Na$_2$CO$_3$-Responsive Photosynthetic and ROS Scavenging Mechanisms in Chloroplasts of Alkaligrass Revealed by Phosphoproteomics

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Abstract

Alkali-salinity exerts severe osmotic, ionic and high-pH stresses to plants. To understand the alkali-salinity responsive mechanisms underlying photosynthetic modulation and reactive oxygen species (ROS) homeostasis, physiological and diverse quantitative proteomics analyses of alkaligrass (*Puccinellia tenuiflora*) under Na$_2$CO$_3$ stress were conducted. In addition, Western blot, real-time PCR, and transgenic techniques were applied to validate the proteomic results and test the functions of the Na$_2$CO$_3$-responsive proteins. A total of 104 and 102 Na$_2$CO$_3$-responsive proteins were identified in leaves and chloroplasts, respectively. In addition, 84 Na$_2$CO$_3$-responsive phosphoproteins were identified, including 56 new phosphorylation sites in 56 phosphoproteins from chloroplasts, which are crucial for the regulation of photosynthesis, ion transport, signal transduction and energy homeostasis. A full-length *PtFBA* encoding an alkaligrass chloroplastic fructose-bisphosphate aldolase (FBA) was overexpressed in wild-type cells of cyanobacterium *Synechocystis* sp. Strain PCC 6803, leading to enhanced Na$_2$CO$_3$ tolerance. All these results indicate that thermal dissipation, state transition, cyclic electron transport, photorespiration, repair of photosystem (PS) II, PSI activity, and ROS homeostasis were altered in response to Na$_2$CO$_3$ stress, and they have improved our understanding of the Na$_2$CO$_3$-responsive mechanisms in halophytes.

KEYWORDS: Chloroplasts; Na$_2$CO$_3$ stress; ROS scavenging; phosphoproteomics; *Puccinellia tenuiflora*

Introduction

Soil salinization and alkalization frequently occur simultaneously. In northeast China, more than 70% of the land area has become alkaline grassland [1]. Alkali-salinity is one of the most severe abiotic stresses, limiting the productivity and geographical distribution of plants. Saline-alkali stress exerts osmotic stress and ion damage, as well as high-pH stress to plants [2]. However, little attention has been given to the sophisticated tolerance mechanisms underlying plant response to saline-alkali (e.g. Na$_2$CO$_3$ and NaHCO$_3$) stresses [3,4]. As the organelle for photosynthesis, chloroplasts are extremely susceptible to saline-alkali stress [5]. Excessive accumulation of Na$^+$ reduces the CO$_2$ diffusion through stomata and mesophyll, negatively affecting plant photosynthesis [6]. As a consequence, excessive excitation energy causes generation of reactive oxygen species (ROS), resulting in damage to the thylakoid membrane [6].
Current high-throughput proteomic approaches are powerful to untangle the complicated mechanisms of chloroplast development, metabolism and stress response [7–10]. More than 522 NaCl-responsive chloroplast proteins were found in different plant species, such as tomato (Solanum lycopersicum) [11], wheat (Triticum aestivum) [12], and other plant species [13–18]. The presence of these proteins indicate that the light harvesting, photosynthetic electron transfer, carbon assimilation, ROS homeostasis, energy metabolism, signaling, and membrane trafficking were modulated in chloroplasts in response to NaCl stress. However, only about 53 salinity-responsive genes encoding chloroplast proteins have been characterized [5], which are insufficient to address the sophisticated salinity-responsive networks in chloroplasts. Additionally, NaCl stress altered phosphorylation levels of several chloroplast proteins in Arabidopsis [19,20], Brachypodium distachyon [21] and sugar beet (Beta vulgaris) [22], implying that state transition, PSII damage repair, thermal dissipation, and thylakoid membrane organization were crucial for plant acclimation to salt stress [23]. However, the critical roles of reversible protein phosphorylation in salinity-/alkali-responsive metabolic networks are virtually unknown.

Alkaligrass (Puccinellia tenuiflora) is a monocotyledonous halophyte species belonging to the Gramineae, and is widely distributed in the Songnen Plain in Northeastern China. It has strong ability to survive in extreme saline-alkali soil (pH range of 9-10). Several salinity-/alkali-responsive genes and/or proteins in leaves and roots of alkaligrass have been reported [24–26]. A previous transcriptomic study also revealed that a number of Na₂CO₃ responsive genes were overrepresented in metabolism, signal transduction, transcription, and cell rescue [27]. Despite this progress, the precise alkali-responsive mechanisms in chloroplasts are still poorly understood. Analyses of the photosynthetic and ROS scavenging mechanisms in chloroplasts regulated by the reversible protein phosphorylation and the expression of nuclear and chloroplast genes are critical for understanding the Na₂CO₃-responsive mechanisms in alkaligrass. In this study, we investigated the alkali-responsive characteristics in chloroplasts and leaves of alkaligrass. By integrative analyses of protein phosphorylation, patterns of protein abundance, gene expression, photosynthesis parameters, antioxidant enzyme activities, and chloroplast ultrastructure, we revealed several important Na₂CO₃-responsive strategies in the halophyte alkaligrass. These results have yielded important insights into the alkali-responsive mechanisms in halophytes.
Results

**Na₂CO₃ treatment decreased seedling growth and biomass**

Na₂CO₃ treatment clearly affected the morphology and biomass of alkaligrass seedlings. The leaves withered with the increase of Na₂CO₃ concentration and treatment time (Figure S1). The shoot length and relative water content decreased significantly at 200 mM Na₂CO₃ of 24 h after treatment (24 HAT200) (Figure 1A). The fresh and dry weights of leaves also clearly decreased under 200 mM Na₂CO₃ (Figure 1B).

**Na₂CO₃ treatment changed ionic and osmotic homeostasis, cell membrane integrity, and abscisic acid (ABA) level**

Na₂CO₃ treatment perturbed the ion and pH homeostasis in leaves. Na⁺ in leaves was gradually accumulated, but K⁺ content did not show obvious changes, resulting in the sharp decline of the K⁺/Na⁺ ratio (Figure 1C, D). In addition, the Mg²⁺ and Ca²⁺ contents gradually decreased under the Na₂CO₃ treatment (Figure 1E). Malondialdehyde content and relative electrolyte leakage significantly increased under different Na₂CO₃ treatments, indicating that the membrane integrity was affected by Na₂CO₃ treatment (Figure 1F). In addition, proline and glycine betaine gradually accumulated with the increase of Na₂CO₃ concentrations (Figure 1G), while the soluble sugar content only showed marked accumulation at 200 mM Na₂CO₃ (Figure 1H). The endogenous ABA content in leaves increased significantly (Figure 1H).

**Photosynthesis and chlorophyll (Chl) content decreased under Na₂CO₃**

In seedlings, net photosynthetic rate, stomatal conductance, and transpiration rate (Figure 2A, B) gradually decreased, while the intercellular CO₂ concentration did not exhibit obvious changes under the Na₂CO₃ (Figure 2B).

To evaluate the photosynthetic performance, we investigated the changes of Chl fluorescence and the polyphasic fluorescence transients (OJIP). The maximum quantum efficiency of PSII photochemistry and PSII maximum efficiency significantly decreased at 24 HAT (Figure 2C), and the actual PSII efficiency and electron transport rate were declined remarkably at 200 mM Na₂CO₃ (Figure 2D). In addition, the non-photochemical quenching did not change and the fraction of open PSII centers significantly decreased at 24 HAT200 (Figure 2E). The fluorescence transient gradually decreased, reaching the lowest level at 24 HAT200 (Figure 2F). After normalization, the relative fluorescence intensities of Vₐ and Vₚ,
two specific indicators of thylakoid dissociation and oxygen-evolving complex (OEC) damage increased at 24 HAT (Figure 2G). \( V_I \) and \( V_b \), however, obviously increased. The relative variable fluorescence intensity of \( V_I \) and \( V_b \) can be considered as a measurement of the accumulation of \( Q_A^\ominus \) and the proportion of the \( Q_B \)-non-reducing reaction center. This suggests that the accumulation of \( Q_A^\ominus \) and increased proportion of \( Q_B \)-non-reducing reaction center in the \( \text{Na}_2\text{CO}_3 \)-stressed leaves (Figure 2H). In addition, the contents of total Chl and Chl \( a \) decreased at 24 HAT (Figure 2I), and the ratio of Chl \( a/b \) also decreased under the \( \text{Na}_2\text{CO}_3 \) treatment (Figure 2J).

**Na\(_2\)CO\(_3\) treatment affected chloroplast ultrastructure**

\( \text{Na}_2\text{CO}_3 \) treatment changed the chloroplast ultrastructure in mesophyll cells and bundle sheath cells from lateral veins, minor veins and midveins (Figure 3). Under normal conditions, chloroplasts in mesophyll cells and bundle sheath cells exhibited long ellipsoidal or shuttle-shaped, double membrane compartment, with only a few osmophilia plastoglobules in the stroma (Figure 3A, F, K, and P). Thylakoids were dispersed in the chloroplasts, and the fully developed thylakoid membrane systems were well organized in grana and stromal lamellae (Figure 3A, F, K, and P). At 12 HAT, slight swelling of the chloroplast stroma occurred, and the membranes of the individual thylakoid fused, eliminating the intraspace (Figure 3C, H, and M). While at 24 HAT, chloroplast volume increased obviously to become round-shaped. The thylakoid membrane systems in various cells became distorted and incomplete, showing a dilated intraspace (Figure 3D, E, J, and O). The size and number of grana somewhat decreased, and some grana completely disappeared (Figure 3D, E, and J). At 24 HAT, numerous plastoglobules were observed in chloroplasts, and the size and number of plastoglobules appeared to be \( \text{Na}_2\text{CO}_3 \) concentration-dependent (Figure 3). This implies that lipid peroxidation-mediated destruction of the thylakoid membranes takes place in chloroplasts. In addition, the aforementioned changes in thylakoids appeared more drastic in mesophyll cells than in the bundle sheath cells (Figure 3D, E).

**Na\(_2\)CO\(_3\) treatment changed antioxidant enzymes in leaves and isolated chloroplasts**

To evaluate the level of oxidative stress in leaves and chloroplasts under \( \text{Na}_2\text{CO}_3 \) treatment, the \( O_2^- \) generation rate, \( \text{H}_2\text{O}_2 \) content and four metabolites (i.e. ascorbate (AsA), dehydroascorbate (DHA), glutathione (GSH), and oxidized glutathione (GSSG)), and the activities of nine antioxidant enzymes in ROS scavenging system were monitored (Figure 4).

In leaves, the \( O_2^- \) generation rate and \( \text{H}_2\text{O}_2 \) content increased under the \( \text{Na}_2\text{CO}_3 \) treatment.
The contents of several metabolites (e.g., reduced AsA, DHA, and GSSG) did not change, but GSH increased at 24 HAT (Figure 4B, C). Importantly, the activities of superoxide dismutase (SOD), catalase (CAT), and dehydroascorbate reductase (DHAR) decreased at 24 HAT, and CAT activity was inhibited at 12 HAT (Figure 4D, E and F). The activities of peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione S-transferase (GST) showed increased patterns under the Na₂CO₃ treatment (Figure 4D, E, F, G and H). These results indicate that the superoxide dismutation by SOD and reduction of H₂O₂ to H₂O by CAT decreased, but the APX/POD pathway, AsA-GSH cycle, and GPX pathway were enhanced to cope with the Na₂CO₃-induced oxidative stress.

We isolated chloroplasts with high purity for activity and proteomics analyses (Figure S2A, B). In isolated chloroplasts, the AsA content decreased, but the DHA content increased under Na₂CO₃. The contents of GSH and GSSG stayed at relative stable levels at 24 HAT (Figure 4I, J). The activities of SOD, POD, APX, and MDHAR increased at 24 HAT, and APX activity increased at 12 HAT (Figure 4K, L, and M). The activities of DHAR, GPX, and GR were inhibited, however GST activity did not change significantly under Na₂CO₃ (Figure 4L, M and N). These results indicate that ROS in chloroplasts were mainly dismutated by SOD, and subsequently reduced in APX/POD pathway under the Na₂CO₃ treatment.

Na₂CO₃-responsive proteome revealed modulation of photosynthesis and ROS scavenging to cope with the stress

A total of 104 Na₂CO₃-responsive proteins in leaves were identified and classified into ten functional categories (Figure 5A, Figure S3, Tables S1-S3). Cluster analysis generated two main clusters (Figure 5B). In Cluster I, 63 Na₂CO₃-decreased proteins were involved in photosynthesis, carbohydrate and energy metabolism, protein synthesis and turnover, and cell wall metabolism. In Cluster II, 41 Na₂CO₃-increased proteins were related to energy metabolism, Chl metabolism, membrane and transport, and cell cycle. Importantly, subcellular localization prediction suggested that 63 proteins (60.6%) were specially localized in chloroplasts, and six proteins (6%) in either chloroplasts or other subcellular locations (Figure 5C; Tables S3 and S4). The changes of 37 photosynthesis-related proteins indicate that the balance of excitation energy between PSII and PSI was disrupted and the efficiency of electron transfer and CO₂ assimilation were inhibited. In contrast, photorespiration was induced under the Na₂CO₃ treatment. Aside from this, changes of 11
proteins involved in ROS and ion homeostasis as well as signaling pathway were triggered under Na\textsubscript{2}CO\textsubscript{3} treatment (Table S3).

Furthermore, we identified 121 Na\textsubscript{2}CO\textsubscript{3}-responsive proteins in chloroplasts (Table S5). Among them, there were 102 chloroplast-localized proteins belonging to eight functional categories (Figure 5D; Tables S6 and S7). Of these, 49 were photosynthetic proteins accounting for 48% of the total. This included five chlorophyll a/b binding proteins, 14 PSII-related proteins, seven PSI-related proteins, nine photosynthetic electron transfer chain proteins, four subunits of ATP synthase and ten Calvin cycle enzymes. Most of them were obviously altered at 12 HAT200 and 24 HAT (Table S6). Besides, nine photosynthetic electron transfer chain proteins and four subunits of chloroplast ATP synthase were changed (Table S6). This indicates that although Na\textsubscript{2}CO\textsubscript{3} inhibited the light harvesting, the PSII and PSI were not changed much at 12 HAT, but were enhanced at 24 HAT. However, ATP synthesis decreased at 12 HAT200, and then recovered to normal or enhanced at 24 HAT. Changes of the ten Calvin cycle-related proteins imply that carbon assimilation was inhibited at 24 HAT (Table S6). In addition, among the five ROS scavenging enzymes, thioredoxin peroxidase, 2-Cys peroxiredoxin BAS1, and GR increased at 12 HAT and decreased at 24 HAT, while APX and GST decreased at 12 HAT and not changed at 24 HAT (Table S6).

**Phosphoproteomics revealed novel Na\textsubscript{2}CO\textsubscript{3}-responsive phosphorylation sites**

We identified 63 Na\textsubscript{2}CO\textsubscript{3}-responsive phosphoproteins in leaves. Of these, 39 proteins showed increased phosphorylation levels and 21 had decreased phosphorylation levels. These proteins were classified into seven functional categories (Figure 5E, Table S8). Thirty-four phosphoproteins were predicted to be chloroplast-located, and involved in light harvesting, PSII, Calvin cycle and ATP synthesis (Table S8).

In chloroplasts from the Na\textsubscript{2}CO\textsubscript{3}-treated leaves, 161 unique phosphopeptides were identified, and 137 were quantified by dimethyl labeling (Figure 6A and Table S9). Among them, 50 proteins were found to be Na\textsubscript{2}CO\textsubscript{3}-responsive with 57 phosphorylation sites, including 33 increased and 15 decreased (Figure 6B and Table 1). The increased phosphoproteins include seven light harvesting proteins, six PSII proteins, five PSI proteins, three electron transfer chain proteins, two Calvin cycle-related proteins, a Na\textsuperscript{+}/H\textsuperscript{+} antiporter, a villin-2, and two thylakoid organization related proteins. The decreased include five ATP synthase subunits and sucrose-phosphate synthase. In addition, two signaling related proteins and six proteins involved in gene expression and protein turnover increased in phosphorylation at 24 HAT (Table 1).
In summary, we identified a total of 84 Na$_2$CO$_3$-responsive, chloroplast-localized phosphoproteins in leaf and chloroplast phosphoproteomes (Figures 6C and S4; Table 1). We identified 56 novel phosphorylation sites in the Na$_2$CO$_3$-responsive chloroplastic phosphoproteins, which may be crucial for regulating photosynthesis, membrane and transport, signaling, stress response, and protein synthesis and turnover (Table S10).

**Three-dimensional (3D) structure modeling of phosphoproteins**

We built thirteen homology-based 3D models of chloroplast-localized phosphoproteins using the SWISS-MODEL program (Figure S5A, B, C, D, F, G, I, J, K, L, M and N). We also accepted two experimentally solved 3D structures as homology models by the significant amino acid sequence similarity and conserved phosphorylation sites with our phosphoproteins (Figure S5E, H). The 3D models showed the numbers of helices and beta sheets, and the phosphorylation sites of each protein (Figure S5 and Table S11).

**Twenty-eight homologous genes of Na$_2$CO$_3$-responsive phosphoproteins exhibited diverse expression patterns**

In order to evaluate the gene expression patterns of the Na$_2$CO$_3$-responsive phosphoproteins, 28 homologous genes were analyzed through quantitative real-time (qRT-PCR) analysis with ubiquitin as an internal control (Figure S6, Table S12). Ten down-regulated genes were involved in light harvesting, PSII and PSI assembling, photosynthetic electron transfer, ATP synthesis, and thylakoid organization (Figure S6). Besides, three genes (*i.e.* photosystem I reaction center subunit II (PsaD), serine/arginine-rich splicing factor 33-like, and Na$^+$/H$^+$ antiporter) maintained stable levels under the Na$_2$CO$_3$. Interestingly, 15 up-regulated genes were involved in photosynthesis, Na$^+$/H$^+$ transport, calcium sensing, gene expression and protein turnover.

**Immunodetection of seven representative Na$_2$CO$_3$-responsive proteins**

To further evaluate the protein abundances of representative photosynthetic proteins under Na$_2$CO$_3$ treatment, Western blotting was conducted using available antibodies. The abundances of PSII subunits (photosystem II 22 kDa protein (PsbS), photosystem II D1 protein (D1) and oxygen evolving enhancer protein (PsbO)) and PSI subunit of PsaD increased at 24 HAT200 (*Figure 7*). RuBisCO large subunit (RBL) decreased at 24 HAT (Figure 7). Photosynthetic electron transfer chain related cytochrome f (Cyt f) and Calvin
cycle related phosphoglycerate kinase (PGK) maintained stable protein abundances at 24 HAT. Calvin cycle related sedoheptulose-1,7-bisphosphatase (SBPase) was used as the loading control (Figure 7).

Over-expression of PtFBA enhanced cell alkali tolerance

In our proteomics results, chloroplast-localized FBA increased significantly at phosphorylation level under Na₂CO₃ treatment. Therefore, FBA was selected as a representative Na₂CO₃ responsive protein for functional analysis. The full length cDNA of PtFBA was ligated into PpsbAII expression vector, and then transformed to wild-type (WT) cells of a model cyanobacterium Synechocystis 6803, generating an OX-PtFBA strain (Figure 8A). As expected, PCR analysis confirmed a complete segregation of the FBA over-expression (OX-PtFBA) strain (Figure 8B). Transcript analysis of PtFBA gene demonstrated the presence of gene product in the OX-PtFBA cells (Figure 8C). Western blotting analysis using a generic antibody against FBA also demonstrated that the FBA significantly increased in the OX-PtFBA strain when compared with WT (Figure 8D). The growth of the OX-PtFBA cells, as deduced from cell density and Chl a content, was much higher than the WT strain under the treatment of 0.4 M Na₂CO₃ for 4 days, although their growth was similar under normal conditions (Figure 8E, F). Thus, we conclude that overexpression of PtFBA resulted in enhanced Na₂CO₃ tolerance of Synechocystis 6803.

Discussion

Diverse photoprotection mechanisms to counteract Na₂CO₃-induced photoinhibition

Photosynthesis modulation is critical for plant stress response. PSII supercomplex was very sensitive to environmental changes [28,29]. In Na₂CO₃-treated alkali grass, PSII (e.g., OEC and the reaction center proteins) was oxidatively damaged, resulting in the decreases of photochemical efficiency and electron transport [25,30]. Our results indicate that diverse photoprotection mechanisms were employed in alkali grass to counteract alkali-induced photoinhibition. First, the accumulation of PsbS, chlorophyll a/b binding protein (CP) 24, and CP29, as well as induction of CP24 gene at 24 HAT may contribute to PsbS-protonation-dependent conformation conversion of PSII antenna system, suggesting that PsbS-dependent thermal dissipation was enhanced to minimize the potential for photo-oxidative damage under the Na₂CO₃ treatment (Figure S8A) [31]. Consistently, CP24 and CP29 also displayed high abundances in salt-sensitive plants (Arabidopsis, oilseed rape (Brassica napus), and
potato (*Solanum tuberosum*) and salt-tolerant plants (Indian mustard (*Brassica juncea*), mangrove (*Kandelia candel*), wild tomato (*Solanum chilense*) and Sugar beet) under salt stresses [17,32–37]. Second, the phosphorylation at Ser186 of CP24, Thr165 and Ser172 of CP26, as well as Ser95 and Thr108 of CP29 were enhanced in alkaligrass at 24 HAT (Table 1), while CP24 became dephosphorylated in NaCl-treated *B. distachyon* [21]. The reversible phosphorylation of CP24 was supposed to regulate the alternative mode of phosphorylation-independent thermal dissipation and phosphorylation-dependent energy spillover in lycophytes [38]. Third, the state transition between PSII and PSI was regulated by protein phosphorylation in the Na$_2$CO$_3$-treated alkaligrass (Figure S8B). A serine/threonine-protein kinase (STN7) and protein phosphatases modulate the reversible phosphorylation of LHCII (*i.e.* Lhcb1 and Lhcb2) and thylakoid soluble phosphoprotein of 9 kDa (TSP9) [23]. The phosphorylation of Lhcb1 was reported to be salinity-increased in *B. distachyon* [21]. Besides, TSP9 interacts with LHCII and the peripheries of PSII and PSI, facilitating dissociation of LHCII from PSII for regulating photosynthetic state transitions [39]. Thus, the Na$_2$CO$_3$-enhanced gene expression of *Lhcb1*, abundances of Lhcb1, Lhcb2, and STN7, as well as phosphorylation of Lhcb1 and TSP9 may facilitate the state transition in alkaligrass (Figure S8B). Similarly, Lhcb1 and Lhcb2 increased in abundances by salt in salt-tolerant plants (*e.g.*, *K. candel*, moss (*Physcomitrella patens*)), *B. juncea*, soybean (*Glycine max*), and sugar beet [17,34,35,40,41]. Furthermore, Lhcb3 phosphorylation also increased in alkaligrass (Table 1). The rate of state transition was induced in Arabidopsis *Lhcb3* knockout mutant [42]. This implies that Lhcb3 is probably involved in the state transition, but the underlying regulatory mechanism needs to be further investigated (Figure S8B).

Cyclic electron transport (CET) is critical for protecting photosynthetic apparatus and additional ATP supply [43]. Several decreased electron transport-related proteins indicate that electron transport was slowed down in alkaligrass at 12 HAT (Figure S8C), and this may alleviate the damage of plastoquinone over-reduction. However, at 24 HAT, when the photosynthetic capacity and electron transport rate were inhibited, CET was induced due to the Na$_2$CO$_3$-increased gene expression of Cyt $f$, accumulated protein levels and enhanced phosphorylation of ferredoxin-NADP (+) reductase (FNR), Cyt $f$ and cytochrome $b_{6}f$ complex iron-sulfur subunit (Figure S8C). Among them, FNR phosphorylation may modify its thylakoid membrane localization to regulate the electron transport [44]. Additionally, several CET-related proteins (*e.g.* Cyt $f$, cytochrome $b_{6}f$ complex iron-sulfur subunit, FNR, NAD(P)H-quinone oxidoreductase subunit M (NdhM), and NdhJ) were accumulated in the NaCl-stressed halophytes [14,45]. Therefore, the Na$_2$CO$_3$-stressed alkaligrass employed
NDH-dependent CET to alleviate photo-oxidative damage and provide extra ATP.

Photorespiration is critical for GSH synthesis, nitrogen and carbon assimilation, and feedback regulation of photosynthetic activity to cope with alkali stress (Figure S8D) [4,25,46]. In addition, the decrease of photosynthesis was not resulted from stomatal limitation, but from inhibition of Calvin cycle because most Calvin cycle enzymes decreased significantly (Figure S8D). This is consistent with a previous report in halophytes and salt-tolerant cultivars [4,47].

Enhancement of PSII repair machinery to minimize photodamage

PSII repair machinery was efficiently and dynamically employed to minimize photodamage in alkaligrass (Figure S8E). Na$_2$CO$_3$-induced STN7 and STN8 can promote the phosphorylation of PSII core proteins and CP29. The protein phosphorylation loosens the attractive forces among the subunits of PSII-LHCII supercomplex, enabling migration of the damaged PSII to stroma thylakoids for subsequent detachment of damaged D1 from the core complex during the repairing process [48]. Subsequently, the thylakoid lumen 18.3 kDa protein can function as an acid phosphatase to dephosphorylate the damaged D1 protein, and this process is facilitated by the Na$_2$CO$_3$-accumulated PsbO with GTPase activity. The dephosphorylated D1 is recognized and degraded by the Na$_2$CO$_3$-induced ATP-dependent zinc metalloprotease FtsH 2 (FtsH) (Figure S8E). Besides, Na$_2$CO$_3$-induced ToxA-binding protein 1 may contribute to the D1 degradation process through its positive regulatory role in the stable accumulation of FtsH protease in chloroplast stroma [49]. Simultaneously, nascent copies of D1 protein are synthesized and processed rapidly (Figure S8E). Under Na$_2$CO$_3$ treatment, the D1 maturation and co-translational insertion into PSII complexes were prompted by the induced PsbH gene, accumulation of PsbH and low PSII accumulation 1 protein, as well as the induced phosphorylation of Thr3 or Thr5 in the PsbH [50,51].

OEC (PsbO, PsbP and PsbQ) is peripherally bound to PSII at the luminal side of the thylakoid membrane, which can stabilize the binding of inorganic cofactors, maintain the active Mn-cluster, and enhance oxygen-evolution in PSII [52]. The expression of PsbO and PsbP decreased in leaves at 24 HAT, and the abundance of OEC was affected in alkaligrass and mangrove [45]. Interestingly, phosphorylation of PsbO and PsbP was Na$_2$CO$_3$-decreased in alkaligrass (Figure S8C), and PsbP and PsbQ have been reported to be phosphorylated in thylakoid lumen of Arabidopsis [53]. This indicates that the Na$_2$CO$_3$-regulated OECs change will facilitate the PSII assembly and oxygen evolution. Besides, photosystem II subunit L
(PsbL) and TL29 also participate in the assembly of the PSII complex [54,55]. The phosphorylation of PsbL was induced and the phosphorylation of TL29 was inhibited in response to Na$_2$CO$_3$ (Figure S8E). Although the phosphorylation of PsbL was also reported in Arabidopsis [23], their regulatory mechanisms are not known.

**Reverse phosphorylation of Na$_2$CO$_3$-responsive proteins regulated the activities of PSI and ATP synthase**

In contrast to PSII, PSI drew little attention due to difficulties in accurately measuring its activity [56]. The abundance changes of several PSI proteins (e.g. Lhca1 and Lhca5) and Na$_2$CO$_3$-enhanced phosphorylation of Lhca2 and Lhca4 in alkaligrass allow the regulation of light absorption through the antenna modulation to prevent PSI damage (Figure S8C) [25]. The STN7-regulated phosphorylation of Lhca4 was also induced in Arabidopsis when the plastoquinone overly reduced [57]. Therefore, the enhancement of Lhca4 phosphorylation would be favorable for trapping and dissipation of excitations, working as a photoprotective mechanism of PSI [58].

Among the PSI core proteins, PsaA, PsaB, and PsaC [59] were decreased in alkaligrass (Figure S8C), barley (*Hordeum vulgare*) [60], and other halophytes [33,61]. The phosphorylation of PsaC was enhanced in alkaligrass at 24 HAT (Figure S8C), which has been reported in Arabidopsis, green algae (*Chlamydomonas reinhardtii*), and spikemoss (*Selaginella moellendorfii*) [23]. The decreased abundances of PsaA, PsaB, and PsaC imply that saline-alkali stress inhibited the energy transfer of PSI. In addition, PsaD and PsaE provide docking sites for ferredoxin, and PsaF is important for interaction with the luminal electron donor plastocyanin, while PsaG and PsaH participate in stabilizing PSI complex [62]. In this study, these proteins were significantly accumulated, and the phosphorylation of PsaD, PsaE, and PsaH were also enhanced at 24 HAT of Na$_2$CO$_3$ (Figure S8C), although only *PsaH* gene transcription was obviously induced at 24 HAT. Their phosphorylation events have been reported in Arabidopsis, green algae, and *Synechocystis* 6803 [23], and the phosphorylation of PsaD was supposed to regulate the electron transfer from PSI to the electron acceptors in Arabidopsis chloroplast stroma [63]. Therefore, we suggested that the salinity-/alkali-increased abundances or phosphorylation of these proteins facilitate stabilization of the PSI complex (thus protecting it from photodamage), and activate the CET around PSI [62].

The gene expression and protein abundance of different subunits of ATP synthase were altered by Na$_2$CO$_3$ stress in alkaligrass (Figure S6) and other plant species [4]. The activity of
ATP synthase is salinity-responsive, being regulated by the reversible protein phosphorylation [4,64]. Several phosphorylation sites in ATP synthase subunits (α, β, δ, and ε) of spinach (*Spinacia oleracea*) chloroplasts have been reported [64]. In this study, Na$_2$CO$_3$ inhibited the phosphorylation of α (Ser125), β (Ser497, and Thr52), and ε subunit (Thr110), but enhanced the phosphorylation of α (Ser9, Ser21, and Thr43) and β subunits (Thr53 and Ser445) in alkaligrass at 24 HAT (Figure S8C). This implies that the stability and rotation of F1 head of ATP synthase are modulated for the dynamic regulation of its activity to cope with alkali stress.

Different ROS homeostasis pathways employed in chloroplasts and other subcellular locations to cope with Na$_2$CO$_3$ stress

Na$_2$CO$_3$ treatment disrupted the electron transport in chloroplasts, as well as tricarboxylic acid cycle and respiration chain in mitochondria (Figures 2 and S8F), leading to the increases of H$_2$O$_2$ and O$_2^-$ in leaves [65]. A previous proteomic study reported that many ROS-scavenging enzymes were altered in salinity-stressed leaves [4]. Our results indicated that parts of AsA-GSH cycle (*i.e.* APX and GPX pathways) were induced in leaves, but most ROS scavenging pathways in chloroplasts were inhibited, except for the APX pathway and SOD pathway (Figure 4). This implies that different pathways are employed in chloroplasts and other subcellular locations in leaves to cope with the short-term Na$_2$CO$_3$ stress (12 h). While under long-term NaCl or Na$_2$CO$_3$ stress (7 d), the pathways of SOD, POD, and CAT were all induced in leaves of alkaligrass [24, 25].

Various non-enzymatic antioxidants are important for ROS scavenging [66]. In this study, although the balance of AsA and DHA in chloroplasts was perturbed, the contents of AsA and DHA in leaves were stable. Additionally, the ratio of GSH/GSSG was stable in chloroplasts, and increased in leaves at 24 HAT (Figure 4). Furthermore, Na$_2$CO$_3$ also increased glyoxalase I, but decreased chloroplast-localized cysteine synthase. Both enzymes are involved in GSH/GSSG balance (Figure S8F). All these imply that the AsA-GSH cycle is inhibited in chloroplasts, but enhanced in other organelles and cytoplasm of leaf cells to cope with the alkali stress. In addition, a chloroplast-localized activator of bc1 complex kinase increased at 24 HAT, which would facilitate tocopherol cyclase phosphorylation to stabilize it at plastoglobules for vitamin E synthesis [67]. The thylakoid membrane-localized vitamin E is a lipid antioxidant. This result is consistent with our previous finding of NaCl-increased vitamin E content and tocopherol cyclase abundance in leaves of alkaligrass [25].

Additionally, the atlas of Na$_2$CO$_3$-responsive proteins indicated that modulation of Chl
synthesis (Figure S8G), chloroplast movement and stability (Figure S8H) were critical for alkali adaptation, and ABA-dependent alkali-responsive pathways were employed to regulate both nuclear and chloroplastic gene expression and protein processes for osmoprotectant synthesis and signaling pathways in alkaligrass (Figure S8I and J).

Conclusion

Although NaCl-responsive mechanisms have been well-studied in various halophytes using proteomics approaches [4], the Na₂CO₃-responsive proteins and corresponding regulatory mechanisms in halophytes were rarely explored. This study is the first detailed investigation of Na₂CO₃-responsive proteins in chloroplasts using proteomics and phosphoproteomics approaches, which revealed several crucial Na₂CO₃-responsive pathways in halophyte chloroplasts (Figure S8). Our study showed that maintenance of energy balance between PSII and PSI, efficiency of PSII damage repair, cyclic electron transport, dynamic thylakoid membrane architecture, as well as osmotic and ROS homeostasis were essential for photosynthetic modulation in response to Na₂CO₃. Both the nuclear- and chloroplast-encoded proteins were critical for the Na₂CO₃-responsive chloroplast function. More importantly, the newly-identified protein phosphorylation sites suggest that the reversible protein phosphorylation is important for regulating multiple signaling and metabolic pathways in chloroplasts to cope with the Na₂CO₃ stress. Some of these Na₂CO₃-responsive proteins and phosphoproteins are potential saline-alkali stress biomarkers for further functional characterization and biotechnological application.

Materials and methods

Plant material treatment and biomass analysis

Seeds of alkaligrass (*Puccinellia tenuiflora* (Turcz.) scribn. et Merr.) were sowed on vermiculite and grown in Hoagland solution in pots under fluorescent light (220 μmol m⁻² s⁻¹, 12 h day and 12 h night) at 25 °C day and 20 °C night, and 75% humidity for 50 days. Seedlings were treated with 0 mM, 150 mM, and 200 mM Na₂CO₃ (pH 11) for 12 h and 24 h, respectively (Figure S7). After treatment, leaves were harvested, either used immediately or stored at -80 °C for experiments. Shoot length and leaf fresh weight of seedlings were immediately measured. Dry weight, relative water content, as well as ion contents of K⁺, Na⁺, Ca²⁺ and Mg²⁺ were determined according to the method of Zhao et al. [26].
Membrane integrity, osmolytes, and ABA analysis

The malondialdehyde content and relative electrolyte leakage were determined using previous methods [68,69]. Free proline and total soluble sugar contents were quantified with a spectrometer at 520 nm and 630 nm, respectively [26]. The content of endogenous ABA was measured by an indirect ELISA method [70].

Photosynthesis and chloroplast ultrastructure analysis

Photosynthesis and Chl fluorescence parameters were measured using previous methods [25,69]. Net photosynthetic rate, stomatal conductance, and transpiration rate were determined at 10:00 a.m. using a portable photosynthesis system LI-COR 6400 (LI-COR Inc., Lincoln, Nebraska, USA). Chl fluorescence parameters were recorded using a pulse-amplitude-modulated (PAM) Chl fluorometer (Dua-PAM-100) (Heintz Walz, Effeltrich, Germany) and an emitter-detector-cuvette assembly with a unit 101ED (ED-101US). For the rapid fluorescence induction kinetics analysis, the OJIP were measured at room temperature (25 °C) with a portable fluorometer PAM-2500 (Walz, Effeltrich, Germany). The fluorescence measurement and calculation were performed according to the protocol of Strasser et al. [71]. Chl contents were determined according to the method of Wang et al. [69]. The ultrastructure of chloroplasts were observed under a JEOL-2000 transmission electron microscope (JEOL, Tokyo, Japan) according to Suo et al. [72].

Measurements of ROS and antioxidant contents, and antioxidant enzyme activities

The O$_2^-$ generation rate and H$_2$O$_2$ content were measured according to Zhao et al. [26]. Reduced AsA, total AsA, GSSG, and total GSH contents were determined according to methods of Law et al. [73]. The activities of SOD, POD, CAT, APX, GR, and GST were measured as previously described [25,26]. The activities of MDHAR, DHAR, and GPX were assayed according to Zhao et al. [26].

Chloroplast isolation, protein extraction and purity assessment

Intact chloroplasts were isolated according to Ni et al. [74], and the chloroplast protein for iTRAQ and phosphoproteomics analysis was extracted according to Wang et al. [75]. The purity of chloroplast protein was assessed by Western blot analysis with antibodies against marker proteins for different subcellular compartments according to Dai et al. [76]. Primary antibodies against marker proteins and protein loading amounts were listed in Table S13.
Proteomic analysis chloroplasts and leaves

The protein samples were extracted, fractioned, lyophilized, and resuspended for MS/MS analysis according to Zhao et al. [26]. The peptides of control (0 mM Na₂CO₃), 12 HAT150, 12 HAT200, 24 HAT150, and 24 HAT200 were labeled with iTRAQ reagents 116, 117, 118, 119, and 121 (AB Sciex Inc., Foster City, CA, USA), respectively. Three biological replicates were performed. The LC-MS/MS analysis was performed by Triple TOF™ 5600 LC-MS/MS (AB Sciex Inc., Concord, Canada) according to Zhao et al. [26]. The MS/MS data were submitted to database searching using the Paragon algorithm of ProteinPilot (version 4.0, AB Sciex Inc.). The databases used were the Uniprot Liliopsida database (320 685 entries) and the National Center for Biotechnology Information (NCBI) non-redundant database (7,262,093 sequences). The proteomics data was available in the Proteomics Identifications (PRIDE) database [77] under accession number PXD005491.

Total protein samples from leaves were prepared and analyzed using two-dimensional gel electrophoresis according to the method of Suo et al. [72] and Dai et al. [76]. The MS and MS/MS spectra were acquired on a MALDI-TOF/TOF mass spectrometer (AB Sciex Inc.) [69]. The MS/MS spectra were searched against the NCBI non-redundant green plant database (http://www.ncbi.nlm.nih.gov/) (3,082,218 sequences) using the search engine Mascot (version 2.3.0, Matrix Science, London, UK) (http://www.matrixscience.com) according to Meng et al. [78]. The proteomics data was deposited in the PRIDE database [77] under accession number PXD005455.

Phosphoproteomic analysis of proteins from chloroplasts and leaves

After digestion, the chloroplast peptide samples of control, 24 HAT150, and 24 HAT200 were labeled with stable isotope dimethyl labeling in light, intermediate, and heavy, respectively, according to the method of Boersema et al. [79]. In addition, the peptides from each leaf protein sample were labeled with iTRAQ reagents (113 and 116 for control, 114 and 117 for 24 HAT150, as well as 115 and 119 for 24 HAT200) according to the manufacturer’s instructions, respectively (AB Sciex Inc.). The phosphopeptides were enriched using TiO₂ micro-column [80]. LC-MS/MS analysis was performed using a nanoAcquity ultraperformance LC (Waters, Milford, MA, USA) coupled with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) [81].

For database searching, raw data files from chloroplast phosphoproteome were processed using Mascot server (version 2.3.0, Matrix Science) and searched against the NCBI green plant database (7,262,093 sequences) using an in-house Mascot Daemon (version 2.4, Matrix
For phosphopeptide relative quantification, Mascot Distiller (version 2.5.1.0, Matrix Science) was used. Precursor ion protocol was used for peptide quantification and the ratios were calculated using the peak areas of extracted ion chromatograms based on the trapezium integration method [82]. For leaf phosphoproteins, MS/MS data and peak lists were extracted using ProteinPilot (version 4.0, AB Sciex), searched against the NCBI green database (7,262,093 sequences) at a 95% confidence interval (unused ProtScore > 1.3). After database searching, reliable quantification of individual phosphopeptide was achieved by the mean ± S.D. of triplicate experiments, the peptides with more than 1.5-fold changes in at least two replicates were considered to be changed at phosphorylation level. The chloroplast and leaf phosphoproteomics data have been deposited to PRIDE [77] under the accession numbers of PXD005472 and PXD005471, respectively.

**Protein classification, hierarchical clustering, subcellular location prediction, and 3D structure analysis**

Functional domains of proteins were analyzed using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/), and then were clustered by cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). The prediction of protein subcellular location was determined according to Suo et al. [72]. The SWISS-MODEL comparative protein modeling server (http://swissmodel.expasy.org/) was employed to generate 3D structural models of phosphoproteins [83].

**qRT-PCR analysis of homologous gene expression**

Total RNA was isolated from leaves using the pBiozol plant total RNA extraction reagent (BioFlux, Hangzhou, China). A first-strand cDNA was obtained from 1 μg of total RNA using a PrimeScript® RT reagent kit (Takara Bio, Inc., Otsu, Japan). The sequences of candidate genes were obtained from the local alkaligraass EST database using a BLASTn program. qRT-PCR amplification was performed using the specific primer pairs (Table S12) on a 7500 real time PCR system (Applied Biosystems Inc., USA). The amplification process was performed according the method of Suo et al. [72].

**Western blot analysis**

Western blotting was conducted according to Dai et al. [76]. The primary antibodies were raised in rabbits against the Arabidopsis PsbS, Cyt f, D1, PsbO, PsaD, RBL, PGK, and SBPase. Signals were detected with ECL Plus™ reagent (GE Healthcare) according to the
manufacturer's instruction. Relative abundances were analyzed using the Image Master 2D Platinum Software (version 5.0, GE Healthcare). For the immunodetection of the FBA level in the WT and OX-PtFBA Synechocystis 6803, antibodies against FBA and RBL was used, 15 μg total protein aliquots of WT and OX-PtFBA (including the indicated serial dilutions) were loaded, and RBL was used as a loading control.

Overexpression of PtFBA in Synechocystis 6803

Full length cDNA of PtFBA was amplified by PCR using appropriate primers (Table S14). The PsbAII expression vector was used to generate OX-PtFBA strain. A fragment containing the PtFBA gene was amplified by PCR (Table S14) and then inserted into Nde I sites of PsbAII to form the PsbAII-PtFBA expression vector construct, which was used to transform the WT Synechocystis 6803 using a natural transfer method [84]. The transformants were spread on BG-11 agar plates containing 10 μg·ml⁻¹ of spectinomycin, then incubated in 2% (v/v) CO₂ in air and illumination at 40 μmol photons m⁻²·s⁻¹. The OX-PtFBA cells in the transformants was segregated to homogeneity (by successive streak purification) as determined by PCR amplification (Table S14), reverse transcription (RT-PCR) analysis (Table S14), and immunoblotting [85]. Cell growth and Chl a content analysis were conducted according to Gao et al. [86].

Statistical analysis

All the results are presented as means ± standard deviation of at least three replicates. The physiological and proteomics data were analyzed by Student's t test. A p-value < 0.05 was considered statistically significant.

Authors’ contribution

SD and ZQ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. JS and HZ carried out physiological experiments. HZ, QZ, BS, JY, JL, and LG participated in proteomics analysis. JS, JM, YS, and XZ carried out the phosphoproteomics analysis. JC carried out the chloroplast ultrastructure analysis. JS and LP carried out the immunoassays. NZ, YZ, YL, and WM carried out the molecular genetic studies. SC, TW, YM, and SG helped to draft the manuscript. All authors read and approved the final manuscript.
Competing interests
The authors have declared no competing interests.

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**Figure Legends**

**Figure 1** Leaf physiological characteristics in alkaligrass under Na$_2$CO$_3$ treatment

A. Relative water content (RWC) in leaves and shoot length (SL) of seedlings. B. Fresh weight (FW) and dry weight (DW) of leaves. C. K$^+$ and Na$^+$ contents. D. K$^+$/Na$^+$ ratio. E. Ca$^{2+}$ and Mg$^{2+}$ contents. F. Malondialdehyde (MDA) content and relative electrolyte leakage (REL). G. Proline (Pro) and glycine betaine (GB) contents. H. Soluble sugar (Sug) and abscisic acid (ABA) contents. The values were determined after plants were treated with 0, 150, and 200 mM Na$_2$CO$_3$ for 12 and 24 h, and were presented as means ± standard deviation (n ≥ 3), respectively. The asterisks indicate significant differences (*, P < 0.05; **, P < 0.01).

**Figure 2** Photosynthetic characteristics of alkaligrass under Na$_2$CO$_3$ treatment

A. Net photosynthetic rate (Pn) and stomatal conductance (Gs). B. Transpiration rate (Tr) and intercellular CO$_2$ concentration (Ci). C. Maximum quantum efficiency of PSII photochemistry (Fv/Fm) and PSII maximum efficiency (Fv'/Fm'). D. Actual PSII efficiency (φPSII) and electron transport rate (ETR). E. Non-photochemical quenching (NPQ) and the fraction of open PSII centers (qL). F. Chlorophyll fluorescence OJIP transient, fluorescence intensity (F$_i$) was recorded between 0.01 and 1000 ms time period. G. Relative fluorescence intensity (RFI) of band L (V$_L$) and K (V$_K$) after double normalization between the two fluorescence extreme F$_O$ and F$_K$, F$_O$ and F$_I$ phases: V$_L$ = (F$_L$ - F$_O$)/(F$_K$ - F$_O$), V$_K$ = (F$_K$ - F$_O$)/(F$_I$ - F$_O$). H. Relative fluorescence intensity (RFI) of step J (V$_J$) and I (V$_I$) after double normalization between the two fluorescence extreme F$_O$ and F$_P$ phases: V$_J$ = (F$_J$ - F$_O$)/(F$_P$ - F$_O$), V$_I$ = (F$_I$ - F$_O$)/(F$_P$ - F$_O$). I. Total chl and chl a contents. J. Chl b content and Chl a/b ratio. The values were determined after plants were treated with 0, 150 and 200 mM Na$_2$CO$_3$ for 12 and 24 h, and were presented as means ± standard deviation (n ≥ 3). The asterisks indicate significant differences (*, P < 0.05; **, P < 0.01).
Figure 3 Ultrastructure of chloroplasts in alkaligrass leaves under Na$_2$CO$_3$ treatment

Figure 4 Effects of Na$_2$CO$_3$ treatments on antioxidant systems in leaves and chloroplasts of alkaligrass
A. O$_2^-$ generation rate and H$_2$O$_2$ content in leaves. B. Ascorbate (AsA) and dehydroascorbate (DHA) contents in leaves. C. Glutathione (GSH) and oxidized glutathione (GSSG) contents in leaves. D. Superoxide dismutase (SOD) and peroxidase (POD) activities in leaves. E. Catalase (CAT) and ascorbate peroxidase (APX) activities in leaves. F. Monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) activities in leaves. G. Glutathione peroxidase (GPX) and glutathione reductase (GR) activities in leaves. H. Glutathione S-transferase (GST) activity in leaves. I. AsA and DHA contents in chloroplasts. J. GSH and GSSG contents in chloroplasts. K. SOD and POD activities in chloroplasts. L. GST and APX activities in chloroplasts. M. MDHAR and DHAR activities in chloroplasts. N. GPX and GR activities in chloroplasts. The values were determined after plants were treated with 0, 150 and 200 mM Na$_2$CO$_3$ for 12 and 24 h, and were presented as means ± standard deviation (n ≥ 3). The asterisks indicate significant differences (*, P < 0.05; **, P < 0.01).

Figure 5 Functional categorization, hierarchical clustering analysis, and subcellular location prediction of the Na$_2$CO$_3$-responsive proteins
A. Functional category of Na$_2$CO$_3$-responsive leaf proteins. The percentages of proteins in different functional categories are shown in the pie. B. Heat map of Na$_2$CO$_3$-responsive protein species from leaf proteome. Two main clusters (I–II) are shown in the figure, functional categories indicated by lower-case letters (a, photosynthesis; b, carbohydrate and energy metabolism; c, other metabolisms; d, stress and defense; e, membrane and transporting; f, signaling; g, protein synthesis and turnover; h, cell wall metabolism; i, cell cycle; j, miscellaneous and function unknown), spot numbers and protein name abbreviations are listed on the right side (detailed information on protein names and abbreviations can be found in Table S1). C. Predicted localization of proteins from leaf proteome by internet tools
and literatures. The numbers of protein species with different locations are shown in the pie. Chl, chloroplast; Cyt, cytoplasm; Mit, mitochondria; Nuc, nucleus. D-E. A total of 102 Na₂CO₃-responsive protein species and 84 Na₂CO₃-responsive phosphoprotein species in chloroplasts were classified into eight and seven functional categories, respectively. The percentages of proteins in different functional categories are shown in the pie.

**Figure 6 Summary of alkaligrass chloroplast phosphoproteome**

A. Venn diagrams depicting overlap in phosphoproteins, phosphopeptides, and phosphorylation sites identified in three biological replicates. Numbers in parentheses indicate the quantified phosphoproteins, phosphopeptides, and phosphorylation sites, respectively. B. Na₂CO₃-responsive phosphoproteins, phosphopeptides, and phosphorylation sites in at least two replicates; a,b Phosphorylation level increased/decreased in certain treatment condition; c Phosphorylation level increased in one treatment but decreased in the other, or the changed peptide with two phosphorylation sites; Pro, protein; Pep, peptide. C. Example of a representative MS/MS spectrum of phosphopeptides identified in the chloroplast phosphoproteome (fragmentation spectrum shown of m/z 614.82, +2, dimethyl-labeled, Accession No. EMT19581); Asterisk (*) represents the phosphorylation site.

**Figure 7 Western blot image of eight alkali-responsive chloroplast proteins.**

Eight chloroplast proteins from plants under different treatment conditions (0, 150, and 200 mM Na₂CO₃ for 24 h) were loaded with equal amounts. They were photosystem II 22 kDa protein (PsbS), cytochrome f (Cyt f), photosystem II D1 protein (D1), oxygen evolving enhancer protein (PsbO), photosystem I reaction center subunit II (PsaD), RuBisCO large subunit (RBL), phosphoglycerate kinase (PGK), and sedoheptulose-1,7-bisphosphatase (SBPase). These proteins were separated by 15% SDS-PAGE and analyzed by immunoblotting, and SBPase was used to control for equal loading. The relative abundances of these proteins were shown on the right. Results are the average of three independent experiments.

**Figure 8 Transgenic analysis of chloroplast-localized PtFBA gene in a model cyanobacterium Synechocystis sp. strain PCC 6803**

A. Construction of an over-expression vector PpsbAII-PtFBA, to generate the PtFBA over-expression (OX-PtFBA) strain in S. PCC 6803. B. PCR segregation analysis of the OX-PtFBA cells. C. The transcript levels of PtFBA in the wild-type (WT) and OX-PtFBA strains.
The transcript level of 16S ribosomal RNA (rRNA) in each sample is shown as a control. The absence of contamination of DNA was confirmed by PCR without reverse transcriptase reaction. D. Immunodetection of FBA levels in WT and OX-PtFBA strains. Protein blotting was performed with antibody against FBA. Lanes were loaded with 15 μg total proteins and RBL was used as a loading control. E. Growth of WT and OX-PtFBA strains under 0.4 M Na₂CO₃. Three μL of cell suspensions with densities corresponding to A₇₃₀ nm values of 0.1 (upper rows), 0.01 (middle rows), and 0.001 (lower rows) were spotted on agar plates with normal medium and 0.4 M Na₂CO₃ medium. F. A₇₃₀ nm values and chl a contents in WT and OX-PtFBA strains after cultivation in the medium with 0 M (controls) and 0.4 M Na₂CO₃ for 1 and 4 days, respectively. Mean ± S.D., n= 3.

Tables

Table 1 Na₂CO₃-responsive phosphoproteins in chloroplasts from alkaligrass leaves

Supplementary material

Figure S1  Morphological changes of alkaligrass seedlings grown under Na₂CO₃ treatment
50-day old seedlings were treated with different concentrations, 0 mM, 150 mM and 200 mM of Na₂CO₃ (pH 11) for 0, 12 and 24 h, respectively. Bar = 3.23 cm.

Figure S2  Integrity and purity determination of chloroplasts isolated from alkaligrass leaves
A. Autofluorescence of the purified chloroplasts were visualized under a fluorescence microscope, Bar=10 μm. B. Western blot analysis of proteins from purified chloroplasts. Proteins from the leaves and purified chloroplasts were separated on 12.5% SDS-PAGE, and immunodetected with rabbit polyclonal antibodies against VHA (vacuolar-type H⁺-ATPase, vacuole marker), CAT (catalase, peroxisome marker), COXII (mitochondrial cytochrome oxidase subunit II, mitochondrial marker), Sar1 (secretion-associated and ras-related protein 1, endoplasmic reticulum marker), and FBPase (cytosolic fructose 1,6-biphosphatase, cytosolic marker), respectively.

Figure S3  Coomassie brilliant blue-stained two-dimensional gel electrophoresis gels
Protein was extracted from leaves of *Puccinellia tenuiflora* under Na$_2$CO$_3$ treatment conditions and separated on 24 cm IPG strips (pH 4-7 linear gradient) using isoelectric focusing in the first dimension, followed by 12.5% SDS-PAGE gels in the second dimension. A total of 104 Na$_2$CO$_3$-responsive proteins identified by mass spectrometry were marked with numbers on the representative gels. Detailed information can be found in Supplementary Table S2.

**Figure S4** A list of MS/MS spectra of phosphopeptides derived from alkali grass leaf and chloroplast phosphoproteins

**Figure S5** Homology based structural models of the chloroplast phosphoproteins from alkali grass

The ribbon images of A-D, F, G, and I-N are generated by SWISS-MODEL program based on the comparative protein structure modeling approach, and the 3D models based on their own amino acid sequences. While images of E and H are the experimentally resolved 3D structure that has a significant amino acid sequence similarity to our phosphoproteomic identified sequence by searching the SWISS-MODEL template library. By using the Swiss-PdbViewer (version 3.7), the phosphorylation sites are shown with balls in red (enhanced phosphorylation) and green (inhibited phosphorylation). Domains from the phosphoprotein are highlighted in different colors. A. Lhca2, light-harvesting chlorophyll a/b binding protein of LHCI type II (PDB ID: 4xk8). B. Lhca4, photosystem I light-harvesting complex type 4 protein (PDB ID: 4y28). C. Lhcb1, chlorophyll a/b binding protein of LHClI type 1 (PDB ID: 1rwt). D. PsbS, photosystem II subunit S (PDB ID: 4ri2). E. PsbO, oxygen-evolving enhancer protein 1 (PDB ID: 3jcu). F. TL29, thylakoid lumenal 29 kDa protein (PDB ID: 3rrw). G. PsAC, photosystem I subunit VII (PDB ID: 4y28). H. PsaD, photosystem I reaction center subunit II (PDB ID: 2wsc). I. PsaE, photosystem I reaction center subunit IV (I and II representing two protein species of PsaE) (PDB ID: 4y28). J. Cyt f, cytochrome f (PDB ID: 1q90). K. FNR, ferredoxin-NADP reductase (PDB ID: 1qg0). L. ATP synthase alpha subunit (PDB ID: 5cdf). M. ATP synthase beta subunit (PDB ID: 1fx0). N. PDH, pyruvate dehydrogenase E1 component subunit alpha (PDB ID: 1ni4). Detailed information can be found in Table S11.
Figure S6  Cluster analysis of the homologous gene expression patterns of alkaligrass chloroplast phosphoproteins under 24h of Na₂CO₃ treatment

Heat map of the 28 homologous genes from *Puccinellia tenuiflora* leaves under 24 h of Na₂CO₃ treatment (0, 150, and 200 mM), three main clusters (I–III) are shown in the figure. The scale bar indicates log₂ transformed relative expression levels of genes. The up-regulated and down-regulated genes are presented in red and green, respectively. Homologous gene names are listed on the right side.

Figure S7  Workflow of plant treatment and sampling

Seeds of *Puccinellia tenuiflora* were sowed on vermiculite and grown in Hoagland solution in pots. Seedlings about 50-day-old were treated with 0, 150, and 200 mM Na₂CO₃ (pH 11) for 12 and 24 h, respectively. After treatment, leaves from both control and treatments were harvested for experiments.

Figure S8  Schematic presentation of Na₂CO₃ responsive mechanisms in leaves from alkaligrass

The Na₂CO₃ responsive proteins from both leaf and chloroplast proteomes were integrated into subcellular pathways. A. Thermal dissipation. B. State transition. C. Photosynthetic electron transfer. D. Photorespiration and Calvin cycle. E. PSII repair cycle. F. ROS scavenging. G. Chlorophyll biosynthesis. H. Chloroplast movement and thylakoid membrane stability. I. Ionic and osmotic homeostasis. J. Gene expression, protein turnover and transport. The scale bar indicates log (base 2) transformed protein abundance, enzyme activity, and substrate content ratios (compared with 0 mM Na₂CO₃ treatment). The ratio was ranging from -3.0 to 3.0 with 7 different colors from green to red. P in a red (increased in phosphorylation level), green (decreased in phosphorylation level), and blue (can be phosphorylated by other protein, but did not identified in our phosphoproteomic study) circle indicates phosphorylated protein. The solid line indicates single-step reaction, dashed line indicates multistep reaction, and the dotted line indicates movement of proteins or other substances. Abbreviations: 1,3-BPG, 1,3-bisphosphoglyceric acid; 13¹-Hydroxy-Mg-Proto ME, 13¹-hydroxy-magnesium-protoporphyrin IX 13-monomethyl ester; 3-PGA, 3-phosphoglyceric acid; 30S/50S, 30S/50S ribosomal protein; 40S/60S, eukaryotic small/large ribosomal subunit; ABA, abscisic acid; ABC1K, activator of bc1 complex kinases; ALA, 5-aminolaevulinc acid; AlaRS, alanine-tRNA ligase; APX, ascorbate peroxidase; AsA, ascorbic acid; CA, carbonic anhydrase; CAT, catalase; Coprogen III, corproporphyrinogen III;
CP, chlorophyll a/b binding protein; CPOX, coproporphyrinogen-III oxidase; CSase, cysteine synthase; CURT1A, curvature thylakoid 1A protein; Cyt f, cytochrome f; D1/D2, photosystem II D1/D2 protein; DHA, dehydroascorbic acid; DHAP, dihydroxyacetone phosphate; DHAR, dehydroascorbate reductase; DLD, dihydrolipoyl dehydrogenase; DLP, dynamin-2A-like protein; DnaJ, chaperone protein DnaJ-like; E4P, erythrose 4-phosphate; EF-Tu, elongation factor Tu; eIF4B, eukaryotic initiation factor 4B; eIF5A, eukaryotic translation initiation factor 5A1; F6P, fructose 1,6-diphosphate; FBA, fructose-bisphosphate aldolase; FBP, fructose 1,6-bisphosphate; Fd, ferredoxin; FNR, ferredoxin-NADP(+) reductase; FtsH, ATP-dependent zinc metalloprotease FtsH 2; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDC, glycine decarboxylase; GGDR, geranylgeranyl diphosphate reductase; GGPP, geranylgeranyl pyrophosphate; GLO I, glyoxalase I; GPX, glutathione peroxidase; GR, glutathione reductase; GS-, xenobiotic substrates; GSA, glutamate 1-semialdehyde aminotransferase; GSA-AT, glutamate-1-semialdehyde 2,1-aminomutase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; Hsc70, heat shock cognate 70 kDa protein; Hsp70/90, 70/90 kDa heat shock related protein; IDH, isocitrate dehydrogenase (NADP)-like; IPP, isopentenyl pyrophosphate; ISP, rieske-like iron-sulfur (2Fe-2S) protein; LHCII, light harvesting complex II; Lhca1/2/4/5, light harvesting chlorophyll a/b binding protein1/2/4/5; Lhcb1/2/3, light harvesting chlorophyll a/b binding protein 1/2/3; LIL3, light harvesting-like protein 3; LPA1, low PSII accumulation 1 protein; MDA, malondialdehyde; MDH, malate dehydrogenase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; Mg-pro IX, Mg-proporphyrin IX; Mg-pro ME, Mg-protoporphyrin IX monomethyl ester; MgCh, magnesium-protoporphyrin IX chelatase ChlII subunit; MgCy, magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase; MgMT, magnesium-protoporphyrin O-methyltransferase; NACA, nascent polypeptide associated complex subunit alpha-like protein; NDF1, NDH-dependent cyclic electron flow I; NdhJ/M/O, NAD(P)H-quinone oxidoreductase subunit J/M/O; OAA, oxaloacetate; P-EAMeT, phosphoethanolamine N-methyltransferase; PAP, plastid-lipid-associated protein; PC, plastocyanin; PG, phosphoglycolate; PGP, phosphoglycolate phosphatase; Phytyl-PP, phytyl pyrophosphate; PPIase, peptidyl-prolyl cis-trans isomerase CYP20-2 domain; PPOX, protoporphyrinogen oxidase; PQ, plastoquinone; PQH2, plastohydroquinone; PRK, phosphoribulokinase; Prx, 2-Cys peroxiredoxin BAS1; PsaB, photosystem I P700 chlorophyll A apoprotein A2; PsaC, photosystem I subunit VII; PsaD, photosystem I reaction center subunit II; PsaE, photosystem I reaction center subunit IV; PsaF, photosystem I reaction center subunit III;
PsaG, photosystem I subunit V; PsaH, photosystem I reaction center subunit VI; PSAT, phosphoserine aminotransferase; PsbH, photosystem II reaction center protein H; PsbL, Photosystem II subunit L; PsbO, oxygen-evolving enhancer protein 1; PsbP, photosystem II 23kDa oxygen evolving protein; PsbQ, oxygen evolving enhancer protein 3 domain; PsbS, photosystem II 22 kDa protein; R5P, ribose 5-phosphate; RaiA, ribosome-associated inhibitor A domain; RBL, RuBisCO large chain; RBP, RuBisCO large subunit-binding protein; RBS, RuBisCO small chain; RCA, RuBisCO activase; RCC1, regulator of chromosome condensation repeat; RNA-BP, RNA binding protein; RNP, 31 kDa ribonucleoprotein; ROS, reactive oxygen species; RPE, ribulose-phosphate 3-epimerase; RRM, RNA recognition motif; RT, reverse transcriptase domain; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; SAHH, S-adenosyl-L-homocysteine hydrolase; SBP, sedoheptulose 1,7-bisphosphate; SDH, succinate dehydrogenase flavoprotein; SecY, preprotein translocase subunit SecY; SHMT, serine hydroxymethyltransferase; SOD, manganese superoxide dismutase; SRSF, serine/arginine-rich splicing factor 33-like; STN7/8, serine/threonine-protein kinase STN7/8; TCA, tricarboxylic acid; Tic62, Tic 62 domain containing protein; TK, transketolase; TL15, thylakoid lumenal 15 kDa protein; TL29, thylakoid lumenal 29 kDa protein; TLP18.3, thylakoid lumen 18.3 kDa protein; TOC/TIC, translocon at the outer/inner envelope membrane of chloroplasts; ToxABP1, chloroplast-localized ToxA binding protein 1; TPI, triosephosphate isomerase; TPx, thioredoxin peroxidase; Trx, thioredoxin; TSP9, thylakoid soluble phosphoprotein; URO III, uroporphyrinogen III; UROD, uroporphyrinogen decarboxylase; VHA, vacuolar H⁺-ATPase; Xu5P, xylulose 5-phosphate; YCF54, YCF54 domain containing protein; ZF, zinc finger protein.

Table S1  Detailed information of Na₂CO₃-responsive proteins in alkaligrass leaves identified by MALDI-TOF MS/MS

Table S2  Protein spots with multiple proteins identified in a single spot on two-dimensional gels of alkaligrass leaves

Table S3  Na₂CO₃-responsive proteins in alkaligrass leaves

Table S4  Subcellular localization prediction of the Na₂CO₃-responsive proteins in alkaligrass leaves
Table S5  Detailed information on Na$_2$CO$_3$-responsive proteins in alkaligrass chloroplasts revealed by iTRAQ-based proteomic analysis

Table S6  Na$_2$CO$_3$-responsive proteins in alkaligrass chloroplasts

Table S7  Subcellular localization prediction of the Na$_2$CO$_3$-responsive proteins in alkaligrass chloroplasts

Table S8  Na$_2$CO$_3$-responsive phosphoproteins in alkaligrass leaves

Table S9  Phosphoproteins identified in the chloroplast phosphoproteome of alkaligrass under Na$_2$CO$_3$ treatment

Table S10  A comparison of the phosphorylation sites/phosphoproteins identified in alkaligrass chloroplasts with phosphoproteins reported in plant species

Table S11  Summary of homology models of phosphoproteins in alkaligrass chloroplasts

Table S12  List of primer pairs used in quantitative real-time PCR

Table S13  Information on antibodies used for chloroplast purity assessment

Table S14  Primers used for overexpression of PtFBA in Synechocystis 6803
**A** Relative water content (%)

**B** Fresh weight (g)

**C** K⁺ content (mg·g⁻¹ DW)

**D** K⁺/Na⁺ ratio

**E** Mg²⁺ content (mg·g⁻¹ DW)

**F** Ca²⁺ content (mg·g⁻¹ DW)

**G** MDA content (mmol·g⁻¹ FW)

**H** Sug content (mg·g⁻¹ FW)

**E** Ca²⁺ content (mg·g⁻¹ DW)

**F** MDA content (mmol·g⁻¹ FW)

**G** Pro content (mg·g⁻¹ FW)

**H** ABA content (ng·g⁻¹ FW)

**A** RWC SL

**B** FW DW

**C** K⁺ Na⁺

**D** K⁺/Na⁺

**E** Mg²⁺ Ca²⁺

**F** MDA REL

**G** Pro GB

**H** Sug ABA

*Note: Data are presented as mean ± standard deviation. Significant differences are indicated by asterisks: *p < 0.05, **p < 0.01.*
Mesophyll cell

Lateral veins bundle

Minor veins bundle

Midveins bundle

Na₂CO₃ treatment

0 mM 150 mM 12 h 200 mM 12 h 150 mM 24 h 200 mM 24 h
Cluster I

A

Functional categorization
- Photosynthesis
- Carbohydrate and energy metabolism
- Other metabolisms
- Stress and defense
- Membrane and transporting
- Signaling
- Gene expression, protein synthesis and turnover
- Cell wall metabolism
- Cell cycle
- Miscellaneous and function unknown

GFP, Chl
- Chl
- Chl
- Cyt
- Cyt
- Nuc
- Nuc

Cluster II

B

Functional categorization
- Photosynthesis
- Carbohydrate and energy metabolism
- Other metabolisms
- Stress and defense
- Membrane and transporting
- Signaling
- Gene expression, protein synthesis and turnover
- Cell wall metabolism
- Cell cycle
- Miscellaneous and function unknown

GFP, Chl
- Chl
- Chl
- Cyt
- Cyt
- Nuc
- Nuc
### Quantitative Analysis

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<td>&gt; 2 = 15</td>
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<tr>
<td>Replicate 2</td>
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<td>Replicate 3</td>
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<td>&gt; 2 = 14</td>
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### Phosphoproteins

- Replicate 1: 26 (16)
- Replicate 2: 23 (23)
- Replicate 3: 7 (6)

### Phosphopeptides

- Replicate 1: 36 (30)
- Replicate 2: 37 (29)
- Replicate 3: 7 (6)

### Phosphosite

- Replicate 1: 50 (57)
- Replicate 2: 21 (26)
- Replicate 3: 21 (26)

### Phosphorylation Sites

- m/z = 614.82, 2+  
  - y1: 175.12  
  - b2: 187.14  
  - b2+: 272.17  
  - y2: 329.19

### Relative Abundance

- Relative Abundance (%)
- m/z: 200, 400, 600, 800, 1000, 1200

### Phosphorunion

- Phosphorunion 1: 1139.56
- Phosphorunion 2: 914.45
- Phosphorunion 3: 1042.51

---

This page contains data on the quantification of phosphoproteins, phosphopeptides, and phosphosite changes. The data is tabulated and presented in Venn diagrams for visual analysis. Relative abundances are shown in the accompanying figure.
21 kDa

35 kDa

31 kDa

33 kDa

20 kDa

53 kDa

45 kDa

38 kDa

0 150 200 (mM)

0 15 200 (mM)

PsbS

Cyt f

D1

PsbO

PsaD

RBL

PGK

SBPase

24 h

200

150

24 h

(mM)

(mM)

21 kDa

35 kDa

31 kDa

33 kDa

20 kDa

53 kDa

45 kDa

38 kDa

24 h

200

150

24 h

(mM)

(mM)
A. Expression of PtFBA in A. baumannii AB0158

B. RT-PCR analysis of PtFBA expression in WT and OX-PtFBA

C. Western blot analysis of PtFBA expression in WT and OX-PtFBA

D. FBA and RBL expression in WT and OX-PtFBA

E. Growth assay of WT and OX-PtFBA in normal and 0.4 M Na₂CO₃ medium

F. Chl production in WT and OX-PtFBA in normal and 0.4 M Na₂CO₃ medium

** and * indicate significant differences (p < 0.05).
Table 1  Na$_2$CO$_3$-responsive phosphoproteins in chloroplasts from alkaligrass leaves

<table>
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<tr>
<th>Protein name(a)</th>
<th>Accession no.</th>
<th>Biological function(a)</th>
<th>Peptide sequence(b)</th>
<th>Ratio ± S.D.(c)</th>
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<td>Light harvesting complex I chlorophyll a-b binding protein (Lhcc2)</td>
<td>*C4A59049</td>
<td>Light harvesting</td>
<td>APERPIWFPGS$^{25}$TPFPWDLGS$^{17}$L</td>
<td>0.660 ± 0.040</td>
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<td>Hypothetical protein, photosystem I ERM94529</td>
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<td>Light harvesting, energy dissipation</td>
<td>NPG$^{49}$VQNDPPFK</td>
<td>1.87 ± 0.06</td>
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<td>Light harvesting complex type 4 protein (Lhcc4)*</td>
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<td>Light harvesting, state transition</td>
<td>QSL$^{36}$YLDSGLPGDFDPLGLS</td>
<td>1.830 ± 0.01</td>
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<tr>
<td>Light harvesting complex type 3 protein (Cp25, chloroplastic (Cp25))</td>
<td>*XP_003618083</td>
<td>Light harvesting, state transition</td>
<td>ACP$^{32}$SSPVGWGDSR</td>
<td>1.26 ± 0.01</td>
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<tr>
<td>Predicted protein, chlorophyll a-b binding protein (Lhcc2)</td>
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<td>Light harvesting, state transition</td>
<td>VAASS$^{16}$SPWYGDSR</td>
<td>1.23 ± 0.03</td>
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<tr>
<td>Photosystem I reaction center subunit IV (PsaE)</td>
<td></td>
<td>Light harvesting, state transition</td>
<td>VAASS$^{16}$SPWYGDSR</td>
<td>1.12 ± 0.02</td>
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<tr>
<td>Photosystem II subunit L (PsbL)</td>
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<td>-</td>
<td>AKPV$^{18}$SSPVGWGDSR</td>
<td>1.25 ± 0.10</td>
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<tr>
<td>Photosystem II reaction center protein H (PsbH)</td>
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<td>-</td>
<td>AKPV$^{18}$SSPVGWGDSR</td>
<td>1.25 ± 0.10</td>
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<td>Photosystem II related protein (CP26, chloroplastic (CP26))</td>
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<td>-</td>
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<td>1.25 ± 0.10</td>
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<tr>
<td>Chlorophyll a-b binding protein of LHCII type 1-like (Lhcb1)*</td>
<td></td>
<td>-</td>
<td>ACP$^{32}$SSPVGWGDSR</td>
<td>1.25 ± 0.10</td>
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<td>OsO3gp095200, chlorophyll a-b binding protein BAF12500 of LHCII type I (Lhcb1)*</td>
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<td>TVKS$^{21}$APQSIWYDPPFK</td>
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<td>Predicted protein, chlorophyll a-b binding protein BAI85110 of LHCII type I-like (Lhcb1)*</td>
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<td>QVGS$^{27}$GPSWYGARD</td>
<td>1.59 ± 0.05</td>
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<td>Light harvesting chlorophyll a-b binding protein (Lhcc1)</td>
<td>*CDI44335</td>
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<td>VAGGPLGEVDPILPGDS$^{6}$LDP</td>
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<td>*EM550795</td>
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<td>VLYLGPLSPES$^{47}$LYTGEFGGD</td>
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<td>Light harvesting chlorophyll a-b binding protein (Lhcc1)</td>
<td>*EAT11232</td>
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<td>0.440 ± 0.05</td>
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<td>VLYLGPLSPES$^{47}$LYTGEFGGD</td>
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<td>*C4A32109</td>
<td>Light harvesting, state transition</td>
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<td>P27523</td>
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<td>LGDLPSPEAELK</td>
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<td>Light harvesting chlorophyll a-b binding protein CP29, chloroplastic (CP29)</td>
<td>*I908421A</td>
<td>PSII disassembly, energy dissipation</td>
<td>PAELYQVDVSDLQDLAQNL</td>
<td>1.320 ± 0.26</td>
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<td>Light harvesting chlorophyll a-b binding protein CP29, chloroplastic (CP29)</td>
<td>*CDI44415</td>
<td>PSII disassembly, energy dissipation</td>
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<td>Energy dissipation</td>
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<td>Chlorophyll a-b binding protein CP24</td>
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<td>Photosystem II related protein (11)</td>
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<td>Oxygen-evolving enhancer protein 1 (PsbO)</td>
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<td>Photosynthetic oxygen evolution, PSI D1 repair</td>
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<td>3.28 ± 0.50</td>
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<td>Predicted protein, oxygen-evolving enhancer protein 1, chloroplastic (PsbO)*</td>
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<td>Photosynthetic oxygen evolution, PSI D1 repair</td>
<td>ATQTVEDSSKPR</td>
<td>3.69 ± 0.54</td>
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<td>Photosystem II subunit L (PsbL)</td>
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<td>QTVEDSSKPR</td>
<td>0.32 ± 0.11</td>
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<td>Photosystem II reaction center protein (PsbH)</td>
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<td>Photosynthetic oxygen evolution, PSI activation</td>
<td>QTVEDSSKPR</td>
<td>0.32 ± 0.11</td>
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<td>Photosystem II subunit L (PsbL)</td>
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<td>PSI assembly and PSI dimerization</td>
<td>QTVEDSSKPR</td>
<td>0.32 ± 0.11</td>
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<td>Predicted protein, thylakoid luminal 29 kDa BAI87776 protein (TL29)*</td>
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<td>0.63 ± 0.00</td>
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<td>Predicted protein, containing pfm11403, thylakoid soluble phosphoprotein of 9 kDa domain (TPSP)*</td>
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<td>PSI D1 repair</td>
<td>TLYSAVGSS$^{36}$GQWGFDPK</td>
<td>0.63 ± 0.00</td>
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<td>Predicted protein, thylakoid luminal 29 kDa BAI87776 protein (TL29)*</td>
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<td>PSI D1 repair</td>
<td>TLYSAVGSS$^{36}$GQWGFDPK</td>
<td>0.63 ± 0.00</td>
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<td>Photosystem I iron-sulfur center (PsaC)</td>
<td>POC539</td>
<td>Carries the [4Fe–4S] F$_x$ and F$_y$ clusters</td>
<td>VLYLGEFT</td>
<td>1.51 ± 0.01</td>
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<td>Photosystem I reaction center subunit II (PsaD)</td>
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<td>VLYLGEFT</td>
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<td>VLYLGEFT</td>
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(a) Bold indicates that the protein was identified in the alkaligrass leaves.
(b) Peptide sequence: The sequence is followed by the accession number of the protein.
(c) Ratio ± S.D.: The ratio is calculated based on the relative expression levels of the proteins.

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<th>Biological function(c)</th>
<th>Peptide sequence(c)</th>
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<td>EMT19581</td>
<td>Stabilizing PSI complex</td>
<td>GPQLPPPT^5PGPR</td>
<td>5.82 ± 3.57 4.35 ± 1.89</td>
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<td>Photosynthetic electron transfer chain related protein (3)</td>
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<td></td>
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<tr>
<td>Cytochrome f(Cyt f)</td>
<td>AIEA21</td>
<td>Electron transport</td>
<td>VQLY^17EMF</td>
<td>1.77 ± 0.15 1.91 ± 0.17</td>
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<td>Predicted protein, containing psam13806, BAK01659</td>
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<td>Rieske-like (2Fe-2S) domain (ISP)^</td>
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<tr>
<td>Ferredoxin-NADP+ reductase, chloroplastic KerH37858 (EPR)</td>
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<td>Electron transport</td>
<td>LLYT^30DAEGVVR</td>
<td>1.23 ± 0.35 2.18 ± 0.35</td>
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<td>Calvin cycle (11)</td>
<td></td>
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<tr>
<td>Ribulose bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>EHE75948</td>
<td>Activate Rubisco</td>
<td>LGIAPIM^66AGELESGNAGEPAK</td>
<td>0.27 ± 0.01 0.41 ± 0.01</td>
</tr>
<tr>
<td>Hypothetical protein, fructokinase</td>
<td>ABA91552</td>
<td>Activate Rubisco</td>
<td>GLAYDIS^66DQQQDTR</td>
<td>0.87 ± 0.10 0.60 ± 0.02</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)^</td>
<td>ABB03412</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>GLDP^77KKDENVNSQPFR</td>
<td>1.33 ± 0.08 1.56 ± 0.06</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>CAB85674</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>1.59 ± 0.10 1.10 ± 0.38</td>
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<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>CAA90418</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>ES^77GSTWTWTVDGLTSLSR</td>
<td>1.78 ± 0.19 1.48 ± 0.48</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>CAC04358</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>TTVWTWTVDGLTSLSR</td>
<td>2.00 ± 0.66 1.34 ± 0.02</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>AAG43994</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>TTVWTWTVDGLTSLSR</td>
<td>1.77 ± 0.22 1.62 ± 0.21</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>CAA93205</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>TTVWTWTVDGLTSLSR</td>
<td>0.51 ± 0.14 0.80 ± 0.07</td>
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<tr>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBL)</td>
<td>AFA72686</td>
<td>Combines CO2 to produce 3-phosphoglycerate</td>
<td>TTVWTWTVDGLTSLSR</td>
<td>2.56 ± 0.54 2.53 ± 0.90</td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (A)^</td>
<td>EMT31124</td>
<td>Reversibly converts 1,3-BPG to GAP</td>
<td>GDSS^76PLEVNDTGGVK</td>
<td>1.72 ± 0.08 0.99 ± 0.53</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase (FBA)</td>
<td>AAB70542</td>
<td>Reversibly catalyze FBP to GAP and DHAP</td>
<td>GLVPGSA^26NINESWQCGDLG</td>
<td>31.27 ± 25.90 33.01 ± 10.64</td>
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<tr>
<td>Carbohydrate and energy metabolism (12)</td>
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<tr>
<td>ATP synthase (9)</td>
<td>KEH17711</td>
<td>ATP synthesis</td>
<td>GEIHAS^17ESR</td>
<td>0.47 ± 0.02 0.75 ± 0.02</td>
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<tr>
<td>ATP synthase subunit alpha, chloroplastic</td>
<td>EMS64844</td>
<td>ATP synthesis</td>
<td>NPLAA^18NVIAGLAVGLAS^29PGP</td>
<td>1.18 ± 0.37 1.62 ± 0.13</td>
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<td>ATP synthase subunit alpha, chloroplastic</td>
<td>EAI10071</td>
<td>ATP synthesis</td>
<td>TI^2^GLGQVMGSLVEQPAERT</td>
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<td>ATP synthase subunit alpha</td>
<td>ABH02573</td>
<td>ATP synthesis</td>
<td>AITLLEENKS^26KK</td>
<td>0.46 ± 0.01 0.47 ± 0.05</td>
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<td>ATP synthase subunit alpha</td>
<td>CAB89989</td>
<td>ATP synthesis</td>
<td>GRNTGGQPNA^28TCEVQQLGNNR</td>
<td>3.72 ± 0.55 3.75 ± 1.84</td>
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<tr>
<td>ATP synthase subunit alpha</td>
<td>AAK72724</td>
<td>ATP synthesis</td>
<td>DT^8^VRQOQYCEVQQLGNNR</td>
<td>1.07 ± 0.05 0.48 ± 0.06</td>
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<tr>
<td>ATP synthase subunit alpha</td>
<td>ABR67212</td>
<td>ATP synthesis</td>
<td>GEGILLS^336GDLAQPQAFYLV</td>
<td>1.06 ± 0.03 1.97 ± 0.38</td>
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<tr>
<td>Hypothetical protein, ATP synthase subunit B^</td>
<td>ACP08646</td>
<td>ATP synthesis</td>
<td>AEG^3^KELVEAK</td>
<td>0.56 ± 0.07 -</td>
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<tr>
<td>Succrose and fatty acid biosynthesis (2)</td>
<td>EAY89475</td>
<td>ATP synthesis</td>
<td>AELGCVK^46DA^26EEVR</td>
<td>0.59 ± 0.07 -</td>
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<tr>
<td>Succrose-phosphate synthase (SPS)</td>
<td>AAQ14552</td>
<td>ATP synthesis</td>
<td>LVS^34^DDEDQESK</td>
<td>0.76 ± 0.02 0.62 ± 0.03</td>
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<tr>
<td>Pyruvate dehydrogenase E1 component subunit alpha-2 (PDH)</td>
<td>QHHXYO</td>
<td>Fatty acid biosynthesis</td>
<td>YIEGHS^15MSDPGYVR</td>
<td>2.28 ± 0.26 2.74 ± 0.20</td>
</tr>
<tr>
<td>Phosphoglycerate kinase, chloroplastic (PGK)</td>
<td>XP_003568189</td>
<td>Reversibly catalyzes 3-PG to produce 1,3-BPG</td>
<td>PGVVALIDAEVTYSV^41</td>
<td>1.12 ± 0.01 0.51 ± 0.10</td>
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<tr>
<td>Stress and defense (2)</td>
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<tr>
<td>Predicted protein, containing pfam12481, BAK01943</td>
<td></td>
<td>Stress response</td>
<td>QVAHAPQELNS^78PR</td>
<td>1.77 ± 0.16 2.53 ± 0.18</td>
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<tr>
<td>Predicted protein, containing pfam12481, XP_004960054</td>
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<td>Stress response</td>
<td>QVAHAPQELNS^8PR</td>
<td>2.05 ± 0.15 2.87 ± 0.03</td>
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<tr>
<td>Membrane and transport (8)</td>
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<tr>
<td>Na^+/H^+ antipporter</td>
<td>BAI06107</td>
<td>Na^+ compartmentalization</td>
<td>GFVPFP^22G^26PVER</td>
<td>1.19 ± 0.11</td>
</tr>
<tr>
<td>Villin-2</td>
<td>EMT05628</td>
<td>Actin reverse polymerization</td>
<td>AAAAALSVLTAEQQS^30SD NLR</td>
<td>1.79 ± 0.24</td>
</tr>
<tr>
<td>Predicted protein, villin-2-like^</td>
<td>BAJ119166</td>
<td>Actin reverse polymerization</td>
<td>AAAAALSLVTAEQQS^30SD NLR</td>
<td>1.68 ± 0.12</td>
</tr>
<tr>
<td>Predicted protein, protein curvature thylakoid 1A (CURTIA)^</td>
<td>BAK02001</td>
<td>Induce thylakoid membrane curvature</td>
<td>ASDDTDSTSA^26GDELVDLDDLK</td>
<td>2.13 ± 0.30</td>
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<tr>
<td>Predicted protein, protein curvature thylakoid 1A (CURTIA)^</td>
<td>XP_004965129</td>
<td>Thylakoid organization</td>
<td>QSHS^3^DSLDTMARR</td>
<td>0.96 ± 0.02</td>
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<tr>
<td>Hypothetical protein, fructokinase-like 2 (FLN)^</td>
<td>EMT047290</td>
<td>Chloroplast thylakoids development</td>
<td>VAEQLS^3^DDEGDQSK</td>
<td>0.65 ± 0.03 0.57 ± 0.05</td>
</tr>
<tr>
<td>Hypothetical protein, fructokinase-like 2 (FLN)^</td>
<td>EMT047290</td>
<td>Chloroplast thylakoids development</td>
<td>VAEQLS^3^DDEGDQSK</td>
<td>0.58 ± 0.01 0.76 ± 0.06</td>
</tr>
</tbody>
</table>
Table 1 (continued from previous page.)

<table>
<thead>
<tr>
<th>Protein name(^a)</th>
<th>Accession no(^b)</th>
<th>Biological function(^c)</th>
<th>Peptide sequence(^d)</th>
<th>Ratio ± S.D.^e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc finger protein VAR3, chloroplastic (VAR3)</td>
<td>*EMS67681</td>
<td>Chloroplast development</td>
<td>SDS<strong>PQVFAN</strong>SK</td>
<td>1.69±0.18 1.29±0.06</td>
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<tr>
<td>Putative protein phosphatase 2C (PP2C)</td>
<td>EMT08603</td>
<td>Signal transduction</td>
<td>SIS**ADGLNSLR</td>
<td>0.98 ± 0.00 2.67 ± 0.97</td>
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<tr>
<td>Calcium sensing receptor, chloroplastic (CAS)</td>
<td>AAS00828</td>
<td>Signal transduction</td>
<td>KLPGS**VDG</td>
<td>0.73 ± 0.01 2.46 ± 0.19</td>
</tr>
<tr>
<td>Gene expression, protein synthesis and turnover (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA binding protein (RNA-BP)</td>
<td>EMT29522</td>
<td>Transcription</td>
<td>GQDPQGSMSS**PGPGR</td>
<td>1.89 ± 0.21 1.98 ± 0.07</td>
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<tr>
<td>Hypothetical protein, containing cd12432, RNA recognition motif (RRM)*</td>
<td></td>
<td>Transcription</td>
<td>SDSTASGDS**PKER</td>
<td>3.02 ± 0.16 1.75 ± 0.11</td>
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<tr>
<td>Hypothetical protein, containing pflm07727, CAN161094</td>
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<td>Transcription</td>
<td>PIDT**FIDVNIK</td>
<td>0.90 ± 0.07 7.99 ± 1.01</td>
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<tr>
<td>Predicted protein, zinc finger protein (ZF)*</td>
<td>BAP99149</td>
<td>Transcription</td>
<td>LQS<strong>PGAQQYYGT</strong>SR</td>
<td>- 2.14 ± 0.48</td>
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<tr>
<td>Serine/arginine-rich splicing factor 33-like</td>
<td>XP_004980077</td>
<td>RNA processing</td>
<td>RGY**GGGGGGGGGGGGGGG</td>
<td>5.55 ± 0.78 0.62 ± 0.04</td>
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<tr>
<td>Predicted protein, probable alanine-tRNA ligase (AlaRS), chloroplastic*</td>
<td>ACO61321</td>
<td>Protein synthesis</td>
<td>MAS**AASSSTETAPK</td>
<td>0.04 ± 0.02 -</td>
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<tr>
<td>Predicted protein, probable alanine-tRNA ligase (AlaRS), chloroplastic*</td>
<td>ACO61321</td>
<td>Protein synthesis</td>
<td>MAS**AASSSTETAPK</td>
<td>0.04 ± 0.02 -</td>
</tr>
<tr>
<td>30S ribosomal protein 1, chloroplastic</td>
<td>*XP_003559644</td>
<td>Protein synthesis</td>
<td>EWQTAAASSF**EDVEED</td>
<td>0.91±0.27 0.63±0.02</td>
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<tr>
<td>ATP-dependent zinc metalloprotease FtsH</td>
<td>EMT25919</td>
<td>Protein degradation</td>
<td>QVS<strong>VDVP</strong>DR</td>
<td>1.52 ± 0.06 1.86 ± 0.04</td>
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<tr>
<td>NHL repeat-containing protein 2</td>
<td>EMS60685</td>
<td>Function unknown</td>
<td>TLDL<strong>TVQPP</strong>S**PKPK</td>
<td>0.49 ± 0.07 -</td>
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<tr>
<td>Unnamed protein, no putative conserved CBF59389</td>
<td>Function unknown</td>
<td>RESLY<strong>GSLS</strong>LED**IVR</td>
<td>0.01 ± 0.00 0.80 ± 0.10</td>
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<tr>
<td>Hypothetical protein, containing pflm04398, protein of DAA41082</td>
<td>Function unknown</td>
<td>NLF<strong>F</strong>RLAGR</td>
<td>0.01 ± 0.00 0.03 ± 0.01</td>
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<tr>
<td>Hypothetical protein</td>
<td>XP_010104076</td>
<td>Function unknown</td>
<td>EEEPIEQYWQ<strong>T</strong>AGER</td>
<td>11 ± 3.25 54 ± 23.02</td>
</tr>
</tbody>
</table>

Note: ^a The name and functional categories of the proteins identified by LC-MS/MS, protein names marked with an asterisk (*) have been edited by us depending on searching against NCBI non-redundant protein database for functional domain. The abbreviations for the protein names are indicated in the bracket after protein names.

^b Database accession number from NCBI nr, Pounds (#) indicate that the chloroplast-localized phosphoprotein was identified in the leaf phosphoproteome.

^c Biological function of the identified proteins.

^d Identified phosphopeptide, the phosphorylated amino acids sites are highlighted.

^e The average ratios of phosphopeptides from three biological replicates, error bar indicates ± standard deviation (S.D.). Protein samples were prepared from leaves and chloroplasts of *Puccinellia tenuiflora* that treatment with 0 mM, 150 mM, and 200 mM Na2CO3 for 24 h, respectively. '-' indicate that the phosphorylation site without quantitative information in three replicates.

Abbreviations: 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate.