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Research article

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

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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.

1. Introduction

About one half of the saline soils that cover the land surface contain alkaline salts (FAO: Land and Water Divisi, 2013). Soil alkalinity is a serious agricultural problem as it limits crops yield by reducing the availability of plant nutrients, among other effects (Marschner2nd Ed., 1995; Mirsa and Tyler, 1999). Common alkaline stress symptoms in plants that are alkaline-sensitive include leaf chlorosis[4] and stunting (Bie et al., 2004).

Bicarbonate (HCO_3-) is one of the principal contributors to soil alkalinity (PetersenReed, 1996). High HCO_3^- concentrations in soil may interfere with the uptake of macro elements, particularly P, K and Mg (Pissaloux et al., 1995), and essential micronutrients such as Fe and Zn (AlamPessarakli and Second Edition, 1999). 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 (Cakmak, 2000; Sun et al., 2007; Tewari et al., 2007). 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 (Hirayama and Shinozaki, 2010). Suppression Subtractive Hybridization (SSH) is a

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simple and efficient method, which has been widely used for the identification of differentially expressed genes (Diatchenko et al., 1996). By using SSH, many abiotic stress-inducible genes have been identified and characterized in several plant species (Boominathan et al., 2004; Zhang et al., 2007; Lata et al., 2010).

The legume *Lotus tenuis* (Waldst. & Kit., syn. *Lotus glaber*; (Kirkbride, 2006)), 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; (Stoffella et al., 1998; Correa et al., 2001)). *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 (Paz et al., 2012). 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 sulfur 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) ×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 (Hoagland and Arnon, 1950). 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 (Kotchoni et al., 2003) 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 (Sanchez et al., 2008).

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 21st 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), comprising a total of 158 unigenes in leaves, and 92 unigenes in roots (with an average size of 454 and 357 pb respectively). Five of these unigenes were common to leaf and root SSHs (asparagine synthase, BURP, K(+)/H(+) antiporter-like and two unknown, Fig. 3). The obtained ESTs were deposited in the DDJJ database (DNA Data Bank of Japan, www.ddbj.nig.ac.jp) under Accession N° AB862603-AB862770 and AB863290-AB863455 for leaves (Supplementary Table 1), and N° AB862771-AB862877 and AB863456-AB863461 for roots (Supplementary Table 2).

Blast searches of all unigenes against the nucleotide and protein databases, and the *L. japonicus* database showed significant similarity to known genes. The best alignments in NCBI database were with legume sequences such as *L. japonicus, Glycine max,* and *Medicago truncatula.*

3.3. Functional classification of unigenes isolated from leaf and root SSH libraries of L. tenuis

Unigenes from both libraries were classified into 11 categories according to their putative biological function by using the Gene Ontology database (Fig. 4; Supplementary Tables 1 and 2). The unigene classification of both subtractive cDNA libraries included the following functional categories: metabolism (9% leaf; 11% root); lipid metabolism (1% leaf; 6% root); transcription (1% leaf; 2% root), ion homeostasis and transport (9% leaf; 12% root); signal transduction (2% leaf; 4% root); cell rescue and defense (17% leaf; 9% root); growth and development (9% leaf; 9% root); protein synthesis (4% leaf; 4% root) and unclassified (10% leaf; 9% root). It should be noted that 32 and 21% of the identified sequences in leaf and root SSH libraries, respectively, had homology with proteins of unknown function. Furthermore, 6% and 13% of these sequences from leaf and root SSH libraries, respectively, had no matches in the databases searched, indicating novel gene fragments or UTR regions of *L. tenuis*. Ostensibly, some of the unknown and no-matching transcripts identified in the present study might be associated with alkalinity tolerance. A complete list of BLAST results 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 Fig. 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

Table 1
Oligonucleotides designed for qRT-PCR analysis.

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.

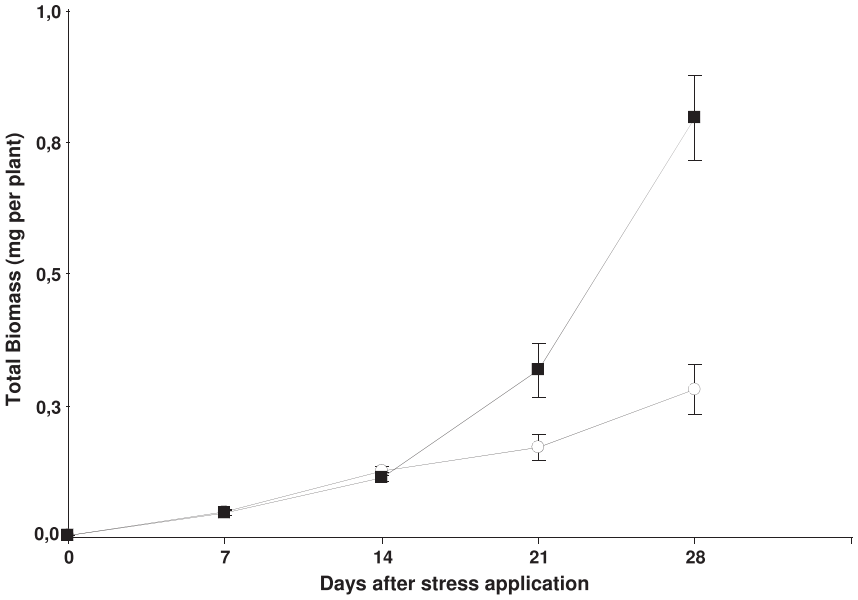


Fig. 1. Time-course analysis of growth performance of *L. tenuis* under control (■) and alkaline (○) stress conditions. Fifteen day-old plants were watered with nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment 10 mM $NaHCO_3$ were added to 0.5 X Hoagland's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

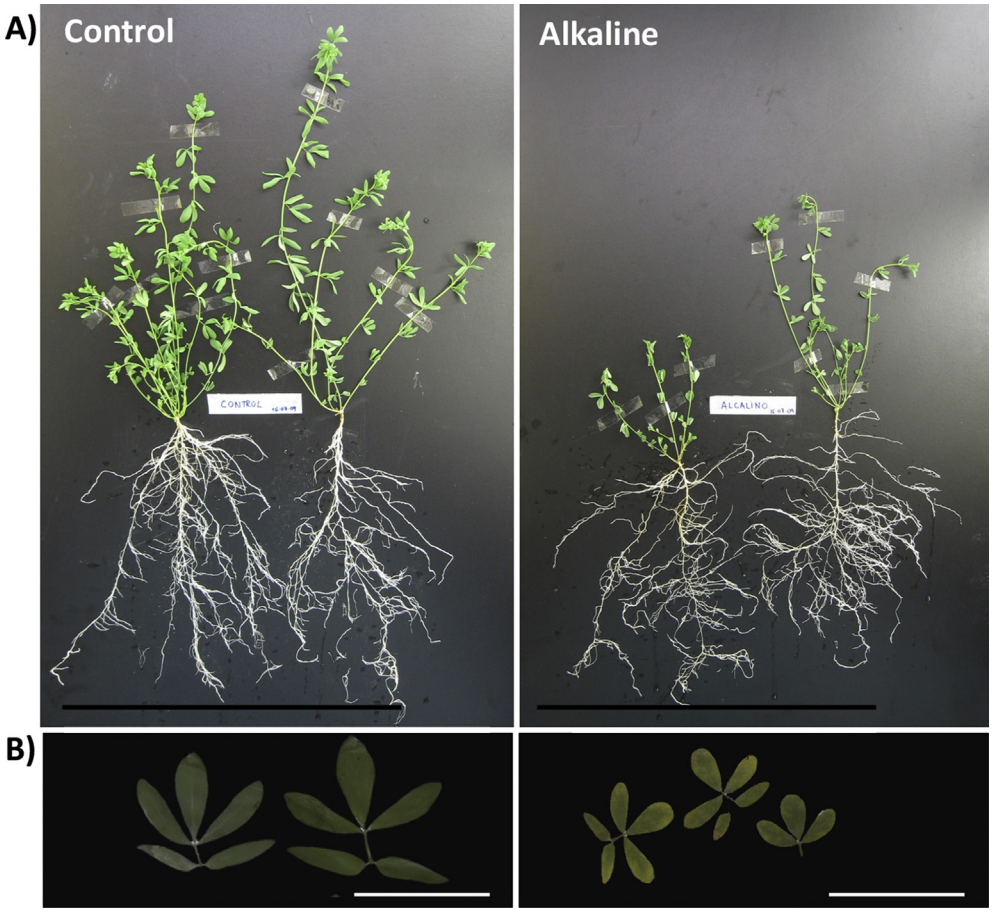
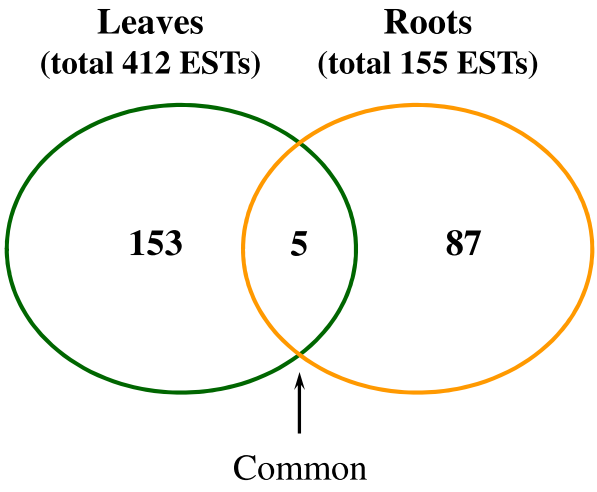


Fig. 2. Plant growth response of *L. tenuis* plants exposed to control (left panel) and alkaline (right panel) stress conditions. Fifteen day-old plants were watered with nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment 10 mM $NaHCO_3$ were added to 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.



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

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

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 (Coelho et al., 2002).

3.5. Validation of SSH by qRT-PCR analysis

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

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

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

4. Discussion

To gain insight into the molecular responses to long-term alkaline stress that are activated in *L. tenuis*, we generated two subtractive cDNA libraries and identified 158 unigenes from leaves and 92 unigenes from roots that are potentially regulated in *L. tenuis* plants subjected to 10 mM of $NaHCO_3$ for 14 and 28 days. Blast searches showed that most of the isolated ESTs had significant homology to nucleotide sequences deposited in the GenBank, and were likely to encode proteins involved in plant stress responses. These genes collectively play a role in growth, development, plant nutrition, detoxification and the maintenance of critical cellular metabolic processes.

The expression pattern of genes identified by SSH and selected by their putative function in nutrient uptake and growth was further characterized by qRT-PCR upon alkalinity response.

4.1. Genes related with nutrient metabolism and oxidative stress relief

LtPT and *LtNTR2* are high-affinity transport systems of phosphorous and nitrogen, respectively. In rice, these genes belong to an alternative transport system induced when nitrogen and inorganic phosphorous (Pi) concentrations are lower than 250 and 15 μM, respectively (Aono et al., 2001; Cai et al., 2008). Both macronutrients are required at high levels for plant growth, and it is expected that plant challenged by alkalinity stress activate mechanisms to ensure their acquisition. Indeed, the expression of these genes in alkalinized *L. tenuis* roots was maximal at day 14, followed by a reduction in expression at day 28 (Fig. 6). This result suggests that plant N and P levels probably decreased below a critical level during the first days of stress, leading to the induction of *LtPT* and *LtNTR*. In turn, these genes possibly contributed to restore N and P levels by day 28.

Methallothioneins and Nicotianamines are chelators responsible of the metal homeostasis in plants. Methallothioneins are small, cysteine-rich and heavy metal-binding proteins, which participate in the regulation of Zn distribution in the intracellular space and in an array of protective stress responses, including alkalinity (Nishiuchi et al., 2007; Wang et al., 2008). It is also known that the specific metals sequestered by metallothioneins vary according to the protein structure among different organisms (Blindauer and Leszczyszyn, 2010). Nicotianamines are small

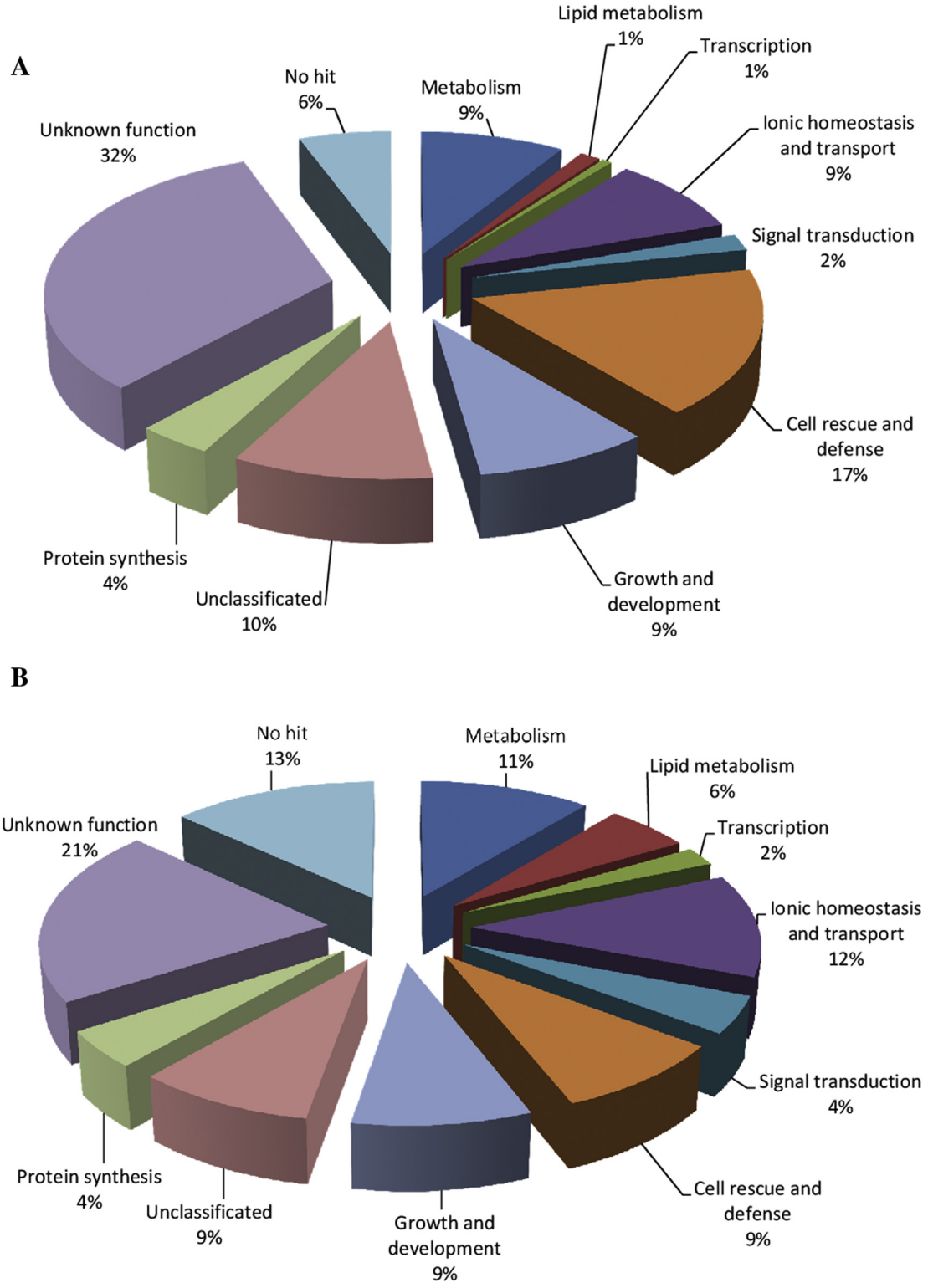


Fig. 4. Functional classification of *Lotus tenuis* genes expressed at long term alkaline response stress. A total of 158 unigenes of leaves (A) and 92 unigenes of roots (B) were classified into 11 functional categories according to their putative biological function reported by Gene Ontology (www.geneontology.org)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

chelators synthesized by the enzyme Nicotinamine synthase and transport micronutrient metal ions like Fe, Zn, Cu and Ni (Scholz et al., 1992). 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

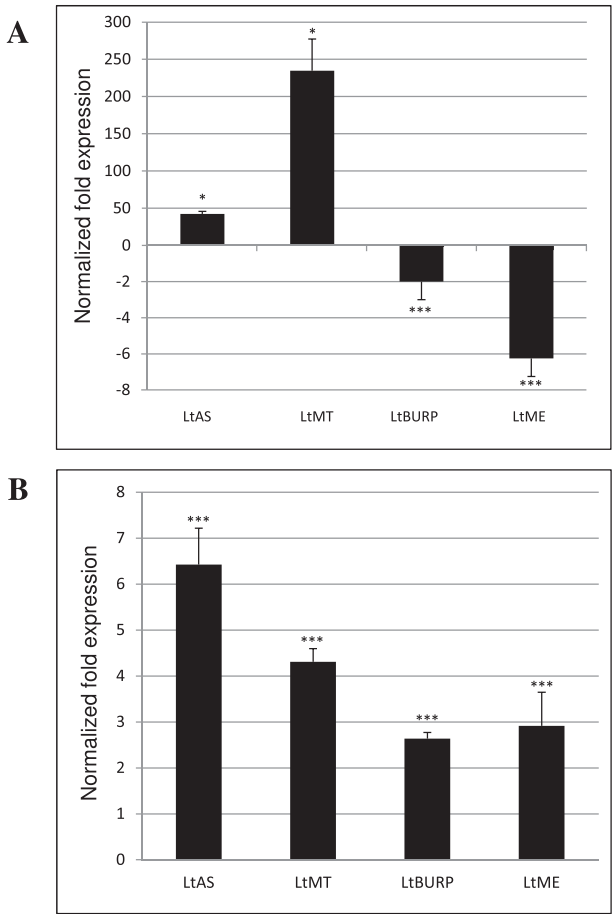


Fig. 5. Differential expression analysis by qRT–PCR of 4 selected cDNA clones from leaf SSH, following 14 and 28 days of alkaline treatment. Fifteen day-old plants were watered with nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment, 10 mM NaHCO3 were added to 0.5 X Hoagland's solution. Total RNA was isolated from leaf tissues at 14 days after stress application (A) and 28 days after stress application (B) and RT-PCR was performed using gene-specific primers. LtBURP: BURP domain (AB862734; AB862827; AB862828); LtME: NADP-dependent malic protein (AB862678); LtAS: Asparagine synthetase (AB862747; AB862785; AB862812); LtMT: Type 1 metallothionein (AB862686). Bars represent SE of mean ($n = 3$) and asterisks indicate significant 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 (*, $p < 0.05$; **, $p < 0.01$). The relative expression levels of all the analyzed unigenes was normalized to the previously described housekeeping gene LjGPI-anchored protein. Ratios lower than 1 (i.e., genes repressed in stress conditions) are represented as minus the inverse of the ratio.

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 (Guerinot, 2000). 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 (Gaufichon et al., 2010). 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 (Paz et al., 2012). 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 (Gaufichon et al., 2010). 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 (Moller et al., 2003). 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*; (Walter et al., 1994)). In fact, it has been suggested that the NADPH produced by NADP-ME provides the reducing power required for ROS metabolism and scavenging (Minard and McAlister-Henn, 2001; Møller, 2001; Mittler, 2002). Interestingly, the over-expression of rice NADP-ME2 in Arabidopsis plants increased tolerance to long term osmotic, alkali and neutral saline stress (Liu et al., 2007).

The expression of methionine synthetase has been reported to be induced under different abiotic stress such as Zn and Cd toxicity (Sarry et al., 2006; Fukao et al., 2009), NaCl (Palmgren et al., 2008) and alkaline conditions (Xie et al., 2002). 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 (Matthews and Singh, 1999; Hesse and Höfgen, 2003). 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 (Amir et al., 2002; Wittstock and Halkier, 2002; Rebeille et al., 2006; Goyer et al., 2007). 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* (Figs. 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 (Ding et al., 2009).

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 (Knoblauch and Peters, 2004). *In vitro* studies of isolated forisomes demonstrated that the volume of this structure is influenced by Ca^{+2} and pH levels (Knoblauch and Peters, 2004). 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

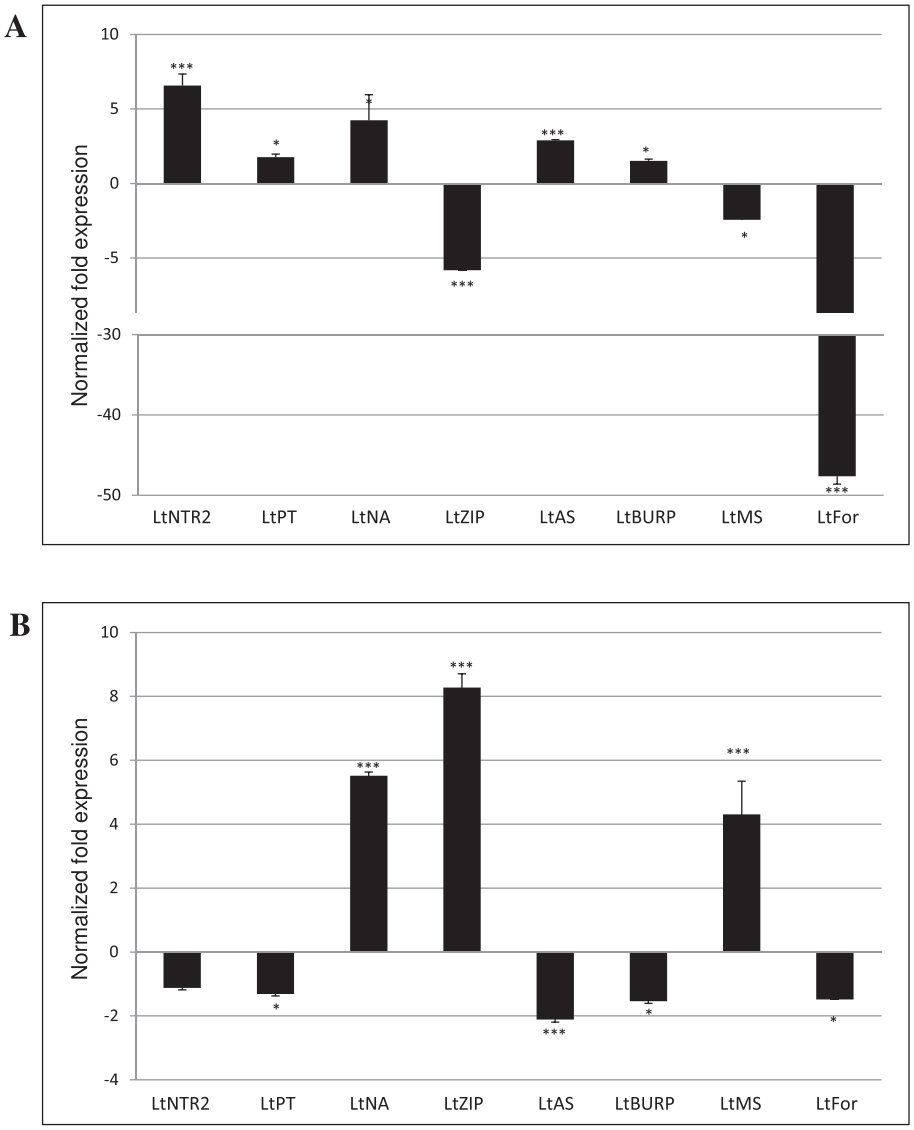


Fig. 6. Root differential expression analysis by qRT–PCR of 9 selected cDNA clones following 14 and 28 days of alkaline treatment. Fifteen day-old plants were watered with nutrient solution containing or lacking salt addition during 28 days. For alkaline stress treatment, 10 mM $NaHCO_3$ were added to 0.5 X Hoagland's solution. Total RNA was isolated from root tissues at 14 days after stress application (A) and 28 days after stress application (B) and RT-PCR was performed using gene-specific primers. LtBURP: BURP domain (AB862734; AB862827; AB862828); LtFor: Forisome (AB862866); LtGS: Cytosolic glutamine synthetase (AB862789; AB862790); LtMS: Methionine synthase (AB862813; AB862814); LtAS: Asparagine synthetase (AB862747; AB862785; AB862812); LtPT: Phosphate transporter (AB862815; AB862816); LtNA: Nicotinamine synthase 2-like (AB862818); LtZIP: ZIP transporter (AB862829; AB862830); LtNTR2: High affinity nitrate transporter (NTR2) (AB862844). Bars represent SE of mean ($n = 3$) and asterisks indicate significant 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.

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 (Paz et al., 2012, 2014).

5. Conclusion

In the present study, we used Subtractive Hybridization for the identification of alkali responsive genes in *L. 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.06.009.

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.

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