MICHELE VALQUÍRIA DOS REIS

GENE EXPRESSION PROFILES IN ROSES UNDER STRESS CONDITIONS

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor

Orientadora
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À Minha Família, que é a razão do meu viver.

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GENERAL ABSTRACT

There are limited information about gene regulation and signaling pathways related to stress response in ornamental plants. Rose flowers represent a good experimental model to investigate these responses, due to the short generation time and small genome size. Thus, the objective was to identify the pattern expression of some genes in response to stress in *Rosa rugosa* and *Rosa hybrida* cv. Knock out. An apple microarray had been used to investigate global gene expression profiles in rose's floral buds before cold exposure (0 h) and in two different times after cold temperature exposure (4^0 C) at 2 and 12 h. In this study was revealed 318 differentially expressed genes, in which 134 genes were up-regulated and 184 down-regulated. The expression patterns of the cold responsive transcripts identified by Microarray were confirmed by qRT-PCR analysis. The AP2/ERFs genes were more inducible in leaves compared with floral buds tissues. A set of the differentially expressed genes identified in this study will facilitate the better understand of cold stress response of rose floral buds. Finally, we analyzed changes in transcript level between plants in different salt stress condition (0; 25; 50 and 100 mM NaCl) for long exposure (30 days). In addition, the effect of salt shock stress was evaluated by the exposition to high concentration (200 mM NaCl) for short time (3 h). Relative reverse transcription polymerase chain reaction (RT-PCR) was performed to compare the expression levels of selected differentially expressed genes. Plants in long salt stress exposure showed no signal of stress in the leaves and roots. In addition, the expression of RhNHX1 in *Rosa rugosa* increased in the presence of NaCl. The transcription of genes EXP4, GPP, NHX1, NAC and DREB increased in the presence of higher concentrations of NaCl. In contrast, MYB and TIR decreased the expression level in salt shock treatment. NHX1 had a high expression level in leaves of plants in both salt stress and salt shock, suggesting that this gene plays important role in salt stress tolerance in *Rosa rugosa*. These genes may enable the exploration of newer avenues for engineering salt tolerance in roses and other member of Rosaceae family.

Keywords: Flowering, Gene expression, Microarray analysis, Temperature, Salt Stress.

RESUMO GERAL

Existem poucas informações sobre a regulação de genes e vias de sinalização relacionadas às respostas aos estresses em plantas ornamentais. As rosas representam um bom modelo experimental para investigar essas respostas, devido ao curto tempo de geração e pequeno tamanho do genoma. Assim, o objetivo deste trabalho foi identificar o padrão de expressão de alguns genes em resposta ao estresse em *Rosa rugosa* e *Rosa hybrida* cv. Knock out. Um microarray foi usado para investigar os perfis de expressão gênica global em botões florais de *Rosa hybrida* cv. Knock out antes da exposição ao frio (0 h) e em dois momentos diferentes após a exposição a temperatura fria (4 º C) em 2 e 12 h. Neste estudo foram revelados 318 genes diferencialmente expressos, nos quais 134 genes foram up-regulados e 184 down-regulados. Os padrões das transcrições dos genes responsivos ao frio identificados pelo Microarray tiveram a expressão confirmada por análise de qRT-PCR. Os AP2/ERFs genes, foram mais induzíveis nas folhas em comparação com os tecidos botões florais. Um conjunto de genes diferencialmente expressos identificados neste estudo permitirá o entendimento de respostas ao estresse de frio em botões florais de rosas. Ainda foram analisadas as alterações no nível de transcrição entre *Rosa rugosa* sob diferentes condições de estresse salino (0, 25, 50 e 100 mM de NaCl), exposição por longo período (30 dias). Além disso, o efeito do choque salino foi avaliado pela exposição à concentração elevada (200 mM de NaCl) para o curto período de tempo (3 h). Os qRT-PCR foram realizados para comparar os níveis expressão de selecionados genes. Plantas expostas a longos períodos de estresse salino não mostraram sinais de estresse nas folhas e raízes. Além disso, a expressão de RhNHX1 em *Rosa rugosa* aumentou na presença de NaCl. A transcrição de genes EPX4, NHX1, NAC e DREB aumentaram na presença de concentração elevada de NaCl. Em contraste, TIR diminuiu o nível de expressão com choque de salinidade. NHX1 teve um alto nível de expressão nas folhas, tanto sob estresse salino quando em choque salino, sugerindo que este gene desempenha papel importante na tolerância ao estresse salino em *Rosa rugosa.* Estes genes podem permitir a exploração de novos caminhos de melhoramento para tolerância de rosas e de outro membro da família Rosaceae ao estresse salino.

Palavras-chave: Florescimento, Expressão gênica, Microarray, temperatura, Estresse salino.

SUMÁRIO

FIRST PART

General introduction

1. INTRODUCTION

Roses are considered one of the most important ornamental plants, cultivated worldwide for use in gardens, medicinal purposes, perfum production, also as food and, mainly, for cut flowers. This plant has been cultivated for centuries and it is the most important species for the ornamental plants market.

In addition to commercial value, the genus *Rosa* is a good model for studying flowering in perennial plants, as it shows a short juvenile period and diversity in timing and flowering model. It would be advantageous to supply traditional breeding practices with a better understanding of how rose genomics and environmental conditions could influence the flower quality.

The genome size of roses is relatively small, but it remains unsequenced. This is attributed mostly to the polyploidy, the higher levels of heterozygosity, and large segments of repetitive elements in the cultivated roses, which difficults the genome assembly. This has been decreasing the ability to perform further genetic studies, such as the molecular base alterations during the changes occurred in environment. Knowledge of how environmental conditions can influence flowering is very important to improve the expression of the important commercial characteristics.

The first goal of this study is to generate global gene expression profiles in *Rosa hybrid cv* Knock Out, an important commercial species, under cold stress. For this it was used the Microarray technology. The results will be of great interest for both the rose genomics community and eventually to growers looking for using sustainable energy strategies in a production of high quality rose plants and flowers.

The second goal was the analysis of salt stress tolerance mechanism of *Rosa rugosa* in different conditions of salinity and also, to understand the molecular mechanism to salt tolerance of this species.

2. LITERATURE REVIEW

2.1 Roses characteristics

Roses are perennial plants that present prominent position among others ornamental plants. Also, have symbolic and culture importance in human history. This species fascinate several civilizations being considered a symbol of beauty. Another different meanings can be ascribed to the rose, as love, religious and politics (BARBIERI; STUNPF, 2005).

Globally, rose present high economic importance as a floriculture commodity, with a wide range of uses, garden ornamentation, rootstock, miniature pot plants, and mainly, as cut flower. Besides of the cultivated for ornamental propose, this species has been cultivated for production of essential oils for the perfum and cosmetic industries (HIBRAND-SAINT et al., 2008; KAWAMURA et al., 2011).

Rose cultivation has been recorded for more than 2000 years. Nowadays, roses are not only appreciated all around the world, but also are cultivated in different climates. Due to the popularity of this crop, roses are cultivated across broad geographic regions (DEBENER; LINDE, 2009).

The domestication of roses have a long and complex history. Asian and Europe areas are the major areas for rose domestication. For centuries rose domestication processes selected several flower traits affecting floral quality, plant architectures, such as recurrent flowering, double flowers, petal colours, fragrance and resistance to biotic and abiotic stress. Nowadays, more than thousand cultivated rose varieties have been comercialized (SMULDERS et al., 2009). Despite of the genus *Rosa* presents more than hundreds species with a large diversity in morphology and physiology, most modern roses are hybrids derived only from few species (DUGO et al., 2005).

The diversity of genus *Rosa* need to be more explored, for that is necessary a better understanding of genetic characteristics of others roses species. For example, wild rose species have greater tolerance to biotic and abiotic stress but the genetics bases for that are unknown. Also, the genus *Rosa* present high polymorphism in important ornamental traits as short juvenile phase, diversity in terms of timing and mode of flowering and recurrent blooming. These characteristics make the genus *Rosa* a good model for studies in perennial plants (FOUCHER et al., 2008).

2.2 Rose genome

The genome size of roses is relatively small, varying from 0.3-0.8 pg per haploid genome. In base pairs, it is estimated to be 300-585 million bps. Chromosome numbers are based on multiples of seven. Wild species are often diploids (2n=2x=14), but almost all cultivated roses are polyploidy, from 2n=4x=28 to 2n=8x=56 (KORBAN; GASIC; LI, 2006; KORBAN, 2007; DEBENER; LINDE, 2009). Modern roses are grouped into horticultural classes that include Polyanthas (2n=2x), Hybrid Teas and Floribundas (2n=3x, 4x), and miniatures (2n=2x, 3x, 4x), the genomic origins of which have been partially obscured by intercrossing (YOKOYA et al., 2000).

Generation and maintenance of the genetic diversity in natural populations can be investigated in diploid and polyploidy species. The segregation pattern of traits is more complicated in polyploids, thereby rendering the genetic analysis of rose difficult (RAJAPAKSE et al., 1992). The difficulty is also attributed to higher levels of heterozygosity, segregating progeny among and among species, problems with sexual hybridization and reproduction, low seed production, and low germination (KORBAN, 2007).

The use of genetic engineering strategies in addition to traditional breeding may prove to be highly useful for rose breeding as they facilitate the introduction and/ or modification of single gene traits without disruption of pre-existing commercially valuable phenotypic characteristics of the target variety. A range of genes have already been highlighted for potential use in the genetic improvement of rose. They include those for pest and disease resistance, flower color, morphology and vase life, as well as plant architecture (MARCHANT et al., 1998).

Rosaceae family, comprised over 100 genera and 3,000 species. This family includes species with different values, ornamental, fruit, nut and wood crops (DIRLEWANGER et al., 2002; SHULAEV et al., 2008). Recent division of subfamilies with base phylogenetic analyses was performed, combining data from six nuclear and four chloroplast loci was realized. The three new subfamily are Dryadoideae (Cercocapus, Dryas and Purshia; x= 9), Spiraeoideae (Kerria, Spiraea, and others; x=8, 9, 15 or 17) and Rosoidae (Fragaria, Rosa, Potentilha, Rubus and others; x=7) (POTTER et al., 2007). It is well-supported that *Rosa* as the closest sister taxon to a clade containing the genus *Fragaria*. In fact, all *Fragaria* pseudo-chromosome contain sufficient markers to infer syntenic relationship between roses e strawberries. An autotraploid linkage map of *Rosa hybrid* was validated using the *Fragaria vesca* (strawberry) genome sequence (GAR et al., 2011).

2.3 Cold stress

2.3.1 The effects of temperature on rose growth

Temperature is an important factor in rose growth and production. The knowledge of how the temperature influences flowering represents great interest

to growers because they could determinate the best conditions for plants grown and achieve the maximum performance (NADEEM et al., 2011). Changes in temperature during the roses growth have a direct effect on flowering time and quality of flowers. The typical temperature recommendations for roses are 25°C during the day and 17-18°C at night. For rose cultivation in greenhouses in temperate countries during the winter it is necessary to use heater systems, which contributes to increase the production costs (RAVIV et al., 2010).

Lower temperatures increase the time from bud break to flowering and the largest effect was observed during the time from visible bud stage to flowering. Temperatures from bud break to visible bud stage has a mirror effect. For the cultivar Kardinal, the decrease in temperature from 30°C to 15°C promoted an increase in number of the days necessary from bud break to flowering, from 21 to 63 days respectively (SHIN; LIETH; KIM, 2001). The duration of the bud phase to flower (B-F) is chiefly dependent on temperature, and independent of the previous characteristics like rooting ability, for example (BERNINGER, 1994).

Low temperature can induce reproductive failure. In fact, lower night temperatures (suboptimal) increase the atrophy and abortion of floral apices and are considered a factor for decreasing the number of flowers per rose plant (MOE, 1971). For example, the exposure of rose plants (*Rosa hybrida* cvs. Sonia and Golden Times) to a lower temperature (12° C) during the night influenced the assimilation and metabolism of carbon, increased leaf starch and sucrose levels, and lowered the reducer sugars (KHAYAT; ZIESLIN, 1989).

Typically, the temperature recommendation is cultivar-specific. Some cultivars are more tolerant than others for lower night temperature. The cold tolerance can also be stage-specific: the cultivar Kardinal can tolerate a lower temperature at the time of bud emergence and the cultivar Milva can tolerate lower temperatures at the time of leaflet unfolding (RAVIV et al., 2010).

2.4 Salinity stress

The three major abiotic stress that affect plants growth and crop production are cold, salt and drought (MAHAJAN; TUTEJA, 2005). Salinity stress gets position of feature because of the strong effect in the plant system and progressive increase of lands with salinity problems. It has been estimated that 20% of all cultivated lands are affected by salinity problem (accounting for 10 million hectares in over 100 countries) (SAHI et al., 2006).

Salt stress is the exposure of plants to salinity by gradual application, increasing salt level or exposure of plants to low levels of salinity. Salt shock is extreme form of salt stress. For example, when high concentration of salt (e.g NaCl) application happen in a single step. In this condition, occurs induction osmotic shock (plasmolysis) by the difference between external and internal solutes in the cell cytoplasm (SHAVRUKOV, 2013). When comparing the gene expression there are differences between salt stress and salt shock. Salt shock induces higher and faster changes. During the salt shock, higher concentration of Na^+ is transported to the shoot, and several genes are induced in response to osmotic shock, the plasma membrane damage in the root and to the ionic stress in the shoot cell .

In general, application in one single step of 50 mM NaCl or less induces only osmotic stress. Concentrations of 50 mM and 100 mM NaCl induce response between osmotic stress and osmotic shock (species depended). Higher concentrations, over 100-150 mM NaCl, will induce osmotic shock (SHAVRUKOV, 2013). Consequently, plants responses to salt stress or salt shock are highly complex and involve changes at molecular, cellular, and physiological levels (ATKINSON; URWIN, 2012). The understandings of mechanisms of salt tolerance in plants are important for providing alternatives solution to salinity problem in agricultural lands (WAHOME; JESCH; PINKER, 2001).

The ions, sodium (Na^+) and chloride (Cl^-) are more common in the saline soil solution and excess of these induce toxicity, membrane disorganization and inhibition of photosynthesis. During the development, plants show excessive uptake of these ions that promote injury formation and premature death of leaves (HASEGAWA, 2013). The saline root-zone environment has negatives impacts on osmotic and ionic equilibrium of the cells. In higher salinity conditions are induced the hyperionic and hyperosmotic stress effects in the plants (TUTEJA, 2007).

Plants can present large spectrum in response to salinity stress that will drive for sensitivity or tolerance. Sodium transport processes present major role in plants tolerance or acclimatization to salinity. In general, Na^+ control is carried out by process like organellar sequestration, membrane exchangers, and exclusion from photosynthetic tissues and meristems (QUEIROS et al., 2009; PARDO; RUBIO, 2011). The Na^+/H antiporter is a transmembrane transport proteins that exclude Na^+ from the cytosol in exchange for H^+. The transgenic rice plants overexpressing the AgNHX1 gene showed a strong tolerance to salt stress (OHTA et al., 2002). Variation in salinity tolerance and shoot sodium accumulation in *Arabidopsis* ecotypes linked to differences in the natural expression levels of transporters involved in Na^+ transport. An inverse relationship between AtSOS1 expression in the root and total plant Na^+ accumulation had been reported, supporting a role for AtSOS1 in Na^+ efflux (JHA et al., 2010).

2.4.1 Effects of salinity stress in rose

Several rose's cultivation systems use irrigation with moderately saline irrigation water or salinity soil. Roses are classified as sensitive to salinity. But the salinity effects depends on the type and concentration of the salts, cultivation

system (soil or hydroponic) and plant species or cultivars (LORENZO et al., 2000).

Usually, increases in soil salt concentrations show negative effect over the growth and flowering in several roses species (CABRERA; SOLIS-PEREZ; SLOAN, 2009). *Rosa chinensis* under stress conditions do not blossom and enter dormancy directly (JIANG et al., 2009). Sodium concentration in the medium had positive relation with sodium absorption in roses (LORENZO et al., 2000; MASSA; MATTSON; LIETH, 2008). Also, sodium is reported to play an important role for the stimulation of the electrical conductivity (Ec) in environments in which a higher salinity level (NaCl) occurs in the irrigations water source. Under higher sodium concentration, the nitrate, potassium and phosphate uptake decreased (LORENZO et al., 2000).

Rootstock roses show different levels of salt tolerance. For example, *Rosa fortuniana* was relatively more salt-tolerant than *R. odorata* and *R. multiflora* (NIU et al., 2008). The rootstock *R. chinensis* 'Major' had higher level of Na^+ in lower leaves than other parts and showed more pronounced leaf injury. Otherwise, *R. rubiginosa* had higher concentration of Na^+ in the roots than in all other parts, and a higher tolerance to NaCl stress (WAHOME; JESCH; PINKER, 2001). Another impact of salt stress is that in presence of higher Cl levels the NO^{-3} uptake decreases (DEBOUBA et al., 2007). For example, the rose uptake of NO^{-3} was negatively affected by NaCl concentration in cultivar Kardinal grafited on 'Natal Briar' rootstock (MASSA; MATTSON; LIETH, 2008; (MASSA; MATTSON; LIETH, 2009).

Since *Rosa rugosa* is a salt tolerant candidate, based on its natural occurrence form, growing in sand dune or it can be introduced by the growth environment. In European coastal areas, *R. rugosa* have been planted for sand stabilization, for boundaries of pathways, and it was also used with ornamental propose. The process of emergence and survival of seedling appears to be better

in dwarf-shrub dominated communities in dunes (KELAGER; PEDERSEN; BRUUN, 2013).

When daily treated with 0.25 N NaCl, roses showed no injured and lowest of Na and Cl in leaf content (DIRR, 1978). *Rosa rugosa* wild type had a higher resistance to salt stress than other cultivars,'Ziyan'; 'Purple Branch' and 'Zhongke 2' (YANG; ZHAO; XU, 2011).

Molecular mechanisms for responses for salt stress in rose are unclear. Limited researches have been considering how salinity stress affects the gene expression. A vacuolar Na^+/H^+ antiporter gene in rosa hybrid (RhNHX1) had expression increase in NaCl presence (KAGAMI; SUZUKI, 2005). The NHX1 I plays an important role in the compartmentalization of cytosolic Na+ into the vacuole (OHTA et al., 2002)

2.5 Microarray

The array technology was developed in the 1990s, in Patrick Brown's laboratory utilizing gridding robots to print DNA from purified cDNA clones on glass microscope slides (SCHENA et al., 1995). Microarray is a miniaturized analytical systems version of the tradition blot. New technology in robots permit the disposition of probe molecules (oligonucleotides, PCR-amplified cDNA fragments, proteins, and antibodies) in micrometer distances. The probes are immobilized in solid substrate, this surface usually consists of nylon membranes or glass slides. The probes design are based in genome sequence or on known or predicted open read frame. For that highly sophisticated bio-informatics systems are required (MALONE; OLIVER, 2011).

Microarray study consists of a series of defined phase. First phases, experimental question need to formulated, for example what genes are involved and how does the genome of particular cell types/ species respond to changes in

internal and external stimuli. After the question formulated, starts the design phase, that combines the desired biological objectives with the type of array platform selected and available biological sample to generate a robust experimental design (CHEN et al., 2003).

Microarray correspond to stable platform technologies that allow assay at a genome-wide scale. The primary focus of microarray technology is for genome exploration but is also used in proteins studies. Moreover, microarray analysis is a remarkably successful tool have been used for the generation of gene expression data on a genomic scale. In additional, microarray expression studies are producing massive quantitative expression levels of thousands of distinct genes simultaneously and other function genomic data, which promise to provide key insights into gene function and interaction within and across metabolic pathways (CHEN et al., 2003).

In general, a gene expression microarray analysis aims to identify differences in transcriptional levels between two or more experimental sample. For the analysis of gene expression, also termed gene profiling, the sample RNA is generally converted into stable cDNA, during this process, the sample are labelled. The most frequently used dye are Cy3 and Cy5 (red and green colors). Labeled cDNA is hybridized to the spots containing complementary sequence on the array. After this hybridization, the amount of individual hybridization of each of the two samples to each spot is quantified by scanning with laser for each dye on the array. The intensity of red and green signal measured represent relative gene-expression ratios (MARTIN-MAGNIETTE et al., 2008; MALONE; OLIVER, 2011).

New powerful technologies has been developed as RNA sequencing. However, RNA sequencing technology is new to most research, more expensive than microarray, data store is more challenging and the analysis is more complex. Microarray is easier to use and there are many tools for data analysis

to choose from. Researcher are comfortable to use microarray, sample labeling, array handling and data analysis methods are tried and true. Expression array are still cheaper and easier when processing large number of samples (e.g., hundreds to thousands). The emergence and diffusion of microarray technology is an open system of innovation in action (LENOIR; GIANNELLA, 2006; DUGAT-BONY et al., 2012). Microarray remain useful technologies that have much to offer in the exploration of biological complexity and RNA-seq complements and extends microarray measurements (MALONE; OLIVER, 2011).

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SECOND PART

Original papers

Scientific Article 1

Monitoring the expression profiles in rose under cold stress using cDNA microarray

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ABSTRACT

Low temperatures adversely affects crop production by restraining plant growth and productivity. Several studies have been performed to better understand the complex regulatory processes that occur during the cold acclimation. However, there are limited information about gene regulation and signaling pathways related to cold stress response in ornamental plants, specifically in the reproductive organs. Rose flowers represent a good experimental model to investigate these responses, due to the short generation time and small genome size. So, it was uses an apple microarray to investigate global gene expression profiles in rose's floral buds under low night temperature. Expression profiles were captured in floral buds at two different times after cold temperature exposure (4^0 C) for 2 and 12 h, and a control, 0 h. The qRT-PCR analysis the mRNA accumulation changes were made for a selection of 12 genes, up or down regulated in microarray hybridization. The tubby, dead box and pas 2 amplicons of *Rosa sp*. were cloned into pDrive vector, and nucleotide sequence was determined. Additionally, it was analyzed pattern genes expression of some genes of AP2 family comparing floral buds and leaves during cold stress. In this study was revealed 318 differentially expressed genes, in which 134 genes were up-regulated and 184 down-regulated. The expression patterns of the cold responsive transcripts identified by Microarray were confirmed by qRT-PCR analysis. Sequence analysis of TUBBY, DEAD BOX and PAS 2 revealed a higher level of similarity with *Fragraria vesca* (strawberry). The AP2/ERFs genes were more inducible in leaves compared with floral buds tissues. A set of the differentially expressed genes identified in this study will facilitate the better understand of cold stress response in rose floral buds.

Highlights

- Cold stress induce transcription in rose floral buds
- Tubby, Dead box and Pas 2 genes are orthologous in rose and strawberry
- AP2/ERF gens shows different levels of expression in floral buds and leaves

Keywords: *Rosa hybrid* cv Knockout. Gene expression. Floral bud. Low night temperature. Stress tolerance. AP2 gene family.

1. INTRODUCTION

Rose is the most economically important ornamental crop, being cultivated worldwide in different climates and for different goals. In addition to its commercial importance, the genus *Rosa* is a good model for studying flowering in perennial plants. Indeed, shows a short juvenile period and diversity in timing and kind of flowering.

The genome size of Rose is relatively small (0.3 -0.8 pg per haploid genome). Modern roses are grouped in different horticultural classes that include Polyanthas (2n=2x), Hybrid Teas (2n=3x, 4x), Floribundas (2n=3x, 4x), and miniatures 2n=2x, 3x, 4x), the genomic origins of that have been partially obscured by intercrossing (Yokoya, Roberts et al. 2000). Rose's genome remains unsequenced, that is attributed mostly to polyploidy, high levels of heterozygosis, and for the large segments of repetitive elements in cultivated roses which make assembly of the genome difficult.

Knowledge considering how environmental conditions influence flowering is very important in order to improve the expression of commercially important characteristics. The flowering process depends on several signals, endogenous and environmental rekated. The conditions of environment have strong effect on flowering of some species of plant, make this process occur fastest or fail. But there is a lack of knowledge on topics as genetics regulation and controls during early stages of flower development in roses.

It is known that temperature is an important factor in the growth and yield of rose and how these factors influences flowering shows great interest to growers in order to determine the best conditions for plants and achieving their maximum performance (Nadeem et al. 2011). Changes in temperature during the roses growth effect flowering time and flowers quality. The typical temperature

recommendations for roses are 25^0C during the day and 17-18^0 C at night. Lower night temperatures (suboptimal) increase the number of atrophy and aborted flowers, decreasing the number of flowers per plant (Moe 1971).

This is a complex regulatory network of signaling components that determine how plants respond to the stress conditions. Physiological, molecular and metabolic reprogramming occurs against the stressor to get the homeostasis condition (Atkinson; Urwin 2012). Cold is one of the most important abiotic stresses. Various physiological, molecular, and metabolic changes occur during cold acclimation. This suggests that plants cold stress responses is a complex vital phenomenon that involves more than one pathway (Maibam et al. 2013). Plants homeostasis at cold stress happen in function of the mechanisms that limit damage and maintain growth (Zbierzak et al. 2013). In general, plants mechanisms used in the environmental sensing network, can drive to success or fail in adverse conditions for adaptation (Dodds; Rathjen 2010).

Tropical and temperate plants show different abilities to response the cold stress. For example, plants from temperate zones when are undergo for process of exposure to low temperature (but not freezing) can increase the fitness to freezing tolerance. This process is known as cold acclimation (Maibam et al. 2013).The AP2/ERF protein family contains transcription factors that play a crucial role in plant growth and development and also in response to biotic and abiotic stress conditions. These regulatory proteins are involved in the control of primary and secondary metabolism, growth and developmental programs, as well as in response to environmental stimuli (Artlip et al. 2013; Licausi et al., 2013). The known of how environment condition influence flowering is important for improve expression of commercially traits.

In this study, in order to identify genes involved in responses to low night temperature in rose (R*osa hybrid* cv Pink Knock Out), expression profiles were

investigated using microarray slides. Also, we compared some expression levels genes of AP2 family in leaves and floral buds.

2. MATERIALS E METHODS

2.1 Plant material and stress treatment

Floral buds of *Rosa hybrida* cv Pink Knock Out were collected from established plants. Plants were grown in the greenhouse with 25^0 C/ 18^0 C day/ night temperatures, until showed floral buds in developmental stage 3-4 (Dubois et al. 2011), when they were moved to cold room (4^0C). The total period of exposure to low temperature were 12 h during the night (7 pm to 7 am). Reproductive organs were sampled from three biological triplicates at 0h (before cold exposure); 2 h (at 4^0C) and 12 h (at 4^0C) and immediately frozen in liquid nitrogen and stored at -80^0 C until the use.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using RNase Plant Minikit (Qiagen, Doncaster, VIC, Australia), with modification. Before the tissues be ground into a fine powered, 10% of polivinilpirrolidona was added on the sample. RNA was quantified using NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Three micrograms of RNA was used for 20 uL cDNA synthesis reaction in SuperScript II (Invitrogen, Carlsbad, CA, USA) with an oligodT primer, following the protocol outlined by the supplier. Following coupling with aa-cDNA to Cy-dye ester, cDNA was purified using a QIAquic column to remove unincorporated aa-dUTP and free amines. cDNAs were quantified in a NanoDrop ND-1000 Spectrophotometer.

2.3 Microarray hybridization

Using a 40,000 gene apple microarray created in the Korban's laboratory (Soria-Guerra, Rosales-Mendoza et al. 2011), slides were be prehybridized in a solution containing 20% formamide, 6× SSC, 0.1% SDS and 5× Denhardt's solution, with 25 μg mL^{-1} tRNA (Sigma) during 45 min at 42°C, washed with water sequentially for five times, once in isopropanol, and dried by centrifugation at 400 *g* for 3 min. The cDNA probes were dissolved in 42 μL of hybridization solution 1X (Ambion, Austin, TX), denatured for 1 min in boiling water and cooled to 42°C. Hybridizations were done at 42°C for 16 h using the Maui chamber system (BioMicro systems, Salt Lake City, UT). This was followed by post-hybridization washes done in Coplin jars along with gentle agitation. Subsequently, washes were conducted once in 1× SSC and 0.2% SDS at 42°C for 5 min, 0.1× SSC, 0.2% SDS at 25°C for 5 min, and twice in 0.1× SSC for 5 min. Finally, slides were dipped in 0.01× SSC, and dried by centrifugation at 400 *g* for 3 min. In addition, dye-swap will be done among these two technical replicates.

2.4 Bioinformatic data analysis

Following hybridization, microarray slides were then be scanned with Genepix 4000 B fluorescence reader (Axon Instruments Inc., Foster City, CA) using Genepix 3.0 image acquisition software adjusted for Cy3 and for Cy5. The image files obtained were analyzed using the GenePix Pro 3.0 software package, and visually inspected (www.moleculardevices.com). All nonhomogeneous and aberrant spots will be flagged.

Data files were imported into Beehive suite (**http://stagbeetle.animal.uiuc.edu/Beehive1.0/**) to identify differentially expressed

genes based on samples taken at different time point and dye assignment across microarrays. Data for each spot will be normalized using the Lowess normalization method. In the analysis model it were utilized differences between temperatures and various time points as fixed effect and performed the contrast analysis, whereby we were able to identify various probes that were differentially expressed based on time and temperature. Genes with means of normalized $\log_2$ intensity ratio of ≥ 1 or ≤ -1 were identified as differentially expressed genes.

Functional classification of differentially expressed genes using the data base web site from the Database Annotation and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/).

2.5 Quantitative Real Time-PCR For Microarray Validation

Real time RT-PCR analysis was carried out to validate the relative change in expression of the gene identified of the genes by microarray analysis. Initial RNA sample from floral buds, isolated for microarray analysis, were used for expression analysis.

Total RNA (4 ug) from each sample was treated with DNase I (Invitrogen) and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dt) primer using SuperScript III RT(Invitrogen). cDNA was diluted to 30 ng/ul and used for Real-Time PCR reactions in 96-well plates in a 7,300 Real-Time PCR System (Applied Biosystem, Foster City, CA) using SYBER Greem PCR Master Mix (Applied Biosystems). Gene-specific primers from identified genes for real time RT-PCR were designed using the Primer 3 program (http://frodo.wi.mit.edu/) based on the blast consensus sequence from GeneBank. Each RT-PCR reaction (25 ul) contained 10.5 ul water, 0.5 ul 200 nM of forward and reverse primers, respectively, 12.5 ul of 2 x SYBR Green I

Master, and 5 ul of diluted cDNA. The amplification program, consisted of one cycle of 95 C for 10 min followed by 95 C for 15 s and 60 C for 1 min. After amplification, a melting curve analysis was run using the program for one cycle at 95 C for 5 s, 65 C for 1 min, and 95 C with 0 s held in the step acquisition mode, followed by cooling at 40 C for 10 s. A negative control without cDNA template was run with each analysis to evaluate the overall specify. To normalize the total amount of cDNA in each reaction, one rose RhGADPH gene was co-amplified as an internal control. Each sample was replicated three times, and data were analyzed using the SDS software from 7,300 Real-time PCR System (Applied Biosystem) based on relative standard curves of PCR efficiency of the target and reference genes. The primers used in this study are shown at Supplementary Table 1.

2.6 Cloning and Sequencing Analysis

Gel purified PCR product was ligated into the pDrive cloning vector (Qiagen Inc., Chatsworth, Calif., USA) according to the manufacturer's instructions. The resulting recombinant plasmid was transformed into *E. coli* DH5*a* Cells. The transformants were screened for blue-white colonies by plating on LB medium uspplemented with 100 µg/mL ampicillin, 0.5 mM IPTG and 80 µg X-Gal/mL. White colonies were isolated and subcultured into LB with ampicillin, and screened for the presence of the plasmids carrying the genes (*dead box, pas2* and *tubby*) insert by PCR using the same combination of primers and by restriction digestion with *Eco*R1 restriction enzyme (Promega). The digested plasmids Cloning and Sequence Analysis of DEAD box, PAS2 and TUBBY, and PCR products were subjected to electrophoresis and photographed. A 1 kb DNA ladder (Fermentas, UAB, Lithunia) was used as a standard marker.

Following the determination of the presence for insert in *E. coli* clones, DNA sequencing was performed to confirm the identity of the insert. Both strands of the inserts were sequenced by Applied Biosystems 3730xl (UIUC Core Sequencing Facility)**.** The amino acid sequences determined in this study and those from the databases were aligned by GeneMapper software (Software Programs for Fragment Analysis Data). The aligned nucleotide and amino acid sequences were compared with the sequences present in GenBank (EMBL) using BLAST.

2.7 AP2 family analysis in leaves and floral buds

Rose plants were pruned to get new shoot. After 3 month, plants were exposed to cold stress again. Plants were moved to cold room (4^{o} C) at night (7 pm until 7 am). And samples from leaves and floral buds were collected from the same branch in three different point, 0 h (before cold stress), 2 h and 12 hours of cold night. Samples were immediately frozen in liquid nitrogen then stored -80^{0} C until use. RNA extraction, cDNA synthesis qRT-PCR are performed as described above.

The genes analyzed were *Rosa chinensis* clone 3 putative AP2 domain protein mRNA (GenBank: HQ842597.1); *Rosa chinensis* clone 1 putative DREB protein mRNA, partial cds (GenBank HQ842595.1); *Rosa chinensis* putative CBF/DREB transcription factor mRNA, complete cds (GenBank: EF583559.1); *Rosa hybrid* cultivar dehydration-responsive element-binding protein 1A mRNA, complete cds (GenBank: EU784069.1) and *Rosa hybrid* cultivar dehydration-responsive element-binding protein 1B mRNA, complete cds (EU784070.1).

3. RESULTS AND DISCUSSION

3.1 Microarray analysis of gene expression in rose floral buds

To investigate the molecular control in response of rose floral buds to cold stress, gene expression profiles were analyzed using a 40,000 apple oligo array. Since *Rosa hybrida* cv knock Out is a chilling tolerant, it was chosen for global gene expression profiling under cold stress. Plants grown under regular conditions were moved to cold conditions (4 ^{o}C) at early-nigth and floral buds tissues from three individual plants were harvested at three different time points: 0, 2, and 12 hours.

Normalized data were filtered for expression level for quality control to eliminate genes in the array with no value throughout the study. Filtering criteria was used to define differentially expressed genes in data analysis were a two-fold change (log_2) in transcript levels. Using the results, we examined the differences in transcript abundances that occurred during early hours of cold treatment (4 ^{o}C). Changes in expression of all probe sets on a representative microarray at different times are presented in Fig. 1.

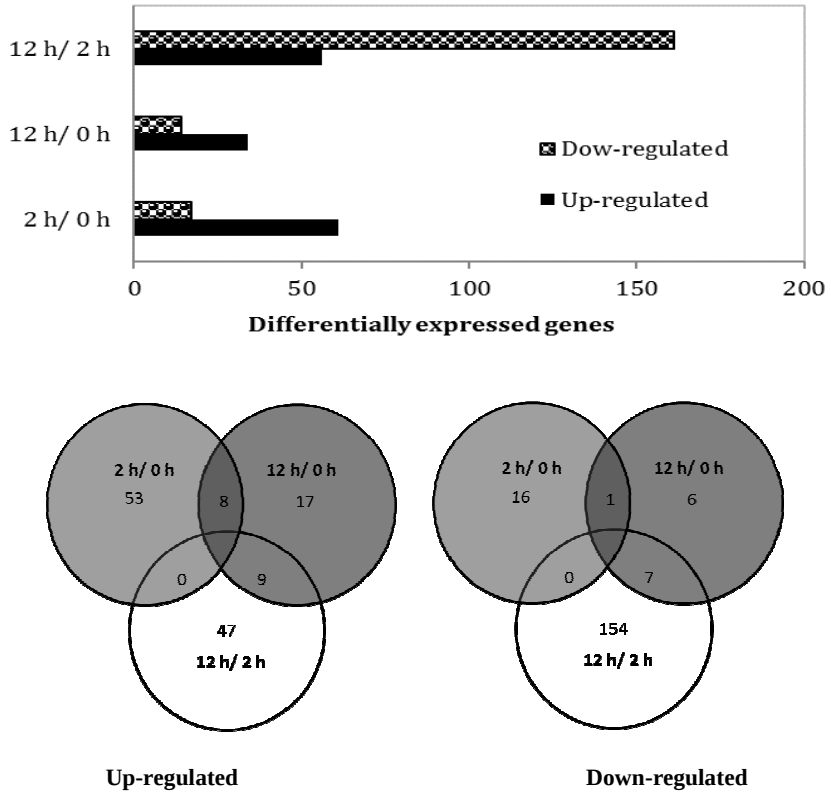

Fig 1: Expression profiling of cold-regulated genes in rose floral buds (A). Venn diagrams showing cold-regulated across three set of comparison, 2 h/ 0 h, 12 h/ 0 h and 12 h / 2 h (B).

Global observation of up-regulated genes at 2 h/0 h, 12 h/0 h and 12 h/ 2h showed 61, 34 and 56 genes respectively that had the transcription level increased; while the number of down-regulated genes at 2 h/0 h, 12 h/0 h and 12 h/ 2h were 17, 14 and 161 genes, respectively (Fig 1A). Among, these a total of 134 unique genes were up-regulate (see supplementary Table 2); while 184 unique genes were down-regulated (see supplementary Table 3). A total of 8

up-regulated and 1 down-regulated genes were common between 2 h and 12 h. So, this suggest that the 8 genes that were common up-regulated at 2h and 12 h, once expressed the transcript of these gene persisted during the low night temperature exposure (Fig 1 B). Only one gene was down-regulated at 2h and 12h, a dynamin-like protein (MdUI29543). Dynamim and dyanamin related proteins are involved in membrane trafficking pathway and plant mitochondrial division (Kang et al. 2003; Arimura et al. 2004).

The results (Figure 1A and B) indicated that different transcriptome level signatures in response to cold stress. Variable numbers of transcripts were up or down regulated during low night temperature exposure. Plant adaptation to environmental stresses is regulated through multiple physiological mechanisms. In special to temperature, that is one of the primary environmental factors that influences and limits the growth and development of plant species. The cultivar Knout Oout ® rose is a species that presents cold hardiness traits and the knowledge about the molecular bases for these traits will be helpful for breeding programs.

3.2 Functional categorization of identified genes

To capture and access the expression profiles genes regulated during the first 12 h following the cold exposure, a total of 151 up-regulated and 192 down-regulated genes were used to identify or assign putative functions. These were found to belong to the following functional categories. As part of the functional classification, different expressed genes were assigned one or more GO (gene onthology) terms (Fig 2).

For biological process, categorization demonstrated that different expressed genes involving flower development (GO:0009908), regulation of ethylene mediated signaling pathway (GO:0010104), signal transduction

(GO:0070297) and reproductive developmental process (GO:0003006) (Fig. 2A). Also a negative regulator in the ethylene signal transduction pathway (CTR1) was induced. Ethylene signaling negatively regulate freezing tolerance by repressing expression of CBF genes (Shi, Tian et al. 2012).

In categorization of cellular component, the terns were proteasome regulatory particle (GO:0008540) and plasma membrane (GO:0005886) (Fig. 2B). Interestingly, 93% different expressed genes classified in cellular component tern were involved in plasma membrane. The differential regulatory activities observed among cold regulated plasma membrane genes might contribute cold stress acclimation. One of the most important adaptation mechanisms to freezing is the alteration in plasma membrane compositions and its functions (McClung, Davis 2010)

Molecular function categorization included process related to calmodulin binding (GO:0005516), protein kinase activity (GO:0004672) (Fig. 2C), suggesting the signal perception at plasma membrane by receptor kinases and activation of signal transductions events. Genes responsive to cold stress were significantly involved in “binding” which broad category that includes different kinds of binding. The Gene Ontology analysis provided an overview of various functions and biological processes in which these genes were involved.

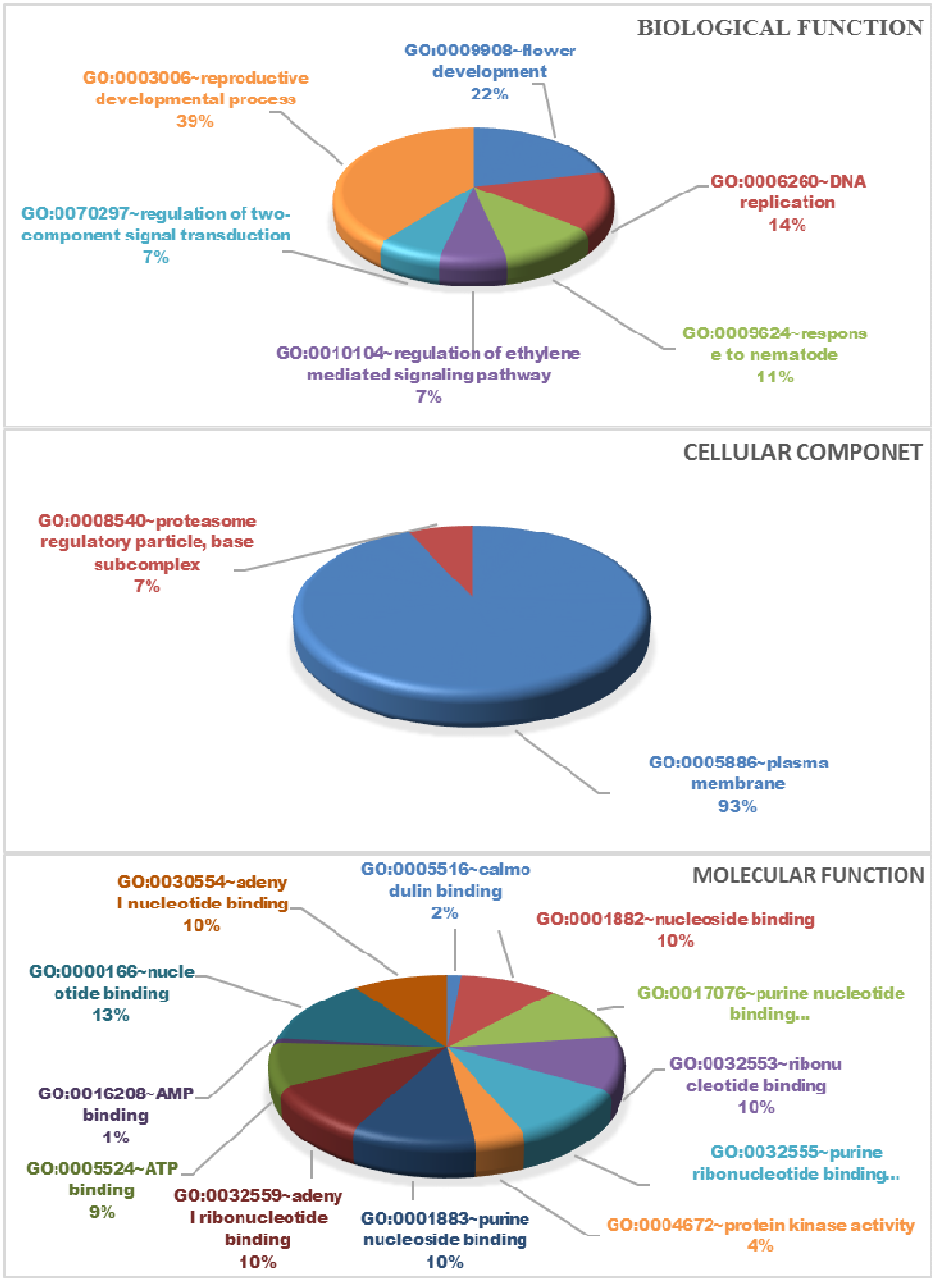

Fig 2: Functional classification of different expressed genes in rose floral buds.

Functional classification of different expressed genes indicate activation of diverse processes. These results indicated that, during exposure to cold stress, rose floral buds extensively reprogrammed gene expression, to limit damage caused by chilling.

3.3 qRT-PCR- Microarray validation

To confirm their transcript abundance profiles thought low night temperature exposure compared with control condition, 12 genes were analyzed by qRT-PCR. Overall, q-RT-PCR results for these genes showed similar pattern of expression profiles to those obtained following microarray hybridization. These results indicate that Microarray analysis was a powerful tool for identification of cold-stress inducible genes in rose floral buds.

We observed that NBS-LRR (nucleotide binding site/leucine-rich repeat- AM411491.1) it was up-regulated after two hours at 4^{o} C, after that the gene expression had decrease expression levels at 12 h (Fig. 3A). The NBS-LRR was first characterized in the diploid *R. multiflora* in response to black spot inoculation (Hattendorf; Debener 2007). NBS-LRR is a type of R or R-like protein (Resistance protein), activated by specific pathogen effectors. As it was known, NBS-LRR receptors trigger local resistance associated with programmed cell death as part of a hypersensitive response, and amplify basal defenses involving the signaling hormone salicylic acid (SA), leading to systemic resistance (Dodds, Rathjen 2010). Substantial evidences indicate that NBS-LRR are also associated with abiotic stress (cold, drought and salt stress) responses (Chini et al., 2004; Takata et al., 2007; Long et al., 2012). Recently was reported that NBS-LRR gene was up-regulated by cold stress in trifoliate orange and *S. mandarin* (Long, Song et al. 2012). These observation indicated that NBS-LRR genes are involved cold resistance.

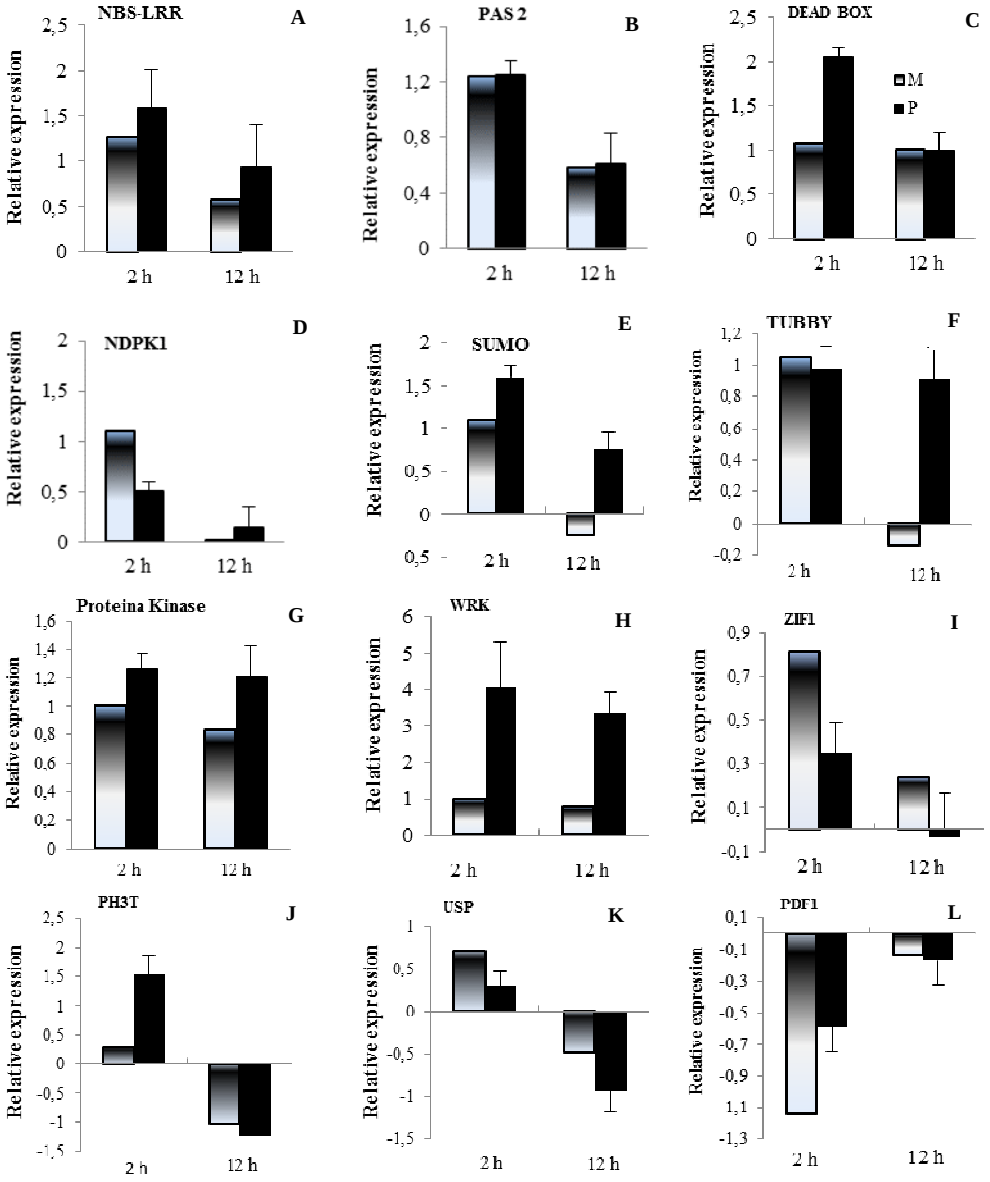

Fig 3: qRT-PCR analysis of expression levels (log_2) of randomly selected genes along with correspond microarray results at 2/0h and 12/0 h. M: Microarray data and P: qRT-PCR data.

In this study the PAS2 (PASTICCINO2- XM_004288469.1**)** showed increase in the expression level in response to the cold stress in the first 2 h of exposure low temperature exposure (Fig. 3 B) The PASTICCINO2 is anti-phosphatase that interact with cyclin dependent kinase (CDK) and belong to protein Tyr phosphatase-like family. They are involved in hormonal (cytokinin and auxin) control of cell division and differentiation in *Arabidopsis* (Baud, Bellec et al. 2004, Bellec, Harrar et al. 2002, Faure, Vittorioso et al. 1998, Harrar, Bellec et al. 2003, Smyczynski, Roudier et al. 2006). The proliferation control by PAS2 happening in both meristimatic e non-meristematic cells (Harrar, Bellec et al. 2003). Transgenic cell of *Arabidopsis*, overexpression of PAS2 gene observed slowed down cell division in suspension cell culture (Da Costa, Bach et al. 2006). Previous studies showed that PAS2 genes are repressors of cytokinin responses. Interesting features of pas mutant plants are that cytokinin response increase and auxin response decrease (Faure, Vittorioso et al. 1998).

In response to cold exposure, rose floral buds increased the transcription level of DEAD Box (XM_004292893.1) gene (Fig 3 C). RNA helicases of the DEAD-box protein family have been shown to participate in every aspect of RNA metabolism, from transcription to RNA decay (de la Cruz, Kressler et al. 1999, Cordin, Tanner et al. 2004, Jung, Park et al. 2013). Observations in *Arabidopsis* lead to proposal that DEAD box RNA helicases are important signaling network in development and stress responses processes (Gong, Lee et al. 2002, Kant, Kant et al. 2007). Substantial evidences indicate that RNA helicase-like proteins, during the plant chilling and freezing responses act as early regulator of transcription factors (Gong, Lee et al. 2002). In other species as Arabidopsis, sorghum and soybean, DEAD-BOX RHs genes were induced by low temperature (Chung, Cho et al. 2009, Guan, Wu et al. 2013).

The transcriptions levels of NUCLEOSSIDE DIPHOSPHATE KINASE (NDPKs- XM_004306930.1) increased under cold stress in rose floral buds (Fig. 3.D). This had already been reported in rice roots, which showed increase in the expression of NDPK gene under cold stress (Chen, Tian et al. 2012). NDPKs are metabolic enzymes that catalyze the transfer of the phosphate group from a nucleoside triphosphate to a nucleoside diphosphate involving a high-energy phosphoenzyme intermediate. They are key metabolic enzymes that maintain the balance between cellular ATP and NTP (nucleoside triphosphates) (Cho, Shin et al. 2004). NDPK has also been implicated in stress adaptation by plants to biotic and abiotic stress (Chen, Tian et al. 2012, Yang, Li et al. 2006). Also, the NDPK1 (a cytosolic protein) plays a significant role in ROS (reactive oxygen species) signaling by interaction with catalases (Fukamatsu, Yabe et al. 2003, Lee, Yoshida et al. 2009).

Cold stress conditions increase the expression of SUMO (small ubiquitin-like modifier) gene in roses (Fig. 3E). SUMO is a small protein, 100-115 amino acids, that can promote protein sumoylation (posttranslational regulatory process). Sumoylation promote the covalent attachment of the SUMO to target proteins, and change the rate of activity, function or subcellular location (Park, Yun 2013, Castro, Tavares et al. 2012, Raorane, Mutte et al. 2013). The sumoylation pathway play important functions in developmental process (growth, flowering and hormonal signaling), biotic and abiotic stresses response (Jin, Hasegawa 2008, Lee, Miura et al. 2007, Miura, Lee et al. 2009, Miura, Lee et al. 2010, Miura, Sato et al. 2011). SUMO, utilizes conjugation and deconjugation mechanisms that act as transcriptional regulator in response to stress (Castro, Tavares et al. 2012, Park, Yun 2013). SIZ1-mediated sumoylation play important function for cold stress response by affect the ICE-CBF-COR transcriptional cascade (Castro, Tavares et al. 2012, Miura, Okamoto et al. 2013, Miura, Jin Jing Bo et al. 2007).

TUBBY-like protein (TLPs) was responsive to temperature change (Fig. 3F). TLPs are conserved across eukaryotic kingdoms and indicate that TLPs may act in fundamental biological functions of TLPs (Reitz, Bissue et al. 2012). TLPs are involved biotic and abiotic stress, ABA signaling, development and ROS signaling (Kou, Qiu et al. 2009, Reitz, Pai et al. 2013, Lai, Lee et al. 2004). The TLPs translate reactive oxygen species currents into signaling not only for transcriptional regulation in the nucleus but also affect plastid-associated functions after release from the plasma membrane (Reitz, Bissue et al. 2012).

The response of Serine/threonine-protein kinase (Ser/Thr- PK) to temperature change was an increase on expression level in floral buds. The network of protein-Ser/Thr kinases is known as “central processor unit” (cpu), due its action, from receptor information (input) until signaling response (output) (Hardie 1999). Up to know, in *Arabidopsis* has nearly 1,000 genes encoding Ser/Thr- PK (Arabidopsis Genome Initiative, 2000; (Kaul, Koo et al. 2000). Recently, SNF1-types Ser/Thr- PK from wheat (*Triticum aestivum L.*), were used to confer enhanced multstress tolerance in Arabidopsis. The overexpression of *TaSnRK* genes can significantly strengthen tolerance to freezing stresses (Mao, Zhang et al. 2010, Tian, Mao et al. 2013, Zhang, Mao et al. 2011). Transgenic tobacco plants overexpressing a TaSRK2C1 (wheat SNF1-Related Protein Kinase 2 Gene) had increase in the tolerance to low temperature and other stress. Also in this study, three putative central regulators (RD29a, DREB1A, and DREB2) were up-regulated in transgenic tobacco plants (Du, Zhao et al. 2013).

In this study, the transcriptor factor WRKY (XM_004303194.1) induction was observed in response to cold stress**.** Autoregulation and cross-regulation by WRKY gene promote signaling network that modulate several plant response (Rushton, Somssich et al. 2010). The gene WRKY are involved in several biological process as plant growth and development (Chi, Yang et al.

2013, Luo, Sun et al. 2013), and biotic stress response (Abbruscato, Nepusz et al. 2012, Babitha, Ramu et al. 2013, Atamian, Eulgem et al. 2012). The WRKY transcription factors have also been observed in response to abiotic stress as thermotolerance (Dang, Wang et al. 2013, Li, Zhou et al. 2010, Li, Fu et al. 2011, Sahin-Cevik 2012), salinity (Li Ming, Ding Bo et al. 2013) and drought (Shen, Liu et al. 2012, Luo, Bai et al. 2013). In rice, 41 WRKY genes were responsive for abiotic stress (cold, drought and salinity), beign 9 cold stress specific (Ramamoorthy et al. 2008). In soybean, from the 64 GmWRKY identified 8 were responsive to cold stress condition. Transgenic Arabidopsis overexpressing GmWRY21 were tolerant to cold stress (Zhou et al. 2008).

It was also identified that transcriptional level of Zinc-Induced Facilitator-Like 1 (ZIFL1) gene increased during low night temperature. ZIFL1 belong to Major Facilitator Superfamily (MFS) transporters that is a major class of transporter in all organisms. ZIFL1 modulates polar auxin transport by potassium and proton fluxes in cells (Remy et al. 2013)**.** ZIFL1 is also involved in Zn (zinc) homeostasis, the ZIFL1 overexpression can confer increased Zn tolerance (Haydon; Cobbett 2007)**.**

The transcript levels of phosphate transporter (PHT) increased at 2h of cold stress, but at 12 h was down-regulated. PHT play role in phosphate homeostasis (Nagarajan, Jain et al. 2011). Other important functions of PhT are in ATP production in plant cells and ethylene signaling (Nagarajan et al. 2011, Zhu et al. 2012). In Sugarcane also PhT1-2 was up-regulated by low temperature. In this study was suggested that increase in gene expression indicate readjusting of Pi status and recover of photosynthetic carbon metabolisms (Nogueira et al. 2003).

The universal stress proteins (*USPs*) showed similar pattern to that observed PHT during cold exposure. At 2h/0h was up-regulated then at 12 h/ 0 h was down-regulated. Similar result was founded in *Solanum pennelli,* after 6 h

of cold stress exposure resulting in accumulation of *SpUSP* transcripts. In this study, *SpUSP* expression also were regulated in response to other abiotic stress conditions (drought, heat, salinity and wounding) and also hormones (ABA and GA3)(Loukehaich, Wang et al. 2012). USPs play important role in plants adaptation under stress conditions, enhancing survival rate (Li, Wei et al. 2010). Up to now, the molecular mechanism of UPS action are not clear.

In this study, a PDF1 (PROTODERMAL FACTOR 1) gene that encodes a putative extracellular proline-rich protein was up-regulated by cold stress. Proline-rich protein (PRP) genes encode a cell wall protein of plant. They are associated to structural integrity of mature tissues and determining cell type-specific wall structure during plant growth and development (Menke, Renault et al. 2000). But also affected by environment stress and the overexpression can confer cold tolerance (Gothandam, Nalini et al. 2010).

3.4 Sequencing

Subsequently, it was proceeded the sequencing of the three gene products. Sequence analysis revealed a higher level of similarity between rose and strawberry. TUBBY-like F-box, DEAD box and PASTICCINO 2A-like, rose product showed 94%, 99% and 92% of similarity with predicted *Fragaria vesca subsp. Vesca,* respectively (Gene Bank Acc: XM_004309647.1).

Table 1: Blast results for genes sequenced

TUBBY	**Query Length:** 106	**Sequence:** CCTAGGTGGCACGAACAACTCCANNTGNTGG TGTCTGAACTTCAATGGACGAGTAACCGTTG CTTCAGTCAAGAATTTTCAGCTGGTTGCTTCT CCAGAGAACGA		
	Total Score: 163	**Query cover:** 99%	**E Value**: 2e-37	**Ident:** 94%
	Accession: XM_004309647.1	**PREDICTED:** Fragaria vesca subsp. vesca tubby-like F-box protein 3-like (LOC101309415), mRNA		
DEAD BOX helicase	**Query Length:** 106	**Sequence:** AAAGGCGTAGTGGTTTGGTGAGANAAAGCTG GACAAGTTCATCAACTTCTTCAGTCATCGTAG CTGAAAATAGCATGGTTTGTCTCCTTTTGGGG CATACACA		
	Total Score: 185	**Query cover:** 99%	**E Value**: 4e-44	**Ident:** 99%
	Accession: XM_004292893.1	**PREDICTED:** PREDICTED: Fragaria vesca subsp. vesca DEAD-box ATP-dependent RNA helicase 28-like (LOC101307177), mRNA		
PASTICCINO 2A-like	**Query Length:** 106	**Sequence:** CAGCTGGTCCATCACTGAGATTATTCNATACT CTTTCTATGGCATGAAAGAGACTCTTGGTTTT GCGCCTTCCTGGCTCCAGTGGCTCAGGTACAG CACCA		
	Total Score: 147	**Query cover:** 99%	**E Value**: 2e-32	**Ident:** 92%
	Accession: XM_004288469.1	**PREDICTED:** Fragaria vesca subsp. vesca very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehydratase PASTICCINO 2A-like (LOC101300284), mRNA		

This is well-supported as the closest sister taxon to a clade containing the genus *Fragaria*. An autotraploid linkage map of *Rosa hybrid* it was validated using the *Fragaria vesca* (strawberry) genome sequence. All *Fragaria* pseudo-chromosomes contained sufficient markers to infer syntenic relationship between rose and strawberries (Gar et al. 2011).

3.5 Expression of AP2/ERF family in reproductive and vegetative tissues

The AP2/ERF family showed to play an important role in triggering transcriptional cascade in the cold stress response in rose. However, gene expression analysis revealed the differential pattern between leaves and floral bud. All the AP2/ERFs were more inducible in leaves compared with floral buds tissues (Fig. 5A). Leaves showed the higher levels of expression at 2h following the decrease at 12 h. Floral buds expression, in general, increased at 2 hours and keep at 12 h. Such situation indicates that the timely response of AP2/ERF pathway to cold night is variable among different rose tissues.

The DREB like (GenBank: HQ842595.1; Fig 5B) expression was induced in the first hours of stress in leave (Fig. 5C). But after 12 h of stress the expression level was almost the same as in plants unstressed. This gene in floral buds showed lower expression level than in leaves, but these were more stable during cold stress. Expression of DREB1 in plants exposed to cold stress was rapidly accumulated in *Phyllostachys edulis* after 3 h (Liu, Cao et al. 2012). DREB subfamily genes induce multiples target genes involved in plant tolerance. Maybe make cross-point or node connecting several via and regulates the expression a set of genes abiotic stress-related to plant tolerance (Agarwal, Agarwal et al. 2006).

Few minutes of plants exposure to low temperatures are enough to CBFs transcript levels start to increase (Fig. 5 C, D and E). In peach leaf and bark tissues showed that genes of CBF-regulon (PpCBFs1-4) were all responsive to LT. Peach leaves showed higher expression levels of the CBFs genes than bark, (Artlip, Wisniewski et al. 2013). The genes in the CBF/DREB subfamily play a crucial role in the resistance of plants to abiotic stresses by recognizing the dehydration responsive or cold-repeat element (DRE/CRT) with a core motif of A/GCCGAC (Yamaguchishinozaki, Shinozaki 1994).

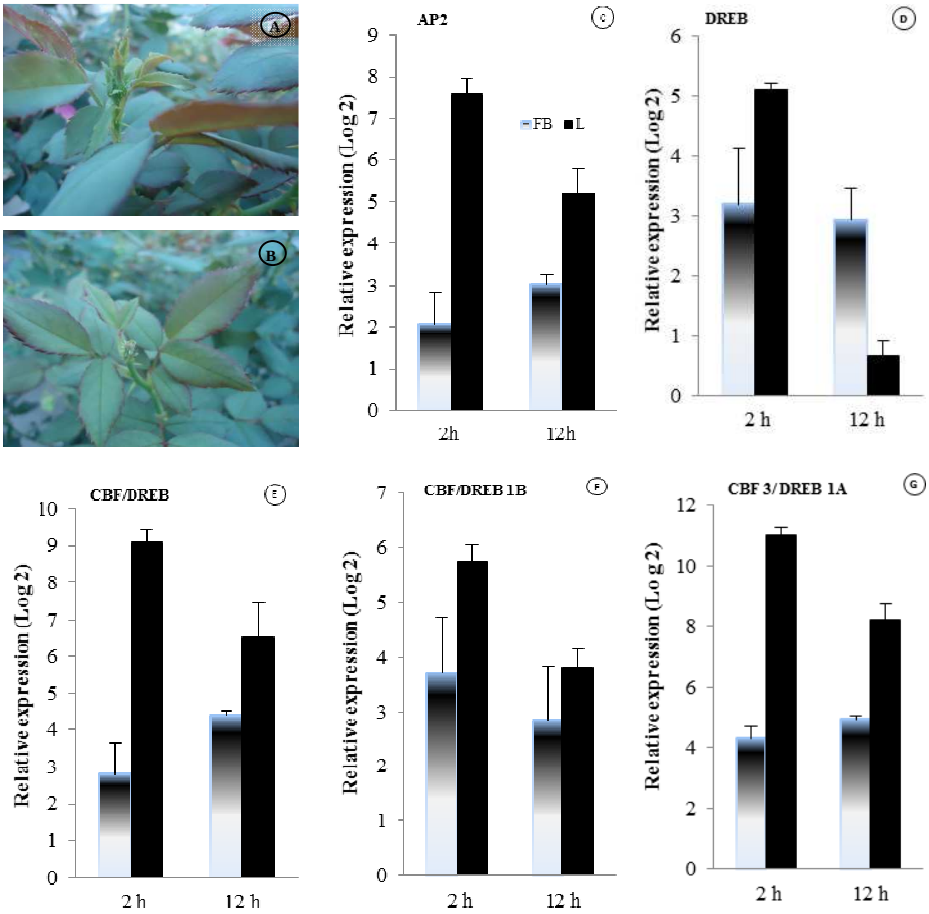

Fig. 5: Expression pattern of important transcripts in response to cold stress in leaves and floral buds

4. CONCLUSIONS

In summary, clarify the transcriptomic changes in flower buds during low night temperature exposure of *Rosa hybrida* cv Knock out.

Among of the up-regulated genes exist those involved in defense-related genes, transcription and signaling pathways.

Sequence analysis revealed that a high level of similarity between rose and strawberry, with orthologous genes.

AP2 family is strongly involved response to cold stress in rose. And this show different pattern of expression between leaves and floral buds.

The identification and characterization of genes involved in the molecular regulation in cold acclimation may enable us to develop plant varieties with improved cold tolerance.

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Supporting Information

Table S1: Primers used in this study (XLSX)

Table S2: List of genes up regulated in floral bud during cold night (XLSX)

Table S3: List of genes down regulated in floral bud during cold night (XLSX)

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Supplementary Data

Table S1: Primer list used to qRT-PCR

Primer name	Sequence	Product (bp)	Species	Gene bank	Gene name
R1	GCTGGCAGGTATCCTTTCTG	190	*Rosa luciae*	EC589884	RhGAPDH
R2	GGCGACAATATCAGCCAAGT				
R3	AGGCTAATGGGTTTGGAGCT	119	*Rosa hybrida*	AM411491.1	Putative NBS-LRR resistance protein, clone brp37-9
R4	CCGCCATTCCTCTCTTGTTT				
R5	TCAGCTGGTCCATCACTGAG	101	*Fragaria vesca*	XM_004288469.1	Protein tyrosina phosphate (PAS2, PASTICCCINO2, PEP, PEPINO)
R6	GGTGCTGTACCTGAGCCACT				
R7	GGAAGTTGTGGCAGATTCGT	100	*Fragaria vesca*	XM_004309828.1	Small ubiquitin-like modifier (SUMO) E3 ligase
R8	GCCCATGTGAAGACAAGGTT				
R9	GTGTATGCCCCAAAAGGAGA	102	*Fragaria vesca*	XM_004292893.1	BEAD box RNA helicase
R10	AAAGGCGTAGTGGTTTGGTG				
R11	CAGGACACCTCGTTCAGGAT	107	*Fragaria vesca*	XM_004309647.1	Tubby-like F-box protein 3-like (LOC101309415), mRNA
R12	GTATCATCCGACGCCTCAAT				
R13	TATCCGGACCACTCATCCAT	115	*Fragaria vesca*	XM_004303060.1	Probable serine/threonine-protein kinase At1g08600-like (LOC101306539), mRNA
R14	GCTGTCGTCCTAGCCAAAAC				
R15	TGATACCAAGAGCCCAAAC	113	*Fragaria vesca*	XM_004303194.1	Probable WRKY transcription factor 40-like (LOC101301598), mRNA
R16	CTGCAAGTGCTTTGGTGAAG				
R17	TCAACGAGGTGCTGCTAATG	97	*Fragaria vesca*	XM_004308136.1	Protein ZINC INDUCED FACILITATOR-LIKE 1-like (LOC101299619), mRNA
R18	TTGTGCCCAAGAAAACACTG				
R19	CGCTTTCCAACACTCGTACA	113	*Fragaria vesca*	XM_004303808.1	Protodermal factor 1-like (LOC101303249), mRNA
R20	CCTGACTTGCGTGGTAGTGA				
R21	GTGATTGCTGGAATGGGATT	111	*Fragaria vesca*	XM_004296316.1	Probable inorganic phosphate transporter 1-7-like (LOC101299463), mRNA
R22	CGGCTTCGGATGAGTTATGT				
R23	ATGTGCCAGCTGAAGAGACC	114	*Fragaria vesca*	XM_004297166.1	Uncharacterized LOC101303795 , mRNA (Adenine nucleotide alpha hydrolases-like superfamily protein)
R24	CAGTGACACCCCTTGCTTTT				
R25	CGCTGAGAAGCACTATGCAG	99	*Fragaria vesca*	XM_004306930.1	Nucleoside diphosphate kinase 1-like
R26	CCCTCCCAGATCATAGCAAC				
R27	GGAACCGGTGTTTTGTATGG	139	Rosa hybrida	EU784070.1	CBF / DREB 1B
R28	CACGTCAGCATAACCTTCCA				
R29	AACGGTCAGAATGCCAAGAC	108	*Rosa chinensis*	HQ842595.1	Rc 1 putative DREB protein mRNA
R30	ATCCCATGGAAGCTGATGAC				
R31	TGTAGCAGCTCTGGCTCTGA	104	*Rosa chinensis*	HQ842597.1	Rc 3 putative AP2 protein mRNA
R32	TCAACTCCGGCAATGACATA				
R33	GCACCATCTTCTCCAGCTTC	93	*Rosa chinensis*	EF583559.1	Putative CBF/DREB TF
R34	CCTCCCAGCTTTCCTCTTCT				
R35	ATGCCTCAATTTCGCTGATT	107	*Rosa rugosa*	EU784069.1	CBF / DREB 1A
R36	CAGGTCGAAATGTCTCAGCA				

Table s2: Total list of up-regulated genes by low night temperature

cDNA_IDs	Acession no	Annotation / Description	Log 2 (Ratio) 0		
			0 vs 2 h	0 vs 12 h	2 vs 12 h
MdUI07383	AT2G05780.1	non-LTR retrotransposon family (LINE)SE	**1,63**	0,68	-0,95
MdUI12743	AT3G28153.1	non-LTR retrotransposon family (LINE)	**1,58**	0,12	-1,46
MdUI16263	AT3G22950.1	ATARFC1 (ADP-ribosylation factor C1); GTP binding	**1,52**	0,43	-1,08
MdUI13829	AT4G21110.1	G10 family protein	**1,36**	1,37	0,01
MdUI09875	AT2G40400.1	similar to unknown protein	**1,33**	1,22	-0,11
MdUI14013	AT2G20900.1	diacylglycerol	**1,30**	0,40	-0,90
MdUI19273	AT5G55780.1	DC1 domain-containing protein	**1,29**	0,11	-1,18
MdUI19879	AT1G53350.1	disease resistance protein (CC-NBS-LRR class) putativ	**1,27**	0,57	-0,70
MdUI07205	AT1G24510.1	T-complex protein 1 epsilon subunit putative / TCP-1-epsilon putative / chaperonin	**1,24**	0,69	-0,55
MdUI23667	AT5G10480.1	PEP PAS2 | PAS2 (PASTICCIN 2)	**1,24**	0,58	-0,65
MdUI36859	AT3G26460.1	major latex protein-related / MLP-related	**1,22**	0,29	-0,93
MdUI09653	AT5G44870.1	disease resistance protein (TIR-NBS-LRR class) putativ	**1,22**	-0,18	-1,39
MdUI02673	AT2G21280.1	ATSULA GC1 | GC1 (GIANT CHLOROPLAST 1); catalytic/ coenzyme bindin	**1,18**	1,98	0,80
MdUI29901	AT1G35390.1	non-LTR retrotransposon family (LINE)	**1,17**	0,01	-1,17
MdUI01057	0,00		**1,16**	1,25	0,09
MdUI30893	0,00		**1,16**	1,11	-0,05
MdUI16417	AT5G50320.1	ELO3 (ELONGATA 3); N-acetyltransferase	**1,15**	0,23	-0,93
MdUI06447	AT1G32220.1	catalytic/ coenzyme binding	**1,15**	0,29	-0,85
MdUI35559	AT4G12100.1	similar to CUL4 (CULLIN4) protein binding / ubiquitin-protein ligas	**1,14**	0,18	-0,97
MdUI33481	AT5G02430.1	WD-40 repeat family protein	**1,14**	-0,02	-1,16
MdUI24627	AT3G27720.1	zinc finger protein-related	**1,14**	0,91	-0,23
MdUI21295	0,00		**1,14**	-0,26	-1,40
MdUI27183	AT3G54280.1	ATP binding / DNA binding / helicase	**1,13**	-0,09	-1,22
MdUI11847	AT2G46610.1	arginine/serine-rich splicing factor putativ	**1,13**	0,70	-0,43
MdUI39855	AT3G57490.1	40S ribosomal protein S2 (RPS2D)	**1,12**	0,61	-0,51
MdUI06629	AT4G35800.1	NRPB1 (RNA POLYMERASE II LARGE SUBUNIT); DNA binding / DNA-directed RNA polymerase	**1,12**	1,15	0,03
MdUI08537	0,00		**1,11**	0,33	-0,78
MdUI30557	AT5G07940.1	similar to dentin sialophosphoprotein-related	**1,10**	-0,38	-1,48

MdUI37749	AT4G09320.1	NDPK1 (nucleoside diphosphate kinase 1); ATP binding / nucleoside diphosphate kinase	**1,10**	0,02	-1,09
MdUI18019	AT4G06735.1	unknown protein	**1,10**	1,57	0,47
MdUI20163	AT5G60410.2	ATSIZ1/SIZ1; DNA bindin	**1,09**	-0,24	-1,33
MdUI35833	0,00		**1,09**	0,13	-0,96
MdUI37129	AT1G08280.1	glycosyl transferase family 29 protein / sialyltransferase family protein	**1,08**	-0,28	-1,37
MdUI38617	AT5G17400.1	ADP ATP carrier protein mitochondrial puta	**1,08**	0,12	-0,96
MdUI10701	AT1G48605.1	ATHAL3B (Arabidopsis thaliana Hal3-like protein B); electron carrier	**1,08**	0,29	-0,79
MdUI23977	AT2G21770.1	| CESA9 (CELLULASE SYNTHASE 9); transferase transferring glycosyl group	**1,07**	0,51	-0,57
MdUI19421	AT4G22190.1	similar to conserved hypothetical protein	**1,07**	0,34	-0,73
MdUI33509	0,00		**1,07**	0,36	-0,71
MdUI38597	AT4G16630.1	DEAD/DEAH box helicase putative	**1,07**	1,00	-0,07
MdUI20889	0,00		**1,06**	0,44	-0,62
MdUI32671	AT3G09320.1	zinc finger (DHHC type) family protein	**1,06**	-0,22	-1,28
MdUI35053	AT1G11180.1	secretory carrier membrane protein (SCAMP) family protein	**1,05**	0,08	-0,97
MdUI12563	AT1G17270.1	unknown protein	**1,05**	0,05	-1,00
MdUI34127	AT2G47900.1	AtTLP3 (TUBBY LIKE PROTEIN 3); phosphoric diester hydrolase/ transcription factor	**1,05**	-0,14	-1,19
MdUI12813	AT4G39560.1	kelch repeat-containing F-box family protein	**1,05**	0,02	-1,03
MdUI16321	AT4G19710.1	AK-HSDH/AK-HSDH II; aspartate kinase/ homoserine dehydrogenase |	**1,04**	0,16	-0,89
MdUI23245	AT2G22040.1	transducin family protein / WD-40 repeat family protein	**1,04**	0,03	-1,01
MdUI10297	AT1G32850.1	ubiquitin carboxyl-terminal hydrolase family protein	**1,04**	-0,13	-1,17
MdUI33517	AT2G31751.1	unknown protein	**1,03**	0,15	-0,88
MdUI16365	AT3G04950.1	similar to Os06g0298500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001057443.1);	**1,03**	-0,01	-1,04
MdUI05725	AT5G65310.1	ATHB5 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 5); transcription factor	**1,03**	-0,19	-1,21
MdUI38025	AT4G21630.1	subtilase family protein	**1,02**	-0,13	-1,15
MdUI22281	AT4G24970.1	ATP-binding region ATPase-like domain-containing protei	**1,02**	0,02	-1,00
MdUI04963	AT3G43583.1	similar to leucine-rich repeat family protein / extensin family protein	**1,02**	-0,08	-1,10
MdUI20981	AT3G21690.1	MATE efflux family protein	**1,01**	0,38	-0,63
MdUI03873	AT5G44290.1	protein kinase family protein	**1,01**	0,83	-0,18
MdUI31945	AT4G15233.1	ATP binding / ATPase/ nucleoside-triphosphatase/ nucleotide binding	**1,01**	0,19	-0,82
MdUI17837	AT1G42100.1	copia-like retrotransposon family	**1,01**	0,21	-0,80
MdUI26741	AT4G31800.1	WRKY18 (WRKY DNA-binding protein 18); transcription factor	**1,01**	0,82	-0,19

MdUI20813	AT4G39090.1	RD19 (RESPONSIVE TO DEHYDRATION 19); cysteine-type peptidase	**1,01**	0,71	-0,29
MdUI37581	AT1G08190.1	vacuolar assembly protein putative (VPS4)	**1,00**	-0,01	-1,01
MdUI02673	AT2G21280.1	GC1 (GIANT CHLOROPLAST 1); catalytic/ coenzyme binding	**1,18**	**1,98**	0,80
MdUI24689	AT4G03330.1	SYP123 (syntaxin 123); t-SNARE	-0,08	**1,73**	1,81
MdUI39943	AT3G48520.1	CYP94B3 (cytochrome P450 family 94 subfamily B polypeptide 3); oxygen bind	0,05	**1,64**	1,58
MdUI18019	AT4G06735.1	unknown protein	1,10	**1,57**	0,47
MdUI29923	AT1G03670.1	ankyrin repeat family protein	0,39	**1,52**	1,12
MdUI13829	AT4G21110.1	G10 family protein	1,36	**1,37**	0,01
MdUI14355	AT1G23410.1	Ubiquitin extension protein putative / 40S ribosomal protein S27A (RPS27aA	0,32	**1,30**	0,98
MdUI20035	AT3G14610.1	CYP72A7 (cytochrome P450 family 72 subfamily A polypeptide 7); oxygen bind	0,38	**1,26**	0,88
MdUI39795	AT5G66110.1	metal ion binding	0,22	**1,26**	1,04
MdUI34493	AT1G32190.1	similar to unknown protein	-0,60	**1,26**	1,85
MdUI01057	0,00		1,16	**1,25**	0,09
MdUI19919	AT3G55720.1	similar to unknown protein	0,29	**1,24**	0,95
MdUI36509	AT1G27780.1	Ulp1 protease family protein	0,62	**1,24**	0,62
MdUI09875	AT2G40400.1	similar to unknown protein	1,33	**1,22**	-0,11
MdUI06629	AT4G35800.1	NRPB1 (RNA POLYMERASE II LARGE SUBUNIT); DNA binding / DNA-directed RNA polymer	1,12	**1,15**	0,03
MdUI15543	AT1G12040.1	LRX1 (LEUCINE-RICH REPEAT/EXTENSIN 1); protein binding / structural constituent of cell wall	0,20	**1,14**	0,94
MdUI37401	AT3G62850.1	Zinc finger protein-related	0,06	**1,12**	1,05
MdUI30893	0,00		1,16	**1,11**	-0,05
MdUI14797	AT5G09440.1	phosphate-responsive protein putativ	0,28	**1,11**	0,83
MdUI37939	AT3G61300.1	C2 domain-containing protein	0,65	**1,11**	0,45
MdUI38305	AT2G22620.1	lyase	0,41	**1,09**	0,68
MdUI15847	AT1G01240.1	similar to unknown protein	0,29	**1,09**	0,80
MdUI03827	0,00		0,53	**1,09**	0,55
MdUI09979	AT3G47500.1	CDF3 (CYCLING DOF FACTOR 3); DNA binding / protein binding / transcription factor	-0,28	**1,08**	1,37
MdUI04815	0,00		-0,06	**1,08**	1,13
MdUI26889	AT1G70300.1	KUP6 (K+ uptake permease 6); potassium ion transporter	0,51	**1,07**	0,56
MdUI34537	AT4G30110.1	HMA2 (Heavy metal ATPase 2); cadmium-transporting ATPase | chr4:14720259-14724583 REVERSE	0,09	**1,07**	0,98
MdUI03905	AT5G38670.1	F-box family protein	0,90	**1,07**	0,17

MdUI04775	AT5G19840.1	Transcription factor jumonji (jmjC) domain-containing protein	0,10	**1,06**	0,96
MdUI00149	AT4G22330.1	ATCES1 (ATCES1); catalytic	0,46	**1,05**	0,60
MdUI20545	AT2G16440.1	DNA replication licensing factor putativ	0,45	**1,05**	0,60
MdUI14363	AT1G07630.1	PLL5 (POL-like 5); protein phosphatase type 2C	-0,13	**1,00**	1,13
MdUI15073	AT3G49350.1	RAB GTPase activator	0,50	**1,00**	0,51
MdUI38951	AT4G16630.1	DEAD/DEAH box helicase		**1,00**	-0,07
MdUI38951	AT2G28080.1	glycosyltransferase family protein	-2,05	**0,65**	**2,70**
MdUI02895	AT4G24220.1	VEP1 (VEIN PATTERNING 1)	-1,42	**0,70**	**2,11**
MdUI34493	AT1G32190.1	similar to unknown protein ; similar to C (GB:BAD82560.1)	-0,60	**1,26**	**1,85**
MdUI24689	AT4G03330.1	SYP123 (syntaxin 123); t-SNARE	-0,08	**1,73**	**1,81**
MdUI39943	AT3G48520.1	Symbols: CYP94B3 | CYP94B3 (cytochrome P450 family 94 subfamily B polypeptid	0,05	**1,64**	**1,58**
MdUI35079	AT1G02335.1	GL22 (GERMIN-LIKE PROTEIN SUBFAMILY 2 MEMBER 2 PRECURSOR.); manganese ion binding	-1,05	0,47	**1,52**
MdUI05145	AT3G52540.1	TOFP18/OFP18 (Arabidopsis thaliana ovate family protein 18)	-0,66	0,79	**1,45**
MdUI09979	AT3G47500.1	CDF3 (CYCLING DOF FACTOR 3);transcription factor	-0,28	1,08	**1,37**
MdUI09781	0,00		-0,64	0,72	**1,36**
MdUI38925	AT3G45220.1	serpin putative / serine protease inhibitor putat	-0,44	0,92	**1,36**
MdUI18003	0,00		-0,83	0,51	**1,34**
MdUI02987	AT3G09390.1	ATMT-K ATMT-1 MT2 MT2A | MT2A (METALLOTHIONEI	-0,96	0,36	**1,32**
MdUI19463	AT1G01620.1		-0,36	0,94	**1,29**
MdUI39791	AT1G76730.1	5-formyltetrahydrofolate cyclo-ligase family protein	-1,23	0,01	**1,25**
MdUI34987	AT1G06130.1	GLX2-4 (GLYOXALASE 2-4); hydroxyacylglutathione hydrolase	-0,26	0,98	**1,24**
MdUI14123	AT3G53890.1	40S ribosomal protein S21 (RPS21B)	-0,46	0,78	**1,24**
MdUI15889	AT3G20570.1	plastocyanin-like domain-containing protein	-0,56	0,64	**1,20**
MdUI30037	AT2G04970.1	heat shock protein binding	-1,28	-0,09	**1,19**
MdUI11135	AT1G21240.1	WAK3 (WALL ASSOCIATED KINASE 3); kinase/ protein serine/threonine kinase	-0,38	0,81	**1,19**
MdUI24393	AT3G26920.1	F-box family protein	-0,39	0,79	**1,18**
MdUI31527	AT5G61060.1	| HDA05 (HISTONE DEACETYLASE5); histone deacetylase	-0,90	0,28	**1,18**
MdUI31281	AT1G03030.1	phosphoribulokinase/uridine kinase family protein	-0,36	0,82	**1,18**
MdUI04241	0,00		-0,39	0,76	**1,15**

MdUI08349	AT1G73480.1	hydrolase alpha/beta fold family protei	-0,45	0,69	**1,14**
MdUI36867	0,00		-0,68	0,46	**1,14**
MdUI14363	AT1G07630.1	PLL5 (POL-like 5); protein phosphatase type 2C	-0,13	1,00	**1,13**
MdUI04815	0,00		-0,06	1,08	**1,13**
MdUI07455	AT3G22120.1	CWLP (CELL WALL-PLASMA MEMBRANE LINKER PROTEIN); lipid binding	-0,77	0,35	**1,13**
MdUI29923	AT1G03670.1	ankyrin repeat family protein	0,39	1,52	**1,12**
MdUI35519	0,00		-0,55	0,55	**1,10**
MdUI26029	AT4G13880.1	leucine-rich repeat family protein	-0,49	0,61	**1,10**
MdUI00155	AT3G01900.1	CYP94B2 (cytochrome P450 family 94 subfamily B polypeptide 2); oxygen bind	-0,57	0,52	**1,09**
MdUI39453	AT2G06040.1	similar to unknown protein	-0,37	0,72	**1,09**
MdUI02505	AT5G21482.1	CKX7 (CYTOKININ OXIDASE 7); oxidoreductase	-0,63	0,45	**1,08**
MdUI32027	AT2G14240.1	similar to unknown protein	-0,34	0,74	**1,08**
MdUI14045	AT1G23410.1	ubiquitin extension protein putative / 40S ribosomal protein S27A (RPS27aA	-0,14	0,94	**1,08**
MdUI39593	AT4G22485.1	Encodes a Protease inhibitor/seed storage/LTP family protein	-0,58	0,49	**1,06**
MdUI14467	AT1G04820.1	TUA4 (tubulin alpha-4 chain)	-0,53	0,53	**1,06**
MdUI19693	AT5G13740.1	ZIF1 (ZINC INDUCED FACILITATOR 1); carbohydrate transporter/ sugar porter	-0,81	0,24	**1,06**
MdUI37401	AT3G62850.1	zinc finger protein-related	0,06	1,12	**1,05**
MdUI11007	AT1G61110.1	ANAC025 (Arabidopsis NAC domain containing protein 25); transcription factor	-0,76	0,29	**1,04**
MdUI39795	AT5G66110.1	metal ion binding	0,22	1,26	**1,04**
MdUI39413	AT1G05640.1	ankyrin repeat family protein	-0,60	0,44	**1,04**
MdUI38309	AT4G39610.1	similar to unknown protein	-0,19	0,85	**1,04**
MdUI19223	AT5G53240.1	similar to unknown protein	-0,15	0,89	**1,04**
MdUI16225	AT5G64970.1	mitochondrial substrate carrier family protein	-0,69	0,35	**1,03**
MdUI10043	AT2G25600.1	SPIK (SHAKER POLLEN INWARD K+ CHANNEL); cyclic nucleotide binding / potassium channel	-0,81	0,22	**1,03**
MdUI39151	AT4G30200.1	similar to VIN3 (VERNALIZATION INSENSITIVE 3) zinc ion bindin	-0,27	0,76	**1,03**
MdUI13775	AT5G01990.1	auxin efflux carrier family protein	-0,73	0,29	**1,02**
MdUI30395	AT4G04940.1	transducin family protein / WD-40 repeat family protein	-0,91	0,12	**1,02**
MdUI01029	AT5G03360.1	DC1 domain-containing protein	-0,46	0,56	**1,02**

MdUI08181	AT1G56610.1	syntaxin-related family protein	-0,16	0,85	**1,01**
MdUI19397	AT1G61490.1	S-locus protein kinase putativ	-0,72	0,28	**1,01**
MdUI32733	AT2G39730.1	RCA (RUBISCO ACTIVASE)	0,01	0,55	**1,01**
MdUI01953	AT2G42840.1	PDF1 (PROTODERMAL FACTOR 1)	-1,15	-0,14	**1,00**
MdUI32801	AT3G06590.1	transcription factor	-0,58	0,43	**1,00**

Table s3: Total List of down-regulated genes

cDNA_IDs	Acession no	Annotation / Description	Log 2 (Ratio)		
			2 / 0h	12 / 0 h	12/ 2h
MdUI08355	AT5G18065.1	similar to unknown protein (contains domain Cysteine proteinases)	**-1.01**	-0.27	0.73
MdUI25003	AT3G23340.1	CKL10 | CKL10 (Casein Kinase I-like 10);	**-1.02**	-0.14	0.88
MdUI12021	AT3G22880.1	ATP binding / DNA-dependent ATPase/ damaged DNA binding;	**-1.02**	-0.19	0.83
MdUI35691	AT1G71696.1	SOL1 (suppressor of LLP1 1); carboxypeptidase A;	**-1.03**	-0.43	0.59
MdUI29653	AT3G22880.1	phosphate translocator-related;	**-1.03**	-0.45	0.58
MdUI32724	AT1G62640.1	KAS III (3-oxoacyl-[acyl-carrier-protein] synthase);	**-1.05**	-0.08	0.97
MdUI35079	AT1G02335.1	GL22 (GERMIN-LIKE PROTEIN SUBFAMILY 2 MEMBER 2 PRECURSOR.)	**-1.05**	0.47	1.52
MdUI29543	AT4G33650.1	ADL2 (ARABIDOPSIS DYNAMIN-LIKE 2); GTP binding / GTPase;	**-1.08**	**-1.21**	-0.14
MdUI16431	AT4G20380.1	LSD1 (LESION SIMULATING DISEASE);	**-1.14**	-0.24	0.90
MdUI01953	AT2G42840.1	PDF1 (PROTODERMAL FACTOR 1)	**-1.15**	-0.14	1.00
MdUI36919	AT4G31610.1	REM1 (REPRODUCTIVE MERISTEM 1); DNA binding / transcription factor ;	**-1.15**	-0.20	0.95
MdUI39791	AT1G76730.1	5-formyltetrahydrofolate cyclo-ligase family protein	**-1.23**	0.01	1.25
MdUI32719	AT1G12040.1	LRX1 (LEUCINE-RICH REPEAT/EXTENSIN 1); protein binding	**-1.27**	-0.36	0.92
MdUI30037	AT2G04970.1	heat shock protein binding;	**-1.28**	-0.09	1.19
MdUI02895	AT4G24220.1	VEP1 (VEIN PATTERNING 1)	**-1.42**	0.70	2.11
MdUI12499	AT1G05280.1	| Symbols: | fringe-related protein	**-1.49**	-0.75	0.74
MdUI38951	AT2G28080.1	glycosyltransferase family protein	**-2.05**	0.65	2.70
MdUI24119	AT1G36980.1	similar to salt tolerant protein [Triticum aestivum] (GB:AAY26392.1)	-0.10	**-1.01**	-0.90

MdUI33021	AT3G60570.1	ATEXPB5 (ARABIDOPSIS THALIANA EXPANSIN B5)	-0.45	**-1.01**	-0.56
MdUI07571	AT5G51600.	ATMAP65-3 PLE | PLE (PLEIA	-0.14	**-1.01**	-0.87
MdUI26299	AT4G15955.1	epoxide hydrolase-related	0.25	**-1.01**	-1.26
MdUI13831	AT1G69740.1	HEMB1; porphobilinogen synthase;	0.13	**-1.02**	-1.16
MdUI12177	AT3G24460.1	TMS membrane family protein	-0.32	**-1.03**	-0.71
MdUI20633	AT5G43360.1	PHT3 (phosphate transporter 3); carbohydrate transporter	0.30	**-1.04**	-1.34
MdUI22237	AT1G29280.1	WRKY65 (WRKY DNA-binding protein 65); transcription facto;	-0.41	**-1.15**	-0.74
MdUI29543	AT4G33650.1	ADL2 (ARABIDOPSIS DYNAMIN-LIKE 2); GTP binding / GTPase;	**-1.08**	**-1.21**	-0.14
MdUI33009	AT1G62935.1	unknown protein	-0.17	**-1.43**	-1.26
MdUI28081	AT4G23650.1	CDPK6 (CALCIUM-DEPENDENT PROTEIN KINASE 6);	-0.45	**-1.65**	-1.21
MdUI04375	AT1G45130.1	beta-galactosidase putative / lactase putat	0.46	-0.54	**-1.00**
MdUI06873	AT1G19110.1	inter-alpha-trypsin inhibitor heavy chain-related	0.61	-0.39	**-1.00**
MdUI03937	AT1G55130.1	endomembrane protein 70	0.75	-0.26	**-1.00**
MdUI12563	AT1G17270.1	similar to unknown protein	1.05	0.05	**-1.00**
MdUI15887	AT2G16890.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	0.39	-0.62	**-1.00**
MdUI22281	AT4G24970.1	ATP-binding region ATPase-like domain-containing protein	1.02	0.02	**-1.00**
MdUI15691	AT1G17370.1	UBP1B (OLIGOURIDYLATE BINDING PROTEIN 1B);	0.62	-0.38	**-1.01**
MdUI28567	AT3G53140.1	O-diphenol-O-methyl transferase putative	0.66	-0.35	**-1.01**
MdUI23245	AT2G22040.1	transducin family protein / WD-40 repeat family protein	**1.04**	0.03	**-1.01**
MdUI01171	AT2G23230.1	terpene synthase/cyclase family protein	0.51	-0.49	**-1.01**
MdUI37581	AT1G08190.1	vacuolar assembly protein	**1.00**	-0.01	**-1.01**
MdUI34673	AT3G28490.1	oxidoreductase 2OG-Fe(II) oxygenase family protein	0.88	-0.13	**-1.01**
MdUI27145	AT5G46250.1	RNA recognition motif (RRM)-containing protein	0.81	-0.20	**-1.01**
MdUI30829	AT1G18690.1	galactosyl transferase GMA12/MNN10 family protein	0.40	-0.61	**-1.01**
MdUI17369	AT1G65080.1	OXA1 family protein	0.85	-0.16	**-1.01**
MdUI12495	AT2G20510.1	ATTIM44-1 (A. thaliana translocase inner membrane subunit 44-1)	0.42	-0.59	**-1.01**
MdUI03561	AT1G23080.1	PIN7 (PIN-FORMED 7); auxin: hydrogen symporter/ transporter	0.72	-0.30	**-1.01**
MdUI26431	AT2G46380.1	similar to unknown protein	0.52	-0.50	**-1.02**

MdUI18927	AT5G31804.1	gypsy-like retrotransposon family (Athila)	0.91	-0.11	**-1.02**
MdUI22349	AT3G61590.1	F-box family protein	0.74	-0.28	**-1.02**
MdUI37151	AT1G47500.1	ATRBP47C' (RNA-BINDING PROTEIN 47C'); **RNA** binding	0.80	-0.22	**-1.02**
MdUI34623	AT2G29820.1	kelch repeat-containing F-box family protein	0.86	-0.16	**-1.02**
MdUI33393	AT4G24890.1	PAP24 (purple acid phosphatase 24)/ protein serine/threonine phosphatase	0.77	-0.25	**-1.02**
MdUI07439	AT5G16000.1	NIK1 (NSP-INTERACTING KINASE 1); kinase	0.40	-0.62	**-1.02**
MdUI38303	AT4G31290.1	ChaC-like family protein	0.60	-0.42	**-1.02**
MdUI32141	AT1G30970.1	SUF4 (SUPPRESSOR OF FRIGIDA4); transcription factor	0.54	-0.48	**-1.02**
MdUI16259	AT1G04810.1	26S proteasome regulatory subunit putative	0.26	-0.77	**-1.02**
MdUI30361	AT2G21410.1	VACUOLAR PROTON ATPASE A2); ATPase	0.68	-0.34	**-1.02**
MdUI34221	AT4G37440.1	similar to unknown protein	0.22	-0.80	**-1.03**
MdUI30617	AT3G16850.1	glycoside hydrolase family 28 protein / polygalacturonase (pectinase)	0.60	-0.43	**-1.03**
MdUI12813	AT4G39560.1	kelch repeat-containing F-box family protein	1.05	0.02	**-1.03**
MdUI24547	AT5G43390.1	similar to unknown protein	0.59	-0.44	**-1.03**
MdUI07649	AT5G14770.1	pentatricopeptide (PPR) repeat-containing protein	0.61	-0.42	**-1.03**
MdUI32409	AT5G07940.1	similar to dentin sialophosphoprotein-related	0.77	-0.26	**-1.03**
MdUI20243	AT2G17600.1	DC1 domain-containing protein	0.48	-0.56	**-1.03**
MdUI08321	AT1G31820.1	amino acid permease family protein	0.77	-0.27	**-1.03**
MdUI20743	AT1G11200.1	similar to unknown protein	0.72	-0.31	**-1.03**
MdUI37631	AT5G44300.1	dormancy/auxin associated family protein	0.74	-0.29	**-1.03**
MdUI29179	AT2G07727.1	cytochrome b (MTCYB) (COB) (CYTB)	0.80	-0.23	**-1.03**
MdUI29199	AT1G50030.1	TOR (TARGET OF RAPAMYCIN)	0.61	-0.42	**-1.04**
MdUI08111	AT2G40420.1	amino acid transporter family protein	0.84	-0.20	**-1.04**
MdUI39585	AT1G22040.1	kelch repeat-containing F-box family protein	0.82	-0.22	**-1.04**
MdUI05275	AT1G27460.1	NPGR1 (NO POLLEN GERMINATION RELATED 1); calmodulin binding	0.57	-0.46	**-1.04**
MdUI13589	AT4G30990.1	binding	0.66	-0.38	**-1.04**
MdUI26783	AT1G59620.1	CW9; ATP binding	0.60	-0.44	**-1.04**
MdUI16365	AT3G04950.1	similar to Os06g0298500 [Oryza sativa]	**1.03**	-0.01	**-1.04**

MdUI17789	AT5G65690.1	phosphoenolpyruvate carboxykinase (ATP)	0.74	-0.31	**-1.04**
MdUI05549	AT5G57590.1	BIO1 (BIOTIN AUXOTROPH 1)	0.63	-0.42	**-1.04**
MdUI01241	AT3G57570.1	binding	0.33	-0.71	**-1.04**
MdUI30237	AT3G53190.1	pectate lyase family protein	0.58	-0.47	**-1.04**
MdUI19113	AT5G58620.1	zinc finger (CCCH-type) family protein	0.51	-0.54	**-1.05**
MdUI07477	AT5G38660.1	APE1 (ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT)	0.78	-0.28	**-1.06**
MdUI06985	AT1G18410.1	kinesin motor protein-related	0.21	-0.85	**-1.06**
MdUI30255	AT5G05980.2	ATDFB (A. THALIANA DHFS-FPGS HOMOLOG B);	0.88	-0.18	**-1.07**
MdUI08385	AT3G44920.1	ATCHX11 (cation/H+ exchanger 11); monovalent cation:proton antiporter	0.45	-0.62	**-1.07**
MdUI15871	AT1G17180.1	ATGSTU25 (Arabidopsis thaliana Glutathione S-transferase (class tau) 25)	0.53	-0.54	**-1.07**
MdUI32387	AT2G47450.1	CAO (CHAOS); chromatin binding	0.59	-0.48	**-1.07**
MdUI13983	AT5G07480.1	oxidoreductase 2OG-Fe(II) oxygenase family protein	**1.00**	-0.07	**-1.07**
MdUI35705	AT4G15053.1	similar to unknown protein	0.33	-0.75	**-1.07**
MdUI07545	AT1G04800.1	glycine-rich protein	0.37	-0.71	**-1.08**
MdUI29551	AT1G17070.1	D111/G-patch domain-containing protein	0.71	-0.37	**-1.08**
MdUI16263	AT3G22950.1	ATARFC1 (ADP-ribosylation factor C1); GTP binding	**1.52**	0.43	**-1.08**
MdUI32411	AT3G27040.1	meprin and TRAF homology domain-containing protein n	0.64	-0.44	**-1.08**
MdUI24549	AT2G17420.1	NTRA (NADPH-dependent thioredoxin reductase 2)	0.80	-0.28	**-1.08**
MdUI37749	AT4G09320.1	NDPK1 (nucleoside diphosphate kinase 1); ATP binding	1.10	0.02	**-1.09**
MdUI33563	AT5G01190.1	LAC10 (laccase 10); copper ion binding / oxidoreductase	0.91	-0.18	**-1.09**
MdUI14221	AT5G33300.1	chromosome-associated kinesin-related	0.75	-0.34	**-1.09**
MdUI18745	AT1G20460.1	similar to unknown protein	0.50	-0.59	**-1.10**
MdUI04963	AT3G43583.1	similar to leucine-rich repeat family protein / extensin family protein	**1.02**	-0.08	**-1.10**
MdUI26753	AT2G41705.1	camphor resistance CrcB family protein	0.88	-0.22	**-1.10**
MdUI26571	AT5G17500.1	glycosyl hydrolase family 5 protein / cellulase family protein	0.24	-0.86	**-1.10**
MdUI24253	AT1G71010.1	phosphatidylinositol-4-phosphate 5-kinase family protein	0.56	-0.54	**-1.10**
MdUI05253	AT3G16580.1	F-box family protein	0.71	-0.40	**-1.10**
MdUI17777	AT1G28670.1	ARAB-1 (Arabidopsis lipase); carboxylic ester hydrolase	0.64	-0.46	**-1.11**

MdUI20369	AT5G08420.1	similar to NAP57 (ARABIDOPSIS THALIANA HOMOLOGUE OF NAP57)	0.39	-0.72	**-1.11**
MdUI36515	AT3G48500.1	PDE312/PTAC10 (PIGMENT DEFECTIVE 312); RNA binding	0.59	-0.52	**-1.11**
MdUI36925	AT1G60995.1	similar to S3 self-incompatibility locus-linked pollen 3.15 protein	0.84	-0.27	**-1.11**
MdUI11431	AT2G45650.1	AGL6 | AGL6 (AGAMOUS LIKE-6); DNA binding / transcription factor	0.73	-0.39	**-1.11**
MdUI29349	AT3G44990.1	XTR8 (xyloglucan:xyloglucosyl transferase 8)	0.22	-0.91	**-1.13**
MdUI08183	AT2G01540.1	C2 domain-containing protein	0.99	-0.14	**-1.13**
MdUI37457	AT1G69350.1	pentatricopeptide (PPR) repeat-containing protein	0.13	-1.00	**-1.13**
MdUI01741	AT2G27360.1	lipase putative	0.77	-0.37	**-1.14**
MdUI17977	AT2G04080.1	MATE efflux family protein	0.59	-0.56	**-1.15**
MdUI38931	AT2G28290.2	SYD (SPLAYED)	0.68	-0.47	**-1.15**
MdUI00517	AT1G16290.1	similar to Os02g0170900 [Oryza sativa (japonica cultivar-group)]	0.66	-0.49	**-1.15**
MdUI34159	AT3G44510.1	similar to esterase/lipase/thioesterase family protein	0.93	-0.22	**-1.15**
MdUI34033	AT5G13650.1	elongation factor family protein	0.69	-0.46	**-1.15**
MdUI38591	AT3G04690.1	protein kinase family protein	0.64	-0.52	**-1.15**
MdUI38025	AT4G21630.1	subtilase family protein	1.02	-0.13	**-1.15**
MdUI29183	AT3G27280.2	ATPHB4 (PROHIBITIN 4)	0.64	-0.51	**-1.15**
MdUI37519	AT5G52170.1	homeobox-leucine zipper family protein /	0.37	-0.78	**-1.16**
MdUI13831	AT1G69740.1	HEMB1; porphobilinogen synthase	0.13	-1.02	**-1.16**
MdUI02103	AT2G31220.1	basic helix-loop-helix (bHLH) family protein	0.57	-0.59	**-1.16**
MdUI33481	AT5G02430.1	WD-40 repeat family protein	1.14	-0.02	**-1.16**
MdUI14651	AT4G27080.1	ATPDIL5-4 (PDI-LIKE 5-4); electron carrier/ protein disulfide oxidoreductase	0.66	-0.51	**-1.17**
MdUI19401	AT2G35630.1	MOR1 (MICROTUBULE ORGANIZATION 1)	0.86	-0.31	**-1.17**
MdUI29901	AT1G35390.1	non-LTR retrotransposon family (LINE)	1.17	0.01	**-1.17**
MdUI25619	AT3G19450.1	CAD4 (CINNAMYL ALCOHOL DEHYDROGENASE 4)	0.83	-0.35	**-1.17**
MdUI10297	AT1G32850.1	ubiquitin carboxyl-terminal hydrolase family protein	1.04	-0.13	**-1.17**
MdUI04871	AT1G30610.1	EMB2279 (EMBRYO DEFECTIVE 2279); binding	0.95	-0.23	**-1.18**
MdUI07339	AT1G25570.1	leucine-rich repeat protein-related	0.67	-0.51	**-1.18**
MdUI19273	AT5G55780.1	DC1 domain-containing protein	1.29	0.11	**-1.18**

MdUI06717	AT1G43205.1	similar to unknown protein	0.43	-0.75	**-1.19**
MdUI18017	AT1G69080.1	universal stress protein (USP) family protein	0.71	-0.48	**-1.19**
MdUI20613	AT3G06880.1	transducin family protein / WD-40 repeat family protein	0.78	-0.41	**-1.19**
MdUI03759	AT4G35725.1	unknown protein	0.53	-0.66	**-1.19**
MdUI34127	AT2G47900.1	AtTLP3 (TUBBY LIKE PROTEIN 3); phosphoric diester hydrolase/	1.05	-0.14	**-1.19**
MdUI35441	AT3G11480.1	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	0.93	-0.27	**-1.20**
MdUI09749	AT3G51520.1	diacylglycerol acyltransferase family	0.38	-0.82	**-1.20**
MdUI28081	AT4G23650.1	CDPK6 (CALCIUM-DEPENDENT PROTEIN KINASE 6)	-0.45	**-1.65**	**-1.21**
MdUI05725	AT5G65310.1	ATHB5 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 5); transcription factor	1.03	-0.19	**-1.21**
MdUI21009	AT1G28240.1	similar to hydrolase acting on carbon-nitrogen	0.49	-0.72	**-1.21**
MdUI27183	AT3G54280.1	ATP binding / DNA binding / helicase	1.13	-0.09	**-1.22**
MdUI19227	AT1G35140.1	PHI-1 (PHOSPHATE-INDUCED 1)	0.70	-0.52	**-1.22**
MdUI37253	AT5G06100.1	MYB33 (myb domain protein 33);	0.75	-0.47	**-1.22**
MdUI28747	AT3G13760.1	DC1 domain-containing protein	0.56	-0.67	**-1.23**
MdUI37863	AT5G10250.1	phototropic-responsive protein putative	0.58	-0.65	**-1.23**
MdUI37643	AT2G04190.1	meprin and TRAF homology domain-containing protein	0.66	-0.59	**-1.25**
MdUI33719	AT4G18900.1	transducin family protein / WD-40 repeat family protein	0.81	-0.44	**-1.25**
MdUI36973	AT2G32560.1	F-box family protein	1.00	-0.26	**-1.26**
MdUI27411	AT5G59305.1	unknown protein	0.90	-0.36	**-1.26**
MdUI26299	AT4G15955.1	epoxide hydrolase-related	0.25	**-1.01**	**-1.26**
MdUI33009	AT1G62935.1	unknown protein	-0.17	**-1.43**	**-1.26**
MdUI32671	AT3G09320.1	zinc finger (DHHC type) family protein	1.06	-0.22	**-1.28**
MdUI26071	AT5G49260.1	unknown protein	0.74	-0.55	**-1.29**
MdUI15973	AT2G47700.1	zinc finger (C3HC4-type RING finger) family protein	0.46	-0.84	**-1.30**
MdUI17163	AT5G21080.1	similar to cyclin-related	0.71	-0.61	**-1.32**
MdUI37857	AT1G75310.1	AUL1 | AUL1 (auxin-like 1 protein); heat shock protein binding	0.48	-0.85	**-1.33**
MdUI20163	AT5G60410.2	DNA binding	1.09	-0.24	**-1.33**
MdUI32867	AT1G02150.1	pentatricopeptide (PPR) repeat-containing protein	0.74	-0.60	**-1.33**

MdUI31765	AT1G76350.1	RWP-RK domain-containing protein	0.90	-0.43	**-1.34**
MdUI20633	AT5G43360.1	(phosphate transporter 3; carbohydrate transporter/ phosphate transporter/	0.30	**-1.04**	**-1.34**
MdUI24265	AT1G79540.1	pentatricopeptide (PPR) repeat-containing protein	0.91	-0.44	**-1.36**
MdUI37129	AT1G08280.1	glycosyl transferase family 29 protein / sialyltransferase family protein	1.08	-0.28	**-1.37**
MdUI28653	AT1G67690.1	peptidase M3 family protein / thimet oligopeptidase family protein	0.58	-0.79	**-1.38**
MdUI13303	AT1G79090.1	similar to unknown protein	0.71	-0.66	**-1.38**
MdUI30091	AT3G23640.1	HETEROGLYCAN GLUCOSIDASE 1); hydrolase hydrolyzing O-glycosyl compound	0.98	-0.40	**-1.38**
MdUI09653	AT5G44870.1	disease resistance protein (TIR-NBS-LRR class)	1.22	-0.18	**-1.39**
MdUI12223	AT4G31900.1	chromatin remodeling factor putative	1.00	-0.43	**-1.43**
MdUI14089	AT3G17850.1	protein kinase putative		-0.77	**-1.44**
MdUI14811	AT4G30710.1	similar to unknown protein	0.90	-0.55	**-1.45**
MdUI12743	AT3G28153.1	non-LTR retrotransposon family	1.58	0.12	**-1.46**
MdUI26569	AT2G25737.1	similar to unknown protein	0.71	-0.75	**-1.46**
MdUI07959	AT1G02580.1	EMB173 MEDEA FIS1 MEA | MEA (MEDEA); transcription fa	0.86	-0.61	**-1.46**
MdUI26497	AT1G54410.1	dehydrin family protein	0.86	-0.62	**-1.48**
MdUI30557	AT5G07940.1	similar to dentin sialophosphoprotein-related	1.10	-0.38	**-1.48**
MdUI28737	AT4G05420.1	DDB1A (UV-damaged DNA-binding protein 1A); DNA binding	0.56	-0.99	**-1.55**

Paper 2:

The vacuolar Na^+/H^+ antiporter (NHX1) function in *Rosa rugosa* salt stress tolerance

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ABSTRACT

Rosa rugosa, a salt tolerant candidate, is native to East Asia and presents economical importance as ornamental plant, for fragrance extraction, medicinal and food purposes. *R. rugosa* is a plant with high tolerance for different stress, however, the molecular mechanisms of its behavior under adverse conditions are unclear. Thus, the objective was to identify pattern expression of some genes in response to salt stress. We analyzed changes in transcript level between plants in different salt stress condition (0; 25; 50 and 100 mM NaCl) for long exposure (30 days). In addition, the effect of salt shock stress was availed by exposition to high concentration (200 mM NaCl) for short time (3 h). Relative reverse transcription polymerase chain reaction (RT-PCR) was performed to compare the expression levels of selected differentially expressed genes. Plants in long salt stress exposure showed no signal of stress in the leaves and roots. In addition, the expression of RhNHX1 in *Rosa rugosa* increased in NaCl presence. The transcription of genes EXP4, GPP, NHX1, NAC and DREB increased in the presence of high concentrations of NaCl. In contrast, MYB and TIR decreased the expression level in salt shock treatment. NHX1 presented a high expression level in leaves of plants in both salt stress and salt shock, suggesting that this gene play important role in salt stress tolerance in *Rosa rugosa*. These genes may enable exploration of new avenues for engineering salt tolerance in roses and other member of Rosaceae family.

Keywords: Salinity. Gene expression. Rosaceae. Salt shock. Ion homeostasis. Signal transduction

1. **INTRODUCTION**

The three major abiotic stress that affect plant growth and crop yield are cold, salt and drought (Mahajan, Tuteja 2005). Salt stress has a prominent position due to the strong effect over plant systems and progressive increase of lands with salinity problems. The estimation is that 20% of all cultivated lands are affected by salinity problem (accounting for 10 million hectares of affected land over 100 countries) (Sahz et al., 2006). Soil salinization represents a serious environmental problem to ecological and agriculture systems. It can limit or inhibit plant growth and development, which will also lead to reduction of agricultural productivity and losses of diversity of species.

Plant responses are highly complex and involve changes at molecular, cellular, and physiological levels (Atkinson; Urwin, 2012). Plants can have wild spectrum of response for salinity that will drive for sensitivity or tolerance for different types of salinity stress. The crosstalk of effectors and signaling components are salt stress tolerance determinant. The functional categories of salt tolerance effectors determinants are ion homeostasis, water uptake and transport, long-distance response coordination. The regulatory molecules are signal transduction pathway components. In this group there are transcription factors that regulate salt tolerance effector (amount and timing) (Shavrukov, 2013).

The saline root-zone environment has negatives impacts on osmotic and ionic equilibrium of the cells. The ions, as sodium (Na^+) and chloride (Cl^-) are more common in the saline soil solution and the excess of these induces toxicity, membrane disorganization and inhibition of photosynthesis (Hasegawa, 2013). The classification of types salinity stress are: 1) salt stress (50 mM NaCl) no induce plasmolysis), 2) salt stress/salt shock (80-100 mM NaCl), and maybe induce osmotic shock and 3) salt shock (higher than 150 mM NaCl) causing osmotic shock (Shavrukov, 2013). In high salinity or osmotic shock are induced

hyperionic and hyperosmotic stress effects in plants (Tuteja, 2007). With the develpment time, plants show excessive uptake of these ions that promote injury formation and premature death of the leaves (Hasegawa, 2013). Also, higher Cl levels decrease NO^{-3} uptake. In tomato seedling, after NaCl stress exposure, the concentrations of Na^+ and Cl^- had increased, and the NO_3^- concentrations decreased (Debouba et al., 2007). Roses are traditional classified with sensitive to salinity (Raviv; Blom, 2001).

Sodium transport processes have major roles in plants success in tolerance or adaption to salinity. Na^+ control is typically carried out by process like organellar sequestration, membrane exchangers, and exclusion from photosynthetic tissues and meristems (Queiros et al., 2009; Khan, 2011; Pardo, Rubio, 2011). Some tolerant plants can use Na^+ and Cl^- for osmotic adjustment that then supports cell expansion in growing tissues and turgor in differentiated organs. In addition, the ability of take up and confine Na^+ can facilitates the water uptake. But for efficient vacuolar deposition, it is required higher cost of H^+ pumping.

Several of rose cultivation systems use irrigation with moderately saline water or salinity soil. But the salinity effect depend of type and concentration of salt, cultivation system (soil or hydroponic) and plant species or cultivar (Lorenzo, et al., 2000). In general, rose species exposed to increase in soil salt concentrations shows negative effects over growth (reduction or stop), flower (quality and productivity) and foliage (Cabrera et al. 2009). The elevated salinities of irrigation water in rose reduced the relative chlorophyll concentration and maximal photochemical efficiency of photosystem II (PSII) (Niu et al., 2008). *Rosa chinensis* under stress conditions do not blossom but initiated a dormancy process directly (Jiang et al., 2009). The cultivar ‘Kardinal’ grafted on ‘Natal Briar’ rootstock presented negatively effect in NaCl concentration in NO^{-3} uptake (Massa et al., 2009).

Rosa rugosa (common names: rugosa rose or Japanese rose) is native to East Asia (Bruun 2005). It is an important species used as ornamental plants, for fragrance extraction, as medicinal and food purposes. Recently, several studies also demonstrated that rose extracts have different important functions (Xie Zhang, 2012; Du et al. 2013; Gao et al., 2013; Wang et al., 2013).

This species is related to growing in sand dune of natural form or introduced and it is salt tolerant candidate (Kim et al., 2004). In European coastal area *R. rugosa* was introduced for sand stabilization, for boundaries of pathways, and it was also used as ornamental (Bruun, 2005). Is considered invasive plant with difficult control in the northern Europe because is highly competitive. It has multiple introductions and no loss of genetic diversity. Those are likely to factor as long distance seed dispersal, coastal habitat connectivity and an outcrossing breeding system (Kelager et al., 2013). *Rosa rugosa* most often occurs in sandy or gravelly soils, occasionally on other well-drained substrates and form natural communities in sand dunes, shingle beaches, and on sea cliffs (Bruun, 2005). When daily treated with 0.25 N NaCl, showed no injured and lowest of Na and Cl content in leaf (Dirr 1978). *Rosa rugosa* wild type had a higher resistance to salt stress than the cultivars ('Ziyan'; 'Purple Branch' and 'Zhongke 2') (Yang et al., 2011). The mechanism underlying salt stress tolerance of rugosa rose unclear.

The understanding of the mechanisms of salt tolerance in plants are important for get alternatives solution to salinity problem in agricultural lands (Wahome et al., 2001). Thus, the purpose of this study was to investigate the molecular mechanism of *Rosa rugosa* salt tolerance. We evaluated the effects of long exposure to salinity stress and salt shock in the transcript levels of some stress-related genes. These results provide details of transcript changes of important change related to salt stress tolerance in plants.

2. MATERIAL AND METHODS

2.1 Plant material and growth conditions

Seeds of *Rosa rugosa* were stored in plastic bag with sphagnum at 4^0 C until start germination (approximately 3 month of stratification). After that, plants were transferred to greenhouse and cultivated in Sunshine Mix. Greenhouse temperature condition was25^0C - day/ 18^0 C - night.

2.2 Salinity stress treatment

Long exposure at salt stress

After 3 month of greenhouse cultivation, plants were irrigated with solution of 0; 25; 50 and 100 mM NaCl. Three biological replicates were used for each treatment. The irrigation were performed each two days, during 30 days. After this period, leaves were collected and immediately frozen in liquid nitrogen and then stored at -80^0 C until use.

Salt shock stress

Plants with 6 month growing in greenhouse were irrigated with NaCl (200 mM) or water. After 3h, leaves were collected and immediately were frozen in liquid nitrogen then stored -80 C until use. Three biological replicates were used for each treatment.

2.3 RNA extraction cDNA synthesis

Total RNA was extracted from leaves with the use of RNase Plant Minikit (Qiagen, Doncaster, VIC, Australia), with modification. Before the floral tissues be ground into a fine powder, 10% of polivinilpriralidone was

added to the sample. RNA was quantified using NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA from each sample was treated with DNase I (Invitrogen) and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dt) primer using SuperScript III RT (Invitrogen).

2.4 Quantitative Real Time-PCR for expression analysis

The cDNA was diluted to 30 ng/ul and used for Real-Time PCR reactions in 96-well plates in a 7,300 Real-Time PCR System (App Real-time RT-PCR). Were carried out qRT-PCR on three independent biological replicates each containing three technical replicates using SYBER Greem PCR Master Mix (Applied Biosystems, Foster City, CA). Gene-specific primers from identified genes for real time RT-PCR were designed using the Primer 3 program (http://frodo.wi.mit.edu/) based on the blast consensus sequence from GeneBank. Each RT-PCR reaction (25 ul) contained 10.5 ul water, 0.5 ul 200 nM of forward and reverse primers, respectively, 12.5 ul of 2 x SYBR Green I Master, and 5 ul of diluited cDNA. The amplification program, consisted in one cycle of 95 C for 10 mim followed 95 C for 15 s and 60 C for 1 min. After amplification, a melting a curve analysis was run using the program for one cycle at 95 C for 5 s, 65 C for 1 min, and 95 C with 0 s held in the step acquisition mode, followed by cooling at 4^0 C for 10 s. A negative control without cDNA template was run with each analysis to evaluate the overall specifity. To normalize the total amount of cDNA in each reaction, the rose genes (Table 1) was co-amplified as an internal control using an RhGADPH (EC589884). Data were analyzed using the SDS software from 7,300 Real-time PCR System (Applied Biosystem) based on relative standard curves of PCR efficiency of the target and reference genes.

The expression levels of NAC, NHX1 and EPX4 genes were analyzed in plants salt treated for long exposure to different concentrations of NaCl. In the salt shock experiment were analyzed the NAC, NHX1, EPX4, GPP, DREB, MYB and TIR genes (Table 1).

Table 1: List of primers used for the Real-time RT-PCR

Probe Name	**Genes**	**GenBank accession number**		**Primer sequencing**
MR1	RhNAC	JN857363.1	F	TTCCCACGAGTCACGTATCA
MR2			R	CCCTCAGATGGTCTTGCATT
MR3	RhEPX4	JN857364.1	F	ACGCTCGTGCCACTTTCTAT
MR4			R	GCTCAGAGCAGCAGTGTTTG
MR5	RhNHX	AB199912.1	F	AATCACCGCCCTTTTGATT
MR6			R	AAAAGCACCACAGATGTAGCA
MR7	RrGPP	HM234683.1	F	GGGGAAGAAACTACTGCTGCT
MR8			R	ACACACACCCTCCAGCTTCT
MR9	RhDREB1B	EU784070.1	F	TGGTGGATTTTCAGCTTTCC
MR10			R	TGCCTCGTCTCCTTGAACTT
MR11	RhTIR like	AM075214.1	F	ACCCAAGGGCTTGGTATCTC
MR12			R	TCTCTTCTCAGGGCATGGTAA
MR13	RrMYB	FR828542.1	F	CCTCTACACTCAAATGGGAAG
MR14			R	CATTGATATCCCAGCAGCATC
MR15	RhGAPDH	EC589884	F	GCTGGCAGGTATCCTTTCTG
MR16			R	GGCGACAATATCAGCCAAGT

3. RESULTS AND DISCUSSION

3.1 NaCl Long-term exposure

The effects of salt stress were examined on *R. rugosa* plants submitted to the long salinity exposure treatment during 30-day in order to understanding the mechanism of salt tolerance of this specie. All the *R. rugosa* plants treated with different concentrations of NaCl did not showed signal of stress (Fig. 1A). Plants treated with 50 and 100 mM showed better leaves and root system development (Fig 1 B). When daily treated with 0.25 N NaCl, showed no injured and lowest of Na^+ and Cl^- content in leaf (Dirr, 1978). These results indicate that *Rosa rugosa* is salt tolerant specie.

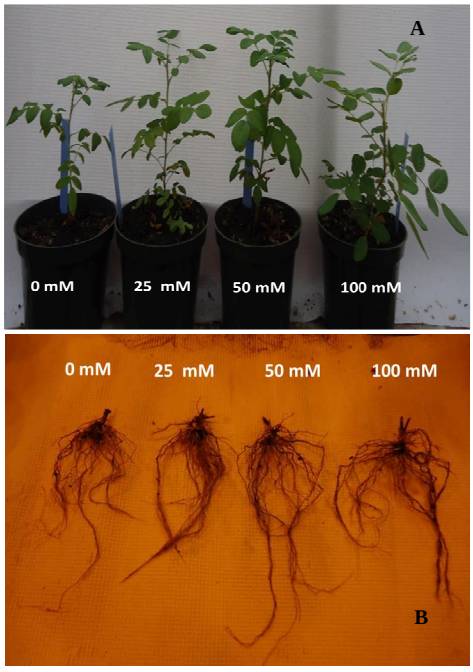

Figure 1*: R. rugosa* plants after 30 days in salt stress. Shoot (A) and root system (B)

Soil salinity occurs naturally and as result of import water management practices associated with intense agriculture can cause substantial salinization. Salt tolerant species after salinity stress imposition have fitness that make with the plants re-establish osmotic and ionic homeostasis. With that, can keep physiological and biochemical reaction to plant growth.

Salt stress induces the expression of many genes in plants. The molecular mechanisms of tolerance for salt stress in *Rosa rugosa* are unclear. RT- PCR-real time analysis were performed to select genes to identify the pattern of responses of these genes to salt stress (Fig 2). The transcript level of NAC increased in plants treated with NaCl more the double of the expression level of control (Fig 2). NAC are involved in different plant biological events as development, biotic and abiotic stress response. The crosstalk between abiotic and biotic stress signaling network, involve NAC regulation (Wang, Dane 2013). Tobacco plants over-expressed DgNAC1 (*Dendronthema grandiform* NAC) exhibited a markedly increased tolerance to salt (Liu et al.; 2011). The higher-level expression of RhNAC genes under NaCl treatments suggests that the NAC transcription factors play important roles in salt stress tolerance and adaptation of *Rosa rugosa*.

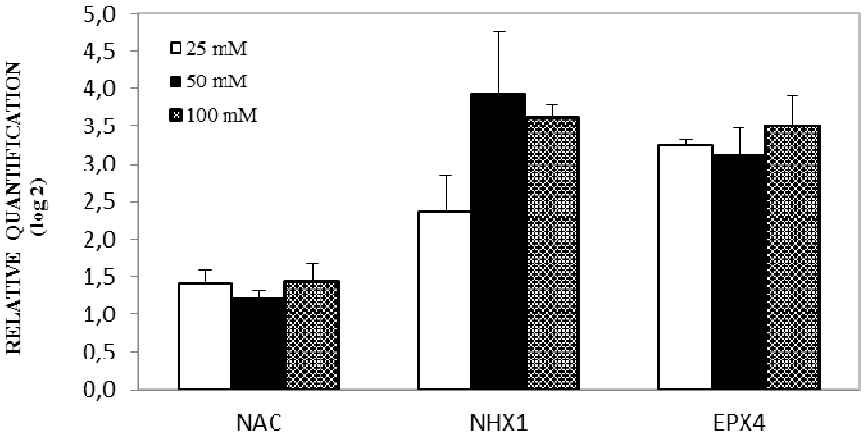

Figure 2: Relative quantification of gene expression in plants treated with different concentrations of NaCl (0, 25, 50 and 100 mM).

The RhNHX1 gene showed increased in its expression in *Rosa rugosa* plants growing under salt stress condition (Fig 2). A vacuolar Na^+/H^+ antiporter gene in *Rosa hybrid* (RhNHX1) had expression increased in the presence of NaCl (Kagami, Suzuki 2005). Plants treated with 25 mM of NaCl had transcript level of 2.37 (log_2), four times more that control. In higher concentration, 50 and 100 mM, showed highest level of expression, 3.92 and 3.62 (log_2) respectively. These values represented that plants treated with high concentration increased more the 12 times the expressions levels of NHX1 than control (no NaCl treated plants).

The Na^+/H antiporter is transmembrane transport proteins that exclude Na+ from the cytosol in exchange for H^+. Intracellular Na^+/H^+ (NHX) are well-known plant cation/proton antiporters, which perform Na^+ and K^+ compartmentalization into the vacuole. It represent an important role in cellular pH, Na^+ (compartmentalization) and K^+ (homeostasis) concentrations (Bassil et al., 2011; Pires et al., 2013; Yokoi et al., 2002). The NHX is a salt

tolerance determinant. This protein, promote the ion homeostasis in saline environments. AtNHX proteins in Arabidopsis acts in facilitate Na^+ ion compartmentalization and maintain intracellular K^+status (Yokoi et al., 2002). Over expression of NHX1 in rice, *Arabidopsis* and soybean was correlated with salt tolerance in these species (Apse et al., 2003; Fukuda et al., 2011; Liu et al., 2010).

Sodium concentration in the medium had positive relation with sodium absorption in roses (Lorenzo et al., 2000; Massa et al., 2008). Sodium is reported to play an important role for the stimulation of the electrical conductivity (Ec) in environments in which a high salinity level (NaCl) occurs in the irrigations water source. Under higher sodium concentration, the nitrate, potassium and phosphate uptake decrease (Lorenzo et al., 2000).

The RhEXPA4 expression level increased when rose was treated with NaCl. This suggests that RhEXPA4 conferred in *Arabidopsis* tolerance to abiotic stresses through modifying cell expansion and plant development (Lu et al., 2013). In addition, *RhEXPA4* was involved in the regulation of dehydration tolerance during the expansion of rose petals (Dai et al., 2012). Other three kinds of RhEXP 1-3 have been identified: RhEXP1 was described as involved in expansion growth of rose petal (Takahashi et al., 2007). The expansions genes act in the cell wall (CW) causing loosing and extension (Abuqamar et al., 2013). This group of genes respond to different biotic and abiotic stress. Cell wall modifying proteins mediate plant acclimatization to biotic and abiotic stresses (Sasidharan et al., 2011). The Arabidopsis expansin-like A2 **(**EXLA2**)** shows important response to various biotic (pathogenesis of necrotrophic pathogens) and abiotic stress (salt, cold, and ABA). And in mutant (exla2 *Arabidopsis*) which is more sensitive to stress (Abuqamar et al., 2013). This indicates that expansions contribute significantly to plant response to stress and impact signaling pathways that regulate gene expression.

To plants tolerate or adapt to salinity, mechanisms as Na^+ vacuole compartmentalization are very important, this adaptation make the plant keep growing in salinity conditions. When the plants are in salt condition they need to coordinate several genes expression to cell get Na^+ homeostasis (Yokoi, Quintero et al., 2002).

3.2 Salt-shock

Salt shock is an extreme form of salt stress (exposure to a high salt concentration by single application of salt). It is represented as a comparison of the 3 h NaCl (200 mM) treatment, since were generally the most responsive to stress at this point. This suddenly exposure induces osmotic shock as primary phases of the stress. In this condition, plants need a strong osmotic adjustment to survive the effects of this salt shock. The second phase is related by ionic component becomes gradually (Shavrukov, 2013). Plants induced expression of different groups of the gene in response to stress as: 1) signal transduction (includes genes encoding transcription factors), 2) enzymes, in response to plant oxidation stress, 3) osmoprotectants and 4) proteins related to water stress. It was analyzed some rose's genes responses during salt shock.

After 3h under salt stress, rose plants showed double of RhEXPA4 expression compared with plants in normal condition. This gene is responsive to osmotic shock and acts in salt tolerance. β-expansin protein levels were higher in a salt-resistant maize cultivar than the levels in a sensitive cultivar. β-expansin transcript abundance was induced in *Sorghum bicolor* under saline conditions (Buchanan et al., 2005).

Galactose-1-phosphate phosphatase (GPP) showed a small increase of mRNA level at 3 h of salt shock. Expression of some genes in response to osmotic shock can be registered within minutes of salt shock. Maybe 3h was long time after stress and *Rosa rugosa* have fast change expression level before

this time, the gene expression backed to normal levels. GPP plays a central role in ascorbic acid biosynthesis in higher plants (Rosa et al., 2013). Ascorbic acid is one of the most important and abundantly occurring water soluble antioxidants in plants. Some studies demonstrated that the use of exogenous application of salicylic acid through the rooting medium or spraying in the shoot can alleviate salt stress (Dehghan et al., 2011; Munir;Aftab 2011; Rafique et al., 2011). *Arabidopsis* mutants (ascorbate-deficient) were more sensitivity to salinity stress (Huang, 2005).

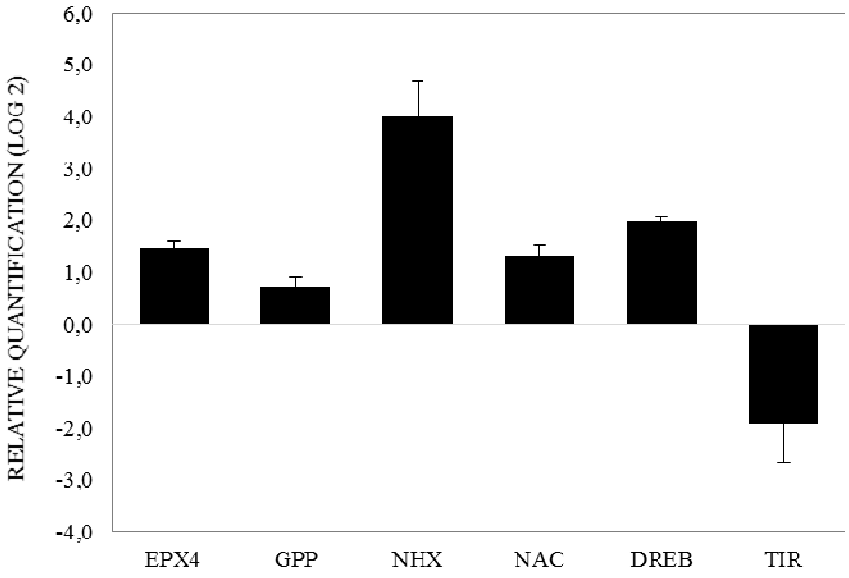

Figure 3: Relative quantification of genes expression in rugose rose leaves during salt shock stress for 3 hours.

In this study, the NAC transcription factor was rapidly induced by salt stress. The mRNA level of RhNAC (*Rosa hybrid* NAC) increased after 3h of salt shock treatment with NaCl (200 mM) (Fig 3). NAC is stress-responsive transcription factor. The gene ENAC1 **(**early NAC-domain protein induced by

abiotic stress 1) in rice, also accumulated in the first hours of stress (Sun et al., 2012).

The RhNHX1 showed higher-expression level in plants treated with high NaCl concentration (salt-shock). The Na+/H+ antiportes play a important role in the compartmentalization of cytoplasmatic Na+ into vacuoles, and it expression is increased in the presence of NaCl (Kagami, Suzuki 2005). These results suggest that vacuolar Na+/H+ antiporters is an element of rose salt tolerance, that plays an important role in the first hours of salt-shock. AtNHX1 and AtNHX2 expression in Arabidopsis increases in response to high salt stress through an ABA-dependent process (Yokoi et al., 2002). Also, the Arabidopsis Na^+/H^+ antiporters (NHX1 and NHX2) control the vacuolar pH and K^+ homeostasis to regulate growth, flower development, and reproduction (Bassil, Tajima et al. 2011). Treatment with higher concentrations of NaCl and KCl increased the transcript levels of OsNHX1 in rice roots and shoots(Mahasal et al., 2011, Fukuda et al., 2011, Kinclová-Zimmermannová et al., 2004, Chen, Polle 2010) .

The transcript level of DREB1 was higher after salt shock. DREB1 is an important TF (that regulates various abiotic stress-responsive genes) by the activation of different downstream gene expression. In the study, *LlaDREB1b* was induced by salt and drought stresses (Gupta et al., 2013). Overexpression of StDREB1 increases tolerance to salt in transgenic potato plants (Bouaziz et al., 2013). Also, *Arabidopsis* plants overexpressing MsDREB2 were more tolerant to salt stress and other abiotic stress (cold, heat, drought and ABA). These transgenic plants exhibited increased root growth, leaf growth and proline level. Also, reduced water loss and stomatal aperture (Zhao et al., 2013).

Rosa rugosa salt shock stressed plants showed in decrease of expression level of brp6 gene putative for TIR-NBS-LRR resistance protein. The same

behavior was related to *Thellungiella halophile*, being TIR down-regulated in response to drought and short-term salinity stress, but up-regulate in long-term (Gao et al., 2008, Wong, Li et al., 2006). These same genes are shared between various stresses, biotic and abiotic (Zhu et al., 2010). This illustrated the complex mechanism of stress response adaptation.

3. CONCLUSION

In conclusion, *Rosa rugosa* leaves did not show the salt injury symptoms after 30 days of salinity stress or 3h of salt shock. These results indicate that this species had a great resistance to salinity stress. The tolerance of *R. rugosa* to salt stress may be is related to high expression levels of NHX1 in both long exposure and salt shock.

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