IMPACT OF GRAFTING ON COLD RESPONSIVE GENE EXPRESSION

IN *CITRUS UNSHIU*

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IMPACT OF GRAFTING ON COLD RESPONSIVE GENE EXPRESSION IN *CITRUS UNSHIU*

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IMPACT OF GRAFTING ON COLD RESPONSIVE GENE EXPRESSION

IN *CITRUS UNSHIU*

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Ying Huang, daughter of Jianming Huang and Lingfeng Gu, was born on October 04, 1982, in Taiyuan city, Shanxi Province, People's Republic of China. She graduated from Nanjing No.9 High School in 2001. She entered Yangzhou University in September 2001 and earned a Bachelor of Science degree in Biotechnology in July 2005. In August 2007, she enrolled in Auburn University to pursue a Master Degree in the Department of Horticulture. She married Ziyuan Mi in December 2005.

THESIS ABSTRACT

IMPACT OF GRAFTING ON COLD RESPONSIVE GENE EXPRESSION

IN *CITRUS UNSHIU*

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Commercial citrus, the most economically important fruit crop in the world, is sensitive to low temperatures. *Poncirus trifoliata* (L.) Raf., a close relative of Citrus species, has been widely used as a cold hardy rootstock for citrus production in low temperature environments. In this study, the expression profiles of 16 cold responsive genes in leaves of *Poncirus trifoliata*, Satsuma mandarin (*Citrus unshiu),* and *Citrus unshiu* scions grafted on *Poncirus trifoliata* rootstock were compared following exposure to cold shock or cold acclimation treatments. Using relative quantitative (RQ) real-time RT-PCR analysis, several genes such as UDP-glycosyltransferase (UGT), trehalose-6-phosphate synthase, chalcone isomerase-like protein, proline transporter, plasma membrane intrinsic protein (PIP) and nitrate transporter, involved in osmotic and oxidative stress, were upregulated in response to cold, suggesting an active role for secondary metabolites with respect to osmotic balancing, except for lipoxygenase and

phosphomannomutase which were down-regulated in response to low temperature. Species-specific expression profiles were detected especially in response to cold acclimation. Levels of auxin responsive GH3 transcript increased to high levels in Poncirus, but decreased in Satsuma. Levels of UGT did not change in Poncirus, while high increases occurred in Satsuma. Similarly levels of nitrate transporter increased 10 fold in Poncirus, more than 100 fold in Satsuma during cold acclimation. Differences in down regulation of transcript levels were observed for lipoxygenase, with higher levels in Satsuma as compared to Poncirus following cold acclimation.

Grafting on Poncirus rootstock did exert a significant effect on gene expression of the Satsuma scion. Expression of genes involved in signal transduction and regulation of gene expression, such as RING finger protein, and auxin-responsive GH3 was positive and increased in grafted as compared to non-grafted Satsuma plants during cold acclimation, while expression of genes involved in nitrate transport, PIP, trehalose 6-P synthase, UGT, were negatively regulated by the rootstock in that expression levels in the grafted Satsuma were reduced as compared to the nongrafted plants during cold acclimation. These results indicate that cessation of growth is more rapidly invoked in cold hardy species than cold sensitive species and that through grafting this response can be activated or induced in the scion. Furthermore, signal transduction between scions and rootstocks leads to significant changes in gene expression during cold stress.

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I. LITERATURE REVIEW

INTRODUCTION

Many environmental conditions can affect plant growth. Temperature (high and low), salinity and drought are the major environmental factors that change from season to season and undergo daily fluctuations. All plant processes are affected by temperature. Consistent with this, the expression of a large number of specific genes is up-regulated or down-regulated during cold acclimation or cold shock in plants. The fundamental question during low temperature is how plants perceive the low but nonfreezing temperatures that activate cold acclimation or cold shock responses. New findings in the past years suggest that changes in membrane fluidity, cytoskeleton rearrangement, and calcium influxes are among the earliest events taking place in many plants upon exposure to low nonfreezing temperatures, especially in Arabidopsis. Many genes have been isolated in response to low temperature stress in herbaceous plants, while very limited information is known for woody plants, such as citrus.

Since most commercially important citrus varieties are cold sensitive and therefore susceptible to freezing, *Poncirus trifoliata* (L.) Raf., an interfertile Citrus relative that can tolerate temperatures as low as −26°C after acclimation, is being used for improving cold tolerance in citrus rootstocks and as a source for the identification of cold-regulated genes (Talon and Gmitter 2008). This hardy trifoliate orange usually grows as a small tree

about 2-5 meters in height. It produces white flowers which typically appear in spring. As compared to citrus, Poncirus is tolerant to cold and many diseases. In general, many studies have been performed comparing expression in sensitive and resistant varieties. It has been shown, for example, that expression of a CBF (C-repeat binding factor) and one of its targets, COR19 (Cold Responsive gene 19) accumulated both earlier and to higher levels in Poncirus. Moreover, COR19, COR11, and COR15 were found to belong to an unusual group 2 LEA (Late Embryogenesis Abundant) gene family responsive to low temperature (Cai et al. 1995). Other up-regulated transcripts that may play a role in cold sensitivity are a novel RING-H2 finger gene, AP2 (Apetala 2) domain containing genes and CLT (Citrus Low Temperature), and a homologue of a low-temperature-responsive gene from Arabidopsis. Genes differentially expressed in the chilling response have mostly been related to lipid membrane and cell wall enzymes, to main regulators of secondary metabolism and hormonal homeostasis, and to oxidative and general stress responses (Sapitnitskaya et al. 2006).

1. Effect of low temperature on plants

Plants encounter a wide range of environmental stress conditions and have evolved mechanisms to tolerate these conditions through various physiological adaptations. Low temperature is one of the most important environmental factors affecting plant growth. It adversely affects the growth and development of plants and significantly constraints the spatial distribution of plants and agricultural productivity. Every year, losses of different crops due to low temperature amount to billions of dollars (Pearce 1999). In addition, it

affects the quality of the produce and storage of fruits and vegetables (Graham and Patterson 1982).

Physiologically, cold stress prevents the expression of the full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses. Cold acclimation is a process by which plants acquire freezing tolerance upon prior exposure to low non-freezing temperatures (Fowler and Thomashow 2002). Most temperate plants can cold-acclimate and acquire tolerance to extracellular ice formation in their vegetative tissues. Structural and biochemical analyses identified several consistent features of cold-acclimation which are thought to have an important role in cold-tolerance. One of the characterized plant temperature responses is the ability of plants to increase their freezing tolerance following a period of low nonfreezing temperatures. However, temperature impinges on all aspects of plant metabolism and physiology, and thus there are probably many important changes in response to cold, and some of them are yet unknown.

Biologically, cold acclimation is complex, involving the remodeling of cell and tissue structures and the reprogramming of metabolism and gene expression (Viswanathan and Zhu 2002). These changes include the increased expression of many genes (Thomashow 1999), the reduction or termination of growth, transient increases in abscisic acid (ABA) concentrations, changes in membrane lipid composition, the accumulation of compatible osmolytes, and increased levels of antioxidants (Browse and Xin 2001). In short, almost every cellular process is altered during cold acclimation.

2. Species of Citrus

Citrus is a common term and genus of flowering plants in the family Rutaceae, originating in tropical and subtropical Southeast Asia. The plants are large shrubs or small trees, reaching 5–15 m in height, with spiny shoots and alternately arranged evergreen leaves with an entire margin. The flowers are solitary or in small corymbs, each flower 2–4 cm in diameter, with five (rarely four) white petals and numerous stamens; they are often very strongly scented. The fruit is a hesperidium, a specialized berry, globose to elongated, 4–30 cm long and 4–20 cm diameter, with a leathery rind surrounding segments or "liths" filled with pulp vesicles. The genus is commercially important as many species are cultivated for their fruit, which is eaten fresh or pressed for juice (Page, 2008; http://en.wikipedia.org/wiki/Citrus).

Many of the named species are clonally-propagated hybrids. Cultivated citrus may be derived from as few as four ancestral species. Numerous natural and cultivated origin hybrids include commercially important fruit such as the orange, grapefruit, lemon, some limes, and some tangerines. The closely related genus *Fortunella,* and perhaps also Poncirus and the Australian genera *Microcitrus* and *Eremocitrus*, are included in Citrus (Page, 2008; http://en.wikipedia.org/wiki/Citrus).

3. Physiological basis of cold injury

Freezing injury in most plants results largely from severe cellular dehydration that occurs from ice formation in intercellular spaces. Since the chemical potential of ice is less than that of liquid water, there is a decrease in water potential outside the cell. Consequently, water moves from inside the cell to the intercellular spaces leading to

cellular dehydration (Fig. 1 Sharma et al. 2005). The process of ice formation in the extracellular spaces was monitored by McCully (2004) in snow gum, a frost-tolerant plant. During frosts, the tissue volume reduced to one-third of its natural volume with the growth of large amounts of extracellular ice in an expanding region of compact tissue known as “fault zone”. Thus the extracellular ice from these zones was reabsorbed into the cells in the form of water, thus restoring their volume and function.

The plasma membrane is the primary site of chilling injury. It undergoes qualitative and quantitative modifications in its lipid component under cold stress. Plasma membrane changes in fluidity, protein structure stability and flexibility are due to alterations in the protein and the lipid composition. Among the various lipid molecules in the bilayer of the membrane, the percentage of phospholipids increases (Uemura et al. 1995). Freezing injury causes lamellar-to-hexagonal-II phase transition in plasma membrane. Molecules of 16:0/16:0 phosphatidyl choline, phosphatidyl glycerol or cerebrosides change from a fluid liquid crystalline phase to the more rigid gel phase, based on their phase transition temperatures (Uemura et al. 1995). The gel phase prevents proper functioning of integral membrane protein in maintaining an efficient permeability barrier, hence, molecular packing defects develop at the boundaries between the distinct liquid crystalline and gel phase micro domains. The gel-phase may inhibit membrane function, and non-lamellar lipids may destabilize the bilayer structure (Williams 1990). These defects promote leakiness of ions across the membrane that can lead to cell dysfunction. An increase in the degree of membrane lipid unsaturation, with accompanying increase in bilayer fluidity, is an important determinant of cold acclimation (Steponkus et al. 1993).

4. Cold stress signaling

Cellular membranes are fluid structures, and cold temperatures can reduce their fluidity, causing increased rigidity. Plant cells can sense cold stress through low temperature-induced changes in membrane fluidity, protein and nucleic acid conformation and/or metabolite concentration. Recently, plasma membrane rigidification has been shown previously to induce COR genes and result in cold acclimation in *alfalfa* and *Brassica napus* (Sangwan et al. 2001). These results add support to the notion that plant cells can sense cold stress through its membrane rigidification effect. Cold-induced Ca^{2+} increase in the cytosol can also be mediated through membrane rigidification-activated mechano-sensitive or ligand-activated Ca^{2+} channels. Subsequently, calcium signal amplification and phospholipid signaling might be involved in cold-stress signaling (Chinnusamy et al. 2006).

Secondary signals, such as ABA, as evidenced by the los5 (low expression of osmotically responsive genes) mutant, and reactive oxygen species (ROS) can also induce Ca^{2+} signatures that impact cold signaling (Komatsuet al. 2007). The los5 mutant is impaired in molybdenum co-factor (MoCo) sulphurase, which synthesizes MoCo for abscisic aldehyde oxidase, and is thus defective in ABA synthesis. The los5 mutant showed significant reduction in cold- and salt/drought-induced expression of COR genes (RD29A, COR15, COR47, RD22, and pyrroline-5-carboxylate synthetase) and is unable to acquire freezing tolerance. Thus, ABA plays a significant role in cold acclimation of plants. ABA may transduce cold-stress signals through second messengers such as H_2O_2 and Ca^{2+} (Xiong et al. 2001). ROS accumulate in cells challenged with various abiotic stresses, and they appear to have a strong influence on cold regulation of gene expression.

The Arabidopsis fro1 (frostbite1) mutant, which constitutively accumulates high levels of ROS, exhibits impaired expression of COR genes and hypersensitivity to chilling and freezing. Besides effect on calcium signatures, ROS signals can also exert their effects directly through the activation of mitogen-activated protein kinases (MAPKs) and redox-responsive proteins, such as transcription factors and protein kinases (Xiong et al. 2001). Cold and other abiotic stresses regulate the expression and activity of various kinases of MAPK pathways, which suggests that MAPK cascades act as a converging point in abiotic stress signaling (Chinnusamy et al.
2004). Under cold stress, ROS activates the AtMEKK1/ ANP1 (MAPKKK)-AtMKK2 (MAPKK)-AtMPK4/6 (MAPK) MAPK cascade that is necessary for cold acclimation in plants (Teige et al. 2004).

Low temperature affects water and nutrient uptake, membrane fluidity and protein and nucleic acid conformation, and it drastically influences cellular metabolism either directly by reducing the rates of biochemical reactions or indirectly through gene expression reprogramming. Metabolic profiling revealed that cold acclimation increases ~75% of the 434 metabolites detected in Arabidopsis plants (Kaplan et al. 2004). In addition to their role as osmoprotectants and osmolytes, certain metabolites (individual metabolites or redox state) induced during cold acclimation might act as signals for reconfiguring gene expression. For example, cold stress induces the accumulation of proline, a well known osmoprotectant. Microarray and RNA gel blot analyses have shown that proline can induce the expression of many genes, which have the proline-responsive element (PRE, ACTCAT) in their promoters (Satoh et al. 2002).

5. Transcriptional regulation

5.1 ICE1–CBF transcriptional cascade

Cold stress induces the expression of CBFs (also known as dehydration-responsive element-binding protein 1s or DREB1s), which can bind to cis-elements in the promoters of COR genes and activate their expression. Analyses in transgenic plants have shown that ectopic expression of CBFs is sufficient to activate the expression of COR genes and induce cold acclimation (Stockinger et al. 1997). CBFs regulate the expression of genes involved in phosphoinositide metabolism, transcription, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone metabolism and signaling and many other known or presumed cellular protective functions (Lee et al. 2005). Microarray analysis of transgenic Arabidopsis plants expressing CBFs revealed a constitutive expression of downstream cold-responsive transcription factor genes RAP2.1 and RAP2.7, which might control subregulons of the CBF regulon (Fowler and Thomashow 2002). Thus, CBFs play a pivotal role in gene regulation during cold acclimation in evolutionarily diverse plant species.

In Arabidopsis, ICE1 (Inducer of CBF Expression1), a MYC-type basic helix–loop–helix transcription factor, can bind to MYC recognition elements in the CBF3 promoter and is important for the expression of CBF3 during cold acclimation. The ice1 mutant is defective in the cold induction of CBF3 and is hypersensitive to chilling stress and incapable of cold acclimation. Constitutive overexpression of ICE1 enhanced the expression of CBF3, CBF2 and COR genes during cold acclimation, and increased freezing tolerance of the transgenic Arabidopsis. ICE1 is constitutively expressed and localized in the nucleus, but it induces expression of CBFs only under cold stress. This

suggests that cold stress-induced posttranslational modification is necessary for ICE1 to activate downstream genes in plants (Chinnusamy et al. 2003) (Fig. 2). The cold induction of genes involved in calcium signaling, lipid signaling or encoding receptor-like protein kinases are also affected by the ice1 mutation (Lee et al. 2005). Bioinformatics analysis of microarray data on the cold-responsive transcriptome of wild type and mutants or of transgenic Arabidopsis plants overexpressing specific transcription factors led to the prediction of a cold-acclimation transcriptional network. In this network, ICE1 is predicted to be a transcriptional inducer of CBFs (CBF1–CBF3), ZAT12, NAC072 and the constitutively expressed transcription factor HOS9 in Arabidopsis (Benedict et al. 2006).

5.2 Negative regulators of the CBF regulon

Feedback repression of transcription factors that regulate cold-responsive gene expression appears to be a key to maintaining an optimal cold-induced transcriptome. Molecular analysis of a cbf2 null mutant of Arabidopsis suggested that CBF2 is a negative regulator of CBF1 and CBF3 expression during cold acclimation (Novillo et al. 2004). Conversely, CBF3 might negatively regulate CBF2 expression, because reduced expression of CBF3 in the ice1 mutant is accompanied by an enhanced expression of CBF2 (Chinnusamy et al. 2003). These results suggest that cross-regulation and, perhaps, self-regulation have an important role in the expression levels of CBFs during cold acclimation (Fig. 2). Furthermore, CBFs are negatively regulated by an upstream transcription factor, MYB15 (an R2R3-MYB family protein) in Arabidopsis. MYB15 is expressed even in the absence of cold stress, and MYB15 can bind to MYB recognition

elements in the promoters of CBFs (Fig. 2). Interestingly, ICE1 can negatively regulate MYB15 as indicated from the increased MYB15 transcript level in ice1 mutant compared with wild-type plants under cold stress (Agarwal et al. 2006).

In Arabidopsis, a cold-induced C2H2 zinc finger transcription factor gene, ZAT12, also appears to function as a negative regulator of CBFs (Fowler et al. 2005). Transgenic overexpression of ZAT12 decreases the expression of CBFs under cold stress. Transcriptome analysis of ZAT12-overexpressing Arabidopsis revealed that the ZAT12 regulon consists of at least 24 COS (Cold Standard Set) genes, of which nine are cold-induced and 15 are cold-repressed genes (Vogel et al. 2005). Molecular analysis of the los2 mutant of Arabidopsis revealed that another C2H2 zinc finger protein, ZAT10/STZ, might act as a negative regulator of CBF-target genes. LOS2, a bifunctional enolase, binds to the MYC recognition elements in the ZAT10 promoter in vitro and los2 mutant plants showed an enhanced and more sustained induction of ZAT10 during cold stress (Lee et al. 2002). Thus LOS2 appears to be a negative regulator of ZAT10 expression during cold acclimation. CBFs might have a role in mediating or modulating cold-stress induction of ZAT10 because transgenic plants overexpressing CBF3 showed an enhanced expression of ZAT10 (Maruyama et al. 2004). Furthermore, impairment of CBF3 expression caused by the ice1 mutation also led to a significant decrease in the cold induction of ZAT10 (Chinnusamy et al. 2006). Thus, ZAT10 could be a subregulon of CBFs and might regulate a subset of genes involved in cold acclimation (Fig. 2).

5.3 non-CBF regulons

Microarray analysis has shown that CBFs regulate only ~12% of the cold-responsive transcriptome (Fowler and Thomashow 2002). Hence, non-CBF transcription factors might regulate the remaining large portion of cold-responsive genes. In soybean, the cold-stress inducible C2H2-type zinc finger protein SCOF1 appears to induce the expression of COR genes, probably by enhancing the DNA binding activity of the cold-inducible basic leucine zipper transcription factor, G-Box-binding factor 1 (Kim et al. 2001). By employing a genetic screen for deregulated expression of the PRD29A::LUC reporter gene, two constitutively expressed transcription factors, HOS9 (a homeodomain protein) and HOS10 (an R2R3-type MYB), were identified. hos9 mutant plants are less tolerant to freezing both before and after cold acclimation.Microarray analysis revealed that the HOS9 regulon is distinct from that of the CBFs (Zhu et al. 2004). Moreover, the hos10-1mutant has much less freezing tolerance despite an enhanced expression of some COR genes under stress. Furthermore, HOS10 might regulate ABA-mediated cold acclimation (Zhu et al. 2005). Overexpression of a cold-stress-inducible rice transcription factor, MYB4 (an R2R3-type MYB), in transgenic Arabidopsis enhances the expression of COR genes, proline levels and freezing tolerance (Vannini et al. 2004). Additionally, the cold-, drought- and salt- upregulated OsMYB3R-2 (an R1R2R3 MYB) appears to regulate positively cold and other abiotic stress tolerance by a CBF-independent pathway in rice (Dai et al. 2007).

6. Post-transcriptional regulation

Post-transcriptional regulation of gene expression is mediated through pre-mRNA splicing, nucleocytoplasmic transport, RNA stability, translation, post-translational modification, and proteolysis. . In wheat, two early COR genes (a ribokinase and a C3H2C3 RING-finger protein) were shown to be regulated by intron retention in their mature mRNAs under cold stress (Mastrangelo et al. 2005). The DEAD-box family of RNA helicases is involved in RNA metabolism, such as transcription, RNA processing, RNA decay and nucleocytoplasmic transport. A role for such a helicase in mRNA export and plant abiotic stress responses was revealed from the analysis of the los4 (low expression of osmotically responsive genes 4) mutant of Arabidopsis (Gong et al. 2002). Cold induction of CBF3 is blocked, while that of CBF1 and CBF2 is delayed in the los4 mutant, and thus, the mutant is impaired in the cold induction of COR genes. Consistent with the cold-sensitive phenotype of los4-1 mutant,mRNAexport is significantly lower in los4-1 under both normal and cold-stress conditions. By contrast, the cold-tolerant but heat-sensitive cryophyte/los4-2 mutant shows normal mRNA export under cold stress but is defective in mRNA export from the nucleus at warm temperatures. These results suggest that the LOS4 RNA helicase is crucial in mRNA export and important in the regulation of CBF and COR gene expression (Gong et al. 2005).

The regulatory small RNAs such as microRNAs (miRNAs) and short interfering RNAs (siRNAs) play a vital role in the post-transcriptional gene regulation. Cold and other abiotic stress-regulated regulatory RNAs have been identified in Arabidopsis (Sunkar and Zhu 2004), which will help in understanding post-transcriptional gene regulation during abiotic stresses.

Although ICE1 is constitutively expressed, it activates CBF gene expression only upon cold treatment (Chinnusamy et al. 2003), which suggests that ICE1 requires either interaction with additional factors induced by cold or post-translational regulation under cold stress for its activity.

7. Prospects for modifying freezing and tolerance

One approach to improving freezing-tolerance would be to modify factors regulating acclimation (Thomashow 1999). This is a powerful approach when applied to freezing-adapted species. The *Arabidopsis thaliana* transcription factor, CBF1, has a central role in acclimation, binding to *cis*-acting regulatory elements of cold-expressed genes (Stockinger et al. 1997). Constitutive expression of CBF1 in *A. thaliana* confers constitutive freezing-tolerance (Jaglo-Ottosen et al. 1998). However, this approach will not necessarily work with species having no innate capacity to acclimate, since they may be impaired in the functional as well as the regulatory components of acclimation.

In susceptible species, a small improvement in freezing-tolerance may be economically important and achievable by manipulating a single aspect of structure or physiology. Thus, an alternative approach for species that cannot acclimate is to try to increase freeze-avoidance. Where plants do avoid freezing by supercooling substantially, the mechanisms involved are not fully understood but they include structural features (Wisniewski and Fuller 1999) that could prove difficult to manipulate with precision by genetic engineering.

Susceptible species can freeze extracellularly, and hence it is possible to aim to improve their tolerance of the consequent dehydration. Small but important

improvements might be achieved by aiming to alter just one area of structure or biochemistry that might be needed for tolerance. A limitation in choosing this area is that the subcellular target for damage in susceptible species is, at present, only speculative. Also, tests of the effect of changes in single areas of function have only been made in non-acclimated plants of species that do, nevertheless, have the capability to acclimate to freezing, so the results are only an indication of what may be possible with susceptible species. As an example, since membrane stability is a key factor in tolerance this is a possible target for improvement (Pearce 2001).

8. Further study

Citrus is an economically important crop throughout the world, and its productivity is seriously affected by low temperature (Porat et al. 2002). Traditional breeding methods of major crops for improving freezing tolerance have had limited success in commercial Citrus species (Yelenosky 1985). An alternative approach is to identify and characterize important cold induced genes and introduce these genes into susceptible crops to enhance freezing tolerance (Owens et al. 2002). *P. trifoliata* is a popular rootstock used in the citrus industry to impart greater cold tolerance to the scion (Yelenosky 1985). We will use molecular biology technologies to enhance freeze resistance. Molecular analysis has focused on genes expressed during cold acclimation. Expressions of a large number of specific mRNAs and proteins are up-regulated and some of them are down-regulated, through comparative studies between species or cultivars differing in cold-acclimation.

In order to characterize a putative regulatory molecular response to cold acclimation, our project is investigation of *Poncirus trifoliata* rootstock on cold-responsive gene

expression patterns in *Citrus unshiu*; and development of full length clones of genes differentially expressed under low temperature stress conditions in *Poncirus trifoliata* and *Citrus unshiu.*

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II. INTRODUCTION

Citrus is one of the most important and widely grown fruit crops, with total global production reported to be 105.4 million tons in 2004-2005 (Food Agriculture Organization of the United Nations 2006). Citrus producing areas of the United States are occasionally subjected to freezing temperatures that can cause yield losses of millions of dollars. The losses to the Florida Citrus Industry alone that resulted from a freeze in 1980 were estimated to be more than US $ 1 billion (Tignor et al. 1998). Several other citrus production areas are similarly subject to high risk of low temperatures. Thus, this abiotic stress has one of the most economically important impacts on plant growth (Jia et al. 2004), and in addition, on fruit quality and quantity. *Poncirus trifoliata* L.(Raf.), an interfertile citrus relative which can withstand temperatures as low as −26 ^{o}C when cold-acclimated, is being used for improving cold tolerance in citrus rootstocks and as a source for the identification of cold-regulated genes (Yelenosky 1985).

Many plants can acclimate to freezing by a pre-exposure to low but nonfreezing temperatures, a process called cold acclimation (Fowler and Thomashow 2002). The cold adaptation process involves a number of biochemical and physiological changes, including increased levels of sugars, soluble proteins, proline, organic acids, alterations in lipid membrane composition (Thomashow 1990; Hughes and Dunn 1996), and the

activation of ion channels (Knight et al. 1996). Moreover, this process simultaneously induces alterations in the protein synthesis pattern as well as changes in gene expression (Guy 1990). Several COR (Cold Responsive) proteins are involved in membrane stability (Thomashow 1998). The expression of cold inducible genes is necessary to elevate levels of freezing tolerance (Knight et al. 1999). In Arabidopsis, a family of transcription factors known either as C repeat-binding factor (CBF1, CBF2, and CBF3) (Stockinger et al. 1997; Gilmour et al.1998) or dehydration-responsive element-binding factor (DREB1B, DREB1C, and DREB1A) (Liu et al. 1998) has been identified. The binding of the CBF transcription factor to the C-repeat/dehydration response element (*CRT/DRE*-motif) can activate the coordinate expression of several COR genes (Jaglo-Ottosen et al. 1998). Expression of CBF in transgenic plants resulted in the induction of genes encoding cryoprotective proteins and enzymes involved in the synthesis of low-molecular weight cryoprotectants such as proline and raffinose, which enhance freezing tolerance (Jaglo-Ottosen et al. 1998; Liu et al. 1998). Expression of the CBF regulon also results in an increase in drought and high salinity tolerance (Kasuga et al. 1999). Recent expression analysis of cold induced genes in *P. trifoliata* showed that genes induced in *P. trifoliata* were similar to those in Arabidopsis, indicating that similar pathways are present and activated during the early stages of exposure to low temperatures (Sahin-Cevik and Moore 2006; Champ et al. 2007). Jia et al. (2004) reported the isolation and molecular characterization of a novel citrus low temperature responsive gene (CLT) from *P. trifoliata*, which produces two transcripts. Up until recently, a few cold-induced genes, mostly dehydrins, in woody plants such as blueberry and rhododendron had been identified during cold acclimation (Dhanaraj et al. 2004; Wei et al. 2005).

Using traditional breeding programs, cold-tolerant rootstocks have been produced by crossing *P. trifoliata* with citrus, resulting in scion varieties with acceptable tree characteristics, but the level of cold hardiness has been limited, mainly because the fruit of the hybrids tends to contain high levels of poncirin which gives it a disagreeably bitter taste (Soost and Cameron 1975; Soost and Roose 1996). Compared with traditional methods, genomics and molecular biology techniques such as gene cloning, gene manipulation and genetic transformation can overcome problems associated with conventional breeding and provide new approaches for understanding and improving cold tolerance in citrus (Cai et al. 1995). Substantial differences in the transcription profiling of *P. trifoliata* during cold acclimation versus cold shock treatment have been observed (Meng et al. 2008). Few reports explore the signal transport and regulation of gene expression between rootstocks and scions. Changes in gene expression during cold acclimation and cold shock of *P. trifoliata*, Satsuma mandarin cultivar ('Owari') and 'Owari' Satsuma grafted onto Poncirus rootstock were analyzed to gain a better understanding of the effects of signal transduction on gene expression between rootstock and scion.

III. MATERIALS AND METHODS

3.1. Plant material and growing conditions

One-year-old seedlings of 'Flying Dragon' (*Poncirus trifoliata*), 'Satsuma Mandarin' (*Citrus unshiu* 'Owari') cultivar and *C. unshiu* scions grafted on 'Flying Dragon' rootstocks were placed in a growth chamber under a 12/12-h (light/dark) photoperiod with light supplied at an intensity of 400 mmol m^{-2} s^{-1}. The cold acclimation treatment was performed by placing 3 plants from the species and graft combination (total: 9 plants) into a growth chamber. The regimen for the temperature decline was as follows: 25°C (day)/20 °C (night) for one week; 20 °C (day)/15 °C (night) for one week; 15 °C (day)/10 °C (night) for one week; 10 °C (day)/5 °C (night) for one week and 5 °C (day)/5 °C (night) for one week, which mimics the natural temperature declines found in the southeastern US. Leaf tissue samples were collected and pooled from at least three different plants at specific time points at the end of each week following the initiation of cold acclimation. Additionally 3 plants from the species and graft combination (total: 9 plants) were subjected to cold shock treatment in the growth chamber set at 4°C following a control period of 25°C (day)/20°C (night) for one week. Leaf tissue samples were harvested after 12h, 24h, 36h, 48h and 60h at 4 °C and immediately stored at –80°C until further use. Plants were uniformly watered every two days.

3.2. Total RNA isolation and cDNA-AFLP

Total RNA was isolated essentially from leaf samples collected from at least 3 individual non-acclimated or cold-acclimated plants obtained from two independent cold treatment experiments according to the manufacturers' protocol for the RiboPure kit from Ambion (Austin, TX). Extracted RNA was purified by digesting any contaminating genomic DNA by treatment with DNase I following the manufacturers' instruction for the TURBO DNA-free Kit from Ambion (Austin, TX). The concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY). RNA extracted from the end of first week was used as a non-acclimated control and RNA collected from leaves of plants treated for 60 h at 4 ^{o}C as treatment group for cDNA-AFLP analysis. cDNA-AFLP analysis was performed according to the protocol of AFLP® kits from LI-COR (LI-COR Biosciences, Lincoln, NE). Sequences of the adapters and primers used for cDNA-AFLP analysis were provided by Li-COR. The selective amplification products were run on 6% polyacrylamide sequencing gel containing urea at 80 W for 5 hrs. The cDNA bands were visualized by silver staining according to the Silver Sequence™ DNA Sequencing System Technical Manual (Promega, Madison, WI) as described by Zhang et al. (2005 a, b).

3.3. Relative quantitative real-time polymerase chain reaction (RQ-PCR)

For the detection of the RNA transcripts in control, cold shock and cold-acclimated leaf tissues, the real-time quantitative PCR of RNA targets was performed using Applied Biosystems 7500 Real-Time PCR System. A 2.5µg aliquot of total RNA was reverse transcribed to cDNA, according to the RETROscript kit manufacturers' protocol

(Ambion, Austin, TX). The resultant cDNA served as the template in the real-time PCR reaction. Real-Time quantitative PCR reaction was amplified in a 20 ul solution with 0.5 mM gene specific primer (Table 1, GenHunter, Huntsville, AL) and 12.5 ul Power SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA). 0.3 mM Poncirus specific actin primer was used as housekeeping gene to ensure a single melting peak during the dissociation step. The use of actin as an internal control ensures cDNA quantity and integrity. The PCR cycle program consisted of 1 cycle of 2 min at 50^{o}C, 1 cycle of 10 min at 95^{o}C, 40 cycles of: 15 s at 95^{o}C and 60 s at 60^{o}C. The data collection was carried out after 40 cycles. Specific oligonucleotide primers were designed for selected cDNA fragments based on cDNA AFLP products.

Quantification is based on a Ct value, which is the PCR cycle number when probe fluorescence exceeds a threshold value which is arbitrarily set in the exponential phase of reaction fluorescence. One dissociation reaction and melting curves were conducted for each primer pair after each real-time reaction followed by confirmation of differentially expressed genes to ensure optimal PCR efficiencies for Relative Quantification Real-Time PCR. Threshold cycle values were recorded for each gene and actin in triplicate at all time points (12 h, 24h, 36h, 48h and 60h at 4^{o}C and cold acclimation). Results for all selected gene primers were normalized with respect to results obtained with actin. According to ABI7500 system SDS v1.4 software (Applied BioSystems), comparison of the expression of each gene between control and cold treatments was analyzed after RQ-PCR reactions. Only reproducible differences in RQ-PCR triplicates were considered positive.

3.4. Cloning of cDNA fragment and rapid amplification of cDNA ends (RACE)

Differentially expressed transcript-derived fragments (TDFs) extracted from cDNA-AFLP gels (Zhang et al. 2005) were used as templates for re amplification by PCR. TDFs were ligated directly into a pGEM-T Easy Vector (Promega, Madison, WI) and then transformed into competent *E. coli* (Promega, Madison, WI). Plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Five colonies were selected for each transformation event. Colony PCR was carried out by taking a small colony and suspending it in 60 µl of H_2O in a PCR tube. The PCR reaction was set up as normal with primers provided by Promega to detect the correct insertion sizes. 10 µl of PCR product was separated on a 1.5% agarose gel. Only fragments larger than 200 bp were selected and sequenced in one direction using either the T7 or SP6 primers (Promega) with an ABI 3100 DNA sequencer (AU Genomics Lab). The analysis of the nucleotide sequences of the selected fragments was carried out using the NCBI BLASTx search tool.

The primers used for the cloning of core cDNA fragments of nitrate transporter, carbonic anhydrase and proline transporter (Table 2) were designed and synthesized according to the conserved regions of the gene sequences. PCR analysis was initiated with hot start method using single strand cDNA template and Taq DNA polymerase (New England BioLabs, Ipswich, MA). The PCR product was subcloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced.

RACE was performed according to the manual of the 5'-RACE System Version 2.0 and 3'-RACE System (Invitrogen, Carlsbad, CA). Gene specific primers for 5'-RACE, and for 3'-RACE (Table 2) were generated based on the cloned conserved core cDNA sequences.

3.5. Sequences analysis

Amino acid sequences encoding nitrate transporter, carbonic anhydrase and proline transporter genes from different species were chosen from the NCBI database. Multiple sequence alignment was carried out with Vector NTI 10 software (Invitrogen, Carlsbad, CA) at default setting. Dendroscope software (version 2.2.1) was used for displaying the phylogenetic trees (Huson et al. 2007).

IV. RESULTS AND DISCUSSION

Twenty-eight putatively differentially expressed TDFs were selected for study based on earlier studies of Meng et al. (2008); Lang et al. (2005); Zhang et al. (2005a, b). Twelve TDFs showed no differential expression in Poncirus or Satsuma during cold acclimation and were not used for further study (Appendix Fig. 35). Sixteen TDFs did show up- or down-regulation in response to low temperature using QR-PCR. These TDFs were resubjected to the NCBI BLASTx program to search for homologues of gene functions in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers of nucleotide sequences along with the results of the BLASTx search are shown in Table 3, or were published earlier.

Cu 98

The inferred amino acid sequence of *Cu98* shows 70% similarity to histone deacetylase 19 from *Arabidopsis thaliana*. Histone deacetylases (HDACs) catalyze the removal of acetyl groups from *N*-acetylated lysine residues in histone tails. They are thought to regulate effects on gene expression by altering the accessibility of nucleosomal DNA to DNA-binding transcriptional activators, other chromatin-modifying enzymes or multi-subunit chromatin remodeling complexes capable of displacing nucleosomes (Fuks et al. 2000). Major groups of HDACs include the Reduced Potassium Deficiency 3

(RPD3) superfamily, the Silent Information Regulator 2 (SIR2) family and the HD-tuins family. In the Arabidopsis genome, 12 of 18 histone deacetylases belong to the RPD3 superfamily, such as AtHDA6, AtHDA7, AtHDA9 and AtHDA19, which are representatives of class I (Hollender and Liu 2008). Antisense knockdown mutants of AtHda19 exhibit pleiotropic developmental phenotypes, such as early senescence, reduced apical dominance, homeotic changes, heterochronic developmental shifts and flower defects coupled with male and female sterility (Tian et al. 2003). Furthermore, knockdown of AtHDA19 causes striking global histone hyperacetylation (Tian and Chen 2001), and AtHDA19 works antagonistically with the histone acetyltransferase AtGCN5 to regulate the maintenance of embryo axis formation and light-responsive gene expression, respectively (Long et al. 2006). Besides during development, AtHDA19 is required for jasmonic acid- and ethylene-mediated plant responses to plant pathogens, for transcriptional repression in response to abscisic acid, drought and salt stress (Song and Galbraith 2006). In Arabidopsis, the HDA19 was highly expressed in inflorescences and young floral tissues but underexpressed in vegetative tissues, pollens, seeds and late-stage flowers (Hollender and Liu 2008). In this study, only the gene expression level of Cu98 in Satsuma decreased during cold acclimation (Fig. 3b). The Cu98 gene in grafted Satsuma and Poncirus rootstock showed little changes in expression profile during cold stress, suggesting that the rootstock might play a role during signal transduction to the scion.

Cu120

The inferred amino acid sequence of *Cu120* shows 90% similarity to phosphomannomutases (PMMs) from *Glycine max*. Phosphomannomutases catalyze the interconversion of mannose-6-phosphate into mannose-1-phosphate that reacts with GTP to form the sugar precursor GDP–mannose by the action of GDP–mannose pyrophosphorylase (Hoeberichts et al. 2008). As such, plant PMMs are involved in ascorbic acid (AsA) biosynthesis and *N*-glycosylation. The AsA content of *Nicotiana benthamiana* leaves was substantially reduced when the PMM gene was silenced (Qian et al. 2007). Previous characterization of PMM has shown that it is a heart-shaped protein with 463 residues comprising four domains (Regni et al. 2002).Very recently, in *Arabidopsis thaliana*, a temperature-sensitive *pmm-12* mutant showed reduced PMM enzyme activity and protein levels, developmental arrest, and subsequent cell death at restrictive temperature (Hoeberichts et al. 2008). In this study, cold acclimation did reduce the transcript level of the Cu120 gene to 0.03 fold in *C. unshiu* (Fig. 4b), while this did not occur in grafted plants, suggesting an effect of the rootstock on gene expression in the scion. Although the exact roles of the PPM protein under environmental stresses are not clear, the reduced level of this gene in grafted Satsuma suggests that cold stress may repress processing of this protein.

CuA12

The inferred amino acid sequence of *CuA12* shares 65% homology with Magnesium-chelatase subunit H in *Rhodobacter capsulatus*. Mg-chelatase (ChlH), a chloroplast enzyme, catalyzes the ATP-dependent insertion of Mg^{2+} into protoporphyrin-IX to form

Mg-protoporphyrin-IX. This is the first unique step of the chlorophyll biosynthetic pathway, and it lies at the branch point for porphyrin utilization; the other branch leads to heme (Willows and Hansson 2003). CHLH has been reported to have multiple functions in plant cells. In addition to its enzymatic function as a subunit of Mg-chelatase in producing the photosynthetic apparatus (Walker and Willows 1997), CHLH has a key function in mediating plastid-to-nucleus signaling (Nott et al. 2006). Shen et al. (2006) reported that Mg-chelatase H subunit also functions as an abscisic acid receptor, suggesting a vital function in plant adaptation to stressful environments. In this study, the transcript level of CuA12 in grafted Satsuma showed significant down-regulation to 0.013 fold during cold shock (Fig. 5a), while transcript levels increased following cold acclimation, mainly in *C. unshiu* seedlings, and the effect was reduced in the grafted plants (Fig. 5b).

CuB04

The inferred amino acid sequence of CuB04 shared significant sequence similarity (81%) to the Zinc finger (C3HC4-type RING finger) family protein in *Arabidopsis thaliana.* The RING (Really Interesting New Genes) finger was defined as a novel zinc finger domain among various types of zinc finger family transcription factors, which can act as DNA-binding or protein-binding proteins, and some functions as RNA-binding proteins (Hall 2005). RING finger domains have been found in proteins involved in various signal transduction and regulatory pathways (Vij and Tyagi 2006), C3HC4-type has a cysteine-rich domain of 40–60 residues that coordinates two zinc ions (Alexandrov et al. 2006). In plants, several zinc finger proteins have been identified and characterized.

These include CaKR1, an ankyrin-repeat domain C3H1 zinc finger protein in *Capsicum annuum* involved in responses to cold and salinity (Seong et al. 2007); *ZPT2-3* in petunia induced by cold, drought and heavy metal treatment (Sugano et al. 2003); *OSISAP1*, a zinc finger gene from rice, induced by cold, salt, desiccation, heavy metal and ABA (Mukhopadhyay et al. 2004). In the present study, the transcript level of CuB04 showed down regulation during cold shock (Fig. 6a), but up regulation following a cold acclimation regime (Fig. 6b), especially in Poncirus (at 10/5 ^{o}C). CuB04 was also upregulated in grafted *C. unshiu*, but not in ungrafted Satsuma seedlings, suggesting an effect of rootstock on expression of this Zinc finger protein in scion during acclimation to low temperature.

CuD09

The inferred amino acid sequence of *CuD09* shared significant sequence similarity (98%) to lipoxygenase in *Citrus jambhiri.* Plant lipoxygenases (LOXs) are a family of non-heme, iron-containing dioxygenases that catalyze the oxygenation of linoleic and linolenic acids (Siedow 1991). The hydroperoxy polyunsaturated fatty acids, synthesized by the action of various highly specialized forms of lipoxygenases are substrates of at least seven different enzyme families, with signaling compounds such as jasmonates and antimicrobial and antifungal compounds (Feussner and Wasternack 2002). In plants, LOX itself as well as different enzymes from the LOX pathway utilize both 9- and 13-fatty acid hydroperoxides to form a series of biologically active molecules, collectively called oxylipins (Feussner and Wasternack 2002). Biochemical and molecular studies of dicot species indicated that LOX transcript, protein and activity levels are modulated in

response to both biotic and abiotic stresses and known as signaling substances that play a role in plant development as well as in plant responses to mechanical wounding, insect feeding, and pathogen attack (Blee 1998). A rice 13-LOX gene (*RCI-1*) was overexpressed in plants and was shown to be responsible for increased expression of the pathogenesis-related protein PR1 (Zabbai et al. 2004). In the present study, the mRNA level of CuD09 showed a dramatic increase in *C. unshiu* (more than 500 fold; Fig. 7a) during cold shock, while decreases were observed during cold shock and during cold acclimation in all other plants under study. The induction of CuD09 decreased to lower levels in grafted Satsuma as compared to ungrafted plants and Poncirus (Fig. 7b). These results indicate that the rootstock does reduce lipoxygenase gene expression levels during low temperature stress.

CuG01

The inferred amino acid sequence of *CuG01* shares 81% homology with a chloroplastic chlorophyll a-b binding protein P4 from *Pisum sativum.* The major light-harvesting chlorophyll *a*/*b* (LHC) complex of the photosynthetic apparatus in green plants can be viewed as a protein scaffold binding and positioning of a large number of pigment molecules that engage in rapid excitation energy transfer (Liu et al. 2008). It is generally assumed that Chl *a* represents the precursor of Chl *b* and that the pathway leading to Chl *b* would proceed sequentially (von Wettstein et al. 1995). The enzyme responsible for Chl *b* biosynthesis is chlorophyllide *a* oxygenase (CAO) (Tomitani et al. 1999). The chlorophyll a-b binding protein superfamily in *Arabidopsis thaliana* consists of 20 different proteins (Jansson 1999). The *LHC* RNA level in Arabidopsis was

suppressed when plants were exposed to low temperature (Strand et al. 1997). In this study, all transcript levels of G01 are down-regulated under low temperatures (Fig. 8), confirming results of Strand et al. (1997) and Zhang et al. (2005 a,b). Little effect of rootstock on scion gene expression of CuG01 was detected.

S3

The inferred amino acid sequence of S3 isolated from *P. trifoliata* (Meng et al. 2008) shares 91% homology with an auxin responsive GH3-like protein from *Arabidopsis thaliana* (Meng et al. 2008). The expression of most GH3 genes is regulated by auxin response factors (ARFs), which can either activate or repress target gene expression (Woodward and Bartel 2005). Marked increases in S3 mRNA levels were observed by Meng et al. (2008) in *Poncirus trifoliata* during cold treatments. In the present study, the mRNA level of S3 showed dramatic increases in *P. trifoliata* (Fig. 9) following cold shock and cold acclimation. This was not the case in grafted or ungrafted Satsuma. In ungrafted Satsuma transcript levels decreased during cold acclimation, but grafting of Satsuma on Poncirus rootstock did increase mRNA transcript levels up to 22.1 fold (Fig. 9b). Although the exact function of the GH3 protein during cold stress is not clear, results suggest that this protein plays an important role in the cold response and impact gene expression level of the scion grafted on a cold-hardy rootstock during cold acclimation.

S7

The inferred amino acid sequence of S7 isolated from *P. trifoliata* (Meng et al. 2008) shows 61% similarity to UDP-glycosyltransferase (UGT) from *Ricinus communis*. In

plants, UDP-glycosyltransferases are a superfamily of enzymes that catalyzes the transfer of a glycosyl residues from an activated nucleotide sugars to a hydrophobic molecule (aglycones), leading either to the formation of a glucose ester or to the formation of a glucoside, thus regulating properties of the acceptors such as their bioactivity, solubility and transport within the cell and throughout the organism (as reviewed by Meng et al. 2008). Meng et al. (2008) detected a 5.5 fold increase in S7 transcript level in Poncirus following cold acclimation, whereas UGT transcript levels in Satsuma grafted on *P. trifoliata* did not result in significant changes during cold shock (Fig. 10a) and cold acclimation treatments. However, increases of more than 150 fold of UGT transcript levels were observed in ungrafted Satsuma upon cold acclimation (Fig. 10b). This suggests that the rootstock has a major impact on reducing UGT transcript levels in the scion during cold stress.

S17

The inferred amino acid sequence of S17 isolated from *P. trifoliata* (Meng et al. 2008) shows 91% similarity to a Mitogen activated protein kinase3 (MAPK3) of *Arabidopsis thaliana*. Mitogen-activated protein kinases (MAPKs), MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) have been reported to function in various signal transduction pathways from yeasts to vertebrates. Mitogen-activated protein kinase cascades are among the first signaling pathways demonstrated to be activated by ROS (Mizoguchi et al. 1996). Compared with mammalian MAPKs, all plant MAPKs have highest homology to the extracellular signal-regulated kinases (ERKs) subfamily (Mora-Álvarez et al. 2004). MAPK3 is activated by upstream kinases, resulting in its

translocation to the nucleus where it phosphorylates nuclear targets as reviewed by Meng et al. (2008). In this study, transcript levels of S17 in Satsuma were increased markedly in response to cold acclimation and cold shock treatment (Fig. 11), but not in *P. trifoliata.* However grafted Satsuma showed increases, especially following cold shock. It is unclear whether this effect is related to different temporal regulation of MAPK3 in Satsuma versus Poncirus.

S47

The inferred amino acid sequence of S47, isolated from *P. trifoliata* (Meng et al. 2008), shows 82% similarity to a trehalose-6-phosphate synthase (TPS) from *Ricinus communis*. TPS can transfer one glucose residue from uridine diphosphoglucose (UDPG) to glucose-6-phosphate to produce a trehalose-6-phosphate (T6P) and UDP (Avonce et al. 2006). The main functions of S47 have been reviewed by Meng et al. (2008). In Arabidopsis, 11 homologs encoding TPSs were identified (Chary et al. 2008). TPS5, TPS6 and TPS7 were found to bind to 14-3-3 protein which was up-regulated in *Citrus unshiu* during cold stress (Lang et al. 2005). In this study, primer sequences used for RQ-PCR were as those described by Meng et al. (2008) (forward primer 5'-ATAGGCCAGCTCCAATCC-3', and reverse primer 5'-CCAAGCATGACAATCTGAC-3'). Transcript S47 showed a 5.3 fold following exposure to 4C (Fig. 12a), a 4.9 fold increase during cold acclimation (Fig. 12b) in Satsuma only, while no changes in gene expression were detected in Poncirus or grafted Satsuma, indicating a negative effect of rootstock on gene expression in the scion during low temperatures.

S126

The inferred amino acid sequence of S126 isolated from *P. trifoliata* (Meng et al. 2008) shows 81% similarity to a chalcone isomerase (CHI)-like protein from *Populus trichocarpa*. Chalcone isomerase, one of the key enzymes in the flavonoid biosynthesis pathway in plants, has been proposed as one plant-specific gene marker, based on the apparent lack of similarity to any non-plant sequences or structures (Jez et al. 2000). Meng et al. (2008) detected a 16.8 fold increase in transcript level in cold acclimated Poncirus as compared to control. In the present study, Meng et al.'s primer sequences for S126 were used for RQ-PCR (forward primer 5'- TCTCCAAATCAAGTTCACGG -3', and reverse primer 5'- GTCATCATCTTGTGCAAGG-3'). The transcript levels of S126 during cold acclimation showed up-regulation in all materials, thus confirming previous results. The highest transcript levels were detected (58.6 fold increase) in Poncirus during cold acclimation (Fig. 13b), while lower levels were indicated in grafted or ungrafted Satsuma plants. Little effect of rootstock on scion was detected in CHI expression levels during cold stress.

O10

The inferred amino acid sequence of O10 isolated from *C. unshiu* (Lang et al. 2005) showed 96% similarity to a 14-3-3 like protein D from *Nicotiana tabacum.* The 14-3-3 proteins are small, highly conserved eukaryotic proteins involved in regulating multiple cellular enzymes, and function as sequence-specific and usually phosphorylation-dependent binding proteins. The main functions of O10 gene have been reviewed by Lang et al. (2005), who observed a 2.3 fold increase in Satsuma leaves during cold

acclimation. In the present study, the 14-3-3 gene in Satsumas was upregulated during cold shock and cold acclimation to about similar levels. Levels of gene expression were similar in grafted Satsuma following cold acclimation, indicating that the rootstock exerted little effect on scion over 14-3-3 gene expression (Fig. 14).

P1

The inferred amino acid sequence of P1 isolated from *P. trifoliata* (Zhang et al. 2005a) shared 78% homology with a proline transporter from *Populus trichocarpa,*77% homology with proline transporters from *A. thaliana*, as illustrated in Fig. 15. The P1 protein of 391 amino acids has a conserved domain of the major facilitator superfamily (Fig. 16). Alignment of the proline transporter in Poncirus with other species indicates a conserved structure across many different plant families (Fig. 17). It is a member of the amino acid permease transmembrane family. Proline, a low molecular weight and compatible solute, accumulates in various kinds of plants and microorganisms in response to environmental stresses as reviewed by Zhang et al. (2005a). In Poncirus, up-regulation of P1 during cold acclimation was observed. The function of proline is thought to be an osmotic regulator under water stress, and its transport into cells is mediated by a proline transporter (Ueda et al. 2008). However, little is known about the integrated function of proline transporter gene under cold stress. RNA transcript level of P1 in Poncirus and Satsuma was noticeable induced during cold acclimation, but no changes were observed in grafted Satsuma (Fig. 18). Similar expression profiles were obtained when primer pairs for amplification of a region at the 5'UTR of the full length clone of the proline transporter were used for RQ-PCR analysis (Fig. 28).

P2

The inferred amino acid sequence of P2 isolated from *P. trifoliata* (Zhang et al. 2005a) shared significant sequence similarity (94%) to Plasma membrane intrinsic protein 1d (Probable aquaporin PIP1-5) in *A. thaliana*. Plasma membrane intrinsic proteins (PIPs) localized in the plasma membranes are a subfamily of aquaporins and seem to control most of the transcellular water flow in roots across the membrane, therefore playing a predominant role in water uptake. The PIP, the most highly conserved subfamily among the members of the plant major intrinsic protein (MIP) family, is further divided into two subgroups named PIP1 and PIP2 that have specific arrays of amino acids at the N- and C-termini (Schaffner 1998). Jang et al. (2004) investigated the expressions of a gene family encoding 13 PIPs in *A. thaliana* under various abiotic stress conditions including drought, cold, and high salinity, or abscisic acid (ABA) treatment, and revealed that only the PIP2-5 was up-regulated by cold treatment, but most of the PIP genes were downregulated by cold stress. Moreover, all but one *AtPIP*s (*AtPIP2.6*) were down-regulated during cold acclimation in wild-type Arabidopsis plants (Peng et al. 2008). In a *Rhododendron catawbiense*, two cDNAs, *RcPIP2.1* and *RcPIP2.2*, were down-regulated as the leaf freezing tolerance increased from -7 to -50 °C (Wei et al. 2006). In the present study, the P2 gene was highly induced in Poncirus during cold acclimation (Fig. 19), confirming results of Zhang et al. (2005a), while lower levels occurred in nongrafted Satsuma, but little changes in grafted Satsuma. The rootstock exerted a negative effect on PIP gene expression in the scion during slow adaptation to low temperature.

P5

The inferred amino acid sequence of P5 identified in *P. trifoliata* (Zhang et al. 2005a) shows high similarity with a nitrate transporter, NRT1.2 from *Glycine max* (67%) and *Ricinus communis* (Fig. 20). The P5 protein of 588 amino acids has a conserved domain of the major facilitator superfamily (Fig. 21). Alignment of the nitrate transporter in Poncirus with other species indicates a conserved structure across many different plant families (Fig. 22). During plant vegetative growth, nitrate is taken up from the soil solution by active transport across the plasma membrane of root cells. Two families of nitrate transporters, NRT1 and NRT2, have been identified in higher plants, and both families transport NO_3^- together with a proton (H^+) in a symport mechanism that is driven by the pH gradients across membranes. The nitrate transporters in the NRT1 family were initially thought to transport nitrate with low affinity, such as NRT1.2 (involved in nitrate uptake), NRT1.4 (involved in petiole nitrate storage), and NRT1.5 (involved in xylem loading) (Tsay et al. 2007). Zhang et al. (2005a) reported a marked increase in P5 gene expression during cold acclimation in Poncirus. In this study, the transcript levels of P5 genes in all plant materials during low temperature dramatically increased (Fig. 23). During cold shock, levels of P5 increased more than 400 fold in Poncirus, only 10 fold in grafted or non-grafted Satsuma, during cold acclimation more than 400 fold in Satsuma, only 10 times in Poncirus. Differences in gene expression profiles of grafted versus non-grafted Satsuma can be attributed to rootstock effects during cold acclimation, not during cold shock. Similar expression profiles were obtained during RQ-PCR analysis with primer pairs designed to amplify a region at the 3' UTR of the full length clone of Poncirus nitrate transporter (Fig. 29).

PD3

The inferred amino acid sequence of PD3 identified in Poncirus (Zhang et al. 2005b) shows high similarity to chloroplastic carbonic anhydrases (CA) from several species, such as *A. thaliana* and *Nicotiana tabacum* (Fig. 24). The PD3 protein of 269 amino acids has a conserved domain of the major facilitator superfamily (Fig. 25). Alignment of the chloroplastic carbonic anhydrases in Poncirus with other species indicates a conserved structure across many different plant families (Fig. 26). Carbonic anhydrases are Zn metalloenzymes that catalyze the reversible interconversion of CO_2 and bicarbonate. In higher plants, carbonic anhydrases have been localized to the chloroplast stroma and to the cytoplasm as reviewed by Zhang et al. (2005b). Chloroplast carbonic anhydrases are important for efficient delivery of CO_2 to ribulose-bisphosphate carboxylase/oxygenase (RubisCO) (So *et al.* 2004). In rice (*Oryza sativa L.*), expression of the gene (*OsCA1*) coding for carbonic anhydrase in leaves and roots was induced by environmental stresses from salts and osmotic stress (Yu et al. 2007). The PD3 gene transcript patterns were up-regulated in Satsuma and grafted Satsuma during cold shock (Fig. 27a), but down-regulated during cold acclimation (Fig. 27b) in all materials, suggesting that there might be some effect of rootstock on scion during cold shock only. Since the full length clone of Poncirus carbonic anhydrase is available, primers for RQ-PCR were designed for amplification of a region at the 5' UTR of the gene. Similar expression profiles were obtained when a different set of primer pairs were used for detection of carbonic anhydrase (Fig. 30).

Poncirus trifoliata is a close relative of citrus and is mainly used as rootstock in the citrus industry where it imparts its cold tolerance ability to the scion in freeze prone areas

(Yelenosky 1985). *Citrus unshiu* is a cold-sensitive commercial citrus species. One of the common consequences of exposure to drought, salt and low temperature is oxidative stress which can cause increased production of ROS. Genes encoding oxidative stress related proteins have been extensively identified in many species under multiple stress conditions to protect cell membranes and increase intracellular osmotic potential (Crowe et al. 1990). Previous studies on Poncirus identified genes acting as cellular component transporters such as hexose carrier, glucose-6-phosphate/phosphate translocator (Sahin-Cevik and Moore 2006), proline/betaine transporter, water-channel protein and nitrate transporter (Zhang et al. 2005a). Efforts have been made to increase the accumulation of proline with over expression of the key enzyme, P5CS, in many species (Kishor et al. 1995). It is well established that freeze induced membrane damage results primarily from severe dehydration associated with freezing. The accumulation of sucrose and other compatible chemicals such as proline contribute to the stabilization of membranes based on in vitro experiments. In our experiment, primers designed for TDFs isolated from either *P. trifoliata* or *C. unshiu* could be used successfully to detect differential gene expression in both species. In a few instances, cDNA regions of proline and nitrate transporter from the different species were compared and showed high sequence homology (Fig. 31). Several genes such as UDP-glycosyltransferase, trehalose-6-phosphate synthase, chalcone isomerase-like protein, proline transporter, plasma membrane intrinsic protein and nitrate transporter, involved in osmotic and oxidative stress, were upregulated in response to cold, suggesting an active role for secondary metabolites with respect to osmotic balancing, except for lipoxygenase and phosphomannomutase which were down-regulated in response to low temperature.

Transcriptional profiling of plant species such as Arabidopsis growing under low temperatures revealed that many genes encoding products related to energy metabolism were down regulated, and many of them encode components involved in photosynthesis (Fowler and Thomashow 2002). The down regulation of chloroplastic chlorophyll a/b binding protein and chloroplastic carbonic anhydrase indicate that the cellular metabolisms are slowed down under cold acclimation and photosynthesis adjustment might be one of the ways to alleviate the photooxidative stress to plant cells.

Cold responsive gene expression profiles of *P. trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus were compared during 4°C treatment and a gradual cold acclimation regime. These comparisons provide additional information on the dynamic changes in gene regulation differences occurring in different species during stress, which were especially noticeable during cold acclimation. While Poncirus leaves showed upregulation of Zinc finger (16 fold, Fig. 6), auxin responsive GH3 (>100 fold, Fig. 9), PIP (15 fold, Fig. 16), only small changes occurred in Satsuma with Zinc finger, auxin responsive GH3 was down regulated (Fig. 9) and PIP showing only a 5 fold increase (Fig. 16). Some genes in Satsuma showed high expression during cold acclimation, such as nitrate transporter (>100 fold, Fig. 17) UGT (>100 fold, Fig. 10), proline transporter (8 fold, Fig. 15), MAPK3 (8 fold, Fig. 11), trehalose 6P synthase (5 fold, Fig. 12), while only small changes were observed in Poncirus during cold acclimation. In some cases, differences in down-regulation of transcripts were observed, especially with regards to lipoxygenase (Fig. 7). It is clear that the Poncirus rootstock did exert a negative effect on gene expression of the Satsuma scion in many instances. Expression of genes involved in signal transduction and regulation of gene expression, such as RING finger protein, and

auxin-responsive GH3 was positive and increased in grafted as compared to non-grafted Satsuma plants during cold acclimation, while expression of genes involved in nitrate transport, PIP, trehalose 6-P synthase, UDP-glycosyltransferase, were negatively regulated by the rootstock in that expression levels in the grafted Satsuma were reduced as compared to the non-grafted plants during cold acclimation. During cold shock treatment, this effect was evident early in the plants response to low temperature. Levels of lipoxygenase, trehalose 6P synthase, chalcone isomerase, 14-3-3 protein, carbonic anhydrase were lower in grafted plants as compared to non-grafted plants during cold shock treatment (Fig. 7, 12, 13, 14, 18). Since the auxin-responsive GH3-like protein showed marked increases in Poncirus and grafted Satsuma, and down-regulation in ungrafted Satsuma, the auxin signaling pathway might be an important component of the cold-tolerance ability of a citrus species.

Cold response in woody plants is a complex regulation of different cellular events. Central to successful cold acclimation is the ability to adjust the photosynthetic process to function at low temperatures, especially in moderate to high light conditions, which otherwise expose the plant to photoinhibition and can lead to the formation of reactive oxygen species (Ke et al. 2004). The ability to cold acclimate is a polygenic trait involving a large number of genes, whose expression is controlled by low temperature. Alterations in the expression levels of these genes lead to numerous molecular and physiological changes. The complexity of the acclimation process is reflected in the amount of genes affected by low temperatures. The analysis of expression profiles in other species has revealed the existence of at least two groups of genes that have different temporal patterns of expression (Fowler and Thomashow 2002). In the first group, the

expression is rapid and transient in response to low temperature, and in the second the expression increases gradually during cold treatment. The analysis of expression profiles also indicated that multiple regulatory pathways are activated during cold acclimation (Fowler and Thosmahow 2002). For horticulturally important woody plants like citrus, low temperature is a serious problem that reduces plant growth and productivity. Modern agriculture makes extensive use of rootstocks for a wide range of tree crops to improve production efficiency and fruit quality. Citrus trees which do not reproduce true to type and desirable cultivars are clonally propagated by grafting usually on trifoliate orange (*P. trifoliata*) rootstocks. Rootstocks are known to affect canopy development, disease incidence and cold hardiness. Interactions between rootstocks and scions are through exchange of resources and via hormonal messengers (Jensen et al. 2003). The signaling process from rootstock to cellular responses in scion is still poorly understood. Our results indicate that cessation of growth is more rapidly invoked in cold hardy species than cold sensitive species and that through grafting this response can be activated or induced in the scion. For improvement of the stress tolerance ability of citrus, different scion/rootstock combinations should be studied to optimize this rootstock-scion interaction for enhanced acclimation to low temperatures.

Table 1. Oligonucleotide primer sequences designed for QR-PCR of 13 gene products

Gene Product	Species	Forward primer (5'-3')	Reverse primer (5'-3')
Cu98	*C. unshiu*	AATTCACGCCAGTTGCTT	GGACTTTCGTTCACCATC
Cu120	*C. unshiu*	GACATTTTCAATTGGAGGAC	TATCCTCAGGGCTGGTAA
CuA12	*C. unshiu*	CAATTCATGCCTGGAAAG	CTGCTTGAGGCCTTTATA
CuB04	*C. unshiu*	CACGGGACACCAGAACGTA	GGCATATTCCTCAGGGAA
CuD09	*C. unshiu*	AATTCAGCTCTGTTGCCT	AGATCAAGGCCATCATTG
CuG01	*C. unshiu*	TAACCAGGGAAGTGGCCT	CGGCTTGCACATACCACT
S3	*P. trifoliata*	CCAGCGTCATTGAGTACACA	AACAAGCAACTCCCAGAATA
S17	*P. trifoliata*	GTCCATGAGTTCCATAGCAA	ATGTTATTCATCGGGATTTG
O10	*C. unshiu*	CTTTGAGGAGGCCATTGCTG	CATGACATTCAAAAGCCCAC
P1	*P. trifoliata*	AAGCTTCCTGCAATCAGTCA	CAGAGCATTTCCTTTAATACCA
P1S	*P. trifoliata*	CCATAGCTGGATGTGATTTA	AAGACAGCCAGCTGCCTATA
P2	*P. trifoliata*	AAGCTTGGAGCTTTATATCATC	GCTATTAATTGAACGGATCGA
P5	*P. trifoliata*	TATTACTTAGTTGCTGCATTAGGG	TGTACCATTTTGCACACAAT
P5S	*P. trifoliata*	TTTTATGAATAGCTTGTGCTGC	GGGTCCAACATTTTCTCAAA
PD3	*P. trifoliata*	AGACTCCAGGGTTTGCCCGT	GCAGCTCCAACTCCAGCATA
PD3S	*P. trifoliata*	CCGGGCAGGTGCTTCGATCAAT	GCCTTGCAACGATGGAGGGA

Table 2. Oligonucleotide primer sequences designed for RACE of 3 genes products

Gene Product	Primer (5'-3')
RACE1-P1	CGTTAAGTTGCGAACAGCCAGAGC
RACE2-P1	CCAAGAACTCGTACATTGGGCTTGC
RACE3-P1	CTGAACGTTAAGTTGCGAACAGCC
RACE1-P5	GCCGAAGTTTAAGAGCCCTAATGC
RACE2-P5	ACTTCCGGGGCGCCGTCACCACTC
RACE3-P5	AAGAAGCCGAAGTTTAAGAGCCC
RACE1-PD3	GAGTGCCAGAGTTTTGTTCACCAAGC
RACE2-PD3	GTAACCACCCTTGAGTGCCAGAG

Table 3. *Citrus unshiu* gene products, accession number, length in bp, assigned function and percentage similarity to known genes using BLASTx search in NCBI

Product	Length (bp)	Plant gene	Source	Similarity	Function
Cu98	236	Histone deacetylase 19	*Arabidopsis thaliana*	70%	Multiply pathways
Cu120	555	Phosphomannomutase	*Glycine max*	90%	Multi-function
CuA12	232	Magnesium-chelatase subunit H	*Rhodobacter capsulatus*	65%	Multi-function
CuB04	415	Zinc finger (C3HC4-type RING finger) family protein	*Arabidopsis thaliana*	81%	Signal transduction
CuD09	222	Lipoxygenase	*Citrus jambhiri*	98%	Multi-function
CuG01	225	Chloroplastic chlorophyll a-b binding protein P4	*Pisum sativum*	81%	Protein binding

Fig. 1 The process of cellular dehydration caused by freezing (adapted from Sharma et al. 2005). Difference in solute potential leads to ice formation in intercellular spaces. This causes a drop in water potential in extracellular space, eventually leading to outward movement of water from the cell causing dehydration of the cell.

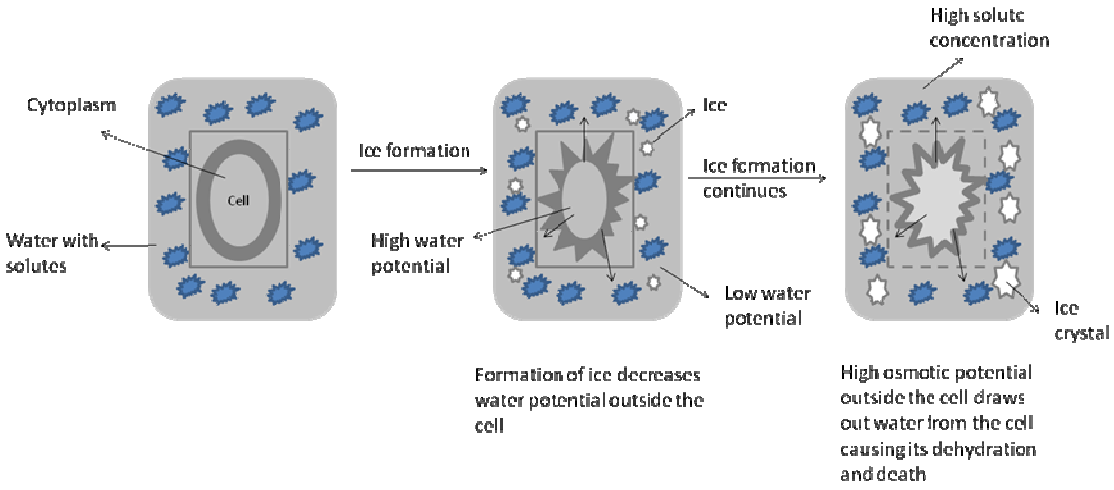


Fig.2 Diagram of cold-responsive transcriptional network in Arabidopsis (adapted from Chinnusamy 2007).

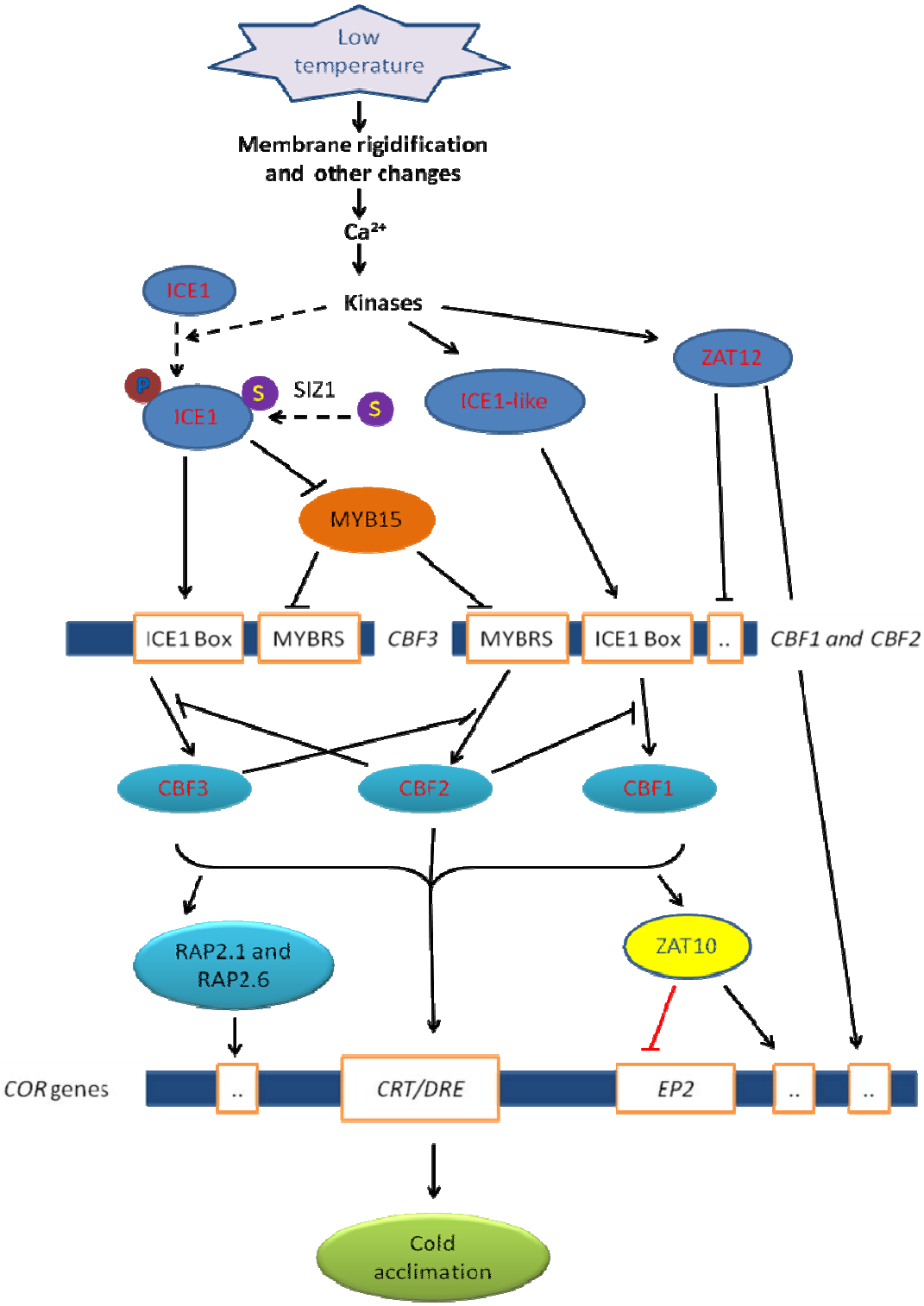
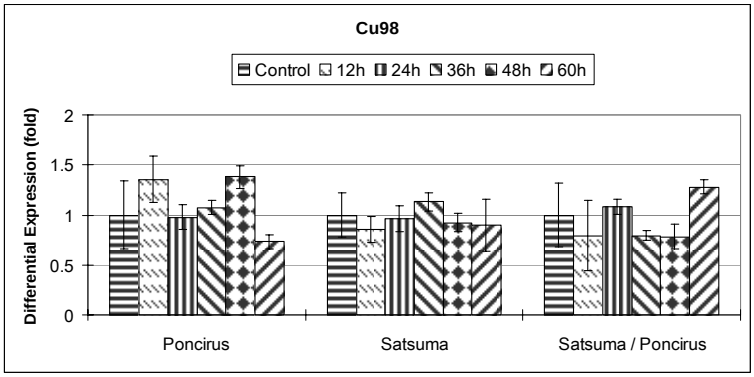
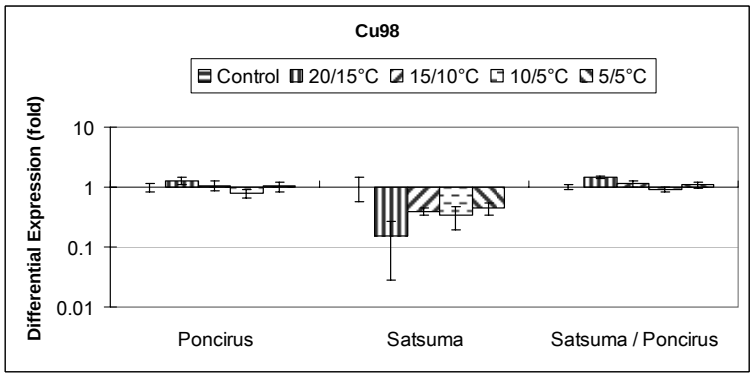


Fig. 3 mRNA expression level comparisons of *Cu98* (histone deacetylase) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



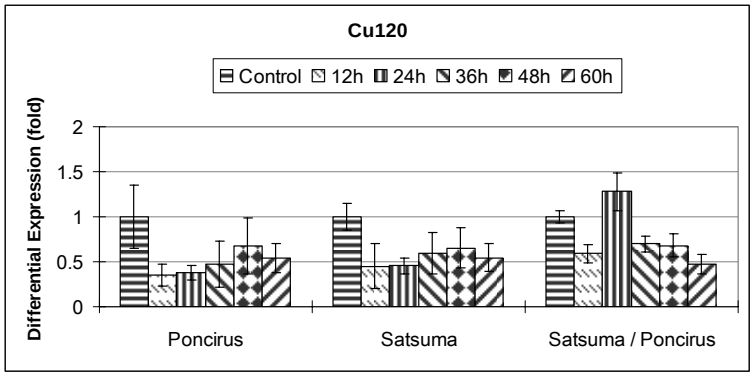
(b)



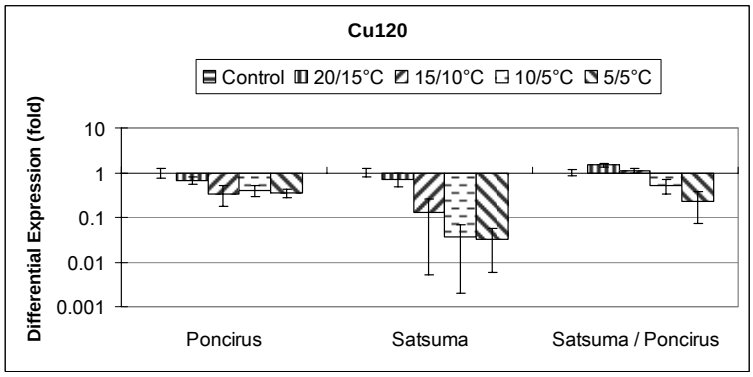
These histograms show relative gene expression levels of *Cu98* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.4 mRNA expression level comparisons of *Cu120* (PPM) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



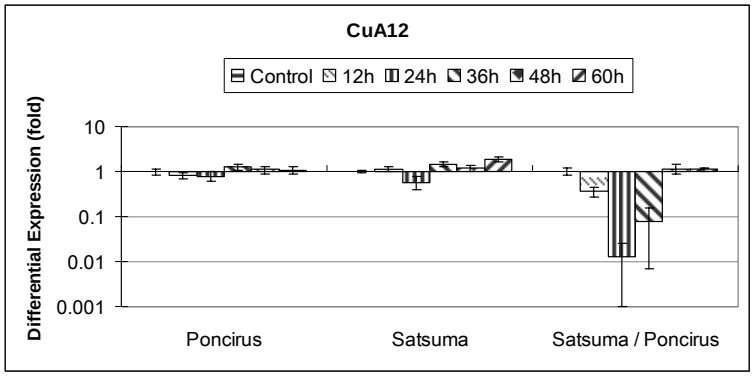
(b)



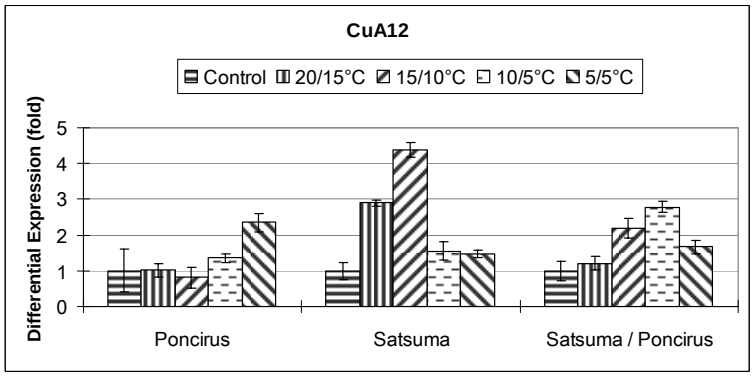
These histograms show relative gene expression levels of *Cu120* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.5 mRNA expression level comparisons of *CuA12* (Mg chelatase) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



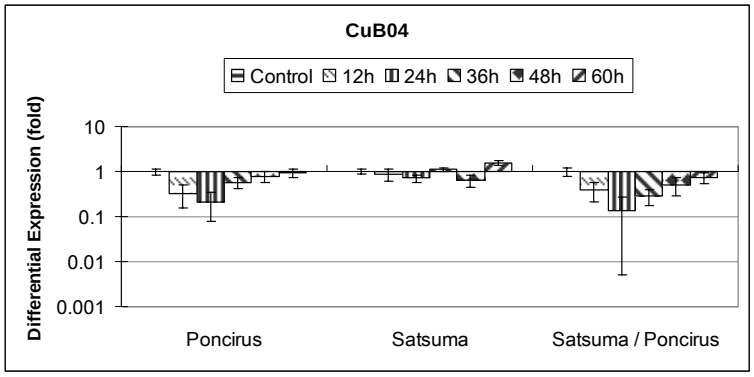
(b)



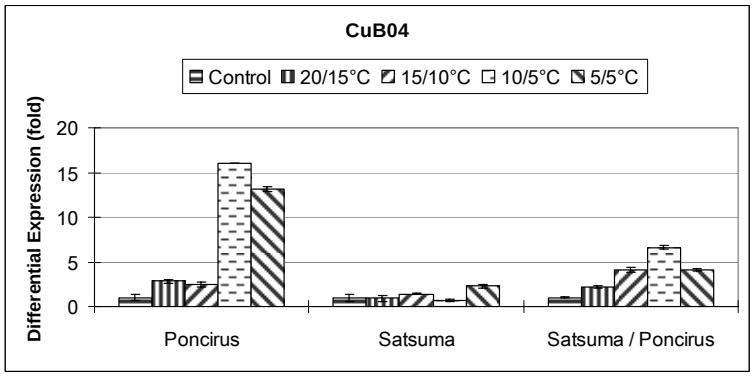
These histograms show relative gene expression levels of *CuA12* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.6 mRNA expression level comparisons of *CuB04* (Zinc finger) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



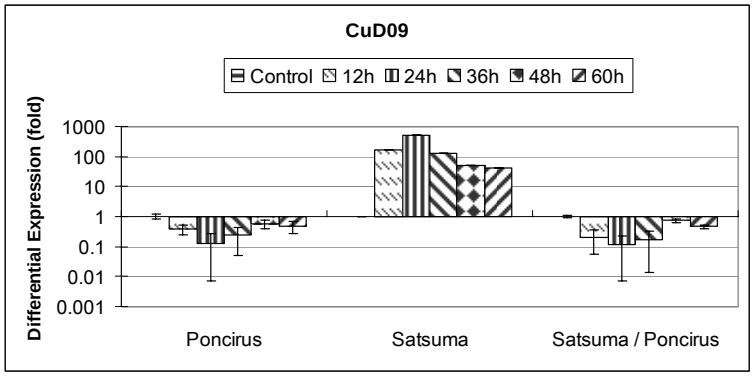
(b)



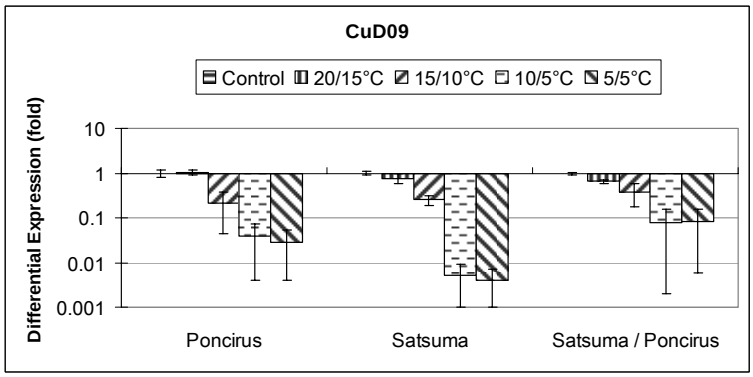
These histograms show relative gene expression levels of *CuB04* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.7 mRNA expression level comparisons of *CuD09* (LOX) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



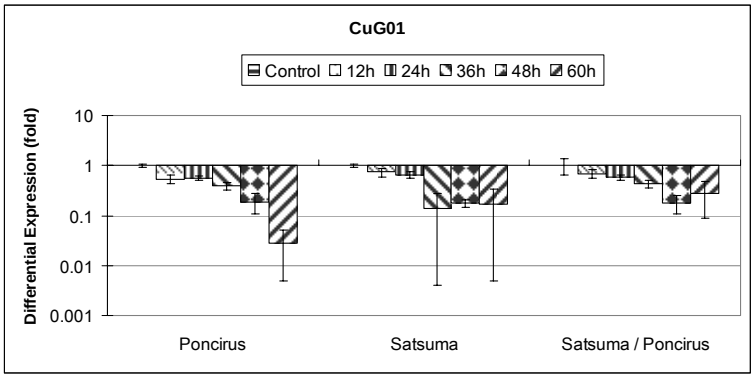
(b)



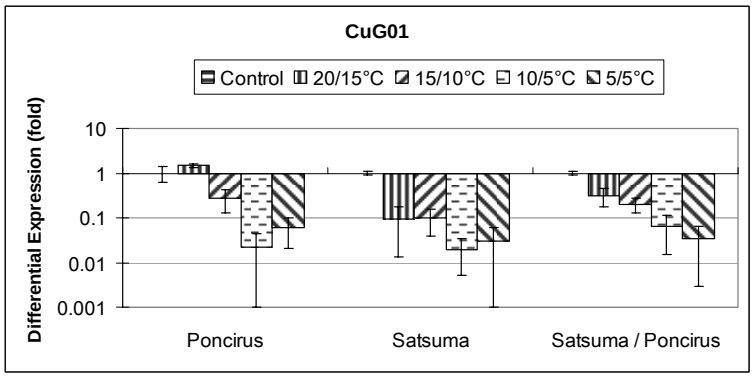
These histograms show relative gene expression levels of *CuD09* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.8 mRNA expression level comparisons of *CuG01* (chlorophyll a-b binding) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



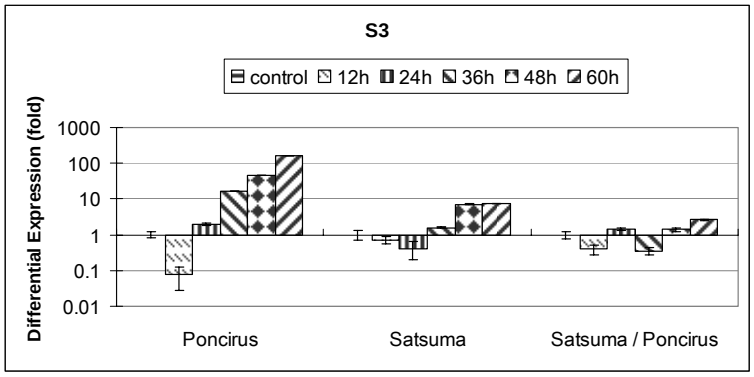
(b)



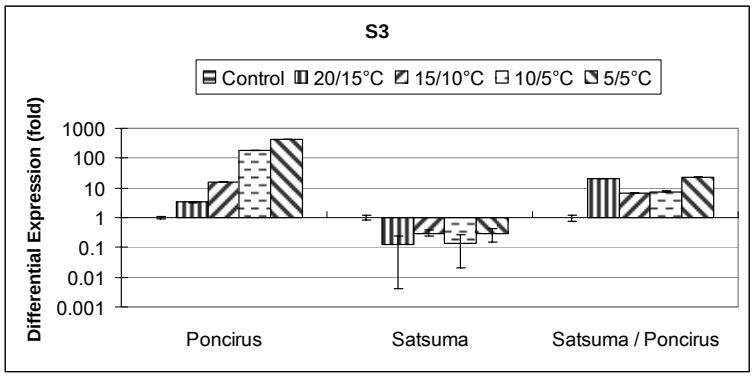
These histograms show relative gene expression levels of *CuG01* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.9 mRNA expression level comparisons of *S3* (Auxin GH3) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



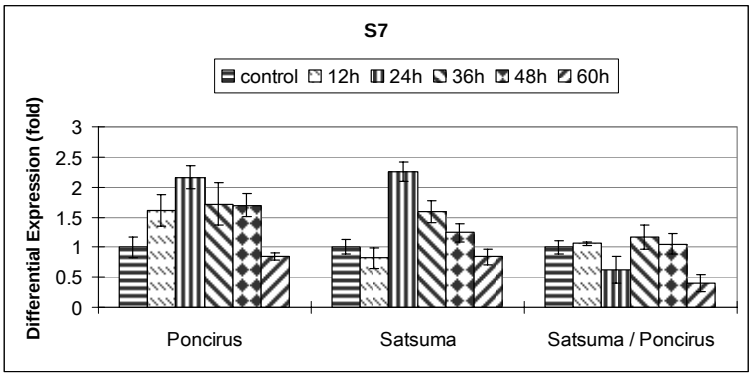
(b)



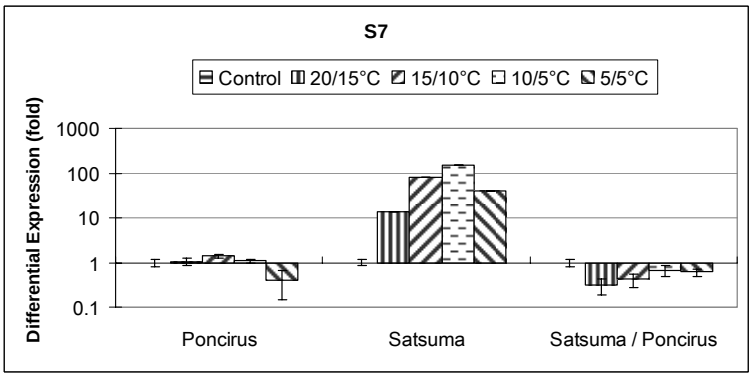
These histograms show relative gene expression levels of *S3* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.10 mRNA expression level comparisons of *S7* (UGT) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



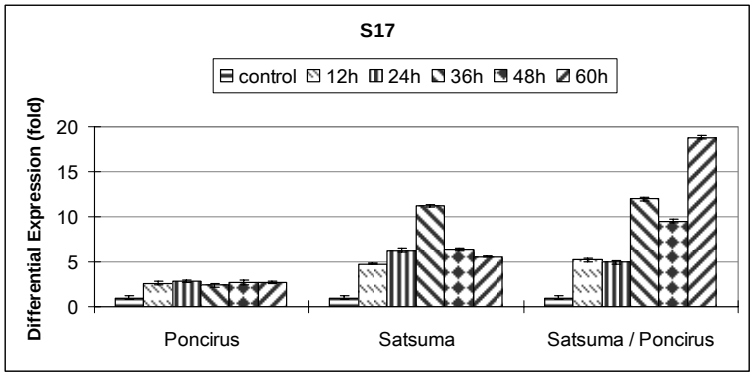
(b)



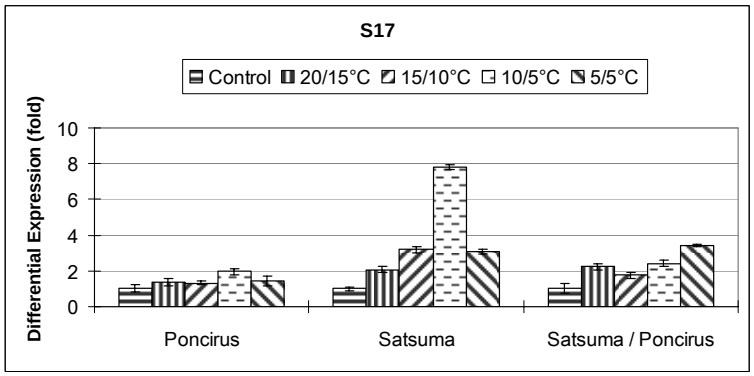
These histograms show relative gene expression levels of *S7* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.11 mRNA expression level comparisons of *S17* (MAPK3) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



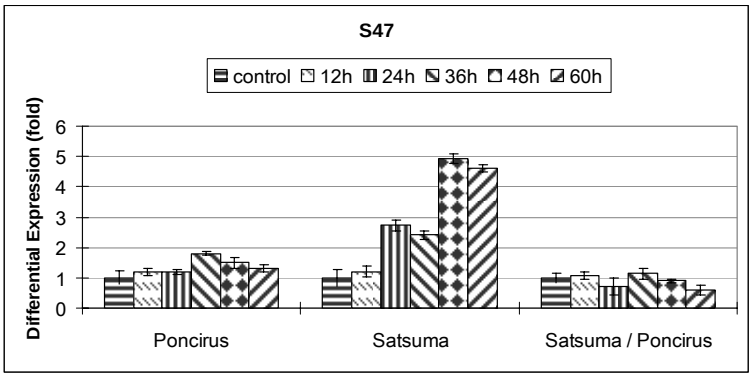
(b)



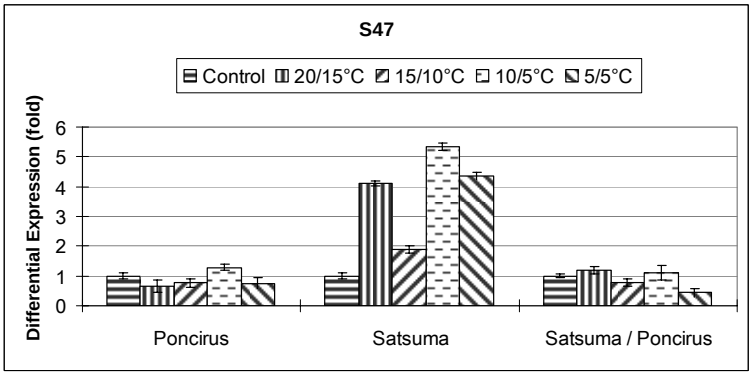
These histograms show relative gene expression levels of *S17* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.12 mRNA expression level comparisons of *S47* (TPS) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



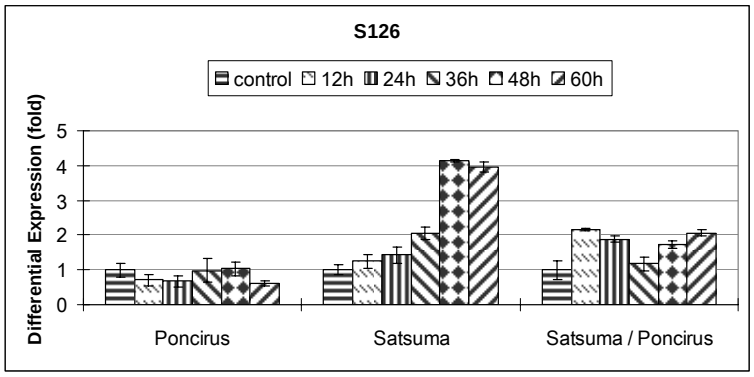
(b)



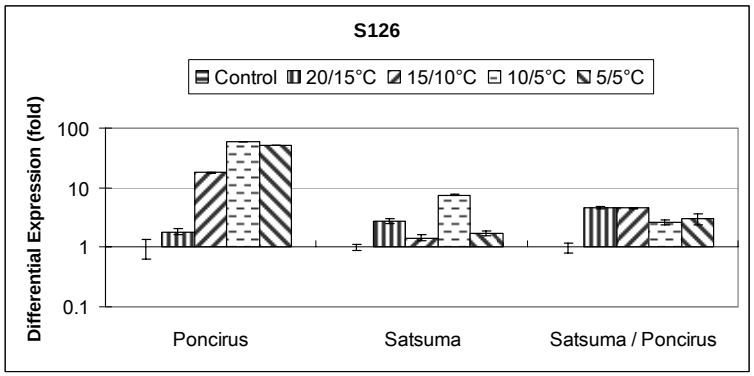
These histograms show relative gene expression levels of *S47* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.13 mRNA expression level comparisons of *S126* (CHI) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



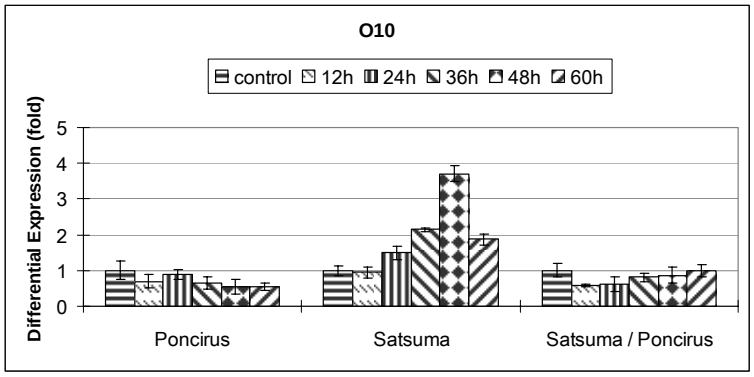
(b)



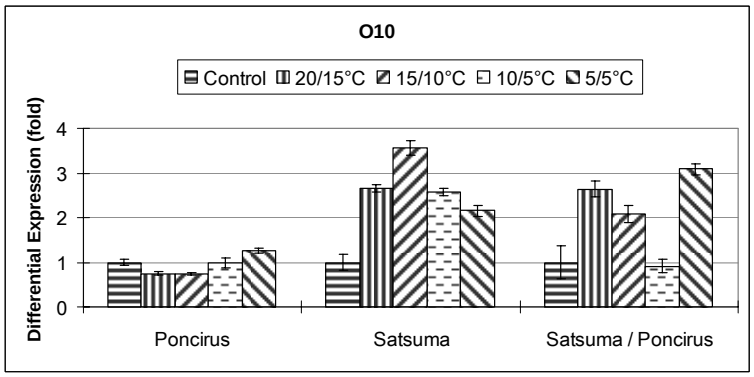
These histograms show relative gene expression levels of *S126* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.14 mRNA expression level comparisons of *O10* (14-3-3) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



(b)



These histograms show relative gene expression levels of *O10* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig. 15 Phylogenetic tree of proline transporter proteins in different species: ProT_PONTR, ProT1_ARATH (NP_181518), ProT2_ARATH (NP_191133), ProT3_ARATH (NP_181198), ProT_POPTR (XP_002316242), ProT_AVIMA (BAB93109), ProT1_LYCES (AAD25160), ProT2_LYCES (AAD25161), ProT3_LYCES (AAD25162), ProT_ATRHO (AAF76897), ProT_HORVU (BAB69951), ProT_ORYSA (BAD31821).

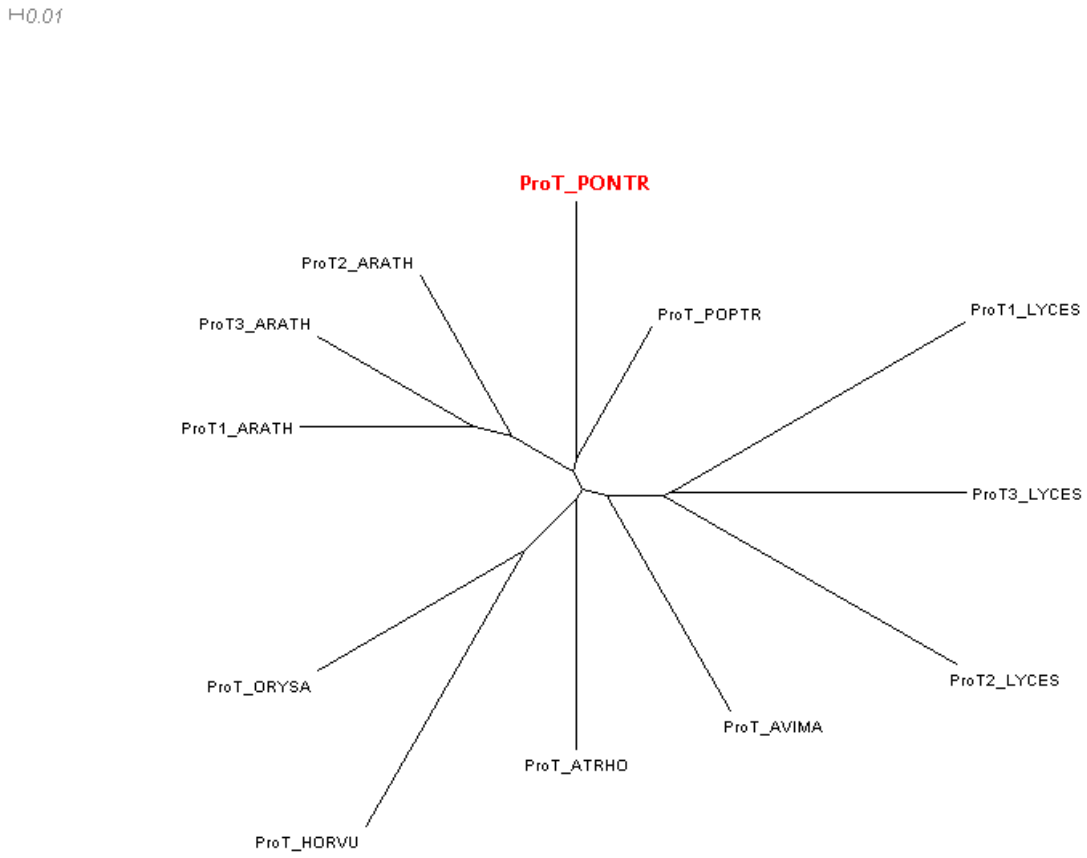


Fig.16 Putative conserved domains of proline transporter protein from *Poncirus trifoliata* by NCBI blastp.

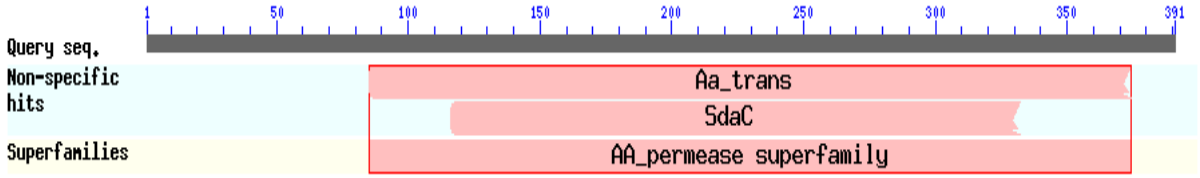


Fig.17 Alignment of the amino acid sequences of proline transporter protein in different species: ProT_PONTR from *Poncirus trifoliata*, ProT1_ARATH (NP_181518) and ProT2_ARATH (NP_191133) from *Arabidopsis thaliana*, ProT_POPTR (XP_002316242) from *Populus trichocarpa*, ProT_ORYSA (BAD31821) from *Oryza sativa*.

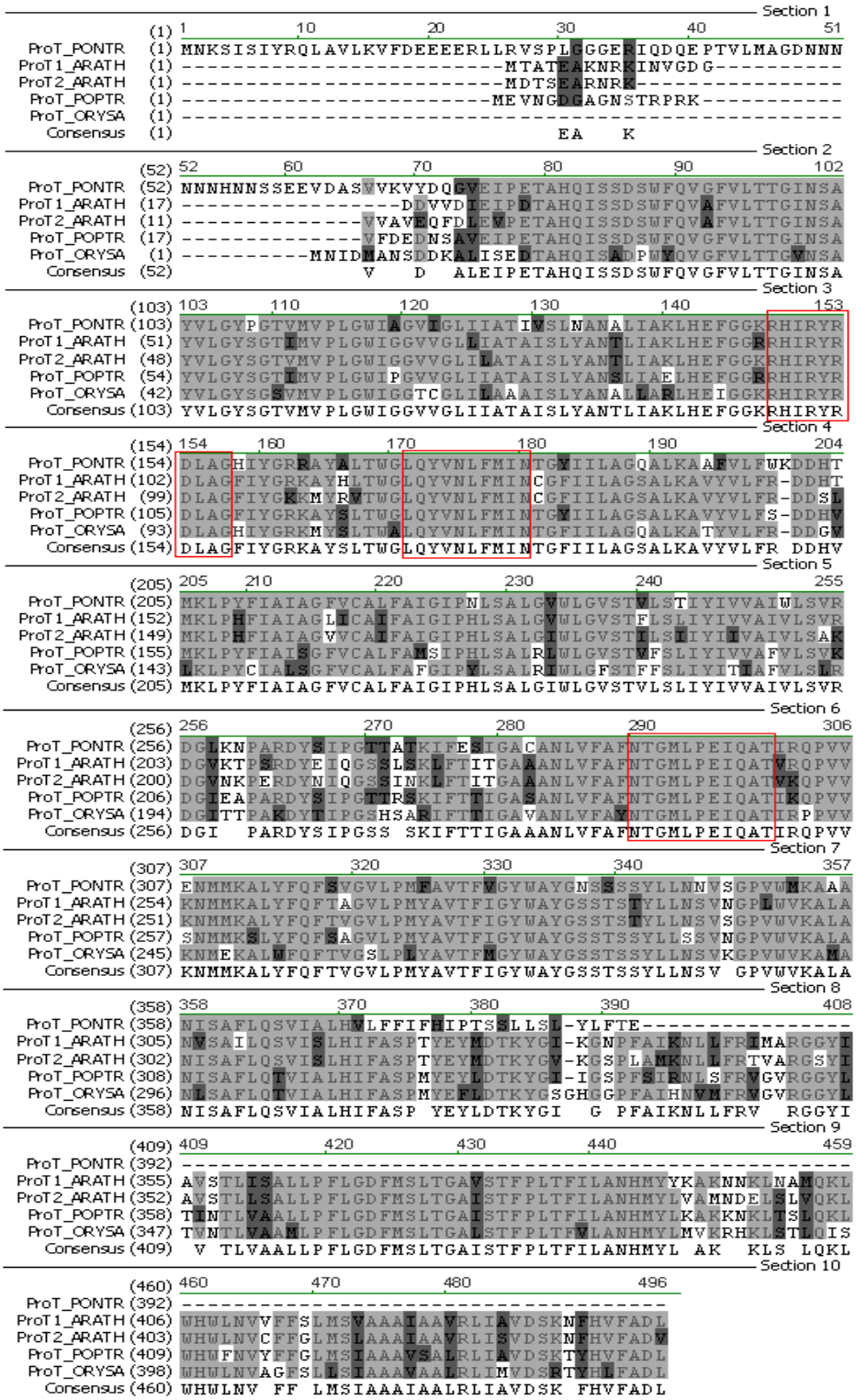
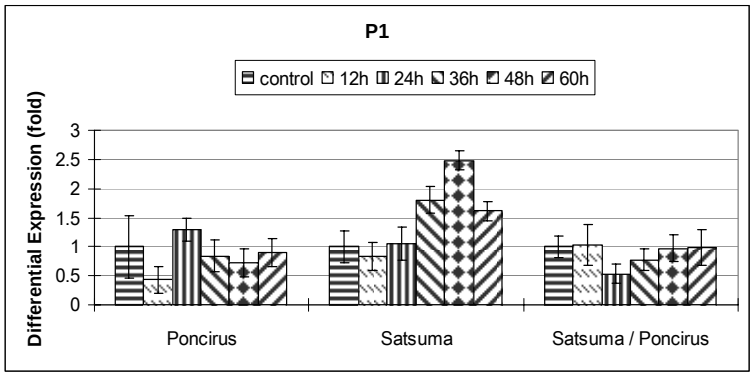
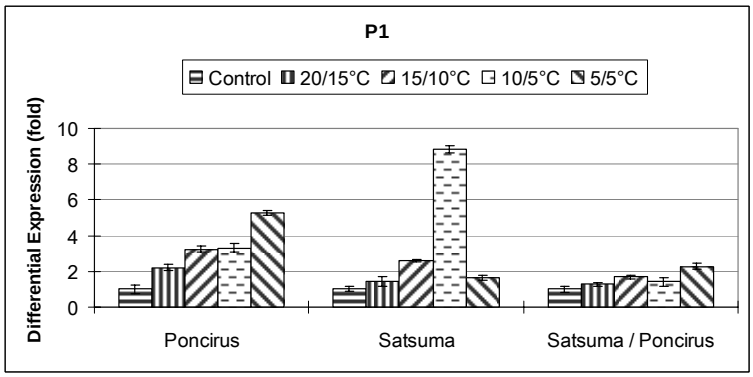


Fig.18 mRNA expression level comparisons of *P1 (*proline transporter*)* gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



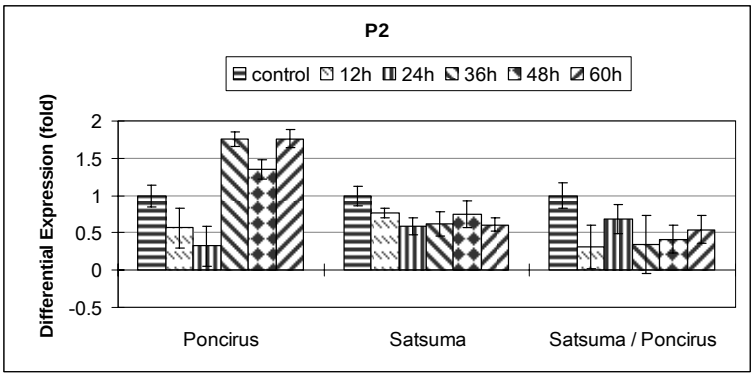
(b)



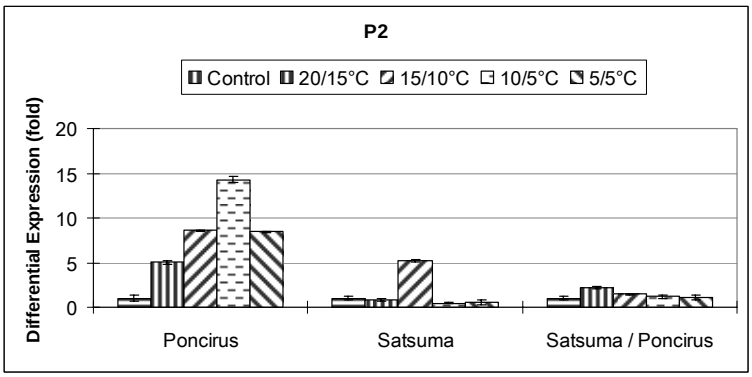
These histograms show relative gene expression levels of *P1* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.19 mRNA expression level comparisons of *P2* (PIP) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



(b)



These histograms show relative gene expression levels of *P2* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.20 Phylogenetic tree of nitrate transporter protein in different species: NRT_PONTR, NRT_RICCO (EEF44932), NRT1.1_GLYMA (BAB19756), NRT1.2_GLYMA (BAB19757), NRT1.5_GLYMA (BAB19760), PTR_HAKAC (ABR32183), NRT_CUCSA (CAA93316), PTR_HORVU (AAC32034), PTR1_VICFA (AAP44102), PTR_ORYSA (BAB62326), PTR2_ORYSA (ABF98582), NRT1.5_ORYSA (BAD82445), PTR2_ZEAMA (ACG28767), PTR2_ARATH (P46032), NRT2.1_ARATH (O82811).

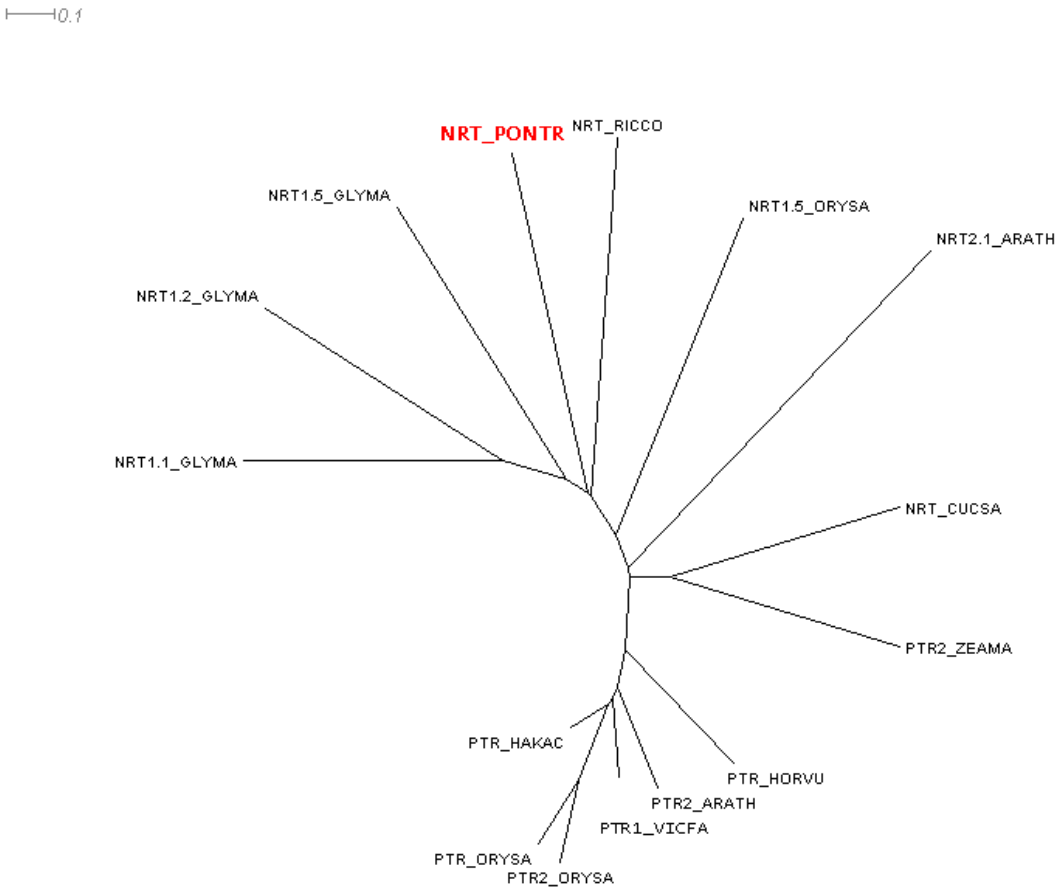


Fig.21 Putative conserved domains of nitrate transporter NRT1.2 protein from *Poncirus trifoliata* by NCBI blastp.

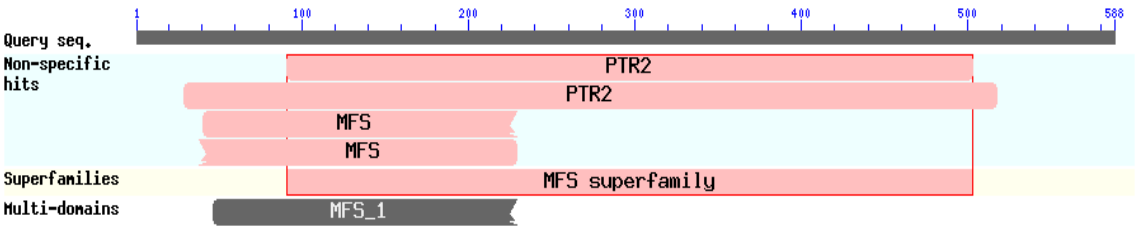


Fig.22 Alignment of the amino acid sequences of nitrate transporter protein in different species. NRT_PONTR from *Poncirus trifoliata*, NRT1.1_GLYMA (BAB19756) and NRT1.2_GLYMA (BAB19757) from *Glycine max*, NRT_RICCO (EEF44932) from *Ricinus communis*, PTR_HAKAC (ABR32183) from *Hakea actites*, PTR_ORYSA (BAB62326) from *Oryza sativa*.

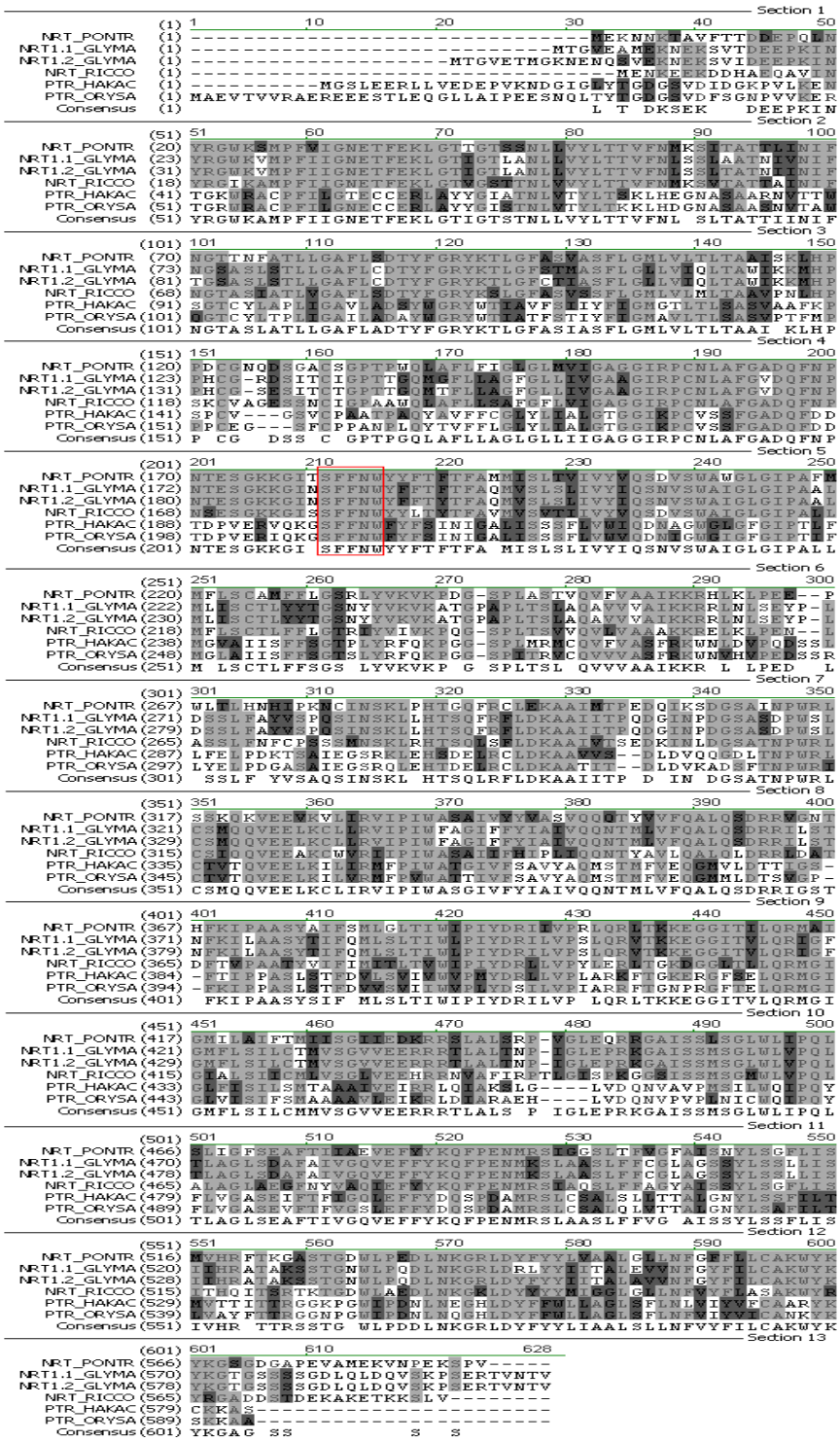
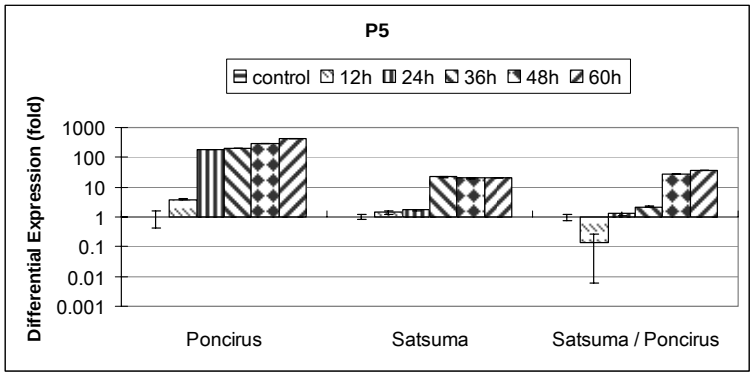
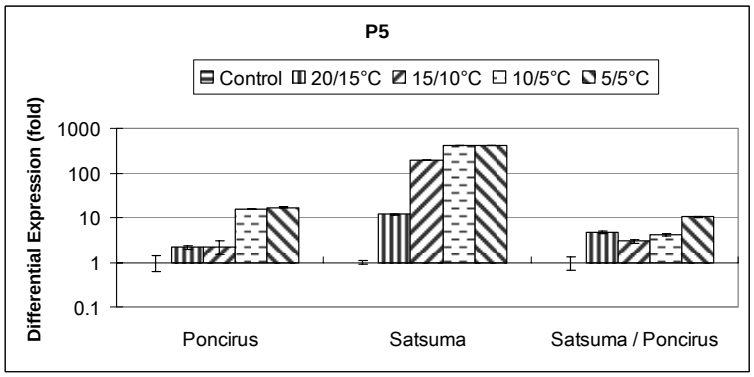


Fig.23 mRNA expression level comparisons of *P5* (nitrate transporter) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



(b)



These histograms show relative gene expression levels of *P5* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.24 Phylogenetic tree of chloroplastic carbonic anhydrase protein in different species: CAHC_PONTR, CAH_ARATH (BAD93915), CAH1_ARATH (NP_850490), CAH2_ARATH (NP_568303), CAH_RICCO (EEF37661), CAH_GOSHI (AAM22683), CAH1_GOSHI (AAD29049), CAH2_GOSHI (AAD29050), CAH_THLCA (AAS65454), CAHC_NICTA (P27141), CAH2_NICTA (AAL51055), CAHC_PISSA (P17067), CAHC_PACTE (ABI14813), CAH_SOLLY (CAH60891), CAH_VIGRA (AAD27876), CAHX_FLABR (P46511), CAH1_FLALI (P46512), CAH3_FLALI (ABC41659), CAHX_FLABI (P46510), CAH3_FLABI (AAO17574), CAHX_FLAPR (P46281), CAH3_FLAPR (ABC41658), CAHC_SPIOL (P16016).

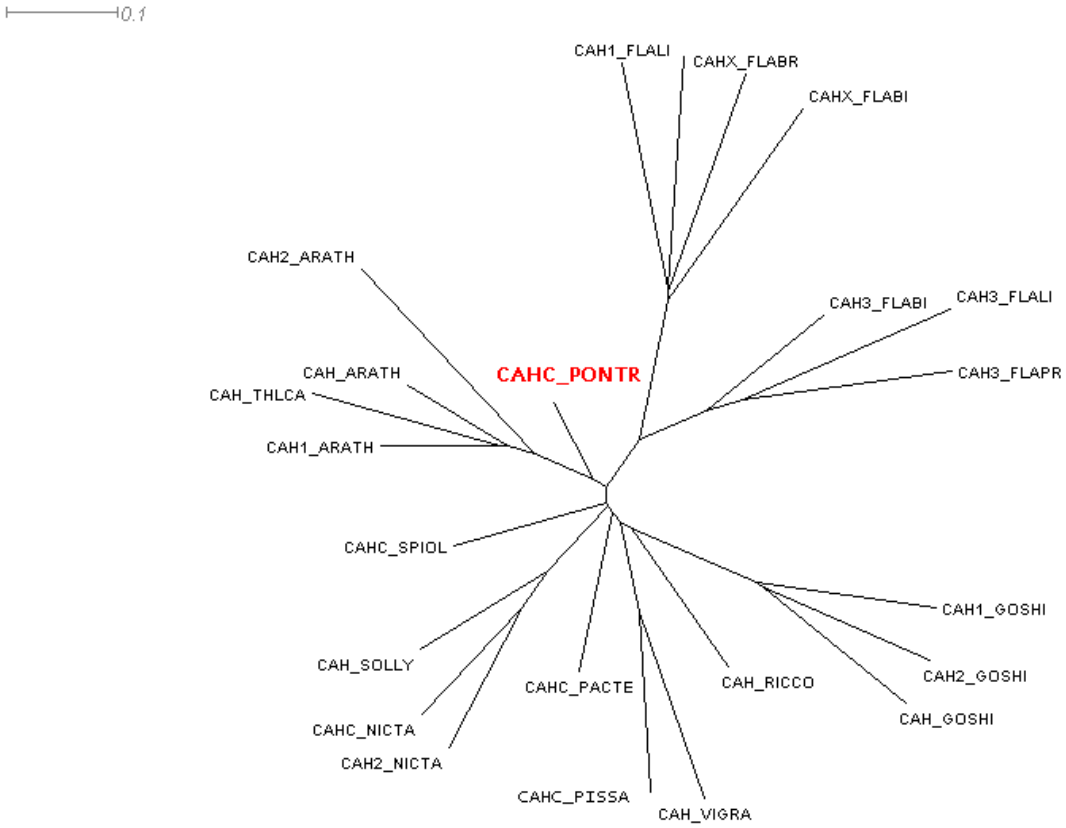


Fig.25 Putative conserved domains of chloroplastic carbonic anhydrase protein from *Poncirus trifoliata* by NCBI blastp.

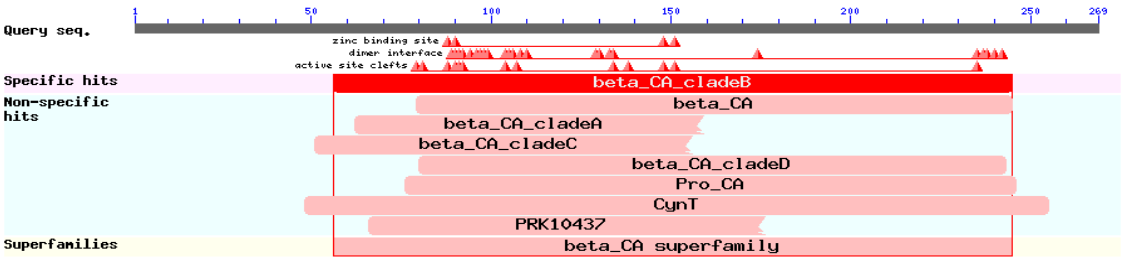


Fig.26 Alignment of the amino acid sequences of chloroplastic carbonic anhydrase protein in different species: CAHC_PONTR from *Poncirus trifoliata*, CAH_ARATH (BAD93915), CAH1_ARATH (NP_850490) and CAH2_ARATH (NP_568303) from *Arabidopsis thaliana*, CAH_THLCA (AAS65454) from *Thlaspi caerulescens*, CAH3_FLAPR (ABC41658) from *Flaveria pringlei*, CAH3_FLALI (ABC41659) from *Flaveria linearis*.

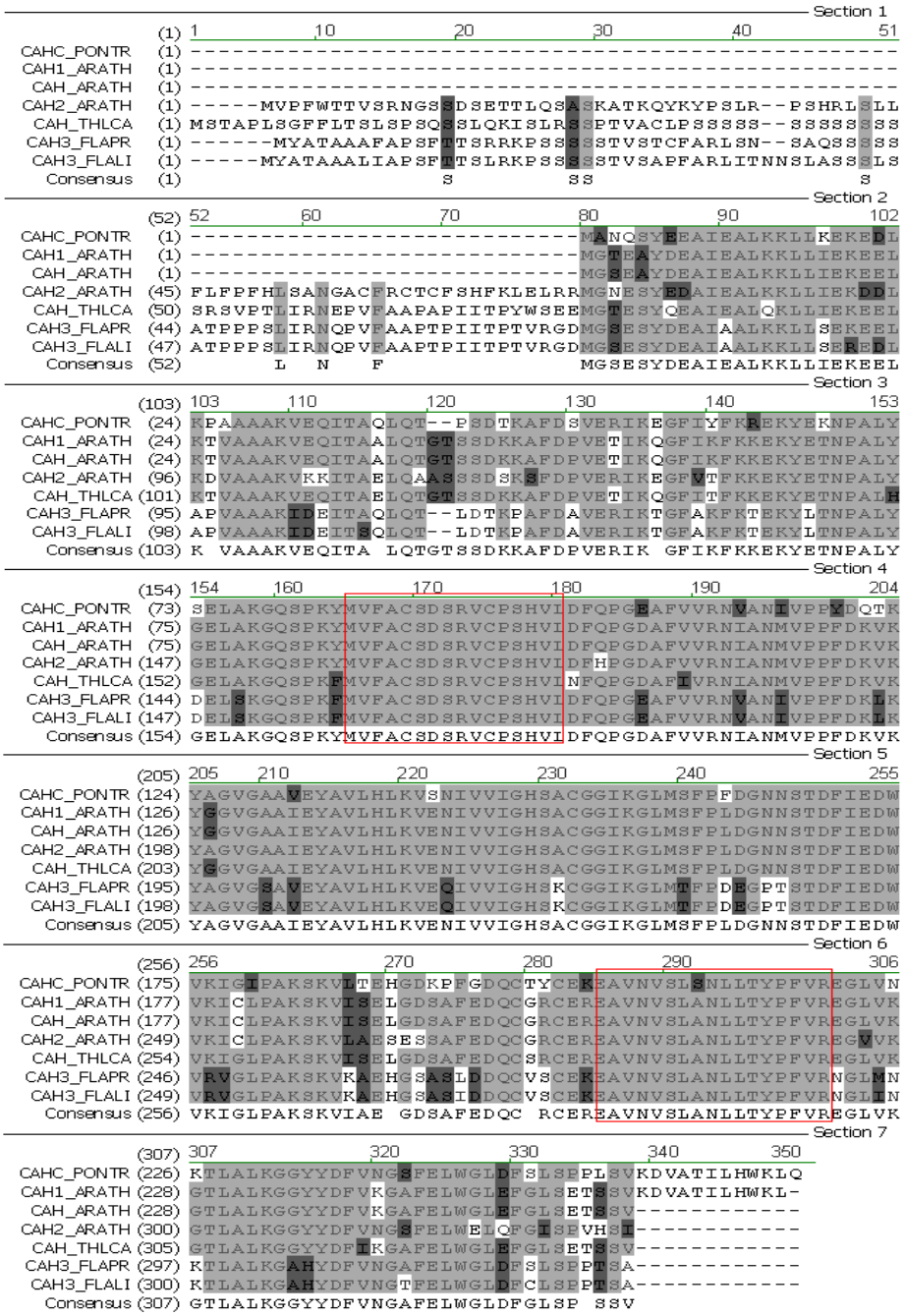
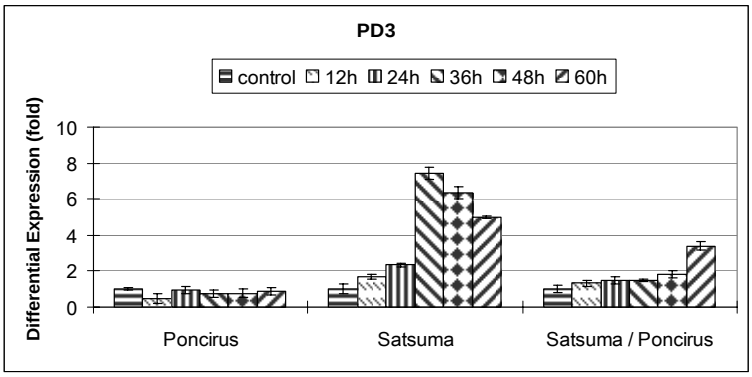
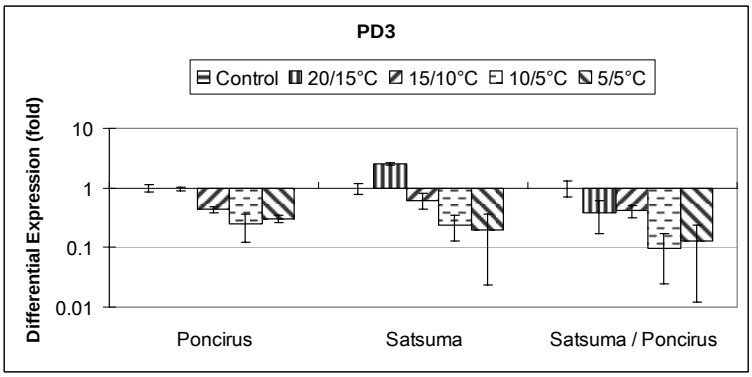


Fig.27 mRNA expression level comparisons of *PD3* (CA) gene between control and cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



(b)



These histograms show relative gene expression levels of *PD3* between control and cold treated plants. Control mRNA of each comparison is considered as 1 and cold treated plant mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

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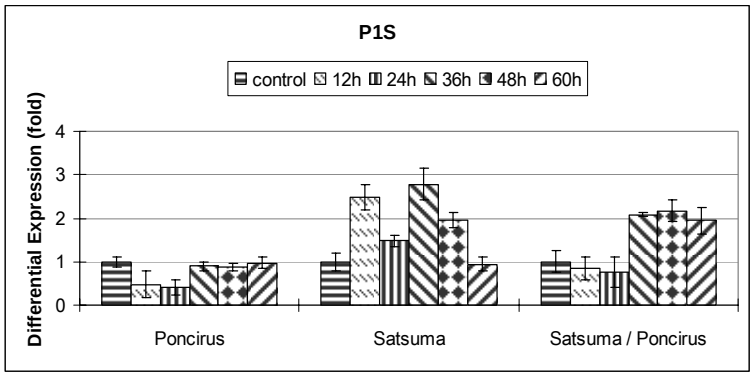
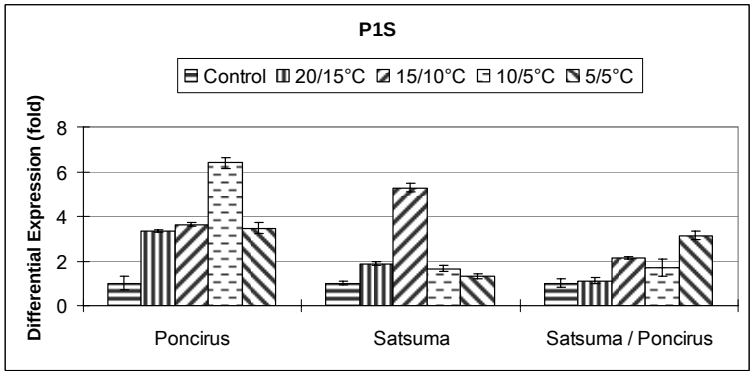
APPENDIX

Fig. 28 mRNA expression level comparisons of *P1* (proline transporter) gene (5' UTR) during cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



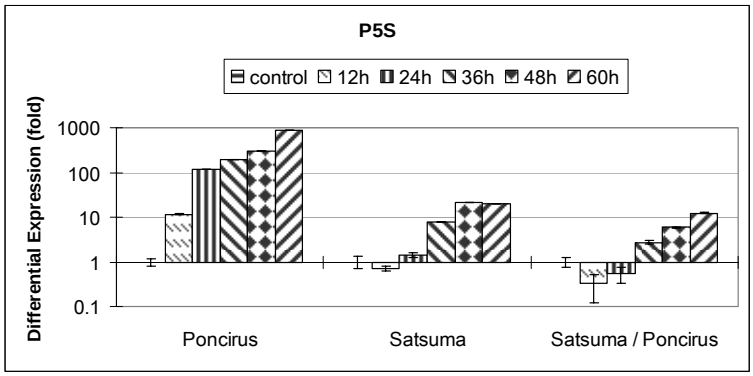
(b)



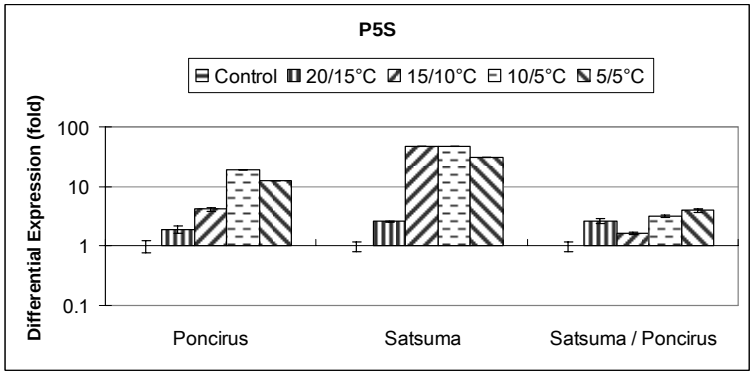
These histograms show relative gene expression levels of *P1S* between control and plants treated under cold stress conditions. Control mRNA of each comparison is considered as 1 and cold treated plants mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig. 29 mRNA expression level comparisons of *P5* (nitrate transporter) gene (3' UTR) during cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



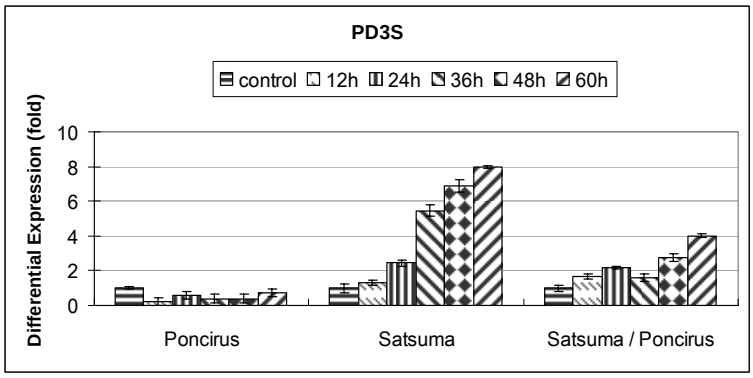
(b)



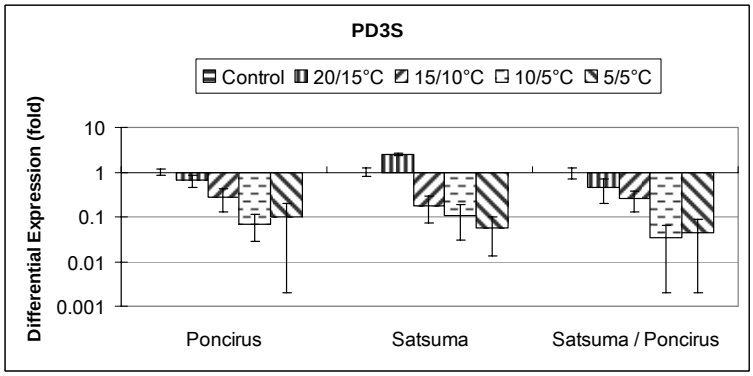
These histograms show relative gene expression levels of *P5S* between control and plants treated under cold stress conditions. Control mRNA of each comparison is considered as 1 and cold treated plants mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig.30 mRNA expression level comparisons of *PD3* (CA) gene (5' UTR) during cold treatments in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted on Poncirus.

(a)



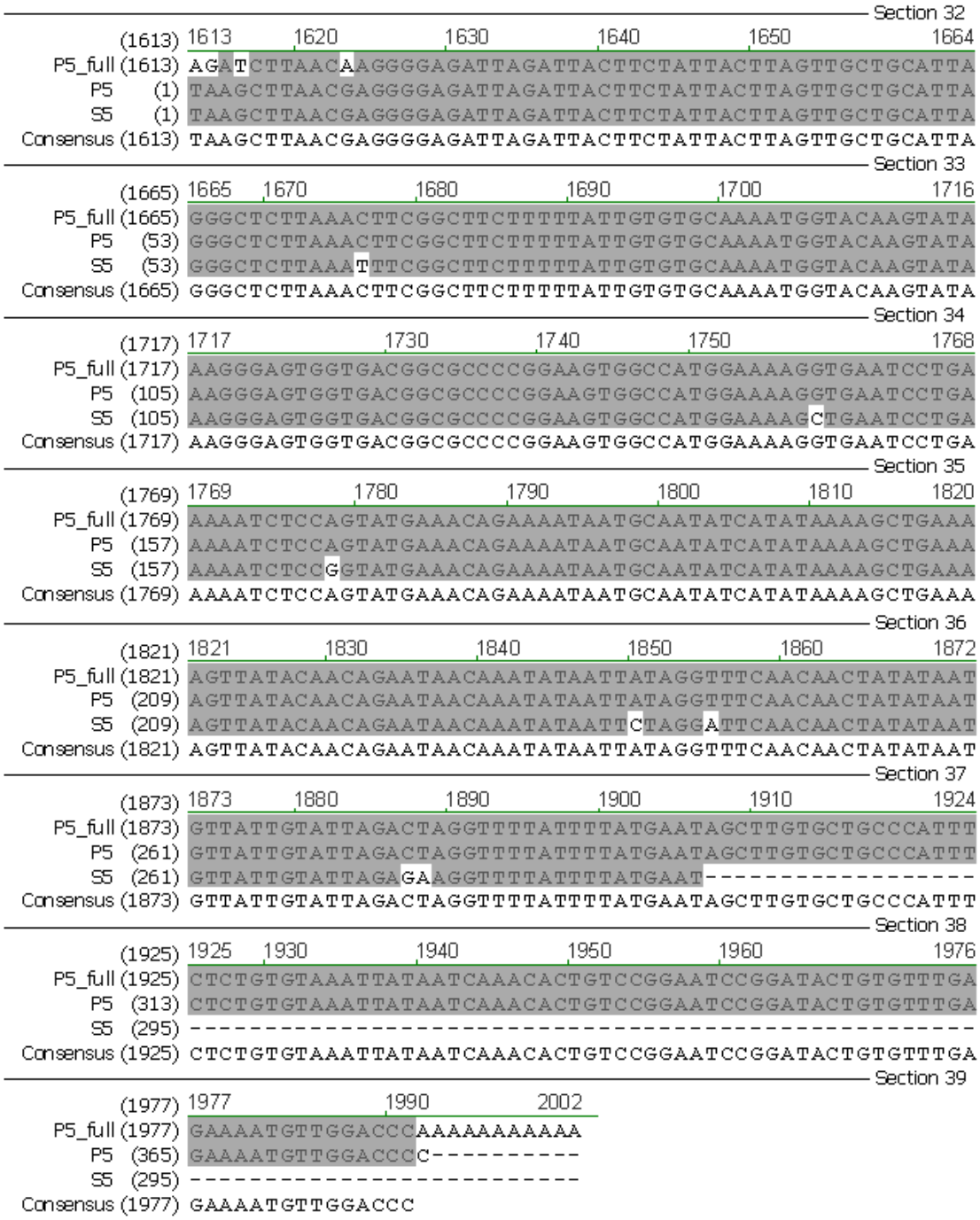
(b)



These histograms show relative gene expression levels of *PD3S* between control and plants treated under cold stress conditions. Control mRNA of each comparison is considered as 1 and cold treated plants mRNA transcript levels were normalized by comparing the ΔΔCt to control. (a): cold shock followed by 12, 24, 36, 48 or 60 h of treatment at 4°C; and (b): cold acclimation. The values are the means of three independent experiments ± SE.

Fig. 31 Alignment of the nucleic acid sequences from (a) full length gene P5 with short regions at the 3' end of Poncirus (P5) or Satsuma (S5), and (b) full length clone of P1 with short regions at 3' end of Poncirus (P1) or Satsuma (S1).

(a) Nitrate Transporter



(b) Proline transporter

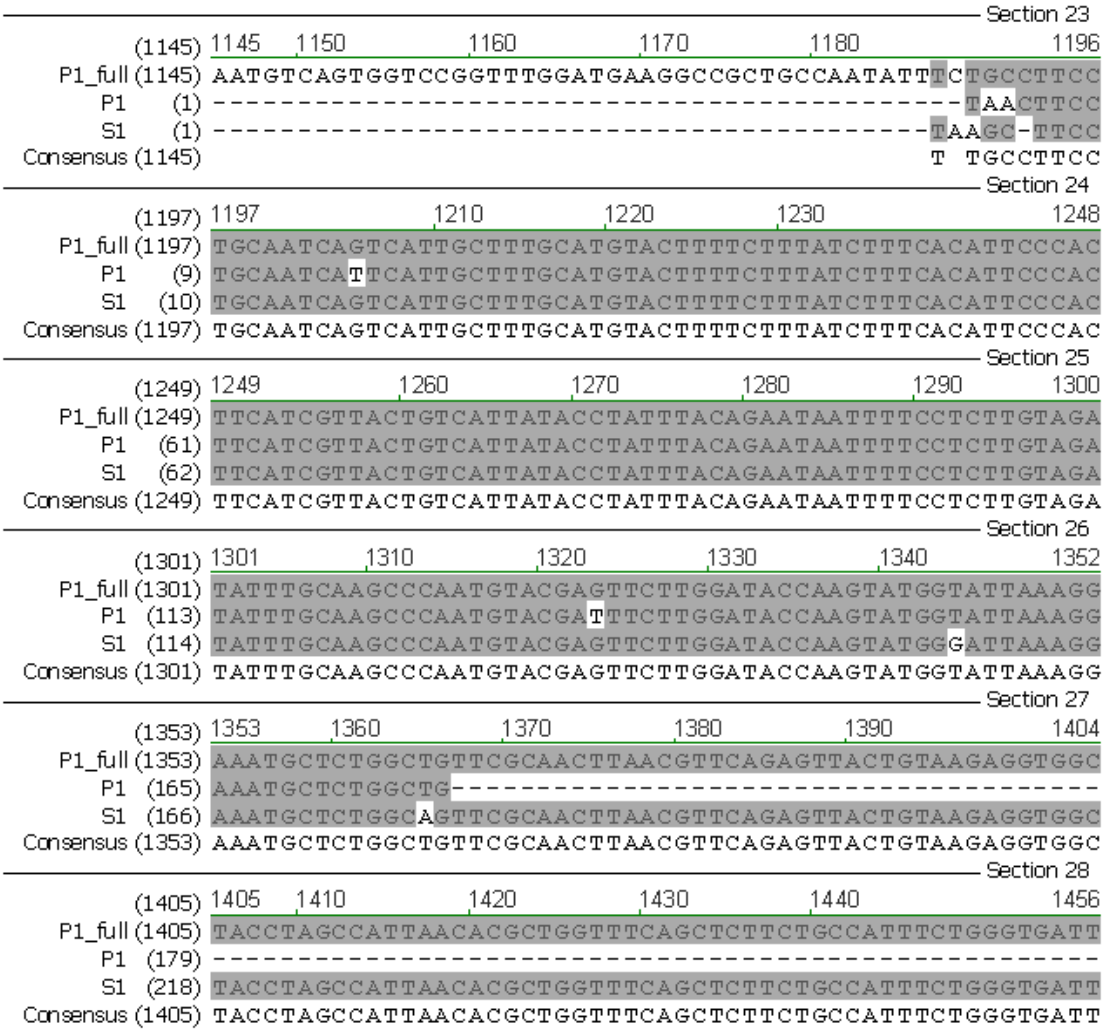
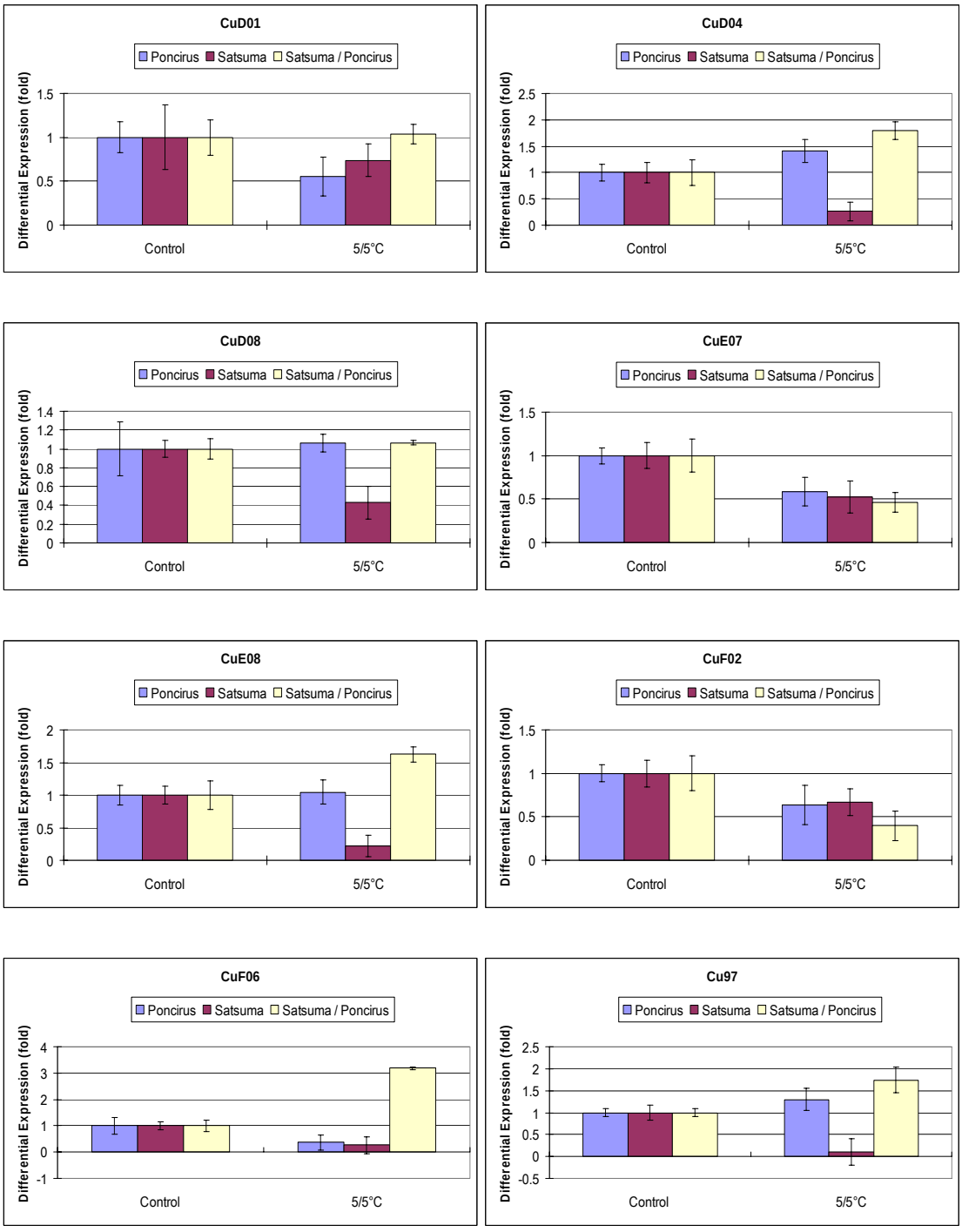
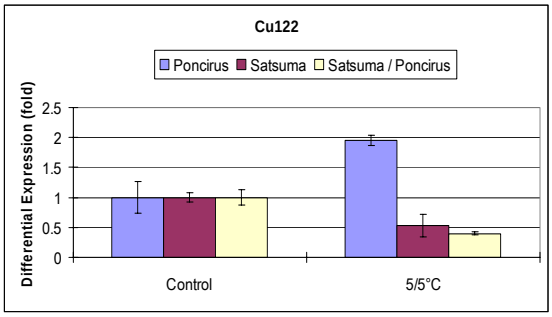
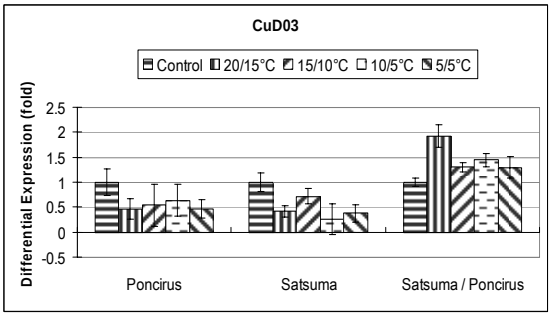
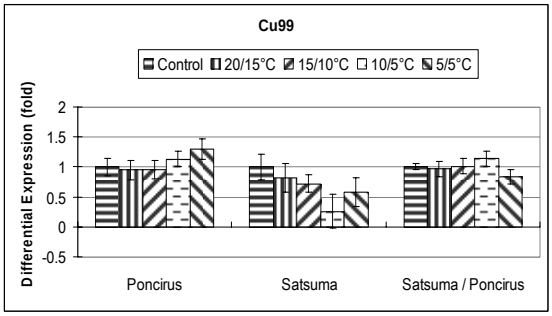


Fig. 32 mRNA expression level comparison of genes which did not show much effect of cold stress in *Poncirus trifoliata*, Satsuma mandarin and Satsuma grafted onto Poncirus. Sequence homology of these TDFs is described in Table 4.




Cu122
Poncirus
Satsuma
Satsuma / Poncirus
Differential Expression (fold)
2.5
2
1.5
1
0.5
0
Control
5/5°C


CuD03
Control
20/15°C
15/10°C
10/5°C
5/5°C
Differential Expression (fold)
2.5
2
1.5
1
0.5
0
-0.5
Poncirus
Satsuma
Satsuma / Poncirus


Cu99
Control
20/15°C
15/10°C
10/5°C
5/5°C
Differential Expression (fold)
2
1.5
1
0.5
0
-0.5
Poncirus
Satsuma
Satsuma / Poncirus

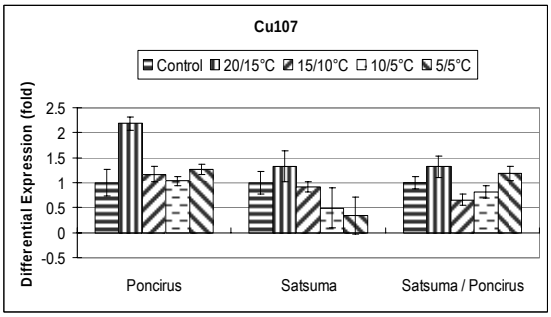

Cu107
Control
20/15°C
15/10°C
10/5°C
5/5°C
Differential Expression (fold)
2.5
2
1.5
1
0.5
0
-0.5
Poncirus
Satsuma
Satsuma / Poncirus

Table 4. Differential gene expression of TDFs isolated from *C. unshiu* in response to cold acclimation.

TDFs	Sequence homology	Organism	*E* value
Cu97	Leucine-rich repeat family protein / extensin family protein	*Arabidopsis thaliana*	1e-32
Cu99	Tubulin alpha chain	*Ricinus communis*	1e-29
Cu107	Structural maintenance of chromosome 1 protein	*Ricinus communis*	1e-20
Cu122	Vacuolar protein sorting-associated protein	*Ricinus communis*	1e-15
CuD01	Brassinosteroid LRR receptor kinase	*Zea mays*	1e-33
CuD03	40S ribosomal protein S6-2	*Arabidopsis thaliana*	5e-21
CuD04	Phosphoglucomutase, cytoplasmic	*Pisum sativum (pea)*	1e-33
CuD08	Glutamate receptor 3.1	*Oryza sativa*	1e-33
CuE07	Glycosyltransferase QUASIMODO1	*Arabidopsis thaliana*	4e-30
CuE08	Eukaryotic translation initiation factor 4G	*Arabidopsis thaliana*	1e-28
CuF02	DEAD-box ATP-dependent RNA helicase 37	*Arabidopsis thaliana*	0.028
CuF06	GTP-binding protein-related	*Arabidopsis thaliana*	2e-26