-GFP and OsJAZ1-GFP transgenic plants. In the absence of JA treatment, we observed clear nuclear localized GFP signals in both OsJAZ6-GFP and OsJAZ1-GFP roots (Figure 5d; Figure S4). However, when we treated the plants with 100 μM MeJA for 20 min, the fluorescent signals were largely eliminated in OsJAZ6-GFP roots, whereas the OsJAZ1-GFP signal persisted up to 40 min after treatment (Figure 5d; Figure S4). These results indicated that OsJAZ6 is more sensitive to JA-induced degradation than OsJAZ1.

DISCUSSION

Rice flower development is critical for grain yield and reproduction, and is orchestrated by environmental and genetic signals (Yuan *et al.* 2020). JA promotes spikelet development by activating the expression of E-class gene *OsMADS1*, a process depending on 26S proteasome mediated degradation of OsJAZ1 (Cai *et al.* 2014, Tian *et al.* 2019, You *et al.* 2019a). However, how OsJAZ1 integrates JA signaling to adapt reproductive development remains elusive. In this study, we revealed that OsJAZ6 interacted with OsJAZ1, and the stability of OsJAZ6 protein levels orchestrated JA signaling output during rice spikelet development.

The fluorescence signals observed from the OsJAZ1-GFP and OsJAZ6-GFP showed that OsJAZ1 and OsJAZ6 distributed in a similar but distinct spatial-temporal pattern during rice spikelet development (Figure 5b). The OsJAZ1 protein was present at low levels; however, OsJAZ6 was completely degraded during spikelet initiation (Figure 5b-d). These differences could explain why over-expression of the *OsJAZ6*, instead *OsJAZ1*, blocked JA signaling transduction and spikelet development (Figure 5a). Given that the OsJAZ6 needs to be degraded via JA, it is plausible that the over-expression of the OsJAZ6 simply overwhelm the tissue and that there is not enough internal regulators, such as JA, to execute the degradation properly, while in the *OsJAZ1-HA* lines, spikelet

develops normally due to lack of OsJAZ6 (Figure 5a). Analogously, the ASYMMETRIC LEAVES1 (AS1) and AS2 complex regulates Arabidopsis leaf polarity during development; however, over-expression of *AS2*, but not that of *AS1*, changed leaf polarity because the increased amounts of AS2 in leaf primordia altered the AS1-AS2 complex formation (Xu *et al.* 2003). With the OsJAZ6, we provide another model for how the levels of different components contribute to protein complex formation.

Based on our biochemical and genetic evidence that substitution of one key amino acid in the OsJAZ6 ZIM domain could abolish the repressive function of OsJAZ6 (Figure 4), and as OsJAZ6 was more sensitive to JA-mediated degradation than OsJAZ1 (Figure 5c and d), we propose that OsJAZ6 may synergistically interact with OsJAZ1 to form a hetero-dimer repressor complex that orchestrates JA signaling output depending on protein stability, even though we cannot exclude that other amino acid in the ZIM domain might also affect OsJAZ1-OsJAZ6 interaction. Here, OsJAZ6 could be degraded, albeit by an unknown mechanism, that determines the stability of the OsJAZ1-OsJAZ6 repressor complex to fine tune JA signaling transduction and activate spikelet development. If so, these results extend our understanding on how an important protein complex titrates signaling input and output responses.

Sub-functionalization or neo-functionalization of proteins, during duplication events in the genome, increase the adaptation of organisms during evolution (Levasseur and Pontarotti 2011). This is exemplified by MADS box family members in floral morphogenesis, WUSCHEL homeobox-containing (WOX) TFs in stem cell homeostasis, AUXIN RESPONSIVE FACTOR (ARF) TFs and the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) family in buffering auxin signaling (Rensing 2014). Similar sub- or neofunctionalization has also been noted for the JAZ gene family where spatio-temporal expression differences drive complementary JA signaling pathways in Arabidopsis (Chini *et al.* 2016, Howe *et al.* 2018, Jin and Zhu 2017). Here, we illustrate neo-functionalization of *OsJAZ* genes in rice JA signaling resilience; however, in contrast to Arabidopsis, the mechanism behind the function is due rather to protein stability than transcriptional regulation. We found that OsJAZ1 and OsJAZ6 interact, and that they have similar expression patterns during spikelet development. These results provide a

374 basis for OsJAZ1 and OsJAZ6 to function synergistically in JA signaling modulation.
375 However, OsJAZ6 is degraded more rapidly upon JA exposure than OsJAZ1. Why these
376 differences occur remain an open question for further investigation. We further reason
377 that the OsJAZ1-OsJAZ6 heterocomplexes may execute a certain repressor function and
378 that once the OsJAZ6 is degraded, the OsJAZ1 may have different effects on plant
379 development. Indeed, formation of different protein complexes could increase the
380 complexity of JA signaling transduction and be related to both expression overlaps and
381 protein stability upon JA exposure (Kazan and Manners 2012, Kombrink 2012, Pauwels
382 and Goossens 2011, Wager and Browse 2012). There are several examples for similar
383 scenarios in other signal transduction pathways. For example, gibberellin and nitrogen
384 co-regulate rice tillering by modulating heteromeric protein complex formation, i.e.
385 GIBBERELLIN INSENSITIVE DWARF1 (GID1)-GID2-DELLA to GID1-GID2-
386 NITROGEN-MEDIATED TILLER GROWTH RESPONSE 5 (NGR5). In "green
387 revolution" lines with *SEMI-DWARF1* (*SD1*) mutation, decreased gibberellin causes
388 DELLA protein accumulation, which lead to more GID1-GID2-DELLA complexes and
389 reduced GID1-GID2-NGR5 complexes, which in turn promote branching (Wu *et al.*
390 2020b). However, how the OsJAZ1-OsJAZ6 complex respond to different environmental
391 signals is also an open question.

392 Phytohormones are central to plant physiology and to applications in agriculture.
393 Several of the well-known phytohormone signaling pathways, i.e. auxin, JA and
394 gibberellin, are mediated via SCF-26S proteasome-mediated degradation of negative
395 regulators, such as Aux/IAA, JAZ and DELLA proteins, respectively (Shan *et al.* 2012).
396 These repressors are pivotal in orchestrating complexity, specificity and sensitivity of
397 signaling input and output of their corresponding pathways (Howe *et al.* 2018, Phokas
398 and Coates 2021, Salehin *et al.* 2015). The degradation of Aux/IAA and DELLA proteins
399 depends on both sequence variants and posttranslational modifications (Calderón
400 Villalobos *et al.* 2012, Fu *et al.* 2002, Itoh *et al.* 2005, Sato and Yamamoto 2008, Winkler
401 *et al.* 2017). We previously showed that sequence variance in the degron sequence of
402 OsJAZ1 modulated JA signaling (Tian *et al.* 2019). This study further validates this
403 concept, i.e. that sequence variance affects protein stability of JAZ proteins. Indeed,

lower COR concentration induced OsJAZ6-OsCOI1b interactions than those between OsJAZ1-COR1b, and the OsJAZ6-GFP under the *OsJAZ1* promoter showed similar protein distribution as when driven by the *OsJAZ6* promoter (Figure 5). One possible reason for this is that there could be subtle changes in the primary amino acid sequence of OsJAZ6 that might allow for a high-affinity binding of OsCOI1b, which would then be reflected in degradation at different JA levels. Although the JA distribution patterns during inflorescence development is unclear, mutation of the JA biosynthetic genes, *EG1* and *OsOPR7*, blocked spikelet initiation (Cai *et al.* 2014, You *et al.* 2019a), indicating that JA content increases when the spikelet initiate and should influence OsJAZ6 stability.

It is also possible that posttranslational modification of OsJAZ6, such as phosphorylation, could impact the stability of the protein. This would then mean that the post-translational modification would influence how tight OsJAZ6 bind to OsCOI1b as this is the driving factor behind its degradation. A recent work on OsJAZ4 found that Glycogen synthase kinase 3 (GSK3)-like kinase OsGSK2 destabilizes OsJAZ4 in an OsCOI1b-dependent manner through phosphorylation during antiviral defense (He *et al.* 2020). OsJAZ6 might perhaps be regulated in a similar manner. Another possibility is analogous to that of JAZ3 in Arabidopsis, where SPL9 could stabilize JAZ3 by preventing COI1-mediated protein degradation (Mao *et al.* 2017). Hence, the stability of either OsJAZ6 or OsJAZ1, or both, might be affected by the interaction with other proteins. Our qPCR analysis showed that, except for *OsJAZ1* and *OsJAZ6*, *OsJAZ3*, *OsJAZ4*, *OsJAZ7* and *OsJAZ8* are highly expressed in rice flower primordium, and that OsJAZ6 can form dimers with OsJAZ1, 7, 8 respectively (Figure 4d). Whether these OsJAZ proteins form higher order protein complexes that impact protein stability and the structural mechanism for their JA sensitivity in rice spikelet initiation remains to be determined.

MATERIALS AND METHODS

Plant materials and growth conditions

Oryza sativa (ssp. *japonica* cv. 9522) was used for generating transgenic plants, and the *Osjaz1-1D/eg2* mutant is kept in the lab. Unless otherwise indicated, plants were grown in the paddy field of Shanghai Jiao Tong University, located in Shanghai (31.03ºN, 121.45ºE), China. *Nicotiana benthamiana* plants were grown in a growth chamber under natural light with 16 h light (23°C)/8 h dark (23°C). Plants were grown for 1 month, the third and fourth of youngest leaves were infiltrated with *Agrobacterium* for protein interaction analysis. For the root-sensitivity assay, seeds were germinated and then grown on 1/2 Murashige and Skoog (MS) medium containing 10 μM MeJA or not containing 10 μM MeJA for 7 days. The length of roots were calculated from ten independent plants. For morphological analysis, fresh spikelets at stage In 9 were photographed with a Leica stereomicroscope (S8 APO).

Generation of transgenic lines

To generate transgenic *UBQ_{pro}:OsJAZ6-6HA, UBQ_{pro}:OsJAZ6^{TIFYI84A}-6HA* and *UBQ_{pro}:OsJAZ1-6HA* lines, the coding regions of *OsJAZ6, OsJAZ6^{TIFYI84A}* and *OsJAZ1* was cloned into pTCK303 vector to fuse with 6HA. To generate the *$OsJAZ1_{pro}$:OsJAZ1gDNA-GFP* transgenic lines, a 6.502-kb genomic sequence containing the 3.284-kb promoter and 3.218-kb genomic sequence of *OsJAZ1* from the wild-type genome was cloned into pCAMBIA1301 vector to fuse with GFP. To generate the *$OsJAZ6_{pro}$:OsJAZ6gDNA-GFP* transgenic lines, a 4.967-kb genomic sequence containing the 3.148-kb promoter and 1.819-kb genomic sequence of *OsJAZ6* from the wild-type genome was cloned into pCAMBIA1301 vector to fuse with GFP. To generate the *$OsJAZ1_{pro}$:OsJAZ6gDNA-GFP* transgenic lines, a 5.103-kb genomic sequence containing the 3.284-kb promoter of *OsJAZ1* and 1.819-kb genomic sequence of *OsJAZ6* from the wild-type genome was cloned into pCAMBIA1301 vector to fuse with GFP. For targeted editing of OsJAZ1 and OsJAZ6, the CRISPR targeting cassette consist of a tandem array of the rice *U3* promoter and specific sgRNA sequence (Xie *et al.* 2015). Target sequences to the *OsJAZ1 and OsJAZ6* genes were designed using the CRISPR-P v2.0 (http://cbi.hzau.edu.cn/CRISPR2/). All the CRISPR/Cas9 construction were transformed

into the *Agrobacterium* strain EHA105 and finally transformed into rice callus as reported previously (Tian *et al.* 2019). Primers are listed in Table S1.

Expression analysis

Total RNA was isolated from wild-type rice inflorescence at different stages using TRIzol™ Reagent (Invitrogen, Cat. no. 15596018), by following the manufacturer's instructions. One microgram of RNA per sample was used to synthesize oligo (dT)-primed first-strand cDNAs using the PrimeScript1 RT reagent kit (Takara). Real-time PCR analyses were performed on a CFX96 (Bio-Rad) instrument using SYBR Green Master mix (QIAGEN), according to the manufacturer's instructions. The rice *ACTIN* housekeeping gene was used as internal control. For RT-qPCR analyses of *OsJAZ1-15*, or *OsMADS1*, cDNA was denatured at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 10 s. Primers used for PCR were listed in Table S1.

Yeast two-hybrid screening and Yeast two-hybrid assay

For screening the OsJAZ1 interactive proteins, cDNA synthesized from the mRNAs of young panicle meristems (<5 mm) were cloned into the prey vector pGADT7, and full-length of OsJAZ1 cDNA was amplified and cloned into pGBKT7 vector, then library screening was performed by following the manual of Matchmaker™ Library Construction & Screening Kits (Cat. PT3955-1). For validation protein interaction, the coding sequences, specific domains or substitution versions of *OsJAZ1*, *OsJAZ3*, *OsJAZ6*, *OsJAZ7*, *OsJAZ8*, *OsJAZ9*, *OsJAZ10*, *OsJAZ11*, *OsJAZ12*, *OsCOI1a*, *OsCOI1b*, *OsCOI2* and *OsMYC2* were amplified and cloned into pGBKT7 or pGADT7 (Takara) and then transformed into the yeast stain AH109. The yeast two-hybrid assays were performed according to the manual of Matchmaker Gold Yeast Two-Hybrid System Kits (Cat. 630489). Briefly, the transformed yeasts were cultured on solid Minimal Synthetic Dropout media SD-TL at temperature 30°C for 3 d. The grown strains were incubated in liquid SD-TL media for 18h and collected by centrifugation, then re-suspended with deionized water to the absorbance value of 1.5 at a wavelength of 600 nm (OD_{600} = 1.5).

The serial decimal dilution was then used for spot assay on selection media SD-TLHA with or without X-α-Gal. Primers used for Y2H were listed in Table S1.

BiFC assays

The coding region of *OsJAZ1*, *OsJAZ6* and *OsMYC2* were amplified and cloned into *p*XY106-nYFP or *p*XY104-cYFP plasmids. The recombinant vectors were transformed into *Agrobacterium tumefaciens* strain GV3101, and cultured to reach OD600 = 0.6~0.8, which were collected by centrifugation and then re-suspended in MS liquid medium (containing pH 5.6, 10 mM MES and 200 μM acetosyringone) until the absorbance value was 0.58~0.61 at a wavelength of 600 nm (OD600= 0.58~0.61). Then incubated the above MS medium at room temperature for 2~4 h. The *p*XY106-nYFP and *p*XY104-cYFP strains were mixed at the ratio of 1:1 before infiltration. The mixture was infiltrated into the leaf of *N. benthamiana.* After 48 h dark incubation, fluorescent eYFP signals were monitored using a Leica SP5 confocal microscope (Leica TCS SP5 X, excitation 514 nm; emission 522–555 nm). Primers used for these assays were listed in Table S1.

Detection of protein degradation *in vivo*

Transgenic $OsJAZ1_{pro}$*:OsJAZ1gDNA-GFP* plants and $OsJAZ6_{pro}$*:OsJAZ6gDNA-GFP* plants were treated with MeJA (100 μM) (Sigma) for 1h, before treatment with or without the proteasome inhibitor MG132 (100 mM) (MERCK) for 1 h. Fluorescent signals of plant roots were monitored using a Leica SP5 confocal microscope.

Statistical Analysis

The root length was measured at 7 d after seed germination, and the "relative root inhibition" was calculated by using equation (0μM MeJA root growth-10μM MeJA root growth)/0μM MeJA root growth. All data in this study were obtained from three independent biological replicates. Data were plotted as mean ± SD, and error bars indicate SD. The data were analyzed with the two-tailed Student's t test using Microsoft Excel 2010.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *OsJAZ6* (Os03g0402800), *OsJAZ1* (Os04g0653000), *OsCOI1a* (Os01g0853400), *OsCOI1b* (Os05g0449500), *OsCOI2* (Os03g0265500), *OsMYC2* (Os10g0575000), *OsMADS1* (Os03g0215400), *OsJAZ2* (Os07g0153000), *OsJAZ8* (Os09g0439200), *OsJAZ3* (Os08g0428400), *OsJAZ4* (Os09g0401300), *OsJAZ5* (Os04g0395800), *OsJAZ7* (Os07g0615200), *OsJAZ9* (Os03g0180800), *OsJAZ10* (Os03g0181100), *OsJAZ11* (Os03g0180900), *OsJAZ12* (Os10g0392400), *OsJAZ13* (Os10g0391400), *OsJAZ14* (Os10g0391801), *OsJAZ15* (Os03g0396500)

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (32170322, 31671260); the Open Research Fund of State Key Laboratory of Hybrid Rice (Hunan Hybrid Rice Research Center), Agriculture Research System of Shanghai, China (Grant No. 202003) and China Innovative Research Team, Ministry of Education and the Programme of Introducing Talents of Discipline to Universities (111 Project, B14016), and SMC Morningstar Young Scholarship of Shanghai Jiao Tong University to Z. Y. S. P. acknowledges funding from a Villum Investigator grant (Project ID: 25915), a Novo Nordisk Laureate grant (NNF19OC0056076) and a DNRF Chair grant (DNRF155).

AUTHOR CONTRIBUTIONS

Z.Y. designed research, L.C.C., J.Q.T., Y.L.L., X.F.C., S.Q.L., D.L., M.J.C., and Z.J.L. performed research, L.C.C., J.Q.T., S.P., D.B.Z. and Z.Y. analyzed data, L.C.C., S.P. and Z.Y. wrote the paper.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

All relevant data are contained within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Figure S1. CRISPR/Cas9-generated *Osjaz6*, *Osjaz1* and *Osjaz6 Osjaz1* mutations.

Figure S2. Relative expression of *OsJAZ1, OsJAZ6* and OsJAZ6^{I84A} in over-expression lines.

Figure S3. Characterization of *OsJAZ* genes.

Figure S4. Different stability of OsJAZ1 and OsJAZ6 in response to JA.

Table S1. Primers used in this study.

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773
774 **Figure Legends:**

775 **Figure 1. OsJAZ6 interacts with OsJAZ1.**

776 (a) and (b) Yeast two hybrid assay (Y2H) to detect interaction between OsJAZ1 and
777 OsJAZ6. Transformed yeast cells were grown on SD/-Trp/-Leu/-His/-Ade media. AD,
778 activation domain; BD, DNA-binding domain.

779 (c) BiFC assay to detect interaction between OsJAZ6 and OsJAZ1 in *Nicotiana*
780 *benthamiana*. OsJAZ6 was fused with the C-terminal fragment of yellow fluorescence
781 protein (cYFP) to form OsJAZ6-cYFP. OsJAZ1 or OsCOI1a was fused with N-terminal
782 fragment of YFP (nYFP) to form OsJAZ1-nYFP or OsCOI1a-cYFP. The leaves of *N.*

benthamiana were infiltrated with *Agrobacterium* strains containing the indicated construct pairs. The data were collected 48 h after infiltration. Scale bars=50 μM.

Figure 2. Overexpression of *OsJAZ6* results in decreased sensitivity to JA signaling.

(a) Spikelet phenotypes of *Osjaz6* mutant and *OsJAZ6-6HA* transgenic line. Arrowhead indicates the extra glume-like organ. Scale bars=2mm. egl, extra glume-like organ; le, lemma; lo, lodicule; lol, lodicule-like tissue; pa, palea; pi, pistil; st, stamen.

(b) Root growth inhibition assay of wild-type (open bars), *Osjaz1-1D*/*eg2* (grey bars), and *UBQ:OsJAZ6:6HA* transgenic plants (black bars) grown on 1/2 MS medium containing 10 μM MeJA. Root length measurements were made at 7 d after seed germination. Statistic data show the mean ± SD, error bars indicate SD (n = 10).

(c) Photograph of representative seedling treated with 10 μM MeJA for 7 d. Scale bar=1cm.

Figure 3. OsJAZ6 interacts with OsCOI1b and is degraded by 26S proteasome.

(a) Y2H assay to detect interaction between OsJAZ6 and OsCOI1a, OsCOIb and OsCOI2. Transformed yeast cells were grown on SD/-Trp/-Leu/-His/-Ade media supplemented with or without 30 μM COR.

(b) JA-dependent proteasome mediated degradation of OsJAZ1-GFP and OsJAZ6-GFP in roots. MG132, a proteasome inhibitor. The 7-day-old seedlings were treated with or without 100 μM MG132 before 100 μM MeJA treatment. Scale bars=50 μM.

(c) Y2H assay to detect interaction between OsJAZ6 and OsMYC2. Transformed yeast cells were grown on SD/-Trp/-Leu/-His/-Ade media supplemented with 25 mM 3-AT (3-amino-1,2,4-triazole).

(d) BiFC assay to detect interaction between OsJAZ6 and OsMYC2 in *Nicotiana benthamiana*. nYFP, N-terminal yellow fluorescent protein; cYFP, C-terminal yellow fluorescent protein. Scale bars=50 μM.

(e) The relative expression of *OsMADS1* in wild type and *OsJAZ6-6HA*. Asterisk indicates significant difference according to Student's t tests (**p<0.01). Error bars indicate SD.

Figure 4. The 84th amino acid residue of OsJAZ6 is critical for OsJAZ repressor complex formation during rice spikelet initiation.

(a) Spikelet phenotypes of wild type, *Osjaz1-1D*/*eg2*, *Osjaz1 Osjaz6, OsJAZ6-6HA eg2* and *OsJAZ6*^{TIFYI84A}*-6HA*. Arrowheads indicate the extra glume-like organs. Scale bars=2mm. egl, extra glume-like organ; le, lemma; pa, palea.

(b) Amino acid sequence alignment of the ZIM domain in 15 rice OsJAZ proteins. The highly conserved TIFY motif (TIFF/YXG) is highlighted by line. The in-color alignment depicts amino acids with similar hydrophobicity, which was generated with the online Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

(c) Y2H assay to detect the interaction between substitution versions of OsJAZ6 and OsJAZ1. Transformed yeast cells were grown on SD/-Trp/-Leu/-His/-Ade media.

(d) Y2H assay to detect the interaction between substitution versions of OsJAZ6 and other OsJAZ proteins. Transformed yeast cells were grown on SD/-Trp/-Leu/-His/-Ade X-αGal media.

Figure 5. OsJAZ6 protein is hypersensitive to JA-induced degradation.

(a) A genetic model for OsJAZ1 and OsJAZ6 actions in spikelet development. In the *OsJAZ1-6HA* transgenic lines, OsJAZ6 degrades and JA signaling release normally. While in the *OsJAZ6-6HA* transgenic lines, increasing of OsJAZ6 proteins form hetero-dimer with OsJAZ1 and block JA signaling output.

(b) Expression pattern of OsJAZ1-GFP, OsJAZ6-GFP and OsJAZ1pro:OsJAZ6gDNA-GFP proteins during spikelet development. The GFP fluorescence were imaged by confocal microscope. The representative images presented were repeated independently

three times. im, inflorescence meristem; le, lemma; pa, palea; rg, rudimentary glume; sl, sterile lemma; st, stamen. Scale bars=50 μM

(c) Y2H assay to detect COR-dependent OsJAZs-OsCOI1b interactions. Transformed yeast cells were grown on SD-Trp/-Leu/-His/-Ade/X-αGal medium that supplemented with different concentrations of COR.

(d) Different stability of OsJAZ1 and OsJAZ6 in response to JA. 10-day-old transgenic seedlings were treated with 100 μM MeJA. Indicated time after treatment, the GFP fluorescence in root tissue were visualized by confocal microscope. The images presented were taken in the same confocal microscope parameters. Scale bars=50 μM.

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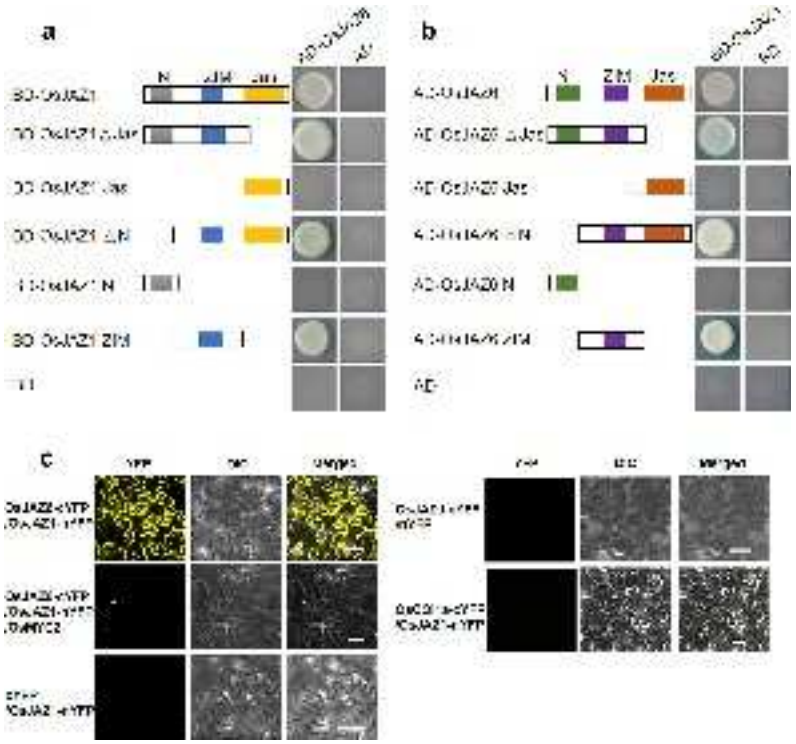

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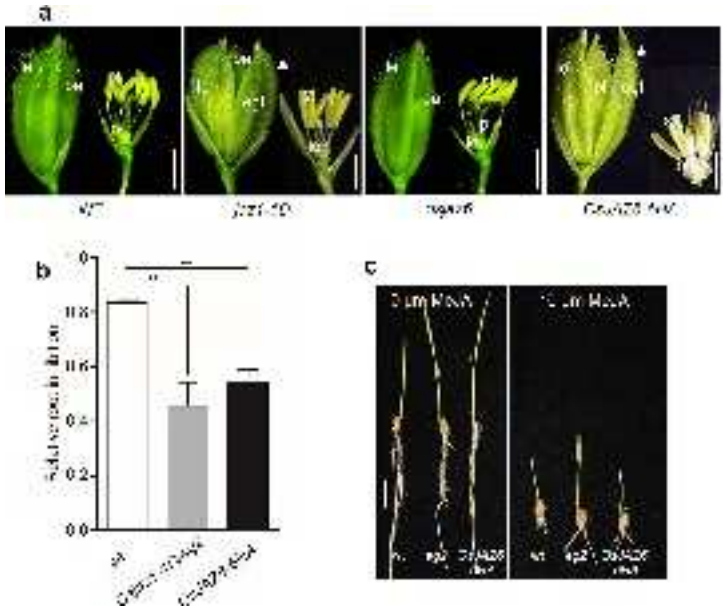

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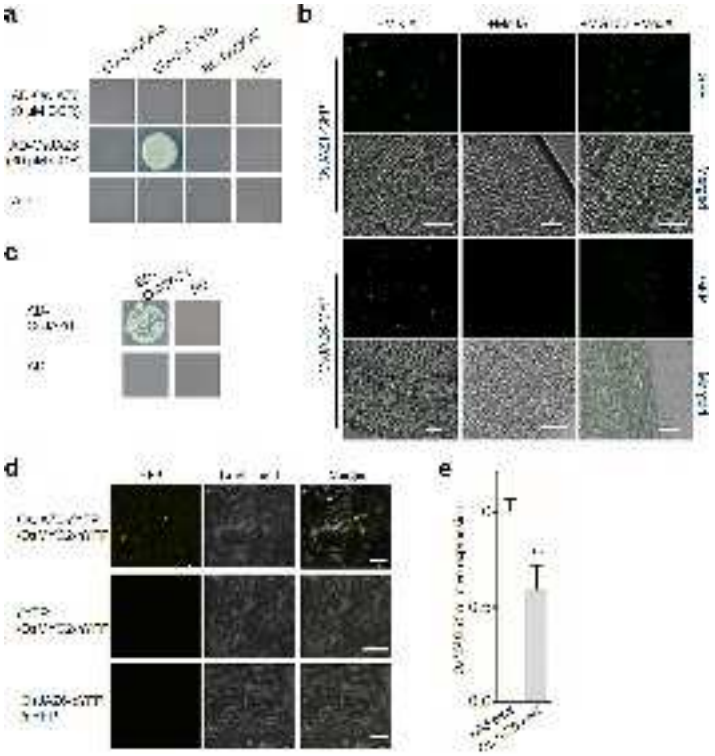

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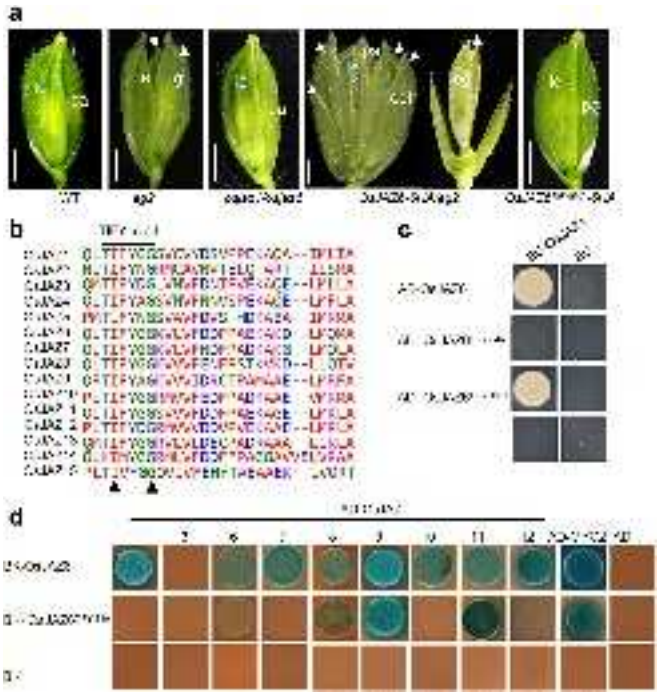

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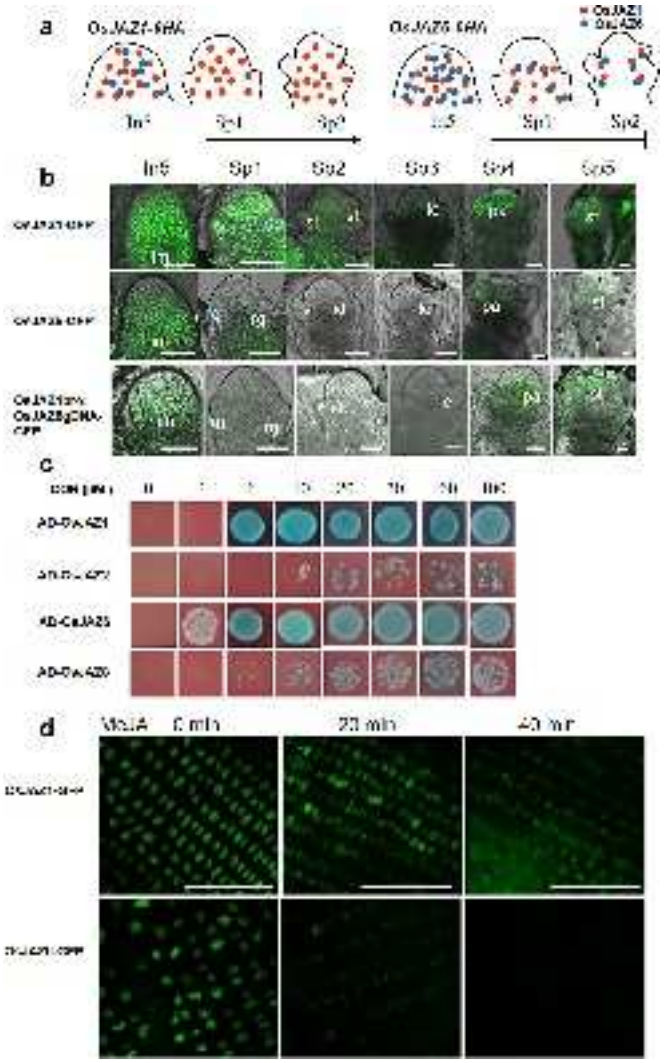

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