ENO2 knock-out mutants in Arabidopsis modify the regulation of the gene expression response to NaCl stress

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Abstract
There is a growing awareness that some dual-function enzymes may provide a directly evidence that metabolism could feed into the regulation of gene expression via metabolic enzymes. However, the mechanism by which metabolic enzymes control gene expression to optimize plant stress responses remains largely unknown in Arabidopsis thaliana. LOS2/ENO2 is a bifunctional gene transcribed a functional RNA that translates a full-length version of the ENO2 protein and a truncated version of the MBP-1 protein. Here, we report that eno2 negatively regulates plant tolerance to salinity stress. NaCl treatment caused the death of the mutant eno2 homozygote earlier than the wild type (WT) Arabidopsis. To understand the mechanism by which the mutant eno2 had a lower NaCl tolerance, an analysis of the expressed sequence tag (EST) dataset from the WT and mutant eno2 Arabidopsis was conducted. Firstly, the most identified up- and down-regulated genes are senescence-associated gene 12 (SAG12) and isochorismate mutase-related gene, which are associated with salicylic acid (SA) inducible plant senescence and endogenous SA synthesis, respectively. Secondly, the differentially regulated by salt stress genes in mutant eno2 are largely enriched Gene Ontology (GO) terms associated with various kinds of response to stimulations. Thirdly, in the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping, we find that knocking out ENO2-influenced genes were most enriched into metabolite synthesis with extra plant-pathogen interaction pathway and plant hormone signal transduction pathway. Briefly, with the translation shifting function, LOS2/ENO2 not only influenced the genes involved in SA synthesis and transduction, but also influenced genes that participate in metabolite synthesis in cytoplasm and gene expression variation in nuclear under salt stress.

Keywords Arabidopsis · Mutant eno2 · NaCl stress · Gene expression

Introduction

In Arabidopsis, LOS2 (low expression of osmotically responsive genes 2)/ENO2 (Enolase 2) is a bifunctional gene. In addition to coding for the full-length ENO2, which catalyzes a key step in glycolysis, it can be alternatively translated (from the second start codon) to a truncated protein, c-MYC...
binding protein (MBP-1), which is a regulator involved in abscisic acid responses [1]. Recently, Eremina et al. argued that MBP-1 could repress the accumulation of LOS2/ENO2 transcription through a negative feedback mechanism [2]. Moreover, LOS2/ENO2 was suggested to be a cold-specific transcriptional repressor [3] that is required for salt stress responses [4]. It is generally accepted that plants actively respond to salt stress by reprogramming their whole metabolism or alternating signal pathways to enhance stress tolerance [5, 6]. The molecular mechanism of ENO2 associated with the salinity tolerance in tonoplasts by Arabidopsis enolase mutant los2 is well demonstrated by Barkla et al. [4]. However, the mechanism by which the nuclear or cytoplasm isoforms of enolase controls the response of Arabidopsis to salinity stress is still unclear.

Finding the upstream or downstream effector genes of LOS2/ENO2 will significantly contribute to the understanding on the mechanism by which metabolic enzymes directly control the gene expression. The 454 GS FLX sequencing platform provides a rapid method for analyzing the differential transcriptomes of the WT and mutant eno2, especially in plants with the eno2/eno2 homozygote for which EST sequences are not currently available for salinity stress. In this work, a mutant eno2 homozygote was identified from two salt-treated seedling EST libraries in which WT and mutant eno2 were sequenced. In all, 2,735 up-regulated and 2,318 down-regulated genes in mutant eno2 were identified. Two SA-associated genes, SAG12 and isochorismate mutase-related, were identified as the most up- and down-regulated genes, respectively. It indicates that LOS2/ENO2 is possible to negatively regulates plant tolerance to salinity stress through SA signaling pathway. Using GO and KEGG-based pathway analysis, we show that the differentially regulated by salt stress genes in mutant eno2 are enriched into the response to stimulations GO terms, but mostly are enriched in metabolite synthesis pathway. High-throughput analysis and the characterization of modulated genes would provide a foundation for detailed studies on the genetic connections between LOS2/ENO2 activity and the Arabidopsis response to salinity stress at the posttranscriptional level.

Materials and methods

Confirmation of homozygous ENO2 T-DNA Insertion Lines

The T-DNA mutagenized Arabidopsis (Columbia-0 ecotype) for ENO2 (SALK_021737, AT2G36530) was confirmed by Beijing Key Laboratory of Gene Resource and Molecular Development. For ENO2 fragment analysis, the primers 5′-AATGGATGTTGCCTCCACAGTTTCC-3′ and 5′-TAAGTACCAATGATGCTGCCTCG-3′ were used. Additionally, the primers of the Arabidopsis housekeeping gene actin-2 (At3g18780) were used as follows: 5′-TAATCT TCCGCTATGTTGCTGCC-3′ and 5′-TTTCTGTGAAGC ATTCCCTGACCT-3′. The PCR program was as follows: denaturation at 94 °C for 5 min; 30 cycles 94 °C for 30 s, 46 °C for 30 s, and 72 °C for 1 min; the 72 °C extension step lasts approximately 10 min. Then, the size of amplified products was analyzed by ethidium bromide staining in agarose gels.

Plant growth

For growth measurements, 4-day-old seedlings of WT and mutant eno2 Arabidopsis were grown on an MS culture [0.7% (w/v) agar] medium containing 3% (w/v) sucrose and transferred to soil in a climate chamber (16 h light and 8 h dark at 21 °C for 3 weeks). Whole plants were assayed for resistance to 300 mM NaCl at various time points using WT and homozygous plants for 18 days. For the gene expression profiling assay, 4-day-old seedlings were also transferred to identical plates with the MS culture. After 6 days of culture, the plants were rinsed briefly with the MS solution and further cultured in the MS medium containing 300 mM NaCl for 24 h. The seedlings were harvested at the 24 h time points for NaCl treatment and were frozen in liquid nitrogen before the start of the sequence experiments.

RNA extraction and cDNA library construction

The total RNA of the whole plants was prepared using EasyPure® Plant RNA Kit (TransGen, Beijing, China). After a DNase treatment, approximately 2.0 µg of poly(A)+ RNA were isolated from the total RNA using the Oligotex mRNA Mini Kit (Qiagen, Chatsworth, CA). One µg of mRNA was mixed with 10 pmol 3′ SMART CDS Primer IIA (BD Clontech, Palo Alto, CA), incubated at 70 °C for 2 min and then quenched on ice for 2 min. One µl of PowerScript RT with the first strand buffer and dNTPs were added, and the reaction was incubated at 42 °C for 1 h. The first strand of cDNA (5 µl) was then used as a template for the second strand polymerase chain reaction with 2 PCR Primer IIA in a 100 µl reaction. The PCR program was as follows: 95 °C for 1 min; 15 cycles of 95 °C for 5 s, 65 °C for 5 s; and 68 °C for 6 min. Amplified cDNA was purified with the PureLink PCR Purification Kit (Invitrogen, Paisley, UK). The resulting cDNA was fragmented and sequenced on a 454 GS FLX Titanium platform (454 Life Sciences, Roche, Branford, CT).

Sequence assembly

DNA sequencing was performed on a 454 GS FLX Titanium Sequencing System according to the manufacturer’s instructions. The cDNA libraries, which ranged from 300 to
800 bp (base pair), were added to the FLX specific adapters (Adapter A and B) and then nebulized and selected for denaturation to generate single-stranded DNA. Approximately 5 µg of single-stranded DNA were amplified by emulsion PCR before sequencing on the 454 GS FLX platform.

The length of the DNA sequencing reads in the dataset is approximately 200 bp (Supplementary Fig. S1). The raw sequencing data stored in the BAM file format contains low-quality sequences and adaptor sequences that were not suitable for the gene scan. First, reads shorter than 30 bp after pre-processing were excluded. Then, after trimming the adapter reads, if the length was less than the length of the set threshold, the reads were also excluded. The raw data were then converted to clean data, which were used for subsequent data analysis.

Unigene function annotation, GO classification, and metabolic pathway analysis

Supplementary Table S1 illustrates the summary from the mapping results (mapping to reference genes). There were 25,582 unigenes identified in WT plants and 26,357 unigenes identified in mutant eno2 under NaCl stress. A total of 17,454,311 (66.13%) and 17,003,457 (65.64%) unique matches in each dataset were obtained. The unigenes were annotated by a BLAST search against GO (http://www.geneontology.org), NCBI GenBank (http://www.ncbi.nlm.nih.gov/), and KEGG (http://www.genome.jp/kegg) databases. The transcripts were assigned a GO term based on the top blast hits in all retrieve queries, and the differentially expressed unigenes were classified into GO categories under the major categories of biological process. We also used BLASTX against the KEGG database, which is a resource for understanding the high-level functions and utilities of the biological system, to assign the differentially expressed unigenes to special metabolic pathways that represent molecular interactions and reaction networks.

Results and discussion

Characterization of eno2 mutants

The T-DNA integration position was at the first intron of the ENO2 gene (Fig. 1a), and our results show that the RT-PCR fragments of the ENO2 transcripts from the mutant eno2 did not produce a signal (Fig. 1b). While the introns are nucleotide sequences that are removed by RNA splicing during maturation, they are integral to the regulation of gene expression. In some cases, they are actually engaged in the regulation of gene expression, such as through nonsense mediated decay [7] and mRNA export [8]. In this work, the broken integrity of the first intron leads to the silencing of the ENO2 gene, indicating that the first intron plays an essential role in ENO2 gene expression.

T-DNA insertion of ENO2 results in decreased NaCl tolerance in Arabidopsis

As NaCl stress activates many signaling pathways, we compared the phenotypes of T-DNA inserted mutant eno2 and WT Arabidopsis under 300 mM NaCl stress. Each experiment was repeated three times, and at least 40 lines of mutant eno2 or WT plants were treated for each time. In visual appearance, mutant eno2 subjected to NaCl stress display no obviously fewer green leaves than WT plants on the 12th day after the salt treatment (Fig. 2, a randomly selected sample). Although the glycolytic enzymes, such as ENO2, that play an important role in regulating salt stress were widely studied [9, 10], little attention has been paid to the
mechanism of the knock-out of ENO2 that results in damage and apoptosis of leaves in salt treatment. The physiological modulation of the plant response to salinity relies on the proteins involved in signaling, changes in gene expression, and protein metabolism. The gene expression profile of these two Arabidopsis lines with different salinity tolerance should be investigated at the transcriptional level to understand the variation between the mutant eno2 and WT plants.

**Differentially expressed genes analysis**

The RPKM (reads per kb per million reads) method was used to measure gene expression levels. Differentially expressed genes (DEGs) with high abundance and differential expression exhibited between the two genotypes were the focus of this work. With NaCl treatment, there were 2302 up-regulated genes and 1126 down-regulated genes with the parameters set as log2 (fold change), ratio ≥ 1 and FDR (false discovery rate) ≤ 0.001 (Fig. 3).

The most up-regulated gene was AT5G45890.1, SAG12, (log2 ratio: 57.05, gi:18422605), which encodes a cysteine protease influenced by cytokinin, auxin, and sugars. The SAG12 gene can be induced by WRKY53, which is a SA inducible transcription factor that promotes plant senescence [11, 12]. Here, we suggest that silencing of ENO2 directly leads to high expression of SAG12, which may play a crucial role in eno2 entering the senescence phase (yellow) earlier than WT under salt stress.

Alternatively, the most down-regulated gene is AT1G35513.1, isochorismate mutase-related gene (log2 ratio: −11.19, gi:297839345). This gene may translate a hypothetical protein that influences the conversion of chorismate to prephenate and then to the products phenylalanine and tyrosine. The isochorismate pathway is the main source of endogenous SA synthesized in chloroplast rather than in the cytoplasm, where the SA is synthesized by the phenylalanine ammonia-lyase pathway [13, 14]. The mutant of the SA induction deficient two (sid2) gene in Arabidopsis down-regulates the expression of the isochorismate synthase (ICS1) gene, leading to hypersensitivity to salt stress [15, 16]. Hence, the suppression of isochorismate mutase-related SA biosynthesis by ENO2 may be an essential factor for salt tolerance in Arabidopsis.

**GO annotations of differentially expressed genes analysis**

To explain all observed gene expression changes for the process that eno2 negatively regulates plant tolerance to salinity stress, GO analysis should be taken in this work. With the corrected p value sorted in ascending order, there are only 12 genes annotated in regulation of response to biotic stimulus and 6 genes in cellular response to abiotic stimulus. But we confirmed that the response to abiotic stimulus and response to abiotic stimulus GO terms, known as a response to salt stress, are classified into the top 10 enriched GO terms with
high significance (Table 1). Nevertheless, all GO terms in top 10 are associated with the response to stimulus.

Except the most up- or down-regulated genes, there may be some other well-characterized salt response genes shows no obviously significance in \( p \) value. In this work, we find that the response to salt stress GO terms contain 8 DEGs as follows: AT3G21370.1, AT1G05675.1, AT5G60270.1, AT5G63990.1, AT5G19000.2, AT5G64000.1, AT1G05680.1 and AT5G09290.1. Among of this, AT1G05675.1 and AT1G05680.1 are also classified into the hyperosmotic salinity response GO term (Table 2). These two GO terms statistical differences are not significant, but the genes annotated into these terms are highly impacted the plant salinity tolerance. The regulation of these well-characterized salt response genes provides a valuable framework that directly links LOS2/ENO2 to the salt response functions.

### Table 1

| Gene ontology term                        | Cluster frequency | Genome frequency of use | Corrected \( p \) value |
|------------------------------------------|-------------------|-------------------------|-------------------------|
| Response to stimulus                     | 1111 out of 2215 genes, 50.2% | 7620 out of 20,969 genes, 36.3% | 4.57E−42 |
| Response to stress                       | 654 out of 2215 genes, 29.5% | 3859 out of 20,969 genes, 18.4% | 7.47E−39 |
| Defense response                         | 238 out of 2215 genes, 10.7% | 1057 out of 20,969 genes, 5.0% | 3.85E−28 |
| Response to oxygen-containing compound   | 189 out of 2215 genes, 8.5% | 789 out of 20,969 genes, 3.8% | 2.91E−25 |
| Response to organic substance            | 390 out of 2215 genes, 17.6% | 2330 out of 20,969 genes, 11.1% | 2.44E−19 |
| Response to biotic stimulus              | 207 out of 2215 genes, 9.3% | 1020 out of 20,969 genes, 4.9% | 3.54E−18 |
| Response to carbohydrate stimulus        | 115 out of 2215 genes, 5.2% | 436 out of 20,969 genes, 2.1% | 4.60E−18 |
| Response to abiotic stimulus             | 388 out of 2215 genes, 17.5% | 2403 out of 20,969 genes, 11.5% | 2.21E−16 |
| Response to chemical stimulus            | 520 out of 2215 genes, 23.5% | 3517 out of 20,969 genes, 16.8% | 5.58E−15 |
| Response to other organism               | 177 out of 2215 genes, 8.0% | 901 out of 20,969 genes, 4.3% | 9.42E−14 |
| Response to endogenous stimulus          | 318 out of 2215 genes, 14.4% | 2013 out of 20,969 genes, 9.6% | 1.64E−11 |
| Multi-organism process                   | 193 out of 2215 genes, 8.7% | 1091 out of 20,969 genes, 5.2% | 1.84E−10 |
| Secondary metabolic process              | 108 out of 2215 genes, 4.9% | 525 out of 20,969 genes, 2.5% | 6.00E−09 |
| Secondary metabolite biosynthetic process | 80 out of 2215 genes, 3.6% | 356 out of 20,969 genes, 1.7% | 3.92E−08 |
| Response to hormone stimulus             | 282 out of 2215 genes, 12.7% | 1864 out of 20,969 genes, 8.9% | 9.05E−08 |
| Signaling                                | 299 out of 2215 genes, 13.5% | 2078 out of 20,969 genes, 9.9% | 5.39E−06 |
| Phenylpropanoid biosynthetic process     | 58 out of 2215 genes, 2.6% | 252 out of 20,969 genes, 1.2% | 7.25E−06 |
| Phenylpropanoid metabolic process        | 64 out of 2215 genes, 2.9% | 293 out of 20,969 genes, 1.4% | 1.10E−05 |
| Cell death                               | 70 out of 2215 genes, 3.2% | 336 out of 20,969 genes, 1.6% | 1.81E−05 |
| Death                                    | 70 out of 2215 genes, 3.2% | 336 out of 20,969 genes, 1.6% | 1.81E−05 |
| Response to reactive oxygen species      | 31 out of 2215 genes, 1.4% | 100 out of 20,969 genes, 0.5% | 1.83E−05 |
| Single-organism biosynthetic process     | 127 out of 2215 genes, 5.7% | 744 out of 20,969 genes, 3.5% | 2.60E−05 |
| Flavonoid biosynthetic process           | 33 out of 2215 genes, 1.5% | 113 out of 20,969 genes, 0.5% | 3.32E−05 |
| Ketone biosynthetic process              | 35 out of 2215 genes, 1.6% | 125 out of 20,969 genes, 0.6% | 4.21E−05 |
| Response to oxidative stress             | 37 out of 2215 genes, 1.7% | 137 out of 20,969 genes, 0.7% | 4.96E−05 |
| Response to osmotic stress               | 153 out of 2215 genes, 6.9% | 965 out of 20,969 genes, 4.6% | 0.00014 |
| Flavonoid metabolic process              | 33 out of 2215 genes, 1.5% | 121 out of 20,969 genes, 0.6% | 0.00019 |
| Programmed cell death                    | 58 out of 2215 genes, 2.6% | 291 out of 20,969 genes, 1.4% | 0.00132 |
| Cellular ketone metabolic process        | 35 out of 2215 genes, 1.6% | 144 out of 20,969 genes, 0.7% | 0.0017 |
| Immune system process                    | 59 out of 2215 genes, 2.7% | 304 out of 20,969 genes, 1.4% | 0.00264 |
| Monocarboxylic acid metabolic process    | 92 out of 2215 genes, 4.2% | 543 out of 20,969 genes, 2.6% | 0.00294 |
| Cell wall modification                   | 29 out of 2215 genes, 1.3% | 112 out of 20,969 genes, 0.5% | 0.00333 |
| Defense response to fungus               | 34 out of 2215 genes, 1.5% | 142 out of 20,969 genes, 0.7% | 0.00341 |
| Response to fungus                       | 34 out of 2215 genes, 1.5% | 144 out of 20,969 genes, 0.7% | 0.00474 |
| Jasmonic acid metabolic process          | 16 out of 2215 genes, 0.7% | 44 out of 20,969 genes, 0.2% | 0.0049 |
| Response to light intensity              | 31 out of 2215 genes, 1.4% | 130 out of 20,969 genes, 0.6% | 0.00982 |
| Immune effector process                  | 17 out of 2215 genes, 0.8% | 52 out of 20,969 genes, 0.2% | 0.01302 |
| Response to radiation                    | 147 out of 2215 genes, 6.6% | 1003 out of 20,969 genes, 4.8% | 0.02057 |
| Cell wall organization                   | 41 out of 2215 genes, 1.9% | 201 out of 20,969 genes, 1.0% | 0.02502 |
| Response to water deprivation            | 21 out of 2215 genes, 0.9% | 79 out of 20,969 genes, 0.4% | 0.04666 |
KEGG pathways of differentially expressed genes analysis

KEGG analysis provides a bioinformatics resource for linking genomes to life and the environment. Pathway enrichment analyses identify significantly enriched metabolic pathways or signal transduction pathways in DEGs. To identify the biological pathways that were changed by the ENO2 mutation, up- and down-regulated genes were assigned to KEGG pathways with enrichment statistics (Fig. 4). The top five enrichment pathways (sorted by q-value) were plant-pathogen interaction (KO04626), plant hormone signal transduction (KO04075), flavone and flavonol biosynthesis (KO00944), stilbenoid, diaroylheptanoid and gingerol biosynthesis (KO00945), and phenylpropanoid biosynthesis (KO00940). The pathways with the highest gene count numbers were sorted as follows: the biosynthesis of secondary metabolites with 321 DEGs, plant-pathogen interaction with 263 DEGs, plant hormone signal transduction with 174 DEGs, starch and sucrose metabolism with 70 DEGs, and phenylpropanoid biosynthesis with 64 DEGs. Unfortunately, only 11 DEGs appeared in the glycolysis pathway while considering ENO2 as a key step in the catalytic process. These results suggest that the decreasing response of the mutant eno2 to NaCl may be due to the role of MBP-1 in the nucleus rather than the role of enolase in glycolysis. Paradoxically, the top 20 statistics of enrichment of pathway have 17 metabolic pathways. Consequently, the re-routing of conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) is expected to interfere with other metabolic processes to adapt to salt tolerance.

There were 263 DEGs identified in the plant-pathogen interaction pathway and 174 DEGs identified in the plant hormone signal transduction pathway. Ten transcription factors (sometimes called sequence-specific DNA-binding factors) were identified in these obviously changed pathways. WRKY25, WRKY29, and MYC2 were identified in the plant-pathogen interaction pathway. ARF, B-ARR, ABF, ERF1/2, BZR1/2, MYC2, and TGA were identified in the plant hormone signal transduction pathway. The variation of these ten genes, which directly target DNA, may control the flow of genetic information from DNA to mRNA along with MBP-1 (a truncated ENO2 localized in the nucleus) and is responsible for the expression of stress-activated genes related to Arabidopsis tolerance and adaptation.

Conclusion

The evaluation of this bifunctional metabolism enzyme in salt stress has been extremely difficult to study, especially as one mRNA generates with two proteins. Our study generated gene expression data for WT and mutant eno2, increasing the available knowledge on how the ENO2 gene is associated with NaCl stress. First, we identified SAG12, a transcription factor that promotes plant senescence, which established the direct relationship between the ENO2 gene and the senescence process. Up-regulated expression of SAG12 consistent with our previous observations in phenotypes of mutant

| Process Ontology term | GeneID | WT_24 h-RPKM | eno2_24 h-RPKM | log2 Ratio | FDR   | Description |
|-----------------------|--------|--------------|----------------|------------|-------|-------------|
| Response to salt stress/ hyperosmotic salinity response | AT1G05680.1 | 7.901282047 | 85.60965475 | 3.437614841 | 0     | Symbols: UGT74E2 | Uridine diphosphate glycosyltransferase 74E2 |
| Response to salt stress | AT5G64000.1 | 4.637864696 | 21.66939811 | 2.224126445 | 7.91E−54 | Symbols: SAL2, ATSAL2 | Inositol monophosphatase family protein |
| Response to salt stress | AT5G60270.1 | 1.156503206 | 7.482285588 | 2.693709767 | 7.27E−44 | Symbols: LECRK-I.7 | Concanavalin A-like lectin protein kinase family protein |
| Response to salt stress | AT5G09290.1 | 1.708224293 | 6.512354541 | 1.930721703 | 1.48E−13 | Symbols: | Inositol monophosphatase family protein |
| Response to salt stress/ hyperosmotic salinity response | AT1G05675.1 | 0.237445936 | 1.87277171 | 2.979504076 | 1.73E−08 | Symbols: UDP-Glycosyltransferase superfamily protein |
| Response to salt stress | AT3G21370.1 | 0.781954259 | 0.001 | −9.610940408 | 2.13E−07 | Symbols: BGLU19 | beta glucosidase 19 |
| response to salt stress | AT5G63990.1 | 0.965002378 | 2.707512504 | 1.488363598 | 4.85E−05 | Symbols: | Inositol monophosphatase family protein |
| Response to salt stress | AT5G19000.2 | 1.836643156 | 0.606054045 | −1.599552992 | 8.21E−05 | Symbols: BPM1 | BTB-POZ and MATH domain 1 |
Additionally, the most down-regulated gene, which leads to hypersensitivity to salt stress, was also identified in this work. Using GO and KEGG of DEGs analysis, we find that the GO terms show more genes response to stimulations terms and KEGG shows more metabolite synthesis pathways. This phenomenon could be rationally explained with the bifunctional molecule of ENO2 gene. Finally, in the process of eno2 mutants adaptation or tolerance to salinity stress, the significant regulated eight well-characterized salt response genes and ten transcription factors (belongs to plant-pathogen interaction and hormone signal transduction pathway) were identified in this work.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.
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