SHORT COMMUNICATION

Plant Signaling & Behavior 8:12, e27674; December; © 2013 Landes Bioscience

Natural antisense transcripts of *Trifolium repens* dehydrins

Irina I Vaseva[1,*] and Urs Feller[2]

[1]Plant Stress Molecular Biology Department; Institute of Plant Physiology and Genetics; Bulgarian Academy of Sciences; Sofia, Bulgaria; [2]Institute of Plant Sciences and Oeschger Centre for Climate Change Research (OCCR); University of Bern; Bern, Switzerland

Keywords: Dehydrins, natural antisense transcripts, sense-antisense pairs, splice variants, Trifolium repens

The recently described complex nature of some dehydrin-coding sequences in *Trifolium repens* could explain the considerable variability among transcripts originating from a single gene.[1] For some of the sequences the existence of natural antisense transcripts (NATs), which could form sense-antisense (SAS) pairs, was predicted. The present study demonstrates that *cis*-natural antisense transcripts of 2 dehydrin types (Y_nK_n and Y_nSK_n) accumulate in white clover plants subjected to treatments with polyethylene glycol (PEG), abscisic acid (ABA), and high salt concentration. The isolated Y_nK_n *cis*-NATs mapped to sequence site enriched in alternative start codons. Some of the sense-antisense pairs exhibited inverse expression with differing profiles which depended on the applied stress. A natural antisense transcript coding for an ABC F family protein (a *trans*-NAT) which shares short sequence homology with Y_nSK_n dehydrin was identified in plants subjected to salt stress. Forthcoming experiments will evaluate the impact of NATs on transcript abundances, elucidating the role of transcriptional and post-transcriptional interferences in the regulation of dehydrin levels under various abiotic stresses.

Dehydrins (group 2 LEA proteins) tend to accumulate late in embryogenesis and in response to stress leading to cell dehydration (e.g., drought, low temperature, and salinity).[2] The expression pattern of group 2 LEA genes is frequently associated with higher tolerance of crop plants to abiotic stresses such as cold[3] and drought.[4-6] All dehydrins have at least one conserved, lysine-rich 15-amino acid domain, EKKGIMDKIKEKLPG, named the K-segment near the C-terminus, and some of them may have a track of Ser residues (the S-segment), and/or a consensus motif (in one or more copies), T/VDEYGNP (Y-segment) located near the N-terminus.[2] The number and order of the Y-, S-, and K-segments define different dehydrin sub-classes: Y_nSK_n, Y_nK_n, SK_n, K_n and K_nS.[2] Detailed analyses of promoter regions of some DHN genes provided evidence for a close relationship between dehydrin expression patterns and the various upstream and downstream *cis*-regulatory elements present in the sequences.[7-11] Published data outlined that the regulation of expression of some dehydrin genes is elaborate and could be a result of several interacting factors.[8,10]

Results from a recent study[1] aimed at the identification of different dehydrin types in white clover (*Trifolium repens*) demonstrated the complex nature of dehydrin-coding sequences, which may lead to a high variability among the transcripts originating from a single gene. The aim of the present study was to confirm experimentally the existence of previously predicted dehydrin NAT transcripts and to compare their abundance in white clover grown under different abiotic stress treatments.

Natural antisense RNAs could potentially regulate the expression of their sense partner(s) at either transcriptional or post-transcriptional level.[12] A recent work described that a rice *cis*-natural antisense RNA acts as a translational enhancer for its cognate mRNA.[13] SAS pairs in plants have the potential to become substrates for the ribonuclease III-like enzyme Dicer to produce short interfering RNAs (siRNAs) and natural antisense microRNAs (nat-miRNAs) with regulatory potential.[14,15] Many studies have confirmed that abiotic or biotic stresses induce production of the so-called nat-siRNA (natural-small interfering RNA) from *cis*-NATs.[14,16,17]

Plants used in the present study (*Trifolium repens,* cv Apis) were grown hydroponically on a standard nutrient solution[18] for 2 weeks (23 °C night / 26 °C day; 80% relative humidity; 14 h photoperiod; photosynthetic active irradiation of 200 μmol m^{-2} s). Some of them were subjected to 72 h treatment with PEG (100 g PEG-6000 added to 1 L nutrient solution), and others were transferred on standard nutrient medium supplemented with 1 μM ABA[19] or 75 mM NaCl[20] and were grown for additional 14 d. Total RNA extracted from differently treated plants was reversely transcribed in sense and in antisense direction, with oligo(dT_{23}) (Sigma-Aldrich) and forward gene specific primers (Y_nK_n F: ATGAATATGGAAACCCAGTG

*Correspondence to: Irina Ivanova Vaseva; Email: irina.vaseva@abv.bg; Urs Feller; Email: urs.feller@ips.unibe.ch
Submitted: 12/17/2013; Accepted: 12/28/2013; Published Online: 12/31/2013
Citation: Vaseva II, Feller U. Natural antisense transcripts of *Trifolium repens* dehydrins. Plant Signaling & Behavior 2013; 8:e27674; PMID: 24390012; http://dx.doi.org/10.4161/psb.27674

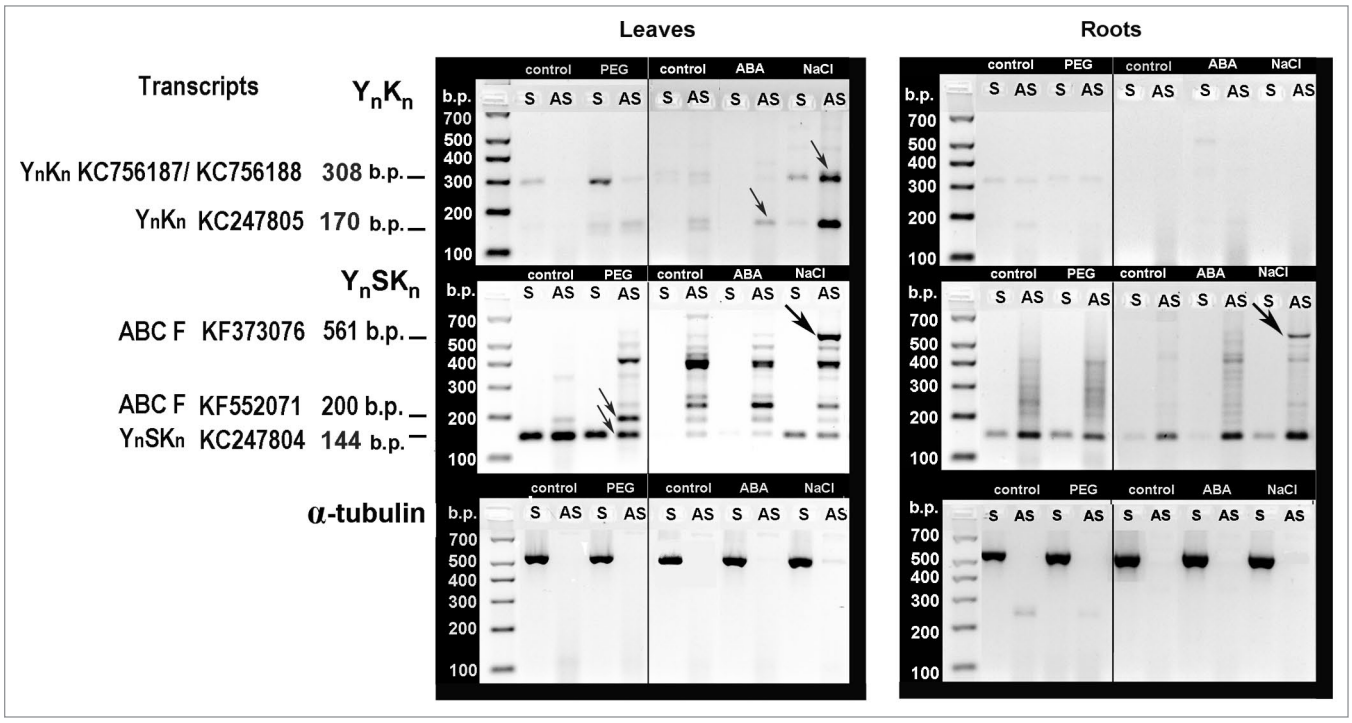

Figure 1. Semiquantitative RT-PCR analysis of sense (S) and antisense (AS) amplification products of Y_nK_n, Y_nSK_n, and tubulin in leaves and roots of 10% PEG-, 1 μM ABA-, and 75 mM NaCl-treated *Trifolium repens*. The positions and the GenBank IDs of the sense Y_nK_n and Y_nSK_n transcripts as well as the isolated ABC F transporter transcripts obtained on the antisense cDNA, are shown to the left. The sequenced *cis* – and *trans*-NATs are indicated by arrows.

and Y_nSK_n F: ACTGTCACCACTCCTAATCCAACTTC), respectively. The expression of *T. repens* α-tubulin (GenBank ID: AY192359.1) in sense and antisense direction (**Fig. 1**) was used as a reference.[21]

It is known that approximately 8–10% of *Arabidopsis* mRNAs have naturally occurring antisense transcripts.[22,23] When the sense and antisense transcripts originate from the same genomic locus but in opposite directions, the natural antisense transcripts are called *cis*-NATs. Usually they are characterized by perfect RNA–RNA sequence complement. As reported earlier, a Y_nK_n dehydrin gene could be expressed in several splice variants.[1] Previously, Jen et al.[22] have noticed that this was a specific characteristic of *cis*-NAT encoding genes where the RNA splicing could be induced by antisense transcripts.

The primer pair (Forward CTTATCAACCATTCCTTTTTCACC/ Reverse CAGCAACAATATGGAGACAGAGG) used in the Y_nK_n RT-PCR of differently treated samples was designed to amplify sense products with expected sizes 308 (transcripts with GenBank IDs: KC756187 and KC756188), 170 (GenBank ID: KC247805), and 140 b.p. (GenBank ID: KC756186, which was not detected in the tested samples). Y_nK_n splice variants formed sense-antisense (SAS) transcript pairs (**Fig. 1**), which was confirmed by direct sequencing of the amplification products. The isolated *cis*-NATs manifested a full overlap with transcript variants KC756187 and KC756188, both coding for Y_2K_4 dehydrin types (**Fig. 2A**). Vector NTI analysis of *cis*-NATs position on Y_nK_n dehydrin-coding DNA revealed that they both map to a site enriched in alternative start codons (**Fig. 2B**).

Direct sequencing confirmed that the antisense Y_nSK_n 144 b.p. (obtained with primers Forward GGTGCTTATGGTGGCGGTGCA/ Reverse CTTGAACTGGAGGAGCGACGAT) accumulating in PEG- and NaCl-treated leaves (**Fig. 1**), was a full overlap *cis*-NAT of the target Y_nSK_n dehydrin gene showing high homology to transcript variant KC247804, as well as to JF748411 and KF234077 (**Fig. 3A**). The last 2 were previously identified as drought-inducible.[1,24] The 144 b.p. *cis*-NAT exhibited comparable expression to its sense partner in leaf samples from the experiment with younger clovers treated with PEG for 72 h. It has been proposed that the similar sense and antisense transcript abundance is controlled by shared regulatory elements or processes related to chromatin structure.[25] Although a certain level of transcriptional noise resulting from bidirectional promoter activity cannot be excluded, the observed differing expression patterns of SAS under various stresses imply a possible physiological function.

The *trans*-NATs are pairs of overlapping transcripts from different genomic loci and often code for a protein or microRNA.[12,23] A genome-wide screening of *trans*-NATs in *Arabidopsis thaliana* has identified 1320 putative *trans*-NAT pairs, and it has been concluded that 430 transcripts had both putative *cis*- and *trans*-NATs.[26] *Trans*-NATs may have imperfect sequence complementarities. A natural antisense transcript (561 b.p.), sharing short nucleotide homology (24 base pairs) with Y_nSK_n dehydrin gene, was isolated as well (**Fig. 1**). The

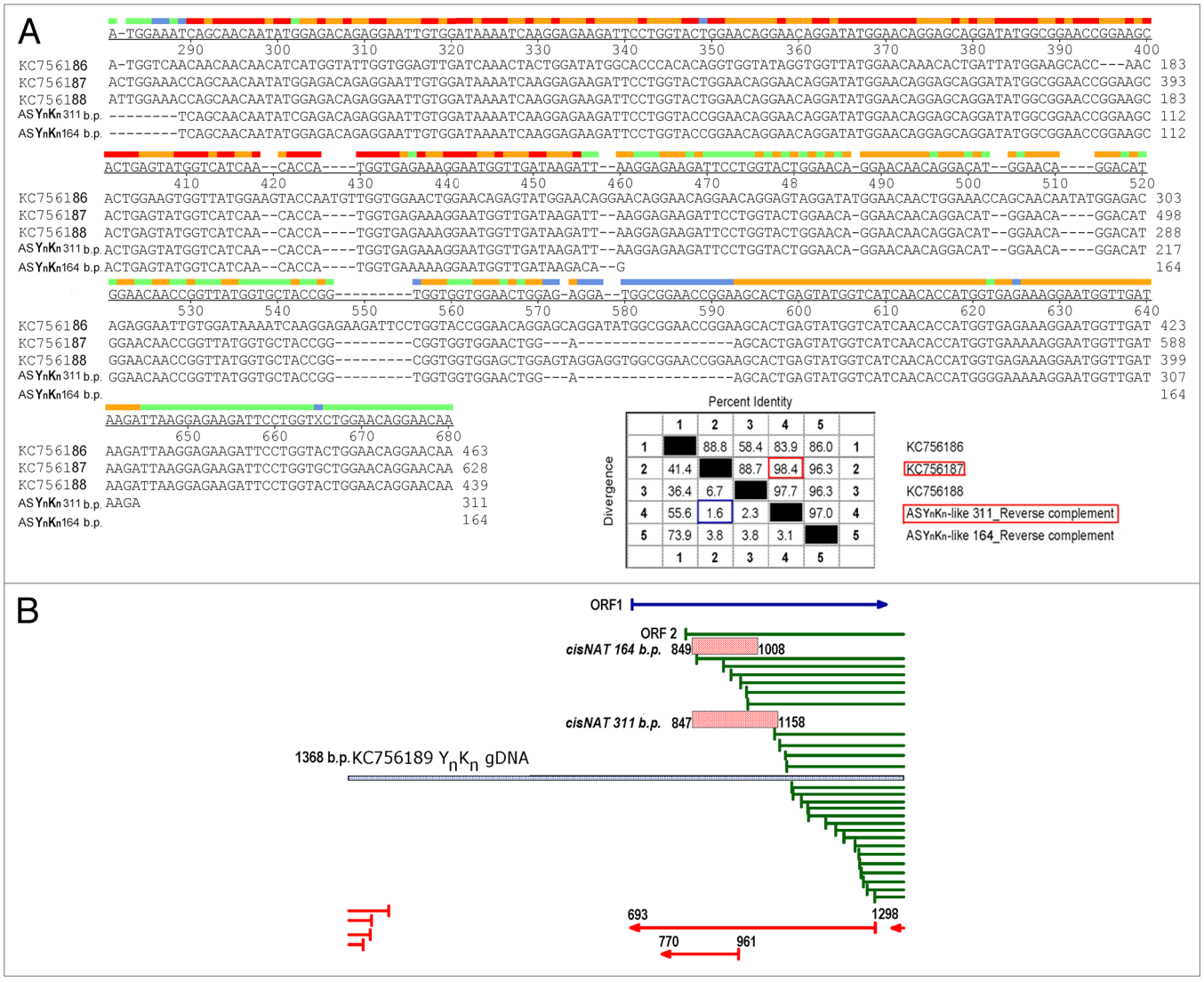

Figure 2. (**A**) Multiple sequence alignment (MegAlign, DNASTAR, Lasergene) of Y_nK_n transcripts with the isolated *cis*-NATs (311 b.p. and 164 b.p.). The alignment showed 98.4% identity (1.6% divergence) between transcript variant KC756187 and the *cis*-NAT (size 311 b.p.). (**B**) Vector NTI analyses of the partial genomic *T. repens* Y_nK_n sequence (GenBank ID: KC756189) and position of the isolated *cis*-NATs (red blocks). The sequence contains one complete ORF1 (699–1325 b.p., blue arrow) and codes for 4 K-segments. The fragmentary ORF 2 (green arrow) has multiple nested start codons. The predicted *cis*-NATs are indicated with red arrows.

sequence codes for ATPase component of ABC transporters (ABC F family, Gen Bank ID: KF373076) which lack a transmembrane domain and function in processes other than transport, most probably in ribosome recycling and translational control.[27,28] The transcript was specifically induced by salt stress both in leaves and in roots (**Fig. 1**). Direct sequencing and BLAST analysis of the antisense RT-PCR band migrating at position around 200 b.p. (GenBank ID: KF552071) visible in controls, ABA- and PEG-treated leaf samples, confirmed that it was a splice variant of ABC F coding sequence. The alignment of the isolated *trans*-NATs and Y_nSK_n-coding sequence showed that the short homology between them lays upstream in the intron and downstream at the beginning of ORF3 (**Fig. 3B**).

The present results will initiate extended studies on the duplex formation between sense and antisense dehydrin transcripts and their potential as functional regulators of diverse gene expression and transcript stability.

Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest.

Acknowledgments

This study was supported by a grant from the Scientific Exchange Programme between Switzerland and the New Member States of the European Union – Sciex-NMS^{ch} (Project No. 11.113: "Identification of Dehydrin Types involved in Abiotic Stress Responses in *Trifolium repens*" – IDAST) and the NCCR Climate (Project PLANT/SOIL).

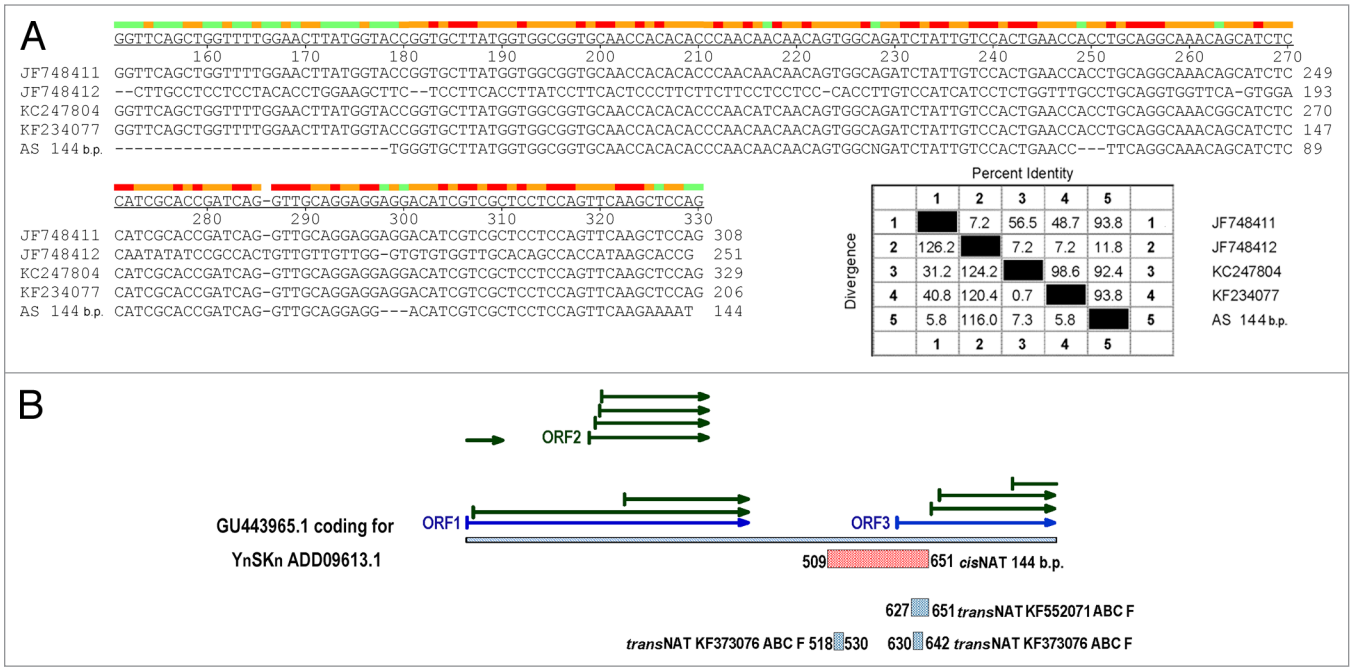

Figure 3. (**A**) Multiple sequence alignment (MegAlign, DNASTAR, Lasergene) of Y_nSK_n transcripts with the isolated *cis*-NAT (144 b.p.). The alignment showed that the isolated *cis*-NAT shares 93.8% identity (5.8% divergence) with transcript variants JF748411 and KF234077. (**B**) Vector NTI analyses of the genomic *T. repens* Y_nSK_n sequence (GenBank ID: GU443965.1) and position of the isolated *cis*-NAT (red block) and *trans*-NATs (GenBank ID: KF373076) coding for ABC F protein (blue blocks). The first exon of Y_nSK_n genomic sequence, which codes for 3 Y-segments and for the stretch of 9 Ser-residues, contains 2 open reading frames – ORF 1 (blue arrow1–399 b.p; alternative starts at positions 10 and 223 b.p., indicated with green arrows) and the nested ORF 2 (173–343 b.p., alternative starts at 182, 188, and 191 b.p., indicated with green arrows). The second exon comprises ORF 3 (blue arrow 607–831 b.p., alternative starts at 655 and 667 b.p., indicated with green arrows), and it codes for 2 K-segments.

References

1. Vaseva II, Anders I, Feller U. Identification and expression of different dehydrin subclasses involved in the drought response of Trifolium repens. J Plant Physiol 2013; http://dx.doi.org/10.1016/j.jplph.2013.07.013; PMID:24054754
2. Close TJ. Dehydrins: a commonalty in the response of plants to dehydration and low temperature. Physiol Plant 1997; 100:291-6; http://dx.doi.org/10.1111/j.1399-3054.1997.tb04785.x
3. Zhu B, Choi DW, Fenton R, Close TJ. Expression of the barley dehydrin multigene family and the development of freezing tolerance. Mol Gen Genet 2000; 264:145-53; PMID:11016844; http://dx.doi.org/10.1007/s004380000299
4. Lopez CG, Banowetz GM, Peterson CJ, Kronstad WE. Dehydrin expression and drought tolerance in seven wheat cultivars. Crop Sci 2003; 43:577-82; http://dx.doi.org/10.2135/cropsci2003.0577
5. Suprunova TT, Krugman TF, Chen G, Shams I, Korol A, Nevo E. Differential expression of dehydrin genes in wild barley, *Hordeum spontaneum*, associated with resistance to water deficit. Plant Cell Environ 2004; 27:1297-308; http://dx.doi.org/10.1111/j.1365-3040.2004.01237.x
6. Vaseva II, Grigorova BS, Simova-Stoilova LP, Demirevska KN, Feller U. Abscisic acid and LEA profile changes in winter wheat under progressive drought stress. Plant Biol 2010; 12:698-707; PMID:20701692; http://dx.doi.org/10.1111/j.1438-8677.2009.00269.x
7. Robertson M, Cuming AC, Chandler PM. Sequence analysis and hormonal regulation of a dehydrin promoter from barley, *Hordeum vulgare*. Physiol Plant 1995; 94:470-8; http://dx.doi.org/10.1111/j.1399-3054.1995.tb00956.x
8. Robertson M. Increased dehydrin promoter activity caused by HvSPY is independent of the ABA response pathway. Plant J 2003; 34:39-46; PMID:12662307; http://dx.doi.org/10.1046/j.1365-313X.2003.01697.x
9. Hinniger C, Caillet V, Michoux F, Ben Amor M, Tanksley S, Lin C, McCarthy J. Isolation and characterization of cDNA encoding three dehydrins expressed during *Coffea canephora* (Robusta) grain development. Ann Bot 2006; 97:755-65; PMID:16504969; http://dx.doi.org/10.1093/aob/mcl032
10. Wisniewski ME, Bassett CL, Renaut J, Farrell R Jr., Tworkoski T, Artlip TS. Differential regulation of two dehydrin genes from peach (*Prunus persica*) by photoperiod, low temperature and water deficit. Tree Physiol 2006; 26:575-84; PMID:16452071; http://dx.doi.org/10.1093/treephys/26.5.575
11. Yang Y, He M, Zhu Z, Li S, Xu Y, Zhang C, Singer SD, Wang Y. Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. BMC Plant Biol 2012; 12:140; PMID:22882870; http://dx.doi.org/10.1186/1471-2229-12-140
12. Sunkar R, Chinnusamy V, Zhu J, Zhu J-K. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends Plant Sci 2007; 12:301-9; PMID:17573231; http://dx.doi.org/10.1016/j.tplants.2007.05.001
13. Jabnoune M, Secco D, Lecampion C, Robaglia C, Shu Q, Poirier Y. A rice cis-natural antisense RNA acts as a translational enhancer for its cognate mRNA and contributes to phosphate homeostasis and plant fitness. Plant Cell 2013; 25:4166-82; PMID:24096344; http://dx.doi.org/10.1105/tpc.113.116251
14. Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu J-K. Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. Cell 2005; 123:1279-91; PMID:16377568; http://dx.doi.org/10.1016/j.cell.2005.11.035
15. Lu C, Jeong D-H, Kulkarni K, Pillay M, Nobuta K, German R, Thatcher SR, Maher C, Zhang L, Ware D, et al. Genome-wide analysis for discovery of rice microRNAs reveals natural antisense microRNAs (nat-miRNAs). Proc Natl Acad Sci U S A 2008; 105:4951-6; PMID:18353984; http://dx.doi.org/10.1073/pnas.0708743105
16. Katiyar-Agarwal S, Morgan R, Dahlbeck D, Borsani O, Villegas A Jr., Zhu JK, Staskawicz BJ, Jin H. A pathogen-inducible endogenous siRNA in plant immunity. Proc Natl Acad Sci U S A 2006; 103:18002-7; PMID:17071740; http://dx.doi.org/10.1073/pnas.0608258103
17. Jin H, Vacic V, Girke T, Lonardi S, Zhu JK. Small RNAs and the regulation of *cis*-natural antisense transcripts in *Arabidopsis*. BMC Mol Biol 2008; 9:6; PMID:18194570; http://dx.doi.org/10.1186/1471-2199-9-6
18. Page V, Blösch RM, Feller U. Regulation of shoot growth, root development and manganese allocation in wheat (*Triticum aestivum*) genotypes by light intensity. Plant Growth Regul 2012; 67:209-15; http://dx.doi.org/10.1007/s10725-012-9679-1
19. Xu X, Zheng G-Q, Deng X-P, Medrano H. Effects of exogenous abscisic acid and water stress on the growth response of subterranean clover of different genotypes. Acta Bot Sin 2002; 44:1425-31

20. Wang J, Drayton MC, George J, Cogan NOI, Baillie RC, Hand ML, Kearney GA, Erb S, Wilkinson T, Bannan NR, et al. Identification of genetic factors influencing salt stress tolerance in white clover (*Trifolium repens* L.) by QTL analysis. Theor Appl Genet 2010; 120:607-19; PMID:19865805; http://dx.doi.org/10.1007/s00122-009-1179-y
21. Asp T, Bowra S, Borg S, Holm PB. Cloning and characterization of three groups of cysteine protease genes expressed in the senescing zone of white clover (*Trifolium repens*) nodules. Plant Sci 2004; 167:825-37; http://dx.doi.org/10.1016/j.plantsci.2004.05.041
22. Jen C-H, Michalopoulos I, Westhead DR, Meyer P. Natural antisense transcripts with coding capacity in *Arabidopsis* may have a regulatory role that is not linked to double-stranded RNA degradation. Genome Biol 2005; 6:R51; PMID:15960803; http://dx.doi.org/10.1186/gb-2005-6-6-r51
23. Zhan S, Lukens L. Protein-coding *cis*-natural antisense transcripts have high and broad expression in *Arabidopsis*. Plant Physiol 2013; 161:2171-80; PMID:23457227; http://dx.doi.org/10.1104/pp.112.212100
24. Vaseva II, Akiscan Y, Demirevska K, Anders I, Feller U. Drought stress tolerance of red and white clover – comparative analysis of some chaperonins and dehydrins. Sci Horticult 2011; 130:653-9; http://dx.doi.org/10.1016/j.scienta.2011.08.021
25. Beiter T, Reich E, Williams RW, Simon P. Antisense transcription: a critical look in both directions. Cell Mol Life Sci 2009; 66:94-112; PMID:18791843; http://dx.doi.org/10.1007/s00018-008-8381-y
26. Wang H, Chua NH, Wang XJ. Prediction of trans-antisense transcripts in *Arabidopsis thaliana*. Genome Biol 2006; 7:R92; PMID:17040561; http://dx.doi.org/10.1186/gb-2006-7-10-r92
27. Braz ASK, Finnegan J, Waterhouse P, Margis R. A plant orthologue of RNase L inhibitor (RLI) is induced in plants showing RNA interference. J Mol Evol 2004; 59:20-30; PMID:15383904; http://dx.doi.org/10.1007/s00239-004-2600-4
28. Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E. Plant ABC Transporters. Arabidopsis Book 2011; 9:e0153; PMID:22303277; http://dx.doi.org/10.1199/tab.0153