**This is the Post-print version of the following article:** ***Rosalía Cristina Paz, Rubén Anibal Rocco, Juan Francisco Jiménez-Bremont, Margarita Rodríguez-Kessler, Alicia Becerra-Flora, Ana Bernardina Menéndez, Oscar Adolfo Ruíz, Identification of differentially expressed genes potentially involved in the tolerance of Lotus tenuis to long-term alkaline stress, Plant Physiology and Biochemistry, Volume 82, 2014, Pages 279-288,*** **which has been published in final form at: https://doi.org/10.1016/j.plaphy.2014.06.009**

**©2014. This manuscript version is made available under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International(CC BY-NC-ND 4.0) license http://creativecommons.org/licenses/by-nc-nd/4.0/**

Accepted Manuscript


# Identification of differentially expressed genes potentially involved in the tolerance of *Lotus tenuis* to long-term alkaline stress

Rosalía Cristina Paz, Ruben Anibal Rocco, Juan Francisco Jimenez-Bremont, Margarita Rodriguez-Kessler, Alicia Becerra-Flora, Ana Bernardina Menendez, Oscar Adolfo Ruiz

PII: S0981-9428(14)00190-9

DOI: 10.1016/j.plaphy.2014.06.009

Reference: PLAPHY 3989

To appear in: *Plant Physiology and Biochemistry*

Received Date: 2 April 2014

Accepted Date: 17 June 2014

Please cite this article as: R.C. Paz, R.A. Rocco, J.F. Jimenez-Bremont, M. Rodriguez-Kessler, A. Becerra-Flora, A.B. Menendez, O.A. Ruiz, Identification of differentially expressed genes potentially involved in the tolerance of *Lotus tenuis* to long-term alkaline stress, *Plant Physiology et Biochemistry* (2014), doi: 10.1016/j.plaphy.2014.06.009.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

# Identification of differentially expressed genes potentially involved in the tolerance of *Lotus tenuis* to long-term alkaline stress.

Paz, Rosalía Cristina 1

E-mail: rpaz@fca.uncu.edu.ar

Rocco, Ruben Anibal 2

E-mail: rubenrocco@intech.gov.ar

Jimenez-Bremont, Juan Francisco 3

E-mail: jbremont@ipicyt.edu.mx

Rodriguez-Kessler, Margarita 4

E-mail: mrodriguez@fc.uaslp.mx

Becerra-Flora, Alicia 3

E-mail: abecerra@ipicyt.edu.mx

Menendez, Ana Bernardina 5,6#

E-mail: anamen@bg.fcen.uba.ar

Ruiz, Oscar Adolfo 2

E-mail: ruiz@intech.gov.ar

1 Grupo INTERBIODES (Interacciones Biológicas del Desierto/Biological Interactions of Desert) CIGEOBIO (FCEFyN, UNSJ/CONICET), Dpto. de Biología. Av. Ignacio de la Roza 590 (Oeste), J5402DCS, Rivadavia, San Juan, Argentina.

2 Unidad de Biotecnología 1, IIB-IINTECH/UNSAM-CONICET, Chascomús, Buenos Aires, Argentina

ACCEPTED MANUSCRIPT

3 Instituto Potosino de Investigación Científica y Tecnológica (IPICyT) Camino a la Presa de San Jose No. 2055 Lomas 4a Seccion CP 78216 San Luis Potosi, S.L.P.

4 Facultad de Ciencias, Universidad Autónoma de San Luis Potosí, Av. Salvador Nava s/n, Zona Universitaria, C.P. 78290 San Luis Potosí, SLP, México

5 Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires

6 PROPLAME-PRHIDEB (CONICET)

# corresponding author

**Abstract**

Soil alkalinity is one of the most serious agricultural problems limiting crop yields. The legume *Lotus tenuis* is an important forage acknowledged by its ability to naturally grow in alkaline soils. To gain insight into the molecular responses that are activated by alkalinity in *L. tenuis* plants, subtractive cDNA libraries were generated from leaves and roots of these plants. Total RNAs of non-stressed plants (pH 5.8; E.C. 1.2), and plants stressed by the addition of 10 mM of $NaHCO_3$ (pH 9.0; E.C. 1.9), were used as source of the driver and the tester samples, respectively. RNA samples were collected after 14 and 28 days of treatment. A total of 158 unigenes from leaves and 92 unigenes from roots were obtained and classified into 11 functional categories. Unigenes from these categories (4 for leaves and 8 for roots), that were related with nutrient metabolism and oxidative stress relief were selected, and their differential expression analyzed by qRT-PCR. These genes were found to be differentially expressed in a time dependent manner in *L. tenuis* during the alkaline stress application. Data generated from this study will contribute to the understanding of the general molecular mechanisms associated to plant tolerance under long-term alkaline stress in plants.

ACCEPTED MANUSCRIPT

**Key words**

Alkaline stress – Suppressive Subtractive Hybridization – Stress induced transcripts – *Lotus tenuis* – Adaptive response – root and shoot tissues – Long-term response

ACCEPTED MANUSCRIPT

## 1. Introduction

About one half of the saline soils that cover the land surface contain alkaline salts [1].Soil alkalinity is a serious agricultural problem as it limits crops yield by reducing the availability of plant nutrients, among other effects [2,3]. Common alkaline stress symptoms in plants that are alkaline-sensitive include leaf chlorosis[4] and stunting [5].

Bicarbonate ($HCO_3-$) is one of the principal contributors to soil alkalinity [6]. High $HCO_3^-$concentrations in soil may interfere with the uptake of macro elements, particularly P, K and Mg [7], and essential micronutrients such as Fe and Zn [4]. In turn, much of the damage at the cellular level due to deficiency of these ions has been attributed to the action of reactive oxygen species [8,9,10]. Thus, the ability of plants to overcome soil alkalinity would relay, at least in part, on transcriptomic changes upon stress imposition, which ultimately balance cell nutrients content and ameliorate oxidative stress.

Gene expression analysis and functional studies of stress inducible genes facilitates the understanding of molecular mechanisms underlying stress tolerance responses [11].Suppression Subtractive Hybridization (SSH) is a simple and efficient method, which has been widely used for the identification of differentially expressed genes [12]. By using SSH, many abiotic stress-inducible genes have been identified and characterized in several plant species [13,14,15].

The legume *Lotus tenuis* (Waldst. &Kit., syn. *Lotus glaber*; [16]), an important forage widely used for cattle production in Argentina, is acknowledged by its ability to naturally grow in highly saline and alkaline soils (pH 8.5–11.5; [17,18]). *L. tenuis* plants subjected to 10 mM$NaHCO_3$ survived after 28 days of treatment, although they showed a 22% decrease in their leaf Zn content and were smaller than non-alkalinized controls [19]. Interestingly, the remaining analyzed ions (Fe, Cu, Ca, Mg, Na, K, Na, and Mn) were not affected by alkalinity, suggesting that *L. tenuis* might activate

mechanisms for improving the uptake and/or the translocation and handling of these nutrients, as part of its response to alkalinity.

The aim of the present study was to identify genes related to nutrients metabolism and oxidative stress relief that were regulated by long-term alkaline stress. For this purpose, we generated two subtractive cDNA libraries (SSH) and identified 158 unigenes from leaves and 92 unigenes from roots that are potentially induced in *L. tenuis* plants subjected to 10 mM of $NaHCO_3$ for 14 and 28 days. The expression pattern of selected genes identified by SSH was further characterized by quantitative real time-polymerase chain reaction (qRT-PCR), upon alkalinity response. The comprehensive analysis of regulated genes in *L. tenuis* under long-term alkaline stress will increase the current knowledge on the plant response to soil alkalinity in legumes, and might contribute to the development of future biotechnological strategies for improving plant tolerance to alkalinity.

## 2. Materials and methods

### *2.1. Plant material and growth conditions*

Seeds of *L. tenuis* cv. Esmeralda were scarified with sulphur acid (100%), washed in distilled water and sown in Petri plates containing water-agar (0.8%). Plates were incubated during 7 days in a growth chamber, with a 16/8 h photoperiod at 24°C/19°C (day/night) and 60/80±5% relative humidity. Light intensity (200 μmol $m^{-2}$ $s^{-1}$) was provided by Grolux fluorescent lamps. Seedlings were transferred to 5.8 (diameter) x 20 cm (length) cylindrical pots containing washed sand (pH 7.0 and E.C.= 0.05 $mS.cm^{-1}$) and irrigated with 0.5μ Hoagland's nutrient solution [20]. Pots were kept at field capacity during the time lapse experiment. An ELGO® drip irrigation system was used in order to avoid variations in pH and salt accumulation due to water evaporation throughout the experiment.

This system allowed a homogeneous distribution of nutrients within the pot and a daily replacement, by percolation, of an amount of nutrient solution equivalent to ¾ of the substrate field capacity.

Alkaline stress conditions in the pot substrate were created by adding 10 mM $NaHCO_3$ to 0.5µ Hoagland's solution. Control treatment consisted of plants irrigated with 0.5µ Hoagland's solution without $NaHCO_3$. The pH and E.C. ($mS.cm^{-1}$) of irrigation solutions were monitored every 3 days with a combined pH meter/conductimeter (HI 255, Hanna Instrument) and maintained at pH-E.C. 5.8-1.2 in control and 9.0-1.9 in alkaline treatment.

Growth parameters were estimated sampling plants from the start of the stress application. At least 10 plants of each treatment were harvest at 0, 7, 14, 21 and 28 days for growth response. From these plants, shoot and root dry biomass per plant were estimated.

*2.2. RNA extraction and Suppressive Subtractive Hybridization library construction*

Leaves and roots were harvested separately at 14 and 28 days after treatment (n=20 plants), frozen in liquid nitrogen, and then kept at -70°C. Frozen tissue samples were ground in liquid nitrogen. Total RNA was isolated from leaves and roots of 14 and 28 days control and alkaline treated plants using TriZOL reagent (GIBCOL/BRL) according the manufacturer's instructions. Quality and concentration of RNAs were analyzed on a formaldehyde-denaturing 1% agarose/EtBr gel and by absorbance measurements at 260 and 280 nm on an UV spectrophotometer. Two Suppressive Subtracted Hybridization (SSH) libraries were generated, one from root tissue and the other from leaves of *L. tenuis*, using the PCR Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the standard protocol provided. For each library, two different RNA mixtures were prepared as source of tester and driver samples. For the tester, equal amounts of the total RNAs isolated from root or leaf tissues treated during 14 and 28 days with alkali stress were mixed. In the case of the driver, RNAs from non-treated roots or leaf tissues, harvested at the same time (14 and 28 days) were mixed.

Double stranded cDNAs were synthesized from one μg of each mixture of total RNAs using the Super SMART™ PCR cDNA Synthesis Kit and, then these double stranded cDNAs were used for the generation of forward cDNA subtractive libraries (Clontech, Palo Alto, CA). The subtracted cDNA population was cloned into the pCR4-TOPO® vector (Invitrogen, Carlsbad, CA) and used to transform One Shot TOP10F electrocompetent *Escherichia coli* cells (Invitrogen, Carlsbad, CA). The Plasmid DNA of individual clones was obtained by the alkaline lysis procedure [21] and digested with *Eco*RI enzyme. Digestion products were analyzed by electrophoresis on 0.8% agarose/EtBr gels to discard fragments lower than 500 bp.

*2.3. Sequencing and bioinformatic analysis of ESTs*

Cloned products were sequenced using the M13 forward primer in an ABI PRISM 377 DNA automated sequencer (Perkin Elmer). All nucleic acid sequences were screened for vector contamination using the Vector Screen program (www.ncbi.nlm.nih.gov/VecScreen) and grouped into contigs (group of overlapping DNA sequences) using the SeqMan program (DNASTAR Lasergene, Madison, WI).

Homology search was conducted using the BLAST program (BLASTN and BLASTX) and the GenBank non-redundant (nr) and the Expressed Sequence Tags (EST) database of the National Center of Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and the *Lotus japonicus* Genome Sequencing Project (www.kazusa.or.jp). A homology assignation criterion was based on maximum probability threshold per sequence and a minimum E-value of $10^{-20}$. Functional classification analyses were performed according to the Gene Ontology database (www.geneontology.org).

*2.4. Differential expression analysis by quantitative RT-PCR*

Total RNA was extracted from leaves and roots of *L. tenuis* at 14 and 28 days after the treatment using TriZOL reagent (GIBCOL/BRL) according the manufacturer´s instructions (n=10 plants per replica, with 3 replicas per treatment and time). First strand cDNAs were synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Promega, WI, USA). Quantitative real-time PCR reactions were performed with specific primers using a FastStart Universal SYBR Green Master with ROX (Roche Applied Science) on a Stratagene Mx3005P Real Time qPCR System (Stratagene, CA, USA) according to the manufacturer's instructions. Two quantitative PCR replicates from three independent biological replications were performed on each cDNA sample. The relative expression was calculated as $2^{-(\Delta Ct \text{ gene of interest}) - (\Delta Ct \text{ reference gene})}$. Primers used for quantitative real-time PCR are listed in Table 1 and were designed using the Bacon designer®. The relative expression levels of all the analyzed unigenes was normalized to the previously described housekeeping gene LjGPI-anchored protein [22].

## 3. Results

### *3.1. Plant growth response*

The effect of alkaline stress on plant biomass was analyzed in control and stressed plants at 0, 7, 14, 21 and 28 days of treatment. The $NaHCO_3$ addition induced a significant reduction in total plant biomass from the $21^{th}$ day onwards, with respect to control (Fig. 1). Leaves in plants treated with $NaHCO_3$ were green, excepting those from the upper nodes, which were slightly yellowish, although they did not senesced or died after 28 days of treatment (Fig. 2).

### *3.2. Differentially expressed genes in leaves and roots of plants exposed to long term alkaline stress*

Overall, 450 plasmids from the leaf subtractive cDNA library (SSH) and 211 plasmids from the root SSH were sequenced and assembled with Seqman II program (DNASTAR Lasergene, Madison, WI),

168 comprising a total of 158 unigenes in leaves, and 92 unigenes in roots (with an average size of 454
169 and 357 pb respectively). Five of these unigenes were common to leaf and root SSHs (asparagine
170 synthase, BURP, K(+)/H(+) antiporter-like and two unknown, Figure 3). The obtained ESTs were
171 deposited in the DDJJ database (DNA Data Bank of Japan, www.**ddbj**.nig.ac.jp) under Accession N°
172 AB862603-AB862770 and AB863290-AB863455 for leaves (Supplementary Table 1), and N°
173 AB862771-AB862877 and AB863456-AB863461 for roots (Supplementary Table 2).
174 Blast searches of all unigenes against the nucleotide and protein databases, and the *L. japonicus*
175 database showed significant similarity to known genes. The best alignments in NCBI database were
176 with legume sequences such as *L. japonicus, Glycine max,* and *Medicago truncatula.*
177
178 *3.3. Functional classification of unigenes isolated from leaf and root SSH libraries of L. tenuis.*
179 Unigenes from both libraries were classified into 11 categories according to their putative biological
180 function by using the Gene Ontology database (Figure 4; Supplementary tables 1 and 2). The unigene
181 classification of both subtractive cDNA libraries included the following functional categories:
182 metabolism (9% leaf; 11% root); lipid metabolism (1% leaf; 6% root); transcription (1% leaf; 2%
183 root), ion homeostasis and transport (9% leaf; 12% root); signal transduction (2% leaf; 4% root); cell
184 rescue and defense (17% leaf; 9% root); growth and development (9% leaf; 9% root); protein
185 synthesis (4% leaf; 4% root) and unclassified (10% leaf; 9% root). It should be noted that 32 and 21%
186 of the identified sequences in leaf and root SSH libraries, respectively, had homology with proteins of
187 unknown function. Furthermore, 6% and 13% of these sequences from leaf and root SSH libraries,
188 respectively, had no matches in the databases searched, indicating novel gene fragments or UTR
189 regions of *L. tenuis*. Ostensibly, some of the unknown and no-matching transcripts identified in the
190 present study might be associated with alkalinity tolerance. A complete list of BLAST results

ACCEPTED MANUSCRIPT

obtained from the NCBI database, *L. japonicus* genome database, along with results of sequences having no hit, from leaf and root tissues are available in Supplementary Tables 1 and 2 respectively.

*3.4. Genes potentially involved in alkaline tolerance*

An important group of the classified and identified genes are described in bibliography as responsive to biotic and abiotic stress. These genes are distributed in all categories described in Figure 4, and represent 27% and 39% of unigenes from leaf and root SSH libraries, respectively (marked with # in Supplementary Tables 1 and 2). Among these are genes encoding enzymes involved in the aromatic metabolism such as chalcone isomerase, 4-coumarate-CoA-ligase and isoflavone reductase. Another group of genes putatively related with alkalinity response at root level were nutrient transport-related genes, like the nitrate high-affinity transporter (NTR2), ZIP transporter, phosphate transporter, metallothioneins and different forms of aquaporines. Genes encoding proteins with a role in cell proliferation regulation were also identified and include the Translationally-Controlled Tumor Protein (TCTP), Cyclin-U2-1, Arabidopsis-Mei2-Like proteins (AML), and the dormancy-associated protein/auxin-repressed protein (ARP/DRM). Components of signaling pathways, such as protein kinase (PK), phosphatidylinositol phosphodiesterase and the mitogen-activated protein kinase kinase kinase (MAKKK) as well as several transcription factors like the homologues of the bZIP91 and the ethylene response element binding protein (EREBP) were also identified. Other genes encoding stress responsive proteins were identified as the BURP domain-containing protein, LEA proteins, NADP-dependent malic protein and sucrose synthase; and cellular detoxification proteins such as glutathione-S-transferase and nonsymbiotic hemoglobin.

One of the most abundant ESTs in leaf SSH (89 sequences, 9 contigs) corresponds to Tar1p (Transcript Antisense to Ribosomal RNA), which encodes a functional protein localized into the mitochondria [23].

215 *3.5. Validation of SSH by qRT-PCR analysis*

216 Five and eight unigenes related to nutrient metabolism and oxidative stress relief were selected from
217 respectively leaf and root SSH libraries to measure their relative expression levels (qRT-PCR), in
218 plants treated during 14 and 28 days with the alkaline salt. Genes selected (marked with *RT* in
219 Supplementary Tables 1 and 2) from the *L. tenuis* leaf library include NADP-dependent malic protein
220 (*LtME*) and Type 1 metallothionein (*LtMT*), whereas genes selected from the root library comprised
221 Forisome (*LtFor*), Methionine synthase (*LtMS*), Phosphate transporter (*LtPT*), Nicotinamine synthase
222 2-like (*LtNA*), ZIP transporter (*LtZIP*), and High affinity nitrate transporter (*LtNTR2*). The genes
223 *LtBURP* and *LtAS* encoding BURP domain protein and Asparagine synthetase, respectively, were
224 tested in both tissues.

225 qRT-PCR analyses of leaf samples revealed that alkalinity induces the expression of *LtAS* (and *LtMT*)
226 at both sampling times (Figure 5), being strongly induced after 14 days of stress application (up 50-
227 fold in leaves and, 3-fold in roots for *LtAS*). On the other side, *LtBURP* and *LtME* were repressed at
228 14 days and induced at 28 days of stress application.

229 Root gene expression analysis revealed that some of the selected nutrition-related genes were alkali
230 responsive. Genes *LtPT* and *LtNRT2* were several-fold induced after 14 days of stress application,
231 whereas *LtZIP* and *LtMS* were only induced after 28 days of treatment (Figure 6b). *LtFor* showed a
232 repression, whereas *LtNA* was induced at both sampling times. In the root, *LtBURP* exhibited an
233 inverse response to that observed in leaves, while *LtAS* was induced only at the first evaluated time.

234

235 **4. Discussion**

236 To gain insight into the molecular responses to long-term alkaline stress that are activated in *L. tenuis*,
237 we generated two subtractive cDNA libraries and identified 158 unigenes from leaves and 92
238 unigenes from roots that are potentially regulated in *L. tenuis* plants subjected to 10 mM of $NaHCO_3$

239 for 14 and 28 days. Blast searches showed that most of the isolated ESTs had significant homology to
240 nucleotide sequences deposited in the GenBank, and were likely to encode proteins involved in plant
241 stress responses. These genes collectively play a role in growth, development, plant nutrition,
242 detoxification and the maintenance of critical cellular metabolic processes.
243 The expression pattern of genes identified by SSH and selected by their putative function in nutrient
244 uptake and growth was further characterized by qRT-PCR upon alkalinity response.
245
246 *4.1. Genes related with nutrient metabolims and oxidative stress relief.*
247 *LtPT* and *LtNTR2* are high-affinity transport systems of phosphorous and nitrogen, respectively. In
248 rice, these genes belong to an alternative transport system induced when nitrogen and inorganic
249 phosphorous (Pi) concentrations are lower than 250 and 15 µM, respectively [24,25]. Both
250 macronutrients are required at high levels for plant growth, and it is expected that plant challenged by
251 alkalinity stress activate mechanisms to ensure their acquisition. Indeed, the expression of these genes
252 in alkalinized *L. tenuis* roots was maximal at day 14, followed by a reduction in expression at day 28
253 (Figure 6). This result suggests that plant N and P levels probably decreased below a critical level
254 during the first days of stress, leading to the induction of *LtPT* and *LtNTR.* In turn, these genes
255 possibly contributed to restore N and P levels by day 28.
256 Methallothioneins and Nicotianamines are chelators responsible of the metal homeostasis in plants.
257 Methallothioneins are small, cysteine-rich and heavy metal-binding proteins, which participate in the
258 regulation of Zn distribution in the intracellular space and in an array of protective stress responses,
259 including alkalinity [26,27]. It is also known that the specific metals sequestered by metallothioneins
260 vary according to the protein structure among different organisms [28]. Nicotianamines are small
261 chelators synthesized by the enzyme Nicotinamine synthase and transport micronutrient metal ions
262 like Fe, Zn, Cu and Ni [29]. We observed that alkalinity induced the *LtMT* gene in leaves and the

*LtNA* gene in roots at both time points. However, the expression level of *LtMT* showed a decrease of two orders of magnitude with time, whereas that of *LtNA* remained approximately invariable. Another gene related with transport and homeostasis of micronutrients, *LtZIP*, was also induced in alkalinized plants but only at day 28. Members of the ZIP protein family are capable of transporting a variety of cations, including Cd, Fe, Mn and Zn [30]. In *M. truncatula*, the identification of six genes of the ZIP family was reported, whereas the expression analysis under different metal deficiency conditions revealed gene specificity to each metal [31]. Previously, it was shown that alkalinity led to reduction of Zn content in leaves of *L. tenuis*, but not in those of other metals, such as Fe or Cu [19]. Further studies addressing the metal specificities of the chellators and transporters here identified are required in order to fully understand their role in the regulation of metal ion homeostasis in alkalinized *L. tenuis* plants.

Another gene related with nitrogen metabolism in plants is *LtAS* encoding asparagine synthetase. This enzyme catalyzes the transfer of an amide group from glutamine to aspartate forming asparagine (Asn) in an ATP-dependent reaction [31]. Asparagine synthetase plays an important role in nitrogen transport and storage in plants. High concentrations of Asn were previously found in various plant tissues under other stress conditions, such as mineral deficiencies, salinity or drought [32]. Our results showed that the *LtAS* gene was greatly induced at day 14 with values of 50-fold in leaves and, 3-fold in roots, although the expression level decreased at the 28-day. Following the same reasoning as for *LtPT* and *LtNTR*, the reduction in the *LtAS* expression level observed between days 14 and 28 constitutes a hint that alkalinized plants were able to balance N nutrition towards the end of the experiment.

Another identified gene that could be relevant for the tolerance to alkalinity by *L. tenuis* is that coding for a NADP dependent malic enzyme (NADP-ME; *LtME*), which was regulated in leaves. This enzyme catalyzes the oxidative decarboxylation of L-malate, producing pyruvate, $CO_2$, and NADPH.

Our results showed that *LtME* was repressed at the 14-day and several-fold induced after 28 days of alkalinity. The *NADP-ME* promoter can be activated by different effectors (UV irradiation, fungal, wounding) and, agents producing redox perturbations in bean (*Phaseolus vulgaris*; [33]). In fact, it has been suggested that the NADPH produced by NADP-ME provides the reducing power required for ROS metabolism and scavenging [34,35,36]. Interestingly, the over-expression of rice NADP-ME2 in Arabidopsis plants increased tolerance to long term osmotic, alkali and neutral saline stress [37].

The expression of methionine synthetase has been reported to be induced under different abiotic stress such as Zn and Cd toxicity [38,39], NaCl [40] and alkaline conditions [41]. Our SSH results showed that *LtMS* was induced at day 28 in *L. tenuis* roots. Methionine synthetase is the last step in the pathway leading to methionine biosynthesis [42,43]. It was estimated that about 80% of methionine is converted to *S*-adenosylmethionine (SAM), which is the methyl group donor for the production of several protective mechanisms such as ethylene, polyamines, DNA methylation, chlorophyll biosynthesis, cell wall biosynthesis, and to a large number of secondary metabolites [44,45,46,47]. Thus, the induction of *LtMS* could be interpreted as one important component of the plant tolerance mechanism that is activated in *L. tenuis* as result of alkalinity-induced metals imbalance.

*4.2. Other genes of interest*

Growth and development of plants under stress conditions are also linked with changes in cell wall composition. The gene encoding the seed coat BURP domain protein was isolated from leaf and root tissues of *L. tenuis* (Fig. 5 and 6). The function of most members of BURP family is largely unknown, although several researches revealed that genes of this family might be crucial not only for plant development but also for response and adaptation to stresses [48].

Finally, one unclassified gene evaluated encodes a stress responsive mechanoprotein that functions as valves in the phloem sieve tubes of the Fabaceae, named Forisome (*LtFor*). Plug formation by forisomes is triggered *in vivo* by plasma membrane leakage induced through injury and by abrupt turgor changes imposed by osmotic shock [49]. *In vitro* studies of isolated forisomes demonstrated that the volume of this structure is influenced by $Ca^{+2}$ and pH levels [49]. Our results revealed an alkalinity-induced down-regulation of *LtFor* in *L. tenuis* roots, suggesting a decrease in plugs formation in the root phloem. This result could be related with the significant reductions in plant growth (Fig. 1) and with the fact that under alkalinity, *L. tenuis* exhibited a decline of phloematic tissues with proportionally higher allocation of resources to root development [19,50].

## 5. Conclusion

In the present study, we used Subtractive Hybridization for the identification of alkali responsive genes in *Lotus tenuis* plants subjected to long-term $NaHCO_3$ treatments. qRT-PCR analysis revealed that genes potentially involved in nutrient metabolism and oxidative stress relief are modulated in a time and tissue dependent manner under alkaline stress. A comprehensive analysis of genes described in this work might lead to a quicker and better understanding of the mechanisms involved in plant response to alkaline stress and could contribute to the design of molecular strategies to improve forage and crop production in soils affected by alkalinity.

## Acknowledgments

This work was supported by the following grants: Consejo Nacional de Ciencia y Técnica (CONACYT, Mexico); PICT, Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), Universidad de San Martin (UNSAM, Argentina), Comisión de Investigaciones Científicas de la

Provincia de Buenos Aires (CIC, Argentina). RCP expresses her gratitude to CONICET and Bilateral Programme of Scientific and Technological Cooperation (MINCYT/CONACYT; Argentina, Mexico) for doctoral fellowships; and for Ing. Agr. Francisco A. González Antivilo for his helpful comments on this paper.

**References**

[1] FAO: Land And Water Division (NRL) http://www.fao.org/ag/agl/agll/spush/intro.htm. Accessed 26 August 2013.

[2] H. Marschner, Mineral Nutrition of Higher Plants. 2nd Ed. Academic Press. London. 889 p, 1995.

[3] A. Mirsa, G. Tyler, Influence of soil moisture on soil solution chemistry and concentrations of minerals in the calcicoles *Phleum phleoides* and *Voronica spicata* grown on a limestone soil. Ann Bot-London, 84 (1999) 401-410. doi: 10.1006/anbo.1999.0941

[4] S.M. Alam, Nutrient uptake by plants under stress conditions. In: Pessarakli M (ed) Handbook of Plant and Crop Stress, Second Edition. CRC Press, pp 285-313, 1999. doi: 10.1201/9780824746728.ch12

[5] Z. Bie, T. Ito, Y. Shinohara, Effects of sodium sulfate and sodium bicarbonate on the growth, gas exchange and mineral composition of lettuce, Sci Hortic-Amsterdam, 99 (2004) 215-224. doi: 10.1016/S0304-4238(03)00106-7

[6] F.H. Petersen, Water testing and interpretation. p. 31-49. In: Reed, D.Wm. (Editor). Water, media and nutrition. Ball Publishing, Batavia, IL, 1996.

[7] A. Pissaloux, P. Morarad, G. Bertoni, Alkalinity-bicarbonate calcium effects on iron chlorosis in white lupine in soilless culture. In Abadía, J. (ed.): Development in Plant and Soil Science. Iron Nutrition in Soils and Plants; Seventh International Symposium on Iron Nutrition and Interactions in

Plants, Zaragoza, Spain, June 27–July 2, 1993. Kluwer Academic Publishers, Dordrecht, pp. 127–133, 1995.

[8] I. Cakmak, Possible roles of zinc in protecting plant cells from damage by reactive oxygen species, New Phytol. 146 (2000) 185-205. doi: 10.1046/j.1469-8137.2000.00630.x

[9] B. Sun, Y. Jing, K. Chen, L. Song, F. Chen, L. Zhang, Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*), J. Plant Physiol 164 (2007) 536-543. doi: 10.1016/j.jplph.2006.02.011

[10] R.K. Tewari, P. Kumar, P.N. Sharma, Oxidative stress and antioxidant responses in young leaves of mulberry plants grown under nitrogen, phosphorus or potassium deficiency, J. Int. Plant Biol, 49 (2007) 313-322. doi: 10.1111/j.1744-7909.2007.00358.x

[11] T. Hirayama, K. Shinozaki, Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J. 61 (2010) 1041–1052. doi: 10.1111/j.1365-313X.2010.04124.x

[12] L. Diatchenko, Y.F.C. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, P.D, Siebert, Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93 (1996) 6025–6030.

[13] P. Boominathan, R. Shukla, A. Kumar, D. Manna, D. Negi, P.K. Verma, D. Chattopadhyay, Long term transcript accumulation during the development of dehydration adaptation in Cicer arietinum. Plant Physiol. 135 (2004) 1608-1620. doi: 10.1104/pp.104.043141

[14] J. Zhang, T. Liu, J. Fu, Y. Zhu, J. Jia, J. Zheng, Y. Zhao, Y. Zhang, G. Wang, Construction and application of EST library from Setaria italica in response to dehydration stress, Genomics, 90 (2007) 121-131. doi: 10.1016/j.ygeno.2007.03.016

[15] C. Lata, P.P. Sahu, M. Prasad, Comparative transcriptome analysis of differentially expressed genes in foxtail millet (*Setaria italica* L.) during dehydration stress, BRRC, 393 (2010) 720-727. doi: 10.1016/j.bbrc.2010.02.068

[16] J.H. Kirkbride, The scientific name of narrow-leaf trefoil. Crop Science 46 (2006) 2169–2170. doi: 10.2135/cropsci2006.02.0084

[17] S.C. Stoffella, G. Posse, M. Collantes, Variabilidad fenotipica y genotipica de poblaciones de *Lotus tenuis* que habitan suelos con distinto pH. Ecol. Aust. 8 (1998) 57–63 [in Spanish].

[18] O.S. Correa, A. Aranda, A.J. Barneix, Effects of pH on growth and nodulation of two forage legumes, J. Plant Nutr. 24 (2001) 1367–1375.

[19] R.C. Paz, R.A. Rocco, H. Reinoso, A.B. Menendez, AB, F.L. Pieckenstain, O.A. Ruiz, Comparative study of alkaline, saline and mixed saline-alkaline stresses, with regard to their effects on growth, nutrients accumulation and root morphology of *Lotus tenuis*, JPGR, 31 (2012) 448-459. doi: 10.1007/s00344-011-9254-4

[20] D. Hoagland, D. Arnon D, The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347 (1950) 1–32.

[21] S.O. Kotchoni, E.W. Gachomo, E.S. Betiku, O.O. Shonukan, AJOB, 2 (2003) 88-90.

[22] D.H. Sanchez, F. Lippold, H. Redestig, M.A. Hannah, A. Erban, U. Krämer, J. Kopka, M.K. Udvardi, Integrative functional genomics of salt acclimatization in the model legume *Lotus japonicus*. Plant J. 53 (2008) 973–987. doi: 10.1111/j.1365-313X.2007.03381.x

[23] P.S.R. Coelho, A.C. Bryan, A. Kumar, G.S. Shadel, M. Snyder, A novel mitochondrial protein, Tar1p, is encoded on the antisense strand of the nuclear 25S rDNA. Genes Dev. 16 (2002) 2755–2760. doi: 10.1101/gad.1035002

[24] T. Aono, N. Kanada, A. Ijima, H. Oyaizu, The response of the phosphate uptake system and the organic acid exudation system to Phosphate starvation in *Sesbania rostrata*, Plant Cell Physiol, 42 (2001) 1253-1264.

[25] C. Cai, J.Y. Wan, Y.G. Zhu, Q.R. Shen, B. Li, Y.P. Tong, Z.S. Li, Gene structure and expression of the high-affinity nitrate transport system in rice roots, J Integr Plant Biol, 50 (2008) 443-451. doi: 10.1111/j.1744-7909.2008.00642.x

[26] S. Nishiuchi, S. Liu, T. Takano, Isolation and characterization of a metallothionein-1 protein in *Chloris virgate* Swartz that enhances stress tolerances to oxidative, salinity and carbonate stress in *Saccharomyces cervisiae*. Biotechol Lett 29 (2007) 1301-1305. doi: 10.1007/s10529-007-9396-4

[27] Y. Wang, H. Ma, G. Liu, D. Zhang, Q. Ban, G. Zhang, C. Xu, C. Yang, Generation and analysis of expressed sequence tags from a $NaHCO_3$-treated *Limonium bicolor* cDNA library, Plant Physiol Bioch, 46 (2008) 977-986. doi: 10.1016/j.plaphy.2008.06.001

[28] C.A. Blindauer, O.I. Leszczyszyn, Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more, Nat Prod Rep 27 (2010) 720–74. doi: 10.1039/b906685n

[29] G. Scholz, R. Becker, A. Pich, U.W. Stephan, Nicotianamine: a common constituent of strategy-I and strategy-II of iron acquisition by plants. A review. J Plant Nutr 15 (1992) 1647–1665.

[30] M.L. Guerinot, The ZIP family of metal transporters, Biochim Biophys Acta 1465 (2000) 190-198. doi: 10.1016/S0005-2736(00)00138-3

[31] L. Gaufichon, M. Reisdorf-Crena, S.J. Rothsteinc, F. Chardon, A. Suzuki, Biological functions of asparagine synthetase in plants. Plant Sci 179 (2010) 141–153. doi: 10.1016/j.plantsci.2010.04.010

[32] M.G. Moller, C. Taylor, S.K. Rasmussen, P.B. Holm, Molecular cloning and characterization of two genes encoding asparagine synthtase in barley (*Hordeum vulgare* L.), Biochim Biophys Acta, 1628 (2003) 123-132.

[33] M.H. Walter, J. Grima-Pettenati, C. Feuillet, Characterization of a bean (*Phaseolus vulgaris* L.) malic-enzyme gene. Eur J Biochem 224 (1994) 999-1009. doi: 10.1111/j.1432-1033.1994.t01-1-00999.x

[34] K.I. Minard, L. McAlister-Henn, Antioxidant function of cytosolic sources of NADPH in yeast. Free Radic Biol Med, 31 (2001) 832-843.

[35] I.M. Møller, Plant mitochondria and oxidative stress: Electron Transport, NADPH Turnover, and Metabolism of Reactive Oxygen Species, Annu Rev Plant Physiol Plant Mol Biol. 52 (2001) 561–591. doi: 10.1146/annurev.arplant.52.1.561

[36] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci, 7 (2002) 405-410.

[37] S. Liu, Y. Cheng, X. Zhang, Q. Guan, S. Nishiuchi, K. Hase, T. Takano, Expression of an NADP-malic enzyme gene in rice (*Oryza sativa* L) is induced by environmental stresses; over-expression of the gene in *Arabidopsis* confers salt and osmotic stress tolerance, Plant Mol Biol, 64 (2007) 49–58. doi: 10.1007/s11103-007-9133-3

[38] J.E. Sarry, L. Kuhn, C. Ducruix, A. Lafaye, C. Junot, V. Hugouvieux, A. Jourdain, O. Bastien, J.B. Fievet, D. Vailhen, B. Amekraz, C. Moulin, E. Ezan, J. Garin, J. Bourguignon, The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses, Proteomics, 6 (2006) 2180-2198. doi: 10.1002/pmic.200500543

[39] Y. Fukao, A. Ferjani, M. Fujiwara, Y. Nishimori, I. Ohtsu, Identification of zinc-responsive proteins in the roots of *Arabidopsis thaliana* using a highly improved method of two-dimensional electrophoresis, Plant Cell Physiol, 50 (2009) 2234-2239. doi: 10.1093/pcp/pcp154.

[40] M.G. Palmgren, S. Clemens, L.E. Williams, U. Krämer, S. Borg, J.K. Schjørring, Zinc biofortification of cereals: problems and solutions. Trends Plant Sci. 13 (2008) 464–473. doi: 10.1016/j.tplants.2008.06.005

[41] G.S. Xie, S.K. Liu, T. Takano, Z.B. You, D.P. Zhang, Cloning and expression of VB12-independent methionine synthase gene responsive to alkaline stress in rice. Yi Chuan Xue Bao. 29 (2002) 1078-84.

[42] B.F. Matthews, Lysine, threonine and methionine biosynthesis. InBK Singh, ed, Plant Amino Acids: Biochemistry and Biotechnology. Dekker, New York, pp 205–225, 1999.

[43] H. Hesse, R. Höfgen, Molecular aspects of methionine biosynthesis in *Arabidopsis* and potato. Trends Plant Sci 8 (2003) 259–262. doi: 10.1016/S1360-1385(03)00107-9

[44] R. Amir, Y. Hacham, G. Galili, Cystathionine gamma-synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. Trends Plant Sci, 7 (2002) 153–156. doi: 10.1016/S1360-1385(02)02227-6

[45] U. Wittstock, B.A. Halkier, Glucosinolate research in the *Arabidopsis* era, Trends Plant Sci, 7 (2002) 263–270. doi: 10.1016/S1360-1385(02)02273-2

[46] F. Rebeille, S. Jabrin, R. Bligny, K. Loizeau, B. Gambonnet, V. Van Wilder, R. Douce, S. Ravanel, Methionine catabolism in *Arabidopsis* cells is initiated by a gamma-cleavage process and leads to S-methylcysteine and isoleucine syntheses, Proc Natl Acad Sci USA, 103 (2006) 15687–15692. doi: 10.1073/pnas.0606195103

[47] A. Goyer, E. Collakova, Y. Shachar-Hill, A.D. Hanson, Functional characterization of a methionine gamma-lyase in *Arabidopsis* and its implication in an alternative to the reverse trans-sulfuration pathway, Plant Cell Physiol, 48 (2007) 232–242. doi: 10.1093/pcp/pcl055

ACCEPTED MANUSCRIPT

516 [48] X. Ding, X. Hou, K. Xie, L. Xiong, Genome-wide identifiication of BURP domain-containing
517 genes in rice reveals a gene family with diverse structures and responses to abiotic stresses. Planta
518 230 (2009) 149–163. doi: 10.1007/s00425-009-0929-z

519

520 [49] M. Knoblauch, W.S. Peters, Forisomes, a novel type of Ca2+-dependent contractile protein
521 motor, Cell Motil Cytoskel, 58 (2004) 137–142. doi: 10.1002/cm.20006

522

523 [50] R.C. Paz, H. Reinoso, A.B. Menéndez, F.D. Espasandin, F.A. González Antivilo, P.A.
524 Sansberro, R.A. Rocco, O.A. Ruiz, Akaline, saline and mixed saline–alkaline stresses induce
525 physiological and morpho-anatomical changes in *Lotus tenuis* shoots. Plant Biology (in press) doi:
526 10.1111/plb.12156

527

ACCEPTED MANUSCRIPT

Figure Legends

**Figure 1. Time-course analysis of growth performance of *L. tenuis* under control (■) and alkaline (○) stress conditions** Fifteen day-old plants were watered with nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment 10 mM $NaHCO_3$ were added to 0.5 X Hoagland's solution. Plants were harvested on days 0, 7, 14, 21 and 28 of stress treatments. Data are mean ± SE (n=10).

**Figure 2. Plant growth response of *L. tenuis* plants exposed to control (left panel) and alkaline (right panel) stress conditions**. Fifteen day-old plants were watered with nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment 10 mM $NaHCO_3$ were added to 0.5 X Hoagland's solution. Plants were harvested on day 28 of stress treatments. Data are mean ± SE (n=10). Scale bars = 20 cm.

**Figure 3. Venn diagrams of non-redundant and common putative -upregulated genes identified on the basis of SSH of roots and shoots.**

**Figure 4. Functional classification of *Lotus tenuis* genes expressed at long term alkaline response stress.** A total of 158 unigenes of leaves (A) and 92 unigenes of roots (B) were classified into 11 functional categories according to their putative biological function reported by Gene Ontology (www.geneontology.org) database and bibliographic reports. The percentage of unigenes included.

**Figure 5. Differential expression analysis by qRT-PCR of 4 selected cDNA clones from leaf SSH, following 14 and 28 days of alkaline treatment.** Fifteen day-old plants were watered with

552 nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment, 10
553 mM NaHCO3 were added to 0.5 X Hoagland's solution. Total RNA was isolated from leaf tissues at
554 14 days after stress application (A) and 28 days after stress application (B) and RT-PCR was
555 performed using gene-specific primers. LtBURP: BURP domain (AB862734; AB862827;
556 AB862828); LtME: NADP-dependent malic protein (AB862678); LtAS: Asparagine synthetase
557 (AB862747; AB862785; AB862812); LtMT: Type 1 metallothionein (AB862686). Bars represent SE
558 of mean (n = 3) and asterisks indicate significant differences of relative gene expression of each gene
559 and time with respect to control according to a simple Student's t-test with Bonferroni correction (*, p
560 < 0.05; **, p < 0.01). The relative expression levels of all the analyzed unigenes was normalized to
561 the previously described housekeeping gene LjGPI-anchored protein. Ratios lower than 1 (i.e., genes
562 repressed in stress conditions) are represented as minus the inverse of the ratio.

563

564 **Figure 6. Root differential expression analysis by qRT-PCR of 9 selected cDNA clones following**
565 **14 and 28 days of alkaline treatment.** Fifteen day-old plants were watered with nutrient solution
566 containing or lacking salt addition during 28 days. For alkaline stress treatment, 10 mM NaHCO3
567 were added to 0.5 X Hoagland's solution. Total RNA was isolated from root tissues at 14 days after
568 stress application (A) and 28 days after stress application (B) and RT-PCR was performed using
569 gene-specific primers. LtBURP: BURP domain (AB862734; AB862827; AB862828); LtFor:
570 Forisome (AB862866); LtGS: Cytosolic glutamine synthetase (AB862789; AB862790); LtMS:
571 Methionine synthase (AB862813; AB862814); LtAS: Asparagine synthetase (AB862747; AB862785;
572 AB862812); LtPT: Phosphate transporter (AB862815; AB862816); LtNA: Nicotinamine synthase 2-
573 like (AB862818); LtZIP: ZIP transporter (AB862829; AB862830); LtNTR2: High affinity nitrate
574 transporter (NTR2) (AB862844). Bars represent SE of mean (n = 3) and asterisks indicate significant

ACCEPTED MANUSCRIPT

differences of relative gene expression of each gene and time with respect to control according to a simple Student's t-test with Bonferroni correction (*, $p < 0.05$; **, $p < 0.01$). Ratios lower than 1 (i.e., genes repressed in stress conditions) are represented as minus the inverse of the ratio.

ACCEPTED MANUSCRIPT

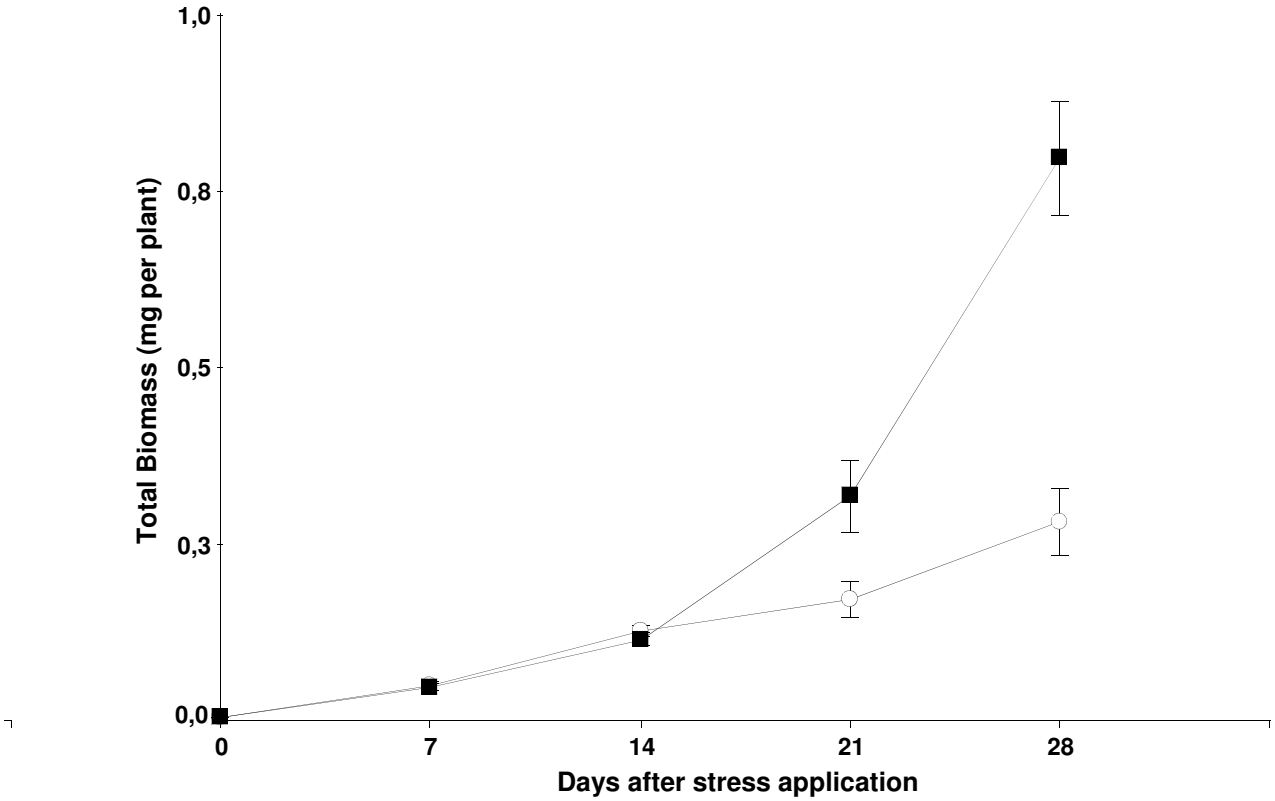
580 **Table 1. Oligonucleotides designed for qRT-PCR analysis**

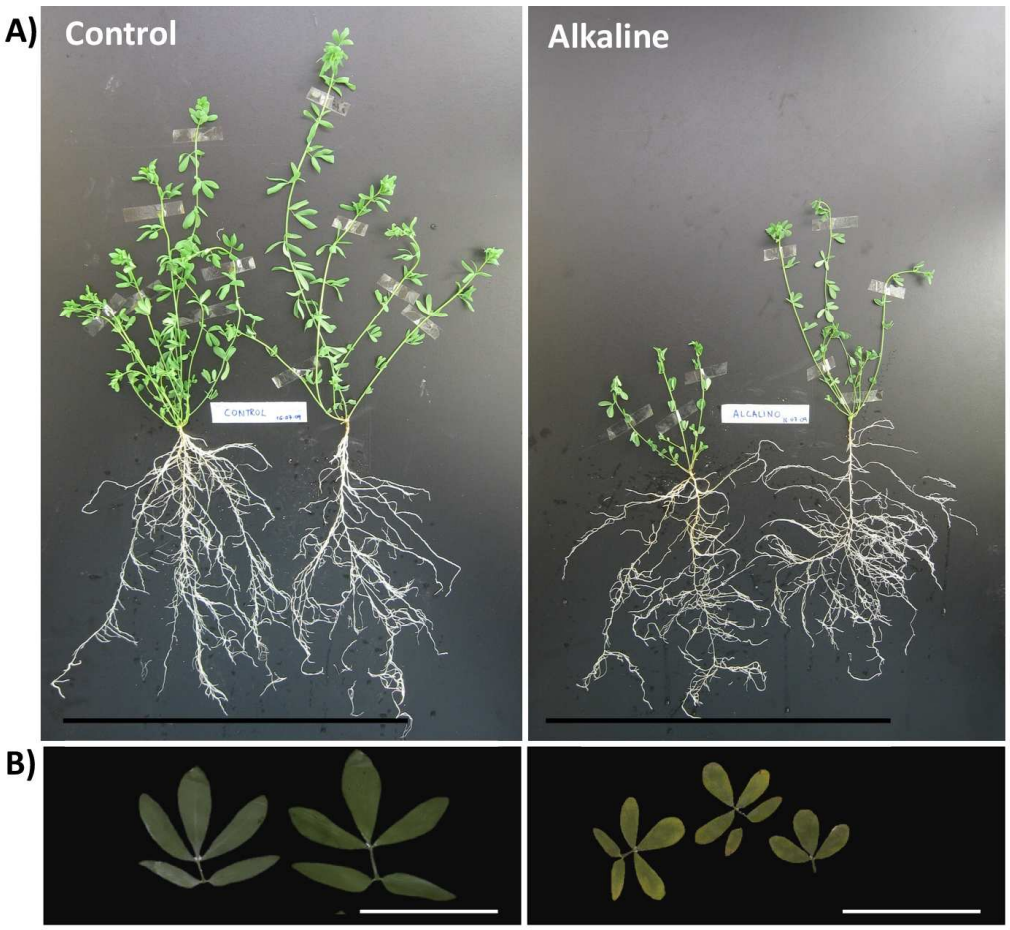
581

| Gene | Oligonucleotid name | Oligonucleotid sequence | Tissue | Amplicon size (bp) |
|---|---|---|---|---|
| LtAS | AspSin128H-Fw: | 5´-CCTTCAGGTAGAGCAGCAC-3´ | L,R | 143 |
| | AspSin128H-Rv: | 5´-CCTTAACTGTGGATGGCAAC-3´ | | |
| LtNADP-ME | ProtMal62H-Fw: | 5´-GTCTCGGGCTAATAATGTC-3´ | L | 104 |
| | ProtMal62H-Rv: | 5´-CCTGTCATAGTCCTCTTGG-3´ | | |
| LtMT | Metalot48H-Fw: | 5´-CTTGAAGCAGAGAGATGGC-3´ | L | 172 |
| | Metalot48H-Rv: | 5´-ACACGCACAACAAATCCC-3´ | | |
| LtBURP | BURP113H Fw | 5´-TGGAAGGAGAAGATGGCGTAAGAG-3´ | L,R | 136 |
| | BURP113H Rv | 5´-GGGAAGAAATGACACACTGGAACC-3´ | | |
| LtGS | GlutSy16R Fw | 5´-GAGAGGATGGTGGCTATG-3´ | R | 116 |
| | GlutSy16R Rv | 5´-GTGTCTTCCTGTCAAACG-3´ | | |
| LtMS | MetSyn39R Fw | 5´-AATGATGGAGTGGATGATGTC-3´ | R | 141 |
| | MetSyn39R Rv | 5´-CTAAGGAAGTCAGAGCAAGC-3´ | | |
| LtPT | PhosphTrans41R Fw | 5´-AGCAAGGTTGAGGTCTAC-3´ | R | 173 |
| | PhosphTrans41R Rv | 5´-TCACACCAAGCATAATAAGG-3´ | | |
| LtNA | NicotSyn43R Fw | 5´-TGACACACAACAATACTAAATCC-3´ | R | 174 |
| | NicotSyn43R Rv | 5´-TGCTTACCATCTTTCATCCC-3´ | | |
| LtZIPT | ZipTransp51R Fw | 5´-TGGGAGTTTCACAGAGTC-3´ | R | 183 |
| | ZipTransp51R Rv | 5´-CAGTTCCAATGCCTATACC-3´ | | |
| LtNTR | NTR262R Fw | 5´-CGGGAGGAAGAGAGGAAGAAGG-3´ | R | 106 |
| | NTR262R Rv | 5´-TTGGAGGAGTTGGAGCAGAGG-3´ | | |
| LtForisome | Forisom85R Fw | 5´-TGCCACAGTGATGCTCCTAATG-3´ | R | 86 |
| | Forisom85R Rv | 5´-GCCGAGTTACAACAACAAGACC-3´ | | |
| LjGPI | LjGPI Fw | 5´-AGGTTGTTCCGTGAATTTCG-3´ | HK | 63 |
| | LjGPI Rv | 5´-GGTCCTTTGCATTTGCTTGT-3´ | | |

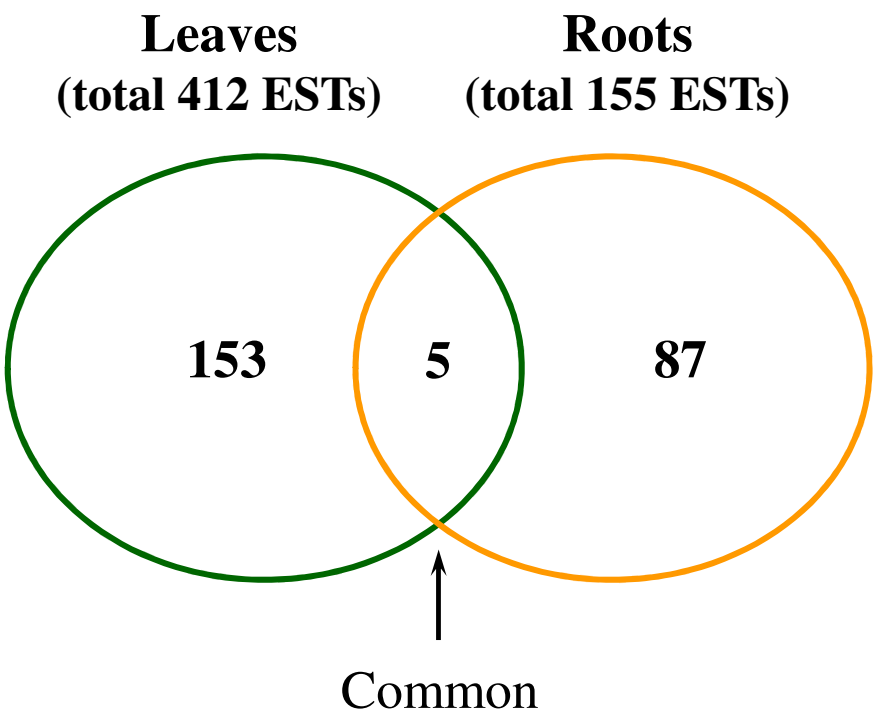
Abreviatures: L, leaf gene; R, root gene; HK, housekeeping gene; NC, negative control gene

ACCEPTED MANUSCRIPT

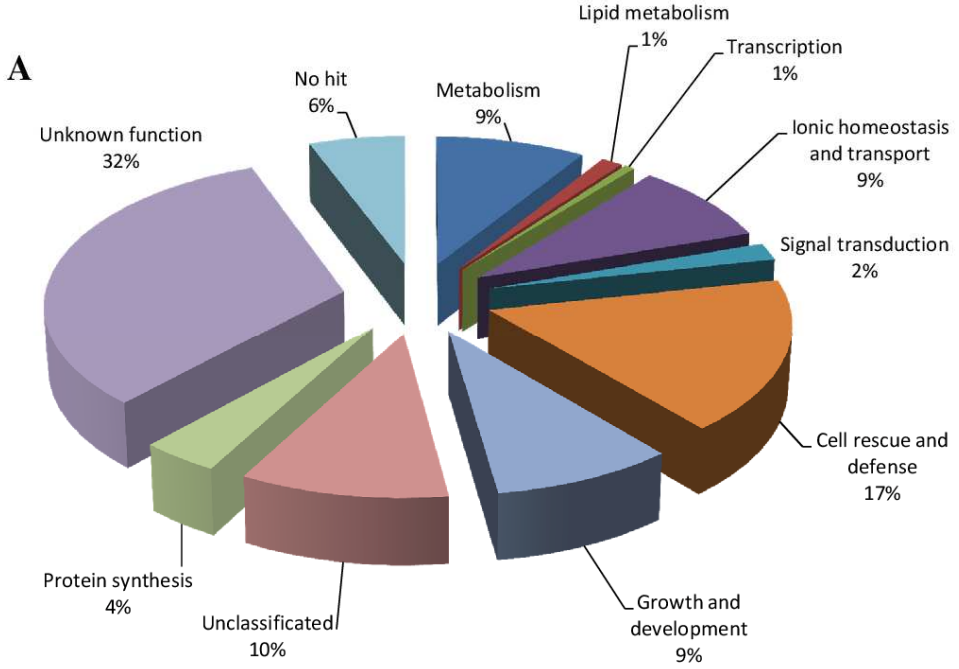

1,0
0,8
0,5
0,3
0,0
Total Biomass (mg per plant)
0
7
14
21
28
Days after stress application

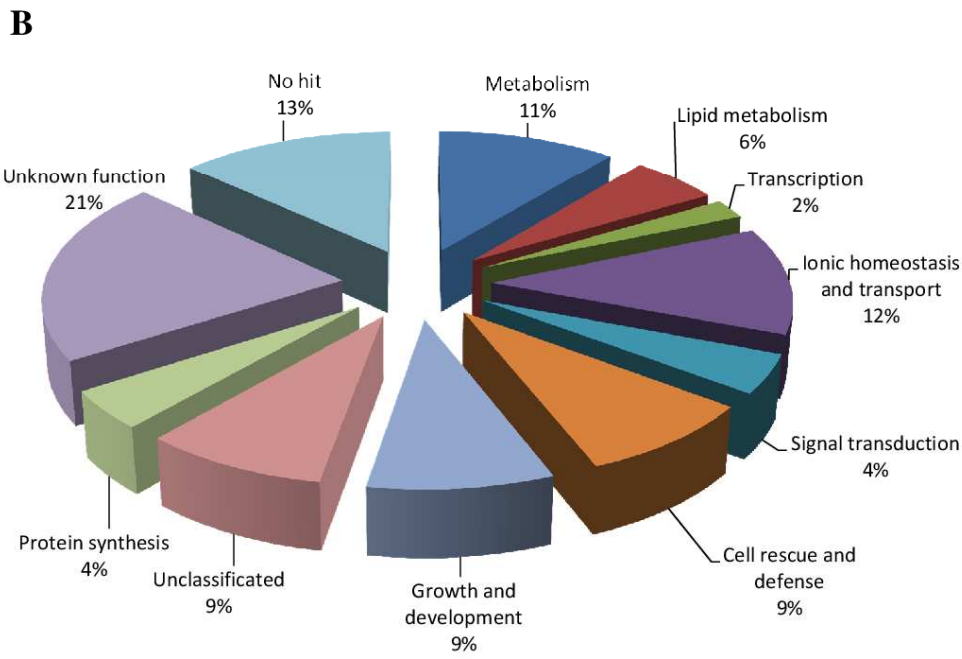



ACCEPTED MANUSCRIPT

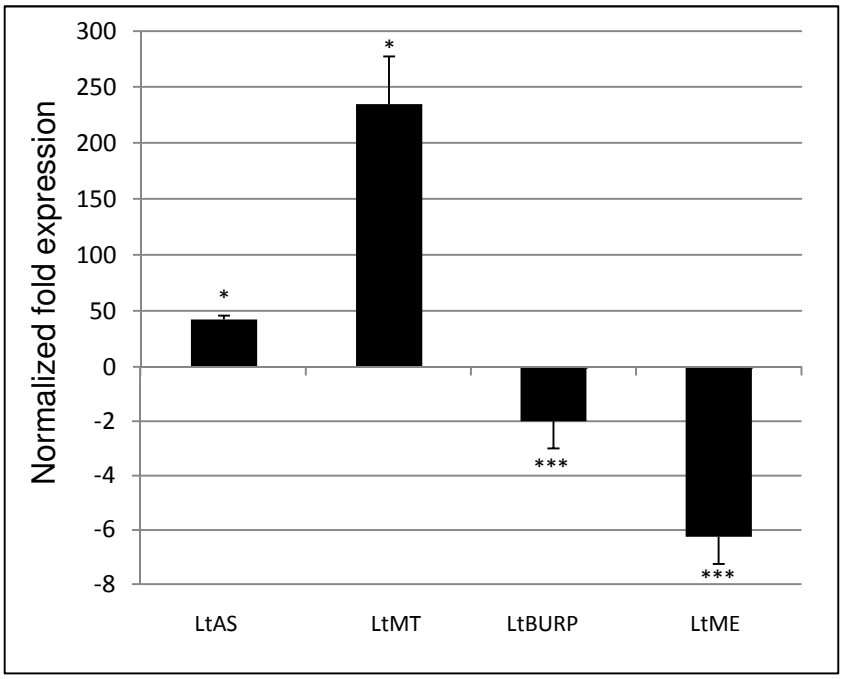



| Common unigenes | Leaf ID | Root ID |
|---|---|---|
| **Asparagine synthase** | AB862747 | AB862785 |
| **BURP** | AB862734 | AB862827; AB862828 |
| **K(+)/H(+) antiporter-like** | AB862674 | AB862836 |
| **Unknown** | AB862629; AB862630; AB862631; AB862632 | AB862793; AB862794 |
| **Unknown** | AB862792; AB862712 | AB862862 |

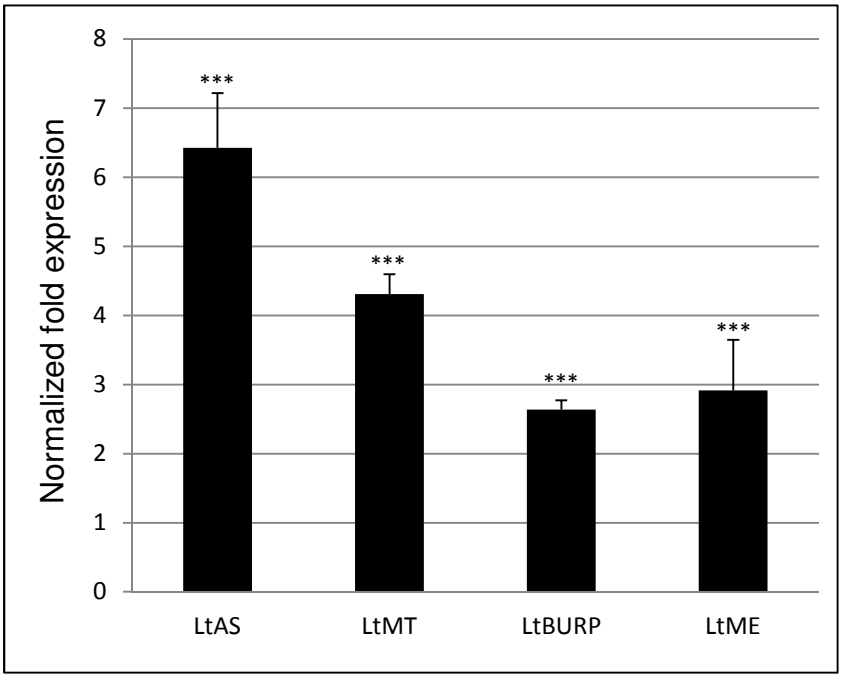

A
Lipid metabolism
1%
Transcription
1%
No hit
6%
Metabolism
9%
Unknown function
32%
Ionic homeostasis
and transport
9%
Signal transduction
2%
Cell rescue and
defense
17%
Protein synthesis
4%
Unclassificated
10%
Growth and
development
9%


B
No hit
13%
Metabolism
11%
Lipid metabolism
6%
Unknown function
21%
Transcription
2%
Ionic homeostasis
and transport
12%
Signal transduction
4%
Protein synthesis
4%
Unclassificated
9%
Growth and
development
9%
Cell rescue and
defense
9%

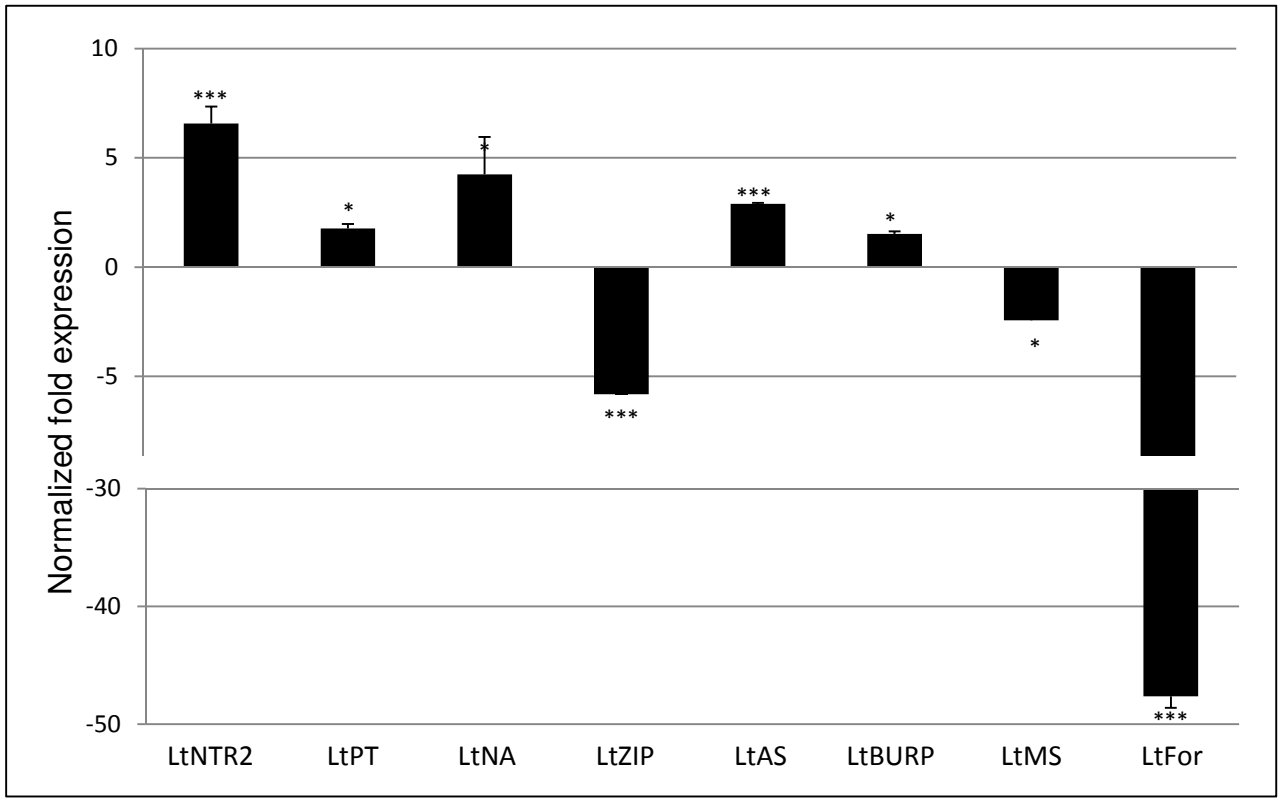
**A**


Normalized fold expression
300
250
200
150
100
50
0
-2
-4
-6
-8
*
*
***
***
LtAS
LtMT
LtBURP
LtME

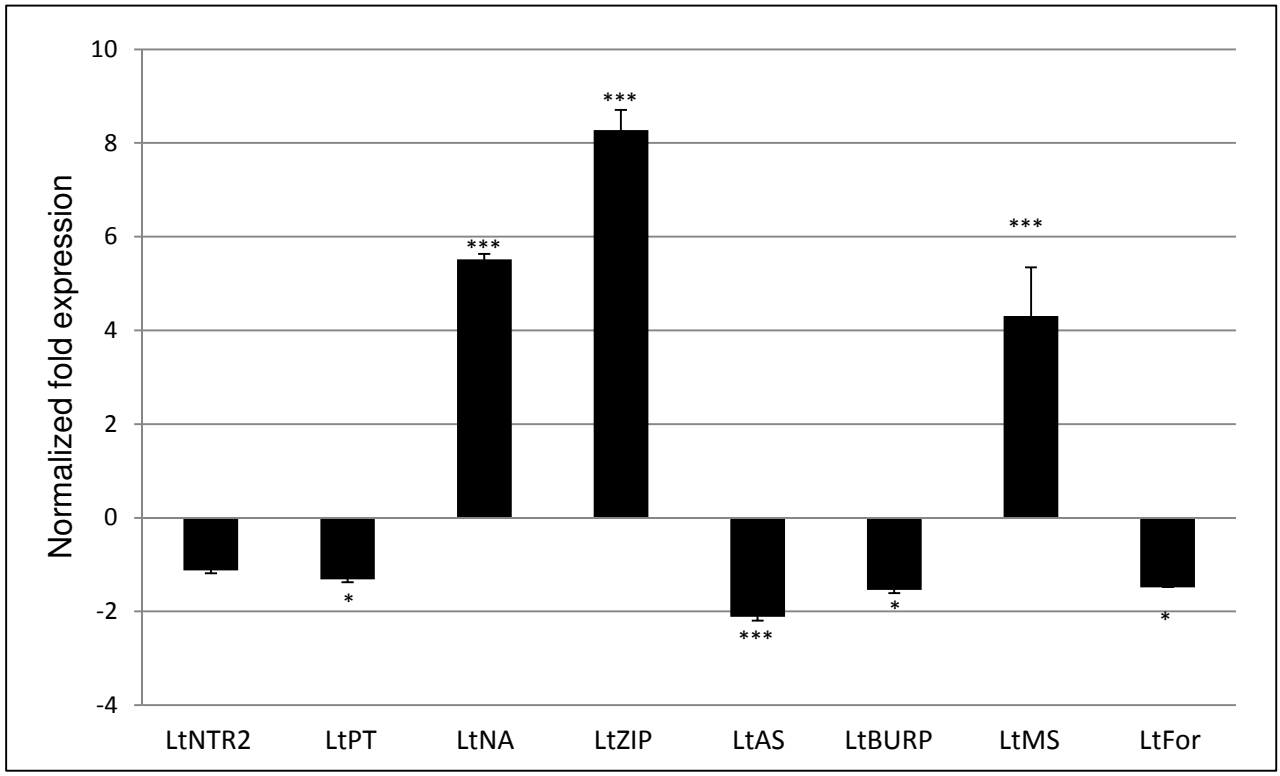
**B**


Normalized fold expression
8
7
6
5
4
3
2
1
0
***
***
***
***
LtAS
LtMT
LtBURP
LtME

**A**


10
5
0
-5
-30
-40
-50
Normalized fold expression
LtNTR2
LtPT
LtNA
LtZIP
LtAS
LtBURP
LtMS
LtFor

**B**


10
8
6
4
2
0
-2
-4
Normalized fold expression
LtNTR2
LtPT
LtNA
LtZIP
LtAS
LtBURP
LtMS
LtFor

ACCEPTED MANUSCRIPT

Highlights

We generated subtractive cDNA libraries from leaves and roots of *L. tenuis* plants.

Total RNAs of non-stressed plants and alkalinized plants were used as source of the driver and the tester samples, respectively.

Uunigenes from leaves and roots were obtained and classified into 11 functional categories.

Unigenes that were related with nutrient metabolism and oxidative stress relief were found to be differentially expressed in a time dependent manner in *L. tenuis* during the alkaline stress application.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Contributions

RP participated of all experimental stages including procedures for SSH and manuscript drafting. RR contributed with bioinformatic analysis and qRT-PCR. JFG-B collaborated with all experimental stages including the design of the study and manuscript preparation. MR-K collaborated with the SSH and general data evaluation. AB collaborated with cloning and sequencing. AM participated in the results interpretation and helped to draft the manuscript. OR conceived the study and participated in its design.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Supplementary Table 1. cDNA clones isolated from a subtractive suppressive hybridization library from leaves of alkaline stressed *Lotus tenuis* plants

| | | | | Top NCBI blast alignment | | | |
|---|---|---|---|---|---|---|---|
| Sequence ID | Clones contained | Length | Annotation | Accession | *Specie* | Score | E-value |
| **Metabolism** | | | | | | | |
| AB863438 | 1 | 304 | Cytochrome P450 like TBP | XM_003614352 | *Medicago truncatula* | 239 | 6.00E-67 |
| # AB862643; AB862644 | 2 | 360 | Fructokinase-2-like | AK339576 | *Lotus japonicus* | 379 | 5E-102 |
| AB862769 | 1 | 192 | Cytochrome P450 like TBP | XM_003614352 | *Medicago truncatula* | 116 | 3.00E-30 |
| # AB862722 | 1 | 443 | 6-phosphogluconate dehydrogenase | AK339500 | *Lotus japonicus* | 375 | 1E-100 |
| *Amino acid metabolism* | | | | | | | |
| RT, # AB862747 | 1 | 489 | Asparagine synthetase | X89409 | *Lotus japonicus* | 596 | 4E-167 |
| *Aromatic metabolism* | | | | | | | |
| # AB862607; AB862608; AB862609 | 3 | 447 | Chalcone isomerase 4 | AY595417 | *Glycine max* | 268 | 2E-68 |
| # AB862711 | 1 | 410 | 4-coumarate:coenzyme A ligase | AF279267 | *Glycine max* | 167 | 7E-37 |
| # AB862727; AB862728 | 2 | 635 | Chalcone synthase-like protein | AK339637 | *Lotus japonicus* | 1678 | 0.0 |
| # AB862761 | 1 | 728 | Chalcone--flavonone isomerase 1 | AB073787 | *Lotus japonicus* | 807 | 0.0 |
| *Energy* | | | | | | | |
| RT, # AB862678 | 1 | 461 | NADP-dependent malic protein | DQ889593 | *Arachis hypogaea* | 257 | 5E-65 |
| # AB862684; AB862685 | 2 | 625 | Thiamin biosynthetic enzyme | AB030493 | *Glycine max* | 381 | 2E-102 |
| AB862743 | 1 | 373 | Ubiquinol-cytochrome c reductase cytochrome c1 subunit | BT052414 | *Medicago truncatula* | 260 | 4E-66 |
| # AB862730 | 1 | 376 | Fructose-bisphosphate aldolase | AK337967 | *Lotus japonicus* | 531 | 1E-147 |
| # AB862732; AB862733 | 2 | 363 | Sucrose synthase | AJ133726 | *Lotus japonicus* | 398 | 4E-96 |
| **Lipid metabolism** | | | | | | | |
| # AB862660 | 1 | 415 | Lipoxygenase | U04526 | *Glycine max* | 233 | 5E-58 |
| # AB862737 | 1 | 554 | Allene oxide cyclase | AJ308489 | *Medicago truncatula* | 434 | 3E-118 |
| **Transcription** | | | | | | | |
| # AB862664 | 1 | 295 | Transcription factor bZIP91 | DQ792731 | *Glycine max* | 118 | 2E-23 |
| **Ionic homeostasis and transport** | | | | | | | |
| # AB862640 | 1 | 366 | Aquaporin (PIP2;1) | BU494164 | *Lotus japonicus* | 496 | 4E-137 |
| AB862654 | 2 | 368 | Aquaporin (TIP2;1)-like | BP077864 | *Lotus japonicus* | 223 | 8E-55 |
| AB862665 | 1 | 431 | High-affinity nitrate transporter (NTR2.4) | BP079708 | *Lotus japonicus* | 556 | 4E-155 |
| # AB862666 | 2 | 451 | Aquaporin (TIP1) | DQ087218 | *Phaseolus vulgaris* | 118 | 3E-23 |
| # AB862671 | 1 | 291 | Aquaporin (TIP) | AF275315 | *Lotus japonicus* | 434 | 2E-118 |
| AB862674 | 1 | 283 | K(+)/H(+) antiporter-like | FS350526 | *Lotus japonicus* | 320 | 4E-84 |
| RT, # AB862686 | 1 | 327 | Type 1 metallothionein | AB176566 | *Lablab purpureus* | 138 | 2E-29 |
| # AB862697 | 1 | 229 | Aquaporin (PIP2;1) | AY995195 | *Phaseolus vulgaris* | 91.5 | 2E-15 |
| AB862667 | 2 | 477 | Aquaporin (TIP1;1)-like | BW619461 | *Lotus japonicus* | 646 | 0.0 |
| # AB862710 | 1 | 642 | V-type proton ATPase catalytic subunit A-like | AK338533 | *Lotus japonicus* | 1034 | 0.0 |
| AB863439 | 1 | 387 | Two pore calcium channel protein 1-like | BT052597 | *Medicago truncatula* | 378 | 2E-101 |
| # AB862724 | 1 | 700 | High affinity nitrate transporter (NTR2) | AB353299 | *Lotus japonicus* | 1065 | 0.0 |
| AB862738; AB862739; AB862740; AB862741 | 4 | 859 | Aquaporin (PIP1;1) | XM_003519335 | *Glycine max* | 508 | 3E-140 |
| AB863445 | 1 | 638 | $K^+$ transporter 7 | XP_002488965 | *Sorghum bicolor* | 132 | 4.00E-35 |
| **Signal transduction** | | | | | | | |
| AB862612; AB862613; AB862614 | 3 | 531 | Cyclin-U2-1 | GO015102 | *Lotus japonicus* | 641 | 1E-180 |
| AB862603; AB862604; AB862605; AB862606 | 4 | 500 | Phosphatidylinositol-4-phosphate 5-kinase family protein | XR_137666 | *Glycine max* | 383 | 6E-103 |
| AB862687; AB862688; AB862689 | 3 | 587 | Protein kinase | XM_002522436 | *Ricinus communis* | 120 | 1E-23 |
| **Cell rescue and defense** | | | | | | | |
| *Oxidative stress* | | | | | | | |
| AB863290 to AB863349 | 60 | 1265 | Tar1p | XM_003614343 | *Medicago truncatula* | 453 | 5.00E-131 |
| AB863350 to AB863362 | 13 | 776 | Tar1p | XM_003614343 | *Medicago truncatula* | 431 | 2.00E-124 |
| AB863363 to AB863373 | 11 | 1012 | NADH dehydrogenase (ubiquinone) Fe-S protein 7 | XM_003614335 | *Glycine max* | 462 | 2E-126 |
| AB863374 to AB863383 | 10 | 532 | Tar1p | XM_003614343 | *Medicago truncatula* | 407 | 2.00E-117 |
| AB863384 | 1 | 329 | Tar1p | XM_003614343 | *Medicago truncatula* | 268 | 6.00E-76 |
| AB863385 | 1 | 311 | Tar1p | XM_003614343 | *Medicago truncatula* | 248 | 7.00E-70 |
| # AB863434; AB863435 | 2 | 769 | Hydroquinone glucosyltransferase | AK337925 | *Lotus japonicus* | 589 | 1E-164 |
| AB863394 | 1 | 221 | Tar1p | XM_003614343 | *Medicago truncatula* | 231 | 6.00E-65 |
| # AB862626 | 2 | 405 | Glutathione S-transferase | AF243377 | *Glycine max* | 140 | 7E-30 |
| AB862638; AB862639 | 2 | 330 | Peroxidase 53-like | GO041603 | *Lotus japonicus* | 470 | 3E-129 |
| AB862698 | 1 | 298 | Peroxidase 72-like | XM_003517158 | *Glycine max* | 524 | 1E-144 |
| AB862702 | 1 | 274 | Lignin-forming anionic peroxidase-like (PR-9) | GO036451 | *Lotus japonicus* | 367 | 2E-98 |
| AB862759 | 1 | 268 | Flavodoxin-like quinone reductase 1 | NM_001254672 | *Glycine max* | 334 | 1E-88 |
| AB863395 | 1 | 226 | Tar1p | XM_003614343 | *Medicago truncatula* | 98.7 | 7E-25 |
| AB863396 | 1 | 278 | Tar1p | XM_003614343 | *Medicago truncatula* | 87.8 | 2.00E-21 |
| # AB862763 | 1 | 439 | Nonsymbiotic hemoglobin class I | AB238220 | *Lotus japonicus* | 262 | 9E-67 |
| AB863397 | 1 | 359 | Tar1p | XM_003614343 | *Medicago truncatula* | 140 | 3.00E-37 |
| *Pathogen response* | | | | | | | |
| # AB862623; AB862624 | 2 | 338 | Pathogenesis-related protein 10 (PR-10) | AJ311049 | *Medicago sativa* | 105 | 1E-19 |
| # AB862625 | 1 | 523 | Disease resistance response protein-like | BT096274 | *Medicago truncatula* | 131 | 2E-34 |
| # AB862655 | 1 | 317 | Osmotin-like (PR-5) | AK337330 | *Lotus japonicus* | 495 | 1E-136 |
| # AB862670 | 1 | 456 | Pathogenesis-related protein 1 (PR-1) | X79778 | *Medicago truncatula* | 199 | 1E-47 |
| # AB863436 | 1 | 310 | Pathogenesis-related protein 10 (PR-10) | AJ311050 | *Medicago sativa* | 104 | 4E-19 |
| # AB862699 | 1 | 431 | Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family | AK337825 | *Lotus japonicus* | 702 | 0.0 |
| # AB862713 | 1 | 371 | LEA protein 3 | DQ097720 | *Arachis hypogaea* | 109 | 1E-20 |
| # AB862742 | 1 | 264 | Glycine-rich RNA-binding protein | AP004542 | *Lotus japonicus* | 136 | 5E-29 |
| # AB862765 | 1 | 449 | Trypsin protein inhibitor 2 | AJ276263 | *Cicer arietinum* | 156 | 3E-42 |
| # AB862767 | 1 | 311 | Cysteine proteinase precursor-like protein (PR-6) | FS359520 | *Lotus japonicus* | 286 | 7.00E-74 |
| **Growth and development** | | | | | | | |
| *Cell wall* | | | | | | | |
| AB862619; AB862620; AB862621 | 3 | 475 | Glucosyltransferase like protein | AB070747 | *Vigna angularis* | 214 | 5E-52 |
| AB862633; AB862634; AB862635 | 3 | 503 | Beta-glucosidase like protein | AK336981 | *Lotus japonicus* | 297 | 7E-77 |
| AB862669 | 1 | 661 | Nucleotide sugar epimerase-like protein | AY062625 | *Arabidopsis thaliana* | 100 | 1E-17 |
| AB862690 | 1 | 696 | Glycosyltransferase | EU561019 | *Hieracium pilosella* | 90.7 | 4E-17 |
| AB862718 | 1 | 442 | UDP-XYL synthase 5 | AK339631 | *Lotus japonicus* | 572 | 5E-160 |
| # AB862719 | 1 | 364 | Hydroxyproline-rich glycoprotein | L22030 | *Glycine max* | 320 | 3E-84 |
| RT, # AB862734 | 2 | 591 | Seed coat BURP domain protein 1 | AF467554 | *Glycine max* | 125 | 1E-32 |
| *Development* | | | | | | | |
| AB862618 | 1 | 324 | MEI2-like protein 1 | XM_003526875 | *Glycine max* | 170 | 5E-39 |
| # AB862712 | 1 | 367 | Dormancy-associated protein/auxin-repressed protein | EF571303 | *Glycyrrhiza uralensis* | 116 | 1E-19 |
| AB862735 | 1 | 915 | WD repeat-containing protein 61-like | AP006737 | *Lotus japonicus* | 596 | 7E-167 |

ACCEPTED MANUSCRIPT

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| # | AB862736 | 1 | 336 | Translationally-controlled tumor protein-like protein | BW597224 | *Lotus japonicus* | 405 | 1E-109 |
| | AB862746 | 1 | 391 | Cytosolic acetoacetyl-coenzyme A thiolase-like protein | AK339753 | *Lotus japonicus* | 410 | 3E-111 |
| # | AB862752 | 1 | 442 | Germin-like protein 3 | EU916251 | *Glycine max* | 425 | 3E-123 |
| | AB862760 | 1 | 287 | CEN/TFL1-like | AY423715 | *Lotus japonicus* | 226 | 1E-63 |
| **Unclassificated** | | | | | | | | |
| | AB862610; AB862611 | 2 | 604 | Alpha-tubulin | XM_003555953 | *Glycine max* | 324 | 6E-85 |
| # | AB862615; AB862616 | 2 | 503 | Heat shock protein-like protein | XM_003610165 | *Medicago truncatula* | 114 | 5E-22 |
| | AB862645; AB862646 | 2 | 326 | 26S proteasome regulatory subunit N2 | AK337975 | *Lotus japonicus* | 176 | 1E-40 |
| | AB862694 | 1 | 411 | Chaperone protein dnaJ 8, chloroplastic-like | AK338735 | *Lotus japonicus* | | |
| | AB862700 | 1 | 268 | Invertase/pectin methylesterase inhibitor-like protein | AK286044 | *Glycine max* | 419 | 5E-114 |
| # | AB862705; AB862706 | 3 | 665 | Protease inhibitor/seed storage/LTP family protein | BT090304 | *Glycine max* | 374 | 4E-100 |
| | AB862707; AB862708; AB862709 | 3 | 398 | Vacuolar protein sorting-associated protein 2 homolog 1-like | AP009847 | *Lotus japonicus* | 527 | 8E-107 |
| | AB862714 | 1 | 412 | Autophagy-related protein 8i | XM_003609808 | *Medicago truncatula* | 430 | 4E-117 |
| # | AB862715 | 1 | 417 | Peptidyl-prolyl isomerase-like | GO022766 | *Lotus japonicus* | 583 | 2E-163 |
| | AB862717 | 1 | 628 | Protease inhibitor/seed storage/LTP family protein | AK245945 | *Glycine max* | 403 | 8E-109 |
| | AB862723 | 1 | 413 | Actine-1 like | AK339288 | *Lotus japonicus* | 634 | 1E-178 |
| | AB862745 | 1 | 464 | Vacuolar protein sorting-associated protein 2 homolog 1-like | XM_003546427 | *Glycine max* | 187 | 6E-44 |
| # | AB862755 | 1 | 322 | Ankyrin repeat domain-containing protein | XM_003630414 | *Medicago truncatula* | 109 | 1E-20 |
| | AB862756 | 1 | 469 | Predicted AT-hook DNA-binding family protein | GO015204 | *Lotus japonicus* | 650 | 0.0 |
| # | AB862757 | 1 | 297 | transaldolase-like | BP042844 | *Lotus corniculatus* | 138 | 2E-29 |
| | AB862762 | 1 | 193 | Phosphate/phosphoenolpyruvate translocator protein | AK337166 | *Lotus japonicus* | 154 | 2E-34 |
| | AB862764 | 1 | 510 | Proteasome subunit alpha type-1-B | BT050776 | *Medicago truncatula* | 89.7 | 2E-14 |
| **Protein shynthesis** | | | | | | | | |
| | AB862627 | 1 | 452 | 60S ribosomal protein L10-2 - like | BP034414 | *Lotus japonicus* | 726 | 0.0 |
| | AB862679 | 1 | 347 | Eukaryotic translation initiation factor 5A2 | AK287336 | *Glycine max* | 129 | 1E-26 |
| | AB862695; AB862696 | 2 | 432 | 40S ribosomal protein S28-like | XM_003543303 | *Glycine max* | 221 | 4E-54 |
| | AB862725; AB862726 | 2 | 591 | 60S ribosomal proteína L7 | NM_001248649 | *Glycine max* | 473 | 5E-130 |
| | AB862729 | 1 | 276 | 60S ribosomal protein L3-like | AK285887 | *Glycine max* | 129 | 1E-26 |
| | AB862754 | 1 | 427 | 40S ribosomal protein S26-3-like | FS343803 | *Lotus japonicus* | 219 | 1E-53 |
| **Unknown function** | | | | | | | | |
| | AB862770 | 1 | 190 | Unknown | GW889000 | *Phaseolus vulgaris* | 86 | 8.00E-14 |
| # | AB863398; AB863399 | 2 | 451 | Unknown | XM_003588307 | *Medicago truncatula* | 394 | 3E-106 |
| | AB863401; AB863402 | 2 | 997 | Unknown | XM_003543991 | *Glycine max* | 455 | 2E-124 |
| | AB863403 to AB863433 | 31 | 743 | Unknown | LOC100819147 | *Glycine max* | 524 | 4E-145 |
| | AB863386 to AB863393 | 8 | 472 | Unknown | BT143077 | *Lotus japonicus* | 614 | 2.00E-172 |
| | AB862673 | 1 | 665 | Unknown | XM_003542258 | *Glycine max* | 500 | 4E-101 |
| | AB863454 | 1 | 316 | Unknown | XM_003614345 | *Medicago truncatula* | 315 | 5.00E-90 |
| | AB863437 | 1 | 240 | Unknown | XM_003614345 | *Medicago truncatula* | 143 | 2.00E-38 |
| | AB863442 | 1 | 221 | Unknown | XM_003543978 | *Glycine max* | 284 | 1.00E-80 |
| | AB863444 | 1 | 270 | Unknown | BT136570 | *Lotus japonicus* | 226 | 8.00E-56 |
| | AB862749 | 1 | 269 | Unknown | BT136570 | *Lotus japonicus* | 168 | 1.00E-38 |
| | AB863446 | 1 | 265 | Unknown | BT136570 | *Lotus japonicus* | 168 | 2.00E-40 |
| | AB863447 | 1 | 315 | Unknown | BT140619 | *Lotus japonicus* | 28 | 2.00E-77 |
| | AB863448 | 1 | 350 | Unknown | XM_003542223 | *Glycine max* | 161 | 2E-36 |
| | AB863451 | 1 | 285 | Unknown | XM_003541133 | *Glycine max* | 280 | 4E-72 |
| | AB862617 | 1 | 503 | Unknown | FS335032 | *Lotus japonicus* | 684 | 0.0 |
| | AB862628 | 1 | 287 | Unknown | FS359865 | *Lotus japonicus* | 459 | 6E-126 |
| | AB862629; AB862630; AB862631; AB862632 | 4 | 409 | Unknown | FS361702 | *Lotus japonicus* | 471 | 1E-129 |
| | AB862636; AB862637 | 2 | 510 | Unknown | NM_001254609 | *Glycine max* | 304 | 5E-79 |
| | AB862641; AB862642 | 2 | 587 | Unknown | AK286343 | *Glycine max* | 417 | 3E-113 |
| | AB862647 | 1 | 291 | Unknown | GO020570 | *Lotus japonicus* | 416 | 6E-113 |
| | AB862648; AB862649 | 3 | 503 | Unknown | AK243800 | *Glycine max* | 262 | 1E-66 |
| | AB862650; AB862651 | 2 | 247 | Unknown | FS349400 | *Lotus japonicus* | 544 | 2E-151 |
| | AB862652 | 1 | 331 | Unknown | GO005273 | *Lotus japonicus* | 499 | 7E-138 |
| | AB862653 | 1 | 444 | Unknown | GO032623 | *Lotus japonicus* | 708 | 0.0 |
| | AB862656; AB862657; AB862658; AB862659 | 4 | 789 | Unknown | FS320122 | *Lotus japonicus* | 145 | 4E-31 |
| | AB862661 | 1 | 401 | Unknown | FS348598 | *Lotus japonicus* | 329 | 1E-86 |
| | AB862662; AB862663 | 2 | 392 | Unknown | FS356980 | *Lotus japonicus* | 435 | 8E-119 |
| | AB862668 | 1 | 283 | Unknown | AK336899 | *Lotus japonicus* | 428 | 1E-116 |
| | AB862672 | 1 | 468 | Unknown | BT096466 | *Glycine max* | 390 | 3E-105 |
| | AB862675 | 1 | 319 | Unknown | XM_003591208 | *Medicago truncatula* | 165 | 1E-37 |
| | AB862676; AB862677 | 2 | 313 | Unknown | AK336932 | *Lotus japonicus* | 288 | 2E-74 |
| | AB862680; AB862681 | 2 | 546 | Unknown | BT094002 | *Glycine max* | 210 | 6E-51 |
| | AB862682 | 1 | 304 | Unknown | AK337041 | *Lotus japonicus* | 471 | 1E-129 |
| | AB862683 | 1 | 205 | Unknown | BT090313 | *Glycine max* | 111 | 2E-21 |
| | AB862691 | 1 | 388 | Unknown | XM_003532293 | *Glycine max* | 192 | 2E-45 |
| | AB862692 | 1 | 357 | Unknown | AK337995 | *Lotus japonicus* | 471 | 1E-129 |
| | AB862693 | 1 | 364 | Unknown | AV773238 | *Lotus japonicus* | 374 | 2E-100 |
| | AB862701 | 1 | 354 | Unknown | XM_003524638 | *Glycine max* | 340 | 1E-89 |
| | AB862703; AB862704 | 2 | 483 | Unknown | XM_003545670 | *Glycine max* | 199 | 1E-47 |
| | AB862721 | 1 | 586 | Unknown | DC596819 | *Lotus japonicus* | 652 | 0.0 |
| | AB863440 | 1 | 313 | Unknown | XM_003614345 | *Medicago truncatula* | 168 | 1E-38 |
| | AB863441 | 1 | 362 | Unknown | AV409845 | *Lotus japonicus* | 262 | 1E-66 |
| | AB862748 | 1 | 443 | Unknown | FS359879 | *Lotus japonicus* | 102 | 3E-18 |
| | AB862750 | 1 | 430 | Unknown | GO010590 | *Lotus japonicus* | 431 | 2E-117 |
| | AB862751 | 1 | 430 | Unknown | AP010398 | *Lotus japonicus* | 352 | 9E-94 |
| | AB862753 | 1 | 350 | Unknown | BP063937 | *Lotus japonicus* | 246 | 6E-62 |
| | AB863450 | 1 | 273 | Unknown | XM_003543978 | *Glycine max* | 1885 | 0.0 |
| | AB862768 | 1 | 354 | Unknown | BP042083 | *Lotus corniculatus* | 244 | 2E-61 |
| **No hit** | | | | | | | | |
| | AB862622 | 1 | 361 | No hit | | | | |
| | AB862716 | 1 | 370 | No hit | | | | |
| | AB862720 | 1 | 271 | No hit | | | | |
| | AB863443 | 1 | 719 | No hit | | | | |
| | AB862731 | 1 | 362 | No hit | | | | |
| | AB863449 | 1 | 224 | No hit | | | | |

ACCEPTED MANUSCRIPT

| | | | |
|---|---|---|---|
| AB862758 | 1 | 192 | No hit |
| AB862766 | 1 | 393 | No hit |
| AB862769 | 1 | 317 | No hit |

RT means selected genes

# means environmental stress responsive

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Supplementary Table 2. cDNA clones isolated from a subtractive suppressive hybridization library from roots of alkaline stressed *Lotus tenuis* plants

| | | | | Top NCBI blast alignment | | | |
|---|---|---|---|---|---|---|---|
| **Sequence ID** | **Clones contained** | **Length** | **Annotation** | **Accession** | ***Specie*** | **Score** | **E-value** |
| **Metabolism** | | | | | | | |
| *Amino acid metabolism* | | | | | | | |
| RT, # AB862785 | 1 | 459 | Asparagine synthetase | X89409 | *Lotus japonicus* | 639 | 3E-180 |
| RT, # AB862789; AB862790 | 2 | 513 | Cytosolic glutamine synthetase | X94299 | *Lotus japonicus* | 758 | 0.0 |
| RT, # AB862812 | 1 | 253 | Asparagine synthetase | X89410 | *Lotus japonicus* | 369 | 5E-99 |
| RT, # AB862813; AB862814 | 2 | 466 | Methionine synthase | NM_001248865 | *Glycine max* | 470 | 4E-129 |
| *Aromatic metabolism* | | | | | | | |
| # AB862773; AB862774; AB862775 | 3 | 634 | Chalcone synthase | AK339635 | *Lotus japonicus* | 807 | 0.0 |
| # AB862824; AB862825; AB862826 | 3 | 566 | Chalcone synthase | AK339522 | *Lotus japonicus* | 787 | 0.0 |
| # AB862838; AB862839 | 2 | 407 | Isoflavone reductase homolog | AK339647 | *Lotus japonicus* | 288 | 3.00E-74 |
| # AB862847; AB862848 | 2 | 473 | Isoflavone reductase homolog | AK339647 | *Lotus japonicus* | 185 | 2E-43 |
| # AB862849 | 1 | 282 | Isoflavone reductase homolog | AK339647 | *Lotus japonicus* | 203 | 6.00E-49 |
| AB862853 | 1 | 235 | Chalcone synthase-like | AK339635 | *Lotus japonicus* | 125 | 1.00E-25 |
| **Lipid metabolism** | | | | | | | |
| # AB862771; AB862772 | 2 | 432 | Epoxide hydrolase-like | XM_003550319 | *Glycine max* | 212 | 2.00E-51 |
| # AB862791 | 1 | 460 | Lipoxygenase-10 (LOX10) | EU003577 | *Glycine max* | 293 | 6E-76 |
| AB862799 | 1 | 242 | Lipoxygenase-like | GO034028 | *Lotus japonicus* | 248 | 5.00E-62 |
| # AB862817 | 1 | 247 | Epoxide hydrolase-like | XM_003550319 | *Glycine max* | 255 | 1.00E-64 |
| # AB862843 | 1 | 479 | Alpha-dioxygenase | AJ784963 | *Pisum sativum* | 343 | 4E-91 |
| **Transcription** | | | | | | | |
| AB862797 | 1 | 298 | Ethylene insensitive 3-like | AK339680 | *Lotus japonicus* | 403 | 4.00E-109 |
| # AB862842 | 1 | 672 | Transcription factor EREBP-like | AB236754 | *Trifolium pratense* | 187 | 9E-44 |
| **Ionic homeostasis and transport** | | | | | | | |
| # AB862781; AB862782 | 2 | 482 | Aquaporin (PIP2;7)-like | XM_003538126 | *Glycine max* | 197 | 5.00E-47 |
| RT, # AB862815; AB862816 | 2 | 371 | Phosphate transporter | AJ286743 | *Sesbania rostrata* | 212 | 1E-51 |
| RT, # AB862818 | 1 | 305 | Nicotinamine synthase 2-like | FS350895 | *Lotus japonicus* | 462 | 4.00E-127 |
| RT, # AB862829; AB862830 | 2 | 373 | ZIP transporter | XM_002324137 | *Populus trichocarpa* | 132 | 9E-28 |
| # AB862835 | 1 | 186 | Ammonium transporter (AMT1;1)-like | AF182188 | *Lotus japonicus* | 100 | 3E-18 |
| AB862836 | 1 | 285 | K(+)/H(+) antiporter-like | FS350526 | *Lotus japonicus* | 325 | 7.00E-86 |
| # AB862840; AB862841 | 2 | 473 | Glycerol-3-phosphate transporter-like | AK245968 | *Glycine max* | 280 | 4E-72 |
| RT, # AB862844 | 1 | 427 | High affinity nitrate transporter (NTR2) | AJ292342 | *Lotus japonicus* | 616 | 4E-173 |
| # AB862860 | 1 | 277 | Aquaporin (PIP2;7)-like | AV429063 | *Lotus japonicus* | 86 | 1.00E-13 |
| # AB862865 | 1 | 270 | Metallothionein-like protein | GO034023 | *Lotus japonicus* | 199 | 6E-48 |
| AB862868 | 1 | 269 | Aquaporin (PIP2;7)-like | BP078931 | *Lotus japonicus* | 96.9 | 6E-17 |
| **Signal transduction** | | | | | | | |
| # AB862787 | 1 | 444 | Phosphatase 2C 73-like | XM_003521628 | *Glycine max* | 289 | 9.00E-75 |
| AB862811 | 1 | 428 | 1-phosphatidylinositol phosphodiesterase-like | BT051997 | *Medicago truncatula* | 143 | 6.00E-31 |
| AB862823 | 1 | 204 | CBL-interacting serine/threonine-protein kinase 4 | AK337210 | *Lotus japonicus* | 273 | 3.00E-70 |
| AB862845 | 1 | 190 | Mitogen-activated kinase kinase kinase alpha | AB167408 | *Lotus japonicus* | 183 | 3E-43 |
| **Cell rescue and defense** | | | | | | | |
| *Oxidative stress* | | | | | | | |
| # AB862822 | 1 | 340 | Peroxidase-like (Peroxidase 52) | XM_003552249 | *Glycine max* | 282 | 1.00E-72 |
| # AB862859 | 1 | 362 | Peroxidase (Peroxidase 12) | XM_003556112 | *Glycine max* | 172 | 1.00E-39 |
| AB862863; AB862864 | 2 | 235 | Peroxidase-like (Peroxidase 39) | FS349768 | *Lotus japonicus* | 304 | 2.00E-79 |
| *Pathogen response* | | | | | | | |
| # AB862788 | 1 | 439 | DNA-damage-repair/toleration protein DRT100 | XM_003627670 | *Medicago truncatula* | 262 | 9.00E-69 |
| # AB862796 | 1 | 482 | Reticuline oxidase-like protein | XM_003546238 | *Glycine max* | 352 | 1.00E-93 |
| AB862800; AB862801 | 2 | 257 | MLP-like protein | XM_003518956 | *Glycine max* | 167 | 4.00E-38 |
| # AB862837 | 1 | 430 | Class I chitinase (PR-3) | BB999930 | *Lotus japonicus* | 655 | 0.0 |
| # AB862861 | 1 | 236 | Pathogenesis-related protein PR-1-like | XM_003550618 | *Glycine max* | 91.5 | 2E-15 |
| **Growth and development** | | | | | | | |
| *Cell wall* | | | | | | | |
| # AB862804; AB862805 | 2 | 268 | EPR1 proline-rich extensin-like | AK336338 | *Lotus japonicus* | 369 | 7.00E-99 |
| # AB862808 | 1 | 299 | Endo-1,4-beta-glucanase | AK339518 | *Lotus japonicus* | 383 | 4.00E-103 |
| RT, # AB862827; AB862828 | 2 | 562 | BURP domain-containing protein | AF467554 | *Glycine max* | 250 | 8E-63 |
| # AB862831; AB862832 | 2 | 476 | Proline-rich cell wall protein | J05208 | *Glycine max* | 201 | 4.00E-48 |
| AB862856 | 1 | 314 | Cinnamyl alcohol dehydrogenase | AK338166 | *Lotus japonicus* | 434 | 2.00E-118 |
| *Development* | | | | | | | |
| AB862862 | 1 | 187 | Dormancy-associated protein/auxin-repressed protein | AP006076 | *Lotus japonicus* | 300 | 1E-78 |
| AB862792 | 1 | 370 | Dormancy-associated protein/auxin-repressed protein | AP006076 | *Lotus japonicus* | 468 | 1E-128 |
| AB862795 | 1 | 369 | Gibberellin receptor GID1B-like | FS345796 | *Lotus japonicus* | 535 | 1.00E-148 |
| AB862820 | 1 | 405 | Gibberellin 20 oxidase 1 | BP076452 | *Lotus japonicus* | 634 | 2.00E-178 |
| AB862821 | 1 | 251 | Gibberellin 20 oxidase 1-like | XM_003528318 | *Glycine max* | 264 | 2.00E-67 |
| AB862869 | 2 | 269 | auxin F-box protein 5 | XM_003544186 | *Glycine max* | 131 | 2.00E-34 |
| **Protein shynthesis** | | | | | | | |
| # AB862776; AB862777 | 2 | 461 | Ubiquitin | DQ249171 | *Lotus japonicus* | 560 | 3E-156 |
| # AB862779 | 1 | 511 | Ubiquitin | DQ249171 | *Lotus japonicus* | 168 | 2E-38 |
| AB862780 | 1 | 470 | Elongation factor 1-alpha-like | AK246053 | *Glycine max* | 205 | 2.00E-49 |
| AB862784 | 1 | 257 | 60S ribosomal protein L35 | AP010402 | *Lotus japonicus* | 315 | 1E-82 |
| **Unclassificated** | | | | | | | |
| # AB862778 | 1 | 473 | CBS domain-containing protein | BT052304 | *Medicago truncatula* | 322 | 1E-84 |
| AB862798 | 1 | 522 | Histone H2A | NM_001252955 | *Glycine max* | 295 | 2E-76 |
| AB862809 | 1 | 391 | Translation machinery associated protein TMA7 | AK245917 | *Glycine max* | 428 | 9E-117 |
| AB862810 | 1 | 368 | Transport inhibitor response 1-like protein | XM_003544186 | *Glycine max* | 235 | 1E-58 |
| AB862819 | 1 | 395 | MtN19-like | AB353308.1 | *Lotus japonicus* | 645 | 0.0 |
| AB862858 | 1 | 317 | Actin-like | NM_001253024 | *Glycine max* | 390 | 2E-105 |
| RT, # AB862866 | 1 | 267 | Calcium-regulated/ATP-independent forisome | GQ478228 | *Pisum sativum* | 190 | 3E-45 |
| # AB862870 | 1 | 389 | Glycine-rich RNA-binding protein7-like | AV769847 | *Lotus japonicus* | 188 | 2.00E-44 |
| **Unknown function** | | | | | | | |
| AB862783 | 1 | 323 | Unknown | BT092041 | *Glycine max* | 132 | 8E-28 |
| AB862786 | 1 | 402 | Unknown | XM_003596926 | *Medicago truncatula* | 167 | 7.00E-38 |
| AB862793; AB862794 | 2 | 351 | Unknown | FS361702 | *Lotus japonicus* | 452 | 9.00E-124 |
| AB863456 | 1 | 416 | Unknown | AK336920 | *Lotus japonicus* | 411 | 1.00E-111 |
| AB862802; AB862803 | 2 | 415 | Unknown | AK339068 | *Lotus japonicus* | 547 | 2.00E-152 |

ACCEPTED MANUSCRIPT

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AB862806 | 1 | 339 | Unknown | FS345277 | *Lotus japonicus* | 500 | 2.00E-138 |
| | AB862807 | 1 | 174 | Unknown | AK243709 | *Glycine max* | 78.8 | 8E-12 |
| | AB863457 | 1 | 297 | Unknown | FS107438 | *Solanum torvum* | 470 | 3E-129 |
| | AB862833; AB862834 | 2 | 317 | Unknown | AP009743 | *Lotus japonicus* | 367 | 2E-98 |
| | AB862846 | 1 | 226 | Unknown | AP005602 | *Lotus japonicus* | 260 | 2E-66 |
| | AB862850 | 1 | 409 | Unknown | AP010495 | *Lotus japonicus* | 96.9 | 8E-17 |
| | AB862854 | 1 | 428 | Unknown | AP004973 | *Lotus japonicus* | 120 | 7E-24 |
| | AB862857 | 1 | 267 | Unknown | NM_001248852 | *Glycine max* | 251 | 1.00E-63 |
| | AB862867 | 1 | 292 | Unknown | AK339068 | *Lotus japonicus* | 342 | 1.00E-90 |
| | AB862871 | 1 | 241 | Unknown | AP009068 | *Lotus japonicus* | 93.3 | 5E-16 |
| | AB862876 | 1 | 268 | Unknown | XM_003542062 | *Glycine max* | 122 | 2.00E-24 |
| **No hit** | | | | | | | | |
| | AB862851 | 1 | 191 | | No hit | | | |
| | AB862852 | 1 | 317 | | No hit | | | |
| | AB862855 | 1 | 354 | | No hit | | | |
| | AB863458 | 1 | 270 | | No hit | | | |
| | AB863459 | 1 | 108 | | No hit | | | |
| | AB863460 | 1 | 148 | | No hit | | | |
| | AB863461 | 1 | 111 | | No hit | | | |
| | AB862872 | 1 | 857 | | No hit | | | |
| | AB862873 | 1 | 269 | | No hit | | | |
| | AB862874 | 1 | 175 | | No hit | | | |
| | AB862875 | 1 | 163 | | No hit | | | |
| | AB862877 | 1 | 160 | | No hit | | | |

RT means selected genes

# means environmental stress responsive

ACCEPTED MANUSCRIPT