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Proteomic analysis of the cold stress response in the leaves of birch (*Betula platyphylla* Suk)

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Abstract

Low temperature is one of the most severe environmental factors that impair growth of plants and agricultural production. To investigate how actively growing birch (*Betula platyphyll*a Suk) adapts to cold stress, two and a half-month-old birch plants were exposed to cold stress (4°C) and compared to plant material maintained at the control temperature (23°C). This treatment did not affect the survival of the plants, but growth was almost arrested. Relative electrolyte leakage (REL) of adult leaves after stress treatment of 4, 7, and 10 days at 4°C was significantly higher than REL of the leaves exposed to the control temperature, There was no significant difference, after 14 days, in REL of leaves exposed either to the control temperature or to cold stress at 4°C. These results show that birch can adapt to cold stress. Proteomic analysis, by bidimensional electrophoresis was performed, and a total of 15 protein spots were identified by mass spectrometry after 14 days of cold stress at 4°C. Proteins that were identified were involved in defense, photosynthesis, biosynthesis, carbon (C)-nitrogen (N) metabolism, and signal transduction. These proteins may be used for the establishment of a new network based on cooperation when plants are subjected to cold stress. Cold stress response proteins such as the β subunit of ATP synthase were mostly related to all aspects of chloroplast physiology, indicating that the cold resistance of birch was influenced, in part at least, by the chloroplast function. It was further found that the protein spots involved in defense responses, biosynthesis, C-N metabolism, and signal transduction were increased in intensity after cold stress, indicating that they played a key role in the cold hardiness mechanism of birch.

Keywords: *Betula platyphylla* Suk; Cold stress; Stress response; Proteome; 2-DE.
Abbreviations: CBB_Coomassie brilliant blue; ESI_MS/MS-Electrospray ionization tandem mass spectrometry; IEF_Isoelectric focusing; LC-MS/MS_Liquid chromatography-tandem mass spectrometry; MALDI-TOF MS_Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; SE_Standard error; PSII_ photosystem II.

Introduction

Low temperature is one of the major limiting environmental factors that affect the distribution, growth, and productivity of crops or wild plants. Plants have formed a series of mechanisms in the long-term evolutionary process to resist such stress. Cold acclimation describes a phenomenon where a series of processes occur at low temperature, or above ice point, which enable plants to survive under subsequent freezing conditions (Bourion et al., 2002, 2003; Ruelland et al., 2009). Cold acclimation involves several processes, such as stability of cell permeability, change in the composition of membrane lipids, increase in antioxidants, activation of main metabolic activities, change in gene expression patterns, synthesis of new proteins (Sharma et al., 2005), among others. One very important aspect is to self-regulate the expression of genes involved in the cold stress response. These cold stress response genes are thought to play an important role in plant responses to low temperature stress. There have been many studies on cold acclimation in plants and the mechanism of cold hardiness in the past few years, especially in model plants such as *Arabidopsis thaliana*, rice (*Oryza sativa*) (Ruelland et al., 2009), and others. These studies have identified a number of cold stress response genes at the mRNA level that help us to further understand the molecular mechanism of plant responses to cold stress. However, intracellular complex regulatory pathways cannot be studied at the genetic level alone, due to the protein changes caused by post-transcriptional and post-translational gene modification and processing. Proteomic techniques can analyze the simultaneous changes in protein expression as well as the temporal changes in protein accumulation during stress perception, adaptation, and cellular defense. Therefore, proteomic analysis has become an important area of functional genomics research. Compared with the transcriptome, a proteome can provide the amount, location, and efficiency of the final cell product that can be included in the corresponding gene annotation. In recent years, high-throughput proteomic techniques have been used to analyze the responses of *Arabidopsis thaliana* (Amme et al., 2009), *Thellungiella halophila* (Gao et al., 2009), rice (*Oryza sativa)* (Lee et al., 2007; Himoto et al., 2007; Atsu et al., 2009), wheat (*Triticum aestivum*) (Sarhadi et al., 2010), Siberian spruce (*Picea obovata*) (Kjellsen et al., 2010), poplar (*Populus tremula*) (Renaut et al., 2004), Barbados nut (*Jatropha curcas*) (Liang et al., 2007), and other plants during low temperature stress, and have shown that the response of plants to adverse processes such as low temperature involves a polygenic synergistic regulatory network. Plants with different degrees of resistance to cold adopt different strategies, through evolutionary development, to adapt to the cold environment. Birch (*Betula platyphylla* Suk) is a species of *Betula* (*Betulaceae*) that is concentrated in China's 3 northeastern provinces, Inner Mongolia

Autonomous Region, Russia (far east of Siberia), Mongolia, Northern Korea, and Japan. With strong cold resistance, good mountain adaptability, fast growth, and high economic value, birch is one of the vital local species. In order to conduct research on the molecular mechanism of cold-adaptation of birch, Puhakainen et al (2004) obtained the low temperature-induced gene *Bplti36*, cloned from birch, and found that the *Bplti36* transcription levels increased significantly after cold treatment. Research data by Martz et al (2006) showed that low temperature could induce the expression of *BpFAD3* and *BpFAD8*, genes, involved in fatty acid biosynthesis in birch, in order to increase the α-linolenic acid (18:3) content and unsaturated fatty acids, which improve their ability to adapt to the cold. Compared with model plants such as rice and *Arabidopsis thaliana*, the large-scale studies on the birch transcriptome are limited by the lack of genome sequence information, resulting in a poor understanding of the molecular mechanisms of adaptation to cold in birch. The post-translational determination of protein expression levels provides a better program for the understanding of how plants respond and adapt to the environment. So far, no research data on proteomic response to cold stress have been reported. Therefore, the present study used the two-dimensional gel electrophoresis (2-DE)-based comparative proteomic approach to evaluate the response of leaves of birch seedlings to cold stress, in order to identify a new cold stress response protein. The cold stress response protein may then reveal the molecular mechanism of adapatation to cold in birch, and lay a foundation for future research on the cold resistance of plants that may be improved by genetic engineering.

Results

Effect of cold stress on height and growth rate of birch

The plant height of birch was 48.4 cm before 4°C cold treatment, and was 49.3 cm after 14 days of cold treatment. As observed in Figure 1, the maximum daily average growth rate was 3 to 3.6 mm during the entire treatment period at the control temperature of 23°C, and was 1.1 mm after 4 days of 4°C cold treatment. Growth rate was less than 1 mm during other treatment periods. The analysis of variance showed that there was no significant difference between the maximum daily average growth rates during the different treatment periods, but there was a significant difference compared with the maximum daily average growth rate at the control temperature. In addition，the leaves of birch became yellow after 14 days of cold stress. It was clear that low temperature stress had a serious impact on the high growth rate of birch.

Effect of cold stress on relative electrolyte leakage (REL) in birch leaves

Relative electrolyte leakage (REL) is an indicator of cold stress causing plasma membrane damage. In order to assess the effect of cold-induced plasma membrane damage in birch, we measured REL after 4, 7, 10, and 14 days of cold stress. The results are shown in Figure 2. As observed in the figure, the RELs of birch seedling leaves showed a trend of initial increase and then decrease after low temperature treatment at 4°C. There was a significant difference between REL of leaves after 4, 7, and 10 days of cold stress at 4°C and REL of leaves for the same time periods at 23°C. The REL after 7 days of cold stress was the highest, and was increased by 24.5% compared with the control. However, there was no significant difference between the REL of leaves following 14 days of cold stress and REL of leaves following 14 days at the control temperature. During the experiment, REL did not change significantly at control temperature (23°C). Therefore, our results showed that 4°C treatment had greater impact on the membrane permeability of birch seedlings. The process of an initial increase of REL levels and then decrease, followed by recovery to the levels observed in the control showed that birch seedlings had a certain ability to adapt to low temperature stress at a later stage of treatment.

Analysis of expression pattern of total proteins in birch leaves by two-dimensional gel electrophoresis

In order to understand the trend of the expression of proteins in birch leaves in response to cold stress, proteome analysis was carried out. The total protein content of leaves at control temperature (23°C) and after cold treatment (4°C) for 4, 7, 10 and 14 days, was separated by two-dimensional gel electrophoresis (2-DE). Two-dimensional gel electrophoresis separation was performed three times for each sample. The results of the two-dimensional gel electrophoresis showed good reproducibility. Since there is no significant change in protein expression in birch leaves after cold treatment (4°C) for 7 days and 10 days compared to cold treatment of birch leaves (4°C) for 4 days and 14 days, we analyzed the difference of expression of proteins in birch leaves that are subjected to cold stress treatment at 4°C for 0, 4, and 14 days, to reflect the changes in protein expression in the early and late phases of cold acclimation. The two-dimensional gel electrophoresis image analysis, by ImageMaster™ 2D Elite (GE Healthcare Life Sciences, PA, USA), can repeatedly detect more than 300 protein spots. Through quantitative image analysis, it was found that there was a change in expression of a total number of 37 protein spots at a given point in time, and the intensities were 2 times ($P < 0.05$) greater than that of the control. Among them, 20 spots with an increased expression are marked in Figure 3. Those marked on gel A are the protein spots that are expressed in the control and disappear after cold stress; those marked on gel B are the protein spots with significantly increased expression after 4 days of cold treatment; those marked on gel C are the protein spots with significantly increased expression after 14 days of cold treatment. Figure 4 is an enlarged view of the dynamic change of a section of differentially expressed proteins in the results obtained. A section of these differentially expressed protein spots differ in the level of expression as observed in 2 spots (spots 23 and 33), while an alternate section of the spots display a qualitative change, for eg, 2 spots (spots 16 and 17) were only detected in the control samples, and disappeared in samples subjected to cold stress; 7 spots (spot 4, spot5, spot9, spot13, spot18, spot 19, spot 20) were not detected in the control samples, but could be detected after 4 days of cold stress treatment. Four spots (spot 25, spot 27, spot 28, spot 37) were not detected in the control samples or in samples subjected to 4 days of cold stress treatment, but were detected after 14 days of treatment at 4°C. Among the 15 differentially expressed protein spots, 2 spots were decreased, and 13 spots were increased in intensity.

Identification of differentially expressed proteins by mass spectrometry under cold stress

Change in protein expression when birch leaves are subjected to cold stress treatment at 4℃ for 4 and 14 days

The differentially expressed protein spots were excised from

Table1. Identification of the differentially expressed proteins by ESI-MS/MS

Spot No	Pretein name	Accession No	P I/Mw(Kda) Theoretical	Experimental	Score	Sequence[a]	C[b] %	Species
4	Ribulose-1,5-bisphosphate carboxylase	Q8HDA1	6.04/52.7	4.9/17.2	440	DDENVNSQPFMR MSGGDHIHAGTVVGKEITLGFVDLLR VALEACVKAREGNEIIRWSPELAAACE XWK	14	*Dactylocladus stenostachys*
5	Ribulose-1,5-bisphosphate carboxylase	Q8HDA1	6.04/52.7	4.4/15.3	402	DDENVNSQPFMR MSGGDHIHAGTVVGKEITLGFVDLLR VALEACVKAREGNEIIRWSPELAAACE XWK	14	*Dactylocladus stenostachys*
9	Ribulose-1,5-bisphosphate carboxylase large subunit	Q33453	6.34/51.7	5.7/27.1	392	DTDILAAFRALRLEDLRIPPAYSKTFKGPPHGIQVER YGRPLLGCTIKPK W SPELAAACEVWK	13	*unclassified sequences*
13	ATP synthase beta subunit	Q3ZU73	5.25/50.5	5.15/60.8	1741	IAQIIGPVLDVAFPPGKMPNIYNALVVKDTAGQQINVTCEVQQLLGNNRAVAMSAT DG LTRGMEVIDTGASLSVPVGGATLGRIFNVLGEPVDNLGPVDTRTTSPIHR LSIFETGIK VVDLLAPYRR IGLFGGA GVGKTVLIME LINNIAKAHGGVSVFGGVGERTREGNDLYMEMKEXGVINEQNIAESKVALVYGQ MNEPPGARVGLTAL TMAEYFRFVQAGSE VSALLGRMPSAVGYQPT QPRIVGEEHYETAQRVKQT LQR	62	*Betula platyphylla*
16	Sc-3 Pretein(Ypr10b protein)	Q39415	5.74/17.4	5.7/22.1	1030	GVFNYEDEATSVIAPARSFVLDADNLIPKVAPENVSSAENIEGNGGPGTIKKITFPEG SHFKHRVDEIDHANF KYCYSIIEGGPLGDTLEKISYEIKIVAAPG GGSILKEEEIKAGKEKGAGLFKAVEN YLVAHPNAYN	93	*Betula pendula*
17	Light harvesting chlorophylla/bbinding protein (Fragment)HW101-LHPII	CAA 48410.1	4.83/20.7	5.1/34.2	201	GLSADPETFAKNRELEVIHS RFGEAVWFK	15	*Hedera helix*
18	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Q9GDL4	6.04/52.5	4.2/21.0	167	LTYYTPXYETKDTXILAAFR	4	*Gunnera monoica*
19	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Q9GDL4	6.04/52.5	4.3/21.2	122	LTYYTPXYETKDTXILAAFR	4	*Gunnera monoica*
20	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Q9MVF4	6.22/52.5	4.4/15.4	399	DTDILAAFRDDENVNSQP FMREITLGFVDLLR DDYIEKDRVALEACVQARIEGREGNEI IRWSPELAAACE	18	*Querc shemisphaerica*
23	Calmodulin	Q8W0Q0	4.07/16.7	4.7.19.3	286	VWKEIKFEFPAMDTL MADPLTDDQISEFKEAFS LFDKDGDGCITTK DTDSEEELKEAFRV FDKDQNGFISAAELR	40	*Stevia rebaudiana*
25	Rubber elongation factor protein	P15252	5.04/14.7	5.7/27.1	185	SGPLQPGVDIIEGPVKNV AVPLYNRDASIQVV SAIR SLASSLPGQTK	34	*Hevea brasiliensis*
27	Rubber elongation factor protein	P15252	5.04/14.7	5.7/33.1	171	SGPLQPGVDIIEGPVKN ©VAVPLYNRDASIQVVSAIR SLASSLPGQTK	34	*Hevea brasiliensis*
28	Protein thylakoid formation1	Q84PB7	8.96/32.1	5.3/33.3	167	GSFSYSRFFAVGLFRSVDRDLDVYR	8	*Oryza sativa (japonicacultivar-group)*
33	Beta-conglycinin	P13916	5.88/50.5	4.7/18.8	167	SPQLENLRLAIPVNKPGRQVQE LAFPGSAQDV ER	10	*Glycine max*
37	Chain A, Prx D (Type Ⅱ)	1TP9A	5.56/17.4	5.3/23.2	197	VILFGVPGAFTPTCSLKGVTEILCISVNDPFVMKRFALLVDDLKV	28	*Populus Tremula*

a : The sequence ofmatched peptides.;b: Sequenoe coverage of matched peptides

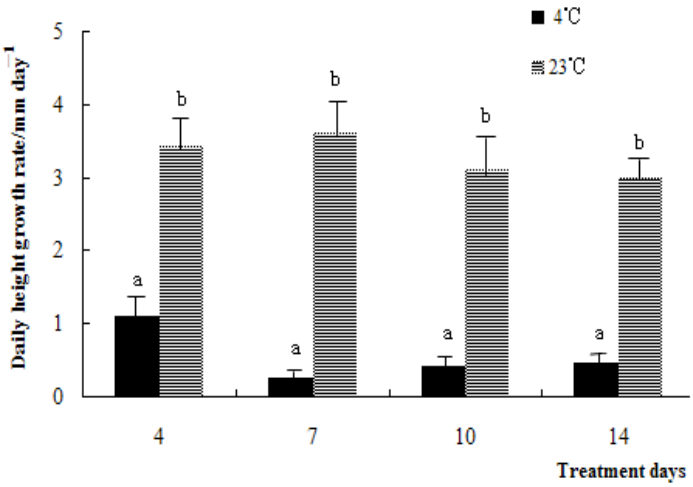

Fig 1. Development of daily height and growth rate of birch during the 14 days of 4°C exposure in comparison with control temperature (23°C) . Each value represents the mean value ± SE from 36 measurements. Different letters indicate significant difference between 4°C and 23°C conditions at $P < 0.05$.

the gel and digested with trypsin on the gel. By mass spectrometric analysis, 15 protein spots were identified. The results are shown in Table 1. Spot 16 identified as Sc-3 protein is also called Ypr10b protein, and belongs to a member of the pathogenesis-related protein PR-10 family. The Sc-3 protein disappeared, through degradation, after the cold stress of 4 days. Spot 17, identified as light-harvesting chlorophyll a/b-binding protein, disappeared through degradation after the cold stress of 4 days. Spot 23, identified as calmodulin, is a signal transduction related protein. The expression of spot 23 was increased after cold stress, and the intensity of protein expression was significantly improved after 14 days. Spot 33 was identified as β-conglycinin, belonging to a member of the seed storage protein family, and continued to increase in intensity after 4 and 14 days of cold exposure at 4°C. The expression of spots 4, 5, 9, 18, 19, and 20 as the degradation fragments of energy-related protein ribulose-l,5-bisphosphate carboxylase/oxygenase (RuBisCO) or the large fragment of RuBisCO were significantly increased after 4 days of cold stress, while the expression level after 14 days was 2 to 3 times as much as that observed after 4 days. Spot 13, identified as ATP synthase β subunit was formed through degradation of ATP synthase at low temperature, and its expression was increased under cold stress conditions with little change after 4 and 14 days. Spots 25 and 27 as rubber elongation factor proteins were expressed with increased intensity after 14 days of cold stress. Spot 28, which was identified as protein thylakoid formation 1 was expressed after 14 days of cold stress.Spot 37 was identified as PrxD (Type II) and was expressed only after 14 days of cold stress.

Identification of the subcellular localization of differential protein expression

Based on their functions, the 15 identified proteins can be classified as follows: defense, photosynthesis, biosynthesis, carbon-nitrogen metabolism, and signal transduction. The major category was the proteins involved in photosynthesis (35.3%). As observed from the database search, protein thylakoid formation 1, the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase, and light harvesting chlorophyll a/b binding protein were located in the plastid and the chloroplast. Beta-conglycinin was located in vacuoles and aleurone. Rubber elongation factor protein was located in the cytoplasm. Calmodulin was located in vacuoles, nucleus, stroma and inclusion bodies of cytoplasm, as well as mesophyll cells and chloroplasts. Sc-3 protein was located in the chloroplasts, endodermis, and matrix of plant mesophyll, the cytoplasm and nucleus. PrxD (Type II) was located in the cytoplasm. ATP synthase beta subunit was located in the chloroplasts and mitochondria. It follows that the majority of the proteins are located in the chloroplasts, indicating that the chloroplast plays an important role in the response mechanism of plants to cold stress.

Discussion

Cold stress affects almost all aspects of plant cell functions. Comparative proteomic analysis can reveal the cold stress-induced complex cellular networks in plants. With a high resistance to cold, birch can be used as a model tree to study the mechanism of cold resistance. The proteomic analysis of the response of birch to cold stress helps to determine the key regulatory factors of plant hardiness. We identified the cold stress response proteins of birch seedling leaves after 4 and 14 days of treatment at 4°C, such as the large subunit of RuBisCO, light-harvesting chlorophyll a/b-binding protein, rubber elongation factor protein, calmodulin, among others as well as 4 new low-temperature response proteins, including PrxD (Type II), Sc-3 protein, β-conglycinin, and protein thylakoid formation 1. These proteins can be divided into 2 broad categories: the first category consists of regulatory proteins, such as proteins involved in signal transduction; the second category consists of functional proteins or proteins that may play a role in the response to resistance, such as the proteins participating in defense, photosynthesis, photorespiration, and C- N protein metabolism. This provides a foundation for identifying the response mechanism of plants to cold stress. The possible physiological functions of these proteins in the cold stress response are discussed as follows.

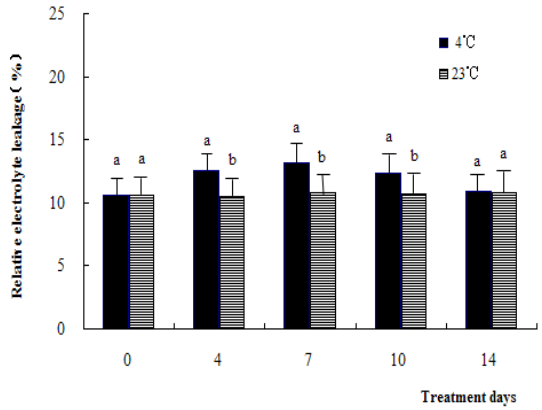

Fig 2. Development of relative electrolyte leakage in the leaves of birch during the 14 days of 4°C exposure in comparison with control termperature (23°C). Each value represents the mean value ± SE from eight measurements. Different letters indicate significant difference between 4°C and 23°C conditions at $P < 0.05$; same letter indicates similar values at $P < 0.05$.

Carbon-nitrogen metabolism-related proteins involved in the response to cold stress

It is reported that many proteins or enzymes play an important role in the cold stress signal transduction pathways. There are significant changes in the gene expression and metabolism of the plants that are subjected to cold stress. The fact that a large number of metabolism-related proteins or enzymes have been identified also shows that these proteins play an important role in the birth or secondary metabolism of plants under cold stress conditions. Adverse conditions such as drought, cold, high salt stress, among others, may cause physiological changes to the plant cells, especially the destruction to plant body homeostasis. In the face of adversity, the changes in plant metabolism such as carbon metabolism, amino acid metabolism, among others, result in adaptation of plants to the environment to achieve a new plant body homeostasis. The seed storage protein, β-conglycinin, is the source of carbon and nitrogen in metabolism and plays a role in plant development. Studies have shown that the expression level of the β subunit of β-conglycinin in soybean and transgenic *Arabidopsis thaliana* was enhanced under S-undernourished conditions (Naito et al., 1994). The β-conglycinin content of soybean root and cotyledons and hypocotyl was increased during salt stress (Aghaei et al., 2008). So far, cold stress-induced plant β-conglycinin expression has not been reported. In this study, the β-conglycinin content of birch leaves gradually increased at the time of cold stress. It was suggested that this protein may be closely related to environmental stress.

Biosynthesis-related proteins involved in the cold stress response

Rubber elongation factor protein relates to plant biosynthesis, and is also a kind of stress-related protein, with environmental stress defense function (Priyaet et al., 2006). In the response of *Thellungiella halophila* to cold stress, the gene encoding rubber extension factor protein was expressed at the transcriptional level (Wong et al., 2006). In the present study, they were significantly expressed only after 14 days of cold treatment, showing that strong low temperature stress induced an elevated expression of rubber elongation factor protein and promoted the synthesis of downstream material. It is possible that the plants need more matter and energy to maintain their survival in order to adapt to the low temperature stress. Protein thylakoid formation 1 (THF1) relates to vesicle-mediated thylakoid membrane, and is required for the entry of the normal tissue of the vesicles into mature thylakoid matrix, ultimately promoting the growth and development of leaves. Furthermore, the THF1 and G-protein (GPA1) of *Arabidopsis* jointly play a role in the sugar signaling processes between plastids and plastid membranes (Huang et al., 2006). In the present study, the chloroplast thylakoid membrane components of birch were greatly influenced by low temperature stress after 14 days. The expression of THF1 was significantly increased in order to maintain the functionality of the thylakoid, which may be the response and adaptation of plants to adverse conditions.

Photosynthesis-related proteins involved in the response to cold stress

Research data have shown that the photosynthesis of a variety of plants is inhibited under low-temperature stress (Allen and Ort, 2001), but the mechanism is not yet fully understood. The photochemical reactions of photosynthesis are conducted in the thylakoid membrane; therefore, the damage to the thylakoid membrane by low temperatures is bound to affect the absorption of light energy, transmission and conversion in plants, thus affecting the metabolism, normal growth, and development of plants. The ketose, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the most abundant protein in the plant and its content accounts for 12% to 35% of all leaf proteins as well as 50% of all the soluble proteins, especially in C3 plants. The RuBisCO participates in photorespiration to maintain the flow of electrons under low temperature stress in order to prevent the occurrence of photoinhibition. Yan et al (2006) identified 19 low temperature-induced protein degradation fragments of the large subunit of RuBisCO. The 2 RuBisCO and 4 RuBisCO large subunits that were identified were also different from the complete RuBisCO in isoelectric point and molecular weight, suggesting that they may be the degradation fragments of complete RuBisCOR or the large subunit of RuBisCO. Continued increase after 14 days of the initiation of cold stress is consistent with the findings of Yukio and Matsuo (2003) with regard to the expression of proteins in response to cold stress in *Arabidopsis thaliana*. This shows that the expression level of enzymes, associated with energy metabolism such as photosynthesis and respiration, is increased under cold stress but does not necessarily signify an increase in plant energy metabolism. Wilson et al (2002) found that the large subunit of RuBisCO was expressed at an increased level in the early part of plant leaf senescence, and the level of expression was significantly decreased in the latter part of senescence. The enhanced expression of RuBisCO in *Thellungiella* leaves was decreased at 4°C after 24 days of cold stress (Gao et al., 2009). These results show that senescence or cold stress induced adversity-related mechanisms in plants, and some proteins in plants show similar changes in expression when adapting to adverse conditions. Green plants accept solar energy for final assimilation of CO_2 through light-harvesting chlorophylla/b binding protein of photosystem II (LHCP II). Studies have found that the LHCP II in birch leaves were completely degraded after 4 days of cold stress. Similarly, Pedron et al (2009) observed that the mRNA expression of LHCP II in cypress was inhibited at low temperatures.

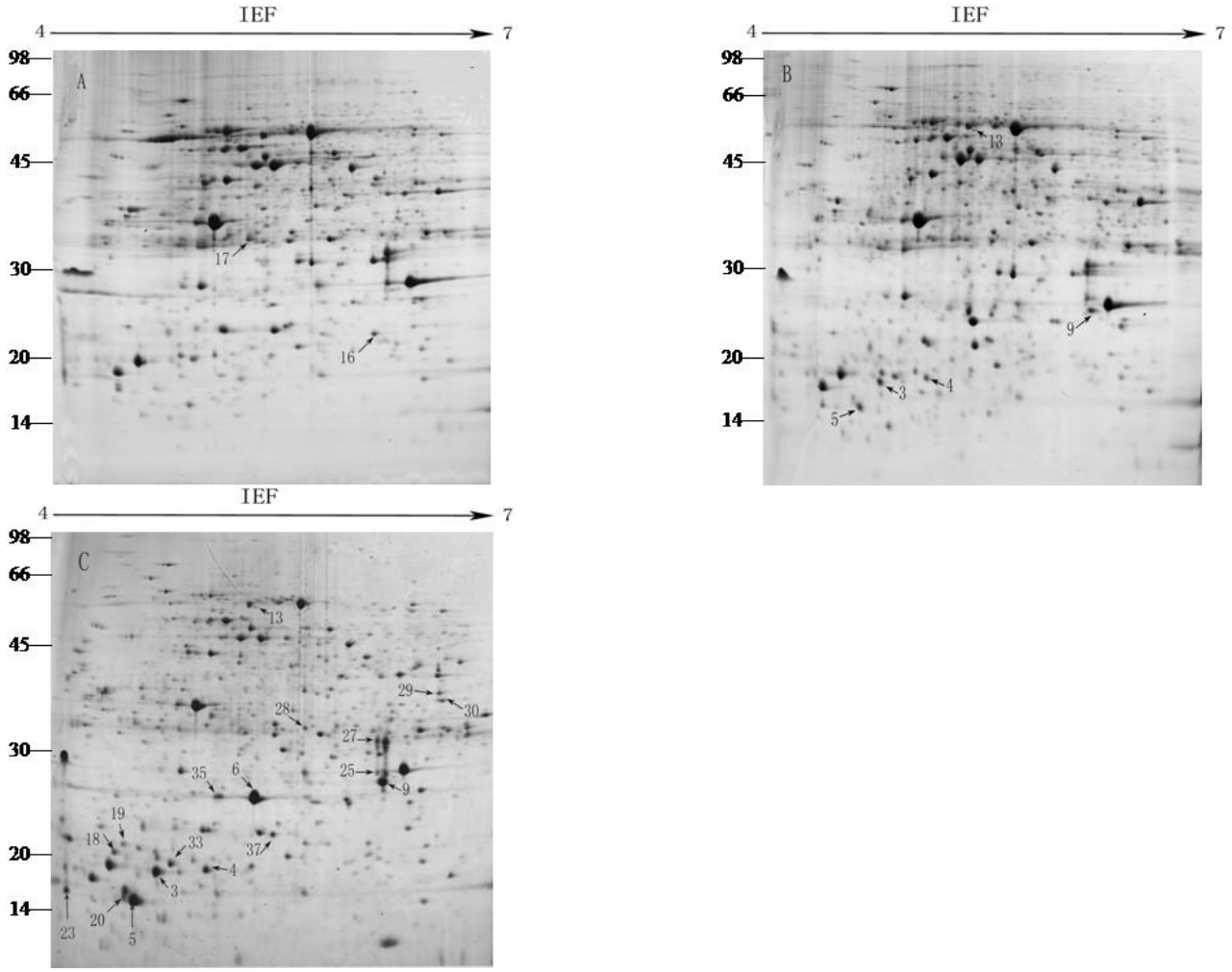

Fig 3. 2-DE gels of birch leaf proteins under cold stress. Total leaf proteins were extracted and separated by 2-DE. In IEF, 1000 μg of proteins were loaded onto pH 4 to pH 7 IPG strips (13 cm, linear). SDS gel electrophoresis was performed with 12.5% gels. The spots were visualized by silver staining. Quantitative image analysis revealed a total of 37 spots that changed their intensities significantly ($P < 0.05$) by more than 2-fold at least at one time point. 3a. 2-DE gel of the control sample. The downregulated spots are numbered. 3b, 3c. 2-DE gel of samples treated at 4°C for 4 d and 14 d respectively. The upregulated spots are numbered.

During the cold adaptation of peas, a LHCP II protein was inhibited (Dumonta et al., 2011). These results demonstrate that cold stress seriously affects plant photosynthesis. ATP synthase (F1F0-ATPase enzyme) is a key enzyme involved in energy metabolism. In the chloroplast, ATP synthase is composed of 2 parts including CF0 and CF1, among which CF1 consists of 5 subunits (α, β, γ, δ, ε). The main catalytic subunit of CF1, β subunit, forms ATP from ADP in the event of transmembrane proton gradient. In this study, the expression of ATP synthase β subunit was induced after 4 days of treatment of birch leaves at 4°C . This is consistent with the result that the β subunit content of the ATP synthase CF1 in *Arabidopsis* chloroplast stroma was increased after 10 days of treatment at 5°C (goulas et al., 2006). ATP synthase β subunit, induced under cold stress, inevitably formed more and more ATP products for the cold defense process.

Defense-related proteins

Peroxiredoxins (Prxs) are very important members of the reactive oxygen scavenging system in plants that participate in the complex regulation of transcription and protein levels by endogenous and external stimuli. Peroxiredoxins can react with many peroxide substrates to clear away intracellular accumulated reactive oxygen species, regulating the balance of the free radical metabolism *in vivo* and improving the stress resistance in plants. Besides the antioxidant defense role in photosynthesis, respiration, and stress response, Prxs may also be involved in redox signaling during development and adaptation (Dietz. 2011). Studies have shown that some types of Prxs play a role in the process of resistance to abiotic stress; for example, the PrxF protein in *Arabidopsis* is slightly increased in cold conditions (Gama et al., 2007). The levels of 2-Cys peroxiredoxin and peroxiredoxin-2E in the *Thellungiella halophilla* with strong cold resistance were increased, while that of 2-Cys peroxiredoxin in peas with poor cold resistance was decreased after cold stress. PrxD was significantly increased in undernourished conditions (Dietz. 2011). PrxD protein was expressed after 14 days of cold stress in this study, demonstrating its defensive role in cold stress. We observed from the previous study that membrane permeability returned to control levels after 14 days of cold stress, showing that the control of the chloroplast redox balance is an important part of the cold adaptation mechanism of birch.

Signal transduction protein involved in the response to cold stress

Calmodulin (CaM), a widely distributed regulatory protein

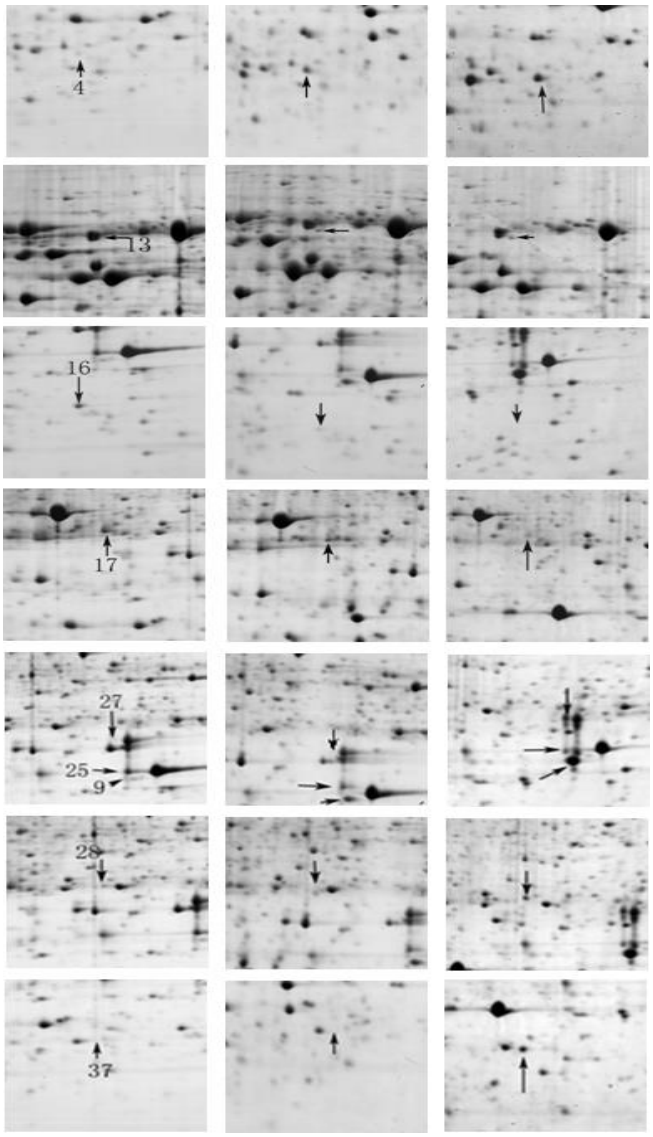

Fig 4. Expression changes of some cold-responsive protein spots. 2.5 month-old seedlings were treated at 4°C for 0, 4, and 14 days. Total birch leaf proteins were extracted and separated by 2-DE. Arrows indicate proteins showing changes in abundance during cold exposure. Spots 4, 9, 18, 19, and 20 identified as the large subunit of ribulose-1,5-bisphosphate carboxylase. Spots 13 (ATP synthase β subunit), Spots 25 and 27 identified as rubber elongation factor protein, spots 28 (protein thylakoid formation1) and 37 (Chain A, Prx D (Type Ⅱ)) were newly induced after the exposure to cold stress for 24-14 days. Spots 16 (Sc-3 protein) and 17 (light-harvesting chlorophyll a/b-binding protein) were downregulated by cold exposure.

with multiple functions, plays an important role in the Ca^{2+} messenger system transfer process, and can regulate the activity of many enzymes dependent on Ca^{2+} levels. The change in calmodulin levels may be one of the important mechanisms of the response of plants to external stimuli. The study on Ca^{2+}/calmodulin-regulating receptor kinase CRLK1 revealed the indirect regulation of cold acclimation response via Ca^{2+} / calmodulin, that is, Ca^{2+}/ calmodulin was bound to CRLK1 in order to increase its activity (Yang et al., 2010a). Subsequent research has shown that the interaction of CRLK1 with MEKK1 (MEKK1, a mitogen-activated protein (MAP) kinase kinase kinase 1 family member) in plants regulated a variety of stress response processes (Yang et al., 2010b). Studies have shown that the 2 isoforms of phosphorylated calmodulin in *Oryza sativa* leaf sheaths were respectively increased and decreased after the cold stress (Komatsu et al., 2009). Cold acclimation increased the CaM content of seedling leaves and the activity of glucose-6-phosphate dehydrogenase (G6PDHase) and ATPase and improved the survival rate and frost resistance of seedlings, thus inferring that CaM may participate in regulating the activity of G6PDHase and ATPase (Lin et al., 2001). In this study, the abundance of calmodulin in birch with strong cold hardiness was increased in the cold adaptation process, inferring that it may participate in regulating the activity of enzymes dependent on Ca^{2+}, and ultimately improve cold hardiness through the regulation of multiple response processes.

Hypothetical proteins expressed during cold stress

Sc-3 protein (Ypr10b protein) is one of the PR-10 protein family members that is expressed at the transcriptional level in European white birch (*Betula pendula*). Sc-3 protein belongs to the BeTV1 family and plays a role in plant defense. PR-10 protein was found to exist in a variety of

plants, which was demonstrated with RNase and protease activity. PR-10 protein was not only involved in the mechanism of antiviral reaction in plants, but also played a role in the development of the plants themselves (Walter et al., 1996). Some PR10 proteins were constitutively expressed in plant growth, developmental processes such as pollen, flowers, fruits, seeds, vegetative organs such as roots, stems and leaves. In the present study, Ypr10b protein was constitutively expressed in the normally growing leaves of birch. Renaut et al (2008) obtained low temperature-induced PR-10 family proteins from the peach tree. The above results may be due to the different subtypes of Ypr10 protein or the different functions of the same subtype in different plants. Research by Martijn et al (2006) showed that Ypr10 protein was the main component of the European birch pollen allergens, but Ypr10b protein function of birch leaves was unclear and needed further study. Cold stress is one of the most significant factors leading to a decrease in the crop yield. It is important to understand how plants will cope with this environmental stress, which is essential for survival and productivity and is a key for its development. Plants respond to environmental constraints in the form of a network that regulates gene expression during stress. By regrouping proteomic observations and based on previous physiological results (Wu et al., 2008) it was observed that (1) plantlets of birch are able to cold acclimate and (2) diverse stress coping mechanisms were observed in sensitive organs. Indeed, 100% survival, growth cessation, perturbation of PS II and adaptation to cold stress (relative electrolyte leakage returned to control levels after 14 days of cold stress) may be related to the accumulation of macromolecules with protective functions (eg, proteins, carbohydrates) or instead related to a stress response. Overall, this work shows that cold exposure, besides inducing different types of responses, triggers several pathways leading to differential gene expression and, consequently, protein expression. It was found that the protein spots associated with defense, C-N metabolism, biosynthesis and signal transduction were significantly increased during cold treatment, indicating that these cellular processes were enhanced due to cold stress. The identified protein spots associated with photosynthesis and energy metabolism showed different changes in patterns, indicating that these processes were reorganized under cold stress. It could be inferred that these proteins were involved in multiple physiological processes *in vivo*, and their combined action enabled birch to establish a new protein balance under low temperature conditions, promoting the formation of cold hardiness.

Materials and Methods

Plant materials

Experimental materials and low-temperature treatment

Seeds of birch from stands of open pollinated trees, originating from Maoershan (45°N, 128°E), in North China, were sown on wet tissue paper in Petri dishes. After germination, the seedlings were transplanted to plastic pots containing a mixture of peat, sand, and pig manure (2:1:1) as the growth medium and grown for two and a half-months at the greenhouse of Northeast forestry university. The 72 birch seedlings with consistent growth and the seedling height of 45.5 ± 0.5 cm were transferred to an artificial climate chamber at 23°C for one week. Then 36 seedlings were respectively placed in the artificial climate chambers at either 4°C treatment temperature or control temperature (23°C) for 14 days. The seedlings were grown in a 16 h/8 h (light/dark) photoperiod with artificial light to give a photon flux density of 160 μmol/$m^{-2}s^{-1}$ at 400 to 740 nm, which was provided by fluorescent lamps (Osram GmbH, Germany, Fluora, L36W/77) with an ambient humidity of 60% to 80%. Samples were obtained from leaves of the central portion of the seedlings after 4, 7, 10, and 14 days of cold stress treatment at 4°C and control conditions at 23°C. A total of 72 leaves were obtained from each treatment. The above samples were frozen in liquid nitrogen, and then stored at -70°C for protein extraction.

Determination of height and growth rate of seedlings

The height and growth rate of seedlings were determined 0, 4, 7, 10, and 14 days, at 23°C and, after treatment at 4°C respectively.

Determination of relative electrolyte leakage (REL)

The relative electrolyte leakage (REL) assay was performed according to the method previously described (Yan et al., 2006) and 8 replicates were performed for each sample. Briefly the leaves, after a treatment period of 0, 4, 7, 10, and 14 days at 23°C and 4°C, were rinsed under running cold deionized water for 15 s. We utilized 8 test tubes of 10 mL capacity. For the blank, we added 5 mL of deionized water to the test tube, and measured the conductivity of a blank using a conductivity meter (Model 1461-81, Cole-Parmer®, IL, USA). Leaves weighing 0.1 g were placed in 8 test tubes (10 mL) and vacuum-infiltrated for 30 min. The tubes were incubated at room temperature for 3 h, and the electrical conductivity was then measured. Electrical conductivity was measured again after autoclaving at 120°C for 30 min. Conductivities were corrected by subtracting the mean conductivity of blanks. REL was expressed as the ratio of initial to final conductivity.

Total soluble protein extraction and two-dimensional gel electrophoresis

Trichloroacetic acid /acetone approach (Yan et al., 2005) was used for the extraction of total soluble proteins, and proteins were quantified with a 2D quantification kit. For 2-DE, 1000 μg of extracted proteins were loaded onto semi-prepared gels. For isoelectric focusing, the Ettan™IPGphor™3 system (GE Healthcare Life Sciences, PA, USA), and pH 4 to pH 7 IPG strips (13 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 13 h in 450 μL rehydration buffer containing protein samples. Focusing was performed in 7 steps: 30 V for 8 h, 50 V for 4 h, 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 12 h. The gel strips were equilibrated for 15 min in 10 ml equilibration buffer (50 mM Tris–HCl buffer, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT and 0.002% w/v bromophenol blue). SDS-PAGE was performed with 12.5% gels using the Ettan Six system (GE Healthcare Life Sciences, PA, USA). The gels were run at 15 mA per gel for the first 30 min and followed by 30 mA per gel for 5 to 6 h. The gels were visualized with Coomassie brilliant blue (CBB) R-250. The gels were scanned using an optical scanner in transmissive mode (Umax®, Willich, Germany) at 400 dpi and analyzed with ImageMaster™ 2D Elite software (GE Healthcare Life Sciences, PA, USA). Each sample was replicated by 2-DE at least three times for further analysis. The abundance of each protein spot was estimated by the percentage of volume (%

vol). Only those protein spots with significant and reproducible changes were determined as differentially expressed proteins.

In-gel digestion, protein identification and database* (MasSeq, Swiss Prot/TrEMBL, TargetP and Gene Ontology)*retrieval

The 200 µl Eppendorf suction head with the front tip, which was cut flat, was used to place different protein spots in the 500 µl eppendorf tube, and the peptides were extracted through trypsin hydrolysis. The Nano-ESI-MS/MS was completed on the Micromass Q-TOF II LC/MS/MS hybrid quadrupole/orthogonal TOF mass spectrometer (Micromass, Manchester, UK), with a nanoflow Z-spray source. Polypeptide sequence analysis used palladium-plated borosilicate needle (Protana A/S, Odense, Denmark) according to the approach of Yan et al (2000). The mass spectrophotometer was operated in the positive ion mode, a temperature source was maintained at 80°C, and a voltage of 800 V to 1000 V was applied to the nano-flow probe. Polypeptide amino acid sequence was deduced with the aid of the MasSeq program (Http://www.matrixscience.co.uk). The function of the protein was retrieved and analyzed by UniProtKB/ TrEMBL database. All proteins that were identified were submitted to tted to TargetP (http://www.cbs.dtu.dk/services/TargetP) to predict protein localization.

Statistical analysis

Unless otherwise specified, all experiments were repeated at least three times. The treatment in each experiment was repeated three times, and each repetition contained 5 to 8 plants. Using SPSS 12.0 statistical analysis software, one-way analysis of variance (one-way ANOVA) was performed on the data, and LSD approach was used for multiple comparisons. Differences were considered significant if *P*-values were less than 0.05 ($P < 0.05$).

Conclusions

Using the 2-DE method, we found the expression level of 37 protein spots changed significantly during the cold adaptation of *Betula platyphylla* leaves, and the 15 protein spots with increased expression were identified by mass spectrometry. Fifteen protein spots were successfully identified, and 4 novel cold response proteins were detected for the first time in our study. These proteins were involved in multiple physiological processes *in vivo*, and their combined action helped to establish a new protein balance in order to allow *Betula platyphylla* to adapt to cold stress. The proteins were involved in defense, photosynthesis, biosynthesis, carbon-nitrogen metabolism and signal transduction. Most of the identified proteins were localized intracellularly, and the majority was located in the proteins-chloroplast, indicating that chloroplasts play an important role in the response mechanism to cold stress in plants. The identification of a new cold response protein provides new insights into the cold stress response. Further research involving functional analysis of the protein is required to understand the relevance of the cold response protein within the genome when plants are subjected to cold stress.

References

Aghaei K, Ehsanpour AA, Shah AH, Komatsu, S(2008) Proteome analysis of soybean hypocotyls and root under salt stress. Amino Acids. 36: 91 – 98.

Allen DJ, Ort DR(2001) Impacts of chilling temperatures on photosynthesis in warm hypocotyls Trends. Plant Sci. 6: 36 – 42.

Amme S, Matros A, Schlesier B, Mock HP (2006) Proteome analysis of cold stress response in Arabidopsis thaliana using DIGE technology. J Exp Bot. 7: 1537 – 1546.

Atsu S, Yamada E, Furukawa K (2009) Cold stress changes the concanavalin A positive glycosylation pattern of proteins expressed in the basal parts of rice leaf sheaths. Amino Acids. 1:115 – 123.

Bourion V, Fouilloux G, Signor C, Lejeune-Henaut I (2002) Genetic studies of selection for productive and stable peas. Euphytica. 2: 261 – 273.

Bourion V, Lejeune-Hénaut I, Munier-Jolain N, Salon C (2003) Cold acclimation of winter and spring peas: carbon partitioning as affected by light intensity. Eur J Agron.4: 535 – 548.

Dietz KJ (2011) Peroxiredoxins in plants and cyanobacteria. Antioxid Redox Sign. 4: 1129 – 1159.

Dumont E, Bahrman N, Goulas E, Valot B, Sellier H, Hilbert JL, Vuylsteker C, Lejeune-Hénaut I, Delbreil B (2011) A proteomic approach to decipher chilling response from cold acclimation in pea (*Pisum sativum* L.). Plant Sci. 1: 86 – 98.

Gama F, Keech O, Eymery F, Finkemeier I, Gelhaye E, Per Gardestro¨m P, Dietz KJ, Rey P, Jacquot JP, and Rouhier N (2007) The mitochondrial typeII peroxiredoxin from poplar. Physiol Plant. 1: 196 – 206.

Gao F, Zhou YJ, Zhu WP, Li XF, Fan LM, Zhang GF (2009) Proteomic analysis of cold stress responsive proteins in Thellungiella rosette leaves. Planta.5: 1033 – 1046.

Goulas E, Schubert M, Kieselbach T, Kleczkowski LA, Gardestrom P, Schroder W, Hurry V (2006) The chloroplast lumen and stromal proteomes of Arabidopsis thaliana show differential sensitivity to short-and long-term exposure to low temperature. Plant J. 5: 720 – 734.

Himoto M, Komatsu S (2007) Proteomic analysis of rice seedlings during cold stress. Proteomics. 8: 1293 – 1302.

Huang J, Taylor JP, Chen JG, Uhrig JF, Schnell DJ, Nakagawa T, Korth KL, Jones AM (2006) The plastid protein THYLAKOIDFORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar signaling mechanism in arabidopsis. Plant Cell. 5: 1226 – 1238.

Jenny R, Jean FH, Carole B, Timothy A, Henry Mi C, Erwin W, Michael W (2008) Quantitative proteomic analysis of short photoperiod and low temperatu rerespon barkt issues of peach (*Prunus persica* L Batsch). Tree Genetics Genomes. 4: 589 – 600

Kawamura Y, Uemura M (2003) Mass spectrometric approach for identifying putative plasma membrane proteins of Arabidopsis leaves associated with coldacclimation. Plant J. 2: 141 – 154.

Kjellsen TD, Shiryaeva L, Schröder WP, Strimbeck GR (2010) Proteomics of extreme freezing tolerance in Siberian spruce (*Picea obovata*). Proteomics. 5: 965 – 975.

Komatsu S, Yamada E, Furukawa K (2009) Cold stress changes the concanavalin positive glycosylation pattern of proteins expressed in the basal parts of rice leaf sheaths. Amino Acids. 1: 115 – 123.

Lee DG, Ahsan N, Lee SH, Kang KY, Lee JJ, Lee BH (2007) An approach to identify cold induced low abundant proteins in rice leaf. C R Biol. 3: 215 – 225.

Liang Y, Chen H, Tang MJ, Yang PF, Shen SH (2007) Responses of Jatropha curcas seedlings to cold stress: photosynthesis-related proteins and chlorophyll fluorescence characteristics. Physiol Plant. 3: 508 – 517.

Lin SZ, Li XP, Zhang ZY (2001) Changes of content of CaM, and activities of G6PDHase and ATPase during low temperature-induced freezing tolerance of Populus tomentosa seedlings. Journal of Beijing Forestry University. 5: 4 – 9.

Martz F, Kiviniemi S, Palva TE, Sutinen ML (2006) Contribution of omega-3 fatty acid desaturase and 3-ketoacyl-ACP synthase II (KASII) genes in the modulation of glycerolipid fatty acid composition during cold acclimation in birch leaves. J Exp Bot. 4: 897 – 909.

Naito S, Hirai MY, Chino M, Komeda Y (1994) Expression of a Soybean (*Glycine max* L. Merr.) Seed storage protein gene in transgenic *Arabidopsis thaliana* and its response to nutritional stress and to abscisic acid mutations. Plant Physiol. 2:497 – 503.

Pedron L, Baldi P, Hietala AM, La Porta N (2009) Genotype-specific regulation of cold-responsive genes in cypress (*Cupressus sempervirens* L.). Gene. 1(2): 45 – 53.

Priya P, Venkatachalam P, Thulaseedharan A (2006) Molecular cloning and characterization of the rubber elongation factor and its promoter sequence from rubber tree (*Hevea brasiliensis*): A gene involved in rubber biosynthesis. Plant Sci. 4: 470 – 480.

Puhakainen T, Li C, Boije-Malm M, Kangasjärvi J, Heino P, Palva ET (2004) Short-day potentiation of low temperature induced gene expression of a C-repeat binding factor controlled gene during cold acclimation in silver birch. Plant Physiol. 4: 4299 – 4307.

Renaut J, Lutts S, Hoffmann L, Hausman JF (2004) Responses of poplar to chilling temperatures: proteomic and physiological aspects. Plant Biol. 1: 81 – 90.

Renaut J, Hausman J-F, Bassett C, Artlip T, Cauchie H-M, Witters E, Wisniewski M (2008) Quantitative proteomic analysis of short photoperiod and low temperature responses in bark tissues of peach (*Prunus persica* L. Batsch). Tree Genet Genomes. 4:589 – 600.

Ruelland E, Vaultier MN, Zachowski A, Hurry V (2009) Cold signaling and cold acclimation in plants. Adv Bot Res. 49: 35 – 150.

Sarhadi E, Mahfoozi S, Hosseini SA, Salekdeh GH (2010) Cold acclimation proteome analysis reveals close link between the up-regulation of low-temperature associated proteins and vernalization fulfillment. J Proteome Res. 11: 658 – 5667.

Schenk MF, Gilissen LJ, Esselink GD, Smulders JM (2006) Seven different genes encode a diverse mixture of isoforms of Bet v1, the major birch pollen allergen. BMC Genomics. 7: 168 – 182.

Sharma P, Sharma N, Deswal R (2005) The molecular biology of the low temperature response in plants. Bioessays. 10: 1048 – 1059.

Walter MH, Liu JW, Wünn J, Hess D (1996) Bean ribonuclease-like pathogenesis-related protein genes (YPR-10) display complex patterns of developmental, dark-inducedand exogenous stimulus-dependent expression. Eur J Biochem. 2: 281 – 293.

Wilson KA, McManus MT, Gordon ME, Jordan TW (2002) The proteomics of senescence in leaves of white clover, *Trifolium repens*(L). Proteomics. 9: 1114 – 1122.

Wu FZ, Wang BC, Liu GF, Yang CP (2008) Growth and physiological responses of *Betula platyphylla* seedlings to low temperature stress. Journal of Northeast Forestry University. 9: 8 – 10.

Wong CE, Li Y, Labbe A, Guevara D, Nuin P, Whitty B, Diaz C, Golding GB, Gray GR, Weretilnyk EA, Griffith M, Moffatt BA (2006) Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in *Thellungiella*, a close relative of *Arabidopsis*. Plant Physiol. 4: 1437 – 1450.

Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH, Dunn MJ (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electro-spray ionization-mass spectrometry. Electrophoresis. 17: 3666 – 3672.

Yan SP, Tang ZC, Su WA, Sun WN (2005) Proteomic analysis of salt stressresponsive proteins in rice root. Proteomics. 1: 235 – 244.

Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006) Comparative proteomic analysisprovides new insights into chilling stress responses in rice. Mol Cell Proteomics. 3: 484 – 496.

Yang T, Chaudhuri S, Yang L, Du L, Poovaiah BW (2010a) A calcium/calmodulin-regulated member of the receptor-like kinase family confers member of the receptor-like kinase family confers coldtolerance in plants. J Biol Chem. 10: 7119 – 7126.

Yang T, Shad Ali G, Yang L, Du L, Reddy AS, Poovaiah BW (2010b) Calcium/calmodulin-regulated receptor-like kinase CRLK1interacts with MEKK1 inplants. Plant Signal Behav. 8: 991 – 994.