A Survey on Cell wall Proteins of C. *Sinensis* Leaf by Combining Cell Wall Proteomic and N-Glycoproteomic Strategy

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Abstract

Background: *Camellia sinensis* is an important economic crop with fluoride over-accumulation in the leaves, which pose a serious threaten to human health due to its leave being used for making tea. Recently, our study found that cell wall proteins (CWPs) probably play a vital role in fluoride accumulation/detoxification in *C. sinensis*. However, CWPs identification and characterization were lacking up to now in *C. sinensis*. Herein, we aimed at characterizing cell wall proteome of *C. sinensis* leaves, to develop more CWPs related to stress response. A strategy of combined cell wall proteome and N-glycoproteome were employed to investigate CWPs. CWPs were extracted by sequential salt buffers, while N-glycoproteins were enriched by hydrophilic interaction chromatography method using *C. sinensis* leaves as a material, afterwards all proteins were subjected to qualitative analysis via UPLC-MS/MS.

Results: 501 and 195 CWPs were identified by cell wall proteomic and N-glycoproteomics profiling, respectively, with 118 CWPs being in common. Notably, N-glycoproteome is a feasible method for CWPs identification and consequently enhance CWP coverage. Among identified CWPs, proteins acting on cell wall polysaccharides constitute the largest functional group with most of them possibly being involved in the remodeling of cell wall structure. The second abundant group encompass mainly various proteases, being considered to be related to CWPs turnover and maturation. Oxidoreductases represent the third abundance with most of them especially Class III peroxidases being known to be implicated in defense response. As expected, identified CWPs emphasized on plant cell wall formation and defense response.

Conclusion: This was the first large scale survey of CWPs by cell wall proteome and N-glycoproteome in *C. sinensis*. The results not only provides a database that will aid deep research on CWPs, but also improve the understanding underlying cell wall formation and defense response in this important economic specie.

Background

Plant cell walls are a primary subcellular structure and locate in the outside of the cells, which offer the skeletal framework to tissues and play essential roles in protection, cell-to-cell adhesion and communication. Cell walls are mainly composed of complex polysaccharidic networks of celluloses, hemicelluloses and pectins, with a smaller proportion of cell wall proteins (CWPs), lignins and lipids [1]. Among them, CWPs constitute around 10% of cell wall dry weight [2–4], but play important roles in various kinds of biological events including cell wall metabolism, cell wall structure and architecture, cell enlargement, cell wall composition modification, signal transduction, biotic and abiotic stress response and other physiological processes [5–10].

In view of the importance of CWPs function, the identification and characterization of CWPs have been performed in some plant species such as Arabidopsis [11–19], *Brachypodium distachyon* [20–22], flax [23, 24], sugarcane [10, 25, 26], rice [27–29] and others in the last decades by cell wall proteomic strategy using destructive and non-destructive extraction methods. The studies have greatly contributed to a

broader knowledge of CWPs. However, to our knowledge, it still remains rare understanding about CWPs due to difficult extraction and high contamination of intracellular proteins.

N-Glycosylation is a common form of eukaryotic protein post-translational modification, and most plant proteins are N-glycosylated through the conventional endoplasmic reticulum (ER) – golgi apparatus (GA) secretory pathway [30, 31]. Consequently, N-glycosylation of plant CWPs is particularly prevalent and extensive. Conversely, large scale and detailed characterization of N-glycoproteins had considerable potential in the understanding of CWPs, and therefore N-glycoproteome can be employed to investigate CWPs [32–36]. Certainly, a suggestion was proposed that combined N-glycoproteome and cell wall proteome could enhance CWPs coverage.

Camellia sinensis is an important woody economic crop cultivated widely from tropical to temperate regions, its leaves are usually used for making tea. It is reported that the leaf of *C. sinensis* can accumulate much higher fluoride (F) than those of most plants without appearing any toxicity symptoms under normal soil conditions [37–40], which suggesting special mechanisms might be responsible for F accumulation/detoxification. Previous researches addressed cell wall immobilization and vacuolar compartmentation contribute to F accumulation/detoxification [41, 42], and recently we found CWPs probably play important roles in F accumulation/detoxification by a comparative proteomics analysis [43]. However, CWPs identification and characterization were lacking in *C. sinensis* up to now.

Herein, to board the knowledge of CWPs and provide a base for the molecular mechanisms underlying CWPs being associated with F accumulation/detoxification, cell wall proteomic and N-glycoproteomic profiling of *C. sinensis* leaf was performed. In this work, $CaCl_2$, EGTA and LiCl were used sequentially to extract CWPs, while hydrophilic interaction chromatography (HILIC) was also employed to enrich N-glycoproteins. The peptides of obtained proteins were analyzed by ultrahigh performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). Afterwards, identified proteins were subjected to the corroboration as CWPs by multiple bioinformatics methods. All in all, 578 CWPs were identified by a combined method of cell wall proteome and N-glycoproteome. This study is not only the first cell wall proteome, but also is the first N-glycoproteome in *C. sinensis*, which will expand the understanding of CWPs and emphasize on plant growth, development and defense response.

Results

CWPs identification

To identify more CWPs, a combined strategy of cell wall proteome and glycoproteome was employed in this work as showed in Fig. 1. After UPLC-MS/MS analysis and database search, 3618 ECWPs and 262 N-glycoproteins were identified in *C. sinensis* leaves by cell wall proteomic and N-glycoproteomic profiling, respectively (Additional file 1, 2: Table S1, S2). To pick out CWPs, all identified proteins (3880) were subjected firstly to WallProtDB database searching. Among them, 627 ECWPs and 187 N-glycoproteins were considered as potential CWPs.

Previous papers addressed that only the proteins (□) having a predicted SP, (□) lacking ER retention signal (KDEL or HDEL) and (□) no more than one TMD were named as CWPs [11, 12, 25]. To verify possible CWPs and get new CWPs, all identified proteins were subjected to multiple bioinformatics analyses including SP, TMD, ER retention signal and subcellular localization. Based on the above-mentioned principles and the report of Day et al [24], 501 ECWPs and 195 N-glycoproteins were given as CWPs. Among them, 17 ECWPs and 8 N-glycoproteins were absent in WallProtDB database and considered as new CWPs (Table 1; Additional file 3: Table S3). As for the remaining proteins, 38 ECWPs were assigned as plasma membrane proteins (Additional file 4: Table S4), others including 3079 ECWPs and 67 N-glycoproteins have been regarded as intracellular proteins. Taken together, 501 CWPs and 195 CWPs were identified by cell wall proteomics and N-glycoproteomic analysis, respectively, and 118 CWPs were in common (Table 1; Fig. 2A; Additional file 3: Table S3).

Table 1
578 CWPs identified in the leaves of *C. sinensis*

	Cell wall proteome	Glycoproteome	In common
Number of identified CWPs	**501**	**195**	**118**
Proteins acting on cell wall polysaccharides	**132**	**43**	**28**
Glycoside hydrolases (GHs)	97	36	23
Carbohydrate esterase family 8 (CE8)	14	2	2
Glycosyl transferases (GTs)	4	0	0
Expansins	6	0	0
PNGase A	2	3	1
Pectin acetylesterases (PAEs)	3	1	1
Pectate lyases (PLs)	2	0	0
homologous to A. thaliana PMR5 (Powdery Mildew Resistant) (carbohydrate acylation)	4	1	1
Proteins involved in signaling	**41**	**30**	**15**
Leucine-rich repeat receptor-like protein kinases (LRR-RLKs)	14	10	3
Receptor-kinases (RLKs, Gnk-2 homologous domain)	5	4	3
S-locus receptor kinases (SD-1)	2	1	0
Lectin receptor kinases (malectin domain)	2	5	1
Wall-associated receptor kinases (WAKLs)	2	2	1
Fasciclin-like arabinogalactan proteins (FLAs)	8	6	5
Expressed protein (LRR domains)	4	0	0
Expressed protein	2	2	2
Homologous to rapid alkalinization factor (RALF)	2	0	0
Proteases	**85**	**34**	**25**
Serine carboxypeptidase S10	22	8	7
Serine carboxypeptidase S28	5	2	2
Asp protease(Peptidase family A1)	23	10	5

	Cell wall proteome	Glycoproteome	In common
Cys proteases(Peptidase family C1) (Papain family)	13	4	3
Ser protease (Peptidase family S8)(Subtilisin)	18	7	6
Subfamily M20A unassigned peptidases	1	2	1
Peptidase M28	1	1	1
Peptidase C13 (legumain family)	1	0	0
DUF239	1	0	0
Proteins with interaction domains (with proteins or polysaccharides)	**31**	**7**	**4**
Plant invertase/pectin methylesterase inhibitors (PMEI)	3	1	0
Proteinase inhibitor family I25 (cystatin family)	5	0	0
Expressed proteins (X8 domain)	3	1	1
PGIPs	2	0	0
Kunitz-P family	3	0	0
Expressed proteins (LRR domain)	5	1	1
Lectin receptor kinases (legume lectin domain)	2	2	1
Serpin (Serine protease inhibitor)	1	0	0
Trypsin and protease inhibitor	1	0	0
lysM domain	1	1	0
Ribosome inactivating protein	5	1	1
Oxido-reductases	**58**	**23**	**19**
Class III peroxidase subfamily	26	9	6
Laccases	5	1	1
BBE (S)-reticulins	6	3	3
Multicopper oxidases	12	6	5
Copper amine oxidases	2	0	0
Thiol reductase (GILT family)	1	0	0

	Cell wall proteome	Glycoproteome	In common
Expressed protein (glyoxal oxidase domain/DUF1929)	1	0	0
Expressed protein (thioredoxin fold)	1	1	1
Expressed proteins (GMC oxido-reductase domain)	2	1	1
Expressed protein (DUF568)	0	2	2
Cytochrome b5-like Heme/Steroid binding domain	2	0	0
Proteins related to lipid metabolism	**39**	**12**	**8**
lipid-transfer proteins (LTPs)	10	1	1
GDSLs	14	4	2
GDPDs	3	2	2
MD-2-related lipid-recognition (ML) domain	1	0	0
Phosphoesterases	2	0	0
Expressed protein (lipase/lipooxygenase domain, PLAT/LH2)	4	0	0
Phospholipase C	1	2	1
Phosphodiesterase/phosphate transferase	1	1	1
Lecithin	1	0	0
Ceramidase	1	1	1
BPI/LBPs	1	1	0
Miscellaneous proteins	**61**	**23**	**9**
Thaumatins (PR5)	5	3	1
Germins	5	0	0
Metallophosphoesterases (PAPs)	10	12	5
Blue copper binding proteins	8	1	0
Dirigent proteins	6	3	1
Phosphate-induced (phi) proteins	3	0	0
SCP-like extracellular proteins (PR-1)	2	0	0
Phosphorylases	3	1	0

	Cell wall proteome	Glycoproteome	In common
Strictosidine synthases	1	0	0
Gibberellic acid-stimulated Arabidopsis (AtGASA1) proteins	3	0	0
Homologous to dienelactone hydrolase	1	1	0
Aldose-1-epimerases	2	0	0
Homologous to phosphatidylinositol transfer protein	1	0	0
Hexokinase	1	0	0
Glucose/sorbosone dehydrogenaes	1	0	0
Carbonic anhydrases	3	0	0
Expressed proteins (cupin domain)	5	2	2
Expressed proteins	1	0	0
Unknown function	**50**	**21**	**9**
Expressed proteins (Gnk2-homologous domain, antifungal protein of Ginkgo seeds)	3	4	2
Expressed proteins (DPBB domain)	4	0	0
Expressed proteins (DUF642)	2	0	0
Plant basic secretory protein (BSP) family proteins	2	0	0
Expressed protein (alpha/beta hydrolase fold)	1	0	0
Expressed proteins (WD40-like beta propeller domain)	3	1	1
NADPH-dependent FMN reductases	2	0	0
Homolog TC173720	2	0	0
Expressed proteins (PA domain)	3	1	1
Expressed proteins (glyoxal oxidase domain/DUF1929)	2	0	0
Expressed proteins (saposin domains)	2	1	1
Expressed proteins (Ole e1 allergen domain)	2	0	0
Expressed protein (cyclase domain)	1	2	1
Expressed protein (BURP domain)	1	0	0

	Cell wall proteome	Glycoproteome	In common
Expressed protein (Xylose isomerase-like TIM barrel)	1	1	1
Expressed protein (human brain CREG protein domain)	1	0	0
Expressed protein (ferritin-like domain)	1	0	0
Expressed protein (DUF303)	1	0	0
Expressed protein (DUF538)	1	0	0
Expressed protein	15	11	2
Structural proteins	**4**	**2**	**1**
LRR-extensins	3	1	1
homologous to AGP/proline-rich protein	1	0	0
hydroxyproline-rich glycoprotein	0	1	0

Functional Classification Of Cwps

To better understand the biological processes, CWPs were categorized on the basis of their functional domains proposed by Jamet et al [44]. 578 CWPs (501 + 195 − 118) can be divided into nine groups (Fig. 2B). Among them, proteins acting on polysaccharides (PACs; 147) was the first largest class, occupying 25.4% of total CWPs. Proteases (Ps; 94) was the second largest class, representing 16.3% of total CWPs. Oxido-reductases (ORs; 62) was the third abundance, taking up 10.7% of identified CWPs. Next, proteins involved in signaling (PSs; 56), proteins related to lipid metabolism (PLMs; 43) and proteins with interaction domains (PIDs 34) were less abundance, representing 9.7%, 7.4% and 5.9% of identified CWPs, respectively. Structural proteins (SPs; 5, 0.9%) were lowest abundant, only containing three LRR-extensises, homologous to AGP/proline-rich protein and hydroxyproline-rich glycoprotein. Other CWPs related to various functions were pooled into miscellaneous proteins (MPs; 75, 13.0%), and CWPs contain previously uncharacterized domains were grouped into proteins of unknown function (PUFs; 62, 10.7%).

Discussion

Identification and functional classification of identified CWPs

Totally, 3618 ECWPs were identified in *C. sinensis* leaves by sequential salt extractions and UPLC-MS/MS. Among them, 501 ECWPs and 3079 ECWPs were considered to be CWPs and intracellular proteins, respectively, via multiple bioinformatics analysis. Notably, intracellular proteins represent 85.1% of ECWPs, indicating ECWPs were subjected to the contamination during ECWPs preparation. Similarity,

high contamination of intracellular proteins was also detected d in sugarcane [25] and rice [29], accounting for 81.6% and 80.5%, respectively. However, the study of cell wall proteome was still rare in plant species and thus CWPs extraction need be improved. However, our study still led to an enlargement of CWPs and offer new knowledge in *C. sinensis* in spite of the high contamination of intracellular proteins.

At the same time, 262 N-glycoproteins were identified in the leaves of *C. sinensis*. As expected, most N-glycoproteins (195, 74.4%) were targeted into the cell wall/extracellular/plasma membrane and thus were assigned as CWPs. The result was in good accordance with that in tomato fruit [35] and *Brachypodium distachyon* leaf [45] which 65% and 60% of N-glycoproteins were reported to be located in the apoplast/cell wall/plasma membrane, respectively, demonstrating that N-glycoproteome is a feasible method to identify and characterize CWPs.

Taken together, 501 CWPs and 195 CWPs were identified by cell wall proteomic and N-glycoproteomic analysis, respectively, and 118 of which was in common. Excitingly, 25 new CWPs being absent in WallProtDB were assigned in this study (Additional file 3: Table S3). The result suggested cell wall proteome is more effective method than N-glycoproteome for CWPs identification. However, it should be noted that the use of N-glycoproteome enhanced CWP identification. As a result, the combined strategy of cell wall proteome and N-glycoproteome should be considered during CWP identification and characterization.

To obtain a global view of the biological processes in which the identified CWPs were involved, 578CWPs were divided into nine functional groups according to their functional domains in this study. Unsurprisingly, PACs (147, 25.4%), Ps (94, 16.3%) and ORs (62, 10.7%) represent top three functional groups. The function distribution of CWPs was in good concordance with that of *A. thaliana* rosettes and *B. distachyon* leaves (Additional file 5: Fig. S3). Notably, the proportion of PSs (9.7%) in *C. sinensis* was obviously higher than that of *A. thaliana* rosettes and *B. distachyon* leaves with 3.7% and 4.0%, respectively [12, 20], which maybe account for the long lifecycle of the evergreen leaf in *C. sinensis.*

Possible roles of identified CWPs

Glycoside hydrolases (GHs). PACs constitute the first largest functional class, with GHs as the major representatives (Table 1). 110 GHs were identified in this experiment corresponding to 74.8% of PACs group, which can be divided into 23 families including GH1, GH3, GH5, GH9, GH10, GH13, GH16, GH17, GH18, GH19, GH20, GH27, GH28, GH29, GH31, GH32, GH35, GH37, GH38, GH51, GH65, GH79 and GH127 according to CAZy nomenclature based on sequence homology (Fig. 3). As expected, the most representative families were GH3 and GH17, as previously documented [12, 20, 26]. Moreover, GH1, GH5, GH16, GH18, GH19, GH27, GH28, GH31, GH35 and GH38 were also well represented families, with at least five members of each (Fig. 3).

Possible substrates of most of GHs families were hemicelluloses (xyloglucan, xylans, glucomannans) and pectin (galactans, homogalacturonan). Out of GHs identified in this study, GH16, GH29, GH31 and

GH65 potentially act on xyloglucans, GH10 and GH51 show possibly action on xylans, and GH28 and GH35 could hydrolyze homogalacturonan and galactans, respectively [46–48] (Additional file 6: Table S5). Moreover, GH1, GH3 and GH5 possess broad substrates range, their enzymes are reported to be involved in the modification and/or breakdown of cell wall hemicelluloses and pectins [49, 50], and also be implicated in lignification and secondary metabolism [51]. Identification of these GHs families suggested that hemicelluloses and pectins might undergo important structural changes in the leaves of C. *sinensis*. Furthermore, GH127 (DUF1680 domain protein), being characterized recently as a novel beta - L-arabinofuranosidase, might be implicated in the degradation of cell wall polysaccharides and hydroxyproline-rich glycoproteins [52], and GH9 was known to catalyze the endohydrolysis of cellulose.

Some identified GHs could participate in defense against pathogens and various stress. Chitin and beta − 1,3- or beta − 1,6-glucan are main components of cell walls of various fungi. GH17 acts as beta − 1,3-glucanase, together with chitinases (GH18 and GH19) and GH20 that function as key hydrolyzed enzyme of chitin, have shown to possess antifungal activity by degrading their cell walls and then participate in defense against pathogens [47, 53]. Intriguing, chitinases being in response to abiotic stress were also reported [54, 43]. GH37, a non-reducing sugar, was identified to be a new CWP in this work without being documented as CWPs in WallprotKB. GH37 acts as a universal stabiliser of protein conformation, might contribute to various stress defense [55].

Several identified GHs including GH13, GH27 and GH32 might be implicated in mobilization, allocation and partitioning of storage reserves. GH13 is was associated with the hydrolysis of starch and glycogen to yield glucose and maltose [56], GH27 is one of three hydrolyzed enzymes of galactomannans as a cell wall storage polysaccharide [57], and GH32 as invertases is involved in long distance nutrient allocation and carbohydrate partitioning [58, 59]. Additionally, a couple of GHs enzymes including GH3, GH18, GH19, GH35, GH38 and GH79 were known to be involved in post-translational modifications (PTMs) of glycoproteins [32, 47]. Here, GH3, GH35, GH38 and GH79 were verified as N-glycoproteins.

Collectively, identified GHs potentially give rise to complex cell wall carbohydrates remodeling, pathogen and stress response, mobilization and allocation of storage reserves as well as glycoproteins PTMs. The high number of GHs associated with cell wall metabolism and defense response were found in this work, which is consistent with published reports of sugarcane stems and leaves [26], *B. distachyon* grains [21], *Saccharum officinarum* cell suspension [25]. The results might be attributed to sustainability remodeling during plant growth and development and terrestrial habit of plants.

Other CWPs acting on polysaccharides. Less represented CWPs acting on polysaccharides including carbohydrate esterase [11 pectinesterase, knows as pectin methylesterases (PMEs) and 3 pectinesterase inhibitor (PMEIs)], GTs (4), expansins (6), PNGase A (4), PAEs (3), PLs (2) as well as carbohydrate acylation (trichome birefringence-like proteins, 4) were also identified.

PMEs, PAEs and PLs are pectin modifying enzymes. PMEs that catalyse the demethyl-esterification of homogalacturonan domain of pectin [60]. The degree of pectin methylation/demethylation impacts on cell wall stiffening and access to enzymes [61]. Demethyl-esterificated pectin more favor the cleavage of

the acidic polygalacturonic chains by GH28 and PLs. In parallel, PAEs can regulate pectin deacetyltation by cleaving the acetylester bond from pectin [62]. Overall, these enzymes play a major role in controlling cell wall plasticity/rheology by affecting pectin metabolism [63].

Trichome birefringence-like proteins and PNGase A are also two modification enzyme families of cell wall. The former was characterized as xylan acetyltransferases, and was believed to be implicated in the mediation of xylan O-acetylation, which being required for secondary wall deposition and pathogen resistance [64]. The latter is one of deglycosylation enzyme and has been considered to be involved in the release of N-glycans from glycopeptides generated by the proteolysis of denatured glycoproteins [65].

Regarding expansins, known as non-enzymatic and the most important structural proteins, are believed to play a central role in cell wall extension via their action on the cellulose-hemicellulose network, suggesting be essential for primary cell wall structure during plant growth and development related processes [66]. Besides, 4 cell wall GT families are represented including GT2, GT31, GT48 and GT68, which is associated with the biosynthesis of cell wall polymers.

Identified CWPs involved in proteases. Proteases (Ps; 94) were the second largest class, representing 16.8% of total CWPs, with Asp proteases (28), Ser carboxypeptidases (28), Ser proteases (19) and Cys proteases (14) as main families. Although the biological roles of proteases are remarkable diverse, proteases certainly play crucial roles in the plant developmental and in response to environmental stresses through turnover and maturation of CWPs, the generation of active peptides in the cell wall [67]. Overall, the importance of this class was expected because proteases are responsible for the degradation or the maturation of cell wall modifying enzymes.

Identified CWPs involved in redox. The third abundant functional class found in tea leaves was ORs (62, 10.7%), mainly comprises class III peroxidase (PODs, 29), multicopper oxidases (13), BBE (berberine bridge enzyme) (S)-reticulin (6) and laccases (5). Class III PODs, a large multigene families, corresponded to one half of the OR functional class.

Class III PODs are known to be involved in lignin metabolism by catalyzing the oxidative polymerization of monolignols [68], stress responses and signaling via consuming hydrogen peroxide and generating reactive oxygen species [69]. Class III PODs also could mediate cross-linking of cell wall compounds such as structural proteins, monolignols as well as of aromatic amino acids with polysaccharides [70–72]. Laccases, like Class III PODs, are candidates for polymerizing monolignol unit into lignin, suggesting be required for cell wall lignification [73, 74].

BBE-like proteins, act as monolignol oxidoreductases, may participate in the mobilization and oxidation of monolignols required for polymerization processes [75]. All in all, three high represented enzyme families in the class were considered to be involved in ligin production and subsequence the reinforcement of cell walls strength and rigidity, which favoring plant defense against adverse environmental factors.

Other CWPs related to redox processes including monocopper oxidase-like proteins (SKU5 and SKS1), blue copper proteins and ascorbate oxidases were identified, which probably play a role in both cell wall loosening, expansion and reticulation processes[24, 76].

Identified CWPs involved in signaling. Identified CWPs from the class mainly contain fasciclin-like arabinogalactan proteins (FLAs, 9) and receptor-like protein kinases (RLKs) superfamily proteins (38). FLAs, heavily O-glycosylated CWPs, have been found to be correlated with cell wall formation [77], cell-to-cell adhesion and communication [78] and abiotic stress response [79]. In the present study, RLKs comprise 21 LRR-RLKs, 6 cysteine-rich receptor-like protein kinases, 3 S-locus receptor kinase subfamily proteins, 2 wall-associated receptor kinases and 6 lectin receptor kinase (LRK) subfamily proteins. RLKs, primary cell wall “sensors”, are responsible for the control of diverse signaling events [80], has been found to possess important functions in a wide variety of developmental and defense-related processes by recognition of an extracellular ligand which leads to activation of the intracellular kinase domain and subsequent transduction of downstream signaling pathways [81].

Identified CWPs related to lipid metabolism. The class of CWPs is that of proteins predicted to be related to lipid metabolism, mainly consist of lipid-transfer proteins (LTPs, 10) and GDSL esterase/lipases (GDSLs, 16). Besides, other CWPs related to PLMs like glycerophosphodiester phosphodiesterases (3), phosphoesterases (2), embryo-specific protein ATS3B-like (4) as well as neutral ceramidase (1) were identified.

LTPs have been shown to be required for lipid export to the cell surface and be closely associated with cutin and wax formation [82]. A LTP was also suggested to be involved in cell wall extension by interacting with the cellulose/xyloglucan network [83]. GDSLs, a newly discovered subclass of lipolytic enzymes, possess multifunctional properties which are assumed to play important roles not only in the formation of surface cutin and epi-cuticular wax [84], but also function in tolerance to biotic and abiotic stresses [85, 86]. In summary, numerous LTPs and GDSLs might play important roles in cuticle assemble during the growth and development of *C. sinensis* leaf. The identification of CWPs related to PLMs is easy to understand for leathery leaf of *C. sinensis*.

Identified CWPs related to other function. Identified MPs mainly encompasses purple acid phosphatases (PAPs, 17), blue copper binding proteins (BCPs, 9), dirigent proteins (DIRs, 8), germin-like proteins (GLPs, 5), Thaumatins (7) and proteins having a cupin domain (5).

PAPs, might associated with the degradation of xyloglucan and oligosaccharides via dephosphorylating CWPs like alpha xylosidase and beta glucosidase [87]. DIRs, are linked to lignin polymerization [88, 89] and play important roles in various stress responses and controlling cell wall modification/reinforcement during cell wall integrity maintenance [90]. Regarding BCPs, GLPs, cupins and Thaumatins were previously reported to be associated with stress responses in plants [91–94].

Five structure proteins were identified in present study including three leucine-rich repeat extensin-like protein (LRR-EXTs), non-classical arabinogalactan protein 31-like (AGP) and hydroxyproline-rich

glycoprotein. LRR-EXTs have known to influence mechanical properties of cell wall by their ability to form insolubilized, covalently crosslink to cell wall components [95], as well as function as perceive extracellular signals and indirectly relay into the cytoplasm to regulate plant growth and salt tolerance, thereby suggesting they are important for cell wall development, plant growth and stress tolerance [96]. Non-classical AGPs have both a proline-rich domain and a non-proline-rich domain, may be function in metal ion-binding, defense response and interact with pectin [97, 98]. As for hydroxyproline-rich glycoprotein, which is an important structural components of plant cell walls and are thought to be implicated to structural integrity, cell-cell interaction and intercellular communication [99].

Several enzymes of CWPs inhibitor were also detected in this study. PMEIs that inhibited partly the activity of PMEs, adjust the degree of pectin methyl-esterification. PGIPs (polygalacturonase inhibitor-like) specifically bind with polygalacturonases (GH28), thereby they can inhibit the hydrolyzation of pectin and then regulate pectin degradation, which can trigger defense against microbes and insects [100]. In summary, two couple of PMEIs and PME, PGIPs and PG occurred coincidentally and modulate precisely pectin metabolism. As for Cys proteinase inhibitor possess inhibitory activities against specific Cys proteases, probably play a role in insect predation [101].

Identified CWPs emphasizing on plant cell wall formation and defense response

Under dynamically changing environmental conditions, plant grow and develop continuously, and always encounter variable stresses and deleterious attack of insects and microbes. To acclimate, plant cell walls that acting as the first barrier change constantly, whereas CWPs play central roles in altering cell wall properties.

Doubtlessly, to meet normal growth and development, a large amount of CWPs could be triggered to adjust vigorously cell wall structure. Here, identified numerous CWPs related to PACs, mainly including GH1, GH3, GH5, GH9, GH10, GH16, GH28, GH29, GH31, GH35, GH51 and GH65, might contribute to the rearrangement of cell wall structure. In contrast, expansins probably lead to cell wall extension. Certainly, several CWPs associated with the formation and metabolism of secondary cell wall, like Class III PODs, BBEs, laccases, LTPs, GDSLs and DIRs, maybe favor to the reinforcement/modification of cell wall (Fig. 4).

Facing to adverse environment, *C. sinensis*, a terrestrial plant, have no ability to escape. Therefore, they have evolved in the context of altering cell wall properties for improved defense responses. Today, ample identified CWPs were potentially involved in various defense. GH17, GH18, GH19 and GH20 were reported to be involved mainly in against pathogens as well as abiotic stress by hydrolyzing chitin. Class III PODs, monocopper oxidase-like proteins, blue copper proteins and ascorbate oxidases were known to be implicated in respond to various biotic and abiotic stresses by redox reaction. LTPs, GDSLs and DIRs were also associated with defense response through the regulation of secondary cell wall. PGIPs and Cys proteinase inhibitor might function in improving protection against insects and pathogens [102] via inhibiting the activity of degradation enzymes of invaders. Likewise, BCPs, GLPs, cupins and Thaumatins also serve functions in defense response (Fig. 4).

To sense changed environment and the status of complex cell wall structures, plants have developed cell wall integrity-sensing pathway to transduce signals into cytoplasm. A number of sensors at the plasma membrane including RLKs and FLAs were identified in present study, which enable *C. sinensis* to coordinate the processes of the cell wall and the cytoplasm (Fig. 4).

In summary, a work model of identified CWPs were proposed (Fig. 4), which emphasizing on plant cell wall formation and defense response, and further making a bit explanation for plant internal activities during normal growth under natural environment.

Conclusions

In this experiment, a study of combined cell wall proteome and N-glycoproteome was performed to depict CWPs in *C. sinensis*. 3880 proteins were identified by sequential salt extraction and UPLC-MS/MS. Meanwhile, 262 N-glycoproteins were identified by HILIC enrichment coupled to UPLC-MS/MS. Subsequently, 501 of 3880 proteins and 195 of 262 N-glycoproteins were assigned as CWPs by multiple bioinformatics analysis. Out of designated CWPs, 118 were in common. In total, 578 CWPs were identified in *C. sinensis* leaves, 25 of which being absent in WallprotKB were named as new CWPs. Altogether, this was the first large scale survey of CWPs by cell wall proteome and N-glycoproteome in *C. sinensis*, which not only provide a major contribution to CWPs identification and characterization, but also improve the understanding underlying cell wall formation and defense response in this important economic specie.

Methods

Plant materials

The first to fifth leaves of 20 uniform 2-year-old cutting seedlings of the Echa 1 variety (*Camellia sinensis* cv. ‘Echa 1’) were collected from tea germplasm bank located in Wuhan city of Hubei province (China), then washed three times with Milli-Q water, grinded into fine power in liquid nitrogen immediately, and finally stored at -80 °C for further use.

Cell wall enrichment

Cell wall fraction was obtained from the leaves of *Camellia sinensis* using sequential washes as described by Printz et al [103] with slight modification. Briefly, 5g fine power of the leaves were homogenized with 3-fold volumes of 0.4 M sucrose buffer for 10 min, vortexed for 2 min, shaken overnight at 250 rpm at 4 °C, and then centrifuged. Subsequently, 0.6 M sucrose buffer was added into the precipitations, shaken for 30 min at 250 rpm at 4 °C and centrifuged. After that, 1 M sucrose buffer was added into the precipitations again, suspended and centrifuged. Finally, the precipitations were washed twice using 5 mM sodium acetate buffer. The final precipitations were cell wall fraction (pellet). Sucrose buffers contained 5 mM sodium acetate and 1% protease inhibitor cocktail (ApexBio), all buffers (pH 4.6) were precooled at 4°C and the centrifugation was operated at 1000 rpm for 15 min at 4 °C.

Cell wall protein extraction

CWPs were extracted successively using $CaCl_2$, EGTA and LiCl according to the method reported by Printz et al [103]. Briefly, 0.2 M $CaCl_2$ buffer was firstly added into cell wall pellet, shaken for 30 min at 200 rpm at 4°C followed by centrifugation, then the supernatants were collected. This step was repeated once and the supernatants were pooled as $CaCl_2$ fractions. Afterwards, cell wall pellet was again mixed with 50 mM EGTA buffer followed by shaking for 1 h at 300 rpm at 37 °C, centrifugation and supernatants collection. This step was repeated twice and all supernatants were pooled as EGTA fractions. Cell wall pellet was finally resuspended in 3 M LiCl buffer, homogenized overnight at 250 rpm, 4 °C. After centrifugation, the supernatants were collected. The proteins were once again extracted from the pellet with 3 M LiCl buffer by shaking for 6 h at 250 rpm, 4 °C. The obtained supernatants were pooled and stored as LiCl fractions. Finally, $CaCl_2$, EGTA and LiCl fractions were combined as extracted CWPs (ECWPs) fractions. All extraction buffers were precooled at 4°C and the centrifugation was performed for 15 min at 10000 rpm at 4 °C.

Whole protein extraction

Whole protein was extracted from *C. sinensis* leaves according to several published paper [43, 104, 105]. Briefly, about 0.5 g fine powder were firstly homogenized with 5 ml pre-cooled homogenization buffer [20 mM Tris-HCl (pH7.5), 250 mM sucrose, 10 mM EGTA, 1 mM PMSF, 1 mM DTT, 1 % (v/v) Triton], and then centrifuged at 12000 g for 20 min at 4 °C. The obtained supernatants were pooled and stored as whole protein fractions.

Protein precipitation and cleaning

According to our previous study [43], whole protein fractions and ECWPs fractions were precipitated severally by Tris-phenol (pH ≥ 8.0) and ammonium acetate. In brief, the fractions were mixed with equal volume of Tris-phenol, vortexed followed by centrifugation at 12000 g for 20 min at 4 °C. Afterwards, the phenol phases were transferred carefully into other tubes, mixed thoroughly with 5 volumes of 0.1 M ammonium acetate in 100 % methanol and incubated at -80 °C overnight. The precipitated proteins were washed twice with 0.1 M ammonium acetate and acetone, respectively. The protein pellets were lyophilized and then dissolved into lysis buffers [7 M urea, 2 M thiourea, 4 % CHAPS, 250 mM DTT, 0.2 % (v/v) Bio-Lyte]. The protein concentration was determined with BCA kit according to the manufacturer's instructions.

Protein digestion

Before trypsin digestion, whole proteins and ECWPs were reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness followed by the dilution to urea concentration less than 2 M by the addition of 100 mM triethylammonium bicarbonate, respectively. Afterwards, whole proteins and ECWPs were digested severally by trypsin (1:50 trypsin/protein) overnight at 37°C followed by trypsin (1:100 trypsin/protein) for 4 h. Finally, tryptic

peptides were desalted by Strata X C18 SPE column (Phenomenex, USA) and concentrated by centrifugal concentrator.

HPLC fractionation

After tryptic digestion, the peptides from whole proteins and ECWPs were fractionated severally by the use of high pH reversed-phase HPLC (high-performance liquid chromatography) with Agilent 300 Extend C18 column (5 μm particles, 4.6 mm inner diameter, 250 mm length). Briefly, the digested peptides were first separated into 60 fractions with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min. Subsequently, the peptides were pooled into 4 fractions and dried by vacuum centrifugation for further use.

Affinity enrichment of N-glycopeptides

To enrich N-glycosylation peptides, the dried peptides from whole proteins were firstly dissolved in 40 μL enrichment buffer (80% acetonitrile, 1% trifluoroacetic acid) and then loaded into HILIC microclumn to divide into glycopeptides and non-glycopeptides by centrifugation for 15 min at 4000 g. To remove nonspecifically adsorbed peptides, HILIC microclumn was washed three times with enrichment buffer. Subsequently, the bound peptides were eluted from the microclumn with 10% acetonitrile and then vacuum-dried. The lyophilized N-glycopeptides were reconstituted in 50 μL 50 mM NH_4CO_3 buffer in heavy oxygen water and incubated with 2 μL PNGase F at 37 °C overnight. Finally, the resulting N-glycopeptides were desalted with C18 ZipTips (Millipore) according to the manufacturer's instructions and lyophilized for LC-MS/MS analysis.

UPLC-MS/MS analysis

For LC-MS/MS analysis, the peptides were firstly dissolved in subjected to solvent A (0.1% (v/v) formic acid and 2% acetonitrile) and then subjected to a gradient elution by EASY-nLC 1000 UPLC system. Peptides separation were conducted with home-made reversed phase column (25 cm length, 100 μm ID). For ECWPs peptides, the following procedure was set for the elution: 450 nL/min constant flow; start from 7%~25% solvent B (0.1% formic acid in 90% acetonitrile) for 0-40 min, 25%~35% for 40-52 min, 35%~80% for 52-56 min, 80% for 56-60 min. For deglycosylated peptides, the following parameters were set: 500 nL/min constant flow, start from 4% to 20% solvent B for 0-24 min, 20% to 32% for 24-32 min, 32% to 80% for 32-36 min and then hold at 80% for 36-40 min.

Subsequently, the separated ECWPs peptides and deglycosylated peptides were injected into a nanoelectrospray ion source followed by MS/MS analysis in Q ExactiveTM and Orbitrap Fusion mass spectrometer (Thermo Fisher scientific), respectively. Briefly, the applied electrospray voltage was 2.0 kV, the intact peptides and their secondary fragments were detected and analyzed by Orbitrap and a data-dependent acquisition mode that automatically altered between MS scan and MS/MS scan was adopted.

For ECWPs peptides, which were detected at a resolution of 70,000 with m/s scan range of 350-1800 for full scan. After that, the 10 most intense parent ions per scan were selected for higher-energy collisional dissociation fragmentation (HCD) at 28% collision energy. The generated fragments were further analyzed at a resolution of 17,500 with a fixed first mass of 100 m/z. To improve the effective utilization rate of mass spectrometry, automatic gain control of 5E4, 30 s dynamic exclusion, 100 ms maximum inject and signal threshold of 20000 ions/s were applied. Likely, deglycosylated peptides were detected at a resolution of 60,000 with m/s scan range of 350-1550 for full scan. The 20 most intense parent ions per scan were selected for HCD at 35% collision energy, and then the resulting fragments were analyzed at a resolution of 15,000 with a fixed first mass of 100 m/z. Likewise, automatic gain control of 5E4, 15 s dynamic exclusion, 200 ms maximum inject and signal threshold of 5000 ions/s were used.

Database Search

The resulting raw MS/MS data was processed using MaxQuant search engine (v.1.5.2.8) with the following query parameters: (□) tea tree genome database (Camellia_sinensis_4442 with 53512 sequences; [106]) concatenated with reverse decoy database and mass spectrometry contaminants database for MS/MS search; (□) Trypsin/P for enzyme cleavage and 2 for missing cleavages; (□) mass tolerance of 20 ppm and 5 ppm for peptide ions in first search and main research, respectively, and 0.02 Da for fragment ions; (□) 7 amino acid residues for minimum peptide length and 5 for maximum modification number in a peptide; (□) Cysteine alkylation as fixed modification; (□□) Variable modification: methionine oxidation and N-terminal acetylation of protein for ECWPs, and methionine oxidation and deamidation (NQ), asparagine deamidation (^{18}O□for N-glycoproteins; (□□□) FDR≦1% for protein identification and peptide-spectrum matches identification.

Multiple bioinformatics analyses

CWPs were predicted and functionally categorized using WallProtDB database [107]. Glycoside hydrolases (GHs) and carboxylesterases (CEs) were grouped according to CAZy database [108]. N-terminal signal peptide (SP) of identified proteins was predicted using SignalP [109]. Transmembrane domain (TMD) was evaluated by TMHMM server [110]. Subcellular localization predication was performed using TargetP [111], WoLF PSORT [112], Loctree 3 [113] and Plant-mPLoc [114]. Endoplasmic reticulum (ER) retention signal was checked using Prosite[115].

Abbreviations

CWPs: cell wall proteins; ECWPs: extracted cell wall proteins; GHs: glycoside hydrolases; PACs: proteins acting on polysaccharides; Ps: proteases;ORs: oxido-reductases; PSs: proteins involved in signaling; PLMs: proteins related to lipid metabolism; PIDs: proteins with interaction domains; PODs: peroxidases.

Declarations

Author's contributions

LYL conducted the experiment and drafted the manuscript. LLM and DC assisted in material culture and collection; JF and HJH assisted in partial data analysis. ZMG and XFJ provided the overall supervision. All author have read and approved the final version of the manuscript.

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Ethics approval and consent to participate

Collection of plant materials in this study complied with institutional, national or international guidelines.

Consent for publication

Not applicable.

Competing interests

The authors have declared no competing interests.

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Figures

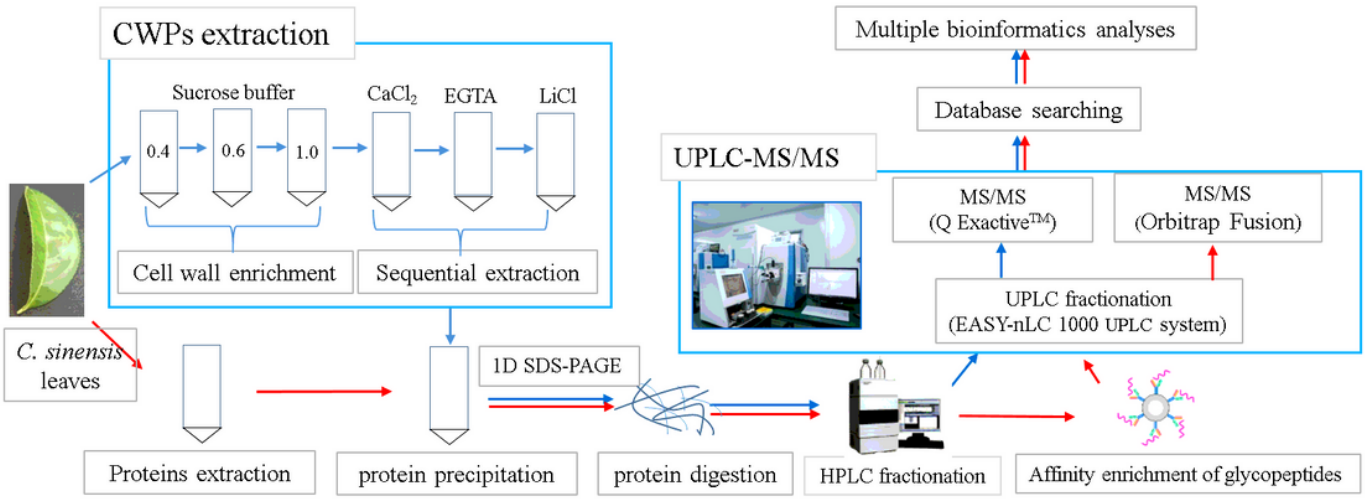

Figure 1

Experiment workflow using in this work. The extraction, precipitation, digestion, fractionation, and MS/MS and data analyses of ECWPs were operated according to blue arrow instruction. Likely, those of glycoproteins were operated according to red arrow instruction.

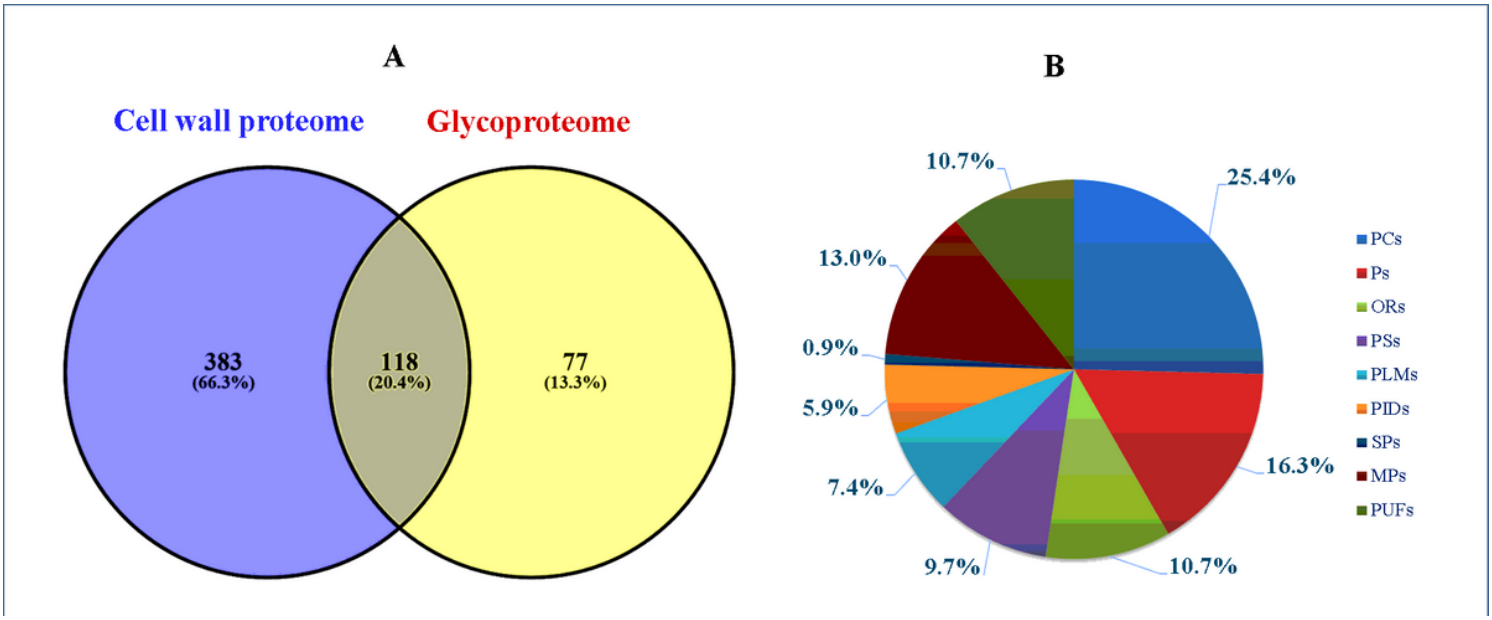

Figure 2

Identification (A) and functional classification (B) of CWPs in the leaves of C. sinensis

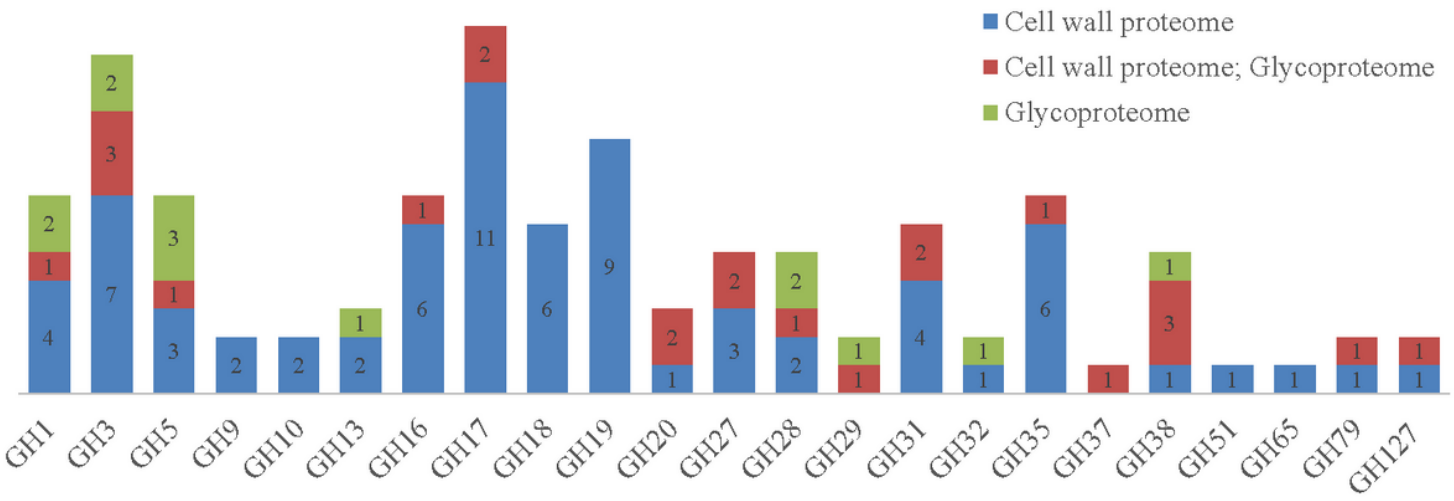

Figure 3

Identified glycoside hydrolases in the leaf of C. sinensis

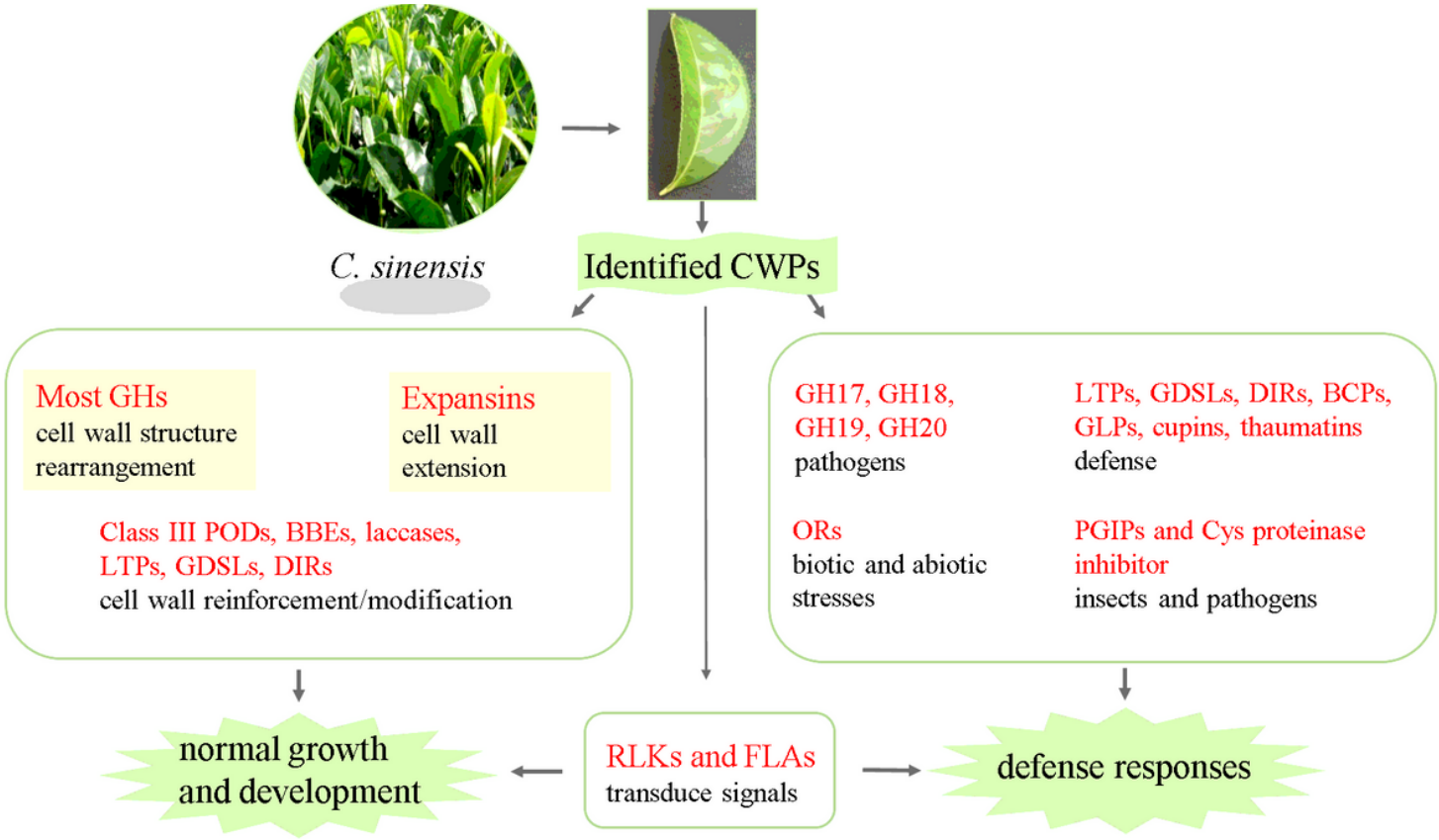

Figure 4

A work model of identified CWPs in C. sinensis

Supplementary Files

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