Exogenous calcium regulates the growth and development of Pinus massoniana detecting by physiological, proteomic, and calcium-related genes expression analysis

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Abstract

In the presented study, a combined physiology, proteomics and gene expression study was performed using *P. massoniana* seedlings cultivated at various calcium levels. The aim of the study is to investigate the impacts of exogenous calcium on *P. massoniana* seedling growth and development and to reveal the underlying molecular mechanisms. The results showed that calcium deficiency lead to severe seedling growth and development inhibition while adequate exogenous calcium markedly improved the growth and development. The underlying mechanisms involved diverse calcium influenced biological processes and metabolism pathways including photosynthesis, carbohydrate metabolism and energy production, protein metabolism, secondary metabolism and calcium signal transduction and calcium ion homeostasis. In general, calcium deficiency inhibited or impaired these pathways and processes, while sufficient exogenous calcium improved and benefited these cellular events through regulating a number of related enzymes and proteins. Besides, adequate exogenous calcium supply relieved oxidation stress which occurred at low calcium level. Enhanced cell wall formation and consolidation and cell division also play a role in exogenous calcium improved *P. massoniana* seedling growth and development. Our study facilitates the elucidation of the potential regulatory role of calcium in *P. massoniana* physiology and biology and is of guiding significance in pinaceae plants forestry.

Title page

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Abstract

In the presented study, a combined physiology, proteomics and gene expression study was performed using *P. massoniana* seedlings cultivated at various calcium levels. The aim of the study is to investigate the impacts of exogenous calcium on *P. massoniana*seedling growth and development and to reveal the underlying molecular mechanisms. The results showed that calcium deficiency lead to severe seedling growth and development inhibition while adequate exogenous calcium markedly improved the growth and development. The underlying mechanisms involved diverse calcium influenced biological processes and metabolism pathways including photosynthesis, carbohydrate metabolism and energy production, protein metabolism, secondary metabolism and calcium signal transduction and calcium ion homeostasis. In general, calcium deficiency inhibited or impaired these pathways and processes, while sufficient exogenous calcium improved and benefited these cellular events through regulating a number of related enzymes and proteins. Besides, adequate exogenous calcium supply relieved oxidation stress which occurred at low calcium level. Enhanced cell wall formation and consolidation and cell division also play a role in exogenous calcium improved *P. massoniana* seedling growth and development. Our study facilitates the elucidation of the potential regulatory role of calcium in *P. massoniana* physiology and biology and is of guiding significance in pinaceae plants forestry.

Key words :Exogenous calcium; *Pinus massoniana* ; growth and development; proteomic

Introduction

Pinus massoniana , a member of the pinaceae family, is widely distributed in the forest area of central and southern China, and northern Vietnam.[1] Due to its fast-growing, strong adaptability and tolerance to barren environments, it plays a critical role in forest ecological integrity restoration and construction.[2,3]In addition, it is an important economic tree species commonly used for timber, wood pulp, rosin and turpentine, which

occupies a vital position in forestry production industry.[2,4]Besides, some tissues/organs of *P. massoniana* including needles, bark, pollen and turpentine, and extractives extracted from above parts have been used as traditional Chinese medicines for disease control and treatment purpose.[5,6]Moreover, *P. massoniana* is also used in landscape and gardening design, and ornamental horticulture.[7]

Calcium (Ca) is an essential nutrient element in plants which occurs in plant tissue as free Ca^{2+} or as Ca^{2+}binding to carboxylic, phosphoric and phenolic hydroxyl groups.[8] It has been reviewed that Ca exhibits diverse functions in various plant biological processes and cellular events.[9-11] Primarily, it acts as a structure component of plant cell wall and membrane, which impacts cell wall rigidity and cell membrane stability and permeability.[9] Thus, plant growth and development progresses can be directly influenced by Ca supply.[10] Moreover, it plays an intracellular second messenger role in kinds of plant physiological and biological processes, such as growth and development, ion homeostasis, metabolism regulation, signal transduction and a variety of biotic/abiotic stress response.[9-12] The latest studies have demonstrated it also participates in programmed cell death, plant immunity and photosynthesis in plant.[13-15] When subjecting to a calcium deficient condition, a variety of disorders and stunned symptoms were observed in horticulture crops.[16] However, after applying excessive calcium, plants may suffer Ca toxicity and result in seed germination inhibition and plant growth reduction.[16]

It was reported that Ca addition alleviated simulated acid rain induced seed germination and seedling growth inhibition in *P. massoniana* .[17] Further proteomic analysis indicated calcium regulated diverse cellular events and biological processes in acid rain treated *P. massoniana* , and resulted in the tolerance improvement to acid rain.[18] Chen et al. demonstrated that exogenous hormones and Ca effectively improved the resistance of *P. massoniana* to *Dendrolimus punctatus*attacks.[19] Li et al. studied the impact of various Ca concentrations on the growth and physiological index of *P. massoniana* seedlings, and concluded 1.0-2.0 mM soil Ca concentration is the optimal condition for *P. massoniana* seedlings growth.[20] Nevertheless, the knowledge of molecular regulatory mechanisms of Ca on *P. massoniana* physiological processes, development and growth is still relatively limited. Considering the important role of *P. massoniana* in both forestry economy and forest ecosystem, it is of significance to address this issue.

In the present study, the combined physiological and proteomic studies were performed using *P. massoniana* seedling cultivated at various Ca levels to investigate the impact of exogenous Ca on growth and development, and finally to reveal the underlying molecular mechanisms. In addition, bioinformatics analysis was implemented to demonstrate the biological functions and roles of the differential expressed proteins (DEPs) identified in proteomics experiments, while Ca-related gene expression was analyzed as well. Our study may facilitate the elucidation of the potential regulatory role of Ca in *P. massoniana* physiology and biology.

Materials and Methods

Experiment design and workflow

The purpose of this study is to explore the effects and the potential molecular mechanisms of different soil Ca supplies on *P. massoniana* growth and physiology. The overall experimental design and workflow is descripted as follow. Briefly, after soil Ca leaching and soil nutrient recovery, the *P. massoniana* seedlings were transplanted into the prepared soil containing high Ca (H), medium Ca (M) or low Ca (L). Medium Ca level was used as the control group; low or high Ca level were used as treatment groups, which simulated Ca deficiency condition or Ca adequacy condition, respectively. After two months cultivation, the growth and development phenotype were observed and photographed. The fresh leaf tissues were harvested for physiological index measurement. A comparative proteomics analysis was performed with the collected leaves to discovery the DEPs under various Ca treatments. Various bioinformatics analyses were implemented to systematically interpret these DEPs. The qRT-PCR analyses for several Ca related genes were performed to reveal the mechanism at transcription level.

Substrate soil preparation

In the present study, the substrate soil for *P. massoniana* growth was collected from southern forest area

of China where *P. massoniana* are naturally distributed. The soil was subjected to a Ca leaching process to minimize the soil Ca content according to previous report.[21] Then soil nutrients were recovered through a two months nutrient solution supplementation with modified Hoagland solution containing different Ca concentrations (20.0, 2.0, or 0.1 mM), respectively.[17] The restored soil samples were used for the following *P. massoniana*seedling cultivation at high Ca level (20.0 mM), medium Ca level (2.0 mM) and low Ca level (0.1 mM), respectively. The final soil pH was approximate 5.0 for all the prepared substrate soil.

Plant materials

Six-months-old *P. massoniana* seedlings with similar size were selected and transplanted into the flowerpots filled with approximate 10 kg the aforementioned substrate soil containing different Ca levels. The transplanted seedlings were cultivated in a greenhouse with normal watering condition. Other cultivation conditions as follows: light/dark regime 15/9 h, temperature 21/27°C (night/day), relative humidity 60-70% and photosynthetically active radiation 210 μmol m^{-2} s^{-1}. After two months growth, the fresh leaf tissues were collected for the subsequent experimental analysis. Three biological replicates were performed for each treatment group.

Physiological and growth indexes determination

Chlorophyll content in the *P. massoniana* seedling leaves was determined referring previous report.[18] Leaf net photosynthetic rate (Pn) was measured with a portable photosynthesis system (Li-6400, Li-Cor, Lincoln, NE, USA) following our previous instrument operation.[18]At least 10 samples were randomly selected from the seedlings under each Ca level.

For the measurement of Ca content in the leaves, the leaf tissue were initially dried at 80°C, then ashed at 550°C and dissolved in concentrated HNO_3 and digested using a microwave digestion machine (MARS6, CEM, USA). Finally, an ICP-MS based element analysis was performed.[22]

To access the biomass of the seedlings grown at different Ca levels, the dry weight of seedling were measured. Seedlings were oven-dried (80°C) to constant weight and measured with an analytical balance.

Soluble protein content were measured referring Qiao et al.[23] Oxidation stress related parameters, leaves hydrogen peroxide (H_2O_2) content, total ascorbate peroxidase (APX) activity and total superoxide dismutase (SOD) activity were detected follow previous procedures.[24]

Proteomic analysis

The proteomic analysis of the leaf tissues including protein extraction, two-dimensional electrophoresis (2-DE) separation, gel analysis and MALDI-TOF/TOF analysis were performed according to our previous reports.[18]

Briefly, total leaf proteins were extracted by phenol method, and quantified by a 2-D Quant Kit (GE Healthcare Amersham Bioscience) according to the manufacturer's instructions. Then a 2-DE process was performed for protein separation and 2-DE gel map acquisition. The protein suspended in the lysis buffer (8 M urea, 2 M thiourea, 4 % CHAPS, 1 % DTT, 1 % IPG buffer pH 4–7) was loaded to strips (Immobiline Dry Strip, pH 4-7,18 cm; GE Healthcare) by overnight rehydration. Then the stripes were transferred into an Ettan IPGphor system (GE Healthcare Amersham Bioscience) for isoelectric focusing (IEF). After the IEF, reduction and alkylation reaction were carried out sequentially. For the second dimension electrophoresis separation, 12.5% SDS polyacrylamide gels were prepared. The strips were placed on top of the prepared SDS-PAGE gels and electrophoresis was implemented in an electrophoresis system (Bio-Rad).

The obtained 2-DE gels underwent a staining procedure with Coomassie Brilliant Blue R-250, and were scanned by an image scanner at a resolution of 600 dpi. PDQuest software (Version 8.01, Bio-Rad, United States) were used for the gels analysis and the screening for protein spots showing changed intensity among the gels. The threshold for the changed protein spots: intensity variation no less than twofold, Student's t-test with $p < 0.05$.

The significant changed protein spots were excised manually from 2-DE gels, trypsin digestion, peptide extraction and vacuum drying were performed sequentially. The resulted peptides were mixed with saturated matrix solution (saturated ∝-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA) and were in order spotted on a stainless steel target plate for MS analyses.

The MALDI-TOF-TOF mass spectrometer (AB SCIEX TOF/TOF, 5800 system) was used for protein identification. The parameters and procedures for MS acquisition and database searching were performed referring previous report.[18] Briefly, positive scan mode was selected and mass range was set as 850-4000 Da for MS1. Five precursors were selected for MS2 analysis. The MS data were searched against the National Center for Biotechnology Information non-redundant (NCBInr) database, and the taxonomy of green plants was selected using Mascot search engine (*http://www.matrixscience.com*). The search parameters were listed as follows: Trypsin/P was specified as the cleavage enzyme and one missed trypsin cleavage was allowed. Fixed modification was defined as carbamidomethylation on cysteine and variable modification was defined as oxidation on methionine. Mass errors for precursors and fragments were 100 ppm and 0.3 Da, respectively. The false discovery rate was defined as 1% for positive identification. All the rest parameters followed the default settings in Mascot.

Bioinformatics analysis

The function annotation and classification of the identified DEPs were performed based on Gene Ontology (http://www.geneontology.org/) and Uniprot (http://www.uniprot.org/) databases.[25,26]Subcellular location prediction analysis was conducted with WoLF PSORT tool (http://wolfpsort.org/).[27]The hierarchical clustering analysis of the protein expression profiles under different Ca levels was performed referring Hu et al.[28] Fold changes of protein abundances were calculated by dividing the density value of DEPs at low Ca level or high Ca level by that at media Ca level. The resulted values were log2 transformed and inputted into the Cluster software (version 3.0) with complete linkage algorithm setting. The hierarchical cluster results were visualized with TreeView software version 1.1.3. For PPI network construction, the STRING database and Cytoscape software was used.[29]

Gene expression analysis

Relative gene expression analysis was performed through total RNA extraction and quantitative real-time PCR (qRT-PCR) analysis following our previous operations.[30] Briefly, fresh leaves were ground into powder in liquid nitrogen and total RNA was extracted using RNA purification kit (Invitrogen Inc., CA, USA). Following concentration measurement by ultraviolet spectrophotometer (Cary 50, Varian, USA) and integrity determination by agarose gel electrophoresis, a reverse transcription procedure was performed with the M-MLV reverse transcriptase (TaKaRa, Dalian, China) and the resulted cDNA mixture was used for subsequent PCRs analysis.

The qRT-PCR analysis was carried out in a Rotor-gene-6000 real-time PCR system (Corbett Research, Mortlake, Australia). The selected genes and designed primers were listed in Supplementary Table S2. After the preparation of the reaction mixture containing primers, cDNA and SYBR Green Master (ROX, Mannheim, Germany), the PCR was performed under the following temperature procedure: 10 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 52–55°C (based on the Tm of the primers) and 20 s at 72°C. The relative abundance of gene expression were determined by comparative threshold cycle (Ct) method and actin was used as an internal control.[31]Three replicates were performed for each sample.

Statistical Analysis

The software SPSS version 22.0 was used for statistical analysis. The values in figures were presented as mean ± SE. The statistical significance was analyzed using a univariate analysis of variance (one-way ANOVA, $p < 0.05$).

Results

Phenotype and physiological responses of *P. massoniana*seedlings to various Ca levels

As shown in Figure 1A, under low Ca condition, significant withering and necrosis, and collapsed lesions were observed in the needles of *P. massoniana* compared with medium Ca level, indicating Ca deficiency resulted in the inhibition of needles growth and development. High Ca treatment significantly alleviated this situation as dark green needle colour and fatter needle morphology showed the flourish growth of needles with adequate Ca supply. The decreased total leaf chlorophyll content (Figure 1B) and Pn (Figure 1C) under low Ca treatment, especially the sharply reduced Pn (approximately 50% reduction), suggested photosynthesis was suppressed by Ca deficiency. Under high Ca level, these two parameters recovered and appeared even a little higher than medium Ca level.

There was only a little decline trend in the leaf Ca content under low Ca level, while the leaf Ca content almost doubled when supplying sufficient exogenous Ca (Figure 1D), which is an interesting phenomenon. As to the biomass analysis (Figure 1E), the changing patterns showed similar trend with that in photosynthesis parameters. Ca deficiency reduced soluble protein content while high Ca treatment led to enhanced soluble protein content (Figure 1F), suggesting environmental Ca level influenced protein turnover significantly in *P. massoniana*seedlings.

Three redox response related parameters including H_2O_2 content (Figure 1G), total APX activity (Figure 1G) and SOD activity (Figure 1I) were determined. As the result shown, these three parameters exhibited a little decrease at low Ca level compared to the control. However, high Ca treatment induced a slight increase of H_2O_2 content, total APX activity and SOD activity.

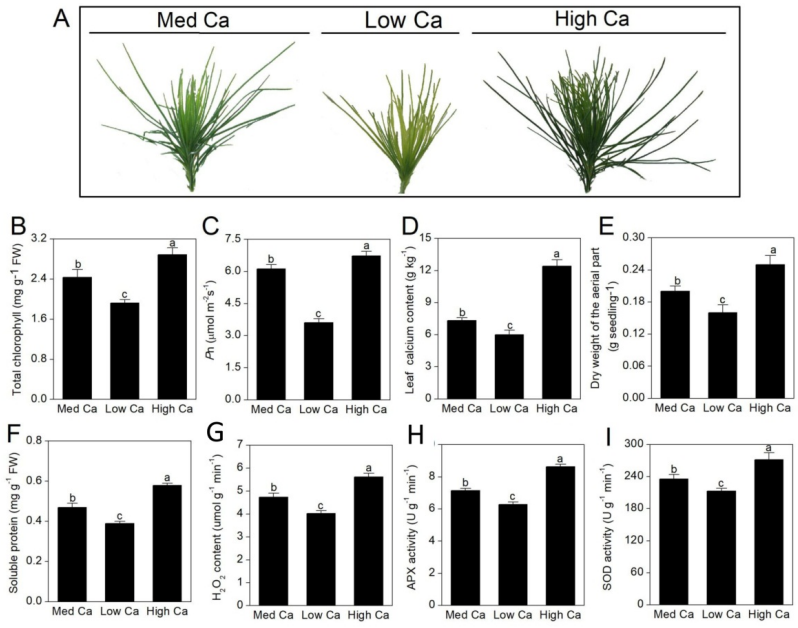

Figure 1 Physiological parameters of *P. massoniana* seedlings cultivated at various calcium levels . (A) Photographs of plant leaves. (B) Total chlorophyll content. (C) Net photosynthetic rate (Pn). (D) Leaf Ca content. (E) Seedling aerial part dry weight. (F) Soluble protein content. (G) Hydrogen peroxide (H_2O_2) content. (H) Total APX activity. (I) Total SOD activity. Data are indicated as means ± SE from six replicates.

Proteome response of *P. massoniana* seedling leaves to various Ca levels

To illustrate the potential mechanisms underlying Ca regulated response of *P. massoniana* , a comparative proteomics analysis for the leaves collected from the seedlings cultivated at various Ca levels was performed. As a result, approximate 1000 protein spots were reproducibly resolved on each gel. In total, 74 DEPs were

identified among various Ca cultivated seedlings by 2.0 fold and $p < 0.05$ (Figure 2A and Table 1). As shown in Supplementary Figure S1, Compared with medium Ca group, low Ca induced 25 DEPs (16 up-regulated and 9 down-regulated) and high Ca resulted in 62 DEPs (46 up-regulated and 16 down-regulated). In the comparison between low Ca group and high Ca group, 65 DEPs were found (44 up-regulated and 21 down-regulated). A close-up view of several representative DEP spots was shown in Figure 2B.

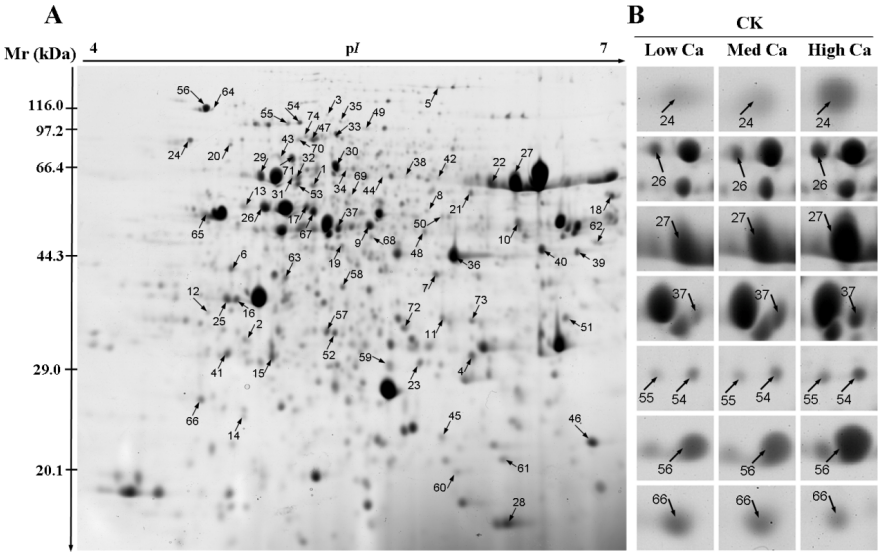

Figure 2 Effects of various calcium levels on the proteome of *P. massoniana* seedlings . The assigned protein spot numbers in the gel corresponded to that listed in Table 1. (A) A representative CBB-R250 stained 2D gel indicating protein profile. (B) Zoom in view for several representative differentially expressed protein spots.

Table 1. Differentially expressed proteins in *P. massoniana* cultivated at various exogenous calcium levels.

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h] L/M	Fold change H/M
Photosynthesis	**Photosynthesis**	**Photosynthesis**						
11	gi|1168738	carbonic anhydrase 1	35.95/5.70	35.56/5.88	5	85	2.3	0.3
13	gi|488888859	chloroplast rubisco activase 1	48.37/7.66	53.41/4.72	7	223	0.8	2.8

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
14	gi|334186238	Mog1/PsbP/DM13 like photosystem II reaction center PsbP family protein	16.37/5.61	24.13/4.75	2	86	0.7	3.1
15	gi|223540996	chlorophyll A/B binding protein	31.10/5.52	30.14/4.89	5	96	0.8	0.5
16	gi|474352688	oxygen-evolving enhancer protein 1	34.64/5.75	37.64/4.72	7	540	2.9	0.0
17	gi|514810582	ribulose bisphosphate carboxylase/oxygenase activase	52.13/7.75	53.10/5.08	10	314	1.1	3.2
18	gi|449455619	glyceraldehyde 3-phosphate dehydrogenase B	48.45/8.29	56.71/6.93	7	169	4.1	2.6
19	gi|413943044	plastid high chlorophyll fluorescence 136 precursor	43.04/8.71	45.36/5.33	6	249	0.9	2.1
20	gi|470110607	RuBisCO large subunit-binding protein subunit alpha	62.03/5.03	82.57/4.66	7	218	1.0	3.1

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
21	gi|449450436	glutamate-glyoxylate aminotransferase 2-like	52.90/5.62	57.72/6.04	7	223	1.7	0.8
22	gi|356998718	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	53.20/6.19	59.41/6.11	25	1050	0.6	2.0
23	gi|470114574	flavoprotein WrbA-like	21.74/5.80	30.15/5.80	2	165	0.9	5.4
24	gi|1351030	RuBisCO large subunit-binding protein subunit alpha	57.71/4.84	83.65/4.36	6	228	1.0	5.5
25	gi|474352688	oxygen-evolving enhancer protein 1	34.64/5.75	37.72/4.67	9	565	2.1	2.1
26	gi|514810582	ribulose bisphosphate carboxylase/oxygenase activase	52.13/7.60	53.00/4.83	8	280	1.1	2.8
27	gi|132016	ribulose bisphosphate carboxylase large chain	53.19/6.19	59.12/6.24	23	1080	1.0	3.3

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/pI[d]	Exper. kDa/pI[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
28	gi|132150	ribulose bisphosphate carboxylase small chain	19.58/8.80	16.79/6.24	9	426	1.0	2.6
Carbohydrate metabolism and energy production	**Carbohydrate metabolism and energy production**	**Carbohydrate metabolism and energy production**						
1	gi|223536136	glucose-1-phosphate adenylyltransferase	57.47/6.48	58.27/5.12	11	317	0.4	2.0
2	gi|222850988	triosephosphate isomerase family protein	27.47/6.00	32.38/4.75	4	195	3.1	1.5
3	gi|332196984	phosphogluconate/	63.67/5.56	110.34/5.21	6	154	0.0	2.8
4	gi|508774665	D-ribulose-5-phosphate-3-epimerase isoform 3	21.55/5.81	31.19/6.06	4	122	1.0	410.7
36	gi|473848356	fructose-bisphosphate aldolase	42.20/5.94	44.79/6.03	6	175	0.8	0.5
39	gi|414876603	fructose-bisphosphate aldolase	41.97/8.39	45.67/6.74	4	200	1.7	5.8
38	gi|110288667	enolase	51.89/5.72	63.33/5.65	10	242	1.3	0.4
53	gi|403241995	UDP-glucose pyrophosphorylase	52.33/5.87	58.43/5.04	5	263	0.6	0.8
37	gi|1170895	malate dehydrogenase (NADP)	50.00/6.42	49.03/5.29	2	70	0.4	2.5
40	gi|527204987	malate dehydrogenase	35.85/6.33	46.38/6.39	4	183	0.5	3.4

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
29	gi|452848969	ATP synthase CF1 beta subunit	52.83/5.18	60.32/4.79	11	1120	1.2	2.6
30	gi|222851303	H^+-transporting two-sector ATPase family protein	60.05/5.91	65.55/5.26	16	836	0.4	1.1
31	gi|452848969	ATP synthase CF1 beta subunit	52.83/5.18	59.78/4.99	10	849	1.3	3.1
32	gi|452848969	ATP synthase CF1 beta subunit	52.83/5.18	60.42/5.02	11	1020	0.9	3.0
33	gi|449455581	ATP-dependent zinc metallo-protease FTSH	76.75/5.81	92.10/5.29	14	515	1.3	2.9
34	gi|475548007	ATP synthase subunit beta	59.15/5.85	65.82/5.32	15	464	1.0	3.0
35	gi|350537129	vacuolar H^+-ATPase A1 subunit isoform	68.81/5.2	105.79/5.31	15	349	0.6	2.7
Nitrogen assimi-lation and protein metabolism	**Nitrogen assimi-lation and protein metabolism**	**Nitrogen assimi-lation and protein metabolism**						
9	gi|426263229	glutamine synthetase	39.35/5.74	50.12/5.47	8	548	0.5	2.6
10	gi|452085037	glutamine synthetase	38.90/5.64	51.54/6.26	5	266	1.0	3.0

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
49	gi|502138744	asparagine-tRNA ligase	63.82/6.00	99.98/5.43	8	60	1.0	6.6
50	gi|502127199	glycine-tRNA ligase 1	81.24/6.43	53.75/5.76	8	154	1.0	3.1
41	gi|460412635	20 kDa chaperonin	26.63/8.55	30.64/4.66	5	126	1.1	2.1
42	gi|302425183	26S protease regulatory subunit 6B homolog	46.79/5.40	63.15/5.81	16	367	0.9	0.4
43	gi|75311214	chaperonin 60 subunit beta 2	63.70/5.6	72.90/4.92	6	118	2.6	1.2
44	gi|223542767	chaperonin-60kD	61.64/5.84	63.00/5.50	9	98	0.3	0.2
45	gi|508786621	HSP20-like chaperones superfamily protein	18.12/5.98	22.92/5.97	6	84	1.3	0.1
46	gi|449445564	peptidyl-prolyl cis-trans isomerase CYP20-2	26.88/8.99	22.11/6.90	3	114	10.1	14.5
54	gi|508784217	heat shock protein 70B	71.85/5.25	103.28/5.04	10	547	1.0	2.3
55	gi|527187624	heat shock protein 70	71.62/5.06	102.52/4.97	16	531	1.0	2.7
56	gi|473939759	stromal 70 kDa heat shock-related protein	66.40/4.79	116.00/4.47	13	445	1.0	2.6

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
64	gi|473939759	stromal 70 kDa heat shock-related protein	66.40/4.79	115.08/4.52	12	521	0.9	8.7
47	gi|508708032	FtsH extracellular protease family isoform 1	74.45/6.11	86.88/5.12	14	388	1.0	0.4
Secondary metabolism	**Secondary metabolism**	**Secondary metabolism**						
6	gi|460373807	lactoylglutathione lyase	32.95/5.95	41.47/4.65	5	156	0.8	3.0
63	gi|470116356	thiamine thiazole synthase 2	37.93/5.72	40.39/4.95	5	136	1.1	2.5
12	gi|460383331	isopentenyl-diphosphate delta-isomerase II-like	38.59/6.14	35.38/4.48	7	136	0.5	1.1
Redox homeostasis and stress response	**Redox homeostasis and stress response**	**Redox homeostasis and stress response**						
52	gi|403084321	ascorbate peroxidase	28.16/5.69	32.96/5.26	3	138	1.2	0.6
57	gi|192912966	cytosolic ascorbate peroxidase	27.55/5.42	33.28/5.22	3	252	2.7	3.2
58	gi|354459054	chloroplast stromal ascorbate peroxidase	35.13/9.04	38.94/5.31	4	98	1.2	2.5
59	gi|440573484	tau class glutathione S-transferase	26.36/5.56	29.66/5.64	5	150	1.1	0.3

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
60	gi|134621	superoxide dismutase (Cu-Zn)	15.48/5.75	20.33/6.01	5	233	1.1	0.1
61	gi|134621	superoxide dismutase (Cu-Zn)	15.48/5.75	20.94/6.21	4	289	2.1	0.9
62	gi|323366911	Mn-superoxide dismutase	24.16/6.49	47.58/6.93	3	83	60.5	5.5
66	gi|460365357	2-Cys peroxiredoxin BAS1	29.41/6.18	25.60/4.46	8	401	2.7	0.3
65	gi|475606711	abscisic stress ripening protein 2	22.87/5.34	51.02/4.41	2	98	2.8	0.8
48	gi|332008500	transcription factor bHLH145	35.26/5.08	48.71/5.78	10	60	3.2	1.0
5	gi|459942337	lipoxygenase	104.70/6.18	140.00/5.88	3	78	1.1	0.2
Cell structure and division	**Cell structure and division**	**Cell structure and division**						
67	gi|380469910	actin 2	41.85/5.31	52.83/5.13	16	819	0.5	1.0
68	gi|474042704	alpha-1,4-glucan-protein synthase 1	63.96/5.55	48.06/5.46	5	108	0.9	2.4
8	gi|3334135	cinnamyl alcohol dehydrogenase	39.47/5.80	53.75/5.76	9	412	1.0	3.1
7	gi|38492949	pinoresinol-lariciresinol and phenyl-coumaran benzylic ether reductases	33.56/5.76	41.85/5.88	10	454	0.9	2.8
69	gi|545363342	xylose isomerase	52.46/5.39	56.41/5.33	4	77	0.9	2.5

Spot[a]	NCBI accession[b]	Protein identity[c]	Thero. kDa/p*I*[d]	Exper. kDa/p*I*[e]	Peptide. Count[f]	Score[g]	Fold change[h]	Fold change
70	gi|223540420	cell division protein ftsH	75.50/6.43	84.74/5.03	14	490	2.4	0.1
Signal transduction and calcium homeostasis	**Signal transduction and calcium homeostasis**	**Signal transduction and calcium homeostasis**						
51	gi|334183678	calmodulin	17.93/4.06	35.97/6.63	4	80	1.0	135.6
Unknown protein	**Unknown protein**	**Unknown protein**						
71	gi|297743227	unnamed protein product	65.26/5.62	72.23/5.01	10	216	0.6	1.3
72	gi|162683812	predicted protein	27.71/5.48	34.61/5.71	3	88	0.6	2.5
73	gi|550337311	hypothetical protein POPTR_0006s289202g	28.36/9.11	35.36/6.07	8	62	2.0	0.5
74	gi|557113008	hypothetical protein EUTSA_v10024591mg	73.55/5.77	84.74/5.03	11	416	3.0	0.0

[a] The spot number corresponding to the number listed in Figure 2.

[b] Database accession numbers according to NCBInr.

[c] The name of the proteins identified by MALDI-TOF/TOF MS.

[d] Theoretical mass (kDa) and pI of identified proteins.

[e] Experimental mass (kDa) and pI of identified proteins.

[f] Number of the matched peptides.

[g] The Mascot searched score against the database NCBInr.

[h] Different protein spots intensity ratios of low calcium treatment (L) or high calcium treatment (H) to the control (medium calcium, M).

Function classification and subcellular localization analysis of DEPs

Function classification and subcellular localization predication were conducted to demonstrate these DEPs in biological process level and cellular component level. As shown in Figure 3A, the majority of these DEPs were involved in photosynthesis (23.0%), carbohydrate metabolism/energy production (23.0%) and nitrogen

assimilation/protein metabolism (20.3%), followed by redox homeostasis and stress response (14.8%), cell structure and division (8.1%) and secondary metabolism (4.0%). Other protein groups included signal transduction and Ca homeostasis (1.4%) and unknown protein (5.4%).

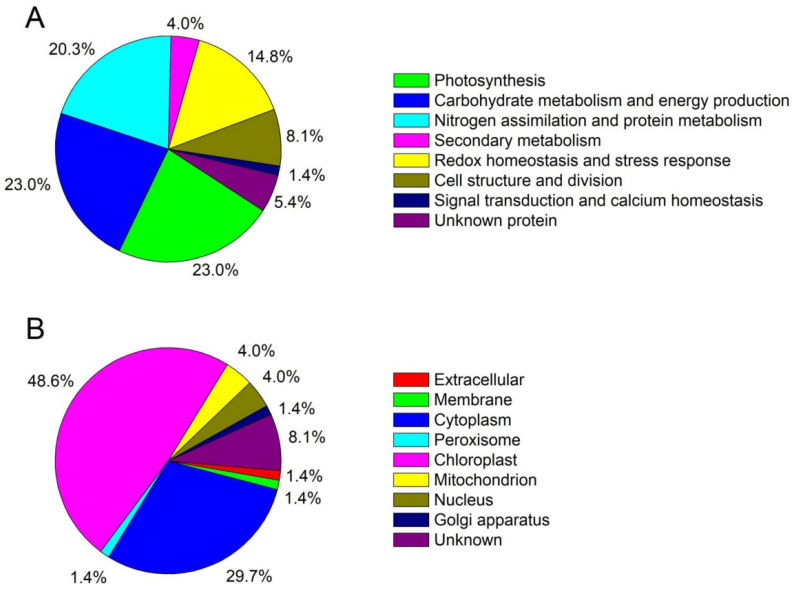

Figure 3 Function classification and subcellular localization analysis for differentially expressed proteins in *P. massoniana* seedlings growing at various calcium levels. (A) Function classification. (B) Subcellular localization predication.

These DEPs showed a wide subcellular localization and cellular component distribution (Figure 3B), among which chloroplast (48.6%) and cytoplasm (29.7%) were the dominant subcellular distribution compartments. Mitochondria localized proteins and nucleus localized proteins accounted for 4.0% and 4.0%, respectively. A small proportion of proteins were predicted to localize to extracellular (1.4%), membrane (1.4%), peroxisome (1.4%) or Golgi apparatus (1.4%). Besides, 8.1% proteins had no definite subcellular localization distribution.

Protein hierarchical clustering analysis for DEPs

A hierarchical clustering was carried out to gain a systematic overview of the expression dynamics of the 74 DEPs resulted from different Ca level treatments. In this analysis, proteins showed similar expression patterns were grouped together. As shown in Figure 4, approximate two-thirds of the 74 DEPs were down-regulated or remain stable under low Ca treatment. While, the expression abundances of these proteins increased under high Ca application. These proteins mainly belong to photosynthesis, energy production, material nitrogen, transcription and signaling, and heat shock proteins. The expression dynamics of these proteins suggested Ca deficiency probable distributed these biological processes while adequate exogenous Ca supply facilitated these processes. We also observed that some proteins showed stable or even increased abundance under low Ca condition, while displayed significantly reduced abundance in high Ca level environment. The numbers of this group included a variety of redox homeostasis related proteins and chaperonin proteins.

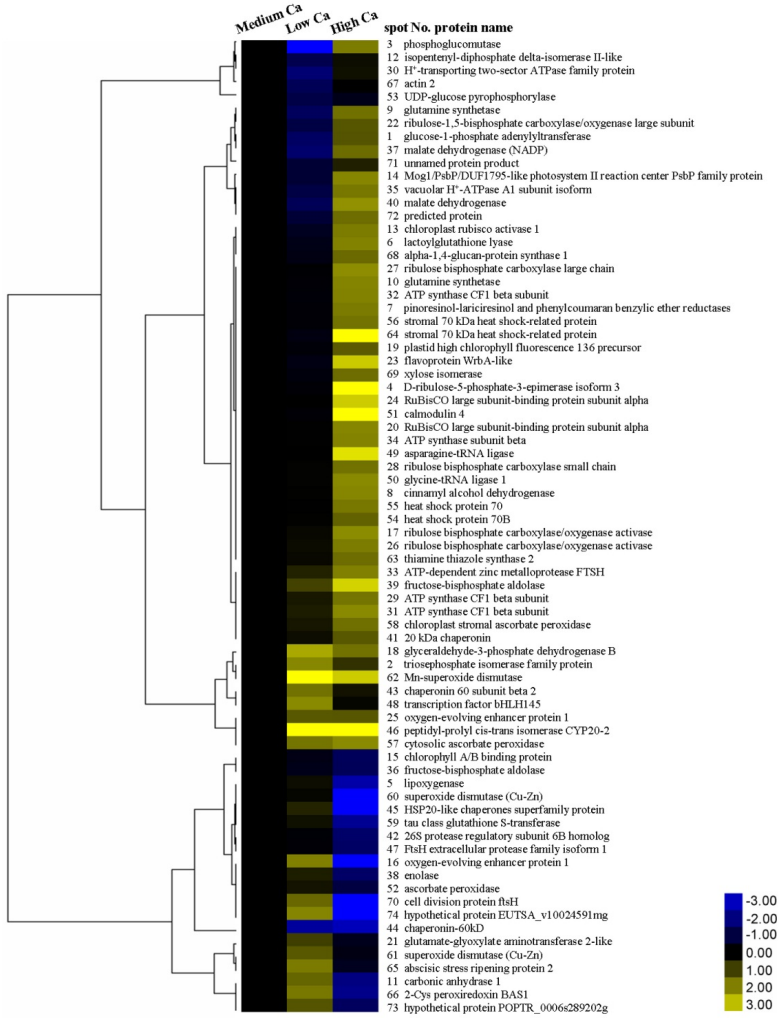

Figure 4 Hierarchical clustering analysis for the protein expression profiles in *P. massoniana* seedlings cultivated at various Ca levels . Fold changes of protein abundance under low or high Ca levels compared with the control (medium Ca level) were log2 transformed and delivered to Cluster and Treeview software. The protein cluster is on the left side and the treatment cluster is on the top. The rows represent each differentially expressed protein. The proteins with increased abundance or decreased abundance are indicated in yellow or blue, respectively. The color intensity was shown in the bar at the right bottom corner of the figure.

Protein-protein interaction (PPI) network

To illustrate the interactions among the DEPs proteins, we carried out a PPI network analysis with all the DEPs at different Ca levels. A total of 28 proteins were matched to the PPI network (Figure 5, Supplementary Table S1). Node degree is a critical index to evaluate the importance and correlation of proteins in the PPI network. As shown in Figure 5 and Table S2, ten proteins with a degree over 6 were observed. The top 5 proteins according to their degrees were ranked as heat shock protein 70 (HSP70), enolase (LOS2), cytosolic ascorbate peroxidase (APX1), 2-Cys peroxiredoxin BAS1 (AT3G11630) and ATP synthase subunit beta (PB), suggesting their central roles in the interaction network. Multiple biological processes may be

influenced by these central nodes.

A total of 4 highly enriched interaction function modules including photosynthesis, carbohydrate metabolism, oxidation stress response and chaperones were observed in the PPI network, suggesting exogenous Ca possibly mediated these physiological and metabolic processes in growing *P. massoniana* seedlings. The links among proteins belonging to different biological processes and pathways indicate there are potential crosslinks among Ca influenced diverse biological processes and metabolism pathways in *P. massoniana* seedlings.

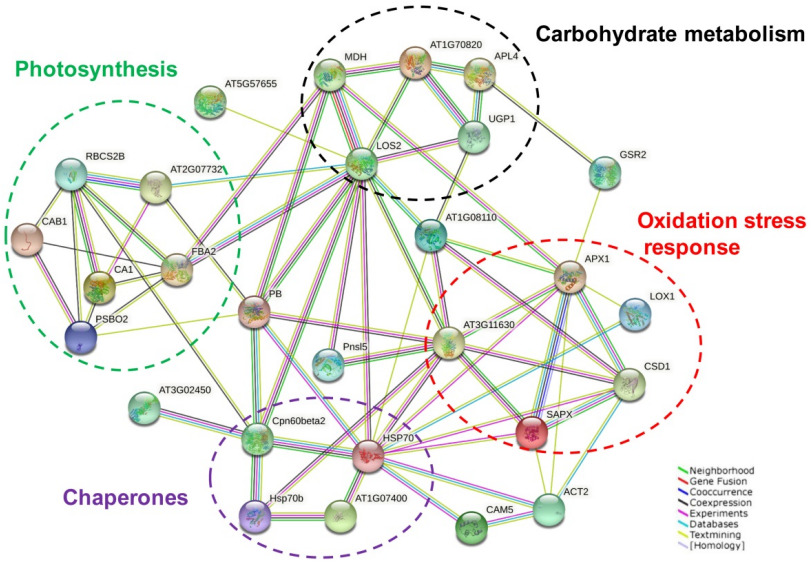

Figure 5 Interaction network for the different expressed proteins . Lines with different colors indicate different evidence types for the association among the protein nodes. Protein abbreviations used in the figure and degrees of these nodes are listed in Supplementary Table S2.

Change patterns of Ca related genes under various Ca levels

To illustrate the expression changes of Ca-related genes in *P. massoniana* seedlings under various Ca treatments, a qRT-PCR analysis was performed for the selected eight genes (Figure 6). Among these genes, except for *GDH2* and *RbohA* , the other six genes (Figure 6A-F) are Ca receptor or Ca binding related genes in plant, and involve in Ca mediated signal transduction pathways or Ca ion homeostasis in plant. Both *GDH2* and *RbohA* (Figure 6G and H) are plant redox processes related genes, which can be activated and regulated by Ca ions. As the result shown in Figure 6, Ca deficiency resulted in the down regulation of these genes, implying potential distributed Ca mediated various signal transduction pathways and Ca homeostasis, and impaired redox balance in *P. massoniana*seedlings under insufficient Ca supply condition. However, high Ca treatment recovered their expression to various extents, such as *CRT3* and *RbohA* recovered similar expression abundance with the control (Medium Ca level) and the others even showed dramatically increased abundance compared with the control. These phenomena suggested sufficient exogenous Ca provision may promote various Ca involved signal transductions, recover Ca ion homeostasis and redox balance in *P. massoniana* seedlings.

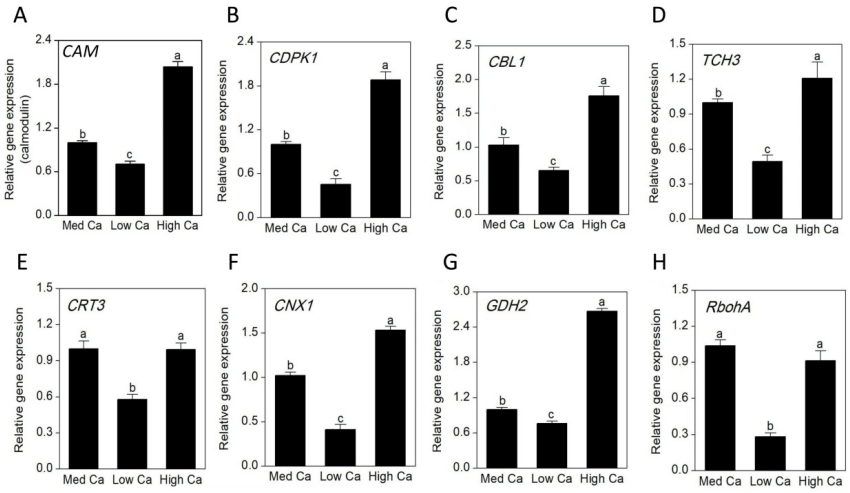

Figure 6 Expression analyses by qRT-PCR for 8 Ca-related genes in *P. massoniana* seedlings cultivated at various Ca levels . (A) Calmodulin, *CaM* . (B) CDPK-related kinase gene, *CDPK1* . (C) Calcineurin B-like Ca sensor protein 1 gene, *CBL1* . (D) Touch 3 gene, *TCH3* . (E) calreticulin 3 gene, *CRT3* . (F) Alnexin 1 gene, *CNX1* . (G) Glutamate dehydrogenase 2 gene, *GDH2* . (H) Respiratory burst oxidase homolog A gene, *RbohA* . Bars indicate the mean value ± SE (n=3).

Discussion

Ca promotes photosynthesis

Our results indicated Ca deficiency significantly inhibited *P. massoniana* photosynthetic process, while sufficient Ca supply markedly facilitated photosynthesis (Figure 1). Consistently, proteomic analysis revealed the down-regulation of a plenty of photosynthesis related proteins under low Ca level and the recovered and even increased expression of these proteins under high Ca level, including both light reaction and dark reaction related proteins (Table 1). PPI network analysis indicated photosynthesis is an important function module of Ca influenced physiological process (Figure 5). A previous review has well characterized the role of Ca in plant photosynthesis.[15] Our study further consolidated the important role of Ca in photosynthesis and demonstrated the mechanism of Ca regulated photosynthesis in*P. massoniana* .

PsbP family protein, one of the key components of photosystem II in higher plants, can enhance oxygen evolution rate at physiological Ca and chloride concentration.[32] The high Ca treatment activated the up-regulation of Mog1/PsbP/DUF1795-like photosystem II reaction center PsbP family protein (spot 14), suggesting photosynthetic oxygen evolution may be accelerated by adequate Ca supply. The changed expression abundance of chlorophyll A/B binding protein (spot 15), plastid high chlorophyll fluorescence 136 precursor (spot 19), flavoprotein WrbA-like (spot 23) and oxygen-evolving enhancer protein 1 (spot 25) imply other light reaction processes could also be affected by Ca level.

Ribose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is the most abundant cellular soluble protein in plant, catalyzes the initial step of CO_2 fixation and is a rate-limiting enzyme for CO_2assimilation.[33] The down regulation of a variety of RuBisCO related proteins (spots 13, 17, 20, 22, 24, 26, 27, 28) indicated CO_2 fixation and assimilation potentially be hindered under Ca deficiency condition. High Ca treatment dramatically reversed this situation; significant up-regulation of these proteins were observed, suggesting the promoting role of Ca in CO_2 assimilation. Chloroplast stroma located carbonic anhydrase (CA), the second richest plant cellular protein apart from RuBisCO, catalyzes the inter-conversion between CO_2 and HCO_3^- and enhance the delivery of CO_2 to RuBisCO.[34] It is generally up-regulated at moderate stress severity to respond stress though partially compensating for decreased CO_2-conducting aquaporin.[35]Accordingly, we

observe the interaction among CA1, Rubisco large subnunit (RBCL) and Rubisco small subnunit (RBSL) in the constructed PPI network (Figure 5). In the presented study, the up regulated CA1 (spot 11) suggests Ca deficiency bring severe CO_2 fixation obstruct in *P. massoniana* seedlings. High Ca treatment lead to the down-regulation of CA1, which is a naturally result of high Ca restored normal photosynthetic carbohydrate fixation.

Glutamate-glyoxylate aminotransferase (GGAT) is a critical enzyme engaged in photorespiration, which is an energy and organic carbohydrate consumption process in plant.[36] Ca deficiency possibly increased photorespiration while inhibited photosynthesis as significantly up-regulation of GGAT 2-like protein were observed at low Ca level. High Ca may lower photorespiration by down-regulating GGAT related proteins.

Collectively, Ca deficiency impaired the photosynthesis process in*P. massoniana* seedlings. Adequate Ca supply improved photosynthesis through promoting CO_2 fixation, regulating light reaction and inhibiting photorespiration processes.

Ca regulates carbohydrate metabolism and energy production

As shown in Table 1, under different Ca levels, the expression of a large scale of carbohydrate metabolism and energy production related proteins were influenced. PPI network also enriched a carbohydrate metabolism function module (Figure 5). The majority of these proteins showed increased abundance under high Ca treatment compared with low Ca or medium Ca treatment. The involved metabolism pathways of these proteins included tricarboxylic acid cycle (TCA), Embden-Meyerhof-Parnas pathway (EMP) and pentose phosphate pathway (PPP).

The critical roles of carbohydrate metabolism to plant growth and development have been well reviewed by previous reports as carbohydrate metabolism provide material and energy premise for plant growth and development.[37,38]High Ca level may benefit carbohydrate metabolism related processes in*P. massoniana* seedlings through up-regulating the expression of relevant protein, and resulted in flourish phenotype and increased biomass (Figure 1). The surging expression of two PPP related proteins, D-ribulose-5-phosphate-3-epimerase isoform 3 (spot 4) and fructose-bisphosphate aldolase (spot 39) under high Ca treatment manifests elevated PPP process play a role in exogenous Ca supported*P. massoniana* seedling growth. PPP is a major source for reduced nicotinamide adenine dinucleotide phosphate (NADPH) and substrates for biosynthetic processes.[39] Exogenous Ca probably accelerated the production of NADPH and metabolic intermediates, which laid a foundation for various biosynthesis processes. The detected up-regulated multiple assimilation and biosynthesis related proteins, such as nitrogen assimilation and amino acid synthesis, secondary metabolites synthesis related proteins (Table 1) verified this speculation to some extent.

Energy metabolism and ATP production are basic biological activities in living organism.[40] The increased expression of a number of ATP synthase subunits (spots 29, 31, 32, 34) revealed high Ca treatment promoted the accumulation of ATP and the storing of energy in *P. massoniana* seedlings. In the PPI analysis, we noticed ATP synthase subunit beta have interactions with all the four obtained function modules (Figure 5). It is broadly accepted that both photosynthesis and some carbohydrate metabolism processes such as TCA are ATP metabolism, especially ATP biosynthesis related processes. Chaperones participate in a number of biological processes in plant, including oxidative phosphorylation process for ATP production.[41] In the oxidative phosphorylation process, the major approach producing ATP, reactive oxygen species (ROS) are unavoidable by-products and can be regulated by anti-oxidation system.[42] High Ca promoted ATP biosynthesis in *P. massoniana* is inferred to be a synergistic result of carbohydrate metabolism and photosynthesis, and probably have crosstalk with other biological processes such as ROS metabolism.

Ca influences nitrogen assimilation and protein metabolism

Plant growth requires a source of nitrogen for the biosynthesis of a variety of nitrogen-containing biomolecules, such as amino acids and nucleic acids. Nitrogen deficiency leads to crops productivity and quality reduction.[43]Previous study has reported Ca mediated nitrogen assimilation in plant.[44] Our study in*P. massoniana* revealed that exogenous Ca may activate the expression of glutamine synthetase (GS; spots 9 and

10), and motivate GS mediated nitrogen assimilation pathway.[45]

Apart from nitrogen assimilation related protein, a number of protein metabolism related proteins were detected with changed expression patterns at different Ca levels (Table 1), which involved in protein synthesis, folding, destination and degradation. The up-regulated mRNA translation and peptide elongation related proteins asparagine-tRNA ligase (spot 49) and glycine-tRNA ligase 1 (spot 50) under high Ca condition suggests Ca perhaps benefit the binding of amino acid and tRNA and contribute to protein biosynthesis. The increased soluble protein content at high Ca level (Figure 1F) is an evidence for this speculation.

Four heat shock protein (HSP) 70 related proteins were identified (spot54-56, 64), whose abundances showed markedly increment at high Ca level (Table 1). HSP 70 is a diverse function protein. Apart from stress response, it also involves in plenty of other biological processes, such as protein folding, assorting and translocation, and the development and differentiation of various plant tissues.[46]Consistently, HSP70 owns the highest degree in the PPI network (Figure 5), showing its versatile roles in diverse biological processes. Adequate Ca treatment stimulated HSP 70 high expression possibly facilitates the aforementioned biological processes in *P. massoniana* seedlings, especially protein folding and transportation related process.

Peptidyl-prolyl cis-trans isomerase (PPIase) catalyzes the reversible conversion of the peptidyl-prolyl bond from cis to trans, which is a rate-limiting step in the folding of proteins.[47] Previous study in *Arabidopsis* reported overexpression of PPIase gene confers stress tolerance to heat, ABA, drought and salt.[48] Our study in*P. massoniana* showed the expression of PPIase CYP20-2 increased ten folds under Ca deficiency condition (Table 1). Up-regulated PPIase may be a potential approach for *P. massoniana* to cope with Ca deficiency, whose underlying mechanism is associated to PPIase in correct protein folding. It is noticeable that high Ca treatment induced drastically soaring of PPIase CYP20-2 as well. This phenomenon can be attributed to high Ca improved protein synthesis and seedling growth, more PPIase is necessary to ensure the proper folding and packaging of accelerating synthesized proteins.

Ca facilitates secondary metabolism

Apart from the aforementioned primary metabolism process, three secondary metabolism related proteins were activated by exogenous Ca (Table 1), whose abundance increased significantly at high Ca level compared with low Ca or medium Ca treatment. The involved metabolites included methylglyoxal, vitamin, isoprenoid and turpentine.

Lactoylglutathione lyase (spot 6) participates in the detoxification of methylglyoxal, and a higher transcription of lactoylglutathione lyase has been reported in aluminum treated tomato root.[49] Methylglyoxal is a by-product of a number of metabolic pathways, especially glycolysis.[50] Plant usually maintains low level methylglyoxal under normal growth conditions while abiotic stresses induce dramatically increase of methylglyoxal, which functions as a toxic molecule and inhibits a variety of biological processes in plant, such as seed germination, photosynthesis and root growth.[51] High Ca treatment induced higher expression of lactoylglutathione lyase possibly accelerated the detoxification and remove of excessive methylglyoxal, which is an unavoidable by-product of Ca improved various metabolism processes, such as glycolysis, in *P. massoniana* seedlings.

Both sopentenyl diphosphate isomerase (IDI)-I and IDI-II participate in the rate-limiting step for the biosynthesis of terpenoid compounds, which are important components of turpentine in pinaceae plant.[52] In the present study, Ca deficiency down-regulated the expression of IDI- II like (spot 12) while high Ca supply recovered its expression to normal level (Table 1). Adequate exogenous Ca supply possibly promoted the biosynthesis and accumulation of turpentine in *P. massoniana* through restoring IDI mediated terpenoid biosynthesis, which is of production guidance significance in *P. massoniana* forest industry for turpentine yield.

Thiamine thiazole synthase involves in the biosynthesis of thiazole, which is the precursor of thiamine (vitamin B1).[53] The significant up-regulated thiamine thiazole synthase 2 (spot 63) implied high concentration of exogenous Ca benefited various glycometabolism and energy pathways may be related to the enhanced bio-

synthesis of thiamine, as thiamine is an essential cofactor for the enzymes activating a plenty of carbohydrate metabolism pathways.[53]

The mechanisms underlying high Ca treatment resulted strengthened secondary metabolism could be attributed to the following potential factors. Primarily, abundant exogenous Ca promoted the processes of photosynthesis and diverse primary metabolism pathways, which laid a sound material and energy foundation for the anabolism of secondary metabolites. Besides, the expression of some secondary metabolism related proteins is a response to high Ca motivated primary metabolism, with the purpose of removing excessive by-products of primary metabolism or synthesizing cofactors for primary metabolism.

In addition, based on the difference in Ca requirement for plants and Ca concentrations in natural habitats, plants can be classified into calcifuge plant and calcicole plant.[16] It can be deduced that *P. massoniana* is a calcicole plant to some extent considering the improved various material metabolism pathways, facilitated photosynthesis, increased biomass and better growth at high Ca level.

Ca restores redox homeostasis and stress response

As shown in Table 1, a plenty of proteins involved in redox homeostasis and stress response changed their expression abundances at various Ca levels. All the identified antioxidant enzymes regulating redox homeostasis showed increased or equal expression abundance at low Ca level compared with the control, including APX (spot 52), cytosolic APX (spot 57), chloroplast stromal APX (spot 58), tau class glutathione S-transferase (GST, spot 59), members of SOD (spot 60-62) and 2-Cys peroxiredoxin BAS1 (spot 66). The higher expression of antioxidant enzymes in plants induced by various abiotic/biotic stresses has been widely reviewed; the up-regulated enzymes enable plant to strengthen stress tolerance and resistance through maintaining redox homeostasis.[54,55]Ca deficiency is a kind of nutrient deficiency stress, and our study hints Ca deficiency may share some common oxidative damage patterns with other abiotic/biotic stresses that cause plant growth inhibition. However, the total APX activity and the total SOD activity showed declined trend at the low Ca level (Figure 1H and I), which looks contradictory with the increased APX and SOD expression. It can be interpreted as follow. Under long time (two months) Ca deficiency stress in our study, the translation of antioxidant enzymes were activated and contributed to accumulated enzymes. Nonetheless, the overall ability of these enzymes to respond oxidative stress may decline gradually with the elongation of stress time, these enzymes showed decreased activity and even deactivated completely as a result of long time Ca deficiency stress.

In the high Ca level, the majority of these antioxidant enzymes showed declined expression. The rationalization to this phenomenon is that adequate exogenous Ca supply relived Ca deficiency stress and subsequent oxidative stress, therefore there is no necessity to sustain high level of antioxidant enzymes.

What's interesting is that cytosolic APX (spot 57), chloroplast stromal APX (spot 58) and Mn-SOD (spot 62) showed increased abundance at both low Ca level and high Ca level compared with the control. Low Ca treatment induced higher APX and SOD expression may probably due to the Ca deficiency resulted oxidative stress. While the up-regulated cytosolic APX, chloroplast stromal APX and Mn-SOD at high Ca level probably be related to the exogenous Ca improved *P. massoniana*seedling photosynthesis (Figure 1B and C). In the process of photosynthetic electron transfer and enzymatic reaction, it is inevitable to produce some by-products such as reactive oxygen species (ROS) including superoxide anion radical (O2-) and hydrogen peroxide (H_2O_2), while H_2O_2 production even acts as an index for photosynthetic electron transport activity evaluation.[56,57]Strengthened photosynthesis signifies accelerated ROS production and accumulation.[57] Within plant cell, especially in chloroplast, O2- is catalyzed to H_2O_2 by SOD, and then H_2O_2 is reduced to water by APX.[46,47]SOD and APX play critical roles in photosynthesis by regulating ROS level.[57,58]High Ca treatment induced the up-regulation of cytosolic APX, chloroplast stromal APX and Mn-SOD manifests exogenous Ca activated the APX and SOD to remove excessive ROS, especially O2- and H_2O_2, and then improved photosynthesis. The interaction between APX and SOD in the PPI network (Figure 5) further proved their collaborative role in ROS elimination. The increased H_2O_2 content, total APX activity and total SOD activity (Figure 1G-I) under high Ca treatment evidenced this deduction at physiological level.

Three stress response proteins, abscisic stress ripening protein 2 (spot 65), transcription factor bHLH145 (spot 48) and lipoxygenase (spot 5) showed similar changing patterns in expression abundances with that in antioxidant enzymes (Table 1). Transcription factor bHLH145 execute a positive regulation of stress response, such as drought and salinity stress response.[59]Lipoxygenase initiate the hydroperoxidation process of polyunsaturated fatty acids and resulted the formation of a variety of oxylipins, such as plant hormone jasmonic acid[60]. Various stresses could activate the expression of lipoxygenase and motivate the sequential stress response pathways.[60] Abscisic stress ripening protein 2 showed increased abundance under drought stress.[61] Compared with low Ca treatment, the down-regulation of the three proteins at high Ca level further proved adequate exogenous Ca can alleviate ROS stress and confer *P. massoniana* seedlings advanced environment adaptability.

Ca modulates cell structure and division

During plant growth and development, cell structure remodeling and cell division are the primary processes, which involve the regulation of a series of cell wall and cytoskeleton related events.[62,63]Our study showed exogenous Ca supply influenced the expression of some cell wall and cytoskeleton related proteins (Table1). Alpha-1,4-glucan-protein synthase 1 (spot 68) have a role in the cell wall polysaccharides biosynthesis process.[64] Xylose isomerase (spot 69) participates in the synthesis of xylose, which is an important structure component of cell wall.[65] Cinnamyl alcohol dehydrogenase (spot 8) is the enzyme catalyzing the last step of lignin biosynthesis and is of vital importance to cell wall formation.[66] In the present study, the abundance of pinoresinol-lariciresinol reductase (PLR, spot 7), a member of the pinoresinol-lariciresinol/isoflavone/phenylcoumaran benzylic ether reductase (PIP) family, that catalyzes two early steps in lignan biosynthesis[67], was found to be increased under high Ca treatment (Table 1). Together, The up-regulation of the above four proteins under high Ca treatment suggest sufficient exogenous Ca potentially accelerate the biosynthesis and accumulation of substrates for cell wall assembly, and further facilitate *P. massoniana* seedling growth and development.

In plants, the actin cytoskeleton system is of vital significance to cell development and morphogenesis.[68] The down-regulated actin 2 at low Ca level implying Ca deficiency suppressed the expression of actin 2, and resulted in distributed *P. massoniana* cell division and development. Under medium or high Ca level, increased expression of actin 2 was observed compared with low Ca level, suggesting cell actin cytoskeleton system is likely recovered.

Collectively, enough exogenous Ca supply is indispensible for *P. massoniana* cell structure reshaping and cell division in the process of plant growth and development.

Role of exogenous Ca in cellular signal transduction and Ca homeostasis

Apart from acts as a nutrient element and structural component, Ca also plays an intracellular second messenger role in plant, and participates in a diverse of signal transduction pathways and biological processes.[9] Our study showed that exogenous Ca markedly increased the expression of an important Ca receptor and Ca signal transducer, calmodulin (CAM), at both gene transcription level and proteomic level (Figure 5 and Table 1). Besides, the gene expression of other three Ca receptors and signal transduction related proteins such as Ca-dependent protein kinase 1 (*CDPK1*), calcineurin B-like protein (*CBL*) and touch 3 gene (*TCH3*) were up regulated dramatically by exogenous Ca (Figure 5). The roles of these Ca receptors in Ca mediated various signal transductions, cellular events and biological processes including plant growth and development have been widely studied.[9,11,69]Adequate exogenous Ca supply ensured sufficient intracellular Ca reservation and Ca ion homeostasis, which is the precondition of various Ca involved signal transduction. On the contrary, Ca deficiency lead to significant down-regulation of these genes (Figure 5), suggesting various Ca signal transduction pathways may be distributed by Ca deficiency, and thereafter result in intracellular Ca ion decline and Ca ion dyshomeostasis.[70]Consistently, physiological parameter measurement (Figure 1D) showed low Ca treatment decreased *P. massoniana* leaf Ca content while high Ca treatment markedly increased leaf Ca content.

Calreticulin 3 (CRT3) and calnexin 1 (CNX1) are another two Ca binding proteins, which localized to endo-

plasmic reticulum (ER) and involved in the transportation and storage of Ca ion within ER.[71] Furthermore, the two proteins also function as molecular chaperones in the regulation of protein folding.[72]Exogenous Ca motivated higher expression of this two genes (Figure 5) implies Ca homeostasis regulation in ER is active under adequate exogenous Ca condition. Moreover, exogenous Ca may accelerate the protein folding and packaging through activating these two molecular chaperones, collaborating with other molecular chaperones such as HSP 70 and PPIase CYP20-2 (Table 1).

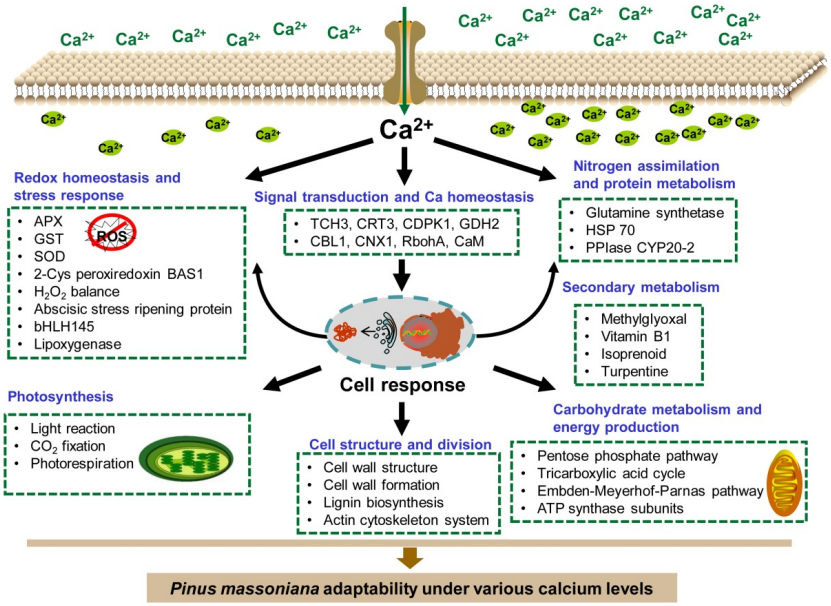

Figure 7. An adaptive strategy of *Pinus massoniana* in response to different calcium levels.

Conclusion

In the present study, the influence of different concentration of exogenous Ca to *P. massoniana* seedling growth was illustrated. Ca deficiency resulted in significant growth and development inhibition while adequate exogenous Ca promoted seedling growth and development. The underling mechanisms were deciphered at physiological, proteomic and transcriptional level (Figure 7). Exogenous Ca supply laid a foundation for various Ca signal transduction pathways and intracellular Ca homeostasis. Various primary metabolisms and basic biological process including photosynthesis, carbohydrate metabolism/energy production and nitrogen assimilation/protein metabolism were distributed by Ca deficiency. Sufficient exogenous Ca restored and promoted these pathways and processes through regulating the expression of enzymes and/or proteins involved in these pathways and processes. Secondary metabolism was activated as a result of improved primary metabolisms at high Ca level. In addition, Ca deficiency leads to severe oxidative stress and redox dyshomeostasis. Affluent exogenous Ca relieved this stress and recover redox homeostasis. Moreover, cell wall structure formation/strengthening and cell division processes were benefited by exogenous Ca. Our study conferred a full view for the demonstration of the potential mechanisms underlying exogenous Ca affected *P. massoniana* seedling growth and development, which may serve as a useful reference and guidance for both pinaceae plant scientific research and pinaceae plant forestry industry.

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Conflict of interest

The authors have declared no conflicts of interest. All authors read and approved the final manuscript.

Authors' contribution

Hailei Zheng, Wenjun Hu and Guoxin Shen supervised the overall study, designed the experiment and revised the manuscript. Wenjun Hu and Jiyun Liu analyzed all data and wrote the manuscript. Wenjun Hu and Tingwu Liu performed the proteomic experiments. Lin Chen, Hongling Lu and Chenkai Jiang conducted the physiological and growth indexes determination. Chunquan Zhu and Qian Wu performed the relative gene expression analysis. Tingwu Liu and Chenkai Jiang carried out plant material cultivation and treatment, and experimental material collection.

Data Availability

All datasets supporting the conclusions of this article are included within the article (and its supplementary material files).

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