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

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Abstract In Solanum lycopersicum (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 JREs are induced by jasmonates (JAs), whereas sodium chloride (NaCl) treatment drastically increases the expression of the JREs 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/ETHYLENE 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 lycopersicum (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
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 JREs, the JREs 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 JREs 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 pIRES-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 Solarium 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 ampicons 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 overexpression 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            | 8.84032267  |      |
| TowgeN_I_18807 | AW648100  | Solyc03g111140   | Malate synthase                                 | 8.23457549  | *2,*3 |
| TowgeN_I_13980 | BE458907  | Solyc08g082970   | FAS-associated factor                            | 7.69010719  |      |
| TowgeN_I_35461 | AW615959  | Solyc11g012200   | Wax synthase                                    | 6.30929135  | *1,*2 |
| TowgeN_I_24215 | BE354617  | Solyc05g007020   | Potassium voltage-gated channel                 | 5.79281547  |      |
| TowgeN_I_20544 | TC180333  | Solyc12g011410   | Unknown protein                                  | 5.37626387  |      |
| TowgeN_I_25911 | TC185720  | Solyc01g080680   | Sugar facilitator protein 3                     | 5.10888383  | *1    |
| TowgeN_I_48778 | AI776728  | Solyc03g043740   | Hydroxyproline-rich glycoprotein                | 4.97591218  |      |
| TowgeN_I_51374 | AW622385  | Solyc01g009933   | SAUR-like auxin-responsive                      | 4.5538965   |      |
| TowgeN_I_44891 | BG132464  | Solyc07g021180   | Adenine phosphoribosyltransferase-like protein  | 4.47143291  |      |
| TowgeN_I_41543 | BP876253  | Solyc06g008920   | AMP dependent ligase                             | 4.11344084  |      |
| TowgeN_I_28695 | AW219535  | Solyc11g006740   | Sulfite exporter                                 | 4.16792363  |      |
| TowgeN_I_47464 | AW621208  | Solyc06g075660   | MYB family transcription factor                 | 4.26302362  |      |
| TowgeN_I_50753 | TC181216  | Solyc05g009170   | Zinc finger family                               | 4.96591863  |      |
| TowgeN_I_49124 | TC187812  | Solyc01g010770   | Hypersensitive-induced response protein          | 4.8211947   |      |
| TowgeN_I_47464 | AW625285  | Solyc01g009933   | SAUR-like auxin-responsive                      | 4.53432714  |      |
| TowgeN_I_44891 | BG132464  | Solyc07g021180   | Adenine phosphoribosyltransferase-like protein  | 4.47143291  |      |
| TowgeN_I_41543 | BP876253  | Solyc06g008920   | AMP dependent ligase                             | 4.11344084  |      |
| TowgeN_I_28695 | AW219535  | Solyc11g006740   | Sulfite exporter                                 | 4.16792363  |      |
| TowgeN_I_47464 | AW621208  | Solyc06g075660   | MYB family transcription factor                 | 4.26302362  |      |
| TowgeN_I_50753 | TC181216  | Solyc05g009170   | Zinc finger family                               | 4.96591863  |      |
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| TowgeN_I_41543 | BP876253  | Solyc06g008920   | AMP dependent ligase                             | 4.11344084  |      |
| TowgeN_I_28695 | AW219535  | Solyc11g006740   | Sulfite exporter                                 | 4.16792363  |      |
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| TowgeN_I_49124 | TC187812  | Solyc01g010770   | Hypersensitive-induced response protein          | 4.8211947   |      |
| TowgeN_I_47464 | AW625285  | Solyc01g009933   | SAUR-like auxin-responsive                      | 4.53432714  |      |
| TowgeN_I_44891 | BG132464  | Solyc07g021180   | Adenine phosphoribosyltransferase-like protein  | 4.47143291  |      |
| TowgeN_I_41543 | BP876253  | Solyc06g008920   | AMP dependent ligase                             | 4.11344084  |      |
| TowgeN_I_28695 | AW219535  | Solyc11g006740   | Sulfite exporter                                 | 4.16792363  |      |
| TowgeN_I_47464 | AW621208  | Solyc06g075660   | MYB family transcription factor                 | 4.26302362  |      |
| TowgeN_I_50753 | TC181216  | Solyc05g009170   | Zinc finger family                               | 4.96591863  |      |
| TowgeN_I_49124 | TC187812  | Solyc01g010770   | Hypersensitive-induced response protein          | 4.8211947   |      |
| TowgeN_I_47464 | AW625285  | Solyc01g009933   | SAUR-like auxin-responsive                      | 4.53432714  |      |
| TowgeN_I_44891 | BG132464  | Solyc07g021180   | Adenine phosphoribosyltransferase-like protein  | 4.47143291  |      |
| TowgeN_I_41543 | BP876253  | Solyc06g008920   | AMP dependent ligase                             | 4.11344084  |      |
| TowgeN_I_28695 | AW219535  | Solyc11g006740   | Sulfite exporter                                 | 4.16792363  |      |
| TowgeN_I_47464 | AW621208  | Solyc06g075660   | MYB family transcription factor                 | 4.26302362  |      |
| TowgeN_I_50753 | TC181216  | Solyc05g009170   | Zinc finger family                               | 4.96591863  |      |
| TowgeN_I_49124 | TC187812  | Solyc01g010770   | Hypersensitive-induced response protein          | 4.8211947   |      |
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 JRE3 in tomato hairy roots had been identities in a similar microarray setting.
Although number of overlapping genes is limited, five genes are commonly identified in both microarray-based screenings: wax synthase \((\text{Solyc}11\text{g}012200)\), sugar facilitator protein 3 \((\text{Solyc}01\text{g}080680)\), phospholipid:diacylglycerol acyltransferase \((\text{Solyc}03\text{g}121960)\) and kinase family protein \((\text{Solyc}01\text{g}067510)\). Interestingly, we also found that six genes in Table 1 [malate synthase \((\text{Solyc}03\text{g}111140)\), hydroxyproline rich glycoprotein \((\text{Solyc}03\text{g}043740)\), sulfate transporter \((\text{Solyc}06\text{g}075660)\), MYB family protein \((\text{Solyc}02\text{g}091350)\) and transmembrane protein \((\text{Solyc}04\text{g}086640)\)] 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.

### 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 |
| signal 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 |

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; \(\text{Solyc}03\text{g}111140\)) and tonoplast dicarboxylate transporter (TDT; \(\text{Solyc}11\text{g}012360\)), 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), \(\beta\)-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.
DWF5-2 and GAME4, in tomato fruits (Thagun et al. 2016; Nakayasu et al. 2018). 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 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 ERFs, 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 ERFs 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), 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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