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RAFFINOSE FAMILY OLIGOSACCHARIDES IN LUPIN (*Lupinus sp.*): POSSIBLE ROLE IN PLANT DEFENSE AGAINST ENVIRONMENTAL STRESS AND CHARACTERIZATION OF GENES ENCODING FOR A KEY ENZYME OF THEIR SYNTHESIS PATHWAY

MASTER THESIS

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VALDIVIA – CHILE

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RAFFINOSE FAMILY OLIGOSACCHARIDES IN LUPIN (*Lupinus* sp.): POSSIBLE ROLE IN PLANT DEFENSE AGAINST ENVIRONMENTAL STRESS AND CHARACTERIZATION OF GENES ENCODING FOR A KEY ENZYME OF THEIR SYNTHESIS PATHWAY

Thesis submitted to the Facultad de Ciencias Agrarias of the Universidad Austral de Chile in partial fulfillment of the requirements to apply for the degree of Master of Sciences

by

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MASTER THESIS FINAL APROBATION REPORT

The Thesis Evaluator Committee communicates to the Director of Escuela de Graduados of the Facultad de Ciencias Agrarias that the Master Thesis presented by the candidate

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has been approved in the defense exam of Thesis presented at day April 30th 2010, as requirement to apply for the degree of Master of Sciences and, so that this may be officially recorded, the signatories for all effects are:

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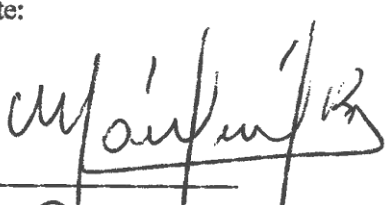

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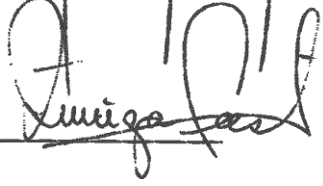

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1 SUMMARY

The accumulation of RFOs was studied in vegetative tissues of four lupin species (*L. luteus*, *L. albus*, *L. mutabilis* and *L. angustifolius*) under water and cold stresses. Genes encoding for the galactinol synthase enzyme, key in the regulation of RFOs synthesis, were isolated from *L. luteus* genomic DNA with the future prospect to breed this species with reduced RFOs seeds content. Raffinose was the only RFO detected in lupin vegetative tissues. Raffinose increased under water deficit in leaves, roots or both tissues (0.45 to 1.3-fold) in almost all lupin species but did not accumulate under cold stress. Sucrose and hexose increased in lupin tissues during both stresses. Two genes encoding galactinol synthase, *LlGolS1* and *LlGolS2*, were identified in *L. luteus* DNA. The *LlGolS1* gene was upregulated in roots but not induced in leaves of *L. luteus* under water stress, whereas *LlGolS2* was not expressed under the stress conditions and tissues evaluated. The different patterns of raffinose accumulation and *LlGolS1* expression suggests that, in lupin, raffinose is involved in plant response to water stress but not to cold stress. Possible roles of raffinose, sucrose and hexose in both stresses are discussed.

Key words: Raffinose – RFOs – Lupin - Abiotic stress – Water deficit – Cold.

2 RESUMEN

La acumulación de RFOs fue estudiada en tejidos vegetativos de cuatro especies de lupino (*L. luteus*, *L. albus*, *L. mutabilis* y *L. angustifolius*) en condiciones de estrés por frío y déficit hídrico. Genes codificando para la enzima galactinol sintasa, clave en la regulación de la síntesis de RFOs, fueron aislados desde ADN genómico de *L. luteus* con perspectivas futuras de mejorar esta especie para reducido contenido de RFOs en semillas. Rafinosa fue el único RFO detectado en tejidos vegetativos de lupino. Rafinosa incrementó bajo estrés hídrico en hojas, raíces, o ambos tejidos (0.45 a 1.3-veces) en casi todas las especies de lupino pero no se acumuló bajo estrés por frío. Sacarosa y hexosas incrementaron en tejidos de lupino durante ambos estreses. Dos genes codificando para galactinol sintasa, *LlGolS1* y *LlGolS2*, fueron identificados en el ADN de *L. luteus.* El gen *LlGolS1* fue regulado hacia arriba en raíces pero no fue inducido en hojas de *L. luteus* bajo estrés hídrico, mientras el gen *LlGolS2* no fue expresado bajo las condiciones de estrés y tejidos evaluados. Los diferentes patrones de acumulación de rafinosa y expresión de *LlGolS1* sugieren que, en lupino, rafinosa está involucrada en la respuesta de la planta a estrés hídrico pero no a stress por frío. Los posibles roles de rafinosa, sacarosa y hexosas en ambos estreses son discutidos.

Palabras clave: Rafinosa – RFOs – Lupino – Estrés abiótico – Déficit hídrico – Frío.

3 INTRODUCTION

Raffinose Family Oligosacharides (RFOs) are secondary metabolites that accumulate abundantly in seeds of leguminous species, producing flatulence and interfering with the correct assimilation of nutrients in humans and in monogastric animals (Dey, 1985; Murphy *et al*., 1972; Price *et al.*, 1988). The lupin genus (*Lupinus sp.*, Fabaceae) is characterized by having the highest levels of RFOs (0.5 to 16.1% of dry weight) in seeds (Petterson, 2004); and thus, limiting its use as foodstuff (Wang *et al*., 2003). Lupin is the leguminous with the most elevated protein content (29 to 50% of dry weight) (Trugo *et al*. 2003) and can be cultivated in regions with mild climates located in South America (Martínez-Villaluenga *et al*., 2006). For these reasons, researchers of the Southern Chile are dealing with the modification of the lupin seed RFOs content by genetic breeding approaches to generate genotypes improved in nutritional quality (Agri Aquaculture Nutritional Genomic Center-CGNA, 2010).

RFOs have been broadly studied in several plant species. Their main roles include protection during environmental stresses such as drought, heat, salinity and cold (Bachmann *et al.*, 1994; Taji *et al.*, 2002; Piotrowicz *et al*., 2005; Salerno *et al*., 1989; Peters *et al.*, 2007), enhancing seed storability (Bernal-Lugo and Leopold, 1992; Horbowicz and Obendorf, 1994), protection during seed desiccation (Clegg *et al*., 1982; Bailly *et al*., 2001), carbon reserve for seed germination (Dey, 1990; Frías *et al*., 1996), scavenging reactive oxygen species (ROS) (Nishizawa *et al*., 2008), and phloem transport of photo-assimilates (Ayre *et al*., 2003; McCaskill and Turgeon, 2007; Sprenger and Keller, 2000).

RFOs are non-reducing sugars of low molecular weight, soluble in water and water-alcohol solutions (Dey, 1985), and consist in lineal chains of galactosyl residues attached to the

glucose moiety of sucrose via an α-(1→6) glycosidic linkage (Avigad and Dey, 1997). The members of this molecular size increasing series are raffinose [α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D- fructofuranoside], stachyose [α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl- (1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside], verbascose [α-D-galactopyranosyl-(1→6)-[α-D-galactopyranosyl-(1→6)]$_2$-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] and ajugose [α-D-galactopyranosyl-(1→6)-[α-D-galactopyranosyl-(1→6)]$_3$-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] (Figure 1) (Obendorf *et al*., 1998). Higher RFOs do not have an assigned name. Sucrose and galactinol are the two substrates needed for the synthesis of the RFOs. The synthesis of galactinol [*O*-d-galactopyranosyl-[1,3,1]-l-*myo*-inositol], is catalyzed by the enzyme galactinol synthase (GolS; EC 2.4.1.123) (UDP-*α*-D-galactose: 1-L-*myo*-inositol-*O*-*α*-galactopyranosyltransferase) using the substrates UDP-galactose and *myo*-inositol (Lehle and Tanner, 1973; Liu *et al*., 1998). Galactinol transfers its galactosyl residue to the glucose moiety of sucrose by means of the raffinose synthase enzyme (RS; EC2.4.1.82). The enzyme stachyose synthase (STS; EC 2.4.1.67) promotes the stachyose synthesis by adding a galactose from galactinol to a raffinose molecule (Peterbauer and Richter, 1998). Stachyose and higher RFOs can also be synthesized in leaves via a galactinol independent pathway by the enzyme Galactan: Galactan Galactosyl transferase (GGT) (Bachmann *et al*., 1994). This enzyme catalyses the transfer of the terminal galactosyl residue of a RFO to a second RFO molecule, yielding a molecule with a higher degree of polymerization and another with a lower degree of polymerization (Figure 2) (Peterbauer and Richter, 2001).

Usually raffinose accumulates in most monocotyledon seeds whereas higher RFOs such as stachyose and verbascose accumulate predominantly in seeds of dicotyledons (Dey, 1985). Raffinose also accumulates in vegetative tissues under drought, cold and salt stresses (Taji *et al*., 2002; Sprenger and Keller, 2000; Peters *et al*., 2007). In leaves *myo*-inositol, galactinol, raffinose, GolS, RS and STS, are extravacuolar. Higher RFOs (stachyose, verbascose and ajugose) are almost exclusively vacuolar (Keller, 1992; Bachmann and

Keller, 1995). Consequently, it has been hypothesized that stachyose synthesis must occur in the cytoplasm followed by stachyose transfer to the vacuole (Peterbauer and Richter, 2001). This hypothesis is in agreement with the stachyose H^+ antiporter found in tonoplasts of *Stachys sieboldii* tubers (Keller, 1992; Greutert and Keller, 1993) and with the presence of GGT in vacuoles (Bachman *et al*., 1994). In seeds, the accumulation is considerably different because of the storage of stachyose seems to be mainly in the cytoplasm (Peterbauer and Richter, 2001).

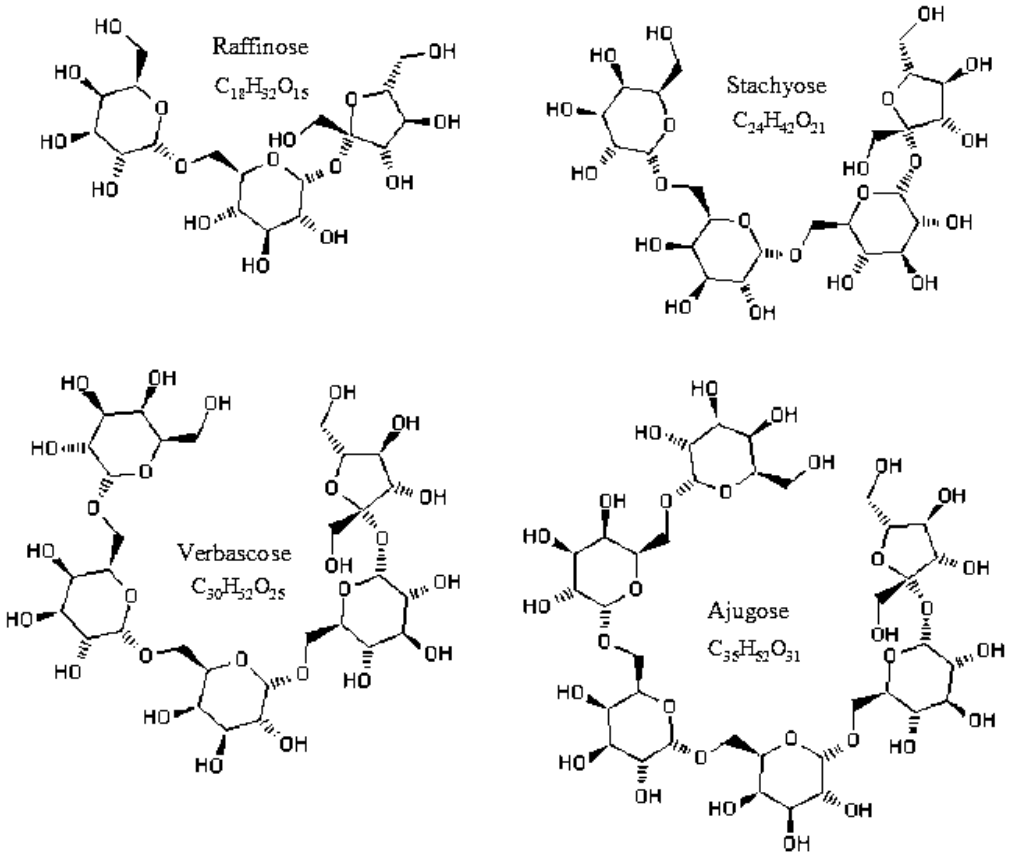

Figure 1. Raffinose family oligosaccharides found in *Lupinus* sp. tissues. Based on information reported by Martínez-Villaluenga *et al*., (2005) and Piotrowics *et al*. (2003). Molecules structures were extracted from Plant Metabolic Network (PMN) (2010).

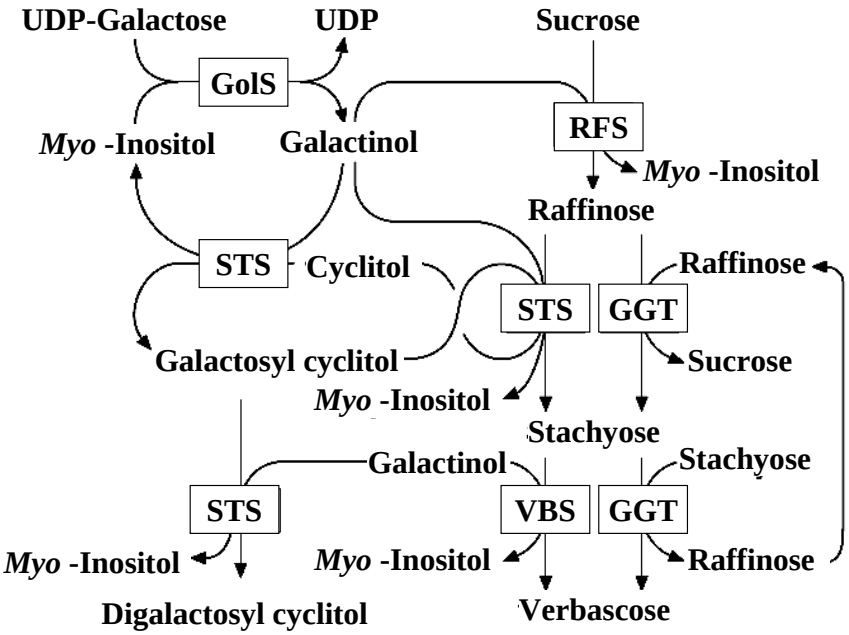

Figure 2. Schematic representation of the biosynthetic pathways of RFOs and galactosyl cyclitols. GolS, galactinol synthase; RFS, raffinose synthase; STS, stachyose synthase; VBS, verbascose synthase; GGT, galactan:galactan galactosyltransferase. Extracted from Peterbauer and Richter (2001).

RFOs concentrations and its allocation in the plant are regulated by a series of enzymatic reactions involved in the synthesis of these compounds. The GolS enzyme has been regarded a key enzyme in the RFOs synthesis pathway (Saravitz *et al*., 1987; Peterbauer and Ritcher, 2001). This assertion was functionally demonstrated by McCaskill and Turgeon (2007) whom suppressed *VpGolS* genes using RNAi, resulting in a pronounced inhibition of RFOs synthesis in the phloem of *Verbascum phoeniceum* (McCaskill and Turgeon, 2007). Galactinol synthase genes of *Arabidopsis thaliana*, *AtGolS1* and *AtGolS2*, seem to be constitutively expressed in seeds and are induced in vegetative tissues during drought, heat and salt stresses while *AtGolS3* is induced only by cold and oxidative stress in vegetative tissues and is expressed weakly in seeds (Taji *et al.*, 2002; Panikulangara *et al*.,

2004; Nishizawa *et al*., 2008). The *AtGolS3* promoter region contains two DRE (TACCGACAT) and two DRE like (A/GCCGAC) core motifs which are target of DREB1A, a transcription factor induced by cold stress (Taji *et al*., 2002). The DREB/CBF (DREB: Dehydration responsive-element binding factor; CBF: C repeat binding factor) genes are rapidly induced in response to low temperature, and encode transcriptional activators that control the expression of stress-inducible genes containing DRE in their promoters. The *AtGolS1* is a target of the heat shock factor 3 (HSF3) (Panikulangara *et al*., 2004) while recent studies have shown that the genes *AtGolS1*, *-2*, *-4*, and RS2 are induced in *A. thaliana* plants overexpressing the gene *HsfA2* encoding HSFA2, as well as the *AtGolS1*, *-2*, *-3*, *-4*, and *-8*, and *RS2*, *-4*, *-5*, and *-6*, are also induced by methylviolongen treatment, a compound that produce oxidative damage (Nishizawa *et al*., 2008).

RFOs studies on lupin species have mainly focused on determining their content in seeds (Martínez-Villaluenga *et al.*, 2005, 2008; Piotrowicz *et al*., 2003). Few efforts have raised the role of these sugars during environmental stress in vegetative tissues. For example, total alpha-galactosides have been determined in leaves and stems of *L. albus* under water deficit, but not the contribution of each specific oligosaccharide (Pinheiro *et al*., 2001, 2004). The effect of water deficit imposed during the pre-storage stage of seed development was determined in seeds of *L. albus* but not in vegetative tissues (Pinheiro *et al.*, 2005). In addition, the sequences of genes participating in the synthesis of RFOs and their expression patterns under abiotic stresses in lupin have still not been determined.

While plant breeders are trying to generate lupin cultivars with low RFOs, physiology studies suggest that these sugars may be related to plant tolerance to various stresses. If different isozymes are responsible of RFOs synthesis in different tissues or stress conditions, thus, it could be possible to generate cultivars with low RFOs content in seeds but still tolerant to stresses. A better understanding of RFOs roles in lupin and the genes involved in their synthesis would be useful to aid the generation of nutritionally improved lupin varieties. The working hypothesis of this work is that RFOs are responsible at least in

part of the response to environmental stresses in lupin vegetative tissues and the regulation of their synthesis is determined by the expression of different galactinol synthase isogenes. The main goal of this research is to study the accumulation of RFOs during water and cold stresses and their relationship with expression levels of galactinol synthase genes in lupin species. The specific goals are:

- To determine the effect of water and cold stresses on the RFOs concentration in several tissues of four lupin species (*L. luteus*, *L. angustifolius*, *L. mutabilis* and *L. albus*).
- To characterize the sequence of galactinol synthase genes in *L. luteus*.
- To study the expression of galactinol synthase genes in *L. luteus* under water and cold stresses.

4 MATERIAL AND METHODS

4.1 Plant material.

Four lupin species were included in the study: *Lupinus albus* L. cv. Rumbo-Baer (Chile), *Lupinus angustifolius* L. cv. Wonga (Australia), *Lupinus luteus* L. (Accession 098117/02, Poland), and *Lupinus mutabilis* Sweet (PI432332, USDA). Seeds stocks of *L. luteus* and *L. mutabilis* accessions were kindly provided by Ivan Maureira.

4.2 Experimental design of stress experiments.

The experiments were carried out using a split-split plot design where the main plot was the stress conditions [water deficit (WD) or cold stress (CS) versus no stress (NS)]. The first subplot in each main treatment was the harvesting time. The first harvest was carried out before the stress treatment, the second harvest at the end of the stress treatment, and the third harvest after plant stress recovery. The second subplot was the lupin species (*L. albus*, *L. angustifolius*, *L. luteus*, and *L. mutabilis)* which was arranged inside each first subplot. Both experiments were replicated three times (WD1, WD2, WD3, or CS1, CS2, CS3; and NS1, NS2, NS3). The water stress experiment was spatially blocked to isolate variability caused by the plants position inside the greenhouse. The cold experiment was temporally blocked staggering each block every four days, which ensured that each replicate was treated independently in the growth chamber. Treatments and replicates were randomly assigned in each experiment. The total size of each experiment was 72 observations where the experimental unit was a pot.

4.3 Water stress experiment.

Plants for the water stress experiment were established in spring under greenhouse conditions (natural photoperiod of 12/12 h day/night with an average temperature of 24/16 °C day/night, 70 to 80% humidity). One seed by genotype was sown in 2 L pots containing composite soil (organic soil:sand, 1:3). Plants were fertilized and watered when needed. When plants were one month old, WD was induced by withholding watering for 14 days. Watering was resume (RW) for stress recovery. Not stressed plants were watered regularly throughout the complete experiment. Tissue samples were collected minutes before the imposition of WD (Day 0), at the end of WD (Day 14), and two days after RW (Day 16). Three fully expanded young leaves and the half of a longitudinally cut root were collected, frozen in liquid nitrogen and stored at -80 °C for later RNA isolation. All remainder leaves and root tissues were collected for sugar extraction, weighed, frozen in liquid nitrogen, lyophilized, weighted again and kept at room temperature in an acrylic dessecator. The water content (WC) was computed by subtracting the dry weights (lyophilized tissues) to the fresh weights, and expressed as percentage of the fresh weight.

4.4 Cold stress experiment.

Plants for the cold experiment were established in summer under greenhouse conditions. Sowing of each replicate was spaced every four days in order to stagger the use of the growth chamber for the stress treatment. Four seeds per genotype were sown in 200 mL pots containing composite substrate (organic soil:sand, 1:3). Plants were grown under natural photoperiod of 14/10 h day/night with an average temperature of 24/16 °C day/night, 70% of relative humidity and standard fertilization and watering. When plants were two weeks old they were transferred at 9:00 h for 24 h to a growth chamber illuminated with cool white fluorescent tubes, set to a photoperiod of 14/10 h day/night with irradiance 600 µmol photons m^{-2} s^{-1}, simulating greenhouse environment. The temperature regimen in growth chamber was 4/4 °C day/night for CS and 24/16 °C for NS

plants. Additionally, CS pots were placed in a plastic container filled with ice to ensure cold conditions during stress treatment. After 24 h of treatment in the growth chamber, plants were replaced in the greenhouse for stress recovery (SR). Plant tissues were collected before transporting plants to the growth chamber (0 h), at the end of the stress treatment (24 h), and finishing SR (48 h). Leaves and roots of two plants were collected for RNA isolation, whereas tissues of the other two remaining plants in the pot were used for sugars extraction. Samples were frozen in liquid nitrogen and stored at -80 °C. Tissues for sugars isolation were later lyophilized and kept at room temperature in an acrylic dessecator.

4.5 Electrolyte leakage determination.

The membrane perturbation in leaf cells was determined by electrolyte leakage using the method described by Murray (1989) with modifications. Two expanded leaf blades were carefully collected in a 15 mL tube containing the same volume of distillated water. The tube was agitated over night in a horizontal shaker at room temperature, and then the electric conductivity was measured. The total cellular damage was estimated measuring the electric conductivity after autoclaving tissues 10 min at 105 °C. The percentage of damage was calculated in relation to the total cellular damage.

4.6 Soluble sugars extraction.

Soluble sugars were extracted according to Morvan-Bertrand *et al.* (2001) with modifications. Lyophilized leaf and root samples were ground with a mortar, and 100 mg of pulverized tissue were weighed and incubated 1 h at 60 °C in a vial containing 1 mL of 80% (v/v) ethanol and 0.1 g/L maltose as internal standard. Samples were centrifuged for 4 min at 5500 g, and the supernatant was recovered in a new vial. Pellet was extracted again 1 h at 60 °C with 1mL of distilled water. Vials were centrifuged for 4 min at 5500 g and supernatant was pooled with the precedent one, and stored at -20 °C for further analysis.

All incubation steps were carried out using a thermoregulated water bath and vials were agitated at 20 min intervals.

4.7 Sugars determination by high performance thin layer chromatography (HPTLC).

Soluble sugars contents were determined by High Performance Thin Layer Chromatography (HPTLC) using CAMAG Scientific Inc. (Muttenz/Switzerland) equipment. A standard of authentic sugars (Sigma) containing 1 g/L of sucrose, D-glucose, fructose, maltose, and 0.1 g/L of raffinose and stachyose, was used as reference to determine sugars identity and quantities in samples. Preceding the analysis, a program was set up using the computational winCAT platform provided by CAMAG. Then, aliquots of 0.1, 0.2, 0.4, 0.5, 1 and 2 μL of sugars standard, and 1 μL of each sample were sprayed on a Silicagel 60 HPTLC glass plate (Merck) using a CAMAG automatic TLC Sampler 4. Sugars were separated in a CAMAG ADC2 automatic chamber by three developments of 30 mm, 50 mm and 60mm, using acetonitrile solutions of increasing concentration. The first development was carried out in acetonitrile 70% (v/v), the second in acetonitrile 80% (v/v), and the third in pure acetonitrile. The HPTLC plates were equilibrated with the respective solvent for 5 min before development and dried for 3 min after each development. Sugars were detected by dipping the plate in a solution containing 1% (v/v) aniline, 1% (w/v) diphenylamine, 9% (v/v) orthophosphoric acid, and 90% (v/v) methanol, using a TLC immersion device III (CAMAG), and then heating for 3 min at 100 °C. Finally, chromatograms were analyzed by densitometry, with absorbance measured at 520 nm in a CAMAG TLC Scanner 3 equipped with Halogen Tungsten lamp. A polynomial regression curve based on the standards sugars concentrations and their respective picks areas was built by the winCAT software (CAMAG). Samples sugar quantities were then automatically calculated. The maltose (internal standard) value was used to correct for the lost of sugars from the sample during extraction.

4.8 Isolation of galactinol synthase genes from *L. luteus* DNA.

In order to isolate *L. luteus* galactinol synthase genes, a sequence alignment was carried out using *GolS* sequences of the others leguminous species *Glycine max* (Accession AY126715), *Phaseolus acutifolius* (CX129890), and *Medicago sativa* subsp. *falcata* (FJ607306). Sequences were obtained by search and BLAST in the Gene Index Project, and in the National Center of Biotechnology Information (NCBI) databases, available online at http://compbio.dfci.harvard.edu/tgi/index.html and http://www.ncbi.nlm.nih.gov, respectively. A set of nine degenerated primers were designed in the most conserved regions of the alignment, and were assayed by PCR in 20 possible combinations. Fragments of expected size were obtained with the forward primer 5´-TGCTRTGYTACCYGATGTTCC-3´ associated with the reverse primer 5´-AACRTTCTCAGGRTGACGCC-3´ (Couple 1), and with the forward primer 5`-GTYGTTGGTTTRGCMAAAGG-3´ associated with the reverse primer 5´-GCATTGAAATAKARAGGAGG-3´ (Couple 2). The PCR reactions were prepared using genomic DNA of *L. luteus* isolated from young leaves according to Fulton *et al.* (1995). The reaction mix consisted in 20 ng of DNA, 1X buffer PCR, 0.25 mM of each dNTP, 3.25 mM $MgCl_2$, 0.4 μM of each primer, and 0.8 U of *Taq* DNA polymerase (Fermentas) in a final volume of 20 µL. The PCR program consisted in a cycle at 94 °C for 3 min; followed by 10 cycles at 94 °C for 45 s, temperature touchdown of 65 to 55 °C (Couple 1) or 55 to 45 (Couple 2) for 45 s, 72 °C for 2 min; 32 cycles at 94 °C for 45 s, 45 s at 55 °C (Couple 1) or 45 °C (Couple 2), and finally 72 °C for 2 min. PCR products were separated on low melting point agarose gels. Expected fragments were purified from the gel using Qiaquick gel extraction kit (Qiagen), and cloned using StrataClone PCR Cloning Kit (Stratagene). Colonies containing the inserts were screened by color in agar plates supplemented with 7 μL of 20% IPTG, and 100 μg/mL of ampiciline. Additionally, white colonies were picked in 10 μL of water, incubated 5 min at 95 °C, and then analyzed directly by PCR. Plasmid DNA of ten positive clones of each transformation (n=20), was isolated using QIAGEN

plasmid mini kit. Aliquots of 2 μL of each plasmid DNA were sequenced using the service of the Brighnam Young University, Salt Lake City, Utah, USA.

4.9 RNA isolation and cDNA synthesis.

The total RNA was isolated according to De Vries *et al.* (1988). RNA quantities of 1 μg were treated with 0.5 U RNAse free DNAse (New England, BioLabs), and 1X DNAse I Reaction Buffer in a final volume of 10 μL. Then, the reaction mix was incubated 10 min at 37 °C, and 10 min at 75 °C to inactivate the enzyme. The treated RNA was reverse transcribed using oligo(dT) and AffinityScript QPCR cDNA synthesis kit (Stratagene), and was stored at -80°C.

4.10 Expression of *LlGolS* under stress conditions.

The expression of *LlGolS* genes under stress conditions was determined by Real Time PCR (QPCR) using Brilliant SYBR Green QPCR Master mix II (Stratagene), and an Mx3000P QPCR Instrument (Stratagene). The analysis of each sample was carried out in triplicate using 4 μL of a 1:100 cDNA dilution including a water negative control. The reaction mix consisted of 1X SYBR green master mix, 0.5 μM of each primer in a final volume of 15 μL. The QPCR reactions were set up in 96 well plates using optical Strip Caps (Stratagene). The QPCR mix and cDNA dilutions were prepared using HyPure molecular biology grade water (HyClone). The QPCR thermal profile consisted in a cycle at 95 °C for 10 min, then 40 cycles at 95 °C for 15 s, and 60 °C for 40 s. The specificity of each primer couple was analyzed in agarose gels, and by observing the dissociation curves calculated at temperatures between 50 and 95 °C. The efficiency of each primer combination was automatically determined by the slope of a standard curve computed using a set of 10X, 100X, 1000X, and 10000X cDNA dilutions. Primers were designed based on the sequences of the two isolated *GolS* genes. Primers LlGolS1-F (5´-TGTGTATCCACCAGAGAACCAA-3´) and LlGolS1-R (5´-

TCAAACAGATGGTCAATGTTCTCA-3´) are located on two different exons separated by an intron of 522 bp. The combination among LlGolS2-F (5´-CCTGTGAACCCACCA CAGAAT-3´) and LlGolS2-R (5´-ACCGTCTAGGTAAATCATCTTCT-3´) primers are located on two different exons separated by an intron of 589 bp. The elongation factor 1 alpha gene of *L. luteus* (*LlEF*-1α) was used as the reference gene to normalize the expression of *LlGolS*, and was amplified with the forward primer 5´-AGGTACTACTGCACAGTCATT-3´ and the reverse primer 5´-CTGACTGTGCCGTCCTTATCATT-3´. These primers amplify an expected fragment of 194 bp of the coding region of the *LlEF-1α*, and were designed using an unpublished sequence contained in a 454-Expressed Sequence Tag (EST) library (L. Parra, J. Udall, S. Straub, H. salvo, and I. Maureira, personal communication). The *LlGolS* and *LlEF-1α* primers were selected among a total of 20 combinations due to their better performance, and their efficiency values near to 100%. Finally, the expression of *LlGolS* gene was expressed as relative quantitative abundance using the delta-delta cp method mathematical model developed by Perkin Elmer Applied Biosystems (Perkin Elmer, Foster CIM, CA).

4.11 Statistical analysis.

Variance analyses as well as the differences among least square means t-tests between control and treated plants were carried out using The SAS System for Windows V8.02 (SAS Institute Inc, Cary, NC, USA). Each point presented in figures corresponds to least square means of three independent experimental replicates, and error bars indicate standard error (±SE). Probability values lower than 0.05 were regarded as statistically significant.

5 RESULTS

5.1 Raffinose, sucrose and hexose accumulation patterns in several vegetative tissues of *Lupinus* sp. under water deficit.

Closing leaves were observed in lupin plants by the 7 d of withholding watering, however, at this stage leaves kept the capacity to reopen fully during the night (picture not shown). The closing leaves state was permanent during day and night by the end of water deficit treatment (Day 14) (Figure 3). Wilted leaves were only observed in the base of *L. mutabilis* plants (Figure 3). All plants maintained their capacity to fully recover after rewatering (Day 16) (picture not shown). Water withholding lead to a significant RWC decline from 91.61 ± 2.89 to 83.14 ± 0.93% of dry weight (%DW) only in roots of *L. mutabilis* (Figure 4). Electrolyte leakage kept invariable during the experiment (result not shown).

The soluble sugars detected in lupin vegetative tissues were sucrose, hexose, and raffinose. Glucose and fructose were not well separated by the method and were thus considered together as hexoses. The basal content of each sugar fraction as well as its course varied depending on the species and type of tissue (Figure 5, 6, 7). *L. albus* (316.83 $mg \cdot g^{-1}DW$) had the higher content of total soluble sugars, followed by *L. luteus* (138.43 $mg \cdot g^{-1}DW$), *L. mutabilis* (109.22 $mg \cdot g^{-1}DW$) and *L. angustifolius* (75.2 $mg \cdot g^{-1}DW$) (Result not shown). The species *L. angustifolius* and *L. mutabilis* showed a similar proportion of sucrose and hexose in leaves with prevalence of sucrose in roots; while *L. albus* and *L. luteus* contained mainly hexoses in leaves, and similar proportion of sucrose and hexose in roots (Figure 5, 6).

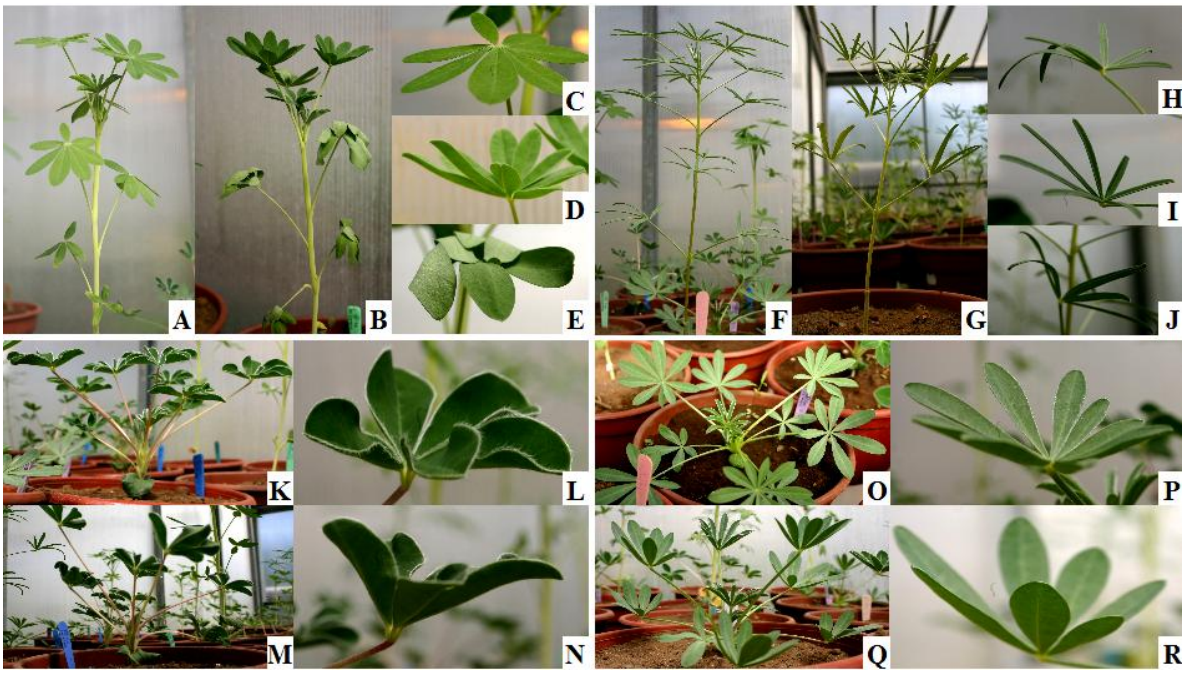

Figure 3. Plant pictures taken on day 14 of the water stress experiment. *L. mutabilis* (A-E), *L. angustifolius* (F-J), *L. albus* (K-N), and *L. luteus* (O-R). Control plants (A, C, F, H, K, L, O, P) present fully open leaves. Leaves of stressed plants (B, D, E, G, I, J, M, N, Q, R) are wilted or closed.

Under water deficit, the soluble sugars content was affected differently depending on tissues and species. Sucrose was constant in leaves of every lupin species but increased significantly in roots of *L. angustifolius* (2.48-fold), and *L. mutabilis* (1.239-fold) (Figure 5). Similar results were observed for hexose content which only increased in roots of *L. angustifolius* (3.6728-fold) (Figure 6). Although raffinose was barely detected in vegetative tissues, these sugars increased significantly in leaves of *L. albus* (0.66-fold) and in roots of *L. mutabilis* (0.617-fold). The same pattern was observed in *L. luteus* (0.8066-fold in leaves; 1.3-fold in roots) but the differences were just above the significant threshold (Figure 7). The extra accumulation of raffinose in the stressed plants disappeared after two days of rewatering (Figure 5, 6, 7).

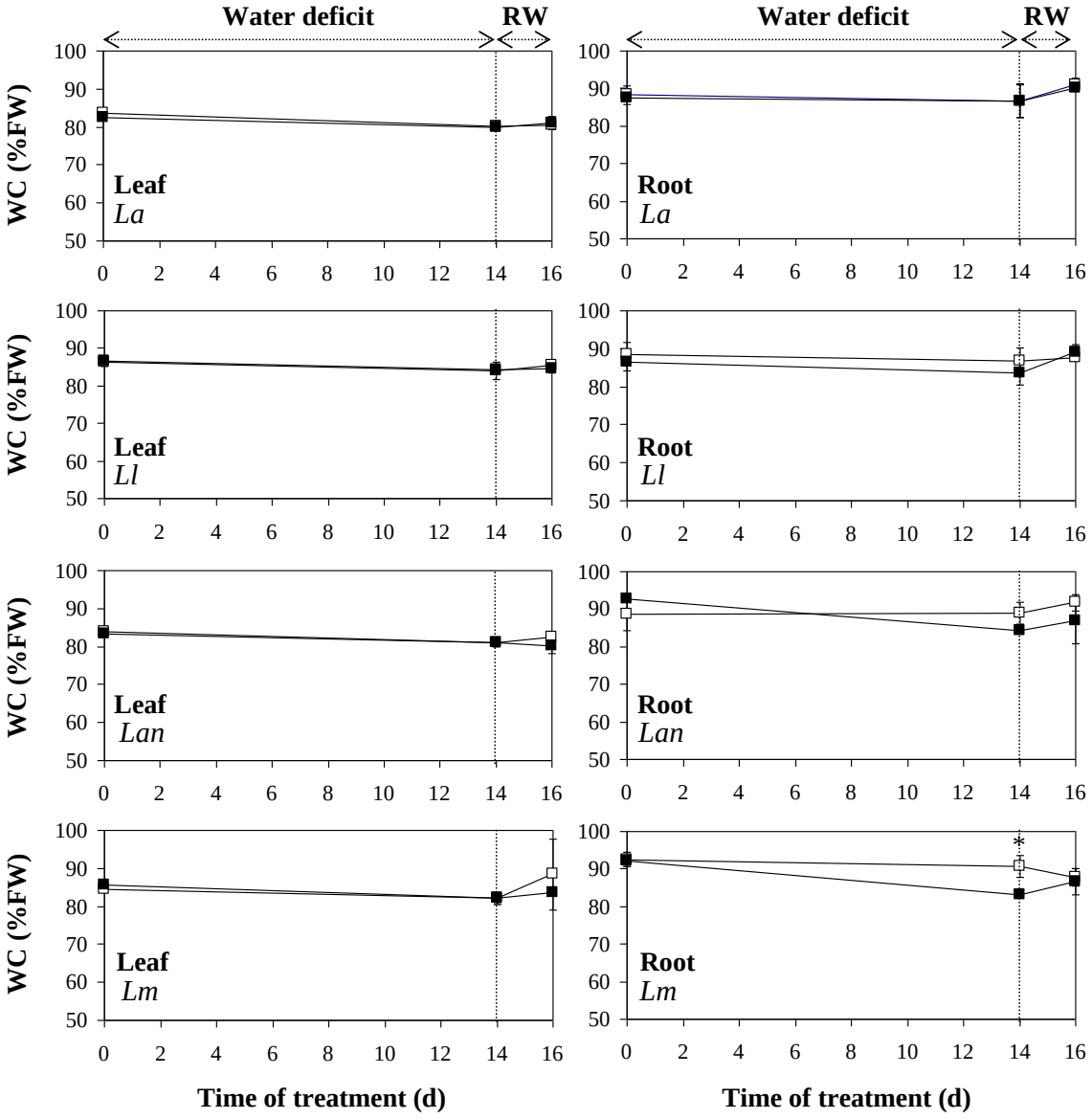

Figure 4. Water content in *Lupinus* sp. tissues during the course of the water stress experiment. Open squares indicate control plants whereas closed squares shows plants subjected to 14 d of water deficit. Each point corresponds to least square means (n=3). Vertical bars represent ± SE. Asterisks show significant differences among control and treated plants with $P < 0.05$. RW: rewatering, DW: Dry weight, *La*: *L. albus*, *Ll*: *L. luteus*, *Lan*: *L. angustifolius* and *Lm*: *L. mutabilis*, WC: water content.

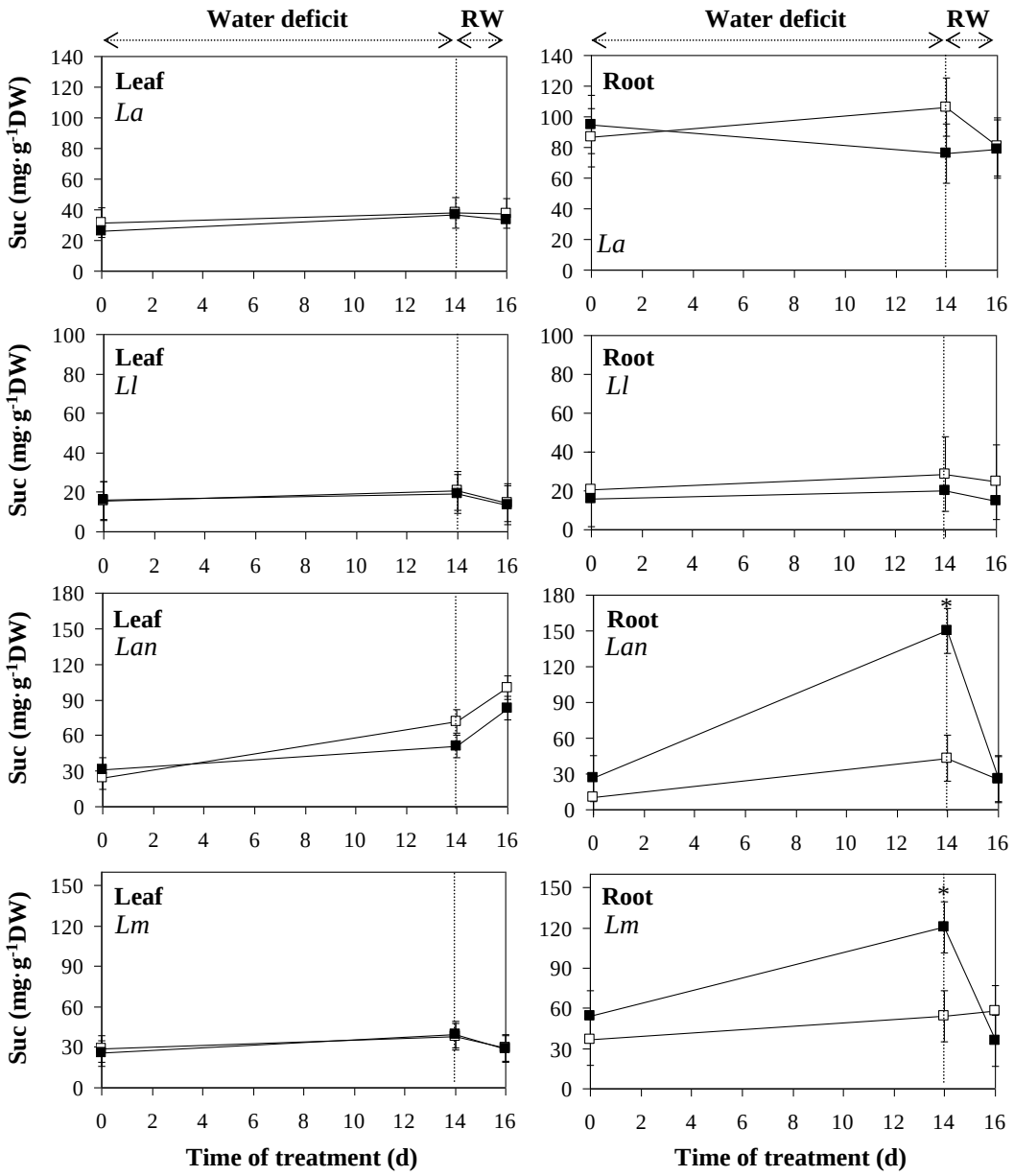

Figure 5. Sucrose content in *Lupinus* sp. tissues during the course of the water stress experiment. Open squares indicate control plants whereas closed squares shows plants subjected to 14 d of water deficit. Each point corresponds to least square means (n=3). Vertical bars represent ± SE. Asterisks show significant differences among control and treated plants with $P < 0.05$. Suc: sucrose, RW: rewatering, *La*: *L. albus*, *Ll*: *L. luteus*, *Lan*: *L. angustifolius* and *Lm*: *L. mutabilis*.

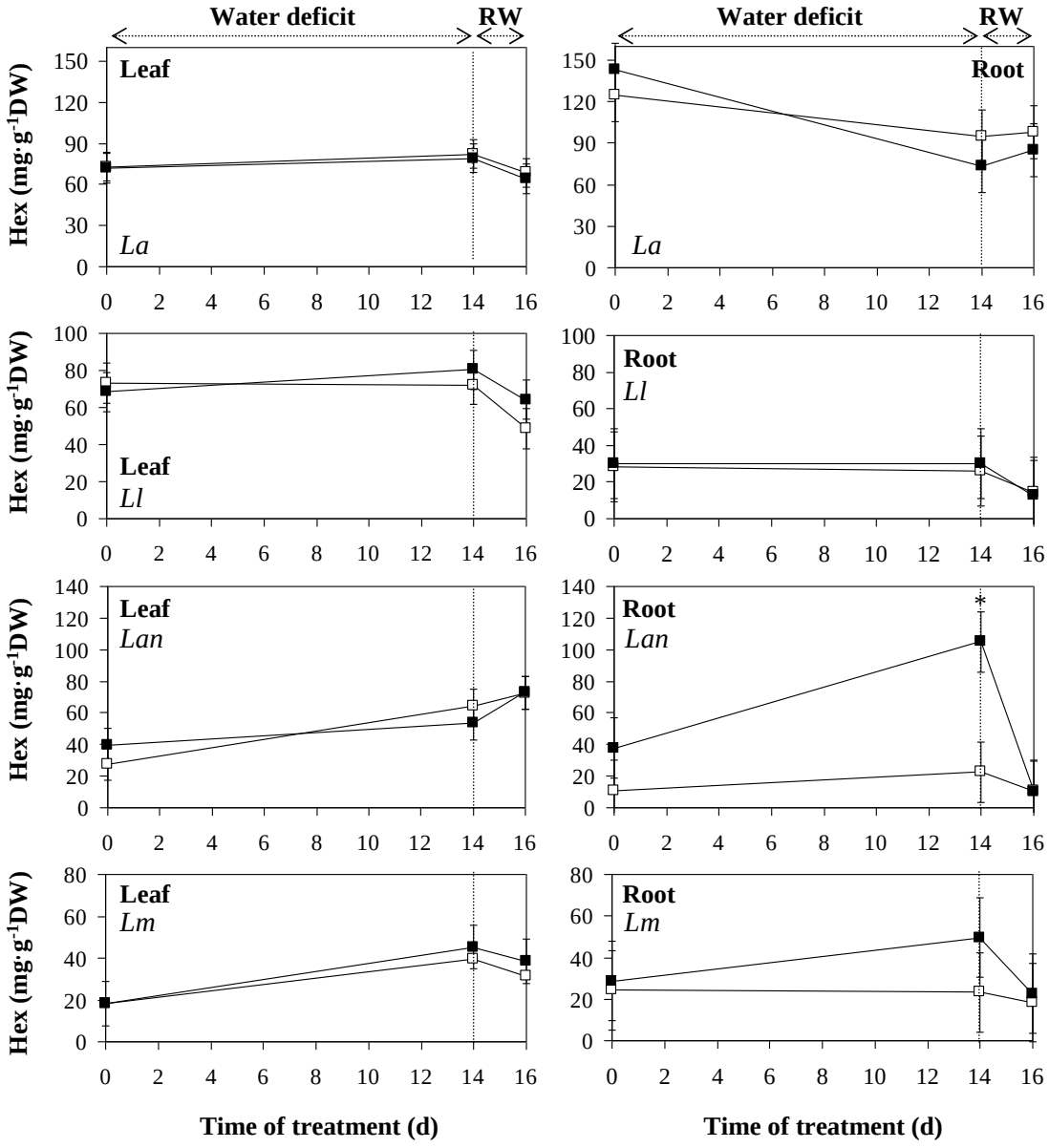

Figure 6. Hexose content in *Lupinus sp.* tissues during the course of the water stress experiment. Open squares indicate control plants whereas closed squares shows plants subjected to 14 d of water deficit. Each point corresponds to least square means (n=3). Vertical bars represent ± SE. Asterisks show significant differences among control and treated plants with $P < 0.05$. Hex: hexoses, RW: rewatering, *La*: *L. albus*, *Ll*: *L. luteus*, *Lan*: *L. angustifolius* and *Lm*: *L. mutabilis*.

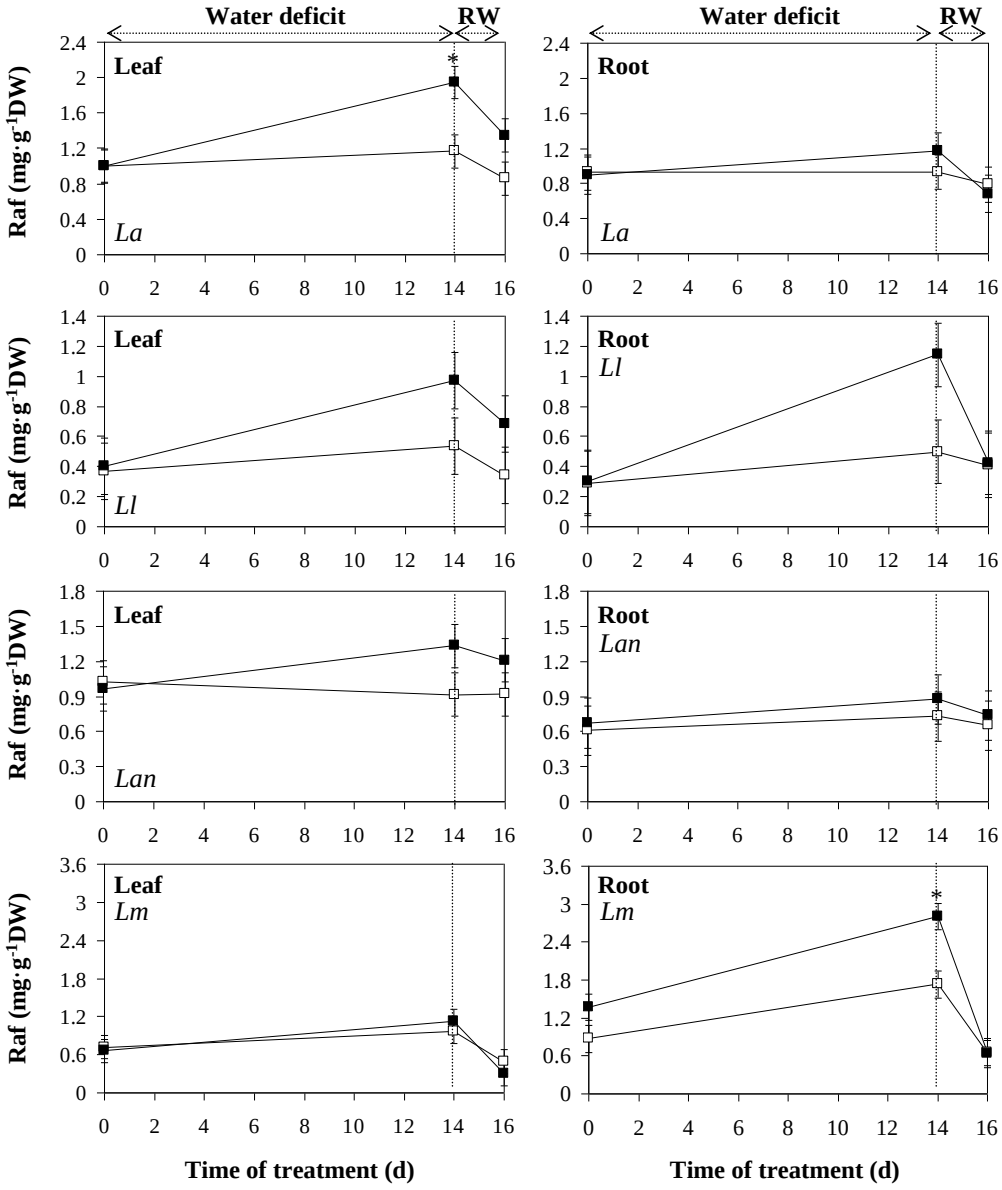

Figure 7. Raffinose content in *Lupinus* sp. tissues during the course of the water stress experiment. Open squares indicate control plants whereas closed squares shows plants subjected to 14 d of water deficit. Each point corresponds to least square means (n=3). Vertical bars represent ± SE. Asterisks show significant differences among control and treated plants with $P < 0.05$. Raf: raffinose, RW: rewatering, *La*: *L. albus*, *Ll*: *L. luteus*, *Lan*: *L. angustifolius* and *Lm*: *L. mutabilis*.

5.2 Sucrose and hexoses but not raffinose accumulated in *Lupinus* sp. vegetative tissues under cold stress.

Two hours after imposition of the cold stress treatment, leaves turned flagged in comparison with control plants. Leaves showed chlorosis symptoms at the end of stress treatment. After the recovery period, control and stressed plants could not be differentiated visually (result not shown). There were no electrolyte leakage differences between cold treated and control leaves (results not shown). Sucrose and hexose were the only soluble sugars detected in leaves and roots of *Lupinus* sp. plants at the age of 14 d. Considering the total soluble sugars in control plants, *L. angustifolius* (86.92 $mg \cdot g^{-1}DW$) contained the highest content followed by *L. luteus* (69.85 $mg \cdot g^{-1}DW$), *L. albus* (62.55 $mg \cdot g^{-1}DW$), and *L. mutabilis* (25.93 $mg \cdot g^{-1}DW$). At day 0, sucrose was more abundant than hexose in leaves of *L. albus* and *L. mutabilis* but hexose was predominant in leaves of *L. luteus* and *L. angustifolius*. In roots of each lupin species there was a higher proportion of sucrose than hexoses (Figure 8, 9). During the cold stress treatment sucrose increased in both leaves and roots tissues of *L. luteus* (2.79/2.042-fold leaves/roots) and *L. angustifolius* (1.25/1.93-fold), and in roots of *L. mutabilis* (1.774-fold). Sucrose kept constant in leaves of *L. mutabilis* and in leaves and roots of *L. albus* (Figure 8). Hexoses increased significantly in leaves of *L. albus* (6.43-fold), *L. luteus* (4.18-fold), *L. mutabilis* (2.312-fold), and *L. angustifolius* (1-fold), whereas it stayed constant in roots (Figure 9).

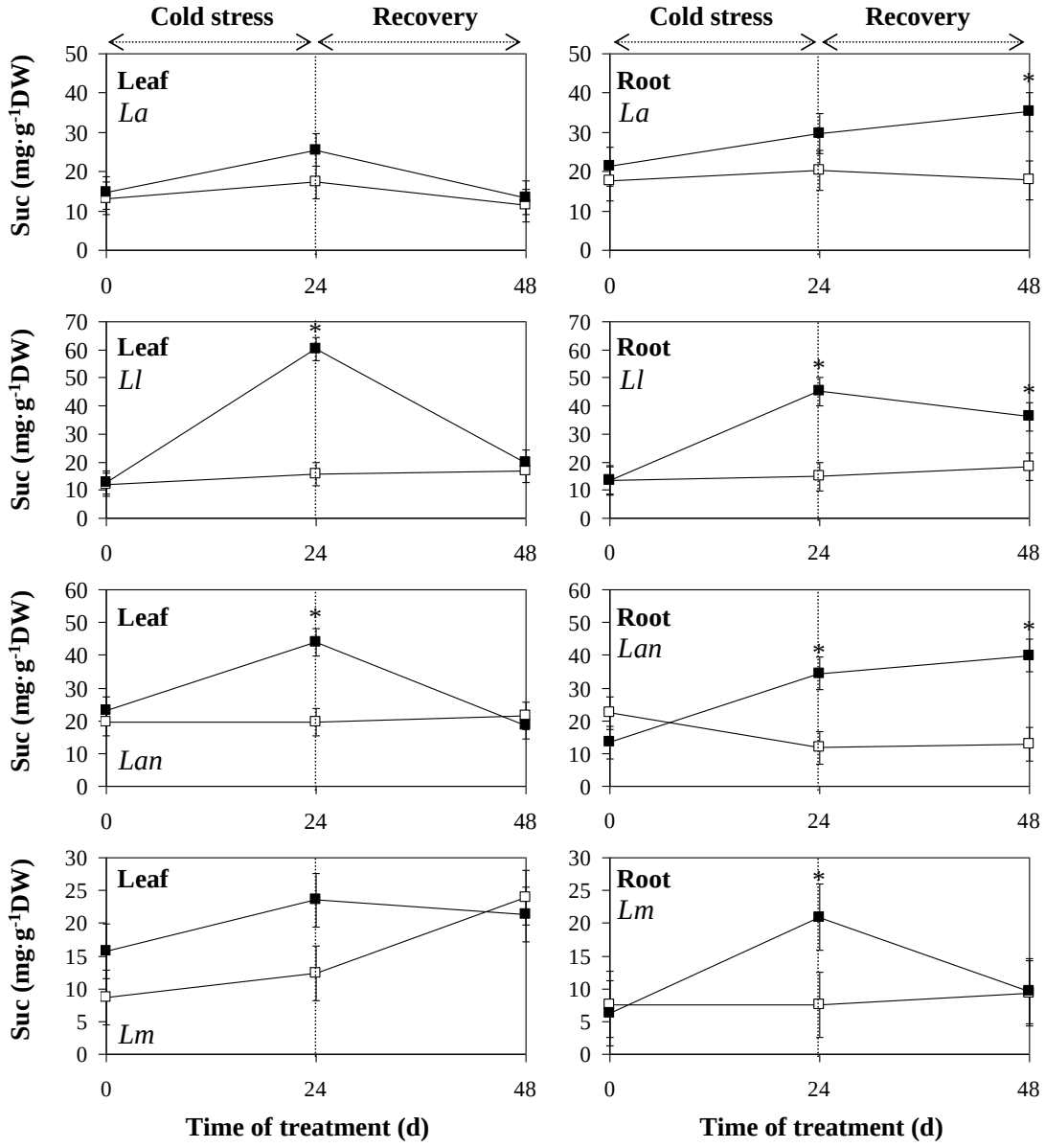

Figure 8. Sucrose content in *Lupinus* sp. tissues during cold stress experiment. Open squares indicate control plants whereas closed squares shows plants subjected to 14 d of water deficit. Each point corresponds to least square means (n=3). Vertical bars represent ± SE. Asterisks show significant differences among control and treated plants with $P < 0.05$. *La*: *L. albus*, *Ll*: *L. luteus*, *Lan*: *L. angustifolius* and *Lm*: *L. mutabilis*.

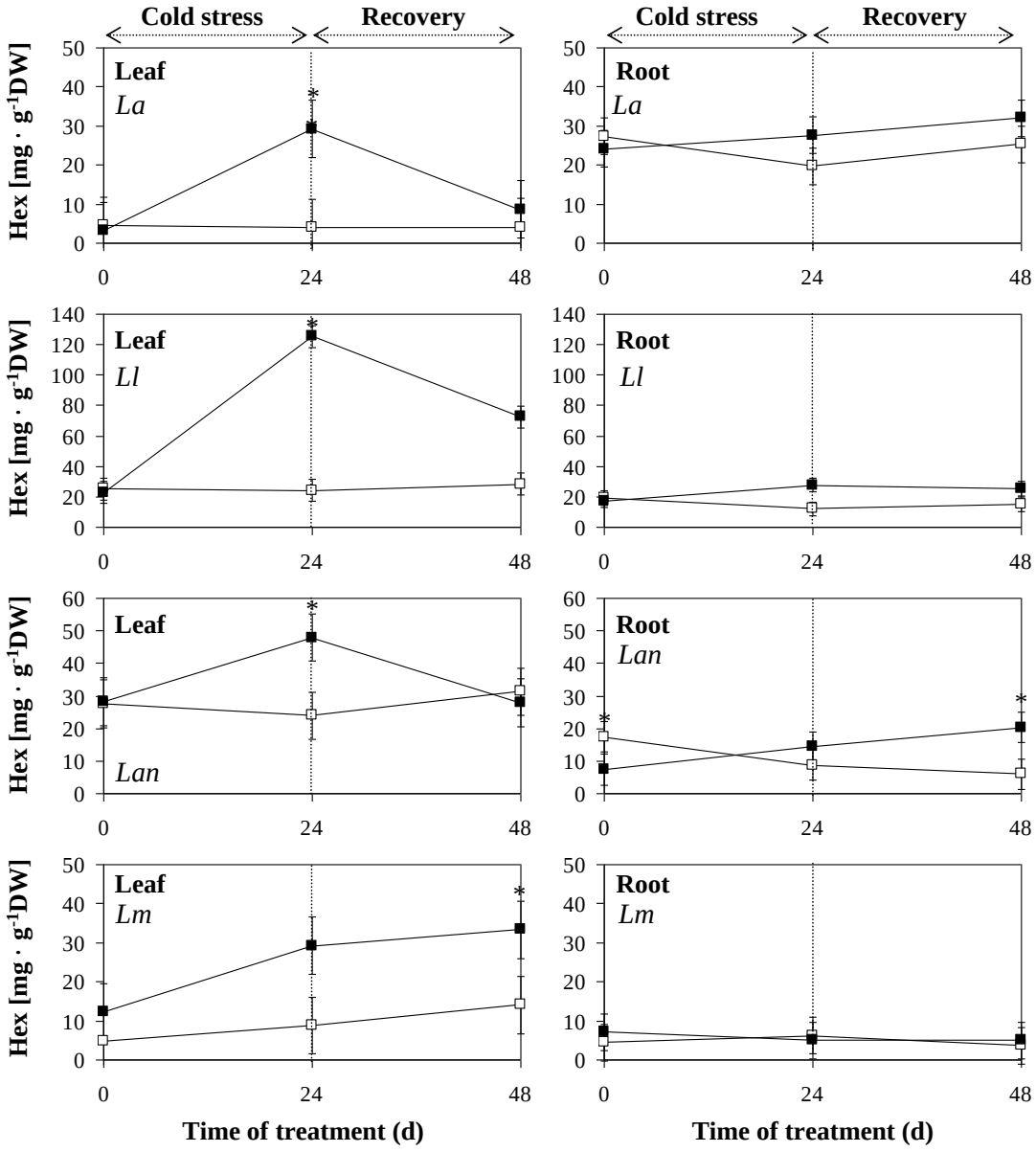

Figure 9. Hexose content in *Lupinus* sp. tissues during cold stress experiment. Open squares indicate control plants whereas closed squares shows plants subjected to 14 d of water deficit. Each point corresponds to least square means (n=3). Vertical bars represent ± SE. Asterisks show significant differences among control and treated plants with $P < 0.05$. *La*: *L. albus*, *Ll*: *L. luteus*, *Lan*: *L. angustifolius* and *Lm*: *L. mutabilis.*

5.3 Sequence characterization of two distinct galactinol synthase genes in *L. luteus* DNA.

Two different galactinol synthase genes were isolated from *L. luteus* genomic DNA. The gene fragments were arbitrary called *LlGolS1* and *LlGolS2* and expanded 1324 bp and 890 bp, respectively. *LlGolS1* and *LlGolS2* were aligned with a *Glycine max* galactinol synthase sequence (*GmGolS*) and gene structure details can be observed in Figures 10 and 11. Both *LlGolS* sequences have over 70% of similarity with *GolS* genes of several plants species belonging to different taxonomic families: *Ajuga reptans* (Lamiaceae), *Arabidopsis thaliana* and *Thellungiella halophila* (Brassicaceae), *Boea hygrometrica* (Gesneriaceae), *Coptis japonica* (Ranunculaceae), *Fagopyrum esculentum* (Poligonaceae), *Populus* sp. (Salicaceae), *Lycopersicon esculentum* and *Capsicum annum* (Solanaceae), *Vitis riparia* (Vitaceae), *Xerophyta viscosa* (Velloziaceae), *Verbascum phoeniceum* (Scrophulareaceae), and *Zea mays* (Poaceae). The similarity among *LlGolS1* and *LlGolS2* coding regions (aligned conserved region) was 85%, while the deduced aminoacids sequences had 88% homology (Result not shown). The alignment of LlGolS proteins using as reference sequences of other species revealed the presence of a putative manganese binding motif-DXD (Takanashi *et al*., 2008) on the lupin sequences (Figure 12). The phylogeny analysis grouped LlGolS sequences in the same subtree that *Glycine max*, *Medicago sativa* and *Ammopiptanthus mongolicus*, all species belonging to the Fabaceae family (Figure 13).

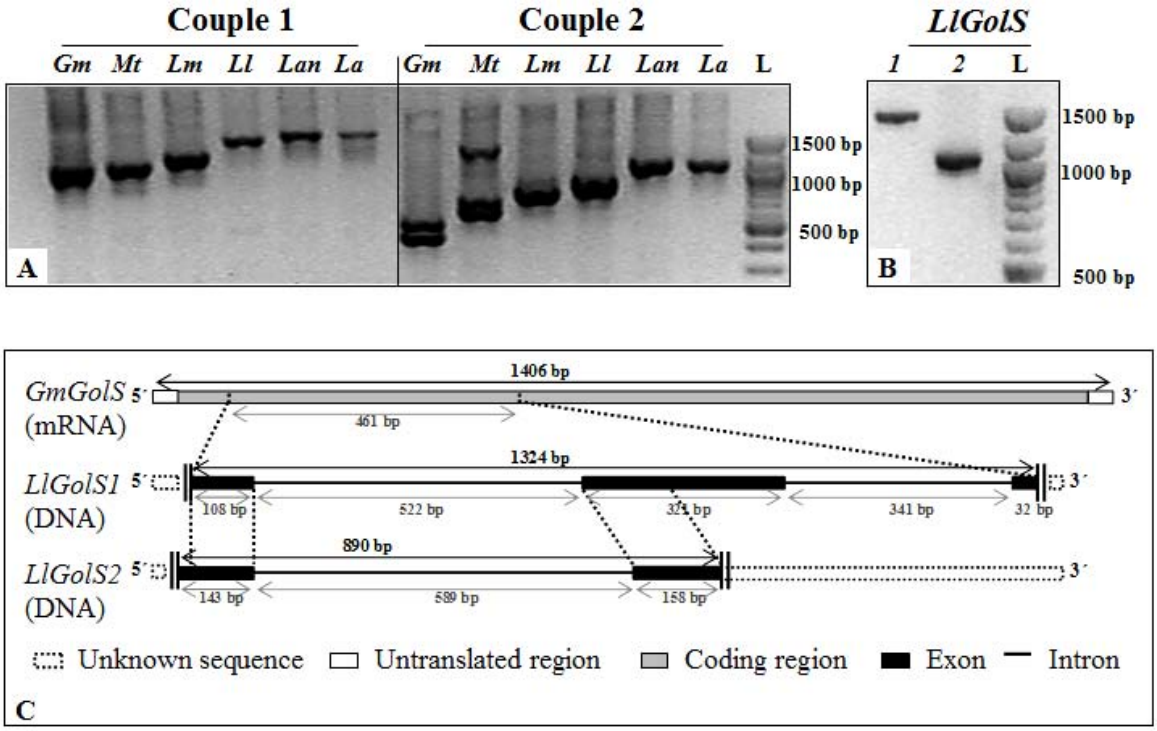

Figure 10. Isolation of galactinol synthase genes from *L. luteus* DNA. The putative *GolS* sequences were amplified by PCR using two couples of degenerated primers designed on conserved regions of other leguminous species. Different fragments sizes were observed in the species *Gm: Glycine max, Mt: Medicago truncatula*, *Lm: Lupinus mutabilis*, *Ll: L. luteus*, *La: L. angustifolius* and *La: L. albus* (A). The *L. luteus* fragments were purified from agarose gels (B) for cloning and sequencing. Two different *GolS* sequences were detected which were arbitrary named *LlGolS1* and *LlGolS2* (C).

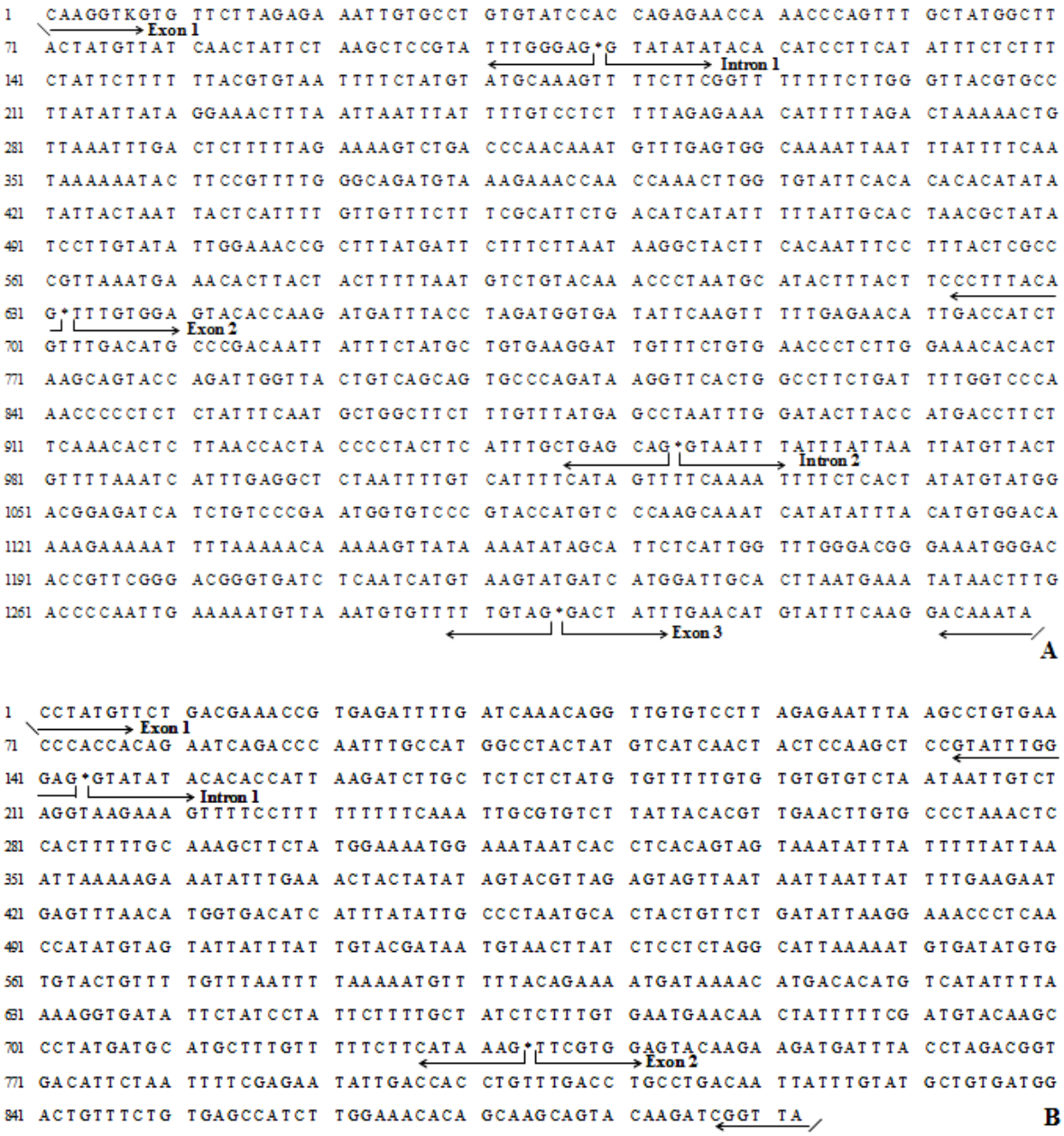

Figure 11. Partial nucleotide sequence of two galactinol synthase genes isolated from *L. luteus* (*LlGolS*) genomic DNA. The sequence obtained for *LlGolS1* comprises 1324 nucleotides (A), whereas the sequence for *LlGolS2* contains 890 bases (B).

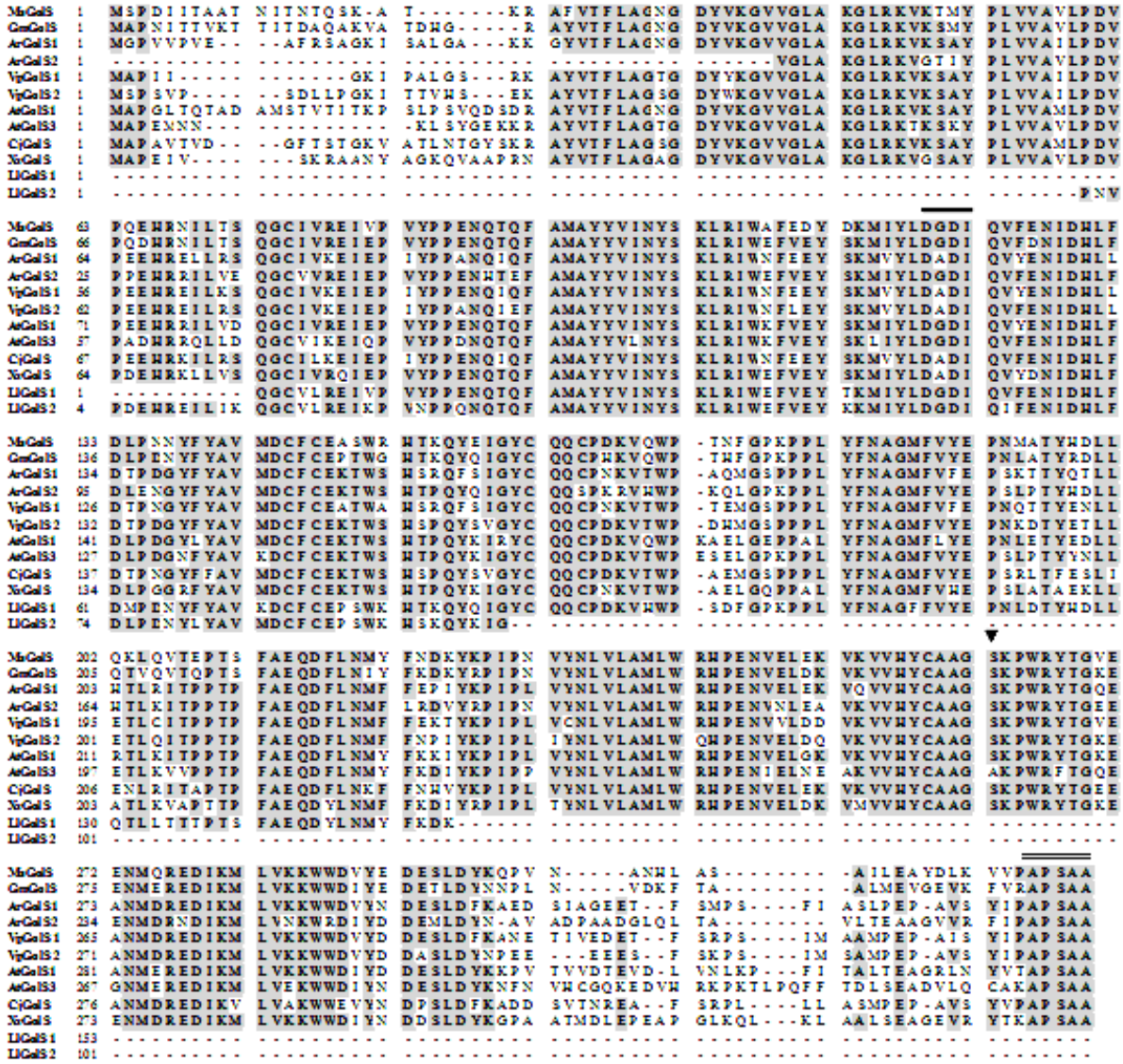

Figure 12. Deduced amino acid sequences of *L. luteus* GolS proteins in comparison with sequences of other plants species. Ms: *Medicago sativa*, Gm: *Glycine max*, Ar: *Ajuga reptans*, Vp: *Verbascum phoeniceum*, At: *Arabidopsis thaliana*, Cj: *Coptis japonica*, Xv: *Xerophyta viscosa*, and *Ll: Lupinus luteus.* The black underline portion indicates a hypothetical manganese binding motif (DXD), and the black triangle shows a conserved serine phosphorilation site. A characteristic hydrophobic pentapeptid is double underlined.

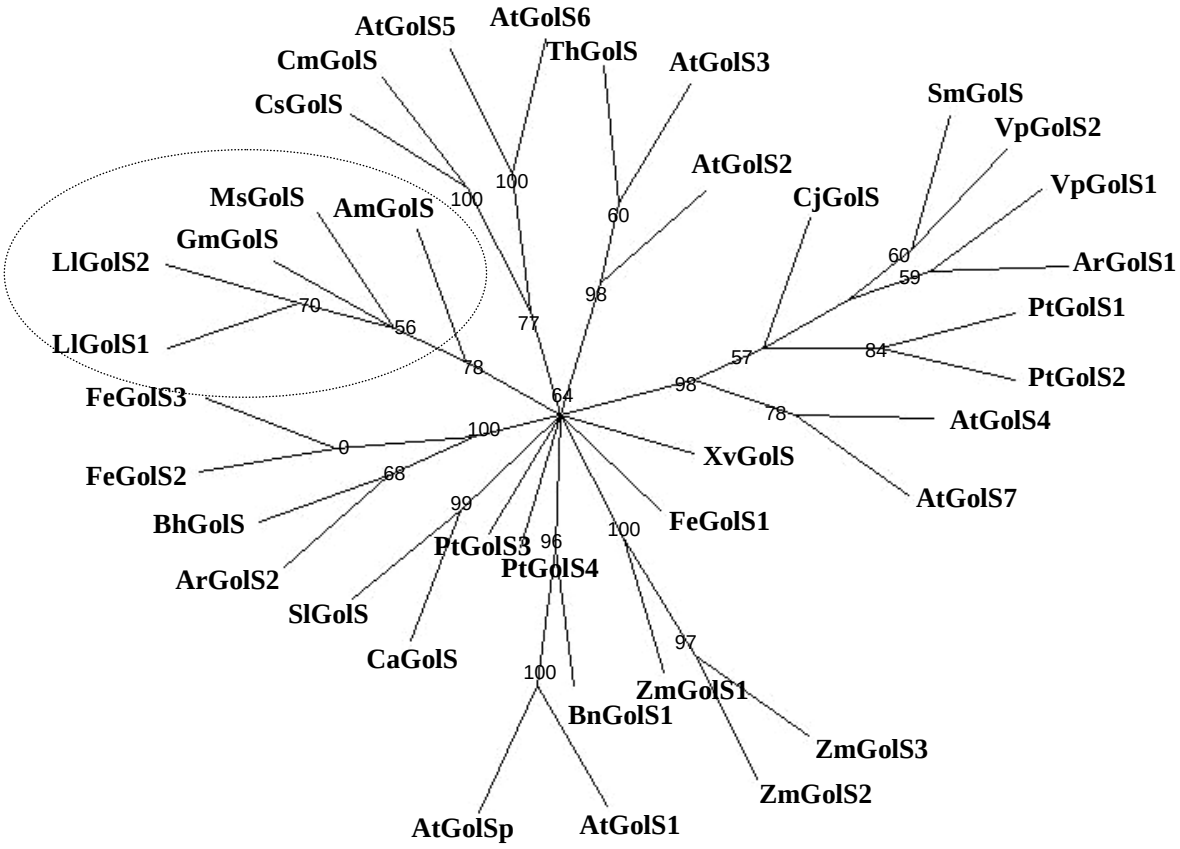

Figure 13. Phylogenetic tree of galactinol synthase proteins (GolS) in plants. The analysis was carried out using maximum parsimony with 1000 bootstrap replicates. The subtree containing the *L. luteus* deduced aminoacid sequences is embraced in a dotted circle. The numbers indicated in nodes correspond to the bootstrap support values. Ll: *Lupinus luteus*, At: *Arabidopsis thaliana*, Pt: *Populus trichocarpa x Populus deltoides*, Ca: *Capsicum annuum*, Sl: *Solanum lycopersicum*, Ar: *Ajuga reptans*, Bh: *Boea hygrometrica*, Fe: *Fagopyrum esculentum*, Gm: *Glycine max*, Ms: *Medicago sativa*, Am: *Ammopiptanthus mongolicus*, Cs: *Cucumis sativus*, Cm: *Cucumis melo*, Th: *Thelungiella halophila*, Cj: *Coptis japonica*, Sm: *Salvia miltiorrhiza*, Vp: *Vervascum phoeniceum*, Zm: *Zea mays*, Bn: *Brassica napus*, Xv: *Xerophyta viscosa.*

5.4 The gene *LlGolS1* is upregulated in roots but not in *L. luteus* leaves under water stress.

A no quantitative RT PCR was first carried out to evaluate the expression of *LlGolS1* and *LlGolS2* under water stress conditions. *LlGolS1* primers succeed to amplify the galactinol synthase fragment from all cDNAs generated from tissues under water stress. On the other hand, *LlGolS2* primers failed to amplify any water stress samples. Both *LlGolS1* and *LlGolS2* were able to amplify *L. luteus* genomic DNA sample (Results not shown). For this reason *LlGolS2* gene was discarded in further analyses. Real Time PCR analysis showed the relative transcript abundance of *LlGolS1* increased during the course of water stress (6.5-fold) in roots, and turned to initial levels by the second day upon rewatering (Figure 14). *LlGolS1* showed no expression differences between control and stressed leaves (Figure 14).

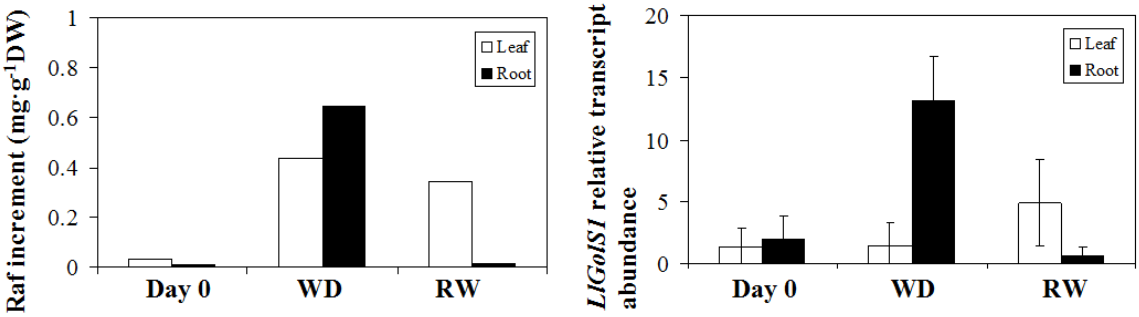

Figure 14. Increment of raffinose and galactinol synthase gene expression during the water stress experiment in *L. luteus* tissues. The raffinose increases were calculated as the difference among treated and control plants. Expression was determined by Real Time PCR using the *EF-1α* gene as reference to normalize expression of *LlGolS1* gene. Raf: raffinose, WD: water deficit, RW: rewatering. The values of relative transcript abundance correspond to means of three experimental replicates, and error bars correspond to standard deviation.

6 DISCUSSION

6.1 Possible role of raffinose under water and cold stresses in *Lupinus* sp. vegetative tissues.

Due the known protective functions of RFOs during abiotic stresses (Taji *et al*., 2002) we hypothesized that these compounds accumulate in vegetative tissues of *Lupinus* sp. as mechanism of defense during water and cold stresses. We demonstrated that, in the four lupin species studied, raffinose was the only RFO accumulated in leaves and roots under water deficit (14 d of water restriction). The accumulation of raffinose followed different patterns among tissues depending on species: *L albus* and *L. angustifolius* accumulated raffinose in leaves whereas *L. mutabilis* only in roots. *L. luteus* exhibited no significant increases of raffinose in leaves or roots (Figure 7). It has been reported that total alpha-galactosides increased in a similar range (0.9 to 1.1-fold) in different organs of *L. albus* (leaf blade, leaf petiole and stem) under different water stress levels, however, the specific oligosaccharides were not determined (Pinheiro *et al*., 2001). In another study, water stress imposed during the pre-storage phase of seed development (15 to 20 d after anthesis) showed an increase in the accumulation of raffinose and stachyose in seeds of *L. albus;* however, the authors did not follow the level of RFOs in vegetative tissues (Pinheiro *et al*., 2005). The accumulation of raffinose during drought in vegetative tissues has been reported in *Arabidopsis thaliana* whole plants (Taji *et al*., 2002) and in dehydrated leaves of the resurrection species *Xerophyta viscosa* (Peters *et al*., 2007) and *Sporobolus stapfianus* (Albini *et at*., 1994). Raffinose has been found to supplement sucrose during desiccation in leaves of desiccation-tolerant species belonging to the families Cyperaceae, Gesneriaceae, Liliaceae, Poaceae, and Velloziaceae (Ghasempour *et al*., 1998).

A different response was observed in the lupin species under cold stress (4 °C, 24 h). RFOs were not detected in both controls and cold stressed tissues, contradicting a number of previous reports where raffinose has been associated to cold stress (Figure 7). This sugar increased under chilling temperature (temperatures below optimal but above freezing) in shoots of *Olea europaea rostrata in vitro* plants (0 to 4 °C, 10 to 30 d) (Rejskova *et al*., 2007), *Arabidopsis thaliana* whole plants (4 °C, 3 to 14 d) (Taji *et al*., 2002), cell cultures of *Chlorella vulgaris* (4 °C, 1 to 46 h) (Salerno and Pontis, 1989), and seedlings of *Oryza sativa* (13/10 °C, 4 d) (Morsy *et al*., 2005). The accumulation of raffinose during cold acclimation under field conditions have been reported in leaves and roots of *Ajuga reptans* (Bachmann *et al*., 1994; Peters and Keller, 2009), shoots apices of *Lonicera coerulea* (Imanishi *et al*., 1998), leaves of *Brassica oleracea* (Santarius and Milde, 1977), crowns and roots of winter hardy *Medicago sativa* accessions (Cunningham *et al*., 2001), and under grown chamber in protoplasts of *Secale cereale* cv. Puma (Koster and Lynch, 1992). Raffinose was accumulated in leaves of *Colobanthus quitensis* stands growing naturally near to Antarctic on King George Island (Piotrowicz *et al*., 2005). Under freezing temperature (-5 to -20 °C, 0 to 24 d) raffinose raised in excised leaves of *A. reptans* (Peters and Keller, 2009).

The fact that raffinose was the most responsive sugar under our mild water stress suggests that its accumulation may be part of a pre-adaptation mechanism in lupin vegetative tissues (Figure 5, 6, 7). Raffinose has been proposed to be a pre-adaptation mechanism in *Ramonda nathaliae* and *Haberlea rhodopensis* (Gesneriaceae) to prevent sucrose crystallization in drying leaves (Müller *et al*., 1997). The role of raffinose in prevention of sucrose crystallization has also been experimentally demonstrated (Caffrey *et al*., 1988). While in *Lupinus* sp. this sugar was found in little quantities and increasing slightly in water stressed plants, in the aforementioned Gesneriaceae species raffinose was detected in higher quantities in leaves but did not vary between control and stressed plants (Müller *et al*., 1997). We propose that raffinose under water deficit may act as a membrane protector. Raffinose has been found to be more effective in membrane stabilization than sucrose or

glucose (Santarius and Milde, 1977). Early studies suggested the hydroxyl group of polyhydroxy compounds can form a hydrogen bond with the polar heads of membrane phospholipids, replacing water, and these hydrophobic interactions are important for membrane stability (Crowe *et al*., 1998; Hincha *et al*., 2002). RFOs have been also associated with increased levels of drought tolerance accompanied of a reduced transpiration rate in *A. thaliana*. Based on these results it has been hypothesized that RFOs may induce ABA which is known to produce stomata closure (Taji *et al*., 2002). The results observed in *Lupinus* sp. suggest a role of raffinose during water deficit; however, further studies are needed to determine if the observed accumulation of raffinose in vegetative tissues is essential for the plant survival during this abiotic stress.

6.2 Galactinol synthase genes in the regulation of raffinose accumulation under stress conditions in *L. luteus*.

Two galactinol synthase genes (*GolS*) were detected in DNA of *L. luteus* which were called *LlGolS1* and *LlGolS2* (Figure 10, 11). Because the lupin genome is not fully sequenced, it is uncertain if these genes belong to a bigger multigene family. In the model species *Arabidopsis thaliana* at least seven *AtGolS* genes have been identified (Taji *et al*., 2002; Panikulangara *et al*., 2004) whereas in the majority of plant species only few *GolS* genes have been found (Table 1).

The expression of *LlGolS* genes was studied in leaves and roots of *L. luteus*. Because the water deficit treatment did not induce a severe stress (Figure 4), and the raffinose increase was not significant in tissues of *L. luteus*, it was hypothesized that these sugars were in an early stage of synthesis (Figure 7). The expression study showed that *LlGolS1* gene was upregulated in roots under water stress but was not induced in leaves (Figure 13). These results suggest the *LlGoS1* expression in leaves occurred at a different time or that another gene is involved in the synthesis of raffinose.

Table 1. Galactinol synthase genes characterized in *L. luteus* and other plants species.

Species	Family	Gene	Stress	Tissue	Expression	Reference
Arabidopsis thaliana	Brassicaceae	*AtGolS1*	Drought, salt, heat.	Whole plant	↑	Taji *et al*. (2002).
		AtGolS2	Drought, salt.	Whole plant	↑	Taji *et al*. (2002). Panikulangara *et al.* (2004).
		AtGolS3	Cold, heat.	Whole plant	↑	Taji *et al*. (2002).
Xerophyta viscosa	Velloziaceae	*XvGolS*	Water deficit.	Leaf	↑	Peters *et al*. (2007).
Coptis japonica	Ranunculaceae	*CjGolS*	Salt.	Cultured cells	↑	Takanashi *et al*. (2008).
Verbascum phoeniceum	Scrophulariaceae	*VpGolS1, -2*	Phloem loading.	Intermediary cells	-	McCaskill and Turgeon (2007).
Ajuga reptans	Lamiaceae	*ArGolS1*	Cold.	Mesophyll	↑	Sprenger and Keller (2000).
		ArGolS2	Cold.	Intermediary cells	↑	Sprenger and Keller (2000).
Zea mays	Poaceae	*ZmGolS1*	Unknown.	-	-	Zhao *et al*. (2003), (2004).
		ZmGolS2	Dehydration upon seed imbibition.	Seed	↑	Zhao *et al*. (2003), (2004).
		ZmGolS3	Developing seed.	Seed	↑	Zhao *et al*. (2003), (2004).
Oryza sativa	Poaceae	*OsGolS1*	Chilling.	Seedling	↑	Takahashi *et al*. (1994).
Lolium perenne	Poaceae	*LpGolS1*	Drought.	Mature leaves	↓	Amiard *et al*. (2003).
Lycopersicum esculentum	Solanaceae	*LeGolS1*	Cold. Developing seed.	Radicle tip. Seed.	↑	Downie *et al*. (2003).
Fagopyrum esculentum	Polygonaceae	*FeGolS1, -2,- 3*	Developing seeds.	Seed	↑	Ueda *et al.* (2005).
Lupinus luteus	Fabaceae	*LlGolS1*	Water deficit.	Roots	↑	Present study.
		LlGolS2	Unknown.		-	Present study.

Unavailable information (-), downregulation (↓) and upregulation (↑) of each respective gene.

The raffinose increment and the expression of *LlGolS1* showed a similar pattern, involving this gene on the synthesis of raffinose in roots (Figure 13). Various studies have shown the same correlation between *GolS* expression and raffinose accumulation. For example, in *Xerophyta viscosa* the *XvGolS* gene was induced in leaves at 96% RWC, reaching a maximum expression at 19% RWC which coincided with the maximum RFOs accumulation (Peters *et al*., 2007). In this study, the accumulation of galactinol, the product of GolS enzyme, reached the maximum value at 70% RWC and then decreased because it is used for the synthesis of raffinose. In *Arabidopsis thaliana* it was observed a similar pattern (Taji *et al*., 2002). The different results obtained among leaves and roots can hardly be compare with other studies as most of them have been dealing only with leaves (Panikulangara *et al*. 2004; Peters *et al*., 2007; Sprenger and Keller, 2000), or with the whole plant, like in the case of *A. thaliana* (Taji *et al*., 2002). The *LlGolS2* gene failed to amplify in all cDNAs suggesting that this gene is expressed at a different time, under different conditions, or in other tissues. Another possibility that would explain these results is the occurrence of alternative splicing or a deletion which can impair the *LlGolS2* amplification with the primers designed. An example of this situation was described in maize where a smaller transcript of the *ZmGolS3,* originated by a deletion, was induced by heat shocking in germinating seeds and callus cells (Zhao *et al*., 2003). The amplification of a pseudogene from genomic DNA may be another explanation of these results. Pseudogenes are nonfunctional genomic sequences with significant sequence similarity to functional RNA or protein-coding genes (Balakirev and Ayala, 2003). Further studies are necessary to obtain the full sequence of *LlGolS1* and *LlGolS2* and understand the regulation mechanism of these genes. Due to the high contents of RFOs, the different patterns of raffinose accumulation, and expression of galactinol synthase genes under water deficit, other *LlGolS* isogenes are expected to be present in lupin.

6.3 Involvement of sucrose and hexose in water and cold stresses in *Lupinus* sp. vegetative tissues.

Hexoses increased in roots of *L. angustifolius*, and sucrose accumulated in roots of *L. angustifoloius* and *L. mutabilis*, suggesting that these sugars may be part of the response mechanism to water stress. These sugars; however, did not accumulate in leaves (Figure 5, 6) which disagree with the study of Pinheiro *et al.* (2001) whom reported abundant sucrose accumulation in leaves of *L. albus* under a similar period of water deficit treatment. This difference could be explained by the fact that in our experiment plants did not reach a severe stress (Figure 4). Sucrose accumulation is one of the known plant responses to water deficit in leaves of resurrecting angiosperms, such as *Boea hygroscopica* (Bianchi *et al*., 1991), *Craterostigma plantagineum* (Bartels and Salamini, 2001), *Ramonda nathaliae* and *Haberlea rhodopensis* (Müller *et al*., 1997), *Xerophyta viscosa* (Ghasempour *et al*., 1998; Peters *et al*., 2007), and of grasses such as *Eragrostis nindensis* (Illing *et al*., 2005), and *Sporobulus stapfianus* (Albini *et al*., 1994). Under water deficit, sucrose increased in roots but not in leaves of *Lolium perenne* whereas hexoses increased in both tissues (Amiard *et al*., 2003). The accumulation of soluble sugars in roots and leaves under water deficit may be explained because growth is more strongly inhibited by this stress than photosynthesis (Chavez, 1991). Moreover, it is well known that drought stress induces a shift in the partitioning of photosynthetic products in favor of sucrose (Hare *et al*., 1998). In *L. albus* a carbon partitioning in favor of sucrose leading to a decrease in starch have been described in leaves during water deficit under field conditions by Quick *et al*. (1992).

Under cold stress it was observed a significant increase of sucrose and hexoses in leaves, and sucrose in roots of almost all species under study (Figure 8, 9), suggesting some participation of these sugars in the response of the plant to this stress. Chilling, freezing temperatures, as well as acclimation to cold all induce synthesis of compounds such as proteins, lipids and soluble carbohydrates (Browse and Xin, 2001; Gupta and Kaur, 2005; Alberdi and Corcuera, 1991). The accumulation of sucrose under cold shock in *Triticum*

aestivum seedlings was accompanied by the expression of sucrose synthase but the participation of this enzyme in the chilling tolerance was not assessed (Crespi *et al*., 2001). Increases of sucrose, glucose, and fructose and of the activity of the sucrose phosphate synthase have been reported in leaves of *Spinacea oleracea* under cold conditions (Guy *et al*., 1992). Sucrose and the activity of sucrose phosphate synthase increased in the Antarctic plant *Colobanthus quitensis* in response to cold (Bascuñán-Gody *et al*., 2007).

One role that has been suggested for sucrose and hexoses under stress conditions is the activation or repression of genes involved in sugar metabolism (UGPase, UDP-glucose pyrophosphorilase; INV, invertase; SPS, sucrose phosphate synthase; SuSy, sucrose synthase), and photosynthesis (Rubisco; CAB, chlorophyll A binding protein) leading to down-regulate photosynthesis (Koch *et al*., 1992; Koch *et al*., 1996; Lalonde *et al*., 1999; Rolland *et al.*, 2002; Gupta and Kaur, 2005). Sucrose and hexose during water deficit are involved on plant osmotic adjustment (Wesgate and Boyer, 1985) and desiccation tolerance, contributing to plant survival (Morgan, 1984; Hare *et al*., 1998). Particularly, glucose (hexose) contribute with the osmotic adjustment in leaves of *Populus deltoides* (Gebre *et al*., 1994), seedlings of *Quercus petraea* (Epron and Dreyer, 1996) and *Pinus taeda* (Meier *et al*., 1992), and leaves of *Fragaria chiloensis* (Zhang and Archbold, 1993). These sugars have also been proposed to operate as membrane protectors during desiccation (Santarius and Milde, 1977). The accumulation of this kind of carbohydrates at low temperatures may have the dual function of storing carbon and providing cryoprotectans (Bachman *et al.*, 1994). In addition, under cold conditions the up-regulation of several genes coding for enzymes of sucrose synthesis (SuSy, SPS, and INV) has been proposed as a part of the acclimation mechanism (Roitsch, 1995, 1999; Wingler *et al*., 2000). The accumulation of sucrose occurring in leaves and roots of *Lupinus* sp. during cold treatment (Figure 8) may also be due to low demand of photosynthates (Pollock, 1986), or breakdown of starch to sucrose that occurs at low temperature in perennials and many herbaceous plants (Pollock and Lloyd, 1987). In *Spinacea oleracea* both starch and soluble sugars increased in leaves under cold conditions; however, sugars were not accumulated as a consequence of starch

breakdown but resulted from a reduction in growth utilization of photosynthates (Guy *et al*., 1992). The accumulation of hexoses in lupin leaves (Figure 9) may be due to degradation of sucrose to amplify a stress signal by the action of INV or SuSy enzymes (Koch, 1996).

7 CONCLUSIONS

Raffinose is the only oligosaccharide belonging to the RFOs series accumulated in leaves of *L. albus* and roots of *L. mutabilis*, under water deficit. Raffinose is not significantly accumulated in tissues of *L. angustifolius* and *L. luteus* under this stress.

RFOs are not accumulated in vegetative tissues of *Lupinus* sp. under cold stress, suggesting that these sugars are not involved in the response of the plant to this stress under the experimental conditions used.

Sucrose and hexoses are accumulated in vegetative tissues of *Lupinus* sp. in different patterns under both stress conditions.

Two different *GolS* isogenes, arbitrarily called *LlGolS1* and *LlGolS2*, are detected in genomic DNA of *Lupinus luteus*.

The *LlGolS1* gene is involved in the regulation of raffinose synthesis in roots of *L. luteus* under water deficit but not in leaves in the time course under study. Sugars accumulation and gene expression patterns observed among different tissues suggest other *LlGolS* isogenes may be involved in the RFOs synthesis under water stress.

The *LlGolS2* gene is not involved in the regulation of raffinose synthesis under the evaluated conditions, suggesting this gene participates in other plant roles or lupin tissues, it is subjected to alternative splicing or corresponds to a pseudogene.

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APPENDIXES

APPENDIX 1 V**ariance analysis for the effect of water, harvesting time and species on the soluble sugars content in *Lupinus* sp. vegetative tissues during the water stress experiment.**

Error source	DF	Leaves			Roots		
		MS	F	p-value	MS	F	p-value
Sucrose							
Water	1	270.937151	0.36	0.5794 ns	2363.06235	1.08	0.3575 ns
Harvest	2	2152.40805	8.79	0.0006 *	8076.36775	8.1	0.001 *
Species	3	5925.72866	24.19	<.0001 *	13754	13.8	<.0001 *
Water*species	3	90.726637	0.37	0.7747 ns	2535.24583	2.54	0.0683 ns
Water*harvest	2	59.988935	0.24	0.7838 ns	2696.03855	2.71	0.0779 ns
Harvest*species	6	1454.12857	5.94	0.0001 *	2270.3468	2.28	0.0531 ns
Water*harvest*species	6	99.816228	0.41	0.8701 ns	1967.33377	1.97	0.0899 ns
Rep(water)	4	746.487203	3.05	0.0266 *	2188.9171	2.2	0.0849 ns
Residual	44	244.944446	-	-	996.4685	-	-
Hexose							
Water	1	76.845918	0.09	0.7766 ns	2157.31657	3.24	0.1461 ns
Harvest	2	1502.22031	5.14	0.0099 *	2973.78338	2.64	0.0826 ns
Species	3	6095.18849	20.85	<.0001 *	25637	22.77	<.0001 *
Water*species	3	76.790878	0.26	0.8519 ns	1498.81804	1.33	0.2765 ns
Water*harvest	2	30.48054	0.1	0.9012 ns	1016.8067	0.9	0.4128 ns
Harvest*species	6	907.345408	3.1	0.0127 *	2240.07393	1.99	0.0877 ns
Water*harvest*species	6	112.993261	0.39	0.8837 ns	875.75007	0.78	0.5918 ns
Rep(water)	4	833.605525	2.85	0.0347 *	665.365435	0.59	0.671 ns
Residual	44	292.282451	-	-	1126.14471	-	-
Raffinose							
Water	1	0.873622	8.97	0.0402 *	0.843268	3.78	0.1239 ns
Harvest	2	0.992443	9.43	0.0004 *	2.528849	19.94	<.0001 *
Species	3	1.706081	16.21	<.0001 *	2.279557	17.98	<.0001 *
Water*species	3	0.159955	1.52	0.2226 ns	0.205025	1.62	0.1991 ns
Water*harvest	2	0.324695	3.09	0.0557 ns	0.45848	3.62	0.0352 *
Harvest*species	6	0.136223	1.29	0.2797 ns	0.761597	6.01	0.0001 *
Water*harvest*species	6	0.034709	0.33	0.9177 ns	0.082327	0.65	0.6904 ns
Rep(water)	4	0.097432	0.93	0.4577 ns	0.223296	1.76	0.1539 ns
Residual	44	0.105247	-	-	0.126807	-	-

DF: degrees of freedom, MS: mean square, ns: no significant differences, *: significant differences with α=0.05.

APPENDIX 2 Variance analysis for the effect of water, harvesting time and species on the soluble sugars content in *Lupinus* sp. vegetative tissues during the cold stress experiment.

Error source	DF	Leaves			Roots		
		MS	F	p-value	MS	F	p-value
Sucrose							
Water	1	76.845918	0.09	0.7766 ns	2157.31657	3.24	0.1461 ns
Harvest	2	1502.22031	5.14	0.0099 *	2973.78338	2.64	0.0826 ns
Species	3	6095.18849	20.85	<.0001 *	25637	22.77	<.0001 *
Water*species	3	76.790878	0.26	0.8519 ns	1498.81804	1.33	0.2765 ns
Water*harvest	2	30.48054	0.1	0.9012 ns	1016.8067	0.9	0.4128 ns
Harvest*species	6	907.345408	3.1	0.0127 *	2240.07393	1.99	0.0877 ns
Water*harvest*species	6	112.993261	0.39	0.8837 ns	875.75007	0.78	0.5918 ns
Rep(water)	4	833.605525	2.85	0.0347 *	665.365435	0.59	0.671 ns
Residual	44	292.282451	-	-	1126.14471	-	-
Hexose							
Water	1	0.873622	8.97	0.0402 *	0.843268	3.78	0.1239 ns
Harvest	2	0.992443	9.43	0.0004 *	2.528849	19.94	<.0001 *
Species	3	1.706081	16.21	<.0001 *	2.279557	17.98	<.0001 *
Water*species	3	0.159955	1.52	0.2226 ns	0.205025	1.62	0.1991 ns
Water*harvest	2	0.324695	3.09	0.0557 ns	0.45848	3.62	0.0352 *
Harvest*species	6	0.136223	1.29	0.2797 ns	0.761597	6.01	0.0001 *
Water*harvest*species	6	0.034709	0.33	0.9177 ns	0.082327	0.65	0.6904 ns
Rep(water)	4	0.097432	0.93	0.4577 ns	0.223296	1.76	0.1539 ns
Residual	44	0.105247	-	-	0.126807	-	-

DF: degrees of freedom, MS: mean square, ns: no significant differences, *: significant differences with α=0.05.