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Original Paper

Identification of genes regulated by a jasmonate- and salt-inducible transcription factor JRE3 in tomato

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Abstract In *Solanum lycoperisicum* (tomato), a transcription factor of APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family, JASMONATE-RESPONSIVE ERF 3 (JRE3), is a closest homolog of JRE4, a master transcriptional regulator of steroidal glycoalkaloid (SGA) biosynthesis. In tomato genome, *JRE3* resides in a gene cluster with *JRE4* and related *JRE1*, *JRE2*, and *JRE5*, while *JRE6* exists as a singleton on a different chromosome. All of the *JRE*s are induced by jasmonates (JAs), whereas sodium chloride (NaCl) treatment drastically increases the expression of the *JRE*s except for *JRE4* and *JRE6*. In this study, to get insights into the regulatory function of the JA- and NaCl-inducible *JRE3*, a series of genes upregulated by *β*-estradiol-induced overexpression of *JRE3* are identified with microarray analysis in transgenic tomato hairy roots. No gene involved in the SGA pathway has been identified through the screening, confirming the functional distinction between JRE3 and JRE4. Among the JRE3-regulated genes, we characterize the stress-induced expression of genes encoding malate synthase and tonoplast dicarboxylate transporter both involved in malate accumulation. In transient transactivation assay, we reveal that both terminal regions of JRE4, but not a central DNA-binding domain, are indispensable for the induction of a gene involved in the JRE4 regulon. Functional differentiation of the JREs is discussed.

Key words: ERF transcription factor, jasmonate, salt, steroidal glycoalkaloids, tomato.

Introduction

Plants have developed tremendous abilities adapting to fluctuating environment, responding to a diverse range of biotic and abiotic stresses mainly by reprogramming gene expression. Transcriptional regulators play a central role integrating upstream signaling steps with expression of downstream genes involved in defense and adaptive mechanisms. A large number of genes encoding transcription factors, grouped into a handful of protein families, are present in plant genomes; the transcription factor families, such as APETALA2/ETHYLERNE RESPONSE FACTOR (AP2/ERF), bHLH, and MYB, have largely expanded in plants (Feller et al. 2011; Nakano et al. 2006). Transcription factors differentially respond to a wide range of environmental and developmental stimuli in multiple layers of signaling networks, ensuring adaptive plasticity of plants in nature.

Jasmonates (JAs) play a key signaling role in a diverse range of plant responses from elicitation of defense-related metabolic pathways to responses to abiotic stresses (Wasternack and Hause 2013). A group of JA-responsive transcription factors of AP2/ERF family, classified in clade II of subgroup IXa (Nakano et al. 2006; Shoji et al. 2013), such as ORCAs from *Catharanthus roseus* (Paul et al. 2017; van der Fits and Memelink 2000), ERF189 from tobacco (Shoji et al. 2010), and JASMONATE-RESPONSIVE ERF 4 (JRE4), or GLYCOALKALOD METABOLISM 9, from *Solanum lycoperisicum* (tomato) (Cárdenas et al. 2016; Nakayasu et al. 2018; Thagun et al. 2016), are involved in transcriptional regulation of unrelated specialized metabolic pathways in various plant species (Shoji 2018).

In tomato, JRE4 is a master transcriptional regulator of steroidal glycoalkaloid (SGA) biosynthesis (Nakayasu et al. 2018). As reported for its homologs from tobacco (Kajikawa et al. 2017) and *C. roseus* (Paul et al. 2017), in tomato genome, *JRE4* resides in a tandem gene cluster with related *JRE1*, *JRE2*, *JRE3*, and *JRE5* on chromosome I, while *JRE6*, also structurally related

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to them, is not in the cluster but present as a singleton on chromosome V (Thagun et al. 2016). In addition to response to JA common to all the *JRE*s, the *JRE*s except for *JRE4* and *JRE6* show basal expression at low levels and are strongly elicited with sodium chloride (NaCl) treatment, suggesting the involvement of these *JRE*s in abiotic resistance rather than SGA regulation (Nakayasu et al. 2018). Indeed, it had been demonstrated that induced overexpression of *JRE3*, the closest functional homolog of *JRE4*, did not result in the induction of SGA pathways in tomato hairy roots (Nakayasu et al. 2018). In this study, to understand the regulatory role of JA- and NaCl-inducible *JRE3*, we conduct the microarray-based transcript profiling and identify a series of genes regulated by *JRE3*, including those involved in malate biosynthesis and accumulation. We discuss the functional differentiation of the mostly clustered *JRE* genes in tomato.

Materials and methods

Hairy root culture

The binary vectors *pXVE-JRE3* for overexpression and *pJRE3-EAR* for dominant suppression had been generated in Nakayasu et al. (2018) and Thagun et al. (2016), respectively. The vectors were introduced into *Agrobacterium rhizogenes* ATCC15834 by electroporation. Sterilized seeds of *Solanum lycopersicum* cv. Micro-Tom were germinated on half-strength Murashige and Skoog (MS) medium solidified with 0.6% (w/v) agar and supplemented with 2% (w/v) sucrose. Hypocotyls from two-week-old seedlings were used for the infection as described (Thagun et al. 2016). Hairy roots emerging were excised and cultured three times every week on solidified MS medium containing 300 mg l^{-1} cefotaxime for disinfection and appropriate antibiotics for drug resistance selection. The selected transgenic lines were cultured every 8 days in 100 ml glass flasks filled with 25 ml of liquid Gamborg B5 medium supplemented with 2% (w/v) sucrose with shaking at 100 rpm in the dark.

qRT-PCR analysis

Total RNA was isolated from the samples using an RNeasy kit (Qiagen) and converted to cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo). Using the cDNAs as templates, PCR was performed using a Light Cycler 96 (Roche) with SYBR Premix Ex Taq (Takara) as described (Shoji et al. 2010). Primer sequences are given in Supplementary Table 1. *EF1α* (*Solyc05g005060*) was used as a reference gene.

Microarray analysis

Total RNA was isolated from hairy roots treated with β-estradiol at 100 μM for 12 h. Checking of RNA integrity, labelling of cRNA probes, hybridization of a tomato custom oligoarray, and data acquisition and processing were done as described (Thagun et al. 2016). Probes with low signal intensity (average values for the two controls <0.2) and variable intensities between lines (differences between the two controls >2.5-fold) were excluded from the analysis. Values relative to the controls were obtained by pairwise comparisons and averaged. Up-regulated (>3-fold) are listed in Table 1.

Transient transactivation assay

The cDNA portions of *JRE3* and *JRE4*, terminal regions and a central DNA-binding domain, were separately amplified with primers (Supplementary Table 2), some of which were designed to contain the sequences that are shared between the two *JREs* and are from the adjacent regions, or partial sequences of *attB1* and *attB2* adapters for subsequent cloning. To join the fragments, certain three out of the six amplicons were combined and amplified by PCR with a pair of most outer primers. The jointed products were amplified once more by PCR with appropriate primers to attach full-length *attB1* and *attB2* sequences at the ends. The chimeric and native fragments for *JRE3* and *JRE4* with the adapter sequences were cloned into *pDONR/Zeo*, and then transferred into *pGWB17* (Nakagawa et al. 2007) using Gateway cloning technology (Invitrogen). The vectors, *p19* for P19 silencing suppressor (Voinnet et al. 2003) and *p35S-GFP* for the *GFP* reference gene, were used.

Transient transactivation assay was performed as described (Thagun et al. 2016). The bacterial suspensions for *JRE*-containing vectors plus those for *p19* and *p35S-GFP* were combined, and the resultant solution was injected into mature green fruits. Gene expression was analyzed by qRT-PCR using fruits harvested 3 day after injection.

Results

A series of genes upregulated by β-estradiol-induced overexpression of JRE3 in tomato hairy roots

We had demonstrated that β-estradiol-induced overexpression of *JRE4* but not *JRE3* activates SGA biosynthesis genes and increases the accumulation of the alkaloids in transgenic tomato hairy roots (Nakayasu et al. 2018). To get insights into the regulatory function of *JRE3*, microarray-based transcript profiling was conducted using transgenic tomato hairy roots. One of the transgenic lines for induced overexpression of *JRE3* (line OX1), which had been established previously (Nakayasu et al. 2018), and two control lines transformed with an empty vector (EV), line EV1 and EV2, were treated with β-estradiol at 100 μM for 12 h. The chemical-induced overexpresion of *JRE3* in the OX1 line was confirmed by qRT-PCR (Supplementary Figure 1). RNAs prepared from the lines were labelled with Cyanine 3 and hybridized to a tomato custom oligo-array representing over 40,000 transcripts. After excluding probes with low and variable signal intensities in the controls, probes and corresponding genes up-regulated over 3 folds in the line OX1 relative to the controls were listed in Table 1; when

Table 1. A list of gene up-regulated by induced overexpression of *JRE3* in transgenic tomato hairy roots. Probes and corresponding genes are ordered according to fold change values.

Probe ID	Accession	Gene ID	Annotation	Fold change	Note
TowgeN_I_39209	AI781582	Solyc12g006380	2-oxoglutarate-dependent dioxygenase	8.840322627	
TowgeN_I_18907	AW648100	Solyc03g111140	Malate synthase	8.234575349	*2, *3
TowgeN_I_13980	BE458907	Solyc08g082970	FAS-associated factor	7.609107197	
TowgeN_I_35461	AW615959	Solyc11g012200	Wax synthase	6.309291351	*1, *4
TowgeN_I_24215	BE354617	Solyc05g007020	Potassium voltage-gated channel	5.792815471	
TowgeN_I_20544	TC180353	Solyc12g011410	Unknown protein	5.376263687	
TowgeN_I_25911	TC185720	Solyc01g080680	Sugar facilitator protein 3	5.108688383	*1
TowgeN_I_48778	AI776728	Solyc03g043740	Hydroxyproline-rich glycoprotein	4.975912183	*2
TowgeN_I_22317	TC182126	Solyc05g009170	Zinc finger family	4.965013863	
TowgeN_I_25497	TC185306	Solyc11g012360	Tonoplast dicarboxylate transporter	4.930313696	*3, *4
TowgeN_I_29299	TC189108	Solyc10g078720	MYB family transcription factor	4.85158986	
TowgeN_I_49124	AI778123	Solyc01g010770	Hypersensitive-induced response protein	4.8211947	
TowgeN_I_47464	AW625285	Solyc01g009935	SAUR-like auxin-responsive	4.55383965	
TowgeN_I_44891	BG132464	Solyc07g021180	ATP synthase subunit beta	4.524328714	
TowgeN_I_41543	BP876253	Solyc08g079020	Adenine phosphoribosyltransferase-like protein	4.474134291	
TowgeN_I_28695	AW219535	Solyc03g031530	Sulfite exporter	4.416792363	
TowgeN_I_42296	BP881103	Solyc06g084140	Sulfate transporter	4.314994722	*2
TowgeN_I_31166	TC190975	Solyc01g006780	Nitrate reductase	4.310660258	*4
TowgeN_I_40284	AW621208	Solyc06g075660	MYB family protein	4.263023623	*2
TowgeN_I_50753	AW036111	Solyc11g051090	Sulfotransferase	4.220919529	
TowgeN_I_47590	BF097299	Solyc01g006660	Subtilisin-like protease	4.205077454	
TowgeN_I_28691	TC188500	Solyc05g056430	Small auxin up-regulated RNA56 SlSAUR56	4.191859756	
TowgeN_I_48471	BG127874	Solyc06g008920	AMP dependent ligase	4.111344048	
TowgeN_I_51374	AW621504	Solyc02g032550	Apyrase	4.078820069	
TowgeN_I_26159	TC185968	Solyc07g047950	GRAS family transcription factor	4.070848818	
TowgeN_I_21911	TC181720	Solyc02g084980	Hexosyltransferase	3.982044619	
TowgeN_I_46572	AW031420	Solyc04g079240	Patatin	3.948301885	
TowgeN_I_27740	TC187549	Solyc04g080820	Cytokinin oxidase4	3.923788624	
TowgeN_I_14317	TC174126	Solyc05g011860	Sulfotransferase	3.917782001	
TowgeN_I_47603	BF096561	Solyc03g113490	HIPL1-like protein	3.914651814	
TowgeN_I_43348	DY523780	Solyc10g084950	Major facilitator superfamily protein	3.894891506	
TowgeN_I_14980	TC174790	Solyc08g081540	1-aminocyclopropane-1-carboxylate synthase	3.878530849	
TowgeN_I_11251	TC171060	Solyc12g096770	Acyl-transferase	3.834529274	
TowgeN_I_21735	TC181544	Solyc11g072800	Respiratory burst oxidase-like protein	3.834514454	
TowgeN_I_49906	AI782504	Solyc05g006740	Glutathione S-transferase	3.81732516	*4
TowgeN_I_51360	AW622335	Solyc03g005820	Purine permease	3.805744513	
TowgeN_I_29033	TC188842	Solyc03g112320	Ferric reduction oxidase 8	3.792445194	
TowgeN_I_29740	TC189549	Solyc08g082440	UDP-glucose 4-epimerase	3.775583825	
TowgeN_I_42041	BP882954	Solyc05g052870	Glycosyltransferase	3.771290476	
TowgeN_I_21955	TC181764	Solyc06g082470	MAP kinase kinase kinase 42	3.769692059	
TowgeN_I_42592	BP888894	Solyc02g093750	C3H4 type zinc finger protein (DUF23)	3.762826536	
TowgeN_I_37530	AW096562	Solyc12g009920	Glycosyltransferase	3.744698232	*1
TowgeN_I_29454	TC189263	Solyc02g091350	Glycosyltransferase	3.737364945	*2
TowgeN_I_48975	BE353810	Solyc04g078340	Cytochrome P450	3.729097043	
TowgeN_I_47164	AW039590	Solyc04g079230	Patatin	3.728356964	
TowgeN_I_48056	AW041547	Solyc03g121960	Phospholipid:diacylglycerol acyltransferase	3.700317129	*1
TowgeN_I_26135	TC185944	Solyc01g010000	ACT domain containing protein	3.688132634	*4
TowgeN_I_13226	TC173035	Solyc12g008800	Myb-like transcription factor family protein	3.568294038	
TowgeN_I_26381	TC186190	Solyc06g007180	Asparagine synthetase 1	3.533410617	
TowgeN_I_51343	AW622109	Solyc09g064910	Adenylate isopentenyltransferase	3.515774315	
TowgeN_I_15800	TC175609	Solyc01g067510	Kinase family protein	3.51500259	*1
TowgeN_I_50449	AW621226	Solyc09g011140	NAD(P)-binding Rossmann-fold superfamily protein	3.506256872	
TowgeN_I_29450	TC189259	Solyc10g008880	Zinc finger transcription factor 56	3.494804048	
TowgeN_I_42846	BW687235	Solyc11g061720	Kinase family protein	3.493840526	
TowgeN_I_43350	DY523803	Solyc12g056920	Sulfate transporter, putative	3.470526638	
TowgeN_I_14379	TC174188	Solyc06g062450	Leucine-rich repeat protein kinase family protein	3.452331472	
TowgeN_I_23218	TC183027	Solyc01g100870	ADP-ribosylation factor	3.449704049	
TowgeN_I_50177	AW649767	Solyc08g078460	Oxidoreductase family protein	3.447480684	
TowgeN_I_26454	TC186263	Solyc07g049480	Cleavage and polyadenylation specificity factor	3.442228191	

Table 1. Continued.

Probe ID	Accession	Gene ID	Annotation	Fold change	Note
TowgeN_I_15970	TC175779	Solyc03g098300	Ornithine decarboxylase 2	3.429649622	
TowgeN_I_21699	TC181508	Solyc02g078140	MAP kinase kinase kinase 18	3.425866243	
TowgeN_I_48767	AI776395	Solyc06g036100	Divalent ion symporter	3.413269463	
TowgeN_I_14074	TC173883	Solyc03g111710	BTB/POZ and TAZ domain protein	3.411298941	
TowgeN_I_20210	TC180019	Solyc06g073780	SMC3	3.40680547	
TowgeN_I_45230	BE460239	Solyc05g053210	Non-specific serine/threonine protein kinase	3.391249538	
TowgeN_I_41066	BP896342	Solyc01g103530	Non-specific serine/threonine protein kinase	3.374259073	
TowgeN_I_18861	TC178670	Solyc07g064990	*S*-adenosyl-L-methionine:carboxyl methyltransferase	3.352060136	
TowgeN_I_20668	TC180477	Solyc04g080640	Transmembrane protein	3.349830423	*2
TowgeN_I_33154	BG134398	Solyc09g092690	Peptidyl-prolyl *cis*-trans isomerase	3.325411622	
TowgeN_I_45161	BG133925	Solyc02g091800	Basic helix-loop-helix (bHLH) DNA-binding	3.302306767	
TowgeN_I_28820	TC188629	Solyc02g065555	Calcium uniporter, mitochondrial	3.290817773	
TowgeN_I_49976	BF051803	Solyc01g005970	F-box/kelch-repeat protein	3.265670529	
TowgeN_I_24560	TC184369	Solyc10g084120	Plasma membrane intrinsic protein	3.255459286	
TowgeN_I_42115	BP883380	Solyc02g080480	Protein DETOXIFICATION	3.249495054	
TowgeN_I_37983	BG124520	Solyc08g007690	Subtilisin-like protease	3.246824931	
TowgeN_I_24063	TC183872	Solyc08g075870	*S*-adenosyl-L-methionine-dependent methyltransferases	3.239419244	
TowgeN_I_36375	AW738299	Solyc07g007550	Heparanase-like protein	3.231822441	
TowgeN_I_47679	AW651102	Solyc03g116060	Exostosin family protein	3.22526528	
TowgeN_I_43501	DV104119	Solyc06g034110	Tomato acid phosphatase	3.216968392	
TowgeN_I_27030	TC186839	Solyc01g108700	Transcription initiation factor	3.213443767	
TowgeN_I_47491	AW616780	Solyc02g070110	FAD-binding berberine family protein	3.211792843	
TowgeN_I_32748	BI422443	Solyc04g074790	RING/U-box superfamily protein	3.193724338	
TowgeN_I_45240	BE435020	Solyc02g086990	Cyclic nucleotide-gated channel	3.190535531	
TowgeN_I_13650	TC173459	Solyc09g091550	*S*-adenosyl-L-methionine: salicylic acid carboxyl methylt	3.17512396	
TowgeN_I_30217	TC190026	Solyc08g067700	Serine/threonine protein kinase	3.172859891	
TowgeN_I_43870	BP907126	Solyc01g059880	ATP-citrate synthase	3.168109684	
TowgeN_I_10534	TC170343	Solyc08g074680	Polyphenol oxidase precursor	3.166421179	
TowgeN_I_20017	TC179826	Solyc05g011890	Sulfotransferase	3.157142167	
TowgeN_I_34040	AW033986	Solyc07g006670	HXXXD-type acyl-transferase family protein	3.153891903	
TowgeN_I_24915	TC184724	Solyc11g009010	Alpha/beta-Hydrolases superfamily protein	3.102475372	
TowgeN_I_30181	TC189990	Solyc07g045040	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	3.092060801	
TowgeN_I_40325	BE450404	Solyc02g079360	Octicosapeptide/Phox/Bem1p family protein	3.078824883	
TowgeN_I_37878	AW932987	Solyc01g109800	Transmembrane protein	3.077517888	
TowgeN_I_27195	TC187004	Solyc12g007320	RING/U-box superfamily protein	3.075999103	
TowgeN_I_46652	BI921560	Solyc10g044783	ATP-dependent Clp protease proteolytic subunit	3.052592382	
TowgeN_I_40348	BE451290	Solyc02g087990	DUF1666	3.052213638	
TowgeN_I_26450	TC186259	Solyc12g036390	Calmodulin binding protein-like	3.051413806	
TowgeN_I_15077	TC174886	Solyc09g009100	Heat shock protein	3.051108382	
TowgeN_I_16183	TC175992	Solyc02g070680	Cytochrome P450	3.049949544	
TowgeN_I_47804	BF096424	Solyc05g050970	Transketolase	3.049737503	
TowgeN_I_15268	TC175077	Solyc07g043590	Amine oxidase	3.040318989	
TowgeN_I_26075	TC185884	Solyc03g118900	WAT1-related protein	3.038199592	
TowgeN_I_15511	TC175320	Solyc03g025290	Hydroxyproline-rich glycoprotein	3.037109971	
TowgeN_I_32692	AW030425	Solyc12g088460	Cytochrome P450	3.035740301	
TowgeN_I_25008	TC184817	Solyc08g014440	BTB/POZ and TAZ domain protein	3.035088135	
TowgeN_I_46677	BI922244	Solyc03g097610	Bidirectional sugar transporter	3.030863435	
TowgeN_I_27020	TC186829	Solyc03g080060	Clade XV lectin receptor kinase	3.029775913	
TowgeN_I_27009	TC186818	Solyc12g088190	Amino acid permease	3.022255618	
TowgeN_I_18723	TC178532	Solyc08g082670	Cellulose synthase	3.017261534	
TowgeN_I_46583	AW031705	Solyc08g029000	Lipoxygenase	3.01425783	
TowgeN_I_50048	AW930492	Solyc01g100630	Catalase	3.010049202	

In the row of note, genes are marked with asterisks associated with numbers; *[1] identified in Thagun et al. (2016), *[2] identified in Lashbrooke et al. (2016), *[3] studied in detail, *[4] represented by mutliple probes.

represented by multiple probes, probes with higher fold change values were adapted, and probes with no match to the coding sequences (BLASTN with cut-off score 1e-10) were excluded.

Consistent with a previous study (Nakayasu et al. 2018), no SGA biosynthesis genes were included in Table 1, confirming that *JRE3* does not regulate the SGA pathway. The genes down-regulated by dominant suppression of *JRE*3 in tomato hairy roots had been identities in a similar microarray setting

Table 2. Gene Ontology analysis of the genes up-regulated by induced overexpression of *JRE3* in transgenic tomato hairy roots.

GO term	GO number	number of genes
Molecular function		
catalytic activity	GO:0003824	37
transporter activity	GO:0005215	10
binding	GO:0005488	9
receptor activity	GO:0004872	3
singal transducer activity	GO:0004871	2
antioxidant activity	GO:0016209	1
structural molecule activity	GO:0005198	1
Biological process		
metabolic process	GO:0008152	31
cellular process	GO:0009987	29
response to stimulus	GO:0050896	8
localization	GO:0051179	7
biological regulation	GO:0065007	5
cellular component organization or biogenesis	GO:0071840	1
Cellular component		
cell part	GO:0044464	21
membrane	GO:0016020	14
organelle	GO:0043226	11
cell junction	GO:0030054	2
macromolecular complex	GO:0032991	1

(Thagun et al. 2016). Although number of overlapping genes is limited, five genes are commonly identified in the both microarray-based screenings; *wax synthase* (*Solyc11g012200*), *sugar facilitator protein 3* (*Solyc01g080680*), *glycosyltransferase* (*Solyc12g009920*), *phospholipid:diacylglycerol acyltransferase* (*Solyc03g121960*) and *kinase family protein* (*Solyc01g067510*). Interestingly, we also found that six genes in Table 1 [*malate synthase* (*Solyc03g111140*), *hydroxyproline rich glycoprotein* (*Solyc03g043740*), *sulfate transporter* (*Solyc06g084140*), *MYB family protein* (*Solyc06g075660*), *glycosyltransferase* (*Solyc02g091350*) and *transmembrane protein* (*Solyc04g080640*)] are among 655 genes reported to be co-expressed with suberin biosynthesis genes in tomato (Lashbrooke et al. 2016).

We subjected the up-regulated genes (Table 1) to Gene Ontology (GO) analysis using the PANTHER classification system (Table 2) (Mi et al. 2013). Significant enrichments were detected for two GO terms, catalytic activity (p=8.11e-11) and its downstream term transferase (p=7.6e-5), in the domain of molecular function.

Regulation of malate synthase and tonoplast dicarboxylate transporter genes

Among the genes in Table 1, we were interested in a pair of genes encoding malate synthase (MLS; Solyc03g111140) and tonoplast dicarboxylate transporter (TDT; Solyc11g012360), which were up-regulated in the microarray analysis about 8.2 and 4.9 folds, respectively (Table 1), and both involved in malate accumulation (Liu et al. 2017). To further characterize the regulation of the genes by *JRE3*, we analyzed the expression levels of *MLS* and *TDT* by qRT-PCR in transgenic tomato hairy roots. In line OX2 (different from the line OX1 used in the microarray analysis), β-estradiol-induced overexpression of *JRE3* resulted in 28- and 4-folds inductions of *MLS* and *TDT* relative to the control (Figure 1A), validating the results of the microarray analysis. The dominant suppressive form of *JRE3* was prepared by attaching an ERF-associated amphiphilic repression motif (Hiratsu et al. 2003) to its C-terminal end and was placed under the control of a constitutive promoter. In two lines CR1 and CR2 for *JRE3* suppression, expression levels of *MLS* and *TDT* decreased markedly to 0.17 to 0.43 folds levels (Figure 1B). These results indicated that functionality of *JRE3* is co-related both positively and negatively with the expression of *MLS* and *TDT*, suggesting the regulation of these genes by the transcriptional regulator.

To further address the functional link between JA- and NaCl-inducible *JRE3* and its downstream genes *MLS* and *TDT*, we examined the responses of the genes to JA, NaCl and high osmolality in tomato hairy roots. As reported (Nakayasu et al. 2018; Thagun et al. 2016), *JRE3* was induced strongly by methyl jasmonate (MeJA) and NaCl, while substantial induction of *JRE4* occurred by MeJA but not by the abiotic stressors (Figure 2). *MLS* was induced by NaCl and mannitol, while the salt treatment induced *TDT* (Figure 2). In contrast to clear induction of *JRE3* in response to JA, the phytohormone did not alter the expression levels of *MLS* and *TDT* (Figure 3).

Regions of JRE4 indispensable for transactivation of MAKIBISHI1 gene

Transient overexpression of *JRE4* but not *JRE3* activate the promoter reporters of SGA biosynthesis genes,

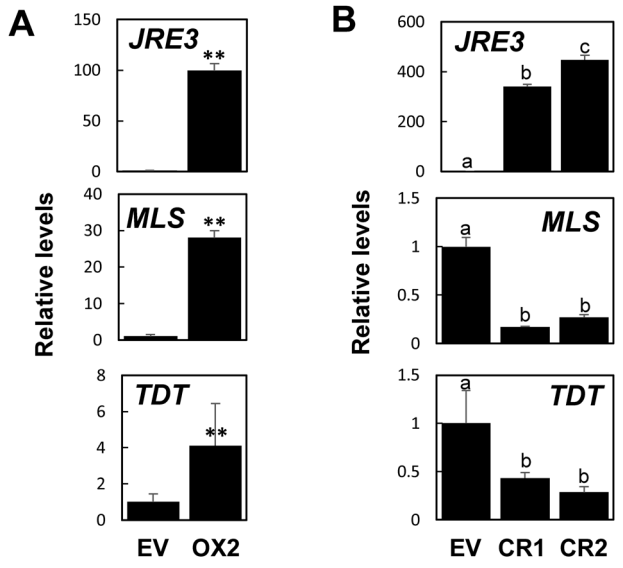

Figure 1. Expression of *MLS* and *TDT* genes in transgenic tomato hairy root lines with altered *JRE3* function. Five-day-old hairy roots of OX2, CR1, CR2, and empty vector (EV)-introduced control lines were used. To induce overexpression of *JRE3* in line OX2, hairy roots were treated with β-estradiol at 100 μM for 12 h. Transcript levels were measured by qRT-PCR, and are shown relative to the EV1 line. Error bars represent standard deviations for three biological replicates. Significant differences between the control and each treatment were determined by Student's *t*-test; ** $p<0.01$, * $p<0.05$. Shared letters indicate no significant difference at $p<0.05$ (ANOVA followed with Tukey–Kramer test).

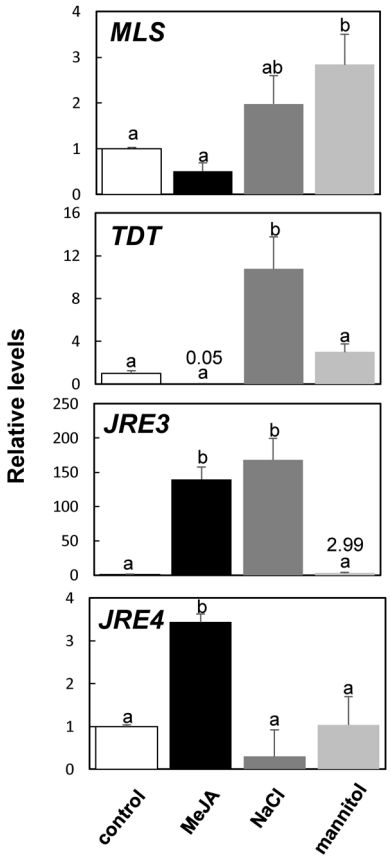

Figure 2. Response of *MLS* and *TDT* genes to JA, NaCl, and high osmolality in tomato hairy roots. Five-day-old hairy roots were treated with methyl jasmonate (MeJA) at 100 μM, NaCl at 300 mM, or mannitol at 300 mM for 5 h. Transcript levels were measured by qRT-PCR, and are shown relative to untreated controls. Error bars represent standard deviations for three biological replicates. Shared letters indicate no significant difference at $p<0.05$ (ANOVA followed with Tukey–Kramer test).

DWF5-2 and *GAME4*, in tomato fruits (Nakayasu et al. 2018; Thagun et al. 2016). We found that an endogenous gene *MAKIBISHI1* (*MKB1*) (Pollier et al. 2013), encoding a RING-finger E3 ubiquitin ligase and being involved in *JRE4* regulon (Thagun et al. 2016), was clearly activated by JRE4 in this experimental system as well, while such induction did not occur with JRE3 (Figure 3).

To examine the transactivation activity of JRE3 in parallel with JRE4 in the same transient system, we tried to find the genes induced by JRE3 among those in Table 1. But unfortunately we could not demonstrate the JRE3-dependent induction for any examined genes, including *MLS* and *TDT*, in the transactivation assay. Such unsuccessful outcome was considered to be in part resulted from difference of tissues, hairy roots and fruits, used in the analyses. Thus we put the focus on the transactivation activity of JRE4 in the following part.

To reveal which portions of JRE4 are important for its transactivation activity, chimeric fusions between JRE4 and JRE3 were generated and the activities of these fusions were examined in the transient expression analysis as *MKB1* expression as an indicator of downstream gene activation. The JREs were divided into three portions, a relatively conserved central DNA-binding domain and more variable terminal regions at the both ends (Figure 3A), and chimera of various combinations (Figure 3B) were constructed. Among the native and chimeric JREs analyzed, native JRE4 and a chimeric construct including both N- and C-terminal regions of JRE4 and a DNA-binding domain of JRE3 (c434) were able to significantly activate the expression of endogenous *MKB1* to 78.6 and 40.1 folds respectively relative to the control, while other fusions, including those containing either terminal region of JRE4 (c433, c334), did not have major impacts on *MKB1* expression levels (Figure 3B). These results indicated that both N- and C-terminal regions of JRE4, but not a DNA-binding domain, are indispensable to JRE4-mediated transactivation of the SGA pathway genes.

Discussion

Clustering of *JRE* genes (Cárdenas et al. 2016; Thagun et al. 2016) and their counterparts in other plants (Kajikawa et al. 2017; Paul et al. 2017), suggest the emergence of the homologous genes through tandem duplication, pointing common ancestry of the genes. Based on the

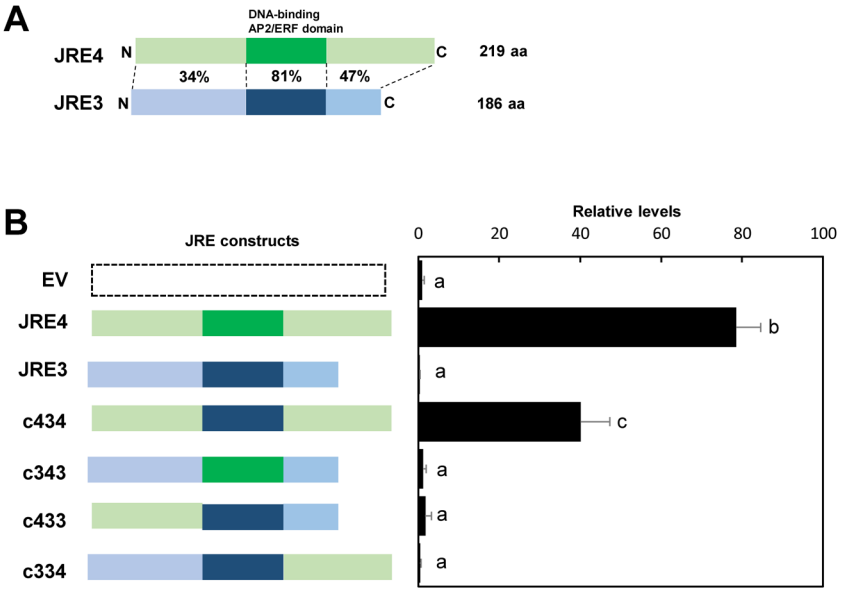

Figure 3. Transient transactivation analysis of JRE4, JRE3, and their chimeric fusions in tomato fruits. Regions, N- and C-terminal and central DNA-binding domains, in JRE4 and JRE3 were depicted schematically and amino acid identities in the same region between the JREs were shown in (A). The vectors for native and chimeric JREs, schematically shown in (B), or empty vector (EV) and GFP reference vector were co-delivered into tomato mature green fruits by agroinjection. Transcript levels of *MKB1*were normalized against those of *GFP*, and are shown relative to those of the EV controls (B). Error bars indicate the standard deviations for three biological replicates. Shared letters indicate no significant difference at $p<0.05$ (ANOVA followed with Tukey–Kramer test).

phylogenetic relationships of the clustered genes from different species, the duplication leading to cluster formation is presumed to occur independently in distinct plant lineages (Shoji 2018). In tomato and tobacco of Solanaceae family, a single gene that commonly resides around a middle of the clusters, such as ERF189 and JRE4, play a predominant role as a regulator of alkaloid biosynthesis in respective species (Kajikawa et al. 2017; Nakayasu et al. 2018; Shoji 2018). On the other hand, other clustered *ERF*s, which are considered more ancestral than the alkaloid regulators because of their relatively marginal positions in the clusters (Shoji 2018) and other structural and expressional evidences (Shoji et al. 2013), do not largely contribute to the alkaloid regulation; the elicitation of the non-alkaloidal *ERF*s by NaCl in tobacco (Shoji and Hashimoto 2015) and tomato (Nakayasu et al. 2018) is one of the circumstantial evidences supporting the notion. The response to NaCl and JA is considered a fundamental and so ancestral property of ERFs of this clade, which is also conserved in a counterpart AtERF13 (At2g44840) in *Arabidopsis* (Lee et al. 2010). Given such expressional conservation of the non-alkaloidal factors, rise of the more specialized alkaloid regulators from them is assumed to rely on adjustment of expression patterns, including the elimination of the response to NaCl.

To gain cues into the functions of JA- and salt-inducible ERFs, which may be ancestral to the alkaloid regulators, we identified a series of gene regulated by JRE3, a closest homolog of JRE4, in tomato hairy roots (Table 1). JRE3 regulates a diverse range of genes associated with various GO terms (Table 2). Such non-specialized manner of gene regulation by JRE3 is stark contrast to the case of JRE4 devoted to SGA regulation. The existence of multiple ancestral factors like JRE3, which may be functionally redundant and not committed to certain processes at least as a single factor and thus ready for assignment to specific roles, seems convenient to allow afterward establishment of metabolic regulons, such as those of JRE4 and ERF189, possibly through repeated recruitments of metabolic genes (Shoji 2018). In the sense of metabolic evolution, it is indicative that GO terms, catalytic activity and transferase, are enriched among the *JRE3*-regulated genes. To reveal possibly frequent rewiring of gene networks (Johnson 2017), which is considered prerequisite for rise of the metabolic regulons (Shoji 2018), it is worth to address whether and if so how much genes regulated by are shared among the non-alkaloidal ERFs from same and different species.

JRE3 regulates a pair of genes encoding MLS and TDT involved in malate accumulation (Table 1, Figure 1). MLS is an acyltransferase involved in glyoxylate cycle, catalyzing an irreversible condensation of acetyl-CoA with glyoxylate to form malate. A tonoplast-localized membrane transporter TDT mediate vacuolar uptake of malate, and altered expression of the transporter gene has major impacts on malate contents in vacuoles (Emmerlich et al. 2003; Liu et al. 2017). In addition to its metabolic function, malate contributes to plant tolerance to aluminum toxicity by chelating the metal

ion (Chen and Liao 2016) and osmotic imbalance as osmoticm (Dong et al. 2018). In *Arabidopsis*, *AtMLS* (*At5g03860*) and *AtTDT* (*At5g47560*) are induced by NaCl and mannitol, reflecting the roles of the organic acid in abiotic tolerance (Arabidopsis eFP Browser 2.0). In line with that, we observed the induction of *MLS* and *TDT* by these stresses in tomato as well (Figure 2). It has yet to be addressed whether altered JRE3 function has significant impacts on malate accumulation in tomato. Suberin is deposited as a protective barrier in response to environmental stresses, such as drought and wounding (Vishwanath et al. 2015). In this regard, it is curious that the *JRE3*-regulated genes were also found among the genes co-expressed with suberin biosynthesis genes (Table 1) (Lashbrooke et al. 2016).

We tried to reveal the molecular basis of functional distinction between JRE3 and SGA-regulating JRE4. To this end, transient transactivation assay was conducted with JRE3, JRE4, and their chimeras in tomato fruits (Figure 3). Unfortunately we could not find the JRE3-dependent transactivation of any genes examined in the transient system, which were regulated by JRE3 in tomato hairy roots, while evident induction of *MKB1* by JRE4 was demonstrated. Using *MKB1* expression as a marker of downstream gene activation, we found that both N- and C-terminal regions variable between JRE3 and JRE4 rather than a relatively conserved central DNA-binding domain are indispensable for the JRE4-mediated transactivation of *MKB1*, suggesting that molecular mechanisms, such as physical interaction with other factors through the terminal regions, rather than DNA binding, is critical for the functional differentiation between the JREs.

In conclusion, a series of genes regulated by JA- and salt-inducible JRE3 were identified through microarray analysis in transgenic tomato hairy roots. JRE3 regulates a diverse range of genes, including *MLS* and *TDT* involved in malate accumulation. These results give insights into the evolution of the alkaloid regulators from ancestral ones, which may be related to JA- and NaCl-inducible ERFs, such as JRE3. Transient transactivation analysis revealed that relatively variable terminal regions at the both ends rather than a conserved central DNA-binding domain of JRE4 is required for its transactivation of a gene involved in JRE4 regulon.

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