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Abstract: Reversely transcribed RNAs coding for YnKn, YnSKn, SKn, and KS dehydrin types in drought-stressed white clover (Trifolium repens) were identified and characterized. The nucleotide analyses revealed the complex nature of dehydrin-coding sequences, often featured with alternative start and stop codons within the open reading frames, which could be a prerequisite for high variability among the transcripts originating from a single gene. For some dehydrin sequences the existence of natural antisense transcripts were predicted. The differential distribution of dehydrin homologues in roots and leaves from a single white clover stolon under normal and drought conditions was evaluated by semi quantitative RT-PCR and immunoblots with antibodies against the conserved K-, Y- and S- segments. Obtained data suggest that different dehydrin classes have distinct roles in drought stress response and vegetative development, demonstrating some specific characteristic features. Substantial levels of YSK-type proteins with different molecular weights were immunodetected in the non-stressed developing leaves. The acidic SK2 and KS dehydrin transcripts exhibited some developmental gradient in leaves. A strong increase of YK transcripts was documented in the fully expanded leaves and roots of drought stressed individuals. The immunodetected drought-induced signals imply that Y- and K-segment containing dehydrins could be the major inducible Late Embryogenesis Abundant class 2 proteins (LEA 2) which accumulate predominantly under drought.

Identification and expression of different dehydrin subclasses involved in drought response of *Trifolium repens*

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ABSTRACT

Reversely transcribed RNAs coding for Y_nK_n, Y_nSK_n, SK_n, and KS dehydrin types in drought-stressed white clover (*Trifolium repens*) were identified and characterized. The nucleotide analyses revealed the complex nature of dehydrin-coding sequences, often featured with alternative start and stop codons within the open reading frames, which could be a prerequisite for high variability among the transcripts originating from a single gene. For some dehydrin sequences the existence of natural antisense transcripts were predicted. The differential distribution of dehydrin homologues in roots and leaves from a single white clover stolon under normal and drought conditions was evaluated by semi quantitative RT-PCR and immunoblots with antibodies against the conserved K-, Y- and S- segments. Obtained data suggest that different dehydrin classes have distinct roles in drought stress response and vegetative development, demonstrating some specific characteristic features. Substantial levels of YSK-type proteins with different molecular weights were immunodetected in the non-

stressed developing leaves. The acidic SK_2 and KS dehydrin transcripts exhibited some developmental gradient in leaves. A strong increase of YK transcripts was documented in the fully expanded leaves and roots of drought stressed individuals. The immunodetected drought-induced signals imply that Y- and K-segment containing dehydrins could be the major inducible Late Embryogenesis Abundant class 2 proteins (LEA 2) which accumulate predominantly under drought.

Keywords

Dehydrins, Drought, Immunoblot, RT-PCR, splice variants, *Trifolium repens*

Abbreviations: DHN, dehydrins; LEA 2, Late Embryogenesis Abundant proteins group 2

Introduction

Climate modeling studies implying changes in average temperatures predict substantial increase of heat and summer drought frequency in Central Europe (Della-Marta et al., 2007; Hansen et al., 2012). Crop yield is intensely affected by adverse environmental conditions, and the understanding of physiological mechanisms that plants have developed to withstand environmental stress will facilitate the selection of suitable genotypes and also will contribute to introduction of novel molecular breeding approaches in the agricultural practice.

Legumes are unique among cultivated plants for their ability to carry out endosymbiotic nitrogen fixation with rhizobial bacteria (Bissuel-Belaygue et al. 2002a; 2002b). As their representatives clovers are usually preferred as a cover crop in organic crop rotations. When sown in summer clover may experience problems with drought which often occurs in these later plantings. Extreme environmental events are likely to shift the adaptive response of forage species, including clover, in the long term. Therefore the identification of suitable diagnostic

markers related to abiotic stress will contribute to develop agronomic strategies to maintain and enhance productivity of grassland crops.

The majority of published data on the effects of drought in clover focus on productivity (Bissuel-Belaygue et al., 2002a; 2002b; Sanderson et al., 2003), morphological parameters (Grieu et al., 1995; Annicchiaricho and Piano, 2004) or compatible solutes and photosynthetic pigments (Singh et al., 2010; Kim et al., 2004; Lee et al., 2009). Dehydrin accumulation under water stress is well-established phenomenon but dehydrin research in white clover, which is a major legume crop, is very limited (Vaseva et al., 2011; Singh et al., 2012). Drought-inducible dehydrins may serve as indicators for alteration in plant water status. The present study intended to monitor the differential accumulation of DHN types under normal and stress conditions and to elucidate dehydrin spatial distribution in the aboveground parts and roots.

Dehydrins (DHN) are specific proteins expressed in plants experiencing extreme environmental conditions such as drought or low temperature (Campbell and Close, 1997; Close, 1997). They are also involved in developmental processes, such as late embryogenesis, which require stabilization of macromolecules (Rorat et al., 2004; Riera et al., 2004; Hinniger et al., 2006). The accumulation of dehydrins is presumably part of the mechanisms protecting plants from protein denaturation under adverse environments. LEA 2 are highly hydrophilic and remain stable under denaturating conditions. Angiosperm DHNs are distinguished by the presence of a consensus sequence, rich in Lys-residues, known as the K-segment (EKKGIMDKIKELLPG). Their molecules consist of one or more K motifs. The K-segment is known to form amphipathic α-helices (Findlater and Graether, 2009; Koag et al., 2009). Therefore, it is able to interact with lipid components of the cell membrane and hydrophobic sites of the partially denatured proteins. Some dehydrins may also have a consensus Y-segment (V/T DEYGNP) near the N terminus or a serine-rich tract (the S-segment) that can be modified by phosphorylation and bind ions. It has been shown that the phosphorylation-regulated ion binding activity is generally conserved in the acidic subfamily of dehydrins (Kovacs et al.,

2008). Phosphorylation may enable dehydrins to interact with actin filaments and as consequence to stabilizethe cytoskeleton under stress conditions (Abu-Abied et al., 2006; Rahman et al., 2011). The Y-segment, when present, occurs in one to three tandem copies near the N-terminus. It is similar to a portion of the nucleotide binding site motif of chaperonins from plants and bacteria (Martin et al., 1993). Recent studies have reported that dehydrins are localized in the nucleus as well as in cytoplasm (Brini et al., 2007; Mehta et al., 2009).

Dehydrins have also some less conserved regions rich in glycine and polar amino acids which are called Φ-segments (Campbell and Close, 1997). The significant amount of charged and hydroxyl group-containing amino acids suggests that dehydrins have high affinity towards phospholipids and sterols, which explains their ability to stabilize cellular membranes. Campbell and Close (1997) have summarized that dehydrins might act at the interface between membrane phospholipids and the cytosol in plant cells and that they most probably interact with hydrophobic surfaces, rather than with a specific class of macromolecules.

The number (n) and order of the Y-, S- and K-segments define five different DHN sub-classes: Y_nSK_n (alkaline dehydrins induced by drought), SK_n (acidic dehydrins responsible for priming of the cells under chill and drought), K_n (usually related to cold tolerance), Y_nK_n (related to drought and cold tolerance) and K_nS (Close 1997). Recent studies have proposed that the basic Y_nSK_n type dehydrins in their phosphorylated form may protect the plant cytoskeleton, particularly the actin microfilament network and might stabilize membranes through lipid binding, whereas the K_nS-type dehydrins might protect the membrane integrity via metal binding and scavenging hydroxyl radicals (Rahman et al., 2010; 2011).

Few studies on the role of dehydrins in vegetative development suggest differential regulation of their expression, dependent not only on stress factors causing desiccation, but also on factors related to organ type and leaf developmental stage (Rorat et al., 2004; Weiss and Egea-Cortines, 2009). Some dehydrins were shown to be more responsive to developmental issues than to abiotic stress (Rorat et al., 2004; Koehler et al., 2007).

Significant number of data on physiological roles of LEA 2 proteins provide clear evidence that dehydrin genes may be key genetic determinants of stress tolerance in a number of species (Campbell and Close, 1997; Brini et al., 2007; Ruibala et al., 2012; Davik et al., 2013). Previously published studies aiming to elucidate the contribution of different dehydrin types to abiotic stress tolerance (Puhakainen et al., 2004; Welling et al., 2004; Hinniger et al., 2006; Šunderlíková et al., 2009) have provided evidence that particular LEA 2 proteins tend to accumulate in different sites depending on the developmental stage and tissue type, and that some of them could be chief players in alleviation of the negative physiological impact of environmental stress. The established concept that dehydrins may serve as potential markers for stress-tolerance selection requires a more detailed view on the specific role of the different DHN classes in plant development under normal and stress conditions and the mechanisms of the regulation of LEA 2 expression.

The complex polyploid nature of white clover (Casey et al., 2010) presents additional complications for identification and characterization of genes responsible for stress tolerance of this important legume crop. The aim of the present study was to identify drought-inducible *T. repens* dehydrin homologues and to evaluate their differential distribution in roots and leaves from a single clover stolon under normal and drought conditions in search for DHN classes induced by unfavorable water availability. A dehydrin of SK_2-type (Dhn b, GenBank ID: EU846208; Hand et al., 2010) was included in the analyses as a representative of LEA 2, which according to previously published data tended to be constantly expressed in unstressed vegetative tissues (Nylander et al., 2001; Bae et al., 2009; Vaseva et al., 2010, 2011).

Materials and methods

Plant material and growth conditions

The experiments were performed with large leafed white clover variety "Apis" (origin described in Boller et al., 2007), characterized by lower tendency to form hydrocyanic acid. The plants (*Trifolium repens*, cv. "Apis", Otto Hauenstein Seeds Ltd, Switzerland) were grown in Klasmann-Deilmann Seedlingsubstrat™ soil (pH 6.5), under 200 μE m^{-2} s^{-1} light, at 23 °C – 26 °C (night/ day) temperature and 14 h photoperiod. Optimal soil humidity (80% of the field capacity) was maintained by gravimetrically controlled watering. Each pot contained four seedlings. Drought stress was imposed on 14-day-old plants (with fully developed first and expanding second leaf) by withholding irrigation for a period of fourteen days when the leaf relative water content (RWC) in the stressed plants decreased to around 50% (Fig. 1). RWC was calculated according to Barrs and Weatherley (1968):

RWC (%) = [(FW-DW)/(TW-DW] x 100, where

FW – Fresh weight, TW – Turgid weight, and DW – Dry weight

Recovery of the stressed plants was performed for three days by resuming daily watering and maintaining the optimal soil humidity (Fig. 1).

All the analyses were made with frozen material derived from distinct leaves of the main stolons of 6-12 clover plants (L1 – first developed leaf, L2 – second developed leaf, L3 – third developed leaf, L4 – fourth developed leaf, L5 – last developing leaf) and from roots.

RNA extraction and synthesis of cDNA

Total RNA was extracted from 100 mg plant material (leaves or roots) with RNeasy Plant mini Kit (QIAGEN). RNA samples (400 ng) were reversely transcribed at 37 °C for 1 h with 2 mM anchored oligo(dT_{23}) primer (Sigma-Aldrich) using Omniscript Reverse Transcription Kit (QIAGEN).

PCR amplification, fragment isolation, and cloning

Different combinations of primers were designed to amplify Y-, K- or other conserved segments from published dehydrin mRNAs of related legume species. The primer sequences are given in Table 1. PCR reactions (50 µL) contained 5 µL RT assay (performed with 400 ng total RNA as template). Amplification was done with HotStart Taq polymerase (QIAGEN) according to the manufacturer's protocol. The cycling conditions were 15 min at 95 °C, 35 cycles of amplification at 94 °C for 1 min, and extension at 72 °C for 1 min. The relevant amplification temperatures were as follows: Ta= 53 °C for K_nS-analogue (amplified with degenerate KS legume primers, Table 1); Ta=50 °C for Y_nSK_n-analogue (amplified with Y-segment Forward and K-segment Reverse, Table 1); Ta= 53 °C for CIG-like (Rémus-Borel et al. 2010) Y_nK_n analogues (primers 3, 4 and 5, Table 1). The obtained PCR bands were cut from the agarose gel and purified with QIAquick® PCR Purification Kit (QIAGEN). The fragments were cloned into pBluescript II SK (+/–) vector (Aglient Technologies), cut with Sma I restriction enzyme (Thermo Scientific) according to the manual. The vectors were transformed into *Escherich coli* strain DH5α. A blue-white screening test on ampicilin containing media was performed to select positive colonies. At least 10 colonies were randomly picked and used for inoculation of overnight liquid cultures, grown at 37 °C with constant shaking. Plasmid DNA was isolated with QIAprep® Spin Miniprep Kit (QIAGEN) and additional PCR screening test with M13 primers (M13 F: GTAAAACGACGGCCAGT and M13 R: CATGGTCATAGCTGTTTCC) was applied to distinguish the plasmids hosting the inserts from the 'false positive' ones. The positive clones were sequenced using M13 forward and reverse primers (Mycrosynth, Switzerland) and were BLASTed against the NCBI database. The sequences exhibiting high homology (E values above E-10) with already published dehydrins from other legumes were selected for the following RT-PCR analyses.

Rapid Amplification of cDNA Ends (3'- and 5'-RACE PCR)

Oligo(dT) anchored (for 3'-RACE) or reverse gene specific primers (for 5'-RACE) were used for the reverse transcription (RT) of total RNA to obtain full length of cDNAs. The following antisense primers were used in 3'- and 5'-RACE PCR reactions:
Oligo(dT) anchored primer: GGCCACGCGTCGACTAGTACT TTT TTT TTT TTT TTT TV
3'-RACE primer: GGC CAC GCG TCG ACT AGT AC

Oligo(dT) anchored primer was added to the RT reactions and 3'-RACE primer was used in the first PCR. The cycling conditions were: initial denaturation at 95 °C for 15 min, 35 cycles of 94 °C for 1 min, amplification for 1 min at 60 °C and 72 °C for 1 min, final extension for 10 min at 72 °C. Gene-specific forward primers 1 (for Y_nSK_n), 7 (for Y_nK_n) and 11 (for K_nS) were used for the second nested PCR (Table 1).

First strand cDNA synthesis in 5'-RACE PCRs was performed with Omniscript Reverse Transcription Kit (QIAGEN) but instead of oligo(dT) primer reverse gene-specific primers "KS legume Reverse" (for K_nS) and 3 (for Y_3SK_2) were used (Table 1). The resulting cDNA was purified with QIAquick PCR purification kit (QIAGEN). Copy DNA 'tailing' reactions were performed with terminal transferase (ThermoScientific) and 2 mM dATP (ThermoScientific) according to the manual. The first PCR amplification of dA-tailed cDNAs was done with oligo(dT) anchored primer (listed above) and reverse gene specific primers 12 (for K_nS) and 4 (for Y_3SK_2, Table 1) using HotStart Taq polymerase (QIAGEN). Gene specific primers 13 (for K_nS) and 5 (for Y_3SK_2), and the 3'-RACE primer (listed above) were used in the following 5' nested PCR. The products from 3'- and 5'-RACE PCR were purified (QIAquick Gel extraction kit, QIAGEN) and sequenced directly (Mycrosynth, Switzerland). The resulting full length cDNAs were submitted to NCBI GenBank.

All nucleotide sequences were examined and charachterized individually with MegAlign, EditSeq of DNASTAR (Lasergene), and Vector NTI TM (InforMaxTM, Frederick,

MD, USA) to identify the most likely open reading frames (ORFs) and putative translational start and stop sites.

Semiquantitative RT-PCR

PCR reactions were carried out with cDNA derived from roots (R) and leaves at different stage of development (L1, L2, L3, L4, L5). The used primers are shown in Table 1. PCR reactions (50 µL) containing 2 µL RT assay (400 ng total RNA was used as template) were performed with HotStart Taq polymerase (QIAGEN) according to the manufacturer's protocol. The cycling conditions were 15 min at 95 °C, 30 cycles of 94 °C for 1 min, amplification for 40 sec and 72 °C for 1 min. The amplification temperatures and primer paires were as follows: Ta= 53 °C for CIG-like Y_2K_4 (primers 6 and 8, Table 1), Y_nK_n (primers 7 and 8, Table 1), and K_nS (primers 11 and 13, Table 1); Ta= 58 °C for Y_3SK_2 (primers 1 and 5a, Table 1); Ta= 60 °C for Y_3SK_2 (primers Y_3SK_2F and Y_3SK_2R, Table 1); Ta= 61 °C for Y_3SK_2 (primers 1 and 5, Table 1); Ta= 62 °C for SK_2 (primers 9 and 10, Table 1). The final extension step was for 10 min at 72 °C. The expression of *T. repens* actin (GenBank ID: AY372368, primer F: CCTGAGGAGCACCCAGTGC, primer R: AAGGGAGAGGACAGCCTGGA) and tubulin (GenBank ID: AY192359.1, primer F: ATCTTGAACCTACCGTCATC, primer R: TGAGAGACAAGGCGATTAAG, Asp et al., 2004) were used as two internal controls for normalization of the expression levels of the studied genes (Thellin et al., 1999).

SDS-PAGE and protein immunodetection

Extraction of soluble proteins, SDS-PAGE and immunodetection were performed with leaf and root samples derived from the third consecuitve biological repeat according to the previously described procedure (Vaseva et al., 2011). The levels of dehydrins were analyzed on

immunoblots developed with primary antibodies against legume K-sequence (GEKKGILDKIKEKLPG); Y- (N-terminal flanking region and the well-conserved Y-segment from *Triticum aestivum* – HDNPANRVDEYGNP) and S- (N-terminal flanking region and internal S-stretch in *Trifolium repens* – SLLEKLHRSDSSSS) segments raised in rabbit. The bands were visualized as described previously (Mitsuhashi and Feller, 1992).

Statistical analysis

The semiquantitative RT-PCR data represented on Figs. 2C, 3C, 4 and 5C are from three independent biological repeats. RT-PCR reactions were loaded on at least three ethidium bromide-containing 2% agarose gels (Suppl. 4) and the mean values of the these technical repeats were used for further analyses. Quantification of bands revealed during the different runs was done with ImageJ 1.30v software (National Institutes of Health, Bethesda, MD, USA). Processed data (Figs. 2C, 3C, 4, and 5C) represent the percentage of average area of the ethidium bromide-stained agarose gel occupied by each band. The graphs depict target dehydrin genes and actin or tubulin expression ratios. The vertical bars indicate the standard deviation from the three independent biological repeats calculated with SigmaPlot for Windows, Version 9.00. Results were subjected to unpaired two-tail Student's t-test to compare the mean values of transcript accumulation under drought stress with their relevant controls. Significant differences are indicated for $p \leq 0.05$ (*), $p \leq 0.001$ (**), and $p \leq 0.001$ (***).

Results

Copy DNAs from control and drought stressed leaves and roots were used as template in PCR reactions with different primer pairs designed to amplify conserved dehydrin sequences. Most of the amplification products showed high homology with published

dehydrins from other species at TBLASTX search (incorporates translated nucleotide databases using a translated nucleotide query, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Y_2K_4 dehydrin – isolation, characterization and RT-PCR

A partial CIG-like Y_nK_n dehydrin homologue (GenBank ID: KC756189) was amplified with primers 6 and 8 (Table 1) on genomic DNA. BLAST, BLASTX, and phylogenetic analyses of the identified genomic sequence confirmed that *T. repens* Y_nK_n had high identity with other previously published dehydrins (Suppl. 1). CIG-like Y_nK_n is 46.7% similar to *Galea orientalis* (GenBank ID: HM7770) and 44.2% to *Vicia monata* dehydrin a (GenBank ID: AB506694.1, Suppl. 1).

Detailed examination of *T. repens* CIG-like Y_nK_n homologue (genomic DNA GenBank: KC756189) identified one complete direct open reading frame ORF 1 (699-1325 b.p.) with three nested alternative start codons (positions 876 b.p., 996 b.p. and 1146 b.p.), coding for four K-segments (Fig. 2A). The fragmentary ORF 2 had multiple nested start codons (Fig. 2A). Two other CIG-like partial genomic sequences differing fom KC756189 were identified (with primers 7 and 8) and sequenced (genomic DNA GenBank: KC247806 and GenBank: KC247807). The examination of KC247806 and KC247807 with VectorNTI revealed similar features as in KC756189 – a compleat direct ORF with one nested alternative start coding for 3 K segments and a fragmentary one with multiple nested start codons (Suppl. 2A). Possible NATs were predicted for the three CIG-like sequences (Fig. 2A, Suppl. 2A).

Several amplification products, most probably originating from different CIG-like genetic sequences, were obtained when cDNA was used as a template (Fig. 2B). Variations in CIG-like transcripts were reported previously for *Medicago* spp. Y_2K_4 homologue as well (Rémus-Borel et al., 2010). *T. repens* CIG-like Y_nK_n cDNAs were sequenced and the translation showed that the peptides contained two Y segments at the N-end of the molecule,

and three (635 b.p. GenBank ID: KC756186 – Y_2K_3), or four K segments (763 b.p GenBank ID: KC756187, 900 b.p. GenBank ID: KC756188 – Y_2K_4) at the C-terminus (Suppl. 2B). Under drought KC756187 and KC756188 transcripts were still accumulating in roots, while the 635 b.p. CIG-like Y_2K_3 transcript (KC756186) was no longer detectable (Fig. 2B). The sequencing and subsequent MegAlign analysis (DNASTAR, Lasergene) of the 1310 b.p. CIG-like transcript isolated from drought stress leaves (Fig. 2B) did not determine any ORF (Suppl. 3).

Another CIG-like Y_nK_n transcript (GenBank ID: KC247805) accumulating predominantly in drought-stressed roots was isolated with primers 7 and 8 (Table 1). The sequence analysis showed that this transcript originated from KC247806 and codes for three K-segments (Suppl. 2B). RT-PCR revealed that KC247805 was scarcely represented in all control samples but drought provoked its accumulation in the fully expanded leaves and in roots (Suppl. 4A, Fig. 2C). The consistency of the expression results were confirmed after normalization with two reference genes – actin and tubulin (Fig. 2C). The expression of CIG-like Y_nK_n variant KC247805 decreased to control level after recovery (Suppl. 4A, Fig. 2C).

KS dehydrin – isolation, characterization, and RT-PCR

The degenerate K_nS primer pair (Table 1) amplified a triplet in samples derived from control and drought-stressed leaves and roots (Fig. 3A). The two bands exhibiting stronger signals (marked with arrows, Fig. 3A) were cut and purified. The corresponding sequences (GenBank IDs: KC247802 and KC247803) were BLASTed against the NCBI database. The transcripts had high homology with a published *Trifolium repens* cold acclimation specific protein (cas15) mRNA containing modified K-motif and S-stretch at the C-end (GenBank ID: JN398458.1 – 91% identity, 6e-40). The K_nS homologues showed also high similarity with several *Medicago sativa* mRNAs coding for different cold related proteins (BudCAR3 mRNA

– GenBank ID: AF220101 – 78% identity, 1e-13; CAR2 mRNA – GenBank ID: AF180373 – 78% identity, 1e-13; CAS15B mRNA – GenBank ID: HQ388395.1 – 67% identity, 1e-19; BudCAR4 mRNA - GenBank ID: AF220456 – 76% identity, 2e-13; BudCAR6 mRNA – GenBank ID: AF220458 – 76% identity, 2e-13; CAR1 mRNA – GenBank ID: AF072932.1 – 76% identity, 6e-13). The alignment of the translated white clover KS transcripts is presented in Suppl. 5.

KS sequence KC247802 encodes a 142 a.a. peptide with estimated Isoelectric Point = 6.643, charge at pH 7.0 = – 2.9, and MW of 14871.96 Da. A downstream nested start codon within the ORF (1>426 b.p.) at position 13 b.p. was detected (Fig. 3B). cNLS Mapper which predicts importin α-dependent nuclear localization signals (Kosugi et al., 2009) predicted a bipartite NLS (underlined) which anticipates cytoplasm localization of the polypeptide:

MAGIMNKIGGALHIGGDKKEGEHKGEQHGHVGGEQQHGHVVGGEQHGFVG 50
GHGGEYKGEQHGLVGGHGGEYKGEQHGLVGGHGGGEYKGEQHHGEEPKEG 100
FVDKIKDKIHGGEGGEKKKKKEKKKHGEGHEHGHDSSSSDSD 142

Analysis of the nucleotide sequence showed that clover KS homologue contained two fragmentary ORFs: ORF 2 (1>161) with undefined start codons and ORF 3 with 12 nested alternative start codons (Fig. 3B). The existence of a fragmentary antisense DNA sequence (402>1), consisting 18 nested start codons, was predicted (Fig. 3B).

Semiquantitative RT-PCR with primers 11 and 13 (Table 1) resulted in amplification of a single 224 b.p. band (named KS224) on genomic DNA (data not shown). The expression of KS224 transcript (ID: KC247802) at the very beginning of the experiment was negligible (Suppl. 4B, Fig. 3C) but later it increased in the older drought-stressed leaves (Suppl. 4B, Fig. 3C) according to actin normalization. This was not validated by the normalization with the other frequently used housekeeping gene tubulin. RT-PCR documented amplification of an

additional band in samples derived from the younger fully expanded control and recovered leaves (Suppl. 4B). The recovered individuals exhibited transcript levels below the age controls (Fig. 3C).

SK_2 dehydrin – RT-PCR

BLASTEXT analyses of the previously published *T. repens* dehydrin b sequence (Hand et al., 2010; GenBank ID: EU846208 = GI:289540937, complement join 91041..91424, 91648..91920) revealed high homology with *P. sativum* dhn-cog gene (Robertson and Chandler, 1994; GenBank ID: Z14145.1) – 65% identity, 5e-49; *Medicago truncatula* (MTR 3g117290) mRNA (XM 003603939, protein BT143429) – 60%, 7e-54; *Phaseolus vulgaris* dehydrin mRNA (GenBank: U54703.1, protein: AAB00554) – 46% identity, 1e-18 – all of them coding for SK_n dehydrins. The predicted amino acid sequence (Hand et al., 2010) has been assigned in NCBI data base as protein ADD09608.1 with MW=24508.74 Da, Isoelectric Point = 5.550, and charge at pH 7.0 = – 10.200 (EditSeq DNASTAR, Lasergene). It contains a stretch of seven serine residues in the middle and two K-fragments close to the C-terminus of the molecule. cNLS Mapper (Kosugi et al., 2009) predicted its localization to both the nucleus and cytoplasm:

MAEENQNKYEDATSTTNSETEIKDRGVFDFLGGKKKDEEHKPQEDAISTD 50
FSHKVTLYEAPSETKVEEAEGEKKHTSLLEKLHRSDSSSSSSSEEEDENG 100
EKRKKKKKEKKEKKEDTSVPVEKVEVVDGTTVGTEEKKGFLEKIKDKLPG 150
HKKTEDVTTPPPVVAPVPTETTTTTTSHDQGEKKGILEKIKEKIPGYHPK 200
TTTDHEEKDHHKDETASH 218

The changing SK_2 transcript profile in the control samples varied according to developmental age of the leaves (Suppl. 4C, Fig. 4). RT-PCR analysis showed that drought did not influence significantly the expression of SK_2 transcript in the younger leaves (L3, L4, L5). SK_2 relative expression marked significant increase in the older leaves and roots (DL1, DL2, and DR) when the results were normalized to actin due to the observed developmental fluctuations in the housekeeping gene in the different samples (Suppl. 4C, Fig. 4). Upon recovery the levels of SK_2 transcripts decreased around or below the controls according to both normalizing standards.

Y_3SK_2 dehydrin – isolation, characterization, and RT-PCR

Amplification of cDNA from drought stressed roots with degenerate primers Y-segment (Forward) and K-segment (Reverse) (Table 1) allowed the identification of a dehydrin homologue which was strongly influenced by drought. The sequenced PCR product was similar to previously identified partial cDNAs (GenBank IDs: JF748411 and JF748412) (Vaseva et al., 2011). BLAST results documented high identity (2e-118, 99%) with a sequence (GenBank: GU443965.1, complement join: 145184>145402; 145682>146014), coding for Y_3SK_2 dehydrin annotated as GenBank ID: ADD09613.1 (Hand et al., 2010). The nucleotide sequence analysis showed that *T. repens* Y_3SK_2 consisted of two exons and one intron (Fig. 5A). The first exon of the gene contained two open reading frames – ORF 1 (1-399 b.p) and the nested ORF 2 (173-340 b.p.). ORF 1 coded for the three Y-segments of the molecule, as well as for the stretch of nine Ser-residues. The second exon comprised only one ORF 3 (607-828 b.p.) coding for two K-segments (Fig. 5A).

The complete cDNA sequence was recovered via 3'- and 5'- RACE PCRs. The isolated mRNA (CenBank ID: KC247804) coded for a 182 a.a. polypeptide with predicted molecular weight of 18866.64 Da, Isoelectric Point = 9.184, and charge at pH 7.0 = 5.082 (EditSeq

DNASTAR, Lasergene). TBLASTX search showed that the isolated Y_3SK_2 cDNA shared high identity with other legume dehydrins from the same class: *Medicago truncatula* dehydrin MTR 3g117190 – 75% identity, 2e-32; *Glycine max* dehydrin (PM12) NM 001250385.1 – 63% identity, 4e-16; *Vitis yeshanensis* dehydrin 4 (GeneBank ID: JQ408444.1) – 45% identity, 7e-11). cNLS Mapper (Kosugi et al., 2009) predicted localization of the protein to both nucleus and cytoplasm:

MAGVQIRDEHGNPIQLTDQFGNPIKLTDEHGNPITLTGVATTVTTPNPTS 50
GSAGFGTYGTGAYGGGATTHPTSTVADLLSTEPPAGKRHLHRTDQVAGGG 100
HRRSSSSSSSSSEDDGQGGRRKKGVKDKVKEKLPGVGGGKDHNSQTTTVP 150
AATATHHPAEPTHEKKGILDKIKEKLPGHHNH 182

The gene-specific Y_3SK_2 primers (Y_3SK_2 F and Y_3SK_2 R, Table 1) amplified three different transcripts in the control and drought-stressed samples: JF748411, which contained retained intron; the compleate ORF 1/ ORF 3 splice form KC247804, and the shortened (107 – 324 b.p.) ORF 1 / ORF 3 splice form KF234077. The last one significantly accumulated only in the drought stressed roots (Fig. 5B), and coded for 142 a.a. polypeptide containing nine serine-residue strech and two K-segments (MW=14697.01 Da, Isoelectric Point of 9.520, and charge at pH 7.0 = 7.878, estimated by EditSeq DNASTAR, Lasergene):

YCHPPNPTSGSAGFGTYGTGAYGGGATTHPTTTVADLLSTEPPAGKQHLH 50
RTDQVAGGGHRRSSSSSSSSSEDDGQGGRRKKKGVKDKVKEKLPGVGGGK 100
DHNSQTTTVPAATATHHPAEPTHEKKGILDKIKEKLPGHHNH 142

cNLS Mapper predicted that Y_3SK_2 polypeptide coded by KF234077 could be localized to both nucleus and cytoplasm.

The splice variant with a retained intron JF748411 (amplified with primers 1 and 5a, Table 1), was represented in almost all tested samples (Suppl. 4D) but the highest levels were detected in the drought-stressed fully expanded leaves (DL3).

The expression of KC247804 variant (primers 1 and 5, size of the expected band 199 b.p., Suppl. 4D) normalized to actin documented comparatively high Y_3SK_2 levels in the fully expanded leaves (DL2, DL3, DL4) and roots (DR) subjected to drought (Fig. 5C). The normalization to tubulin confirmed that significant KC247804 amounts presented only in the third fully expanded leaf (DL3; Fig. 5C). Transcript content decreased to the control levels after 72 h of recovery.

Dehydrin immunoblot analyses

Immunodetection of dehydrins performed with three different antibodies against the conserved K-, Y-, and S-segment allowed to outline the different dehydrin types present in the tested samples. Seven distinct bands (within the range 18-60 kDa) were visualized with the K Ab (Fig. 6). These bands were accepted as a “true dehydrin” reference for the immunosignals obtained with the Y- and S- antibodies. The youngest control leaves (CL0 and CL5; Fig. 6) had a rich dehydrin profile with substantal quantity of immunodetected proteins. The strongest detected signals in the controls, predominantly accumulating in the youngest and second youngest leaves (CL0, CL4, CL5), were $Y_nS_nK_n$-type dehydrins migrating at position around 50 kDa, (Fig. 6A).

A weaker signal at position around 28 kDa was documented with the K- antibody in the fully expanded leaves (Fig. 6A). Drought stress increased the band intensity and the immunosignal diminished considerably after recovery (Fig. 6A).

The samples derived from drought-treated fully expanded leaves (DL2 and DL3) revealed immunosignals with apparent MW between 18 and 22 kDa on the K-probed membrane (Fig. 6A).

The strongest and most consistent signal, cross-detected with the antibodies against K- and Y-segment was documented in drought stressed leaves and roots. The molecular weight of these Y- and K-containing proteins varied between 37 and 48 kDa (Fig. 6A, 6B). The YK immunosignals disappeared in the samples of recovered plants (Fig. 6A, 6B).

Discussion

Previously published studies have demonstrated that the accumulation of certain dehydrins was related to drought stress tolerance in wheat (Lopez et al., 2003) and bermudagrass (Hu et al., 2010). The analysis of dehydrin expression in drought-resistant and drought-sensitive clones from different maritime pine (*Pinus pinaster*) ecotypes showed that only two out of the five identified DHN genes showed an increase in transcription as the drought stress progressed (Velasco-Conde et al., 2012). These data indicate that the involvement of dehydrins in vegetative development and the particular contribution of the different classes in stress responses still hold some unanswered questions.

The protective capacity of different LEA 2 proteins was a subject of numerous studies in different plant species (Rorat et al., 2004; Brini et al., 2007; Koehler et al., 2007; Ruibala et al., 2012). The high hydrophilicity, high glycine content (>20%) and lack of three-dimensional structure define dehydrins as ‘intrinsically disordered proteins’ (IDPs; Kovacs et al., 2008). Being typical intrinsically disordered DHN tend to exhibit abnormal migration during electrophoresis (Receveur-Bréchot et al., 2006) which could be also provoked by the high net charge of certain dehydrin types (Rahman et al., 2010). Some of the multiple dehydrin species may result from post-transcriptional (Wisniewski et al., 2006) or post-translational

modifications such as glycosylation (Levi et al., 1999) or phosphorylation of the serine-strech. IDPs could be involved in regulating signal transduction or gene expression (Tompa, 2002). Previously it has been suggested that some Y_nSK_n dehydrins may act as stress signaling molecules holding the potential to interact with distinct cellular components through their potential chaperone property (Brini et al., 2010). Y_3SK_2 dehydrin was significantly induced by drought in white clover as evident by the high transcript levels in the fully expanded leaves and roots. According to their phosphorylation status Y_nSK_n dehydrins may be transported from the nucleus to the cytoplasm (Riera et al., 2004; Brini et al., 2011). Earlier study on transcriptome profiling of DHN-5 *Arabidopsis* transgenic plants, provided an evidence that this YSK-type confers higher tolerance to oxidative stress via interaction with ascorbate biosynthetic pathway and that the same dehydrin expresses a functional link with jasmonate response which promote them as candidates with important functions in signal transduction or gene expression, perhaps through binding to targets such as proteins, RNA, ions, and membranes (Brini et al., 2011). These properties of YSK-containing dehydrin types show that the substantial amount of these DHNs in the youngest developing leaf identified by immunoblot has its physiological grounds since this is a site of extremely dynamic developmental processes.

White clover is an allotetraploid species with high levels of genetic variation both within and between populations, which are composed of a heterogeneous mixture of highly heterozygous individuals (Casey et al., 2010). Additionally as a result of the high degree of self incompatibility white clover is characterized with very high allelic diversity. The revealed closely migrating Y_nK_n immunosignals (a doublet with MW ~ 38-39 kDa) support this assumption. The existence of Y_nK_n species detected on the immunoblot suggests either an existence of gene homeologues within the genome of the polyploid clover or an existence of a promiscuous start codon choice for the particular dehydrin type (de Mayolo et al., 2006).

White clover plants produce leaves at all stages of development along a single stolon from initiation at the apex, through expansion, maturity, and senescence. The stolon growth of

white clover provides a suitable model to study the roles which different dehydrin types perform under normal and stress conditions in relation to leaf development. The pattern of leaf development (the number of leaves at each stage of development present on the stolon at each stage of development) and dehydrin expression along the white clover stolon was expected to reveal the major sites at which dehydrins are fulfilling their protective functions, and which classes are particularly involved in the drought-stress response. Results demonstrated that the major organs for accumulation of dehydrins under water deprivation were the fully expanded leaves and roots, where the stress-inducible types were exclusively detected. The studied acidic dehydrins SK_2 and KS had the highest transcript levels in white clover organs during vegetative growth demonstrating some developmental gradient in leaves. Transcript accumulation in the control leaves during the experimental period suggests that these dehydrin classes probably have developmental functions. Recent results on sugarcane dehydrins also documented fluctuations of dehydrin profiles (Burrieza et al., 2012), indicating that the pattern of their accumulation has been under developmental control. The studied acidic homologues were less influenced by water deficit, while the basic or neutral dehydrins (YK- and YSK-type) were the main inducible LEA 2 in drought-stressed *T. repens* during vegetative growth.

Detailed analyses of the isolated sequences provided evidence for potential alternative splicing (AS) of dehydrin genes. AS combines different transcript splice junctions that result in transcripts with shuffled exons, alternative 5' or 3' splicing sites, retained introns and different transcript termini. In this way, multiple mRNA species and proteins can be created from a single gene (Ner-Gaon et al., 2004). Studies on tissue-specific forms of alternative splicing demonstrated that it can provide a mechanism to introduce changes in various functional attributes of a single gene, such as different subcellular localization of the coded protein, diverse protein–protein interaction, and even opposing function (Tompa, 2012).

The accumulation of the alternatively spliced non-coding transcript JF748411 in the fully-expanded drought-stressed leaves may have certain regulatory role as suggested by Ner-

Gaon et al. (2004) who have found that the functional distribution of the transcripts with retained introns were skewed towards stress and external/internal stimuli-related functions. The authors proposed that the retained introns could be a prominent feature of AS with a regulatory function.

Some of the *T. repens* dehydrin sequences contain ORFs with nested start codons. A phenomenon earlier identified as 'leaky scanning' is often a part of the complex regulation of expression in genes with multiple start codons (de Mayolo et al., 2006). This may actually be the mechanism for the synthesis of polypeptides with different number of K motives coded by the various mRNA variants originating from one single gene. The presence of many nested potential translation start sites in the KS dehydrin ORFs also supports such a possibility.

It was found earlier that KS-type dehydrins were not enriched in promoter cis-elements similar to the other dehydrins suggesting they may be regulated through pathways distinct from the rest of the dehydrin family (Koehler et al., 2007). As an evidence for the complex regulation of white clover KS homologue could serve the expression profile of *Arabidopsis* KS gene (At1G54410) generated by Arabidopsis eFP Browser (Winter et al., 2007). It demonstrates diurnal changes in expression pattern, suggesting a very short half life of both the transcripts and the polypeptides. This may partially explain the poor immunodetection results for KS proteins. The daily oscillations of protein expression levels require rapid adjustments in mRNA levels. Recently it was established that the unproductive alternative splicing and nonsense mRNAs actually are a very common case in regulation of expression of circadian genes (Filichkin and Mockler, 2012). The analysis of the nucleotide KS sequence gave evidence for the existence of in-frame premature termination codons (PTCs), which is another possibility for the occurrence of an unproductive alternative splicing. Earlier it has been demonstrated that for some circadian genes the ratio of the productive isoform to its PTC counterpart may shift sharply under specific environmental stress conditions (Filichkin and Mockler, 2012).

Another interesting feature of the isolated dehydrin sequences is the allocated natural antisense ORFs. Actually the biogenesis of natural antisense transcript-derived small interfering RNAs (nat-siRNAs) begins with the formation of double stranded RNAs (dsRNAs) by annealing sense and antisense transcripts (Sunkar et al., 2007). The potential accumulation of dsRNAs formed from the mRNAs encoded by natural cis-antisense gene pairs, opens a possibility for regulation of dehydrin expression via small interfering RNAs (siRNAs). Earlier it has been demonstrated that cis-natural antisense transcripts could regulate salt tolerance in *Arabidopsis* (Borsani et al., 2005).

The endogenous sources of dsRNAs could be also microRNA-directed cleavage products of noncoding transcripts, which are then converted into dsRNAs. Apart from the previously isolated non-coding dehydrin Y_3SK_2 transcript with retained intron (Vaseva et al., 2011), a CIG-like Y_nK_n long non-coding transcript from drought stressed leaves was also identified. Long noncoding RNAs (lncRNAs) are increasingly recognized as functional regulatory components in eukaryotic gene regulation (Kim and Sung, 2012). Some lncRNAs are likely precursor molecules that are processed into small RNAs, while others function as intact, long molecules that have the potential to regulate gene expression (Boerner and McGinnis, 2012). All these preliminary results will motivate further detailed study on transcription regulation of dehydrins via interfering RNAs.

As a conclusion the presented data suggest that different dehydrin subclasses perform unique coordinated roles in plant development under normal and drought conditions. Immunoblot analysis attested that water deprivation increases the concentration of dehydrins in the vegetative tissues which are scarcely represented in unstressed plants. The major sites of dehydrin accumulation in white clover under drought are the fully expanded younger leaves and the roots. Studies on dehydrin types and their physiological function in plant growth and development under normal and stress conditions would be helpful to establish a reliable “stress tolerance” profile in economically important crops.

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Table

Table 1. Primers used in PCR, RACE-PCR and RT-PCR experiments.

Primer	Description	Sequence (5'-3')
Degenerate primers		
KS legumes	Forward	ACAAGATTGGKGRKRCYCTTCA
KS legumes	Reverse	CTGCTGCTRTCATGRCCATG
Y-segment	Forward	GAYGAAYWKGGWAACCC
K-segment	Reverse	ATCAAGTATACCCTTCTTCTC
YnSKn gene-specific primers		
Y_3SK_2 F	Forward	ACTGTCACCACTCCTAATCCAACTTC
Y_3SK_2 R	Reverse	GGTGACCAGGCAATTTTTCTTT
1	Forward	ACTGTCACCACTCCTAATCCAACTTC
2	Forward	GGTGCTTATGGTGGCGGTGCA
3	Reverse	TCAGTGGTTGTGGTGACCAGGCA
4	Reverse	GGTGACCAGGCAATTTTTCTTT
5	Reverse	CTTGAACTGGAGGAGCGACGAT
5a	Reverse	TATATGGAAAACAGAAAGTGAGAAGC
YnKn gene-specific primers		
6	Forward	ATGAATATGGAAACCCAGTG
7	Forward	AGAAAGGGGTTATGGACAAGAT
8	Reverse	CTAGTGTCCAGTACATCCTCCAGTAC
SKn gene-specific		
9	Forward	TGGAACAGGAGTAACAACAGGTGGA
10	Reverse	TGCCAGTTGAGAAAGTTGAGGTTGT
KS gene-specific		
11	Forward	GAGACAAGAAAGAGGGAGAACACA
12	Reverse	CTTTTTCTTCTCACCTCCTTCACC
13	Reverse	GTTCTCCTTTGTACTCACCACCAC

Figure captions

Figure 1. Relative water content (RWC) measured in the first fully expanded leaf at the beginning of the experiment (day 14), after 7 (day 21) or 14 days of water deprivation (day 28), and after recovery (day 31). Bars represent standard deviation (n=3 for day 14 – 21, n=8 for day 28 – 31).

Figure 2. (**A**) Vector NTI analysis of the isolated *T. repens* CIG-like Y_nK_n sequence (GenBank ID: KC756189) and locations of the RT-PCR expression assays (marked with arrows and the numbers of the used primers according to Table 1). (**B**) CIG-like Y_nK_n RT-PCR amplification products in control (C) and drought-stressed (D) leaves (L1-L5), and roots (R). The arrows indicate the isolated and sequenced transcripts. (**C**) Expression ratio of Y_nK_n transcript (GenBank ID: KC247805) normalized to actin and tubulin in control, drought-stressed, and recovered leaves (L1-L5) and roots (R). The vertical bars indicate the standard deviation from three independent biological repeats. According to the performed unpaired two-tail Student's t-test significant differences between the samples derived from drought stressed plants and their controls are marked with * ($p \leq 0.05$) or *** ($p \leq 0.001$).

Figure 3. (**A**) RT-PCR amplification products of *T. repens* KS in control (C) and drought-stressed (D) leaves (L1-L5) and roots (R). The arrows indicate the isolated and sequenced transcripts. (**B**) Vector NTI analyses of *T. repens* KS sequence (GenBank ID: KC247802) and location of the RT-PCR expression assay (marked with arrows and the number of the used primers according to Table 1). (**C**) Expression ratio of KS224 transcript (GenBank ID: KC247802) normalized to actin and tubulin in control, drought-stressed, and recovered leaves (L1-L5) and roots (R). The vertical bars indicate the standard deviation from three independent biological repeats. According to the performed unpaired two-tail Student's t-test

significant differences between the samples derived from drought stressed plants and their controls are marked with * ($p \leq 0.05$) or *** ($p \leq 0.001$).

Figure 4. Expression ratio of *T. repens* SK_2 transcript (GenBank ID: EU846208) normalized to actin and tubulin in control, drought-stressed, and recovered leaves (L1-L5) and roots (R). The vertical bars indicate the standard deviation from three independent biological repeats. According to the performed unpaired two-tail Student's t-test significant differences between the samples derived from drought stressed plants and their controls are marked with * ($p \leq 0.05$) or ** ($p \leq 0.01$).

Figure 5. (**A**) Vector NTI analyses of *T. repens* Y_3SK_2 sequence (GenBank ID: GU443965.1) with identified transcript variants and locations of the RT-PCR expression assays (marked with arrows and the names of the used primers according to Table 1). (**B**) RT-PCR Y_3SK_2 amplification products in control (C), and drought-stressed (D) *T. repens* leaves (L1-L5) and roots (R). (**C**) Expression ratio of Y_3SK_2 transcript (GenBank ID: KC247804) normalized to actin and tubulin in control, drought-stressed, and recovered leaves (L1-L5) and roots (R). The vertical bars indicate the standard deviation from three independent biological repeats. According to the performed unpaired two-tail Student's t-test significant differences between the samples derived from drought stressed plants and their controls are marked with * ($p \leq 0.05$).

Figure 6. Immunoblot analysis of *T. repens* control (C) and drought-stressed (D) leaf (L0-5) (**A**) and root (R) dehydrins (**B**). Prestained molecular weight standards (Precision Plus Protein Prestained Dual Color, BioRad) are indicated. Arrows show the position of dehydrin signals.

Figure 1
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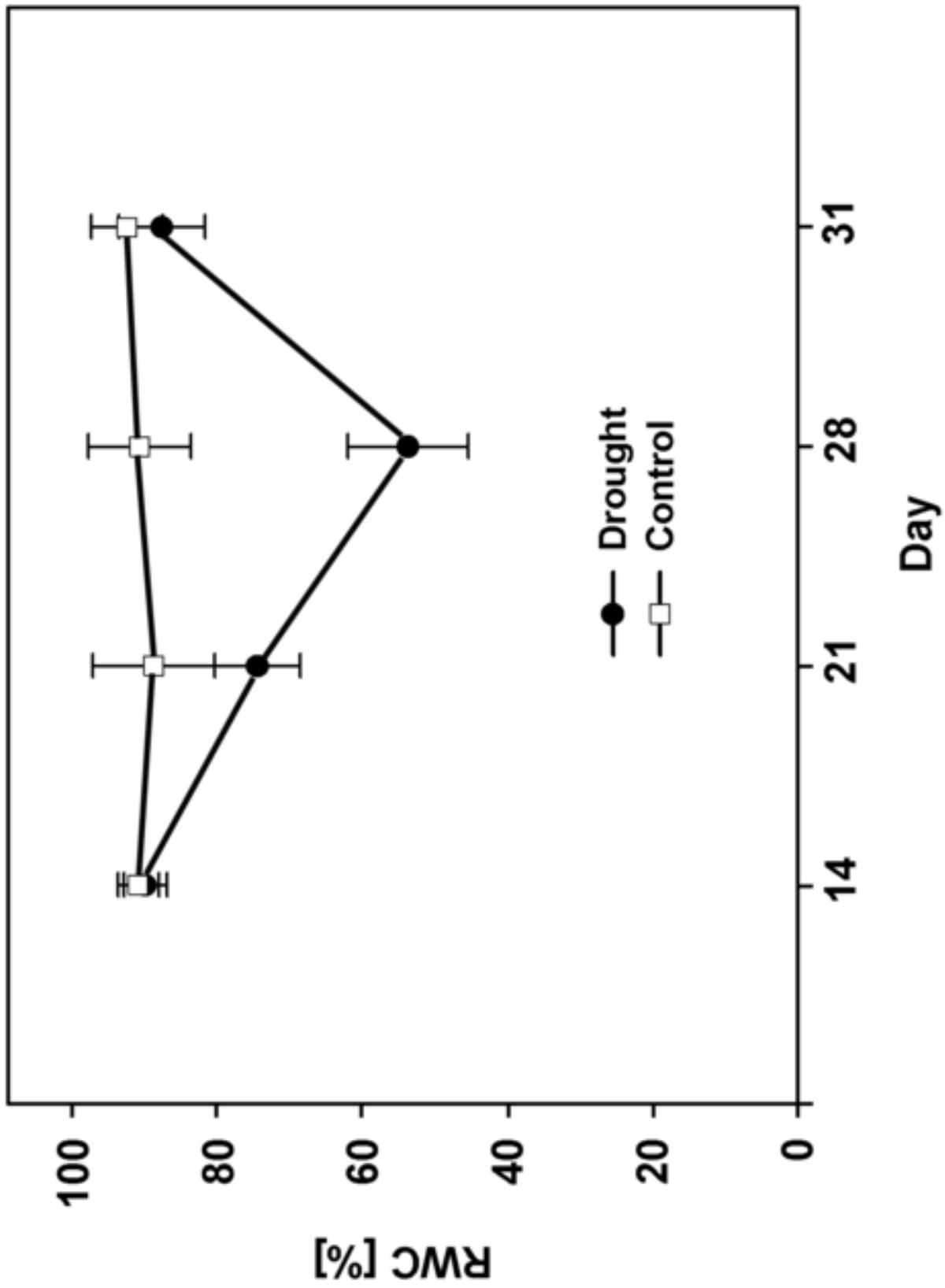

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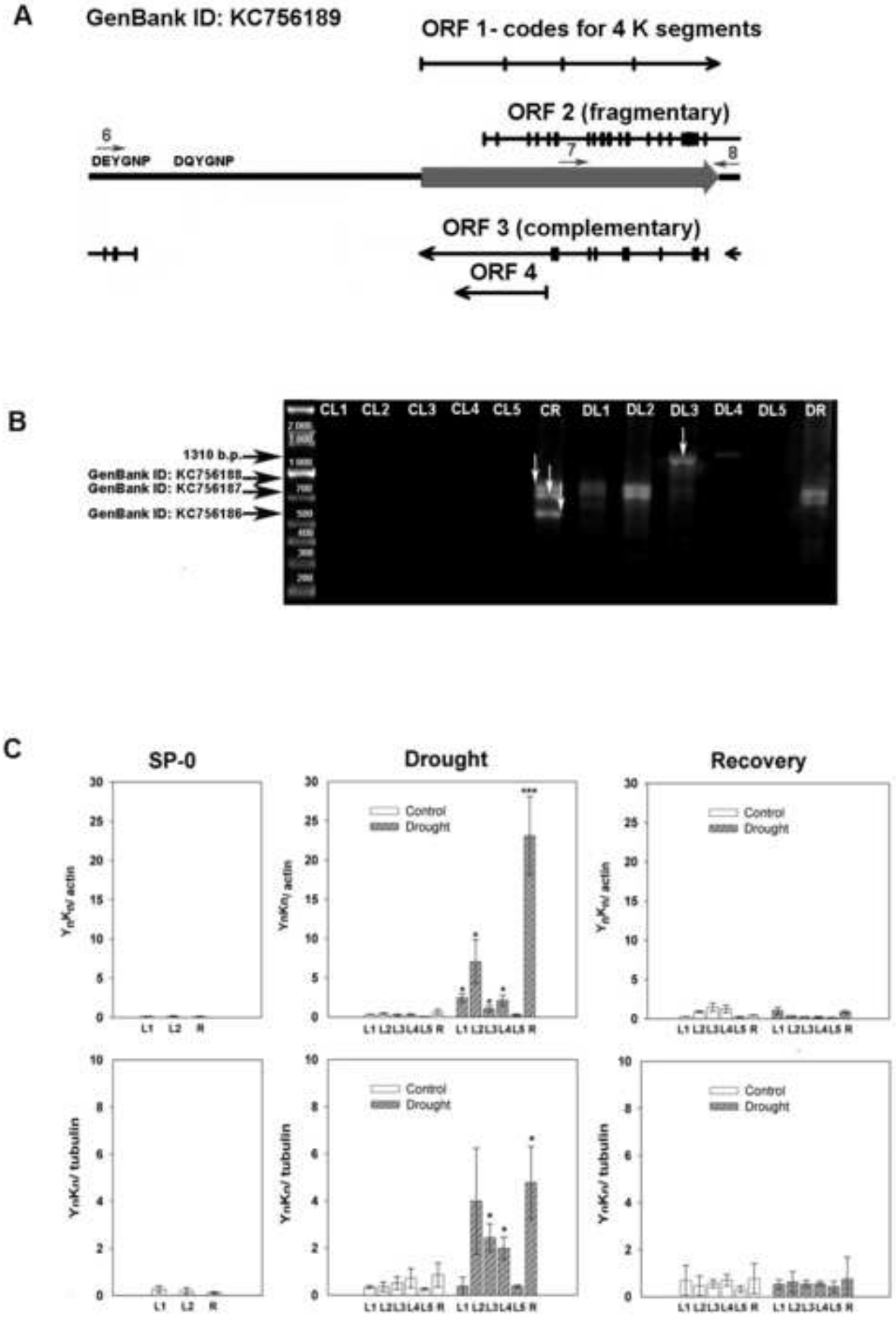

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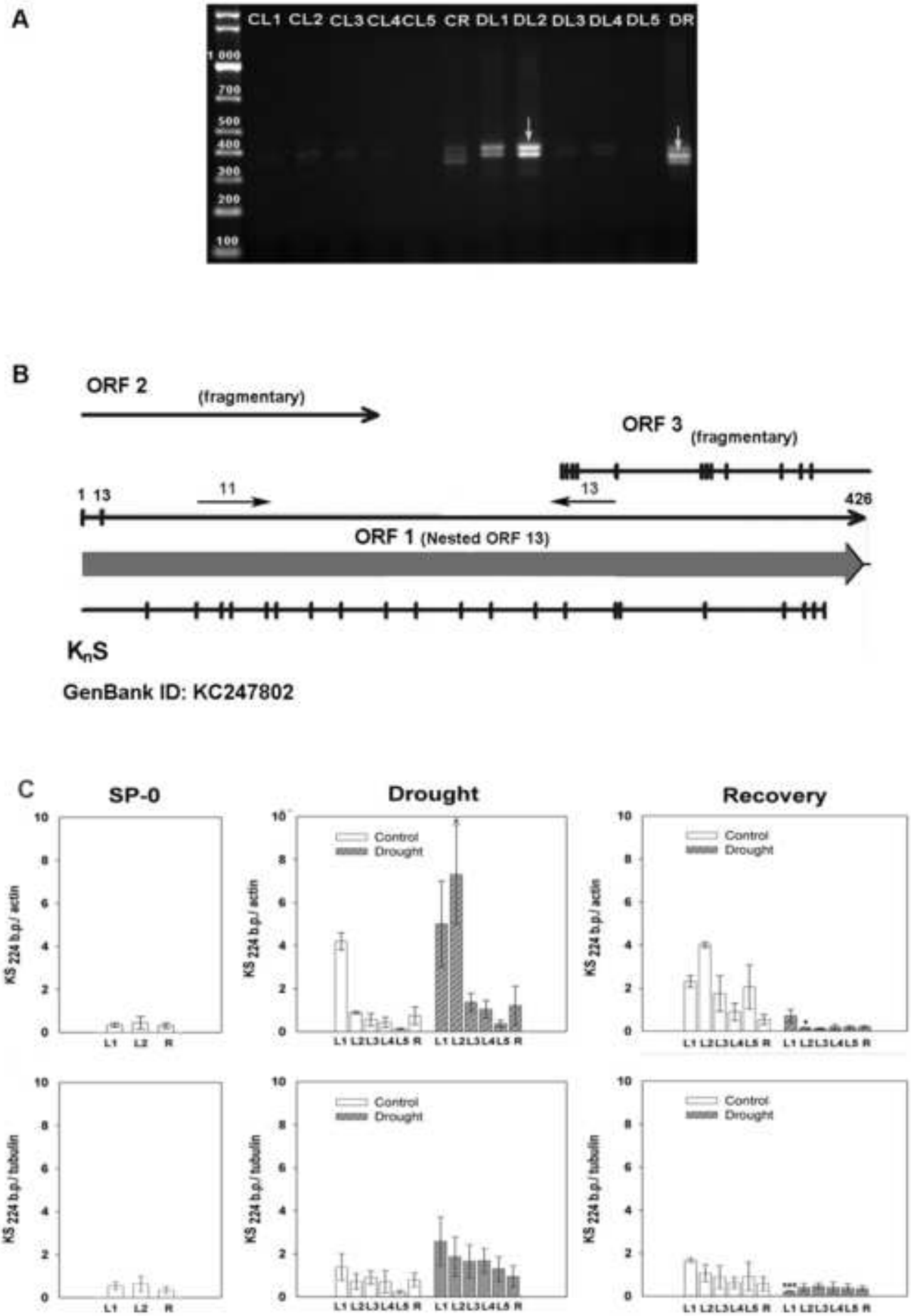

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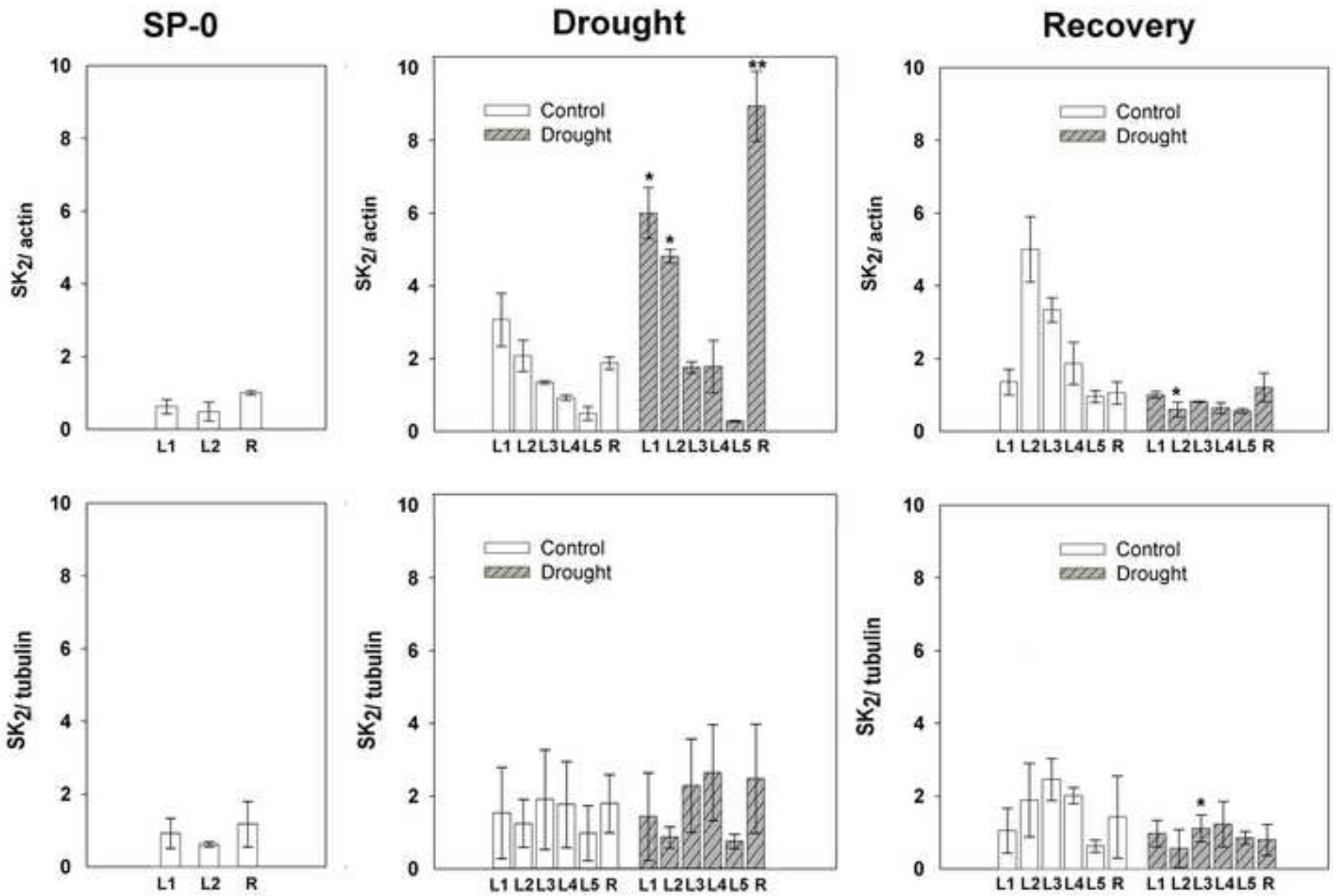

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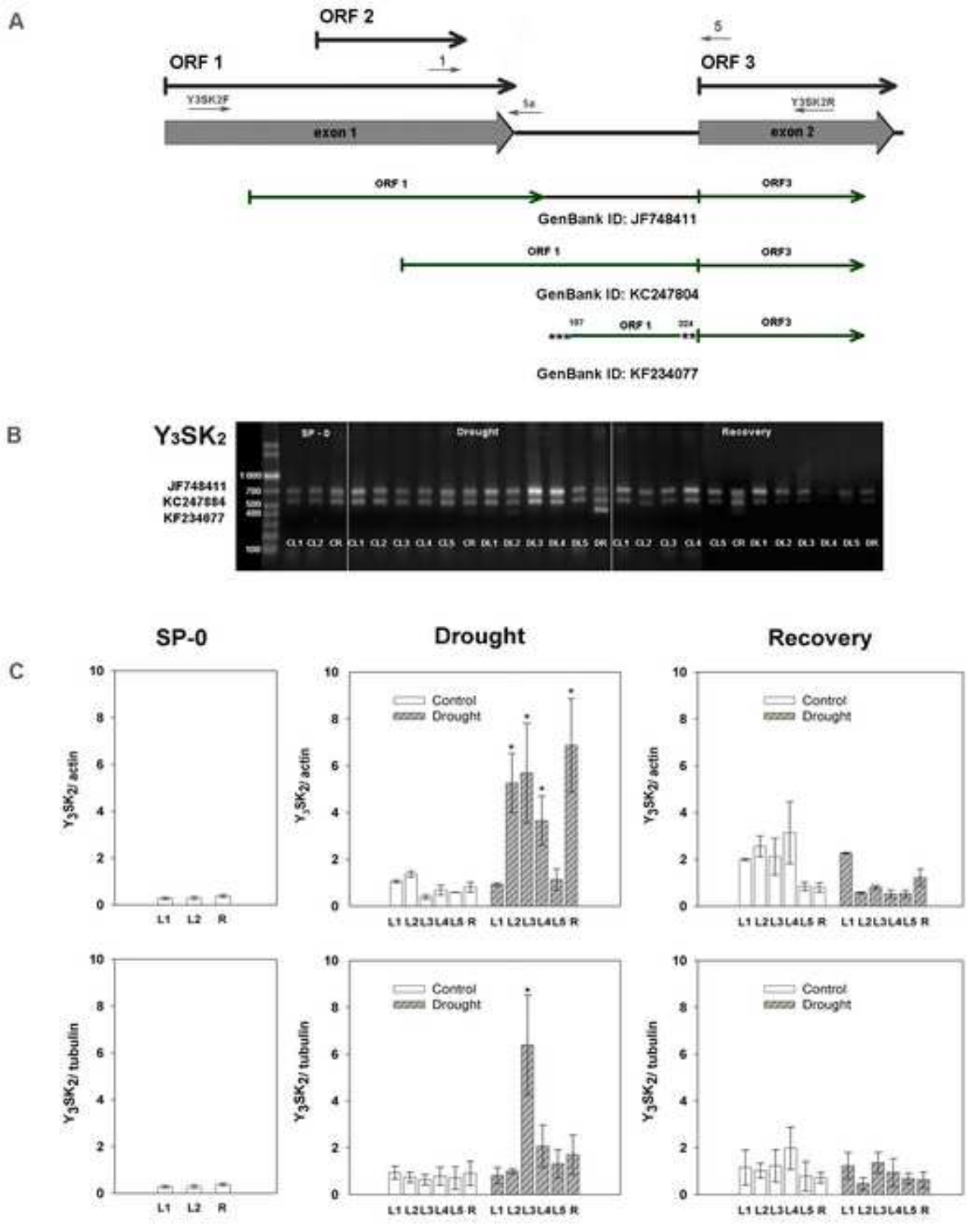

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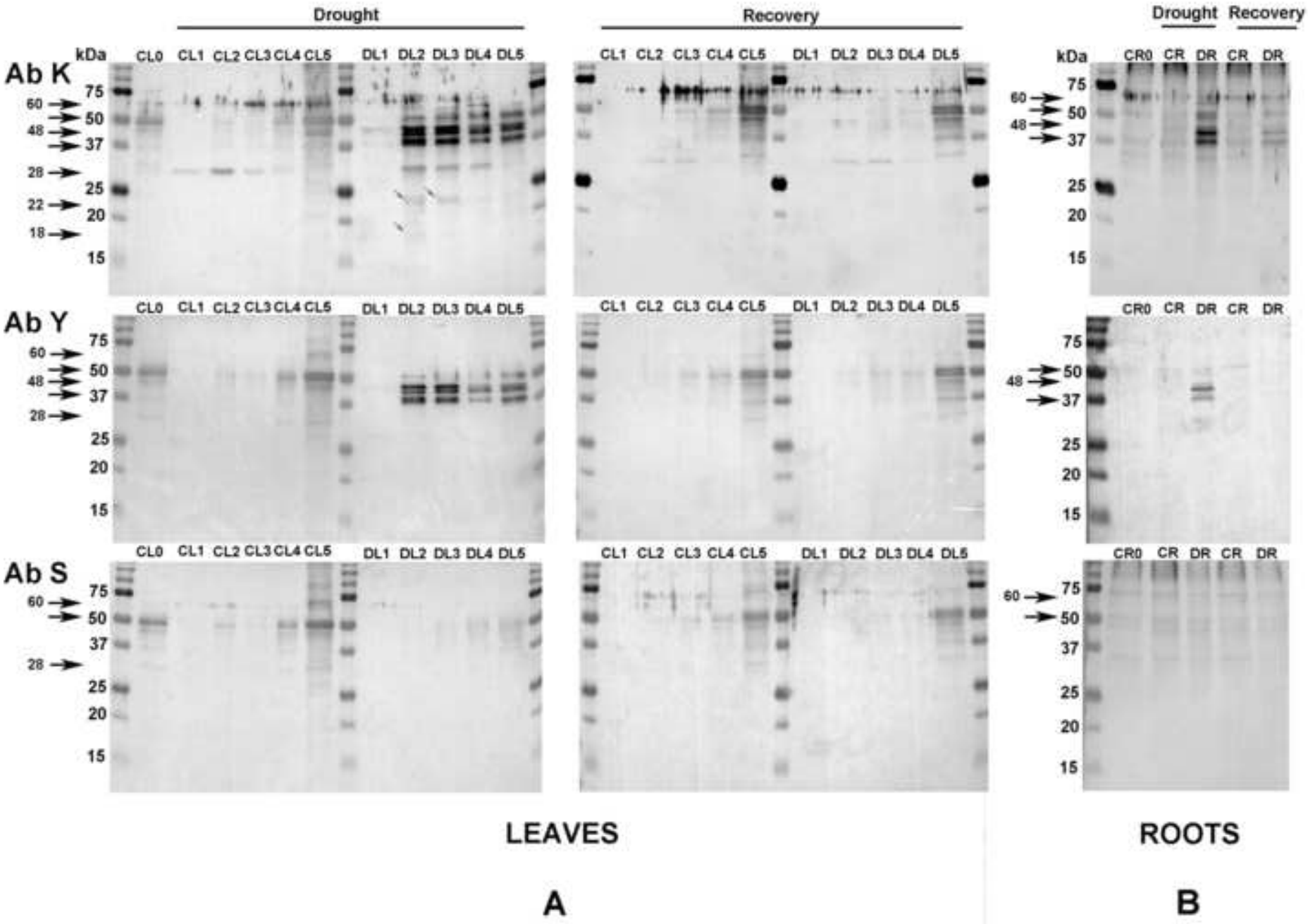

Supplementary file 1. Phylogenetic analysis of *T. repens* CIG-like Y_nK_n homologue with related legume dehydrins. Multiple sequence alignment (MegAlign, DNASTAR, Lasergene) showed that *T. repens* CIG-like Y_nK_n (ID: KC756189) has 46.7 % identity with *Galega orientalis* dehydrin (ID: HM7770 (estimated divergence – 41.9 %) and 44.2 % identity with *Vicia monata* dehydrin a (ID: AB506694.1) (estimated divergence – 73.9 %).

Supplementary file 2.

(**A**) Vector NTI analyses of the isolated partial *T. repens* CIG-like Y_nK_n sequences (GenBank IDs: KC247806 and KC247807) and locations of the RT-PCR expression assays (marked with arrows and the numbers of the used primers according to Table 1)

(**B**) Translation of the isolated partial *T. repens* CIG-like Y_nK_n mRNAs (K-segments are bold and underlined, Y-segments – italics). cNLS Mapper predicting importin α-dependent nuclear localization signals (Kosugi et al., 2009) did not find any evidence for NLS within the four translations, which means that the peptides should be localized in the cytoplasm.

Supplementary file 3. Alignment of 1310 b.p. cDNA derived from drought stressed leaf 3 (DL3) and genomic DNA coding for CIG-like Y_nK_n dehydrin (ID: KC756189).

Supplementary file 4. RT-PCR amplification products of Y_nK_n transcripts (ID: KC247805) (**A**); KS transcript (ID: KC247802) (**B**), SK_2 (ID: EU846208) (**C**), Y_3SK_2 transcripts (ID: JF748411 and ID: KC247804) (**D**); actin (**E**) and tubulin (**F**) in control (C), drought-stressed (D) and recovered leaves (L1-L5) and roots (R).

Supplementary file 5. Alignment of the translated KS transcripts derived from drought stressed leaves (ID: KC247802, DL) and drought stressed roots (ID: KC247803, DR).

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