Natural Antisense Transcripts Associated with Salinity Response in Alfalfa

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Abstract
Natural antisense transcripts (NATs) are long noncoding RNAs (lncRNAs) complementary to the messenger (sense) RNA (Wang et al., 2014). Many of them are involved in regulation of their own sense transcripts thus playing pivotal biological roles in all processes of organismal development and responses to the environment. In our previous study, we have identified a number of differentially expressed genes (DEGs) in alfalfa plants (Medicago sativa L.) subjected to salinity stress (Postnikova et al., 2013). In this work, we selected several experimentally validated DEGs identified in response to salt and analyzed them for the presence of NAT pairs. The majority of the examined DEGs encoded NATs. Expression of some NAT pairs changed in response to salinity, suggesting their involvement in regulating the responses of alfalfa to salt.

Both DNA strands can be transcribed (Werner, 2013). Until recently, natural antisense transcripts of protein coding genes were considered transcriptional noise without a functional role (Struhl, 2007). Development of next-generation sequencing and gene-silencing technologies has changed this opinion, revealing extensive antisense transcription in both eukaryotes and prokaryotes (Pelechano and Steinmetz, 2013). A growing number of NATs are found to be functional, although a mechanism of their action in most cases is far from being understood (Arthanari et al., 2014).

Natural antisense transcripts are ubiquitous in plants; according to Wang et al. (2014), 70% of Arabidopsis thaliana (L.) Heynh. mRNAs produce long noncoding NATs. They play various important roles in plant cells, presumably through transcriptional and posttranscriptional gene regulation (Borsani et al., 2005; Pelechano and Steinmetz, 2013). No information is currently available on NATs and other lncRNAs in alfalfa. Our interests lie in the identification of NATs and lncRNAs and an understanding of regulatory roles they play in this most extensively cultivated forage legume. Earlier, while studying the molecular mechanisms of salt tolerance in alfalfa using RNA sequencing, we described many DEGs associated with response to salt stress (Postnikova et al., 2013). In this work, we have examined if a subset of those genes is able to generate natural antisense transcripts.
in salt-tolerant alfalfa line. In cases where NATs were found, their expression levels under salinity stress were evaluated to consider their possible roles in modulating transcription of the respective sense RNAs.

**Materials and Methods**

Alfalfa germplasm AZ-GERM SALT-II, salt tolerant at the germination stage (Dobrenz et al., 1989), was used in the experiments. Seeds were scarified with H\textsubscript{2}SO\textsubscript{4}, surface sterilized with 70% ethanol for 1 min and with 0.5% sodium hypochlorite solution for 5 min, rinsed with distilled water, and germinated in Petri dishes on wet sterile filters. After 2 to 3 d, seedlings were transferred into individual 4- by 20-cm containers containing Pro-mix BX soil and grown under controlled conditions (temperature, 22°C ± 2; humidity, 70%; light, ~200 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1}; day/night cycle, 16:8) in a growth chamber (Percival Scientific, Inc.). All plants received only nonsaline irrigation (water) for the first 14 d. Three plants per replication (3 replications in total) were irrigated with 200 mM NaCl or received nonsaline irrigation (water) for the next 14 d. Total RNA was extracted from roots as previously described (Postnikova et al. 2013), followed by 30-min treatment with DNase I (Thermo Fisher Scientific) to remove genomic DNA. Extracted RNA was additionally purified on RNaseasy mini spin columns (Qiagen) to eliminate any potential contaminating DNA. Final RNA samples were screened by polymerase chain reaction (PCR) with gene-specific primers to ensure they are free from genomic DNA. Complementary DNA (cDNA) synthesis was performed using SuperScript III First-Strand Synthesis SuperMix kit as directed by the manufacturer (Life Technologies). Complementary DNA was treated with RNase A (Thermo Fisher Scientific) for 30 min to degrade single-stranded RNA. Strand-specific cDNA was made for each of the 10 selected genes (10 NAT-specific cDNAs and 10 sense-specific cDNAs) from each of the RNA samples (three RNA samples in each replicate and three replicates for each condition, mock, and salt).

Strand-specific detection of NATs was done following general outlines described by Ho et al. (2010). Primers for the first-strand synthesis and nested PCR amplification were designed using Geneious software version 6.1.7 (Biomatters LTD, 2012) with integrated Primer 3 program (optimum Tm 50°C for the first strand synthesis) and using IDT (Integrated DNA Technologies, Inc.) online Q-PCR primer design tool (optimum Tm 60°C) (Supplementary Table S1). For the correct estimation of expression levels, outlying genes were identified and removed. Previously detected NP_001237047, an unknown gene with little variation in expression levels, was used as a reference in all real-time PCR experiments (Postnikova et al., 2013). Delta Delta C(T) method (\(2^{-\Delta\Delta C(T)}\)) was used for analysis of relative expression. A ratio between each of the salt treated samples (sense or antisense) and a corresponding average of the mock (control) samples was calculated. To obtain a final ratio for any given gene, an average and standard deviation for all biological replicates were calculated.

**Results**

Strand-specific reverse-transcription PCR (RT-PCR) has emerged as a reliable means to detect NATs (Ho et al., 2010). The absence of priming to the sense strand and successful priming to the complementary strand results in cDNA derived only from the antisense transcript (Ho et al., 2010). We used this straightforward protocol to analyze several DEGs previously identified in our lab in alfalfa lines contrasting in tolerance to salinity (Postnikova et al., 2013). The selected genes, affected by salt stress, are implicated in a broad spectrum of cellular, metabolic, physiological, and defense pathways (Table 1).

We first designed strand-specific primers for synthesis of the antisense cDNAs. These primers are complementary to the predicted 3’ regions of the antisense transcripts (Fig. 1; Supplementary Table S1) and would not anneal to the sense mRNA. Strand-specific primers were also made to produce first strand cDNAs from the respective sense transcripts. First strand cDNA synthesis was followed by PCR amplification with internal nested primers located inside the 5’ ends of both strand-specific primers (Fig. 2). Nested primers generate cDNA overlapping in sense and antisense transcripts and thus can be used for amplification from both strands (Ho et al., 2010). First strand synthesis reactions without addition of strand-specific primers were also performed with each RNA sample as negative controls to account for possible nonspecific priming (Ho et al., 2010). Out of 10 tested DEGs, antisense-specific RT-PCR reproducibly detected NATs in 9 genes (Fig. 2 and Table 1). Both sense and antisense pairs were detectable (Fig. 2A, 2B, respectively). No amplification was observed in no-primer reactions (results not shown). All NAT PCR products were sequenced and found to represent cis-antisense transcripts.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Descriptions</th>
<th>NATs</th>
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<tbody>
<tr>
<td>Medtr2g060880.1</td>
<td>C2H2 zinc finger protein</td>
<td>+</td>
</tr>
<tr>
<td>Medtr3g071740.1</td>
<td>Abscisic acid receptor PTL6</td>
<td>+</td>
</tr>
<tr>
<td>Medtr8g020630.1</td>
<td>Germin-like protein 9</td>
<td>+</td>
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<tr>
<td>Medtr5g024020.1</td>
<td>Seed lipoxgenase</td>
<td>+</td>
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<tr>
<td>Medtr4g098850.1</td>
<td>Inositol-145-trisphosphate-5-phosphatase-like protein</td>
<td>+</td>
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<tr>
<td>Medtr4g021350.1</td>
<td>Aldose reductase</td>
<td>+</td>
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<tr>
<td>Medtr8g013680.1</td>
<td>Aquaporin TIP2-1</td>
<td>+</td>
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<tr>
<td>Medtr1g093100.1</td>
<td>Xyloglucan-specific endoglucanase inhibitor protein</td>
<td>-</td>
</tr>
<tr>
<td>Medtr3g070880.1</td>
<td>Zinc finger CCCH domain-containing protein</td>
<td>+</td>
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To additionally confirm detection of NATs, we performed amplification with primers that were used for strand-specific cDNA synthesis of each pair (sense and antisense). The PCR products of the expected sizes were obtained from all sense RNAs (Fig. 3). As anticipated for antisense transcripts, the reaction led to three different outcomes when NAT cDNAs were used as templates: (i) no PCR products were amplified, NATs terminated before the start of the sense strand-specific primer (lanes 1N, 4N, 5N, and 6N); (ii) NAT PCRs differed in size from PCR products amplified from sense transcripts, antisense pre-mRNA underwent different splicing events as described in Collani and Barcaccia (2012), (lanes 2N, 3N, and 8N); and (iii) NAT PCR products were of the same size as their sense counterparts, NATs are at least as long as the sense transcripts (lanes 7N and 9N). Sequencing of the amplified NAT PCR products showed that they overlap with the corresponding sense RNA.

To test if the detected NATs are associated with alfalfa responses to salinity, their expression in AZGERM SALT-II was evaluated under salt stress using reverse transcription–quantitative PCR (RT-qPCR). Importantly, we found that basal expression levels of NATs (under control, nonsalinity conditions), were 30- to 100-fold less as compared with their respective sense pairs (Fig. 4). Among 10 profiled transcripts, expression changes in six NATs associated with alfalfa orthologs of *Medicago truncatula* Gaertn. annotated genes Medtr2g060880.1, Medtr3g071740.1, Medtr5g024020.2, Medtr4g098850.1, Medtr8g013680.1, and Medtr7g099800.1 were statistically significant (Fig. 5). Expression of four NATs, Medtr3g071740.1 (abscisic acid receptor PYL6), Medtr4g098850.1 (inositol-1,4,5-trisphosphate 5-phosphatase-like protein), Medtr8g013680.1 (aquaporin TIP2–1), and Medtr7g099800.1 (K+[+]/H[+] antiporter), was concordant (changed in the same direction) with their sense counterparts. Three of these NATs were downregulated and one transcript was induced (Medtr8g013680.1). Two other NATs, Medtr2g060880.1 (C2H2 zinc finger protein) and Medtr5g024020.2 (seed lipoxygenase), expressed discordantly with their sense pairs (repressed and induced, respectively). This may
suggest potential roles of these NATs in alfalfa responses to salinity via regulation of the respective sense RNA.

**Discussion**

Long noncoding RNAs can be transcribed in both sense and antisense orientation from any part of the genome: exons, introns, intergenic, and overlapping regions (Kim and Sung, 2012). Natural antisense transcripts represent a type of IncRNAs that are complementary to the protein coding transcripts (sense mRNA) and may originate from the same (cis-NATs) or separate (trans-NATs) genomic loci. Cis-NATs usually exhibit complete sequence complementarity to their sense pairs in contrast to the trans-NATs that can target different sense transcripts (Lapidot and Pilpel, 2006). In eukaryotes, NATs are involved in various biological processes, including response to abiotic and biotic stresses, via regulation of gene expression (Faghihi and Wahlestedt, 2009; Wang et al., 2014).

According to a comprehensive noncoding RNA sequence database fRNAdb (http://www.ncrna.org/), only a few IncRNAs are currently identified in alfalfa. To the best of our knowledge, no information is currently available in the literature on natural antisense transcripts in *M. sativa*. In this study, for the first time, we have experimentally detected several NATs associated with a group of genes differentially expressed during salinity stress. The selected genes, for which NATs were discovered, are implicated in a variety of key biological activities, such as transcription regulation (C2H2 zinc finger protein, Medtr2g060880.1; zinc finger CCCH domain-containing protein, Medtr3g070880.1), abscisic acid (ABA)-mediated stress signaling pathways (abscisic acid receptor PYL6, Medtr3g071740.1; germin-like protein 9, Medtr8g020630.1; seed lipoxygenase, Medtr3g024020.2; inositol-1,4,5-trisphosphate 5-phosphatase-like protein, Medtr4g098850.1; aldosereductase, Medtr4g021350.1; aquaporin TIP2–1, Medtr8g013680.1; K(+)\(/\)H(+) antiporter, Medtr7g099800.1; and plant defense reactions against pathogens (inositol-1,4,5-trisphosphate 5-phosphatase-like protein, Medtr4g098850.1), and other processes.

Reverse transcription–quantitative PCR experiments demonstrated that at least six NATs derived from the genes encoding C2H2 zinc finger protein, abscisic acid receptor PYL6, seed lipoxygenase, inositol-1,4,5-trisphosphate 5-phosphatase-like protein, aquaporin TIP2–1 and
K\((+)/H(+)\) antiporter changed their expression profiles in response to salinity and thus may be directly involved in transcriptional control of sense RNAs. Although proposed mechanisms of such regulation mostly suggest transcriptional intervention (Faghihi and Wahlestedt, 2009; Pelechano and Steinmetz, 2013), both concordant and discordant effects of NATs on sense transcripts appear to be essential for their functional roles (Wang et al., 2014). Since, according to our data, expression of NATs under normal conditions was very low, any changes in it due to salinity stress (or other environmental factors) may be indicative of an exceptionally fine-tuning gene regulation by NATs. More experiments are underway to confirm these findings.

**Supplemental Information Available**

Supplemental information is included with this article. Supplemental Table S1: Primers for strand-specific and conventional PCRs.

**Acknowledgments**

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**References**


