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Differential Proteomic Analysis of Chinese fir Clone Leaf Response to Salicylic Acid

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ABSTRACT : Chinese fir (Latin name: *Cunninghaimia lanceolata*) is one of the major commercial coniferous trees. Most of Chinese fir forests are managed in successive rotation sites, which lead productivity to decline. Autotoxicity is the important reason for soil degradation of Chinese fir plantation, especially, phenolic acids are considered as the major allelopathic toxins which induce autotoxicity in Chinese fir rotation stands. We performed here proteomic approach to investigate the response of proteins in Chinese fir leaves to salicylic acid. The tube plantlets of Chinese fir clone were treated with 120 mg/L salicylic acid for 1, 3 and 5th day. 2-DE, coupled with MALDI-TOF-TOF/ MS, was used to separate and identify the responsive proteins. We found 12, 7, and 12 candidate protein spots that were up- or down-regulated by at least 2.5 fold after 1, 3, and 5th day of the stress, respectively. Of these protein spots, 16 spots were identified successfully. According to the putative physiological functions, these proteins were categorized into five classes (1) the proteins involved in protein stability and folding, including 26S proteome, Grp78, Hsp70, Hsp90 and PPIase; (2) the protein involved in photosynthesis and respiration, including OEC 33 kDa subunit, GAPDH; (3) the protein related to cell endurance to acid, F-ATPase; (4) the protein related to cytoskeleton, tubulin; (5) the protein related to protein translation: prolyl-tRNA synthetase. These results give new insights into autotoxic substance stress response in Chinese fir leaves and provide preliminary footprints for further studies on the molecular signal mechanisms induced by the stress.

Keywords : Chinese fir, Salicylic acid stress, Two-dimensional gel electrophoresis, MALDI-TOF-TOF/MS

INTRODUCTION

Chinese fir (*Cunninghaimia lanceolata*) is one of the most important commercial coniferous tree species, cultured in the sixteen provinces of Southern China. Currently, most of Chinese fir forest had been managed on successive rotation sites. The results showed that the repeatable soil is not suitable for Chinese fir growth, the total biomass consistently decreased by 45% and 50%, the effective component of soil declined by 23% and 28% in second and third rotations, respectively compared to the first rotation (Zhang et al, 1994). Among the reasons of dropped productivity of successive Chinese fir stand, in addition to the decline of soil fertility and soil structure, the accumulated auto-toxic substances in successive site was considered the significant reason for soil degradation of Chinese fir plantation (Lin et al., 1999; Ma et al., 2000; Chen et al., 2003; Du et al., 2003).

Autotoxity, as one special manifestation of allelopathy, produces the direct or indirect harmful effects on plant themselves by the accumulated autotoxins which are released through some ways such as leaves dripping, plant residues decomposing and roots secreting etc. In recent years, phenolic substances have found to be the major allelopathic substances to induce auto-toxicity of Chinese fir. There are some reports about the alleloppathic effects induced by several phenolic auto-toxic substances such as vanillin, P-hydroxybenzoic, ferukic, cinnamic and salicylic acids due to successive rotation. These autotoxins can accumulate to a certain level in the soil, then inhabit the plant growth processes including seed germination, seedling growth, root vitality, photosynthesis, nutrient uptake, osmotic adjustment and so on, as one of the stress factors to reduce Chinese fir productivity in successive sites (Ma

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et al., 1998; Chen et al., 2002a; Chen et al., 2002b; Wang et al., 2002; Cao et al., 2001; Lin et al., 2003; Yang et al., 2006; Yang et al., 2007a). In addition, salicylic acid was considered as the toxic phenolic acid in plant-soil ecosystems, which was elaborated in the research about the global response of the soil bacterium *Bacillus subtilis* to salicylic acid (Duy et al, 2007)

Proteomics has been employed to analyze protein changes in response to environmental changes, and although one plant genome is not available, databases of non-redundant and contiguous ESTs will provide a potentially useful resource for its proteome investigations (Yan Zhen, 2007). The differential expression of proteins in two stressed plants can be compared using differential proteome, which helps to explore the regulation mechanism and response pathways to outside stimulation. This approach is useful for studying plant allelopathy. By the differential proteome technique, He et al (2004) had obtained the differential proteins involved in catalyzing or metabolizing process of secondary substances in rice allelopathy.

To explore the effects of salicylic acid on the differential proteome of Chinese fir, Yang (2007b) optimized the two-dimension polyacrylamide gel electrophoresis technique to obtain the better protein profiles of Chinese fir leaves. In this study, the proteins extracted from Chinese fir leaves were separated with 2-DE after being treated with 120 mg/L salicylic acid, then the differentially expressed proteins were identified by MALDI-TOF/TOF-MS and protein databases. These results would be helpful for investigating the response and regulation mechanism of Chinese fir to the auto-toxic substances.

MATERIALS AND METHODS

Plant Materials and Salicylic Acid Treatement

The tube plantlets of Chinese fir clone, graciously provided by Xinmin seedling company Ltd in Fujian, China, were transferred into the modified MS mediums to grow for ten days. Then the uniformly grown plantlets were exposed to 0 mM and 120 mM Salicylic acid in mediums respectively. The treated samples were harvested at 1, 3, and 5th day. Leaves were cut and frozen in liquid nitrogen and kept at -70℃ until used.

Protein Extraction

Leaves were ground in liquid nitrogen in a mortar to a fine powder, then immediately suspended in eight volumes of 10% (w/v) trichloroacetic acid (TCA) in acetone containing 0.07% v/v 2-mercaptoethanol at -20℃ overnight (Damerval et al., 1986). The homogenates were centrifuged for 20 min at 18 000 g at 4℃. After discarding the supernatant liquid, the pellets were re-suspended in cold acetone containing 0.07% v/v 2-mercaptoethanol, incubated at -20℃ for 1 h and were centrifuged for 20 min at 18 000 g. The washing was repeated twice. The final pellets were lyophilized to remove the residual acetone and then kept at -70℃ overnight. The lyophilized samples were solubilized in lysis buffer according to Yang (2007b). After incubating at 35℃ for 2h, the supernatants were centrifuged at 18 000 g for 20 min to remove precipitation. The protein concentration was determined by Bradford assay using BSA as a standard.

Two-Dimensional Gel Electrophoresis

Before loading for 2-DE, proteins were solubilized in 450 μL rehydration buffer (Yang et al., 2007b). Twenty-four centimeter immobilized pH gradients (IPG) strips linear (pH 4-7) were rehydrated with this solution in each rehydration tray. Rehydration and isoelectric focusing were carried out using an IPG phor at 20℃ to reach a total of 63,000 V h^{-1}. After isoelectrofocusing (IEF), the strips were equilibrated for 15 min in equilibration buffer containing 10 mg mL^{-1} Dithiothreitol (DTT), followed by 15 min in equilibration buffer containing 25 mg mL^{-1} iodoacetamide. Then the equilibrated strips were mounted on top of vertical 13.5 % sodium dodecyl sulfonate and polyacrylamide gels (SDS-PAGE, 18×24 cm) in Ettan™ DALT II performed by 30 mA per gel.

Gel Imaging and Data Analysis

After migration, the gels were stained with Coomassie brilliant blue R-250 and scanned with the Epson Scanner with 300 dpi and 24 bit full color. Image Master 2D Platinum 6.0 (made by GE Healthcare) were applied to analyze image for detecting and matching protein spots between the CK and treatment gels at the same time. The abundance of each protein spot was estimated by the percentage volume (% Vol).Specific spots were described as showing variations when their volumes were significantly different (at least 2.5-fold in relative abundance at one time point). And those spots were identified by MALDI-TOF-TOF/MS (Note: MALDI-Matrix Assisted Laser Desorption/ Ionization, TOF-Time of Flight, MS- Mass Spectrometry).

In-Gel Tryptic Digestion

Spots of interest were excised from the gels and detained in 100 μL buffer containing 50% acetonitrile and 50 mM ammonium bicarbonate for 20 min at room temperature. Then they were washed twice with ultrapure water and shrunk by dehydration in acetonitrile. After removing the supernatant, the gel pieces were dried. The samples were then swollen in a digestion buffer containing 20mM ammonium bicarbonate and 12.5 ng / μL Trypsin at 4℃. After 30 min incubation, the gels were digested overnight at 37℃. Peptides were then extracted twice using 0.1% Trifluoroacetic acid (TFA) in 50% acetonitrile (CAN) and dried under the protection of N_2.

Identification of Proteins by Mass Spectrometry

For MALDI-TOF/TOF-MS, the tryptic peptides were eluted onto the target with 0.7 μl matrix solution (α-cyano -4-hydroxy-cinnamic acid in 0.1% w/v TFA, 50 % v/v ACN) and deposited onto the MALDI target. Before inserting samples into the mass spectrometer, they were allowed to air to dry at room temperature. The tryptic peptides were analyzed with a MALDI-TOF/TOF-MS 4700 Proteomics Analyzer. Protein identification was performed by searching in NCBInr database and using GPS Explorer software and MASCOT (Multiple-Access Space-Time Coding Tested) search engine software. The following search parameters were applied: all species, trypsin digest with one missing cleavage, PMF(peptide mass fingerprint) tolerance of 0.3 Da, MS/MS tolerance of 0.4 Da.

RESULTS AND ANALYSIS

2-DE Analysis of Leave Proteins in Chinese Fir Treated by Salicylic Acid

Proteins were extracted from the leaf samples, respectively, at the 1, 3 and 5th analyzed by 2-DE gel electrophoresis as described in “Materials and Methods”. Previous experiments (Yang et al, 2007b) revealed that most of the Chinese fir leaf proteins were in the 4−7pH and 14.2−66.2 k Da range.

Accordingly, IEF was carried out within the 4−7pH range and SDS-PAGE using 13.5% polyacrylamide. Approximately 500 protein spots per gel were revealed by Image Master 2D Platinum 6.0 software. The proteins were separated well in both dimensions and the gel maps with high quality provided the reference 2-DE maps for Chinese leaf proteins. The representative 2-DE maps were shown in Fig 1, in which differential protein spots are enlarged in Fig 2.

Differential proteomic analysis was used to investigate the protein profiles under salicylic acid stress. There were not changes in protein accumulation profiles between controlled and treated samples. By matching the protein spots between the controlled and treatment gels at the same treated time, most of differential proteins ranged from pH5 to pH6 and located in higher molecular weight scope. At the 1st day, twelve spots showed significant changes in abundance (img101C and img101T in Fig. 1). Six of them were up-regulated (spots 1-6) and seven were down-regulated (spots 7-10). Two protein spots were visible only in the treated samples, suggesting that they were newly synthesized after the treatment or their abundance were too low to be detected in the controlled samples. At

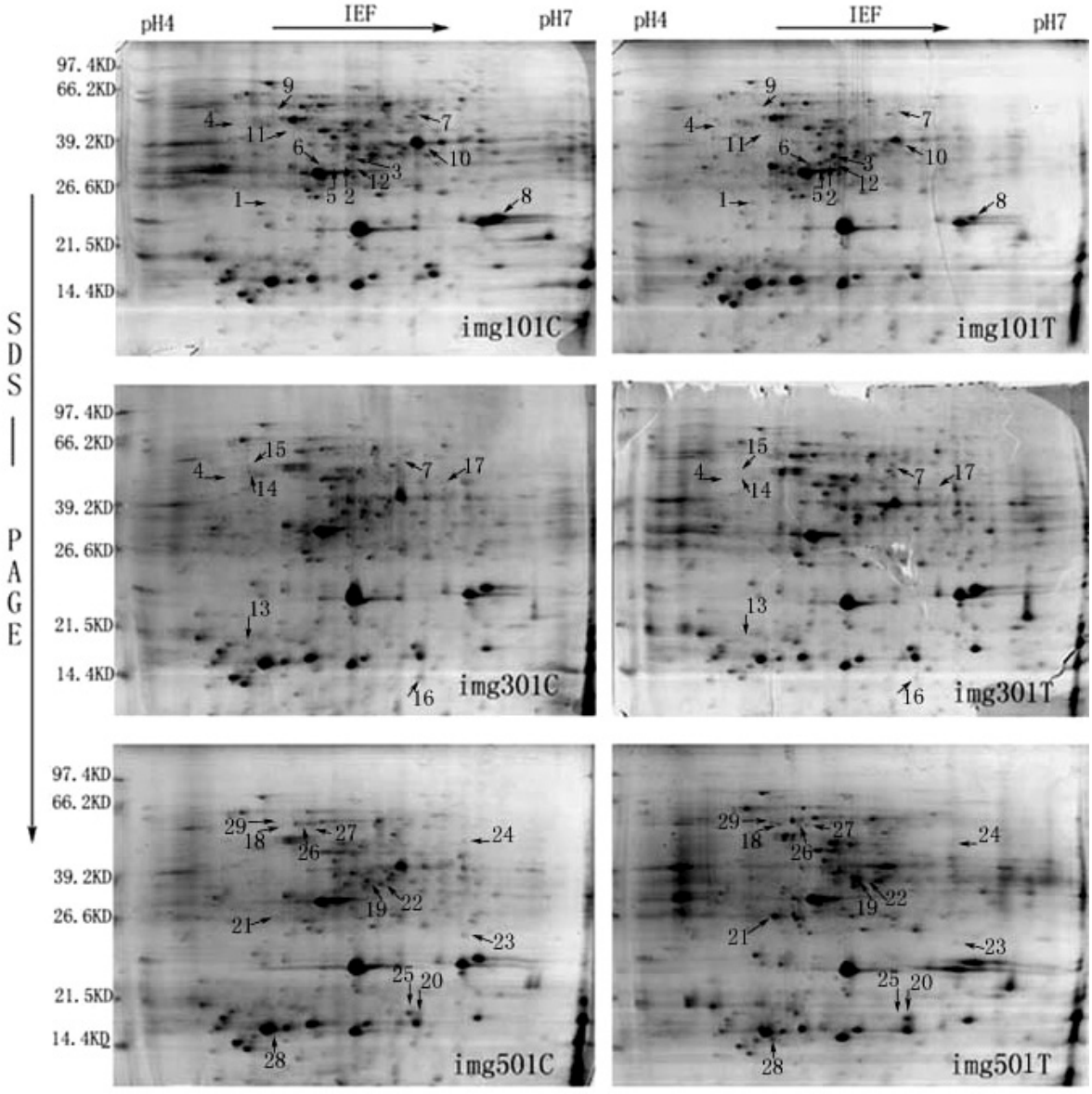

Fig. 1. 2-DE maps of differentially expressing proteins in Chinese fir leaves at 1st, 3rd, 5th days under salicylic acid stress (the number is the sign of each differential protein, the same as in following figures); 101C and 101T, 301C and 301T, 501C and 501T show the 2-DE gel patterns of controlled and treated groups at 1, 3, and 5th day, respectively.

the 3rd day, seven spots showed significant changes in abundance (img301C and img301T in Fig. 1). Spot 7 was up-regulated, Spots 4, 13-16 were down-regulated and the protein spot 17 vanished in the treated sample. At the 5th day, twelve spots showed significant changes in abundance (img501C and img501T in Fig. 1). Four of them were up-regulated (spot 18-21) and three were down-regulated (spots 22-24), Spot 25 disappeared while spots 26-29 appeared after 5th day of salicylic acid stress.

The Identification of Responsive Proteins to Salicylic Acid by MS/MS

In order to identify the differentially expressed proteins, spots were excised from the preparative gels, GPS Explorer using MASCOT as a search engine against NCBInr database. To gain functional information about these proteins, BLAST was used to investigate their homologies with other proteins in the database. Among the 29 proteins

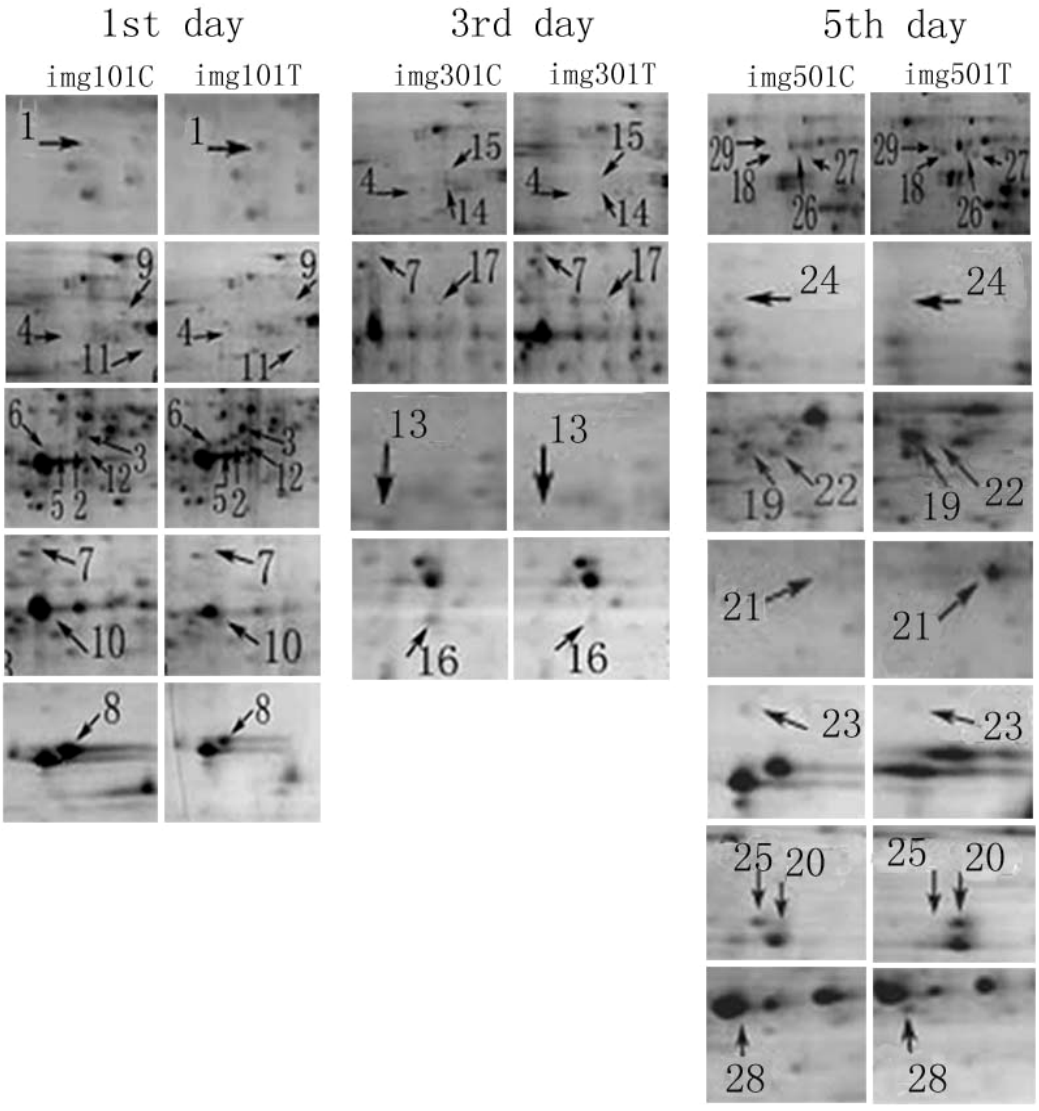

Fig. 2. The partial magnified pictures of the differential proteins Chinese fir leaves at 1, 3, and 5th days under salicylic acid stress

resolved from the samples, 13 spots showed no good matches while other 16 spots were identified with higher probability than 78 score (Table 1). The MS analysis of spot 9 was shown in Fig. 3 as an example. Some of the identified proteins were annotated either as unknown and hypothetical proteins, or without specific functions. The identified proteins were the following eight categories: (1)33 kDa subunit of oxygen evolving system of photosystem II, (2) Heat Shock Proteis(HSPs), (3) Peptidylprolyl cis trans Isomerase (PPIase), (4). 26S proteaome, (5). Proton shifting membrane ATP enzyme (F1 ATPase), (6) Glyceraldehyde -3-phosphoric acid Dehydrogenase (GAPDH), (7) Microtubule protein, (8) Prolyl tRNA synthetase (ProRS).

DISCUSSION

OEC 33 kDa Subunit

In the hydro cracking and oxygen evolving process of photosystem II, 33 kDa subunit is a necessary protein that protects the manganese cluster and the oxygen evolving complex (OEC). Some experiment have demonstrated that Changed environment would lead 33 kDa protein to release from the core part in parallel with Mn losing,

Table 1. The identification of differentially responsive proteins from Chinese fir leaves, in response to salicylic acids, with MALDI-TOF-TOF/MS analysis

Treated Time	Spot No.	Regulated Type after Treated	Expressed Aboundance (%Vol)		Accession No.	Homologous Protein	Putative Mr (Da) / pI	Organism	Protein Score
			Control	Treatment					
1st day	1	up	0.0213	0.0582	gi|131386	oxygen-evolving enhancer protein1 (OEE1) (33 kDa subunit of oxygen evolving system of photosystem II)	35148.9 / 5.58	Spinacia oleracea	232
	2	up	0.0415	0.1313	gi|35213280	gsr2711	9980.2 / 5.91	Gloeobacter violaceus PCC 7421	81
	4	up	0.0159	0.0544	gi|12580859	glucose regulated protein homolog 4 precursor	48187.7 / 4.83	Picea abies	194
	7	up	0.0807	0.0197	gi|68555136	PpiC-type peptidyl-prolyl cis-trans isomerase	54197.2 / 7.88	Ralstonia metallidurans CH34	82
	9	down	0.0273	0.0099	gi|51090590	26S proteosome regulatory particle triple-A ATPase subunit5a	47767.6 / 4.94	Oryza sativa	383
	11	up	–	0.0245	gi|9837422	heat shock protein 90	63241.7 / 5.39	Paramecium tetraurelia	129
	12	up	_	0.0810	gi|35213280	gsr2711	9980.2 / 5.91	Gloeobacter violaceus PCC 7421	78
3rd day	4	down	0.0338	0.0116	gi|12580859	glucose regulated protein homolog 4 precursor	48187.7 / 4.83	Picea abies	194
	7	up	0.0170	0.0798	gi|68555136	PpiC-type peptidyl-prolyl cis-trans isomerase	54197.2 / 7.88	Ralstonia metallidurans CH34	82
	14	down	0.0384	0.0088	gi|19256	heat shock protein cognate 70	71242.3 / 5.15	Lycopersicon esculentum	158
	15	down	0.0373	0.0125	gi|67595854	hypothetical protein Chro.60634	8336.3 / 8.68	Cryptosporidium hominis TU502	80
	17	up	0.0467	_	gi|7620557	F_1 ATPase alpha subunit Metasequoia glyptostroboides	36429 / 6.69	Metasequoia glyptostroboides	247
5th day	18	up	0.0256	0.0832	gi|37529492	beta-tubulin 3	48165.2 / 5.47	Gossypium hirsutum	234
	22	down	0.1580	0.0255	gi|37529490	alpha-tubulin 4	49539.3 / 4.93	Gossypium hirsutum	104
	24	down	0.0623	0.0200	gi|1084372	glyceraldehyde-3-phosphate dehydrogenase	52972.3 / 6.53	Pisum sativum	141
	26	up	–	0.0632	gi|37529490	alpha-tubulin 4	49539.3 / 4.93	Gossypium hirsutum	232
	27	up	–	0.0706	gi|37529490	alpha-tubulin 4	49539.3 / 4.93	Gossypium hirsutum	152
	28	up	–	0.1554	gi|71653235	prolyl-tRNA synthetase	91290.1 / 8.46	Trypanosoma cruzi	88

which caused the depressed oxygen evolving activity of PSII and inhibition of photosynthesis (Zouni et al., 2001; Svensson et al., 2004; Lu et al., 2000; Yamane et al., 1998; Henmi et al., 2004). In addition, OEC33 is one source of carbonic anhydrase (abbreviation: CA) activity and requires manganese as a cofactor for maximal CA activity. Removing the OEC33 from PSII also removed CA activity (Lu et al., 2005). Shutova(Shutova et al., 1997) found OEC33 has the good resistance to low pH value. During the salicylic acid stress period, spot 1 that was up-regulated at 1st day was matched with OEC33. The increment of OEC33 promoted the stability of core complex and the activity of PSII oxygen evolving and CA, indicating the photosynthesis intensity and the endurance to acid stress.

HSPs

HSPs have the typical physiological function of molecular chaperones which help to repair and aid the renaturation of stress-damaged protein so as to maintain their natural function (Guy et al., 1998; Cho et al., 2006; Guy et al., 1998). In addition to temperature, many factors such as salt, anoxic conditions, heavy metals, acid toxic, nutrition, hormones may also induce heat shock response (Wang et al., 2003; Ahsan et al., 2007). But at some stress, not all HSPs act the function at the same time and the cell resistance to stress conditions is related to special stress proteins induced by stress. In the current study, three proteins were identified as the differentially expressed HSPs in Chinese fir leaves, including Hsp cognate 70

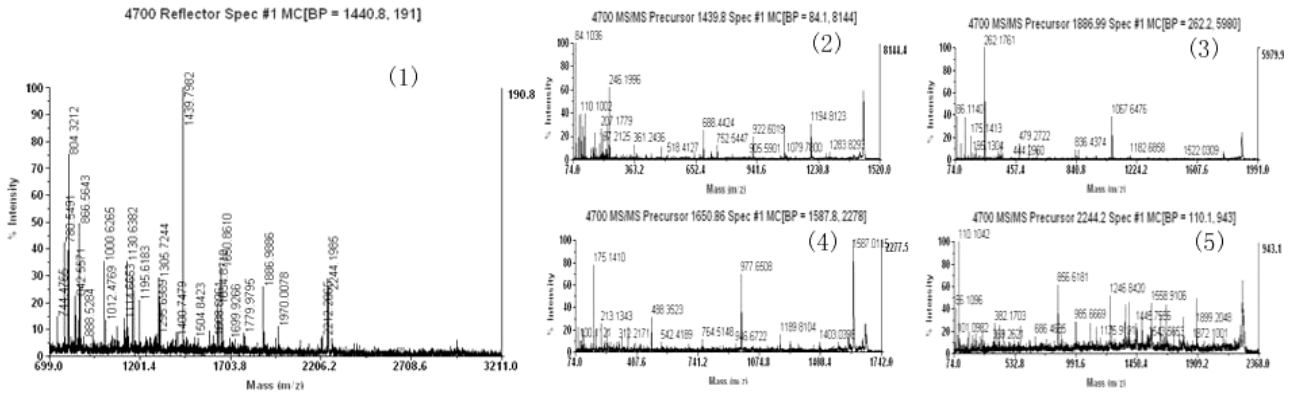

Fig. 3. MAIDI－TOF－TOF/ MS spectra of differential protein spot 9 as an example to show the results by using Proteomics Analyzer. The spectral peaks in (1) picture showed the intensities of different peptides and the four peptides with the most intense spectral peaks were further analyzed by MS/MS which were shown in (2), (3) and (4) pictures.
Note: Spot 9 as an example to show Mascot search results as followed. Search title: SampleSetID: 8338, AnalysisID: 15676, MaldiWellID: 510519, SpectrumID: 1507822, Path=\zxw\060802\com 1.
Database: Ncbi0603 0603 (3466531 sequences; 1190035678 residues)
Warning: A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score: 383 for gi|1174613, 26S protease regulatory subunit 6A homolog (TAT-binding protein homolog 1) (TBP-1)
Probability Based Mowse Score
Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 78 are significant ($p<0.05$).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

(Hsc 70), Hsp90 and glucose regulated protein homolog 4 precursor ("function=''78 kDa, Grp78). Hsc70 and Grp78 belong to HSPs70 family and Hsp90 belong to HSPs90 family. The unusual proteins are transported to endoplasmic reticulum by Grp78 and to be degraded, which avoid the further damage to cells. So Grp78 has the quality of resistance to cell apoptosis and act as the information receptor of cell face (Wang et al., 2005; Li et al., 2006; Fu, 2006). Although the study on Grp78 usually involve in human diseases, those results will help the plant Grp78 research. Hsc70 and Hsp90 can repair those denatured proteins by stress. Hsc70 combining with nascent polypeptides would promote proteins to fold correctly (Beckmann et al., 1990). Hsp90 can combine the wrongly folding proteins and help them to fold again to resume the natural conformation finally or promote the degradation of denatured proteins (Mayer et al., 1999; Yonehara et al., 1996). According to the 2-DE maps, Grp78 was up-regulated at 1st day and down-regulated at 3rd day, Hsc70 was down-regulated at 3rd day and Hsp90 was up-regulated at 1st day. So they were either up-or down-regulated at least one time by treatment, indicating that HSPs played the important roles to degrade the denatured proteins or rehabilitate their natural structure, which assured correct proteins synthesis and prevent cell apoptosis. At the same time, maybe HSPs operated the response function to phenolic.

26S Proteasome

The 26S proteasome has similar functions to molecular chaperones. In eukaryotic cells, 26S selectively prevents the accumulation of non-functional, potentially toxic proteins by degrading those folded wrongly or damaged polypeptides. This process is of particular importance in protecting cells against harsh conditions (for example, heat shock or oxidative stress) (Goldberg, 2003). It is presumed that proteolysis may be a self-controlled system during the process of cell preventing the harm by stress and its role in plant resistance to stress had been revealed. By 2-DE and MALDI-TOF-MS technique, Yamazaki (Yamazaki et al., 2004) identified this proteasome expressed differentially under the darkness stress. In this research, 26S proteasome was down-regulated at 1st day but not differential at 3

and 5th day, which hinted it participated in degrading abnormal proteins by salicylic acid treatment. So 26S proteasome was supposed to mediate Chinese fir response to phenolic acid for its holding back the accumulation of toxic proteins by change its expression in operating the function.

Peptidyl-Prolyl Cis-Trans Isomerase

Spot 7 was identified as peptidyl-prolyl cis-trans isomerase (PPIase). In peptide chains there are cis and trans isomers in the peptide bond between peptide and prolyl. Rotamer is one of the limited speed steps in protein folding reaction, which is extremely essential for the protein to form dimensional structure with the biological function activity (Fruman et al., 1994; Rinfret et al., 1994). In the study, salicylic acid treatment affected protein synthesis. PPIase helped the peptide-prolyl of target protein to transform between cis-trans and trans-cis configuration correctly and decrease the quantity abnormal proteins. The identified protein had the highest homologous to PPIase of Ralstonia metallidurans CH34 with 82 score. Ralstonia metallidurans CH34 is separated and filtrated from the sentiment in a zinc factory and possesses high ability to degrade phenol. In high concentration of heavy metal, it still remained stronger activity to degrade phenol (Gao et al., 2005), which hinted that, as the special protein induced by phenolic acid treatment, PPIase played the key role in remaining the protein's natural conformation and the resistance ability in Chinese fir leaves.

F_0F_1-ATPase

F_0F_1-ATPase widely consists in mitochondria, chloroplast and bacteria. F_0 and F_1 co-operate the biological function to regulate and maintain pH gradient both inside and outside cell. In the study spot 17 was identified as α subunit of F1 ATPase which had the highest homologous to the same protein of Metasequoia glyptostroboides with 247 score.

F_0F_1-ATPase is the main regulation mechanism of adaptation and resistance to acid in bacterial cells (Suzuki et al., 2000; Welin, 2003). The more researches on its function were undertaken about bacteria mainly, especially, F-ATPase of streptococcus sanguis (one of the most endurace to acid, pathogenic bacteria of decayed tooth) had been the emphasis all along for its effective contribution to acid endurace (Kuhnert et al., 2003; Li et al., 2002). In acid circumstance, F_0F_1-ATPase induce an acid tolerance response of cells by pumping out proton to make pH value approach to neutral level, which assured that cell could perform the function in cell growth and metabolism (Sheng et al., 2006), and at the aspect of contribution to aciduricity, F_0 acted better than F_1 (Cotler et al., 2003). Under the salicylic acid stress, it was suggested the differential expression of F_0F_1-ATPase was associated with the maintenance of pH homeostasis by H^+ extrusion, which might be a principal regulation mechanism of aciduricity in Chinese fir leaves.

Glyceraldehyde-3-Phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) relating to ATP synthesis closely is one of the necessary core enzymes during glycolysis process in cellar respiration, which implements important function in producing energy (Pi et al., 2000). The content or activity of GADPH and the transcription level of coding genes were sensitive to adversity circumstance, for example, the mRNA level of GADPH in Arabidopsis thaliana increased 5-10 times (Yang, 1993) and the expression content and activity of GADPH increased in maize roots (Manjunath et al., 1997) under anaerobic treatment. GADPH in the root tips of tomato up-regulated under hungry or excessive Fe stress (Herbik et al., 1996) and, during the rice grain development, GADPH also up-regulated under high temperature, which accelerated glycolysis speed and promoted starch decomposing (Lin et al., 2005).

In this study spot 24 was identified as GAPDH which remained normal level at 1st and 3rd day and down-regulated at 5th day. So we speculated that its differential expression was the results that it participated in both

photosynthesis and respiration, or salicylic acid stress might up-regulate the transcription level but down-regulate translation level of GADPH. In addition, GAPDH maybe have the function to transcript promoter, help tubulin array and induce apoptosis (Berry et al., 2003). The complicated situation and the predictions should be validated by further researches.

Tubulin

Tubulin is the main component of eukaryotic cytoskeleton which is recognized as important elements in plant development, being composed of α- and β- tunits. Tubulin not only plays main roles at maintaining cellular morphology and interior structure but also relates to substance transport in cell, cell movement, cell differentiation and development, cell division and reproduction, signal transduction, and so on (Hepler et al., 1996; Mitsopoulos et al., 2003). Being destroyed Tubulin structure would lead to cellular function obstacle and affect on cell lives, which induces cell to die. So keeping α- and β- tubulin units at appropriate level contributes to normal tubulin function.

Tubulin skeleton is sensitive to environment. Low temperature induced tubulin to depolymerize in guard cells of stoma and root tip cells of some warm-liking corps such as tomato, cucumber and rice, going with the harm to plant (Jian et al., 1989). Chu (Chu et al., 1993) also reported that β-tubulin expression in Arabidopsis thaliana decreased at low temperature. In the research on cold-resistance of cucumber, the tubulin of root tip in the species with weaker resistance depolymerzed earlier, its filamentous structure turned to be more unclear even to disappear; but the content of resistance species increased after cold training (Zhao et al., 2006). But Bongani (Bongani et al., 2005) identified that the tubulin protein down-regulated under salt stress, which related to mRNA of tubulin being suppressed. From above, different plant species have various responses to different stress conditions.

Four spots were identified as tubulin that showed differential expressions at 5th stress day, including spots 18, 26 and 27 up-regulated at 1st day but spot 22 down-regulated at 3rd day, which were suggested that tubulin involved in Chinese fir resistance in response to salicylic acid stress by polymerizing and depolymerizing itself.

Amino acyl-tRNA Synthetase

Amino acyl-tRNA synthetase (aaRS) guarantee the genetic information of mRNA to reflect on the amino sequence exactly for the single and accuracy to discern amino and tRNA, which make the protein synthesize fidelity (Hati et al., 2006). In our study, spot 28 was identified as ProRS that showed differentially expression at 5th stress day.

In theory, each kind of aaRS corresponds to one kind of amino and corresponded tRNA, while later research find that ProRS possesses the unusual bi-catalysis characteristic that a single enzyme can attach two different amino acids, proline and cysteine, to their cognate tRNA species, accordingly, to prevent amino from mismatching with tRNA (Stathopoulos et al., 2001; Lipman et al., 2002). In addition, ProRS often exist as amalgamation form with other aaRS, which improve the catalytic efficiency of enzyme (Rho et al., 1999). Such as glutamyl-prolyl-tRNA synthetase (EPRS), one peptide fragment with GluRS and ProRS catalytic activity can catalyse Glu and Pro to bind the corresponding tRNA, respectively, which elevate the utilization efficiency to tRNA followed by elevating protein synthesis. In addition,the complex of ProRS and LeuRS increaseD the catalytic efficiency of ProRS by five fold while no effect on LeuRS, whose interaction improve the aminoacylation function to assure the fidelity of protein translation(Practorius-Ibba et al., 2005).

Although there is not reported about the change of aaRS under stress conditions, according to the past research on aaRS or aaRS complex, it was suggested that salicylic acid stress might lead proteins to hydrolyze followed by more dissociative Pro, which induced the increment of ProRS synthesis to accelerate ProRS aminoacylation and benefit usual protein synthesis.

CONCLUSION

By using 2-DE coupled with the MALDI-TOF-TOF/MS analysis, a total of 16 differential proteins were identified in response to salicylic acid stress. These proteins were categorized into five types: (1) the proteins involved in protein stability and fold, which suggested that salicylic acid stress has severe effects on protein denaturation and regulation, including 26S proteome, Grp78, Hsp70, Hsp90 and PPIase; (2) the proteins involved in photosynthesis and respiration, including OEC 33 kDa subunit, GAPDH; (3) the protein related to cell endurance to acid, F-ATPase; (4) the protein related to cytoskeleton, tubulin; (5) the protein related to protein translation: prolyl-tRNA synthetase. These results suggested that Chinese fir coped with salicylic acid stress in a complex manner by the signal transduction system and interaction system. These differential proteins formed to play their roles in the Chinese fir resistance to the stress.

Although, we launch here an initial proteomic investigation into Chinese fir response to salicylic acid stress, it is believed that this kind of study provides a good starting point in understanding the responses of Chinese fir to auto-toxic substance and taking further investigation into their functions using genetic and other approaches. With the improving protein staining techniques, the sensitivity of mass spectrometry and the protein database, future work will involve attempting to identify the remaining other auto-toxic substances stress-responsive proteins whose identities were not successfully established in this study could be identified. In addition, further other auto-toxic substance treatments and comparative analysis should be conducted in order to gain a better understanding for the response mechanism or Chinese fir to overall autotoxity.

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