Comparative proteomics analysis of coregulation of CIPK14 and WHIRLY1/3 mediated leaf pale yellowing in Arabidopsis

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Abstract: Leaf variegation pale yellowing is observed in the Calcineurin B-Like-Interacting Protein Kinase14 (CIPK14) overexpression line (oeCIPK14) and double knockout WHIRLY1/WHIRLY3 (why1/3) lines of Arabidopsis, the distribution of WHIRLY1 (WHY1) protein between plastids and the nucleus are affected by the phosphorylation of WHY1 by CIPK14. To elucidate the coregulation of CIPK14 and WHIRLY1/WHIRLY3 mediated leaf pale yellowing, a differential proteomic analysis is conducted between the oeCIPK14 variegated (oeCIPK14-var) line, why1/3 variegated (why1/3-var) line and wild type (WT). More than 800 protein spots are distinguished on each gel, 67 differential abundance proteins (DAPs) are identified by matrix-assisted laser desorption ionization-time-of-flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS), of which, 34 DAPs are in the oeCIPK14-var, 33 DAPs are in the why1/3-var compared to WT. Five overlapping proteins differentially change both in the oeCIPK14-var and in the why1/3-var. They are ATP-dependent Clp protease proteolytic subunit-related protein 3 (ClpR3), Ribulose bisphosphate carboxylase large chain (RBL), Beta-amylase 3 (BAM3), Ribosome-recycling factor (RRF), Ribulose bisphosphate carboxylase small chain (RBS). Bioinformatics analysis show that most of DAPs are involved in photosynthesis, defense and antioxidation pathway, protein metabolism, amino acid metabolism, energy metabolism, malate biosynthesis, lipid metabolism and transcription. Thus, the photosystem parameters are measured that the content of chlorophyll, the photochemical efficiency of PS II (Fv/Fm), and electron transport rates (ETR) decrease in the why1/3-var and oeCIPK14-var, but the non-photochemical quenching (NPQ) increases. Both mutants show high sensitivity to strong light. Based on the annotation of DAPs from both why1/3-var and oeCIPK14-var lines, we conclude that CIPK14 phosphorylation mediated WHY1 deficiency in plastids is related to impairment of protein metabolism leading to chloroplast dysfunction.

Keywords: comparative proteomics analysis; CIPK14; WHIRLY1/WHIRLY3; protein metabolism

1. Introduction

Leaf senescence is a complex process that highly is regulated by genetic materials, and also is induced by internal (such as age and hormones) and external (including multiple biotic and abiotic stresses) factors [1]. Leaf senescence accompanies with the degradation of chlorophyll and various biomacromolecules in plant cells [1], the remobilization of nutrients to seeds and fruits during reproductive growth stage [2]. It has been reported that more than 20 transcription factor families are associated with senescence-regulation, such as NAC, WRKY, MYB, C2H2-type zinc finger and AP2/EREBP proteins families, many members of NAC and WRKY families have been reported to play a central role in leaf senescence regulatory network [3-7]. Among the WRKY families, WRKY53 has been shown to act as a key regulator at early stage of leaf senescence in Arabidopsis [8], while
WHIRLY1 has been reported to repress the high expression of WRKY53 by binding to the promoter of WRKY53, and delay the leaf senescence [9].

WHIRLY (WHY) family proteins are dually located in both the nucleus and organelles, and they perform numerous cellular functions in both locations [10, 11]. There are two WHY members (WHY1 and WHY2) in monocotyledonous plants, but three members (WHY1, WHY2 and WHY3) in dicotyledonous plants [12]. WHY: GFP fusion proteins fluorescence assay shows that the Arabidopsis WHY1 and WHY3 locate in chloroplasts, and WHY2 locates in mitochondria [10]. The intriguing feature of the dual location in plastids and the nucleus of WHY1 in the same cell has been demonstrated by immunogold-labelling assay in barley (Hordeum vulgare) [11]. The translocation of plastidial WHY1 protein from chloroplasts to the nucleus has been confirmed in the transplastomic tobacco plants which synthesized an HA-tagged version of WHY1 in plastids. The WHY1 protein is detected in the nucleus, where it changes the expression of target genes [13]. The characteristics of dual location and regulating nuclear gene transcription make WHY1 as an ideal candidate for plastid-to-nuclear retrograde signaling research.

WHY proteins are first discovered as nuclear transcriptional activator binding at elicitor response element in the promoter regions of pathogenesis-related genes in potato (Solanum tuberosum) and Arabidopsis [14, 15]. Later it is found that they are able to bind to various DNA sequences, including telomeres [16], a distal element upstream of a kinesin gene [17], the promoter region of the early senescence marker gene WRKY53 in a development-dependent manner in Arabidopsis [9], and the promoter region of the senescence-associated gene HvS40, which was induced during natural and stress-related senescence in barley [18]. WHY1 is also proposed to bind to both ssDNA and RNA with a role in intron splicing in maize (Zea mays) chloroplasts [19], while in barley chloroplasts WHY1 is associated with intron-containing RNA [20].

Additionally, in Arabidopsis, plastid-located WHY1 and WHY3 both are identified as two novel plastid transcriptionally active chromosome proteins (pTACs) by mass spectrometry (MS) in transcriptionally active chromosomes of nucleoids [21]. At the same time, WHY3 and WHY1 both are found in the protein complex bound to the promoter of kinesin in Arabidopsis by pull down-MS assay [17]. While WHY3 is discovered as a redox-affected protein in the thiol-disulphide redox proteome of the chloroplast [22], WHY1 is proposed to involve in the perception of redox changes in the photosynthetic apparatus, the relocation of WHY1 from the chloroplasts to the nucleus may be initiated by the redox state of the photosynthetic electron transport chain [23]. A recent research indicates that WHY1 interacts with light-harvesting protein complex I (LHCA1) and affects the expression of genes encoding photosystem I (PS I) and light harvest complexes (LHCI) [24]. Although most double knockout WHY1 and WHY3 (why1/3) plants have no apparent phenotype, about 5% individuals show variegated phenotype which associated with the instability of plastid genome [25]. Furthermore, triple mutant why1why3polIb-1 defective WHY1 and WHY3 and chloroplast DNA polymerase 1B (POLIB) exhibit a grievous variegated phenotype and higher plastid genome instability [26]. The why1why3polIb-1 mutant shows lower photosynthetic efficiency and produces more reactive oxygen species (ROS) in chloroplast, the elevated ROS level is correlated with the intensive expression of oxidation-related nuclear genes [26]. In barley, WHY1 RNAi knockdown mutants are shown to have more chlorophyll and less sucrose than the wild type [27]. Large numbers of genes encoding photosynthesis and protein synthesis proteins exhibit up-regulated expression in the Hvwhy1 mutants [27]. These results suggest that plastid-located WHY proteins participate in plastid-to-nuclear retrograde signaling to maintain plastid function for environmental fluctuations response. Therefore, the dual location and dual function of WHY protein might have special traits in communication between two compartments in one cell.

The latest study has illuminated that the Calcineurin B-like-Interacting Protein Kinase14 (CIPK14) interacts with and phosphorylated WHY1, the phosphorylated WHY1 is imported to nucleus and enhances the binding affinity with the promoter of WRKY53 [28]. CIPK14 overexpression (ocCIPK14) plants show an increased nuclear isoform but decreased plastid isoform of WHY1, fascinatingly, among the CIPK14 overexpression transgenic plants, about 5% individuals show variegated pale-green phenotype which is similar with why1/3 and why1why3polIb-1 [28]. Even more
Furthermore, staining (Figure 1D), it clearly shows that proteins associate with the CIPK14 discovered in the plastids and the nucleus. Among them, 33 differentially expressed protein analyzed by MALDI electrophoresis analysis (2-DE) and the differential expression proteins are identified by matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS). The selected proteins are identified in transcriptional level by quantitative real-time PCR (qRT-PCR) and in protein level by western blot. The chlorophyll content and chlorophyll fluorescence kinetic curve are used to determine for photosynthetic performances analysis of the detail phenotype of different mutants.

2. Results

2.1. Proteomic analysis of why1/3-var and oeCIPK14-var mutants

Based on our previous reports, the dual localization and distribution of WHY1 protein between plastids and the nucleus were affected by WHY1 protein phosphorylation status mediated by CIPK14. The CIPK14 overexpression transgenic plants, about 5% individual oeCIPK14-var showed variegated pale-green phenotype, which was similar with why1/3-var [28]. To evaluate the relationship of CIPK14 and WHY1/WHY3 producing pale yellow leaf. The total proteins of wild type (WT), why1/3-var and oeCIPK14-var rosette leaves are separated by two-dimensional gel and the differential expression proteins are identified with 2-DE. The total proteins of rosette leaves of 4-week-old plants are separated by 2-DE, more than 800 protein spots are detected reproducibly on each gel for WT, why1/3-var and oeCIPK14-var lines. Total 67 differentially expressed protein spots show significant changing (fold≥2, p<0.05) in the why1/3-var and oeCIPK14-var lines (Figure 1A-C). The differential expressed protein spots are analyzed by MALDI-TOF/TOF-MS, total 66 protein spots are identified successfully (identification rate: 99%), only one protein (spot 42) remains unknown. The identified proteins are listed in Table 1. Among them, 33 differentially expressed protein spots (spot 11, 14, 21, 30, 32, 35-62) are detected in the why1/3-var (Figure 1B), 34 differentially expressed protein spots (spot 1-34) are detected in the oeCIPK14-var (Figure 1C). Interestingly, only 5 overlapping proteins (spot 11, 14, 21, 30, 32) are discovered in both why1/3-var and oeCIPK14-var. These five proteins are proposed to intimately associate with the CIPK14 mediating functions of WHY proteins.

In order to evaluate the quality of 2-DE differentially display proteins, two light-dependent reaction complex related proteins are selected to do immunodetection by using consumable antibodies against the ATP synthase subunit alpha (atp A) and the photosystem II (PS II) complex proteins PSBR (Figure 1D). While an antibody against atp A didn’t work, PSBR is barely detectable in the oeCIPK14-var that is in agreement with the changes of protein abundance observed by 2-DE (Figure 1D). We further directly detect the RBL and RBS amount by coomassie bright blue R250 staining (Figure 1D), it clearly shows that both RBL and RBS decrease in the why1/3-var and oeCIPK14-var lines, which is in consistent with the changes of protein abundance observed by 2-DE. Furthermore, two antibodies against RBL and RBS (kindly provided by Krupinska lab) are used to perform western blot. The results show consistently in the why1/3-var and oeCIPK14-var lines that the proteins RBL and RBS both are down-regulated in the oeCIPK14-var and slightly decreases in the why1/3-var plants compared to wild-type plants (Figure1D). It confirms the MS data reliable.
Figure 1. 2-DE and immunoblot analysis of total proteins extracted from rosette leaves of WT and two variegated mutants. (A-C) Representative 2-DE gel images of WT (A), why1/3-var (B), and ocCIPK14-var (C). An equal amount (1.5 mg) of total proteins is loaded on each IEP strips (3-10 NL). The spot numbers indicated proteins that showed significant changes between WT and two variegated mutants. (D) The changing of protein abundance selected from 2-DE are confirmed by western blot and CBB R250 staining. The immunoblot analysis is performed using antibodies against RBL, RBS, and PSBR. CBB R250 staining shows RBL and EBS protein amount and the same amount of loading proteins.

2.2. Functional classification of differentially expressed proteins

Based on Uniprot database (http://www.uniprot.org/) and the description from the literatures, the function of 65 proteins have been annotated. These proteins are categorized into 8 groups based on their biochemical functions as shown in Table 1. The majority of the proteins are photosynthesis-associated proteins, followed by defense and antioxidation proteins, protein metabolism and amino acid metabolism, energy metabolism, malate biosynthesis, lipid metabolism and transcription related proteins (Table 1 and Figure 2). Comparative analysis of differentially expression proteins between why1/3-var/WT and ocCIPK14-var/WT are shown in Figure 2A and 2B, respectively. The five overlapping proteins between why1/3-var/WT and ocCIPK14-var/WT are ATP-dependent Clp protease proteolytic subunit-related protein 3 (ClpR3), Ribulose bisphosphate carboxylase large chain (RBL), Beta-amylase 3 (BAM3), Ribosome-recycling factor (RRF), Ribulose bisphosphate carboxylase small chain (RBS) (Figure 2C).
### Table 1 Differential proteins identified by MALDI-TOF/TOF-MS of why13-var and CIPK14-var

| Spot no | Protein name                     | Accession no | Mascot score | Matched peptides | Theor MW (kDa)/pI | Cov% | Subcellular Loc |
|---------|----------------------------------|--------------|--------------|------------------|-------------------|------|-----------------|
| Light reaction |
| 1       | Fe-S cluster assembly factor HCF101 | HF101_ARATH  | 371          | 13               | 57.728/5.91       | 20%  | plastid         |
| 2       | RCA                              | F4IVZ7_ARATH | 499          | 18               | 48.469/7.55       | 34%  | plastid         |
| 3       | ATP synthase subunit alpha       | ATPA_ARATH   | 927          | 32               | 55.294/5.19       | 50%  | plastid         |
| 4       | Magnesium-chelatase subunit ChlI-2 | CHLI2_ARATH  | 365          | 23               | 46.069/5.36       | 44%  | plastid         |
| 12      | Chlorophyll a-b binding protein CP26 | CB5_ARATH   | 213          | 12               | 30.183/6          | 35%  | plastid         |
| 26      | Magnesium protoporphyrin IX methyltransferase | CHLM_ARATH | 653          | 23               | 33.775/7.68       | 53%  | plastid         |
| 28      | PsbP domain-containing protein 4 | PPD4_ARATH   | 122          | 8                | 28.484/7.02       | 33%  | plastid         |
| 33      | Oxygen-evolving enhancer protein 3-2 | PSBQ2_ARATH | 464          | 16               | 24.628/9.72       | 59%  | plastid         |
| 34      | PSBR                             | A0A178WGP6_ARATH | 112         | 7                | 9.77/10.1         | 39%  | plastid         |
| 35      | TROL                             | A0A178V0X3_ARATH | 444         | 21               | 54.448/5.09       | 29%  | plastid         |
| Calvin cycle |
| 5       | Phosphoglycerate kinase 1        | PGKH1_ARATH  | 642          | 24               | 50.081/5.91       | 37%  | plastid         |
| 6       | Sedoheptulose-1,7-bisphosphatase | S17P_ARATH   | 354          | 18               | 42.388/6.17       | 28%  | plastid         |
| 14      | Ribulose bisphosphate carboxylase large chain (Fragment) | A0A142I795_ARATH | 292         | 12               | 51.833/6.17       | 9%   | plastid         |
| 17      | Ribulose bisphosphate carboxylase small chain | A0A178UL15_ARATH | 326         | 14               | 20.33/7.59        | 45%  | plastid         |
| 18      | Ribulose bisphosphate carboxylase small chain 1A | RBS1A_ARATH | 369          | 15               | 20.203/7.59       | 45%  | plastid         |
| 23      | Ribulose bisphosphate carboxylase large chain (Fragment) | A0A142I795_ARATH | 292         | 12               | 51.833/6.17       | 18%  | plastid         |
| 27      | Ribulose bisphosphate carboxylase large chain | RBL_ARATH | 740          | 31               | 52.922/5.88       | 42%  | plastid         |
| 32      | Ribulose bisphosphate carboxylase small chain | A0A178UL15_ARATH | 326         | 14               | 20.33/7.59        | 45%  | plastid         |
| Entry | Description | Accession | DDBJ ID | Goana | Identity | Similarity | Localization |
|-------|-------------|-----------|---------|--------|----------|------------|--------------|
| 40    | Ribulose bisphosphate carboxylase large chain (Fragment) | A0A142I795_ARATH | 292     | 12     | 51.83/6.17 | 26%        | chloroplast  |
| 43    | Ribulose bisphosphate carboxylase small chain 1A | RBS1A_ARATH | 362     | 13     | 20.20/7.79 | 49%        | chloroplast  |
| 45    | Ribulose bisphosphate carboxylase small chain 1B | RBS1B_ARATH | 348     | 14     | 18.50/8.22 | 53%        | chloroplast  |
| 59    | Beta carbonic anhydrase 1 | BCA1_ARATH | 307     | 15     | 37.42/5.74 | 48%        | plastid/cytoplasm |
| 7     | Glucan endo-1,3-beta-glucosidase, acidic isoform | E13A_ARATH | 568     | 16     | 37.31/4.85 | 33%        | secretion    |
| 9     | Thiamine thiazole synthase | THI4_ARATH | 183     | 10     | 36.64/5.82 | 28%        | plastid     |
| 10    | SAPX | A0A178V0Q5_ARATH | 524     | 24     | 40.44/8.31 | 47%        | plastid     |
| 16    | Glycine-rich RNA-binding protein 7 | RBG7_ARATH | 167     | 9      | 16.88/5.85 | 51%        | cytoplasm/nucleus |
| 20    | Glutathione S-transferase F6 | GSTF6_ARATH | 355     | 15     | 23.47/5.8  | 49%        | cytoplasm   |
| 24    | V-type proton ATPase subunit E1 | VATE1_ARATH | 328     | 30     | 26.04/6.04 | 75%        | vacuole     |
| 25    | L-ascorbate peroxidase 1 | F4HU93_ARATH | 389     | 16     | 27.50/5.85 | 52%        | cytoplasm   |
| 37    | Pyridoxal 5'-phosphate synthase subunit PDX1.1 | PDX11_ARATH | 423     | 23     | 32.84/5.75 | 37%        | cytoplasm   |
| 38    | Thioredoxin-like protein CDSP32 | CDSP_ARATH | 371     | 20     | 33.66/8.65 | 35%        | chloroplast |
| 44    | GRP7 | A0A178VQY8_ARATH | 315     | 9      | 16.93/5.85 | 37%        | cytoplasm/nucleus |
| 46    | V-type proton ATPase subunit G1 | VATG1_ARATH | 304     | 10     | 12.38/5.77 | 70%        | vacuole     |
| 51    | Formate dehydrogenase | FDH_ARATH | 354     | 18     | 42.38/7.12 | 36%        | chloroplast/mitochondria |
| 52    | 12-oxophytodienoate reductase 3 | OPR3_ARATH | 782     | 24     | 42.66/7.71 | 54%        | peroxysome  |
| 54    | Thylakoid luminal 29 kDa protein | TL29_ARATH | 475     | 24     | 37.91/8.59 | 53%        | plastid     |
| 55    | Remorin | REMO_ARATH | 427     | 26     | 20.95/8.63 | 65%        | plasmalemma |
| 57    | VIPP1 | A0A178W0D3_ARATH | 412     | 23     | 28.89/5.9  | 67%        | plastid     |
| 60    | Glutathione S-transferase F7 | GSTF7_ARATH | 552     | 18     | 23.58/6.14 | 52%        | cytoplasm   |
| 61    | Peptide methionine sulfoxide reductase A4 | MSRA4_ARATH | 236     | 12     | 38.62/8.96 | 26%        | plastid     |

**Defense and antioxidation**

| Entry | Description | Accession | DDBJ ID | Goana | Identity | Similarity | Localization |
|-------|-------------|-----------|---------|--------|----------|------------|--------------|
| 22    | Serine hydroxymethyltransferase 4 | GLYC4_ARATH | 504     | 22     | 51.68/6.8 | 46%        | cytoplasm   |
| 48    | Asparagine synthetase [glutamine-hydrolyzing] 2 | ASNS2_ARATH | 235     | 18     | 64.98/6.01 | 27%        | cytoplasm/plasmodesmata |

**Amino acid metabolism**

| Entry | Description | Accession | DDBJ ID | Goana | Identity | Similarity | Localization |
|-------|-------------|-----------|---------|--------|----------|------------|--------------|
|   | Protein Name                                      | Accession Code | Score | E-value | Identity | Location        |
|---|--------------------------------------------------|----------------|-------|---------|----------|-----------------|
| 50| Glutamate-glyoxylate aminotransferase 1          | GGT1_ARATH     | 478   | 20      | 53.267/6.49 | 37% peroxysome  |
| 58| Acetylglutamate kinase                           | NAGK_ARATH     | 424   | 14      | 36.572/9.04  | 36% plastid     |
|   | **Proteometabolism**                             |                |       |         |          |                 |
| 11| ATP-dependent Clp protease proteolytic subunit-related protein 3 | CLPR3_ARATH | 597   | 23      | 36.284/8.64  | 41% chloroplast  |
| 13| Proteasome subunit alpha type-6-A                | PSA6A_ARATH    | 926   | 25      | 27.277/5.6  | 58% cytoplasm/nucleus |
| 15| 30S ribosomal protein S6 alpha                   | RR6_ARATH      | 119   | 8       | 22.746/5.92  | 26% plastid     |
| 19| CPN10                                            | O80504_ARATH   | 567   | 10      | 15.04/8.75   | 45% chloroplast  |
| 29| Proteasome subunit beta type-2-B                 | PSB2B_ARATH    | 715   | 22      | 21.97/6.21   | 70% cytoplasm/nucleus |
| 30| Ribosome-recycling factor                        | RRFC_ARATH     | 497   | 18      | 30.403/9.46  | 44% plastid     |
| 31| Peptidyl-prolyl cis-trans isomerase CYP18-3      | CP18C_ARATH    | 307   | 11      | 18.361/7.68  | 34% cytoplasm   |
| 39| Proteasome subunit alpha type-6-B                | PSA6B_ARATH    | 312   | 19      | 27.333/5.75  | 56% cytoplasm/nucleus |
| 56| Peptidyl-prolyl cis-trans isomerase CYP19-1      | CP19A_ARATH    | 406   | 14      | 18.48/8.65   | 43% cytoplasm   |
| 62| Nascent polypeptide-associated complex subunit beta | A0A178W6R8_ARATH | 332   | 14      | 16.935/5.50  | 45% cytoplasm/nucleus |
|   | **Energy metabolism**                            |                |       |         |          |                 |
| 21| Beta-amylase 3                                   | BAM3_ARATH     | 510   | 24      | 61.314/6.59  | 41% chloroplast  |
|   | **Malate biosynthesis**                          |                |       |         |          |                 |
| 36| Malate dehydrogenase                             | MDHP_ARATH     | 334   | 12      | 42.379/8.66  | 23% chloroplast  |
| 47| NADP-dependent malic enzyme 2                    | MAOP2_ARATH    | 323   | 19      | 64.372/6.01  | 25% cytoplasm   |
| 49| L-galactono-1,4-lactone dehydrogenase            | GLDH_ARATH     | 759   | 35      | 68.513/8.7   | 38% mitochondria |
|   | **Lipid metabolism**                             |                |       |         |          |                 |
| 53| GDSL esterase/lipase At1g29670                   | GDL15_ARATH    | 841   | 19      | 39.847/8.85  | 44% secretion   |
|   | **Transcription**                                |                |       |         |          |                 |
| 8 | RNA-binding protein CP29B                        | CP29B_ARATH    | 602   | 16      | 30.699/5.06  | 52% plastid     |
|   | **Unknow function**                              |                |       |         |          |                 |
|   | Accession  | Molecular Mass | pI   | Identity | Localization       |
|---|------------|----------------|------|----------|--------------------|
| 41| At1g13930/F16A14.27 | Q9XI93_ARATH | 310  | 14       | 16.154/4.82        | 87% chloroplast/plasmalemma |
| 42| Kinesin-like calmodulin-binding protein | KCBP_ARATH | 54   | 35       | 143.359/6.69       | 21% cytoplasm               |

- a. Numbering corresponds to 2-DE gel in Figure 1A-C.
- b. Database accession of the identified proteins in uniprot (http://www.uniprot.org/).
- c. Molecular mass and pI theoretical.
- d. Percentage of predicted protein sequence with match sequence.
- e. Subcellular localization of the identified protein base on uniprot and previous literatures.
Figure 2. Functional classifications of the differentially expressed proteins identified in why1/3-var and oeCIPK14-var compared with WT. (A) Functional classifications of the differentially expressed proteins between why1/3-var and WT; (B) Functional classifications of the differentially expressed proteins between oeCIPK14-var and WT; (C) The Venn diagram analysis between why1/3-var and oeCIPK14-var compared with WT. The Venn diagram is completed by the online tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.3. Hierarchical clustering analysis of differentially expressed proteins

To acquire information of identified proteins, hierarchical clustering analysis is performed in the proteins which appeared on the same branch with the similar expression pattern (Figure 3). It is shown that there are 4 clusters for these proteins. The majority of proteins in the first and second clusters are up-regulated in the why1/3-var and oeCIPK14-var lines, respectively, mainly containing defense and antioxidation related proteins (spot 9, 10, 25, 37, 38, 52, 54, 57, 61), amino acid and protein metabolism related proteins (spot 11, 19, 22, 29, 30, 39, 48, 50), photosynthesis-associated proteins (spot 1, 3, 4, 6, 12, 26, 28, 32, 35) and energy metabolism related proteins (spot 21, 36, 47, 51). Most of proteins in the third and fourth clusters show down-regulated in the why1/3-var and oeCIPK14-var lines, respectively. Among them, some of the key enzymes of Calvin cycle (spot 2, 5, 6, 14, 17, 18, 23, 27, 40, 43, 45) belong to the two branches, suggesting that the fixation rates of CO₂ decrease in the why1/3-var and oeCIPK14-var variegated lines. We also find that other proteins are down-regulated in the why1/3-var and oeCIPK14-var, which are involved in light-reaction, defense and antioxidation mechanisms, amino acid metabolism, proteins metabolism and so on.
Figure 3. Hierarchical cluster analysis of the differentially expressed proteins of why1/3-var and oeCIPK14-var compared with WT. The three columns represent protein expression changes in the (A) WT, (B) why1/3-var, (C) oeCIPK14-var, respectively. The rows represent individual proteins identified in the why1/3-var and oeCIPK14-var lines, the up-regulated or down-regulated proteins are indicated in red or green. The heat map used log2 of fold changes of protein abundance between WT and why1/3-var and oeCIPK14-var mutants.

2.4. Transcriptional level analysis of the encoding genes of differentially expressed proteins

To investigate the correlation between protein and transcript levels, the transcript levels of the encoding genes of differentially expressed proteins are analyzed by quantitative real-time PCR (qRT-PCR). 26 genes are determined in the WT, why1/3-var, and overexpressing WHY1 (oeWHY1) lines; and 27 genes are determined in the WT, oeCIPK14-var, and knockout CIPK14 (cipk14) lines.
respectively. The transcript levels of WHY1, WHY3 and CIPK14 firstly detect in the WHY and CIPK14 mutants (Figure 4). As show in Figure 4, the expression level of WHIRLY1 is barely detectable in the why1/3-var but significant accumulation in the oeWHY1 (Figure 4A). The expression level of WHIRLY3 is lower in the why1/3-var compared with WT (Figure 4B). The expression level of CIPK14 is barely detectable in the cipk14 while it shows significant up-regulation in the oeCIPK14-var (Figure 4C).

![Graphs](https://source.unsplash.com/random/300x200)

Figure 4. The transcript levels of WHIRLY1, WHIRLY3 and CIPK14 in the used lines. (A) The level of expression of WHIRLY1 in the WT, why1/3-var and oeWHY1; (B) The level of expression of WHIRLY3 in the WT and why1/3-var; (C) The level of expression of CIPK14 in the WT, cipk14 and oeCIPK14-var.

We further analyze the transcript levels of the encoding genes of differentially expressed proteins. Relative protein abundance of WT and why1/3-var is represented by percent volume on up panel (a1 and b1) of Figure 5A and 5B panel, the relative expression levels of the encoding genes of differentially expressed proteins in the why1/3-var and oeWHY1 compared WT are shown in the Figure 5A-a2 and Figure 5B-b2 panel, respectively. The encoding genes of differentially expressed proteins in the oeCIPK14-var are analyzed in the WT, cipk14 and oeCIPK14-var. Relative protein abundance of WT and oeCIPK14-var is represented by percent volume on the up panel (c1 and d1) of Figure 5C and 5D panel, the relative expression levels of the encoding genes of differentially expressed proteins in the oeCIPK14-var and cipk14 compared WT are shown in the Figure 5C-c2 and Figure 5D-d2 panel, respectively. The quantitative RT-PCR results of all candidate genes show that the transcription level of 11 out of 26 genes is altered in the why1/3-var or oeWHY1, among them the transcriptional patterns of 5 out of 26 genes are consistent with the protein expression patterns (Figure 5A-a2), whereas the rest 21 genes show inconsistency in the why1/3-var (Figure 5B-b2), however, most of gene expression changings in the why1/3-var are recovered in the oeWHY1 except for spot 21, spot 39, and spot 44. It suggests that WHY1 is not enough to fully rescue the alteration of transcript level of genes in the why1/3-var. The transcript level of 13 out of 27 gene expression is changed in the oeCIPK14-var or cipk14, among them the mRNA levels of 9 out of the 27 genes change in parallel with protein levels (Figure 5C-c2), whereas the rest 18 genes show inconsistency in the oeCIPK14-var (Figure 4D-d2). Unexpectedly, only a few gene expression levels are changed in the cipk14 line, no any gene expression is rescued. This discrepancy between the mRNA and protein levels may be caused by the different half-lives of protein and mRNA, or post-transcriptional or post-translational process and modification.
Figure 5 Comparison of changes in the protein and mRNA levels for selected protein spots. The relative protein abundance is represented by percent volume. (A) The mRNA levels change in parallel with protein levels in the why1/3-var lines; (a1) The relative protein abundance of protein spots in the WT and why1/3-var; (a2) The relative expression levels of the corresponding genes in (a1) are analyzed in the WT, why1/3-var, and oeWHY1; (B) The mRNA levels change independently in the why1/3-var lines; (b1) The relative protein abundance of protein spots in the WT and why1/3-var; (b2) The relative expression levels of the corresponding genes (b1) are analyzed in the WT, why1/3-var, and oeWHY1; (C) The mRNA levels change in parallel with protein levels in the oeCIPK14-var lines; (c1) The relative protein abundance of protein spots in the WT and oeCIPK14-var; (c2) The relative expression level of the corresponding genes in (c1) are analyzed in the WT, oeCIPK14-var, and cipk14; (D) The mRNA levels change independently in the oeCIPK14-var lines; (d1) The relative protein abundance of protein spots in the WT and oeCIPK14-var; (d2) The relative expression of the corresponding genes in (d1) are analyzed in the WT, oeCIPK14-var, and cipk14.

The relative expression level of the gene is normalized to GAPC2, with the WT as 1. The error bars indicated standard error of three biological replications and three technique replicates. Asterisk indicate significant differences (*P<0.05 and **P<0.01) based on Student’s t-test analyzed by Graphpad prism6 software.

2.5. Effects of why1/3-var and oeCIPK14-var on photosynthetic performance

According to above protein annotations, the majority of the differentially expressed proteins are photosynthesis-associated proteins. In order to address the relationship of these protein function and phenotype of pale green leaf appeared in the why1/3-var and oeCIPK14-var lines, the chlorophyll contents and photosynthetic fluorescence parameters of 4-week-old plants of WHY and CIPK14

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mutants are measured to determine the photosynthetic performance. Consistent with the pale-green phenotype, the chlorophyll content is lower in the oeCIPK14-var plants than wild-type, whereas it remains unaltered in the why1/3-var (Figure 6A). Interestingly, the chlorophyll content slightly increases in the oeWHY1 and cipk14 plants (Figure 6A). The maximum photochemical efficiency of photosystem II (Fv/Fm) significantly decreases in the why1/3-var and oeCIPK14-var plants (Figure 6B). The electron transport rates (ETR) in the why1/3-var and oeCIPK14-var are only about 70% and 45% of the WT, respectively (Figure 6C). The nonphotochemical quenching of photosystem II fluorescence (NPQ) displays an increase in the why1/3-var and oeCIPK14-var (Figure 6D), indicating that the two variegated mutants have a lower photosynthetic capacity. It is consistence with plants under high light condition.

Figure 6 The photosynthetic performance analysis of WHY and CIPK14 mutants. (A) Total chlorophyll content. (B) The maximum photochemical efficiency of photosystem II (Fv/Fm). (C) The nonphotochemical quenching of photosystem II fluorescence (NPQ). (D) The electron transport rate (ETR). The error bars indicate the standard error of nine independent measurements. (E) The fluorescence images of the whole plants of WHY and CIPK14 mutants. The fluorescence images are taken by Image-PAM using the plants after 30 minutes dark-adapted.

The fluorescence images of the whole plant of different genotype are taken by using Image-PAM (Pulse-Amplitude Modulation) measuring system, as showed in Figure 6E. The why1/3-var and oeCIPK14-var plants display the smaller size and variegated phenotype as described previously [25, 28]. While the oeWHY1 and cipk14 plants are almost indistinguishable from the wild type, however,
the dark fluorescence yield (F0) of the two variegated lines are higher, but the Fv/Fm decreases remarkably in the why1/3-var and oeCIPK14-var lines.

Taken together, in the why1/3-var and oeCIPK14-var plants higher F0, lower Fv/Fm, and lower ETR, higher NPQ may lead to excessive excitation energy production, which may enhance the level of ROS production. The unbalance of energy may further result in the variation of redox state of the photosynthetic electron transport chain, then trigger the relocation of WHY1 from the chloroplasts to the nucleus and transmitted the signals.

3. Discussion

Leaf pale yellowing phenotype normally appears in plastid dysfunction plants such as plastid ribosome defect plants, chlorophyll synthases abnormal plants or plastid RNA processing abnormal plants. As expected, in this study, most of differential display proteins in the why1/3-var/WT oeCIPK14-var/WT are related plastid dysfunction, such as photosynthesis, amino acids and protein metabolism, or defense and antioxidation.

3.1 Protein involved in light-dependent reaction of photosynthesis

The dual-located protein WHY1 has suggested as an ideal candidate for plastid-to-nucleus retrograde signaling factor [23]. In chloroplast, the WHY1 is located at the boundary between thylakoids and the nucleoids and therefore the WHY1 protein is proposed to link the operation of the photosynthetic electron transport chain to gene expression [23]. WHY3 has found as cofactors of WHY1 playing a function in the plastid genome stability [25], and activators in the nuclear kinesin gene expression [17]. In our study, the results of functional classification and immunoblot analysis reveal that the 7 light reaction related proteins including Fe-S cluster assembly factor HCF101 (HCF101, spot 1), Magnesium-chelatase subunit ChlI-2 (ChlI-2, spot 4), atpA (spot 3), Chlorophyll a-b binding protein CP26 (CP26, spot 12), PsbP domain-containing protein 4 (PPD4, spot 28), TROL (spot 35) and cyt f exhibit more abundance in the why1/3-var and oeCIPK14-var plants. The PPD4 and CP26 are the components of PSII [29, 30]. CP26 is known as an antenna protein which required for the formation of PSII supercomplexes and the energy transition from trimeric light-harvesting complex II (LHCII) to the reaction center of PSII [31]. The Cyt b6f complex modulating the electrons transferring from PSII to PSI are performed by the quinone pool and plastocyanin [28]. The increase in abundance of cyt f may accelerate the ETR that are lower in the why1/3-var and oeCIPK14-var plants. The HCF101 has been shown to serve as a chloroplast scaffold protein for the assembly and transferring of [4Fe-4S] clusters which are essential for the accumulation of the core complex PSI and the soluble ferredoxin-thioredoxin reductases [32]. The ATP synthase is responsible for ATP production, the up-regulated of atpA is observed in our proteomic data. As expected, we also find that the TROL (spot 35) protein is required for tethering of FNR and sustaining efficient linear electron flow (LEF) [33]. The TROL knockout mutant displays lower ETR and increases NPQ, it further confirms our former result which the why1 line showed low TROL protein [24]. Consistent with the pale-green phenotype of two variegated lines, the enzyme of chlorophyll biosynthesis ChlI-2 shows up-regulated and it catalyzes the first committed step toward chlorophyll synthesis, accompanying Mg\(^{2+}\) inserts into protoporphyrin IX and produces Mg-protoporphyrin IX [31]. In addition, another key enzyme of chlorophyll biosynthesis magnesium protoporphyrin IX methyltransferase (spot 26) also exhibits up-regulated in the oeCIPK14-var [35], it suggests that plants may increase the accumulation of chlorophyll and transport to the two PSs for the synthesis of LHC complex, then maintain the efficiency of electron transfer and alleviate the stress of excessive energy. Thylakoid lumenal 29 kDa protein (TL29, spot 54) is targeted in the thylakoid lumen of chloroplasts [36], which was identified as a homology of ascorbate peroxidase associated with PS II [37], it shows more abundance in the why1/3-var and oeCIPK14-var.

3.2 Protein involved in Calvin cycle
Several essential proteins of the Calvin cycle are down-regulated expression in the \textit{why1/3-var} and \textit{oeCIPK14-var}, including ribulose bisphosphate carboxylase large chain (RbcL, spot 14, 23, 27, 40), ribulose bisphosphate carboxylase small chain (RbcS, spot 17, 18, 43, 45), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase (RCA, spot 2), Phosphoglycerate kinase (PGK, spot 5). It is well known that Rubisco, a complex of eight RbcL and eight RbcS subunits containing eight catalytic sites, is the most abundant protein in the earth, utilized by autotrophic organisms to transform CO2 into organic compounds via the Calvin-Benson cycle [38]. Rubisco catalyzes the rate-limiting step of photosynthetic carbon reduction for CO2 assimilation [38]. In this study, except for spot 32, all the other RbcL and RbcS are decreased. The activity of Rubisco is inhibited by RCA, which it can release the ribulose-1, 5-bisphosphate (RuBP) from the active sites of Rubisco by using the energy from ATP hydrolysis by increasing ratios of ADP to ATP in \textit{Arabidopsis} [39], so that CO2 can activate the enzyme [39], indicating that the redox states of photosynthetic electron transport chain are changed, and the production of ATP is inhibited. PGK can catalyze the conversion of 3-phosphoglycerate to 1, 3-bisphosphoglycerate which is a substrate for the synthesis of glyceraldehyde-3-phosphate (G3P) in Calvin-Benson cycle, G3P serves as a substrate for the synthesis of other carbohydrates [40]. Carbonic Anhydrase 1(CA, spot 59) is located in the chloroplast and catalyzes the interconversion of H2O and CO2 into HCO3 which involves in CO2-dependent stomatal closing [41]. The expression of CA is also decreased in the \textit{why1/3-var} and \textit{oeCIPK14-var} lines, implying that the photosynthetic efficiency is changed.

3.3 Protein associated with defense and antioxidation

The results of photosynthetic performance analysis suggest that \textit{why1/3-var} and \textit{oeCIPK14-var} lines show lower photosynthetic electron transport efficiencies. However, several defense and antioxidation related proteins (spot 9, 10, 16, 25, 37, 38, 54, and 61) are up-regulated in the \textit{why1/3-var} and \textit{oeCIPK14-var} for ROS scavenging. Among them, thiamine thiazole synthase (THI, spot 9) and Pyridoxal 5-phosphate synthase subunit PDX1.1 (PDX, spot 37) are reported that they up-regulate expression under oxidative stress [42]. THI (spot 9) is involved in biosynthesis of the thiamine (vitamin B1) precursor thiazole [43]; The PDX (spot 37) catalyzes the formation of pyridoxal 5'-phosphate that is phosphorylated derivatives of VB6, which can act as a coenzyme for numerous metabolic enzymes and has been identified as a potent antioxidant [44, 45]. The identified protein SAPX (spot 10) and L-ascorbate peroxidase APX1 (spot 10 and spot 25) have been proposed to reduce the generation of ROS and enhance the tolerance in oxidative stress [46, 47]. Additional proteins: the thioredoxin-like protein CDSP32 (spot 38) has been reported as a thioredoxin and is involved in plastid responses to oxidative stress [48]; the enzyme peptide methionine sulfoxide reductase (PMSR) (spot 61) can catalyze the reduction of Met sulfoxides back to Met [49], it has been shown to repair oxidative damaged proteins in chloroplasts [48]; the IPP1 (spot 57) is an essential component for thylakoid biogenesis in chloroplasts [50], which it is up-regulated expression for the membrane maintenance, when the envelope membrane integrity of chloroplast was disturbed[51]. Two members of glutathione S-transferase (GST) family proteins like glutathione S-transferase F6 (spot 20, GSTF6) and Glutathione S-transferase F7 (spot 60, GSTF7) are identified in this study. GSTF6 (spot 20) is up-regulated in the \textit{why1/3-var}, but down-regulated in the \textit{oeCIPK14-var}. GST F7 is decreased in the \textit{why1/3-var}. Taken together, it indicates that WHY1/WHY3 or CIPK14 both are involved in ROS balance case. The \textit{why1/3} or \textit{oeCIPK14} affecting the excessive excitation energy may trigger the accumulation of ROS in chloroplasts;

3.4 Proteins related to protein metabolism

Intriguingly, in the present study, several identified protein spots are involved in protein metabolism. Among them, five spots (spot 11, 19, 29, 30 and 39) show an increased abundance in the \textit{why1/3-var} and \textit{oeCIPK14-var}. For example, the ATP-dependent Clp protease is one of plastid protease that plays an essential role in chloroplast development and maintains [52, 53]. ATP-dependent Clp protease proteolytic subunit-related protein 3 (spot 11) is up-regulated in the \textit{why1/3-var} and \textit{oeCIPK14-var} indicating chloroplast dysfunction. In general, selective proteolysis in plants is largely
mediated by the ubiquitin (Ub)/26S proteasome system [54], the abnormal polypeptides are marked
by the covalent attachment of Ub, are degraded by the 26S proteasome [55], the 26S proteasome is
composed of two subparticles, the 20S core protease and the 18S regulatory particle [55], 20S core
protease subunit beta type-2-B (spot 29) and proteasome subunit alpha type-6-B (spot 39) [55] and
protease subunit alpha type-6-A (spot 13) all show down-regulated in the why1/3-var and oeCIPK14-
var. Ribosome-recycling factor (spot 30, RRF) located in the chloroplast [56], increases in the
oeCIPK14-var lines, which is essential for embryogenesis and chloroplast biogenesis, and supposed to
be involved in translation of chloroplast proteins [56]; The chaperonin CNP10 (spot 19) also is shown
to be localized to the chloroplasts [57], and up-regulated in the oeCIPK14-var, which is essential
proteins involved in cellular protein folding [57]. In addition, our data show that RNA-binding
protein CP29B (spot 8) up-regulates in the oeCIPK14-var, which is a kind of a chloroplast RNA binding
proteins and may involve in processing of chloroplast RNA [58]. It demonstrates that CIPK14
mediating plastid WHY1/WHY3 protein might be involved in plastid protein metabolism including
protein translation, synthesis, and proteolysis (Figure 7).

Although the transcription levels of the encoding genes of these proteins are not fully confirmed
their altering trend, it can be explained that in one hand the discrepancy between mRNA and protein
levels may be caused by the different half-lives of protein and mRNA, or post-transcriptional or post-
translational process and modification. In fact, several identified protein spots are involved in protein
metabolism, post-transcriptional or post-translational process and modification. In other hand, it
seems that the alteration of the transcriptional levels in the why1/3-var and oeCIPK14-var could not
rescue in the oeWHY1 or cipk14 mutants. It has been reported that in maize, why1 plants with pale
yellowing phenotype was supposed to be related to impairment of RNA processing [19, 20], however
in Arabidopsis only double mutated why1/3 showed pale yellowing phenotype [25]. It hints that WHY1
protein in monocotyledon plant evolutionary shares functions of both WHY1 and WHY3 in
dicotyledonous Arabidopsis. It will be addressed by genetically rescued experiment in future. It is not
such unexpected that cipk14 plant cannot be rescued the leaf pale yellowing phenotype of oeCIPK14-
var and the gene expression pattern of oeCIPK14-var. Actually, CIPK14 mainly acts as a kinase,
phosphorylates WHY1 and pushes WHY1 enter in the nucleus, mostly showing staygreen
phenotype[28], and only 5% plants showing pale yellowing. The leaf pale yellowing of oeCIPK14-var
resulted from WHY1/WHY3 deficiency can be rescued by overexpressing plastid isoform WHY1 [28].
In fact, in this study, five proteins (ClpR3, RBL, RBS, RRF and BAM3) show an alternative abundance
in the why1/3-var and oeCIPK14-var, interestingly, their mutants all have been reported showing
similar leaf yellowing and small rosette phenotypes [53, 56, 59]. Their speculating mechanism of
action connecting with CIPK14 and WHIRLY protein mediated leaf pale yellowing in protein
metabolism will be detailed addressed in coming future.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Plants of Arabidopsis thaliana Columbia ecotype and the mutants are cultivated in the vermiculite
matrix after vernalization. Plants are grown in a controlled climatic chamber at 13/11 h light/dark
cycle with a periodic temperature 22 °C/18 °C, a light intensity of 60 μmol·m−2·s−1, and a relative
humidity of 65%. The rosette leaves of the plants are collected at 4 weeks after germination for each
genotype. The samples are frozen in liquid nitrogen prior and stored at -80°C before used. Three
biological replicates are used for each experiment.

T-DNA insertion lines SALK_023713 (why1) for WHY1 and SALK_009699 (cipk14) for CIPK14
are provided by NASC. Seeds of WHY1 and WHY3 double mutant (why1/3) are kindly provided by
Prof. Normand Brisson, Department of Biochemistry, Montreal University, Montreal, Canada.
Overexpression of WHY1 (oeWHY1) and CIPK14 (oeCIPK14) lines are prepared from the former work
[28].

4.2. Chlorophyll Content Measurement and Photosynthetic Parameters Analysis
Colored threads are used for rosette leaves labelling after their emergence as previously described [8]. The weight of leaf 5 from 12 independent 4-week-old plants are measured, and each leaf with one milliliter 95% ethanol (v/v) in the 1.5 milliliter (mL) Eppendorf tube. Pigments are extracted after incubating 48 hours in dark. Absorbance of extracts is measured at 470, 649, and 665 nm by the Flexstation 3 Microplate Reader (Molecular Devices, USA), and the total chlorophyll content is determined according the method as described [60].

Chlorophyll fluorescence is measured and chlorophyll fluorescence image is taken using an Imaging-PAM-Maxi (Walz, Germany) as described by Shao [60]. The leaf 5 from 4-week-old plants are selected for measurement after 30 minutes adaptation of darkness, the minimal fluorescence yield (Fo) is measured at a low light intensity and the maximal fluorescence yield (Fm) is measured under saturation pulse, then all of leaves are exposed to a light intensity of 54 μmol·m⁻²·s⁻¹ photosynthetically active radiation (PAR), the kinetic curves are gained according the instruction of the instrument. The maximum photochemical efficiency of photosystem II (Fv/Fm), the nonphotochemical quenching of photosystem II fluorescence (NPQ), the electron transport rate (ETR) are calculated by the control software. The chlorophyll fluorescence images of whole plant are taken after 30 minutes adaptation of darkness. Through one saturation pulse, the values of Fo and Fv/Fm are calculated and formed fluorescence image by Imaging-PAM-Maxi (Walz, Germany).

4.3. Protein Extraction and 2-DE Analysis

The total proteins of rosette leaf are extracted by the phenol method [62]. In brief, approximately 3 g material is ground with liquid nitrogen, then is suspended in 12 mL ice-cold extraction buffer contains 50 mM PBS (PH 7.8), 5mM EDTA, 2% (v/v) β-mercaptoethanol, 0.5% (v/v) NP-40, 1 mM Phenylmethane-sulfonyl fluoride (PMSF), 1% (w/v) polyvinylpolypyrrolidione (PVPP) on ice and vortexed for 5 min. An equal volume of ice-cold Tris-saturation phenol (pH 8.0) adds to suspension and vortexes for 10 min. The phenol phase is collected after centrifugation (4 °C, 15 min, 16000 g), and proteins are precipitated over night with five volumes 0.1 M methanol/ammonium acetate at -20 °C. The protein pellets are collected after centrifugation (4 °C, 15 min, 16000 g) and rinsed three times in ice-cold acetone/13mM dithiothreitol (DTT). Between each wash, the proteins are incubated for 1 hour at -20°C. After centrifugation (4 °C, 15 min, 16000 g), the supernatant is discarded carefully and the protein pellets are air-dried.

The protein pellets are dissolved in sample lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 2% IPG Buffer pH 3-10 NL), and protein concentrations are determined by Bradford method [63]. A total of 1.5 milligram (mg) protein are loaded onto an IPG Strip (24 cm, 3-10 NL) and rehydrated 12 h at 25 °C. Ettan IPGphor system (GE Healthcare, American) is employed for Isoelectric focusing (IEF) in a program manner: 30 V (0.5 h), 100 V (1 h), 200 V (1 h), 500 V (3 h), 1 kV (1 h), 10 kV (2 h, gradient) and finally 10 kV up to 60000 Vhs. Then the strips are equilibrated for 15 min in equilibration buffer I contains 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% DTT, and in equilibration buffer II contains 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2.5% iodoacetamide for 15 min with gentle shaking. After equilibration, the strips are transferred onto 12.5% SDS-PAGE and performed with an Ettan DALT-six System (GE Healthcare, American) at 10 mA per gel for 1 h and then 15 mA per gel overnight. After electrophoresis, the 2-DE gels are stained by the Coomassie Brilliant Blue R250 (CBB R250). Three independent biological replicates are used for each genotype.

4.4. Data analysis in-gel protein digestion and protein identification

Gels are scanned at 600 dpi resolution with a scanner (EPSON Expression 11000XT, Japan), and the images are quantitative analyzed by ImageMaster™ 2D Platinum 7.0 software (GE Healthcare, American). The differential protein spots are in-gel digestion and identification according to the methods as described. In shortly, the protein spots are excised from the CCB R250 stained gels and subjected to in-gel trypsin digestion for 12 h at 37 °C. Peptides are extracted twice within 50% ACN/0.1% TFA, then the extracts dried completely by a vacuum centrifuge. Peptide mixtures are dissolved in 0.1% TFA, 0.8 μl of peptide solution is mixed with 0.4 μl of matrixa-cyano-4-
hydroxycinnamic acid in 30% ACN/0.1% TFA before spotting on the target plate. An AB SCIEX MALDI TOF-TOFTM 5800 Analyzer (AB SCIEX, Foster City, CA) equipped with a neodymium: yttrium-aluminum-garnet laser (laser wavelength was 349 nm) is employed for acquiring peptide mass fingerprint (PMF). All automatic data analysis and database searching are conducted using GPS Explorer TM software (version 3.6, AB SCIEX) running a mascot search algorithm (v2.3, Matrix Science, London, UK) for protein identification. Proteins with protein score confidence intervals (C.I.) above 95% (protein score >61) are considered confident identifications. The identified proteins are then matched to specific processes or functions by searching Uniprot.

4.5. RNA extraction, reverse transcription and qRT-PCR analysis

Total RNA is extracted from the 4-week-old rosette leaves of Arabidopsis using the Trizol Reagents (TransZol up, TRANSGEN BIOTECH, China) according to the protocol of manufacturer. First-strand cDNA is synthesized using the cDNA Synthesis SuperMix with gDNA Removal (TRANSGEN BIOTECH, China) according to the manufacturer’s instructions. Specific oligo nucleotide primers are designed by http://quantprime.mpimp-golm.mpg.de, and synthesized by Sangon Biotech (Shanghai, China). Quantitative real-time PCR using the Ultra SYBR Mixture (CWBIO, China) according to the manufacturer’s instructions, and is performed by a Lightcycler ® 96(Roche, Switzerland). The GAPC2 is used as an internal standard and relative gene expression are analyzed using 2−ΔΔC(T) Method. The primer pairs are shown in supplemental table S1.

4.6. Immunological Analyses

Proteins are extracted from rosette leaves according to the phenol method [62]. Equal amounts of proteins are determined by Bradford method [63]. Proteins are separated on 12.5% polyacrylamide gels [65], transferred to PVDF membranes by semi-dry electroblotting, and immunodetected according the protocols as described [24]. Primary antibodies directed toward the CYTF, PSBP, PSBR and LHCA1 were purchased from Agrisera (Sweden).

5. Conclusions

We concluded that in the why1/3-var or oeCIPK14-var lines most of proteins are involved in photosynthesis, amino acids and protein metabolism, or defense and antioxidation (Figure 7). They are related to photosynthesis and insufficiencies in energy supply. The co-regulation of CIPK14 phosphorylation mediated WHIRLY1/WHIRLY3 deficiency in plastids are speculated that they might be controlled by mechanism of amino acids and plastid protein metabolism at the posttranscriptional level. The detail mechanism will be addressed in future.
Figure 7. Summary of CIPK14 or/and WHIRLY1/3 mediated pathways in chloroplasts, including light reaction, chlorophyll metabolism, ROS scavenging, malate biosynthesis, Calvin cycle, and protein metabolism.

Abbreviations: ADP, adenosine diphosphate; ADT, adenosine diphosphate 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid; APX1, L-ascorbate peroxidase 1; ATP, adenosine triphosphate; ATPA, ATP synthase alpha subunit; BAM3, beta-amylase 3; BPG, 1,3-diphosphoglycerate; CA, carbonic anhydrase; ChlI-2, magnesium-chelatase subunit ChlI-2; CHLM, magnesium protoporphyrin IX methyltransferase; CLPR3, ATP-dependent Clp protease proteolytic subunit-related protein 3; CNP10, 10 kDa chaperonin; CP26, chlorophyll a-b binding protein CP26; CP29B, RNA-binding protein CP29B; Cyt b6f, cytochrome b6f; DHAR, dehydroascorbate reductase; FA, fumarate; Fd, ferredoxin; FDH, formate dehydrogenase; FNR, ferredoxin-NADP (H) oxidoreductase; G3P, glyceraldehydes-3-phosphate; GSH, reduced glutathione; GSSH, oxidized glutathione; GST, glutathione S-transferase; HCF101, Fe-S cluster assembly factor HCF101; Mal, malate; MD, malate dehydrogenase; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; Met, methionine; MetSO, methionine sulfoxide; MSRA4, peptide methionine sulfoxide reductase A4; NADP+/NADPH, nicotinamide adenine dinucleotide; NAD+/NADH, nicotinamide adenine dinucleotide; OAA, oxaloacetic acid; PC, plastocyanin; PDX1.1, pyridoxal 5’-phosphate synthase subunit PDX1.1; PGA, 3-phosphoglycerate; PGK, phosphoglycerate kinase; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; PSA6B, proteasome subunit alpha type-6-B; PSB2B, proteasome subunit beta type-2-B; PsbP, PsbP domain-containing protein 4; RCA, ribulose-1,5-bisphosphate
carboxylase/oxygenase activase; RRF, ribosome-recycling factor; Ru5P, ribulose-5-phosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; THI, thiamine thiazole synthase; TROL, thylakoid rhodanese-like protein; SBP, sedoheptulose-1,7-bisphosphate; SBPase, sedoheptulose-1,7-bisphosphatase; SAPX, stromal ascorbate peroxidase; VB6, vitamin B6; VIPP1, vesicle-inducing protein in plastids.

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Author’s contribution: Y.M. designed the research; Z.G., W.W. and X.Y. performed all experiments. W.L. helped to analyze the data; Z.G and Y.M. analyzed data and wrote the paper.

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Supplemental Table S1 The list of primer pair sequence for qRT-PCR

| TAIR number | Gene name | Primer sequences (5’ to 3’) |
|-------------|-----------|-----------------------------|
| At1g14410   | WHIRLY1   | FP: TTTTACGTGGTGTCATTCGAT  |
|             |           | RP: GTCCACTGTTAAGCAGCTTT    |
|             |           | FP: ACCTTAGAATCCGACGACG     |
| At2g02740   | WHIRLY3   | FP: ATCGAATGACCCAAGTAAAAA   |
|             |           | RP: TCGACGAATCCGACGACG      |
|             |           | FP: TACACACGGACACGACG       |
| AT5G01820   | CIPK14    | RP: TCGTCGAGCCTGGTTGAAACC   |
|             |           | FP: ACCACTGTCACCTCAACACTG   |
|             |           | RP: TACAGGATGAGCAACACTTCCC  |
| AT1g13440   | GAPC2     | FP: ACCTTTGAGCGGTAGGGAAACGC|
|             |           | RP: TGCCGAATTGGCTCCACCCC    |
| AT3G24430   | spot 1    | FP: AGACCGTATCCGTTGTCGCAAG |
|             |           | RP: CCCTCAAAAGCAGGAAAGATCG |
| AT2G39730   | spot 2    | FP: TTCTTCTCCTGGCTAGTAGTG   |
|             |           | RP: GGCCGTCTCAGCTCCACAAAG   |
| ATCG00120   | spot 3    | FP: TGGTGCTGAGCTGGACGTTG    |
|             |           | RP: AGCGCTCTAGCTGTTCCTT    |
| AT5G45930   | spot 4    | FP: CGAGCTTCTTTGGTATTAGTCAG|
|             |           | RP: GGTAGAAGGCCACCAAGCTTTCC|
| AT3G12780   | spot 5    | FP: TCACAAATCTCCGAAATCACGA|
|             |           | RP: ACCATTTCTCCCCGCTGTGTC  |
| AT3G55800   | spot 6    | FP: AGCTAGCCGACGACAGCAGCC  |
|             |           | RP: TGGGAAAGGTATCGCCTAGC    |
| AT3G57260   | spot 7    | FP: GCTCAAGCTCTAGGTATTAGTC |
|             |           | RP: ACCATTTCTCGGGTGTTG      |
| AT2G37220   | spot 8    | FP: TTCTTCCTCGCAGATTGGTCG  |
|             |           | RP: AAACCAATCACCCTCCGCTG   |
| AT5G54770   | spot 9    | FP: TCTCAGGCGAGTGGTCTTG    |
|             |           | RP: ACCACTTTCTGGTTG         |
| AT4G08390   | spot 10   | FP: TATGATGCACGAGCAAGAGCG  |
|             |           | RP: ACATCACCGCCGGAATACCC   |
| AT1G09130   | spot 11   | FP: GCCGTAGTTGCTAGGGTGGTG  |
|             |           | RP: AGCTTGCTCTCAGGAAATCCCG |
| AT4G10340   | spot 12   | FP: AGCTGGTCTCTCAGGGTTTG   |
|             |           | RP: TCTCAGGCAAGCAACTCTCCG  |
| At5g35590   | spot 13   | FP: GGTCTGAGATGACCGCATC    |
|             |           | RP: TCTGCGAAAGCACCACCTCG   |
| AT1G64510   | spot 15   | FP: GGACCAAAAGGGTGAGCTGGAA |
|             |           | RP: CATCGGTCCCAATCGACCT    |
| AT2G44650   | spot 19   | FP: TCTCAGCCTCTGGGAGCTGG  |
|             |           | RP: CATCGGTCCCAATCGACCT    |
| TAIR number | Gene name | Primer sequences (5’ to 3’) |
|-------------|-----------|-----------------------------|
| AT1G02930   | spot 20   | FP: AAGAGCCTTTGATCTTTCGCAACC |
|             |           | RP: TGCTCCTGGAGTTGAGAAGGG    |
| AT4G17090   | spot 21   | FP: AAAGCAGGTCTCAAACCTCAG    |
|             |           | RP: ACTGCAAGAGTCTCTACGTTCC   |
| AT4G13930   | spot 22   | FP: AAAGCCAATGCTGGCCCTTTG    |
|             |           | RP: CAGAGCTCTCAACCTTGTTCG    |
| AT4G11150   | spot 24   | FP: TGGCGTTGATGTGGCATTCC     |
|             |           | RP: GCGAGAACACACCTTGATCAC    |
| AT1G07890   | spot 25   | FP: TGGTCAACACCTTGAGTACATC   |
|             |           | RP: AGGAGCAATGTCTTGACCTCC    |
| AT4G25080   | spot 26   | FP: ATGGTAGTTGTCCTTTGCCTTC   |
| AT1G77090   | spot 28   | FP: ACCAAGATGCAGGATCAGACAG   |
| AT1G81990   | spot 29   | FP: CAGAGCTGTTGACAGTAGGCTAC  |
| AT4G14800   | spot 30   | FP: ACCCGGATTGCAAGTTGAGGTC   |
| AT3G63190   | spot 31   | FP: AGACGTGTGCTACAGGAGCTTC   |
| AT4G38740   | spot 32   | FP: AGGGCCAGCTGCAAGTCTTGAGG  |
| AT4G05180   | spot 33   | FP: ACTTGTAACACCCCTGCTTGC    |
| AT1G79040   | spot 34   | FP: ACCAAGACACACATGGACTTGGAG |
| AT4G01050   | spot 35   | FP: ATGGAGGCCTAAGTCAGGCTAAGC|
| AT3G47520   | spot 36   | FP: ACCCTGCAACTGCTTTCGACTC  |
| AT2G38230   | spot 37   | FP: TCGGCGTTGACACCTTGACG    |
| AT1G76080   | spot 38   | FP: TTCTGATGGGAGGCTACGAGG   |
| AT2G05840   | spot 39   | FP: TTCTGATGGGAGGCTACGAGG    |
| AT2G21660   | spot 40   | FP: TCGGCGTTGACACCTTGACG    |
| AT3G01390   | spot 41   | FP: TCGGCGTTGACACCTTGACG    |

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| TAIR number | Gene name | Primer sequences (5’ to 3’) |
|-------------|-----------|----------------------------|
| AT5G11670   | spot 47   | FP:GGACCTCATTTGCTGCTTAATGC |
|             |           | RP:CCACACCAGATGTCCCAATGAG  |
| AT5G65010   | spot 48   | FP:TGTCTTCCCCGATAACACACCTC|
|             |           | RP:AGTCGCTCTAGCAGCACTCTTC  |
| AT3G47930   | spot 49   | FP:GGCCAACCTAAGGACAAACAAAG|
|             |           | RP:CCTGGACCCTATACTTCACAATGC|
| AT1G23310   | spot 50   | FP:TTACCAACGGAGCTCTCAAG    |
|             |           | RP:GTGTCTCTCAGATGGAACACACC|
| AT5G14780   | spot 51   | FP:AAGGAGGGCCTGTGATTGCAAC  |
|             |           | RP:ACGTTGCTTTCTCATGAGTATC  |
| AT2G06050   | spot 52   | FP:TAAGTACGCGTGGGAAAACACCC|
| AT1G29670   | spot 53   | FP:AGAAGAAACCCGTCGACCAATTTGGG|
|             |           | RP:GCTGCACAACCTTGCGATCGTCTG|
| AT4G09010   | spot 54   | FP:GCAATGTTCTCGTCTGCACTCC  |
|             |           | RP:TCTGGTTCCGGCCGATAGAGTG  |
| AT2G45820   | spot 55   | FP:TTCCAAGCATGCACATCACAGAG|
| AT2G16600   | spot 56   | FP:ACATGACCGTCCG TGCCAATTC|
|             |           | RP:TTCTCGGCGGTTTCTGTTGGA  |
| AT1G65260   | spot 57   | FP:TCTTGCAAGTGGAGGACCCCTAAGAC|
|             |           | RP:TTCAAAGCAGTAGGTGTCCTGAGC|
| AT3G57560   | spot 58   | FP:AATCAGTTGGAGAGGAGCTTGCG|
|             |           | RP:TTCCAGGCCATCGTACAGCCAG  |
| AT3G01500   | spot 59   | FP:AGATGCTTCCGTTGTCGTAAC  |
|             |           | RP:CAACGCCACCGATTTGACCCTTG|
| AT1G02920   | spot 60   | FP:AGCCTTCTCATCTCTCGCAACCC|
|             |           | RP:TTGGAGCGAAGGGAGACAAAGTTGG|
| AT4G25130   | spot 61   | FP:ACTGGCCTACGGGACAATAAC  |
|             |           | RP:ACCCGGAGCTTCAAGGTTGTTG  |
| AT1G73230   | spot 62   | FP:ATCAAGCGGCTTCCGATCCAGAAC|
|             |           | RP:ACCTGGAGCTTGTCTGGAATTG  |

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