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Isolation and characterization of a J domain protein that interacts with ARC1 from ornamental kale (*Brassica oleracea* var. *acephala*)

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Abstract

Key message **A novel J domain protein**, ***JDP1*, was isolated from ornamental kale. The C-terminus of JDP1 specifically interacted with ARC1, which has a conserved role in self-incompatibility signaling**.

Abstract Armadillo (ARM)-repeat containing 1 (ARC1) plays a conserved role in self-incompatibility signaling across the Brassicaceae and functions downstream of the *S*-locus receptor kinase. Here, we identified a J domain protein 1 (JDP1) that interacts with ARC1 using a yeast two-hybrid screen against a stigma cDNA library from ornamental kale (*Brassica oleracea* var. *acephala*). JDP1, a 38.4-kDa protein with 344 amino acids, is a member of the Hsp40 family. Fragment $JDP1_{57-344}$, originally isolated from a yeast two-hybrid cDNA library, interacted specifically with ARC1 in yeast two-hybrid assays. The N-terminus of JDP1 ($JDP1_{1-68}$) contains a J domain, and the C-terminus of JDP1 ($JDP1_{69-344}$) contains an X domain of unknown function. However, $JDP1_{69-344}$ was required and sufficient for interaction with ARC1 in yeast two-hybrid assays and in vitro binding assays. Moreover, $JDP1_{69-344}$ regulated the trafficking of ARC1 from the cytoplasm to the plasma membrane by interacting with ARC1 in *Arabidopsis* mesophyll protoplasts. Finally, Tyr^8 in the JDP1 N-terminal region was identified to be the specific site for regulating the interaction between JDP1 and BoARC1 in yeast two-hybrid assays. Possible roles of JDP1 as an interactor with ARC1 in *Brassica* are discussed.

Abbreviations

ARC1	ARM-repeat containing 1
AtPUB14	*Arabidopsis thaliana* PLANT U-BOX 14
AtPUB17	*Arabidopsis thaliana* PLANT U-BOX 17
CFP	Cyan fluorescent protein
GFP	Green fluorescent protein
HPD	Histidine–proline–aspartic acid
Hsp40	Heat shock protein 40
Hsp70	Heat shock protein 70
JDP1	J Domain protein 1
MBP	Maltose binding protein
MLPK	*M*-Locus protein kinase
PUB	PLANT U-BOX protein
RT-PCR	Reverse transcriptase PCR
SCR	*S*-Locus cysteine-rich
SI	Self-incompatibility
SLG	*S*-Locus glycoprotein
SP11	*S*-Locus protein 11
SRK	*S*-Locus receptor kinase
X domain	DnaJ-X domain
YFP	Yellow fluorescent protein

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Introduction

For flowering plants, pollination is a means to increase genetic diversity and adaptability to new environments. In nature, the majority of angiosperms are hermaphroditic, and their male and female organs are close together within a single flower. Thus, the likelihood of self-pollination, which can lead to loss in genetic diversity and fitness in offspring, is high for these hermaphrodites. To avoid inbreeding depression and promote outcrossing, many plants have adopted self-incompatibility (SI) systems, which enable the plant to discriminate between "self" and "non-self" pollen, to maintain genetic diversity in the population (de Nettancourt 2001; Iwano and Takayama 2012). This discrimination of self and non-self between pollen and pistil is followed by selective inhibition of self-pollen germination and/or growth.

In *Brassica*, SI recognition is controlled by *S* haplotypes (designated S_1, S_2,···S_n) (Bateman 1955). The *S* haplotype is determined by a combination of the pollen determinant gene *SP11* (*S-locus protein 11*)/*SCR* (*S-locus cysteine-rich*) (Schopfer et al. 1999; Takayama et al. 2000), the pistil determinant gene *SRK* (*S-locus receptor kinase*) (Stein et al. 1991; Goring and Rothstein 1992; Takasaki et al. 2000), and the *S*-locus glycoprotein gene *SLG* (Nasrallah et al. 1985). *SP11/SCR* encodes a small cysteine-rich protein that is localized on the pollen coat and functions as the sole determinant of the SI phenotype of the pollen (Schopfer et al. 1999; Takayama et al. 2000). *SRK* encodes a membrane-spanning serine/threonine receptor kinase and functions as the sole determinant of the SI phenotype of the stigma (Takasaki et al. 2000). Upon self-pollination, the SP11/SCR ligand in the pollen coat binds specifically to the extracellular domain of its cognate SRK in the stigma (i.e., with the same *S* haplotype) and activates the SRK kinase domain (Takayama et al. 2001; Cabrillac et al. 2001; Naithani et al. 2007; Ivanov and Gaude 2009).

Several intercellular proteins that interact with the kinase domain of SRK have been identified within the stigmatic papilla cell—thioredoxin h proteins, ARM-repeat containing 1 (ARC1), kinase associated protein phosphatase, calmodulin, sorting nexin, and *M*-locus protein kinase (MLPK) (Bower et al. 1996; Gu et al. 1998; Vanoosthuyse et al. 2003; Murase et al. 2004; Kakita et al. 2007). ARC1 and MLPK function as positive regulators of SRK in SI signaling. The MLPK of *B. rapa* is a receptor-like cytoplasmic kinase localized to the plasma membrane with SRK and is required for the self-incompatibility response (Murase et al. 2004; Kakita et al. 2007).

ARC1 was originally identified with yeast two-hybrid screening as a protein that interacts with the kinase domain of SRK in *B. napus* (Gu et al. 1998). This interaction, which requires the C-terminal ARM-repeat domains of ARC1 and

the kinase activity of SRK, results in the phosphorylation of ARC1 (Gu et al. 1998). MLPK can also phosphorylate ARC1 very efficiently in vitro (Samuel et al. 2008). Suppression of *ARC1* expression by antisense cDNA leads to partial breakdown of the SI response (Stone et al. 1999). ARC1 possesses a U-box-dependent E3 ubiquitin ligase activity, and an increase in ubiquitinated proteins in the stigma accompanies the SI response (Stone et al. 2003).

Recently, *ARC1* was isolated from *A. lyrata*, a naturally self-incompatible species, and shown to be necessary for self-pollen rejection (Indriolo et al. 2012). *ARC1* was also found to be frequently deleted in self-compatible species, including *A. thaliana* (Indriolo et al. 2012). Moreover, when either *ARC1* of *A. lyrata* or *B. napus* was inserted with SCR$_b$-SRK$_b$ into the Col-0 or Sha ecotypes of *A. thaliana*, stronger SI phenotypes resulted (Indriolo et al. 2014).

A model based on these findings proposed that, following the perception of the cognate SP11/SCR ligand, (1) SRK forms a complex with MLPK, (2) the complex activates ARC1, (3) this activated ARC1 then promotes ubiquitination followed by degradation of unknown compatibility factors in the pistil, (4) eventually leading to self-pollen rejection (Ivanov et al. 2010; Indriolo and Goring 2014).

Exo70A1, a predicted exocyst complex subunit in *B. napus*, was identified as an interactor and substrate for ARC1 and shown to be ubiquitinated by ARC1 in vitro (Samuel et al. 2009). Transgenic studies further showed that *Exo70A1* in the stigma was required for the acceptance of compatible pollen and that the gene was negatively regulated by the self-incompatibility response in *B. napus* (Samuel et al. 2009). However, little else is known about the molecular mechanism in the ARC1-mediated self-incompatibility response thus far.

In our previous work, we isolated an *ARC1* orthologue (designated *BoARC1*) from a self-incompatible line of ornamental kale (*Brassica oleracea* var. *acephala*) (Lan et al. 2011). The colorful foliage of ornamental kale and its tolerance to frost and cold environments make it an excellent bedding plant of increasing popularity in northern China and USA. Because most cultivars of ornamental kale are self-incompatible, this characteristic is used to produce hybrid seeds.

In our present work to identify novel ARC1-interacting proteins involved in the SI signaling pathway, we used the yeast CytoTrap two-hybrid library screen. In this system, upon the interaction of cytoplasmic hSos-bait proteins (ARC1) with membrane-bound target proteins (stigma proteins), hSos-bait fusion proteins are recruited to the membrane, thereby activating the Ras-signaling pathway and allowing the temperature-sensitive mutant cdc25H yeast strain to grow at ordinarily restrictive temperatures (Broder et al. 1998; Kim et al. 2008). We identified a J

domain containing protein 1 (JDP1) that interacted specifically with ARC1 in yeast two-hybrid assays. JDP1 is a member of heat shock protein 40 (Hsp40) family that contains a J domain in the N-terminus and an X domain in the C-terminus. We also found that the C-terminus of the protein ($JDP1_{69–344}$) was required for the interaction with ARC1. Thus, we suspect that this novel JDP1 is a potential interactor with ARC1.

Materials and methods

Plant materials and plant growth conditions

The self-incompatible strain containing SRK_{13-b} and SCR_{13-b} was selected based on a self-incompatibility index (i.e., number of seeds/number of self-pollinated flowers) from ornamental kale (*B. oleracea* var. *acephala*) cv. Red Rabbit (Lan et al. 2006). Self-incompatible homozygosity ($S_{13-b}S_{13-b}$) of the strain was confirmed by PCR–RFLP and Southern blot analysis (Lan et al. 2006). The seedlings, which required vernalisation for flowering, were grown during winter at >6 °C in a greenhouse (Li and Yu 2006).

Yeast CytoTrap two-hybrid cDNA library construction

Stigmas from the self-incompatible $S_{13-b}S_{13-b}$ homozygous strain were collected from buds approaching flowering. Total RNA from stigmas was extracted and further purified using the mRNA isolation kit (Amersham Biosciences). The yeast CytoTrap two-hybrid cDNA library was constructed according to the manufacturer's specifications (Stratagene).

Yeast CytoTrap two-hybrid screen

A full-length cDNA clone with *Nco*I and the *Not*I sites corresponding to the open reading frame of *BoARC1* (GenBank: EU344909) was amplified by PCR (Lan et al. 2011). The PCR products were cloned into the pSos vector and sequenced. Library transformation, replication, and yeast two-hybrid screening were performed as described by Kim et al. (2008). A total of 1×10^6 yeast colonies were screened. Positive interactions were verified by plasmid isolation, sequencing, reconstruction of the rescued prey construct, and recapitulation of the yeast transformation. Positive and negative control plasmids were used according to the manufacturer's specifications.

Yeast two-hybrid assays

The full-length JDP1 and fragments $JDP1_{1–68}$, $JDP1_{1–157}$, $JDP1_{57–157}$, $JDP1_{4–344}$, $JDP1_{7–344}$, $JDP1_{8–344}$, $JDP1_{9–344}$, $JDP1_{10–344}$, $JDP1_{11–344}$, $JDP1_{14–344}$, $JDP1_{29–344}$, $JDP1_{23–344}$, $JDP1_{37–344}$, $JDP1_{57–344}$, $JDP1_{69–344}$ and $JDP1_{158–344}$ were cloned into the *Eco*RI and *Xoh*I sites of pMyr for expression of the Myr fusion proteins. For JDP1-Y7F, JDP1-Y8F and JDP1-Y7F-Y8F mutants, site-directed mutagenesis was carried out using an overlap extension PCR strategy. Full-length BnARC1 and fragments AtPUB14 and AtPUB17 were cloned into the *Nco*I and *Not*I sites of pSos for expression of the hSos fusion proteins. All constructs were confirmed by sequencing. Plasmids were used to transform yeast strain cdc25H, which was then plated onto SD/glu (−LU) plates (synthetic dropout glucose agar plates lacking leucine and uracil) and SD/gal (−LU) plates (synthetic dropout galactose agar plates lacking leucine and uracil) at the permissive temperature of 25 °C or the stringent temperature of 37 °C according to the manufacturer's specifications. Positive clones were verified by their ability to grow on SD/gal (−LU) or SD/glu (−LU) at 25 °C or on SD/gal (−LU) plates but not on SD/glu (−LU) plates at 37 °C.

RACE and reverse transcription-PCR assay

A stigma Marathon RACE library was constructed according to the manufacturer's instructions (Clontech). The 5′ RACE-PCR was performed with the JDP1-specific reverse primer (5′-GGCTCATGGGATTCTACTTCAGCG TCG-3′). The PCR products were cloned into the pBS-T vector and sequenced. The RNA samples were reverse transcribed to synthesize first-strand cDNA with ReverTra Ace (TOYOBO, Osaka, Japan) using an oligo $(dT)_{18}$ primer. The single-stranded cDNAs were used as templates for PCR amplification with JDP1-specific primers (5′-A TCCCTTCCTCGCTTTTGTGC-3′, forward) and (5′-CGA AACCTCCACTTCACATAAAC-3′, reverse), designed from the 5′ and 3′ untranslated regions of the annotated *JDP1* sequences, respectively. For the *actin* control amplification, primers (5′-TAAAAAAATGGCTGAGGCTG ATG-3′, forward) and (5′-CAGCTTAGAAGCATTT TCTGTGAA-3′, reverse) were used.

Expression and purification of recombinant proteins and in vitro binding assay

Full-length JDP1 and fragments $JDP1_{1–68}$, $JDP1_{57–344}$, and $JDP1_{69–344}$ were cloned into the *Nde*I and *Bam*HI sites of pET14b vector (Novagen) for expression as 6 × His-tagged fusion proteins. The full-length PCR-amplified BoARC1 fragment was cloned into the *Eco*RI and *Xba*I sites of pMal-c2 vector (New England Biolabs). After sequence confirmation, these constructs were transformed into *E. coli* BL21 cells, and the expression of the fusion protein was induced by 0.5 mM isopropyl-β-D-

thiogalactoside. The induced cells were lysed by sonication in binding buffer (50 mM Tris–HCl, pH 7.5; 200 mM NaCl; 1 % Triton X-100; 5 mM DTT; 2 mM PMSF), and the supernatant was loaded onto a Poly-Prep chromatography column (Bio-Rad) pre-equilibrated with binding buffer. MBP-BoARC1 was purified with amylose resin (New England BioLabs); the $6 \times$ His-tagged JDP1, $JDP1_{1-68}$, $JDP1_{57-344}$, and $JDP1_{69-344}$ proteins were purified with Ni–NTA agarose resin (Qiagen). The proteins were further purified by diafiltration with 10-kD cutoff centrifugal filters (Millipore). For in vitro binding, 2 µg of bait (MBP-BoARC1) and 2 µg of prey ($6 \times$ His-tagged JDP1, $JDP1_{1-68}$, $JDP1_{57-344}$, $JDP1_{69-344}$, or GFP) were added to 1 mL of binding buffer, respectively, and analyzed as described (Seo et al. 2004). Binding proteins were separated by SDS-PAGE and detected by Western blotting using an anti-His monoclonal antibody as described by the manufacturer (Cell Signaling Technology).

Total protein extraction and western blot analysis and pull-down assay

Total protein extracts were prepared by grinding various tissues in liquid nitrogen and analyzed (Stone et al. 2003). The protein concentration for each protein extract was determined using the 2-D Quant kit (Amersham Biosciences) and staining with Coomassie brilliant blue R250 after separation on an SDS-PAGE gel. The polyclonal anti-JDP1 antibody was prepared by immunizing rabbits with recombinant $6 \times$ His-tagged JDP1. IgG was enriched from the immune sera by ammonium sulfate precipitation. The IgG fraction was then passed through a protein A-agarose column (Roche Diagnostics), and bound IgG was eluted with elution buffer (0.1 M Gly-HCl, pH 2.7).

Approximately 10 µg of the total protein extract was fractionated on a 12 % SDS-PAGE mini gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The blots were probed with the anti-JDP1 antibody, washed in TBST buffer three times, probed with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5,000, Cell Signaling Technology), washed, and exposed using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham Biosciences). Images were acquired and analyzed with Kodak 1-D software on a Kodak 2000R imaging station.

For the pull-down assay, 10 µg of MBP-BoARC1 or MBP protein was coupled to 100 µL of a 50 % suspension (v/v) of amylose resin in equilibration buffer (20 mM Tris–HCl, pH 7.4; 200 mM NaCl; 1 mM EDTA) for 30 min at 4 °C. After preclearing with 50 µL amylose resin, approximately 100 µg of the total protein extract of the stigma was transferred to a clean tube containing MBP-BoARC1 or MBP protein coupled to the amylose resin and incubated overnight with constant rotation at 4 °C. Subsequently, the resin was washed four times with washing buffer (20 mM Tris–HCl, pH 7.4; 200 mM NaCl; 1 mM EDTA), and then 60 µL of SDS-PAGE sample buffer was added to each sample. Bound proteins were dissociated by heating at 95 °C for 10 min and separated by 12 % SDS-PAGE and detected by Western blotting using the anti-JDP1 antibody.

Subcellular colocalization assay

The CFP and YFP coding sequences (Liu et al. 2008) were fused in frame to the 5′ end of BoARC1 and to the 3′ ends of JDP1, $JDP1_{1-68}$, and $JDP1_{69-344}$ to generate CFP-BoARC1, JDP1-YFP, $JDP1_{1-68}$-YFP, and $JDP1_{69-344}$-YFP fusions, respectively. *Arabidopsis* mesophyll protoplasts were prepared from 4-week-old wild-type plants (Yoo et al. 2007). The protoplasts were either transfected with a construct expressing CFP-BoARC1, JDP1-YFP, $JDP1_{1-68}$-YFP, or $JDP1_{69-344}$-YFP or cotransfected with constructs expressing CFP-BoARC1 and JDP1-YFP, $JDP1_{1-68}$-YFP, or $JDP1_{69-344}$-YFP. The protoplasts expressing the transfected constructs were imaged using a Zeiss LSM510 META laser scanning confocal microscope.

Results

Identification of a novel protein that interacts with ARC1

To identify novel substrates and/or regulators of *Brassica* ARC1, we performed yeast CytoTrap two-hybrid screens of a stigma cDNA library. The full-length coding sequence of *BoARC1* was cloned into the yeast pSos vector to produce a fusion protein (pSos-BoARC1) as bait. A stigma cDNA library from a self-incompatible $S_{13-b}S_{13-b}$ line was constructed in the yeast pMyr vector to produce target proteins. The pSos-BoARC1 and pMyr stigma cDNA library were used to co-transform the temperature-sensitive cdc25H strain of *Saccharomyces cerevisiae*. Eleven positive clones were obtained by screening 1×10^6 yeast clones in the yeast CytoTrap two-hybrid system.

Among these positive clones, the DNA sequences were identical in 10 of these clones. Since these clones did not contain the full-length cDNA, the remaining 5′ end sequences were determined by 5′RACE-PCR. The nucleotide sequence of the putative full-length cDNA was 1,326 bp, containing a short 5′ (73 bp)- and a long 3′ (218 bp)-untranslated region and an open reading frame of 1,035 bp, which encoded a peptide of 344 amino acids with the predicted molecular mass of 38.4 kDa and calculated pI of

7.04 (Supplementary Fig. S1). In a BLAST search of a non-redundant protein sequence database using the full-length sequence of this protein, 88 % amino acid identity was shared with the DnaJ heat shock N-terminal domain containing protein (J domain containing protein 1, JDP1) of *Arabidopsis thaliana* (At4g39150, AtJDP1) with unknown function. Thus, the isolated protein was named BoJDP1.

The N-terminal region (JDP1$_{1-68}$) contained a DnaJ domain (pfam00226, J domain). The C terminal region (JDP1$_{69-344}$) contained a DnaJ-X domain (pfam14308, X domain) in the SMART database or Pfam database (Fig. 1a). The J domain of BoJDP1 (JDP1$_{1-68}$) lacks the Gly/Phe-rich domain and the zinc finger domain, which is conserved in the type I and type II J domain proteins. Thus, JDP1 could be classified as a type III J domain protein. The J domain of BoJDP1 has the following features—four predicted helices (I to IV) and a histidine-proline-aspartic acid (HPD) tripeptide in the loop between helices II and III

(Supplementary Fig. S2). The function of the X domain (residues 133–328) is unknown (Hettema et al. 1998). Moreover, BoJDP1 has high amino acid identity and similarity of domain organization with other plant JDP1 (Supplementary Fig. S2), suggesting that BoJDP1 represents a novel and conserved type III J domain protein in plants.

Interaction between ARC1 and JDP1 in the yeast two-hybrid system

The original JDP1 clone (JDP1$_{57-344}$, amino acids 57–344) isolated in the screen carried a 1,085-bp insert, which lacked 241 bp at the 5′ end including the J domain (Fig. 1a). The interaction between BoARC1 and JDP1$_{57-344}$ was confirmed by transforming *S. cerevisiae* and plating the transformants on SD/glu (−LU) plates and SD/gal (−LU) to check for growth at the permissive

Fig. 1 Domain structures of JDP1 and JDP1$_{57-344}$ and JDP1$_{57-344}$ isolation, and specific interaction with ARC1. **a** Diagram of structures: J domain is shown in *black*, the X domain in *gray*. JDP1$_{57-344}$ was originally isolated from screening a yeast two-hybrid cDNA library for the stigma. **b** Isolation of JDP1$_{57-344}$ from yeast two-hybrid screen. Interaction between JDP1$_{57-344}$ and BoARC1 was identified using the CytoTrap two-hybrid system. Approximately 1×10^6 cells of yeast cdc25H from a cDNA library made from stigmatic tissues were screened using BoARC1 as bait. Recapitulation of the interaction was done on galactose-containing minimal media in the absence of uracil and leucine at 37 °C. **c** Specific interaction between JDP1$_{57-344}$ and ARC1 identified using the CytoTrap two-hybrid system. The listed combinations of bait (Sos fusion) and prey (Myr fusion) constructs were introduced into the yeast cdc25H cells. Control interactions consisted of pSos-MAFB+pMyr-MAFB (positive control) and pSos-MAFB+pMyr-Lamin C (negative control)
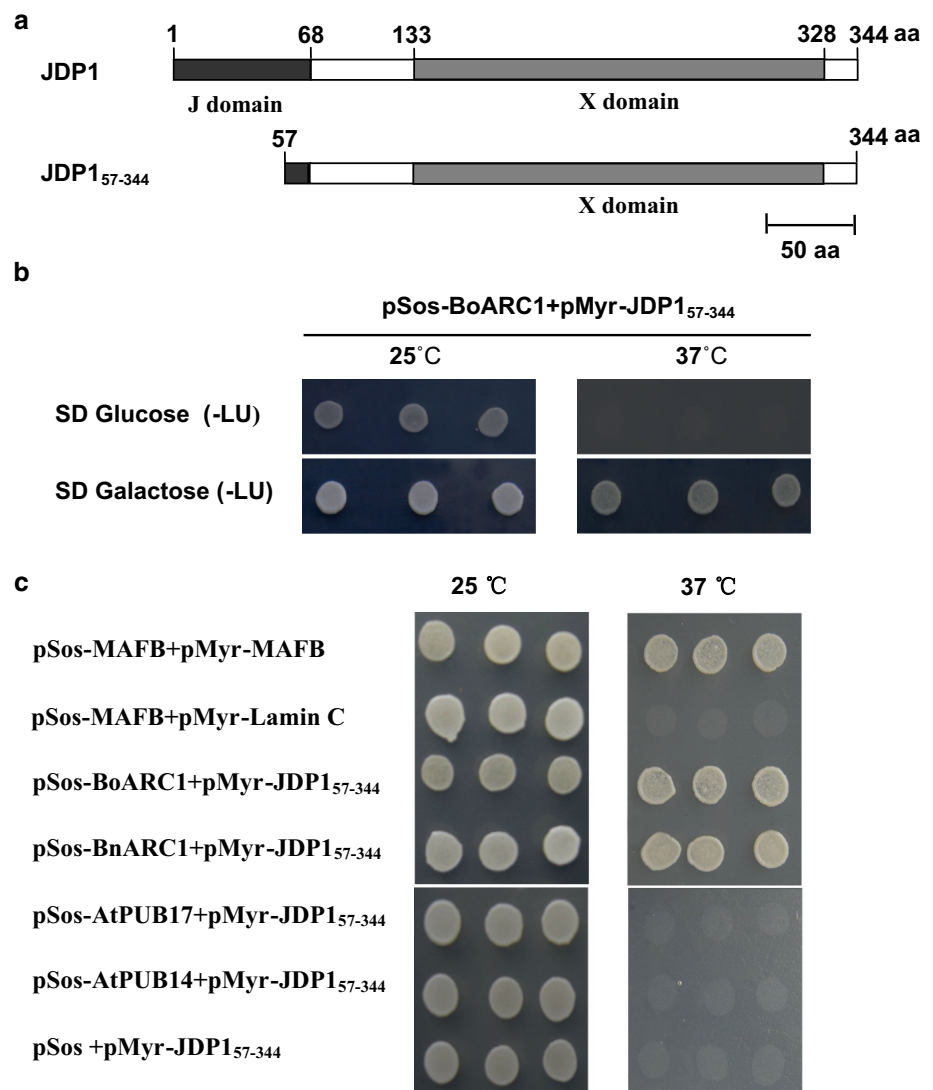
temperature of 25 °C and the stringent temperature of 37 °C. Because galactose can induce the *GAL1* promoter in the pMyr vector to express the prey protein, any clone that grows on SD/gal (−LU) or SD/glu (−LU) at 25 °C or on SD/gal (−LU) plates but not on SD/glu (−LU) plates at 37 °C is an interaction-positive clone. When pSos-BoARC1 and pMyr-JDP1$_{57-344}$ were present in yeast cells, growth was observed on SD/gal (−LU) plates but not on SD/glu (−LU) plates at 37 °C (Fig. 1b).

To identify the specificity of the interactions of JDP1$_{57-344}$ with ARC1 in the yeast CytoTrap two-hybrid system, we used several different pSos fusions to detect non-specific interactions with JDP1$_{57-344}$: (1) BoARC1 and BnARC1, (2) *Arabidopsis thaliana* PLANT U-BOX 17 (AtPUB17) and PLANT U-BOX 14 (AtPUB14), which are the closest *Arabidopsis* homologues of ARC1 (Andersen et al. 2004, Yang et al. 2006; Indriolo et al., 2012), and (3) empty pSos vector. The JDP1$_{57-344}$ fusion interacted with BoARC1 and BnARC1 but not with either of the *Arabidopsis* PUB proteins (AtPUB17 or AtPUB14) (Fig. 1c), indicating that the JDP1$_{57-344}$ interacts specifically with ARC1 in yeast two-hybrid assays.

To determine which region of the JDP1 protein is required for the interaction with BoARC1, several JDP1 deletion mutants were cloned into the yeast pMyr vector and tested for interaction with the full-length BoARC1 protein by the yeast two-hybrid assay. As shown in Fig. 2, the N-terminal truncated fragments (JDP1$_{57-344}$ and JDP1$_{69-344}$) strongly interacted with BoARC1, whereas none of the N-terminal fragments (i.e., JDP1$_{1-68}$ and JDP1$_{1-157}$), the full-length JDP1, JDP1$_{158-344}$, or JDP1$_{57-157}$ bound to BoARC1. These results suggest that the C-terminal fragment of JDP1 (JDP1$_{69-344}$) is required and sufficient for the interaction with ARC1, while the J domain of JDP1 inhibits the interaction with ARC1.

JDP1 interacts with BoARC1 in vitro

To test for the JDP1−ARC1 interaction in vitro, we performed in vitro binding assays using purified fusion JDP1 and ARC proteins. The full-length *BoARC1* was expressed as a fusion protein with maltose binding protein (MBP) in the pMal-c2 expression vector and purified from *E. coli* (Fig. 3a). The full-length JDP1, JDP1$_{57-344}$, JDP1$_{69-344}$, JDP1$_{1-68}$, and green fluorescent protein (GFP) were cloned into another expression vector, pET14b, to produce 6 × His-tagged fusion proteins in *E. coli* (Fig. 3b). Western blotting using an anti-His antibody showed that BoARC1 bound to the full-length JDP1, JDP1$_{57-344}$, and JDP1$_{69-344}$ but not to JDP1$_{1-68}$ or the control polypeptide GFP (Fig. 3c). Also, none of the JDP1 fragments bound to the control MBP protein (data not shown).

Subcellular localization and interaction of JDP1 for BoARC1 in *Arabidopsis* protoplasts

Arabidopsis mesophyll protoplasts were used as a transient expression system to express the fusion protein of the full-length JDP1, JDP1$_{1-68}$ or JDP1$_{69-344}$ with yellow fluorescent protein (YFP) (Yoo et al. 2007). As shown in Fig. 4, cells expressing the full-length JDP1-YFP, JDP1$_{1-68}$-YFP fusion protein or free YFP (control) showed the YFP signal around the nucleus and in the cytoplasm, whereas the signal in cells expressing JDP1$_{69-344}$-YFP fusion came predominantly from the plasma membrane.

When the full-length BoARC1 fused with cyan fluorescent protein (CFP) was expressed, the CFP signal was predominantly detected as characteristic cytoplasmic speckles in the cytoplasm (Fig. 5a), similar to the proteasomal localization of BnARC1 in tobacco BY-2 cells (Stone et al. 2003; Samuel et al. 2009).

Interestingly, when CFP-BoARC1 was co-expressed with JDP1$_{69-344}$-YFP, both the CFP and YFP signals came

Fig. 2 Identification of JDP1–BoARC1 interactions via yeast two-hybrid assays. Constructs for full-length and fragments of JDP1 are shown with the J domain in *black*, the X domain in *gray*. An interaction was detected when yeast cells grew on galactose-containing minimal media in the absence of uracil and leucine at 37 °C (*right*)

a

CBB

b

CBB

c

MBP-ARC1 Binding

WB: His
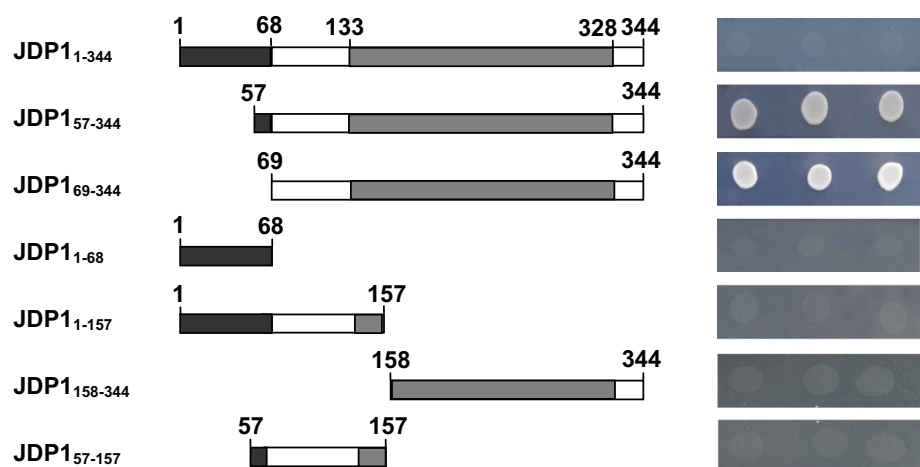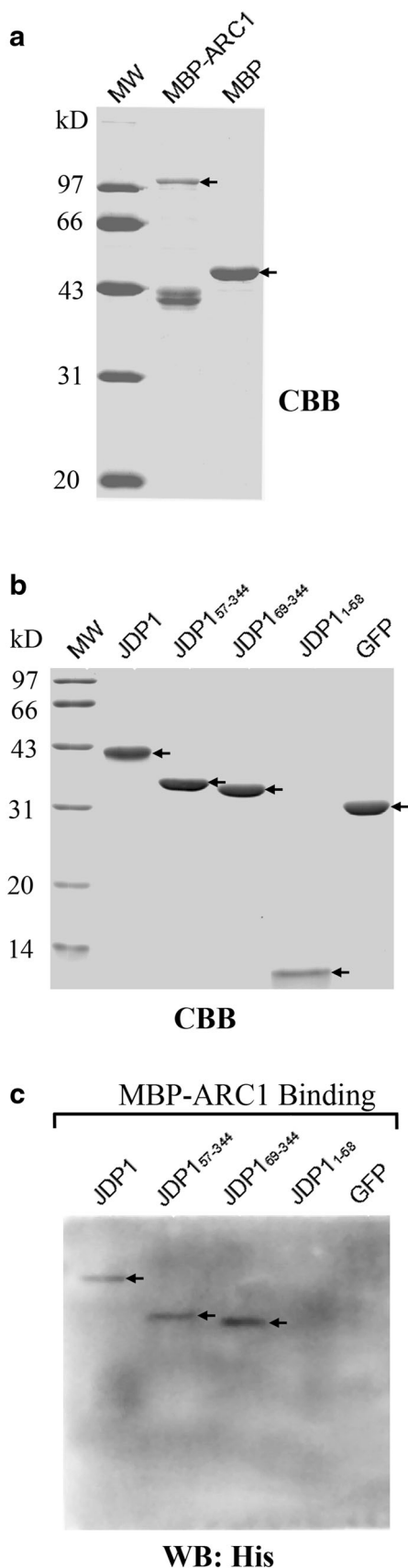
◄ **Fig. 3** JDP1 interacts with BoARC1 in vitro. **a** MBP and the MBP-BoARC1 fusion protein from *E. coli*. Purified proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB) R250. MW molecular weight marker. **b** $6 \times$ His-tagged JDP1, three fragments of JDP1, and green fluorescent protein (GFP) fusions from *E. coli*. Purified proteins were separated by SDS-PAGE and stained with CBB. **c** Binding of BoARC1 and JDP1. The MBP-BoARC1 fusion protein was incubated with $6 \times$ His-tagged JDP1, JDP1$_{57–344}$, JDP1$_{1–68}$, JDP1$_{69–344}$, or GFP proteins, respectively, then amylose resin beads were added and the mixture incubated. Bound proteins (arrow) were eluted and analyzed by western blotting using an anti-His antibody (WB: His)

predominantly from the plasma membrane, but when CFP-BoARC1 was co-expressed with the full-length JDP1-YFP, or JDP1$_{1–68}$-YFP fusion, the localization of CFP-BoARC1 was not affected (Fig. 5b).

Tyr^8 in the JDP1 N-terminal region specifically regulates the interaction between JDP1 and ARC1 in the yeast two-hybrid system

To analyze the J domain sequence that is responsible for inhibiting the ARC1 interaction, we tested a series of deletions in the N-terminal region of JDP1 for their affect on the interaction with ARC1. JDP1$_{37–344}$, JDP1$_{29–344}$, JDP1$_{23–344}$, JDP1$_{14–344}$, JDP1$_{11–344}$, JDP1$_{10–344}$, and JDP1$_{9–344}$ interacted with BoARC1, but JDP1$_{8–344}$, JDP1$_{7–344}$, JDP1$_{4–344}$ and the full-length JDP1 (JDP1$_{1–344}$) did not (Table 1). Tyr^8 of JDP1 is a conserved amino acid residue among homologous JDP1 proteins (Supplementary Fig. S2). To determine whether a single amino acid substitution at this site influences the interaction between JDP1 and BoARC1, the effect of a mutation (Tyr substituted for Phe at position 8, Y8F) in the full-length JDP1 was examined. The mutant (Y8F) interacted strongly with BoARC1, indicating that Tyr^8 activity of JDP1 inhibited the interaction between JDP1 and BoARC1. The mutant at Tyr^7 (Y7F) failed to interact with BoARC1, while the double mutant at Y7F and Y8F did interact with BoARC1.

Genomic structure and expression pattern of JDP1

The *JDP1* gene was composed of 10 exons interspaced with nine introns ranging in size from 84 bp (intron 5) to 238 bp and spanned 2.4 kb in the genome (intron 1) (Fig. 6a). All introns contained a GT boundary sequence at the 5′ end and AG at the 3′ end, complying with the typical "GT-AG rule" in plants (Mount 1982). This genomic structure of *BoJDP1* was almost the same as *AtJDP1* (Fig. 6a).

JDP1 was expressed in stems, leaves, petals, anthers, stigmas, styles and ovaries of non-pollinated flowers as

Fig. 4 Subcellular localization of JDP1-YFP, JDP1$_{1-68}$-YFP or JDP1$_{69-344}$-YFP fusion protein in *Arabidopsis* protoplasts. Protoplasts were transfected with a construct expressing JDP1-YFP, JDP1$_{1-68}$-YFP, or JDP1$_{69-344}$-YFP. YFP channel, bright field, and merged images of the various YFP fusion proteins are shown. *Bar* 10 μm
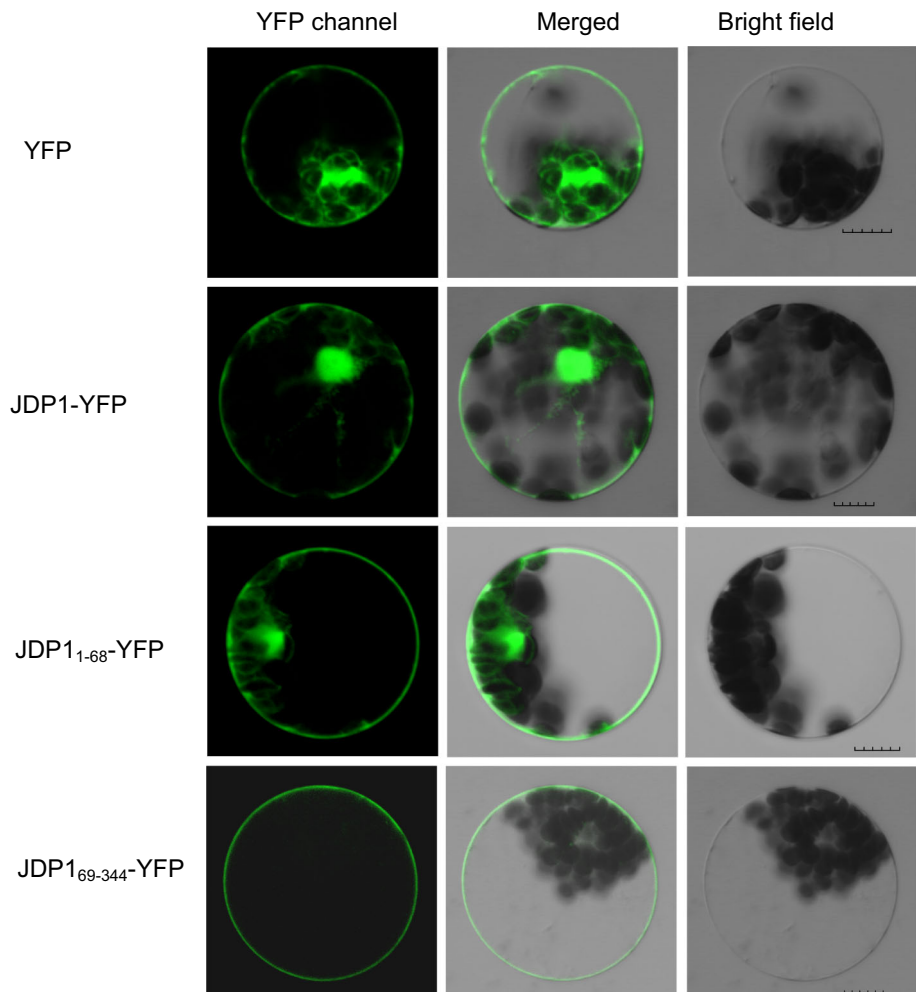
determined by semi-quantitative PCR (Fig. 6b). To analyze the accumulation of JDP1 protein, we raised a polyclonal antibody against recombinant JDP1 protein. The specificity of the antibody was confirmed using a recombinant JDP1 protein expressed in *E. coli* (Supplementary Fig. S3). In all Western blots of all tissues examined, the JDP1 protein was 38 kDa, corresponding to the predicted molecular mass of the full-length JDP1 (Fig. 6c).

To determine whether native JDP1 can form a complex with ARC1, we conducted a pull-down experiment using the bacterially expressed MBP-ARC1 to bind native JDP1 from total protein extract of the stigma. The purified MBP-ARC1 fusion proteins were incubated with total protein extracts of stigma. After washing with buffer, the amylose resin-bound proteins were dissociated by heating at 95 °C, then assayed by SDS-PAGE and examined by Western blotting using polyclonal anti-JDP1 antibody. Similar to that detected in the stigma, a specific protein of 38 kD was detected by the anti-JDP1 antibody when using the MBP-ARC1 fusion protein, and no protein was detected when using the MBP control protein (Fig. 6d). These results

indicated that BoARC1 bound to the native JDP1 protein from total protein extracts of stigma.

Discussion

In the Brassicaceae, down-regulation of *ARC1* impairs SI response and, therefore, ARC1 is considered as a positive regulator of SI signaling pathway (Stone et al. 2003; Indriolo et al. 2014). Searches for ARC1 regulators or its substrates are key steps to dissect the SI signaling pathway in a particular plant. Exo70A1, a member of the exocyst complex in *B. napus*, was identified as an interactor for ARC1 using LexA yeast two-hybrid systems, relying on transcriptional activation of reporter genes in the nucleus to detect interactions. Exo70A1 is required in the stigma for acceptance of compatible pollen, and its overexpression leads to partial loss of SI (Samuel et al. 2009). However, in that system, no protein interaction was detected in the cytosol where the interaction with ARC1 is likely to occur. The conventional LexA or GAL4 two-hybrid system relies
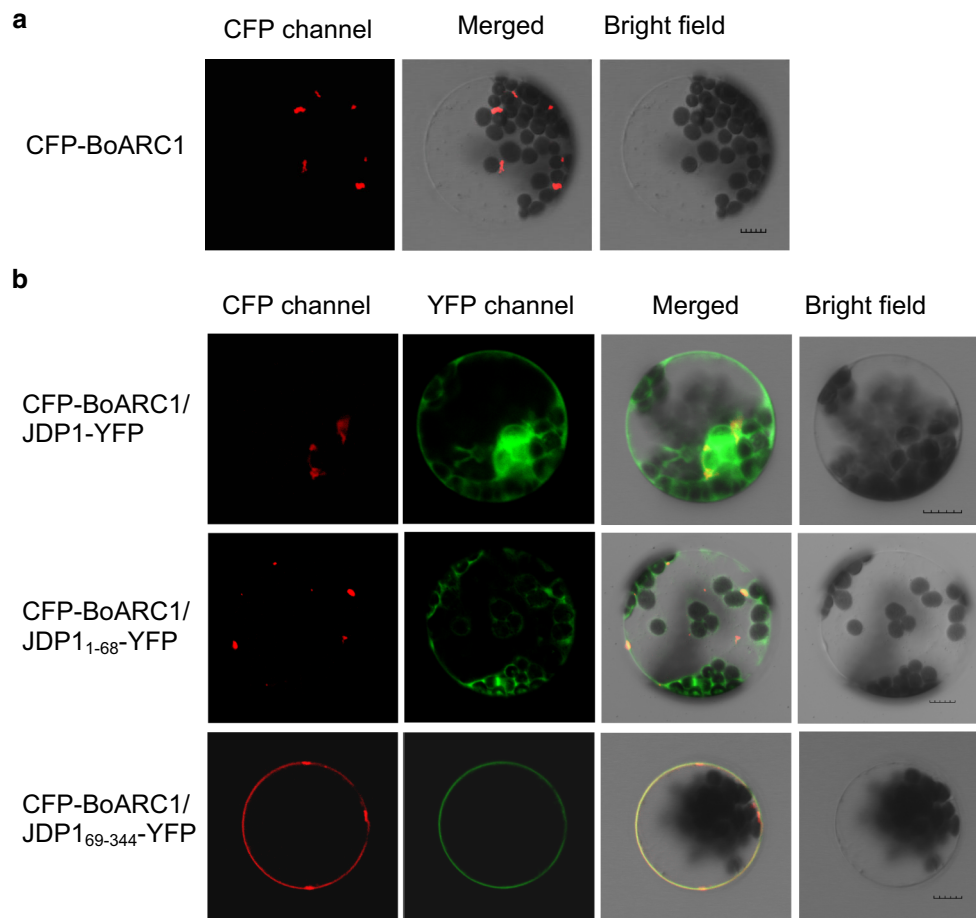
Fig. 5 Subcellular colocalization of JDP1-YFP, JDP1$_{1-68}$-YFP or JDP1$_{69-344}$-YFP with CFP-BoARC1 fusion protein in *Arabidopsis* protoplasts. **a** Subcellular localization of transiently expressed CFP-BoARC1 in *Arabidopsis* protoplasts. **b** Subcellular colocalization of JDP1 and BoARC1 in *Arabidopsis* protoplasts. Protoplasts were imaged using the CFP and YFP channels. The CFP-BoARC1 panels show full-length JDP1-YFP or the JDP1$_{1-68}$-YFP fusion in the cytoplasm, whereas CFP-BoARC1 was present at the plasma membrane with JDP1$_{69-344}$-YFP. *Bar* 10 μm

on transcriptional activation in the nucleus. Since ARC1 was localized predominantly in the cytoplasm of cells (Fig. 5a) (Stone et al. 2003), we used the yeast CytoTrap two-hybrid library screen to find stigmatic proteins that interact with ARC1.

The isolated protein was identified as JDP1, a member of the heat shock protein 40 (Hsp40) family, all of which have a J domain in the N-terminus and an X domain in the C-terminus. In most eukaryotic and prokaryotic cells, the J domain of Hsp40 is known to interact with the heat shock protein 70 (Hsp70) chaperone (Kelley 1998). Hsp40 and Hsp70, which constitute one of the most ubiquitous types of molecular chaperone machinery, function in a wide variety of cellular processes (Barral et al. 2004). The J domain of JDP1 and its ortholog contain a conserved HPD motif in the loop between helices II and III, suggesting that these proteins may interact with partner Hsp70 chaperone. The HPD tripeptide is thought to be crucial for the interaction between Hsp40 and Hsp70, because mutations in this motif abolish binding to Hsp70 (Hennessy et al. 2000, 2005).

The identified JDP1 was classified as a type III J domain protein, which lacks the Gly/Phe-rich domain and the zinc finger domain. A Gly/Phe-rich domain functions in stabilizing the formation of the substrate complex (Perales-Calvo et al. 2010), and the zinc finger domain is believed to participate in protein–protein interactions among DnaJ, DnaK, and target polypeptides (Banecki et al. 1996; Szabo et al. 1996). In plants, very few type III J domain proteins have been biologically characterized. AtJDP1, which showed the highest amino acid identity with BoJDP1, has not yet been functionally characterized. Recently, a type III J domain OWL1 protein was identified as a signaling component dedicated specifically to the very low fluence response (VLFR) in *Arabidopsis* (Kneissl et al. 2009).

An interaction with ARC1 was found for another N-terminal truncated JDP1 protein (JDP1$_{69-344}$), but the presence of the N-terminus prevented its interaction in yeast two-hybrid assays (Fig. 2). Interestingly, we also observed that JDP1$_{69-344}$-YFP was associated mainly with the plasma membrane and affected trafficking of BoARC1 from the cytoplasm to the plasma membrane by interacting with BoARC1 (Fig. 5b). These results indicate that the 69–344 amino acid region (X domain) of JDP1 is required and sufficient for the interaction with ARC1, whereas the N-terminus contains a region that inhibits the interaction. We examined the possibility of the presence of N-terminus-truncated JDP1 in vivo. However, our data did not support the presence of an N-terminal truncation. The originally identified clone contained an insert without the ATG start codon, indicating that the truncation in this clone is artificial. We could not detect any alternative splicing of *JDP1* using 5'RACE-PCR and DNA sequencing. There were no other possible sites for mRNA splicing in the 5' region. Western blot analysis did not show any smaller proteins in any organs analyzed (Fig. 6c).

Interestingly, the full-length JDP1 with a point mutation at Tyr[8] in the N-terminal region interacted strongly with BoARC1 in yeast two-hybrid assays (Table 1), suggesting

Table 1 Effect of a series of N-terminal truncations of JDP1 or mutants of JDP1 on the interaction with ARC1

Constructs	25 °C		37 °C	
	SD/glu (−LU)	SD/gal (−LU)	SD/glu (−LU)	SD/gal (−LU)
JDP1$_{1-344}$	+	+	−	−
JDP1$_{4-344}$	+	+	−	−
JDP1$_{7-344}$	+	+	−	−
JDP1$_{8-344}$	+	+	−	−
JDP1$_{9-344}$	+	+	−	+
JDP1$_{10-344}$	+	+	−	+
JDP1$_{11-344}$	+	+	−	+
JDP1$_{14-344}$	+	+	−	+
JDP1$_{23-344}$	+	+	−	+
JDP1$_{29-344}$	+	+	−	+
JDP1$_{37-344}$	+	+	−	+
JDP1$_{1-344}$-Y7F	+	+	−	−
JDP1$_{1-344}$-Y8F	+	+	−	+
JDP1$_{1-344}$-Y7F+Y8F	+	+	−	+

A series of N-terminal truncations of JDP1 and mutants of JDP1 were cloned into the pMyr vector to co-transform cdc25H yeast cells with pSos-BoARC1. The cdc25H yeast cells were plated onto SD/glu (−LU) plates and SD/gal (−LU) plates at the permissive temperature of 25 °C or the stringent temperature of 37 °C. Protein interactions were determined by yeast growth (+) or lack of growth (−) on SD/gal (−LU) or SD/glu (−LU) at 25 °C or on SD/gal (−LU) plates but not on SD/glu (−LU) plates at 37 °C

that a Tyr[8] modification may be involved in the regulation of JDP1 binding with BoARC1. However, whether the mutation at Tyr[8] of JDP1 affects BoARC1 recruitment to the plasma membrane still needs to be tested. Phosphorylation of a tyrosine residue is a common mechanism to control signaling systems in animal and plant cells (Hubbard and Till 2000). Thus, post-translational modification of JDP1 may be involved in this interaction. Therefore, we suspect that the interaction between JDP1 and BoARC1 could be regulated by a post-translational modification of JDP1 (e.g., phosphorylation or other unknown modifications).

The JDP1$_{57-344}$ truncation, which was isolated using the yeast two-hybrid library screening, specifically interacted with ARC1 in yeast, but failed to interact with either of two *Arabidopsis* U-box E3 ligases (AtPUB17 and AtPUB14) (Fig. 1c). This interaction provides an important clue for the role of JDP1 in the SI response. Moreover, the native JDP1 bound the bacterially expressed ARC1 in the pull-down assay, implying that JDP1 forms a complex with ARC1 in planta. Although the expression pattern of *ARC1* and *SRK* is stigma specific (Stein et al. 1991; Gu et al. 1998), we observed that *JDP1* was ubiquitously expressed in all the examined tissues, suggesting a general biological function for JDP1. It is likely that ubiquitous proteins such as Exo70A1 function as downstream SI signaling components. In addition, we found that JDP1 was not suitable for searching target proteins in the CytoTrap system because it is membrane-localized in yeast cells, thus precluding an extensive functional analysis (data not shown).

Given the role of ARC1 in mediating protein degradation at the proteasome during the self-incompatibility response, the question arises of whether JDP1 is ubiquitinated by ARC1 and subsequently degraded by the 26S proteasome. We observed that in the presence of ubiquitin, rabbit E1, *Arabidopsis* UBC7 (E2), and MBP-BoARC1 fusion proteins, JDP1 did not appear to be modified in in vitro ubiquitination assays (data not shown). One possibility is that a native *Brassica* E2 protein must cooperate with ARC1 to mediate JDP1 ubiquitination. Alternatively, the complex formed between JDP1 and BoARC1 may simply mediate the self-incompatibility response. Importantly, our subcellular colocalization data also appeared to support this view that JDP1 regulated the localization of ARC1 (Fig. 5b).

In summary, we identified a JDP1 that interacts with ARC1 using a yeast two-hybrid screen. The C-terminus of the protein (JDP1$_{69-344}$) is required for the interaction with ARC1, while the J domain of JDP1 regulates the interaction between JDP1 and ARC1. Moreover, Tyr[8] in the JDP1 N-terminal region specifically regulates the interaction between JDP1 and BoARC1 in yeast two-hybrid assays. However, whether the BoARC1–JDP1 complex plays

Fig. 6 Genomic structure and expression pattern of *JDP1* in various tissues. **a** Exon–intron structures of *BoJDP1* and *AtJDP*. Exons: *thick horizontal lines*, introns: *V-shaped dips*. Putative initiation site: ATG, stop site: TAA. **b** Tissue expression of the *JDP1* mRNA. Expression levels of each transcript were analysed by RT-PCR. The *actin* gene was used as a control. **c** JDP1 protein level in various tissues. A strong band at 38 kDa (*asterisk*) was detected in a Western blot (WB) using a specific polyclonal antibody raised against recombinant JDP1 (*top panel*). Equal amounts of the total proteins from various tissues were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB) R250 (*bottom panel*). **d** A pull-down assay for BoARC1 interaction with JDP1. The purified fusion proteins of MBP-BoARC1 and MBP from *E. coli* and amylose resin were incubated with the total protein extracted from the stigma. Bound proteins were pulled down with amylose resin, separated by 12 % SDS-PAGE, transferred to membranes, and analyzed by Western blotting using polyclonal anti-JDP1 antibody (*top panel*). Input proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R250 (*bottom panel*). Total stigma proteins were used as a positive control
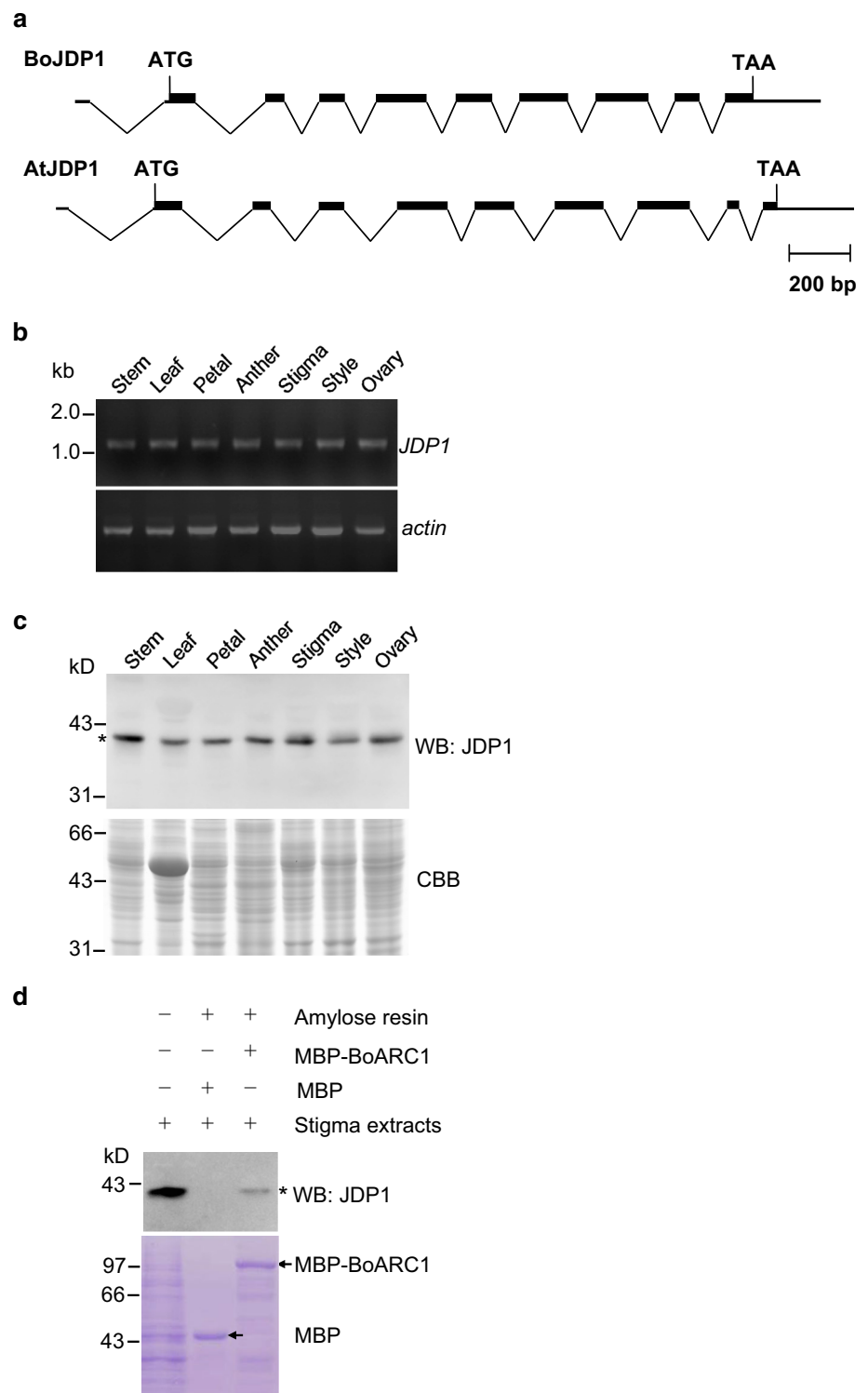
an important role in the self-incompatibility response remains to be shown. Is Tyr[8] phosphorylated as a means to regulate the interaction between JDP1 and BoARC1? Does Hsp70 bind with the BoARC1–DP1 complex as part of this regulatory system? Genetic transformation using *JDP1* and a detailed characterization of the JDP1–ARC1 complex should help us to better understand the function of JDP1 in *Brassica* self-incompatibility.

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