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Cloning, characterization, and subcellular localization of a novel JAZ repressor from *Eleusine coracana*

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Abstract

Jasmonate ZIM domain (JAZ) proteins are key regulators of the jasmonic acid (JA) signaling pathway. Repressors of JAZ remain bound to the myelocytomatosis 2 (MYC2) or MYC3/MYC4 transcription factors in the absence of JA and negatively regulate transcription of the JA responsive genes. In the presence of JA, JAZ proteins interact with coronative insensitive 1 (COI1), the recognition molecule of E3 ubiquitin ligase SCF^{COI1} (COI1 stabilized by Skp, cullin, F-box containing complex), get ubiquitinated, and subsequently degraded by the 26S proteasome. However, there is a dearth of knowledge about this gene family in monocot cereals, specifically its role in finger millet is unknown till date. Here we present the isolation and characterization of a novel JAZ family repressor gene from nonsequenced *Eleusine coracana* (*EcJAZ*) utilizing available genome information of *Oryza*, *Sorghum*, and *Setaria.* The *Ec*JAZ sequence showed the presence of a conserved ZIM domain, the Jas motif, and N-terminal motif 7 like other Group1 TIFY sequence containing proteins. We observed coronatine (an analog of JA-Ile) dependent and time dependent degradation of recombinant *Ec*JAZ that thereby fulfilled the basic characteristic of the JAZ proteins. We found a proteasome inhibitor N-(phenylmethoxy) carbonyl-L-leucyl-N-[(1R)-1-formyl-3-methylbutyl]-L-leucinamide) (MG132) mediated degradation inhibition of *Ec*JAZ that supported its 26S proteasome mediated degradation. Our study shows the nuclear localization of GFP-*Ec*JAZ by *Agrobacterium* mediated transient transformation of onion scale epidermal cells. In *Eleusine* leaves, transcription of *EcJAZ* increased 4.2-fold by salt stress and 5.5-fold by coronatine application; thus ascertained its inducibility by the abiotic stress as well as by bioactive JA-Ile. Taken together, all these results contribute to our understanding of the JA signaling pathway in *Eleusine coracana*.

Additional key words: *Agrobacterium* transformation, coronatine, finger millet; jasmonic acid, salt stress.

Introduction

Jasmonic acid (JA) has been implicated in regulation of root growth (Staswick *et al*. 1992), fertility (Stinzi and Browse 2000), fruit ripening (Perez *et al*. 1997), trichome development (Qi *et al*. 2011), senescence (Xiao *et al*. 2004), and in responses to feeding insects (Howe *et el*. 1996), necrotrophic pathogens (Feys *et al*. 1994), salinity, drought (Zhu *et al*. 2002), ozone, UV radiation (Conconi *et al*. 1996), and mechanical wounding (Farmer *et al*. 1992, Reymond *et al*. 2000). Jasmonic acid is activated by conjugation with the amino acid isoleucine (Ile) that produces a highly bioactive hormonal signal (3R, 7S)-jasmonoyl-L-isoleucine (JA-Ile) (Staswick *et al*. 2004, Fonseca *et al*. 2009). Coronatine, a virulence factor produced by the strains of the hemibiotrophic plant pathogen *Pseudomonas syringe*, is reported to mimic the bioactive hormone JA-Ile both structurally and functionally (Bender *et al*. 1999, Katsir *et al*. 2008).

In Nature 2007, two groups (Chini *et al*. 2007, Thines *et al*. 2007) reported the jasmonate zinc finger (ZIM) domain (JAZ) family of transcriptional repressors as SCF^{COI1} (COI1 stabilized by Skp, cullin, F-box containing complex) substrate targets, which bind to coronatine

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Abbreviations: ABA - abscisic acid; COI1 - coronatine insensitive 1; GFP - green fluorescence protein; Ile - isoleaucine; JA - jasmonic acid; JAZ - jasmonate ZIM domain; MeJa - methyl jasmonate; MG132 - N-(phenylmethoxy) carbonyl-L-Leucyl-N-[(1R)-1-formyl-3-methylbutyl]-L-leucinamide; MYC - myelocytomatosis; RE - restriction endonuclease; SCF^{COI1}- COI1 stabilized by Skp, Cullin, F-box containing complex; TIFY - conserved TIFY sequence containing protein; ZIM - zinc finger protein expressed in inflorescence meristem.

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insensitive (COI1) in a hormone dependent manner. Jasmonate ZIM domain proteins belong to the plant-specific TIFY sequence containing protein family of transcriptional regulators containing a TIF[F/Y]XG motif within a conserved ZIM (or TIFY) domain (~28 amino acids) (Vanholme *et al.* 2007, Chung *et al*. 2009). Alike the ZIM domain, JAZ proteins also contain a multifunctional JA associated (Jas) motif, a C-terminal conserved region. A Jas motif interacts with the basic helix-loop-helix (bHLH) transcription factors MYC2, MYC3, MYC4, transparent testa 8 (TT8), glabra (GL) 3, enhancer of glabra 3 (EGL3), two R2R3 MYB transcription factors, MYB75 and GL1, and most importantly with COI1 (Cheng *et al*. 2011, Fernandez-Calvo *et al.* 2011, Qi *et al*. 2011). Jasmonate ZIM proteins interact with co-repressor topless (TPL) by the ZIM domain through the adaptor protein NINJA (Novel Interactor of JAZ proteins) (Pauwels *et al*. 2010). A recent review depicted how individual isoforms of the *Arabidopsis* JAZ protein interact with a specific set of co-repressors and transcription factors (Kazan and Manners 2012).

A COI1, an F-box protein, is known to function as a component of the ubiquitin E3 ligase complex SCF^{COI1} (Xie *et al*. 1998). Ubiquitin ligases E3 catalyze ubiquitination of target proteins, which results in a rapid degradation of the target proteins by the 26S proteasome (Kepinski 2007). The JA-Ile actually acts as a molecular glue inducing interaction of the SCF^{COI1} complex and JAZ repressors, which are ubiquitinated and targeted to the 26S proteasome for degradation (Sheard *et al.* 2010). In the absence of JA-Ile, JAZ proteins actively repress MYC2, the transcription factor that bind to the *cis*-acting elements of JA responsive genes. Therefore, JA-Ile mediated degradation of JAZs cause derepression of the transcription factor MYC2 and activation of JA responsive genes (Chini *et al*. 2007, Thines *et al*. 2007).

To determine the actual mechanism by which the COI1-JAZ co-receptor senses JA, crystalization and structure elucidation of the COI1-ASK1-JAZ1 degron peptide complex together with either JA-Ile or coronatine were performed. The crystal structure of the COI1-JAZ co-receptor in complex with JA-Ile unveils a direct interaction of the hormone with both COI1 and JAZ1 protein (Sheard *et al*. 2010).

Though the JA signaling pathway is essential for plant survival in nature, most of our current knowledge on this signaling pathway came from the dicotyledonous model plant *Arabidopsis thaliana*. Recent studies with monocots could shed light on the JA biosynthetic and signaling pathways of two monocot cereals, rice and maize (Gao *et al*. 2009, Seo *et al*. 2011,Yan *et al*. 2012, Lee *et al*. 2013). Revelation of the JA-Ile signal perception mechanism in cereal crops is expected to improve their resistance against biotic and abiotic stressors.

Eleusine coracana (finger millet), a highly nutritious grain containing 7 - 14 % of proteins and a high content of methionine, iron, and calcium, is a suitable food for diabetics and infants (Barbeau and Hilu 1993, Vadivoo *et al*. 1998, Devi *et al*. 2011). Therefore, exploration of the JA signaling pathway of finger millet might further potentiate this highly beneficial crop with respect to its stress tolerance.

The aim of this study was cloning, characterization, and subcellular localization of a novel JAZ repressor from *Eleusine coracana*. Owing to the nonavailability of finger millet genome information, we cloned the *Eleusine coracana JAZ* (*EcJAZ*) gene by using the *Setaria* (Zhang *et al*. 2012) genome information. The subcellular localization of *Ec*JAZ was showed by *Agrobacterium* mediated transient transformation of onion epidermal cells. We also investigated the expression patterns of the *EcJAZ* gene in response to salinity, coronatine, salicylic acid, methyl jasmonate, and abscisic acid at different time points.

Materials and methods

Plants and growth conditions: *Eleusine coracana* (L.) Gaertn. cv. GPU28 seeds (obtained from Gandgi Krishi Vigyan Kendra, Bangalore, India) were grown on 1/10 Murashige and Skoog medium (pH 6.0). The plates were kept at room temperature in the dark for one day and then transferred to a chamber with a 16-h photoperiod, an irradiance of 100 µmol m^{-2} s^{-1}, and day/night temperatures of 22/18 ºC. The seedlings (15-d-old) were treated with 100 µM methyl jasmonate (MeJa), 100 µM MeJa + 200 µM abscisic acid (ABA), 1 mM salicylic acid, 200 mM NaCl, 200 pM coronatine (a JA-Ile analog), and water deficit (4 d) and harvested at different time points for cDNA synthesis. For extraction of the total RNA, 200 mg of leaves were washed with diethyl pyrocarbonate water, freezed under liquid nitrogen, and stored at -80 °C.

Extraction of the total RNA and cDNA synthesis: Total RNA was extracted with a *Plant RNA* kit (*Macherey-Nagel*, Duren, Germany) from all treated and untreated samples. Integrity and concentrations were checked by gel electrophoresis and spectrophotometry, respectively. For cDNA synthesis with *Thermoscript* reverse transcriptase (*Invitrogen*, Carlsbad, USA), 3 µg of each of total RNA was used.

Screening of expressed sequence tag (EST) repositories of *Eleusine coracana*: Finger millet EST repositories, the plant genome database (*GDB*) *EST* assembly [1 120 ESTs in PUT-181a series (Plant GDB derived unique transcripts, version 181a, dated 01-12-2011)], *NCBI dbEST* (1 934 ESTs), and the *Clean EST* database (158 water stress EST, 207 drought stress EST, 19 developing seeds EST) have been screened against

JAZ gene sequences of *Oryza* and *Sorghum* (*Os*JAZ1, Sb06g031060.1, and Sb07g020910.1). Though varied sequence identities were obtained, no DNA sequence representing the conserved sequences of JAZ proteins was obtained from the EST screening.

Cloning *EcJAZ* on the basis of genome information of other plants: Owing to scarcity of genome sequence information for finger millet, we attempted to clone *Eleusine coracana JAZ* gene sequences on the basis of *Oryza sativa* JAZ protein sequences reported by Seo *et al*. (2011) and the whole genome sequence of *Sorghum bicolor* (Paterson *et al.* 2009). Primers for PCR were designed according to *Os*JAZ1, *Os*JAZ2, *Os*JAZ4, and *Os*JAZ5 sequences, and two JAZ protein sequences of *Sorghum bicolor*, Sb06g031060.1 and Sb07g020910.1 (Table 1 Suppl.), obtained from the Munich information centre for protein sequence (*MIPS*) database (Mewes *et al*. 2002) and the similarity matrix of proteins (*SIMAP*) database (Rattei *et al*., 2010). The PCR was carried out on different cDNAs prepared from differently treated seedlings at varying annealing temperatures.

More recently, the genome sequence of *Setaria italica* (foxtail millet) was published (Zhang *et al*. 2012). A phylogenetic relationship study revealed that foxtail millet is split from *Sorghum* and maize about 27 million years ago and the highest colinearity of the foxtail millet genome was observed with maize (87.6 %). As our attempts to clone the *Eleusine coracana JAZ* gene on the basis of *Oryza* and *Sorghum JAZ* sequences failed, *Setaria italica* genome data available in the plant genome database *PlantGDB* (www.plantgdb.org/SiGDB) was searched against *OsJAZ* and *SbJAZ* accessions. Primers were designed on the basis of *Setaria italica JAZ* gene sequences *Si-OsJAZ1*, *Si-OsJAZ2*, *Si-OsJAZ5*, and *Si-Sb*07*JAZ1* containing conserved *ZIM* and *Jas* regions.

The DNA sequence of *EcJAZ* was analyzed by *BlastX* (Gish and States 1993) in search of the conserved domains of JAZ proteins. A recent study (Bai *et al*. 2011) on the evolutionary analysis of the TIFY transcription factor family described the presence of seven conserved motifs in the N-terminal domain of the TIFY subfamily in plants and grouped the members accordingly. The *Ec*JAZ was placed into Group1 according to the N-terminal motif present and compared with the DNA sequence of other group members by *Clustal W* multiple sequence alignment (Thompson *et al.* 1994) and the *Boxshade* server (http://ulrec3.unil.ch/software/BOX_html). Comparative representation of conserved domains of Group1 TIFY proteins along with *Ec*JAZ was generated by the *Prosite* software (De Castro *et al.* 2006). Keeping the natural variability of the conserved domains of JAZ proteins in view, logos for all three conserved regions were generated by *WebLogo* (Crooks *et al*. 2004). A phylogenetic tree was drawn with the *MEGA6* program using the neighbor-joining method (Tamura *et al*. 2013). A novel circular tree layout was developed by the *iTOL* software (Letunic and Bork 2011).

Subcloning of *EcJAZ* in the expression vector: The *EcJAZ* PCR product of 615 bp was sub-cloned into the expression vector pHis-TEV (*Biobharati Life Science*, Kolkata, India) using the BamH1 and XhoI restriction endonuclease (RE) sites. The PCR reaction mix contained 1.25 U of *Pfu* DNA polymerase (*BioBharati Life Science*), 5 mm^3 of 10× *Pfu* buffer, 1 mm^3 of 10 mM dNTP, 1 mm^3 of the forward primer (100 ng cm^{-6}) (5′-GCACGTGCGGATCCATGGCGGCCGAGCACC AGCA-3′) and 1 mm^3 of the reverse primer (100 ng cm^{-6}) (5′-CGCACTTGAGGCGCGAGTCGATTGAGCTCC GGACGTC-3′) in 50 mm^3. The PCR reaction was carried out at 94° for 5 min, then 30 cycles (94 °C for 1 min, 69 °C for 1 min, 72 °C for 2 min) followed and it was terminated by 72 °C for 10 min. Both the PCR product and the expression vector were digested with BamHI and XhoI, ligated together, and transformed into the *Escherichia coli* strain Rosettagami2DE3pLysS (*Novagen*, Madison, USA). The transformants were screened by PCR and RE digestion.

The *Ec*JAZ expressed in *E. coli* was purified by a *His-bind* purification kit (*Novagen*) using *His-bind* resin matrix equilibrated with a charge buffer (50 mM $NiSO_4$) and with a binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9). The large scale culture (3 dm^3) of *E. coli* (an absorbance $A_{600} \approx 0.6$) was supplemented with 1 mM isopropyl β-D-1-thiogalctopyranoside (IPTG), grown for 5 h, harvested, centrifuged at 3 000 *g* for 30 min and the total protein was extracted with a *Q-proteome* bacterial protein prep kit (*Qiagen,* Valencia, USA). The extracted bacterial protein was concentrated with *Centriprep* (*Merck-Millipore*, Massachusetts, USA) 3 kDa (10×) and loaded onto equilibrated *His-bind* resin matrix, washed with the binding buffer, a wash buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH7.9), and finally eluted with an elute buffer (1 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and a strip buffer (0.5 M NaCl, 100 mM $EDTANa_2$, 20 mM Tris-HCl, pH 7.9). The eluted protein was checked by SDS-PAGE (15 %, m/v).

***In vitro* degradation of *Ec*JAZ:** A total protein extract was prepared from 15-d-old finger millet seedlings, and protein content was determined by Bradford assay. The total protein extract of 100 µg in 50 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 50 µg cm^{-3} ubiquitin, with or without 30 µM coronatine and MG132 was incubated with 5 µg of the purified His-EcJAZ fusion protein at 30 °C. Aliquots were collected at different time points and reaction was stopped with an SDS-PAGE loading buffer. The His-*Ec*JAZ fusion protein present in each aliquot was determined by SDS-PAGE (15 %, m/v) and by immunoblotting using an anti-His mouse monoclonal primary antibody and polyclonal goat anti-mouse IgG (*Novagen*) as the secondary antibody.

Subcellular localization of *Ec*JAZ: The *Agrobacterium tumefaciens* strain LBA4404 was transformed with

EcJAZ-pCAMBIA1303 following the standard protocol (Gynheung 1987). *Agrobacrterium* cells (an absorbance $A_{600} \approx 0.5$) were pelleted, resuspended with 0.25 M NaCl, again pelleted, resuspended with 20 mM $CaCl_2$, mixed with 1 ug of *EcJAZ*-pCAMBIA1303 DNA, and incubated on ice for 30 min. The cells were briefly freezed in liquid N_2, thawed, resuspended in Luria Bertani (LB; *HiMedia*, Mumbai, India) medium, grown at 28 °C, and selected on LB agar plates containing rifampicin (50 µg cm^{-3}), streptomycin (25 µg cm^{-3}), and kanamycin (50 µg cm^{-3}).

The LB medium (10 cm^3) was taken in a sterile 50-cm^3 tube and supplemented with 20 µM acetosyringone, 10 mM 2-(N-morpholino)-ethane sulfonic acid (MES) buffer, 10 mM $MgCl_2$, 50 µg cm^{-3} rifampicin, 25 µg cm^{-3} streptomycin, and 50 µg cm^{-3} kanamycin and inoculated with transformed *A. tumefaciens*. The cells were kept shaking at 28 °C and 200 rpm for 1 - 2 d till an absorbance A_{600} of approximately 1.0 was reached. The cells were harvested by centrifugation at 3 000 *g* for 10 min and resuspended in the same medium to A_{600} of approximately 0.3 by gentle vortexing. The culture was left at room temperature for 6 h before infiltration. About 0.2 cm^3 of an agroinfiltration liquid was slowly injected into the interface between the adaxial epidermis and the mesophyll of the scale leaves of an onion bulb using a syringe to ensure a high transformation efficiency of the *Agrobacterium* cells (Xu *et al*. 2014). After two days, the onion scale leaves were excised and assayed for reporter gene expression.

Green fluorescent protein (GFP) expression in onion epidermal cells was detected using a fluorescence microscope (*Nikon Eclipse Ti,* Tokyo, Japan) and processed using the *NIS-Elements AR 4.20.00* software.

Expression of *EcJAZ* after different treatments: Amplification by PCR was performed on cDNAs from the differently treated (MeJa, NaCl, ABA, coronatine, wounding) 15-d-old seedlings using Si-OsJAZ1, Si-OsJAZ2, Si-OsJAZ5, and Si-Sb07JAZ1 forward and reverse primers (Table 1 Suppl.) using the *Phusion High Fidelity PCR Master Mix* (*New England Biolabs*, Ipswich, USA) The PCR was done at 98 ºC for 30 s followed by 35 cycles at 98 ºC for 10 s, 45 - 50 ºC (a gradient) for 30 s, 72 ºC for 60 s, and a final extension at 72 ºC for 10 min. The PCR products were gel extracted using a *QIAquick* gel extraction kit (*Qiagen*) and sub-cloned into the *TOPO T/A* cloning vector (pCR2.1, *Invitrogen*) and the sequence of the insert was determined (*SciGenom Labs*, Kakkanad, Kerala, India) using M13 forward and reverse primers.

Results

Eleusine coracana being a nonsequenced plant, mining its EST repositories, was emphasized to get at least some basic information on its *JAZ* sequences. However, unavailability of any such information led us to attempt cloning on the basis of possible sequence identities with *Oryza, Sorghum,* and *Setaria JAZ* equences. Finally, PCR using the Si-OsJAZ2 forward and reverse primers (Table 1 Suppl.) on a cDNA template from plants treated with MeJa for 2 h generated a 615 bp product that was sub-cloned into the T/A cloning vector and sequenced. On *BLAST* search with *BlastX*, the *Ec*JAZ sequence showed the presence of two conserved domains of JAZ proteins: TIFY (pfam 06200; ZIM domain) and CCT_2 (pfam 09425; Jas motif) at 94 - 129 residues and 152 - 178 residues, respectively. The DNA sequence of *EcJAZ* was submitted to the GenBank (acc. No. KJ689791).

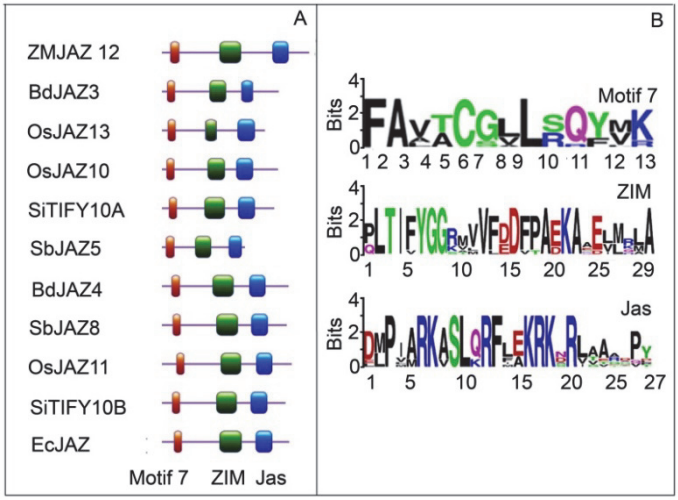
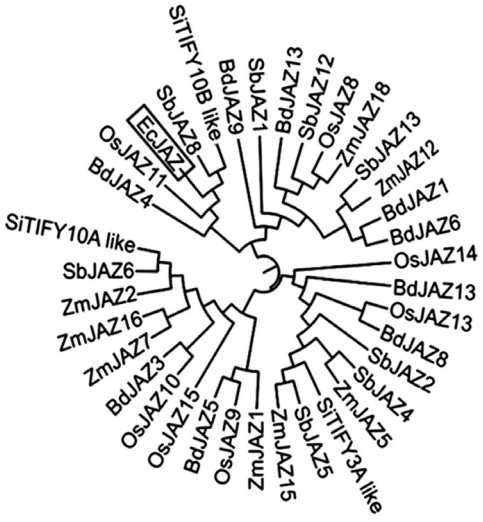


Fig. 1. *A* - Three conserved domain structures of *Ec*JAZ and other Group1 JAZs of *Poaceae*. The name of each JAZ protein is indicated *on the left*. Protein domain structures have been generated by the *Prosite* software. *B* - Sequence logos of three conserved domains have been generated using all JAZ sequences used for protein domain analysis. Logos have been produced using the *WebLogo* software.

This DNA sequence determined for the 615 bp *EcJAZ* was used for further analysis. A large group of plant JAZ proteins containing motif 7 in their N-terminal domain were included into Group1 (Bai *et al*. 2011). Owing to the presence of motif 7 (FAVTCGLLRQYMK) in the N-terminal region of *Ec*JAZ, it was included into Group 1. A comparative domain representation of *Ec*JAZ and other *Poaceae* JAZ proteins belonging to Group1 and logos for all three conserved regions that supported the inclusion of *Ec*JAZ into the Group 1 JAZ proteins were generated (Fig. 1*A*,*B*). We took the sequences of six JAZs of *Oryza sativa*, nine JAZs of *Brachypodium distachyon*, eight JAZs of *Sorghum bicolor*, and six JAZs of *Zea mays* to analyze their phylogenetic relationships (Fig. 2*A*,*B*). From Kyoto encyclopedia of genes and genomes (*KEGG*) pathways: Sita04075, *Setaria italica* JAZ sequences like *Si*TIFY10A like (*UNIPROT: K4AFP5*), *Si*TIFY10B like (*UNIPROT: K4AFD5*), *Si*TIFY3A like (*NCBI GeneID: 101786707*), *Si*TIFY3B like (*UNIPROT: K3Z9Q4*), *Si*TIFY6A like (*UNIPROT: K3ZTZ1*) and *Si*TIFY6B like (*UNIPROT: K3YHT8*) were deduced. Among the *Setaria* JAZ proteins, *Si*TIFY10A like, *Si*TIFY3A like, and *Si*TIFY10B like contained N-terminal motif 7, so we considered them for phylogenetic relationship analysis as the Group1 proteins (Fig. 2*A*,*B*). A circular phylogenetic tree showed a close evolutionary relationship of *Ec*JAZ with *Si*TIFY10B like, *Os*JAZ11, and *Sb*JAZ8 proteins. We also compared the Group1 JAZ proteins of *Poaceae* by *ClustalW*. For *Ec*JAZ, we found the highest alignment score of 61 with *Si*TIFY 10B like protein, followed by *Os*JAZ11, which showed an alignment score of 52. For all other Group1 proteins, the alignment score ranged from 30 to 40 only, supporting an ample sequence variation among the JAZ family members (Fig. 1 Suppl).

A



B

Organism	Gene name	Locus name
Oryza sativa	OsJAZ8	LOC_Os09g26780.1
	OsJAZ9	LOC_Os03g08310.1
	OsJAZ10	LOC_Os03g08330.1
	OsJAZ11	LOC_Os03g08320.1
	OsJAZ13	LOC_Os10g25230.1
	OsJAZ15	LOC_Os03g27900.1
Brachypodium distachyon	BdJAZ1	Bradi1g21490.1
	BdJAZ3	Bradi1g72590.1
	BdJAZ4	Bradi1g72600.1
	BdJAZ5	Bradi1g72610.1
	BdJAZ6	Bradi3g10820.1
	BdJAZ7	Bradi3g23180.1
	BdJAZ8	Bradi3g23190.1
	BdJAZ9	Bradi3g23220.1
Sorghum bicolor	SbJAZ1	Sb01g023290.1
	SbJAZ2	Sb01g023300.1
	SbJAZ4	Sb01g023330.1
	SbJAZ5	Sb01g023431.1
	SbJAZ8	Sb01g045180.1
	SbJAZ12	Sb02g025720.1
	SbJAZ13	Sb02g039190.1
Zea mays	ZmJAZ1	GRMZM2G343157_T01
	ZmJAZ2	GRMZM2G445634_T01
	ZmJAZ5	GRMZM2G145412_T01
	ZmJAZ12	GRMZM2G101769_T01
	ZmJAZ15	GRMZM2G173596_T01
	ZmJAZ18	GRMZM2G116614_T01
Setaria italica	TIFY3A	XP_004983105.1
	TIFY10A	XP_004985485
	TIFY10B	XP_004985487.1
Eleusine coracana	EcJAZ	KJ689791 (GenBank)

Fig. 2. *A* - A phylogenetic tree generated with *Ec*JAZ and other JAZ protein sequences of the *Poaceae* family belonging to Group1 drawn with the help of the *MEGA6* program using the neighbor-joining method. Circular tree layout was developed by the *iTOL* software. The position of *Ec*JAZ has been marked by a *box*. The names of JAZ proteins are mentioned. *B* - The names of JAZ proteins used for phylogenetic tree generation, their plant sources and locus names excepting *Ec*JAZ for which GenBank acc. No. was given.

For further characterization of the *Ec*JAZ protein, the DNA sequence of 615 bp corresponding to *EcJAZ* was sub-cloned into the pHis-TEV vector in the BamHI and XhoI sites and expressed as a His-tagged protein in *E. coli*. The selected transformant was finally screened by RE digestion and release of the 615 bp *EcJAZ* insert DNA. After IPTG induction, His-tagged *Ec*JAZ was purified by a His-bind resin column as a 22 kDa single protein band by SDS-PAGE and silver staining (Fig. 3*A*).

Jasmonate ZIM domain repressor proteins, the key regulators of the JA signaling pathway, get degraded in the presence of bioactive JA-Ile and its analog coronatine. The purified recombinant His-*Ec*JAZ fusion protein was incubated with the finger millet protein extract in the presence and absence of coronatine, and degradation of the protein was checked at 15, 30, and 60 min by immunoblotting with the anti-His antibody. Degradation of the His-*Ec*JAZ fusion protein after incubation for

60 min in the presence of coronatine indicates coronatine-mediated and time dependent degradation of *Ec*JAZ (Fig. 4*B*). In the presence of coronatine, the *Ec*JAZ protein intuitively interacted with the endogenous *Ec*COI1 (from the plant extract) and thereby with the E3 ubiquitin ligase complex, got ubiquitinated, and degraded by the endogenous 26S proteasome. This fact was supported by the reversal of coronatine mediated degradation of *Ec*JAZ in the presence of MG132, an inhibitor of the 26S proteasome (Fig. 3*B*).

In the absence of bioactive JA-Ile, JAZ repressor proteins remain bound to MYC2/MYC3/MYC4, the transcriptional activator for the JA responsive genes. Though the nuclear localization motif was not found in *Arabidopsis* JAZ isoforms, it interacted with COI1 and several other transcriptional regulator proteins through its conserved ZIM domain and the Jas motif and therefore substantiated its localization into the nucleus of the cell (Thines *et al*. 2007). We studied the actual subcellular localization of *Ec*JAZ in onion scale epidermal cells by *Agrobacterium*-mediated transient transformation. In the nucleus of the onion epidermal cells, expression of *Ec*JAZ-*mGFP* was visualized by fluorescence microscopy (Fig. 4).

To elucidate the different responses of *EcJAZ* to treatment with NaCl, MeJa + ABA, coronatine, salicylic acid, and water deficit stress, PCR was done from the cDNAs prepared from the differently treated finger millet seedlings harvested at varying time points. A pronounced increment of *EcJAZ* transcription was found at 2 h of the NaCl stress and 24 h with coronatine, whereas mild increments at different time points of the same treatment and MJ + ABA combined treatment were observed. Expression of *EcJAZ* was not evident after salicylic acid or water stress treatment (Fig. 5).

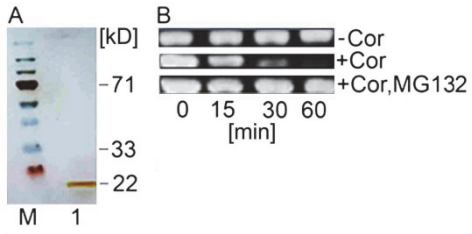


Fig. 3. *A* - Purification of a 22 kD His-*Ec*JAZ fusion protein from pHis-TEV in *E. coli* by His-bind resin column. *B* - *In vitro* degradation of a recombinant *Ec*JAZ in protein extract of finger millet seedlings in a coronatine and time dependent manner.

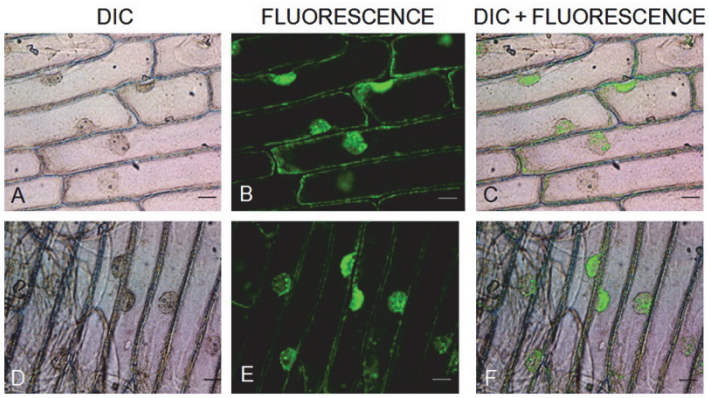


Fig. 4. A differential interference contrast (DIC) image of onion scale epidermal cells transiently transformed with *Agrobacterium tumefaciens* LBA 4404:pCAMBIA1303:*EcJAZ* cells (*A* and *D*). A fluorescence microscopy image of the same onion scale epidermal cells showing green fluorescent protein (GFP) expression (*B* and *E*). A merged image of the same onion scale epidermal cells showing GFP expression (*C* and *F*). *Bars* = 100 µm.

Discussion

Survival of plants depends on their ability to perceive and respond to external challenges through transcriptional reprogramming in response to the external factors (Pauwels *et al*. 2008). Similarly as other phytohormones, JA regulates many developmental and stress responses throughout the entire plant life (Browse 2009). Most of the studies on the JA pathway were performed on the model dicotyledonous plant *Arabidopsis thaliana*, however, *Eleusine coracana* remained understudied till date and phytohormone signaling pathways for further development of its biotic as well as abiotic stress tolerance remain awaited.

In this study, we cloned a gene encoding the JAZ repressor protein of finger millet (*Ec*JAZ) to open up exploring its JA signaling pathway at the molecular level. In the absence of adequate genome information of *Eleusine*, we had to use the *Oryza, Sorghum*, and finally *Setaria* (Zhang *et al*. 2012) genome databases (Table 1 Suppl). As analyzed with *BlastX*, the *Ec*JAZ sequence showed the presence of both the conserved regions, the ZIM domain and Jas motif (Fig. 1). Keeping the N-terminal conserved motifs of TIFY proteins in view, we found N-terminal motif 7 (FAVTCGLLRQYMK) (Bai *et al*. 2011) in *Ec*JAZ and therefore considered it to

belong to the Group1 subfamily (Fig. 1,2 Suppl.).

Our experiment to evaluate the JA-Ile/coronatine mediated degradation revealed *Ec*JAZ degradation in a coronatine and time dependent manner. This observation indicates toward a probable interaction of *Ec*JAZ-*Ec*COI1, which caused ubiquitination and proteasome mediated degradation of *Ec*JAZ (Fig. 3). Jasmonic acid-mediated degradability is an essential feature for JAZ proteins. Coronatine, is an analog of jasmonoyl isoleucine (JA-Ile). Coronatine mediated degradation of *Ec*JAZ would definitely suffice establishing itself as a member of the JAZ repressor family.

Several studies reported the expression of JAZ-GFP fusion proteins in the plant nucleus (Chini *et al*. 2007, Thines *et al*. 2007). Core JA signaling components, including a transcriptional co-repression complex consisting of the JAZ, novel interactor of JAZ proteins (NINJA), and topless (TPL) proteins bound to the JA-responsive transcription factors and COI1-JAZ JA-receptor complex, appeared to be located in the nucleus (Pauwels *et al*. 2010). A recent study of Withers *et al.* (2012) revealed a highly conserved arginine residue in the Jas motif, which could critically couple JAZ9-MYC2 interaction with the nuclear localization of JAZ9 on one way and its repressor functionality on the other. Therefore, their result established the nuclear localization of JAZ9 to be the important criterium for its repressor activity through MYC2 binding. Our experiment clearly shows the expression of *Ec*JAZ in the nucleus of the onion epidermal cells (Fig. 4). Therefore, it appears that *Ec*JAZ was functional in the nucleus as a transcriptional regulator, which indirectly supported its MYC2 binding ability and thereby its repressor activity.

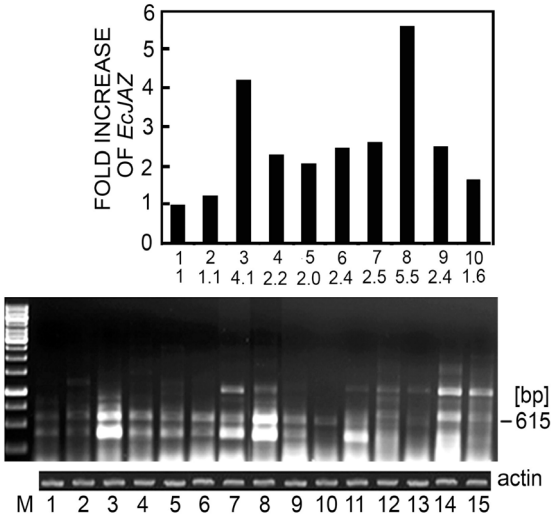


Fig. 5. Study of *EcJAZ* transcription after different treatments. *Lanes*: M - 1 kb DNA ladder; 1 - untreated seedlings; 2,3,4,5 - NaCl stress for 1, 2, 18, and 24 h; 6,7,8 - coronatine for 1, 2, and 24 h; 9,10 - MeJa + ABA for 1 and 2 h; 11,12,13 - salicylic acid for 1, 2, and 24 h; 14,15 - water stress for 18 and 24 h. A 615 bp band of *EcJAZ* is shown. The upper panel shows the fold increase of the *EcJAZ* transcription of the *EcJAZ* band obtained in lanes 1 - 10 as measured by the *Image J* software (data below).

Our experimental result shows a clear increment of *EcJAZ* transcription (4.2-fold) in response to the salt stress for 2 h (Fig. 5). A previous work on rice revealed a lower endogenous JA content in salt sensitive cultivars than in salt tolerant cultivars (Kang *et al*. 2005). Similarly, salt stress results in a high content of endogenous JA in tomato cultivars (Pedranzani *et al*. 2003). Jasmonate ZIM domain proteins not only regulate expression of JA responsive genes, but also belong to the JA responsive group of genes themselves (Chini *et al*. 2007, Thines *et al*. 2007). Intuitively, a salt stress-induced rapid increment of *EcJAZ* transcription might reflect an increase of endogenous JA content, activation of JA-responsive genes through the MYC2 transcription factor and increased expression of *EcJAZ* as a JA-responsive gene. Additionally, our result shows a clear enhancement of *EcJAZ* transcription in response to the 24 h treatment with coronatine (5.5-fold than at 2 h). A similar increment of several JAZ trancsripts (JAZ2, JAZ6, JAZ9, and JAZ12) at the 24 h time point than those of the 2 h time point in herbivore attacked *Arabidopsis* leaves (Chung *et al*. 2008) supports our finding. Jasmonate ZIM domain proteins are the key regulators for "off" and "on" of the JA responsive genes, and we speculate that the onset of delayed transcript increment of *EcJAZ* followed by its degradation might precede synthesis of JA late-response genes. Therefore, in finger millet, existence of a crosstalk of JA signaling and salt stress response involving *Ec*JAZ could not be ruled out.

In conclusion, we have presented here a novel JAZ repressor *Ec*JAZ from finger millet with the aim of exploring its JA signaling pathway. A future work towards delineating protein-protein interaction of *Ec*JAZ would help to unravel the mechanisms of the JA signaling pathway in finger millet.

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