Chloroplast biogenesis depends on an extensive interplay between the nucleus, cytosol, and chloroplasts, involving regulatory nucleus-encoded chloroplast proteins, as well as nucleocytosolic photoreceptors such as phytochromes (phys) and other extrachloroplasmic factors. However, this whole process is only partially understood. Here, we describe the role of VENOSA4 (VEN4) in chloroplast development and acclimation to adverse growth conditions. A 35S:VEN4:eGFP fusion protein localizes to the nucleus in Arabidopsis (Arabidopsis thaliana) protoplasts, and VEN4 homologs are present in a wide range of eukaryotes including humans, where the corresponding homolog (SAMHD1) cleaves dNTPs. Defective photosynthesis in ven4 seedlings results from reduced accumulation of photosynthetic proteins and appears to be caused by a reduction in the translational capacity of chloroplasts. The negative effect of the ven4 mutation on photosynthesis can be phenotypically suppressed by germinating seeds in the presence of excess dCTP or a pool of dNTPs, implying that VEN4, like human SAMHD1, is involved in dNTP catabolism. Moreover, VEN4 activity is also required for optimal responses to cold and salt stresses. In conclusion, our work emphasizes the importance of the nucleocytosolic compartment and the fine-tuning of dNTP levels for chloroplast translation and development.
performance in phyB-9 is attributable to the lack of VEN4 and not PHYB (Yoshida et al., 2018). In pp7l mutants, levels of PHYB mRNA and its protein product are increased (Xu et al., 2019), raising the possibility that overexpression of PHYB might phenocopy the pp7l mutant. In experiments undertaken to test this assumption, our attention was drawn to VEN4, because both overexpression of PHYB (in the phyB-9 background) and a lack thereof in the phyB-9 mutant are associated with reduced photosynthetic performance, suggesting that a second mutation might be present in phyB-9. During our attempts to identify this putative second mutation, the VEN4 manuscript mentioned above (Yoshida et al., 2018) was published.

Here, we describe two further ven4 mutants. We characterize these mutants, together with pp7l-1, a phyB-9 line that still contains the original ven4 mutation, and a phyB-9 line in which that second mutation had been out-crossed, with respect to germination behavior and photosynthetic performance in both light-grown and etiolated seedlings. VEN4 is localized to the nucleus, and feeding experiments with a mixed dNTP pool and each single dNTP suggest that the protein is involved in dNTP metabolism. Additionally, VEN4, but not PHYB, is a positive regulator of chloroplast protein synthesis. Reduced photosynthetic performance was detected in both seedlings and older, but not emerging, leaves. Seed germination in all investigated mutants was reduced by exposure to cold, and both ven4 seedlings and mature plants displayed enhanced sensitivity to salt stress.

RESULTS AND DISCUSSION

PSII Activity Is Reduced in Seedlings and Older Leaves in ven4 Mutants

Because in pp7l mutants, PHYB levels are increased (Xu et al., 2019), we set out to test whether the overexpression of PHYB in pp7l might be responsible for altered chloroplast development in the mutant. To this end, we initially used a phyB-9 mutant line that overexpresses a PHYB-YFP fusion under the control of the 35S promoter (oe-PHYB [phyB-9]; Medzihradzky et al., 2013), and measured the Fv/Fm as an indicator of chloroplast development. Overexpression of PHYB was indeed accompanied by reduced Fv/Fm values in 3-d-old seedlings, although this effect was less pronounced than in the pp7l-1 mutant (Supplemental Fig. S1A). Moreover, in 4-week-old plants, overexpression of PHYB phenocopied the pp7l phenotype with respect to both reduced Fv/Fm and reduced rosette size (Supplemental Fig. S1A). If overexpression of PHYB alone is sufficient to cause the photosynthesis phenotype of the pp7l mutant, then that phenotype should be corrected in the pp7l phyB-9 mutant. However, the double mutant still exhibited reduced photosynthetic performance (Supplemental Fig. S1). In addition, when Fv/Fm values were measured daily in 3- to 7-d-old seedlings (Supplemental Fig. S1B) and 4-week-old plants (Supplemental Fig. S1A), a reduction of Fv/Fm was also seen in the phyB-9 mutant, although the phyA phyB double mutant displayed wild-type–like Fv/Fm values (Xu et al., 2019). The Fv/Fm values were reduced to a similar intermediate extent in phyB-9 and oe-PHYB (phyB-9) seedlings. On the face of it, these findings imply either that a delicately adjusted level of PHYB accumulation is required for proper chloroplast development, or that overexpression of PHYB in the phyB-9 line fails to complement the phyB-9 photosynthesis phenotype, although it rescues the hypocotyl growth phenotype. Taken together, these data point to the presence of a second mutation in the phyB-9 line. Indeed, during the process of double mutant selection, we had observed an unexpected segregation ratio, which supports the supposition that the phyB-9 line might harbor a second mutation. During our attempts to identify this putative second mutation, a manuscript was published as a Letter to the Editor in Plant Physiology in which the second-site mutation was localized to the VENOSA4 (VEN4; AT5G40270) gene (Yoshida et al., 2018). We confirmed that our phyB-9 and oe-PHYB (phyB-9) lines also harbored the mutation in VEN4 (Supplemental Fig. S2A). As already highlighted by Yoshida et al. (2018), phyB is one of the most extensively studied proteins in Arabidopsis, and the phyB-9 mutant line was frequently used because of its Col-0 background and the EMS-induced premature stop codon (Reed et al., 1993). This line was also used in recent studies of chloroplast development and chloroplast-to-nucleus signaling (Ganguly et al., 2015; Lv et al., 2019).

In an experiment designed to determine the roles of phyA and phyB in UV-C radiation stress, control phyB-9 plants had lower Fv/Fm values compared with Col-0, whereas the phyA-211 phyB-9 double mutant (which does not carry the VEN4 mutation; Yoshida et al., 2018) displayed a higher Fv/Fm value (Rusaczon et al., 2015). Therefore, we wanted to gain more insight into the functional consequences on chloroplast development and photosynthetic performance caused by the second-site mutation in VEN4. For the sake of clarity, and in accordance with the nomenclature introduced by Yoshida et al. (2018), from here on we refer to the original phyB-9 line bearing the VEN4 mutation, a back-crossed line that carries only the PHYB mutation, and the line that harbors only the VEN4 mutation as phyB-9GC, phyB-9BC, and bnen, respectively. It was noted before that Fv/Fm is not depressed in phyB-9BC but is reduced in first-node true leaves of 13-d-old phyB-9GC and bnen seedlings (Yoshida et al., 2018). It was also shown that the reduction of Fv/Fm is less pronounced in emerging leaves of phyB-9GC and bnen, which clearly contrasts with the fact that Fv/Fm is strongly reduced in emerging pp7l-1 leaves and young seedlings (Fig. 1, A and F; Supplemental Fig. S1A; Xu et al., 2019). These results raise the question of whether VEN4’s role in chloroplast development is restricted to mature stages, especially because chloroplast development in cotyledons and leaves might differ slightly (Waters and Langdale, 2009). To test this, and to
determine whether phyB has an impact on chloroplast development in early stages of plant development, $F_{\psi}/F_m$ values were measured daily in 3- to 7-d-old phyB-9OG, phyB-9BC, bnen, ven4-1, ven4-2, and pp7l-1 seedlings. The $F_{\psi}/F_m$ was measured with an imaging Chl fluorometer (Imaging PAM). Scale bars = 1 cm. B, Graph displaying $F_{\psi}/F_m$ values measured for 3- to 7-d-old Col-0 and mutant seedlings. The data represent mean values ± SD of three independent experiments, each performed with at least 10 seedlings per genotype. C, Opened green siliques of Col-0 and mutant plants grown under LD (16-h light/8-h dark) conditions. Corresponding $F_{\psi}/F_m$ pictures illustrate $F_{\psi}/F_m$. Scale bars = 0.5 cm. D, Phenotypes (top) and corresponding $F_{\psi}/F_m$ pictures (two at bottom) of 3-d-old etiolated seedlings, which had been exposed to 12-h continuous light. Scale bars = 0.5 cm. E, Graph displaying $F_{\psi}/F_m$ values measured for 3-d-old etiolated seedlings, which had been exposed to 2-24-h continuous light. The data represent mean values ± SD of three independent experiments, each performed with at least 10 seedlings per genotype. F, Images of 4-week-old Col-0 and mutant plants grown under LD conditions (−120 μmol photons m$^{-2}$ s$^{-1}$ on leaf surfaces; 16-h light/8-h dark) together with their Imaging PAM profiles. $F_{\psi}/F_m$ values are indicated for selected leaves of the same age. Scale bars = 1 cm.

Figure 1. Phenotypic characterization of phyB-9GC, phyB-9GC (the original phyB-9 line that also carries the VEN4 mutation), phyB-9GC (a back-crossed line which harbors only the PHYB mutation), bnen (a back-crossed line which bears only the VEN4 mutation), ven4-1, ven4-2, and pp7l-1 seedlings grown under long-day (LD; 16-h light/8-h dark) conditions. The $F_{\psi}/F_m$ was measured with an imaging Chl fluorometer (Imaging PAM). Scale bars = 1 cm. B, Graph displaying $F_{\psi}/F_m$ values measured for 3- to 7-d-old Col-0 and mutant seedlings. The data represent mean values ± SD of three independent experiments, each performed with at least 10 seedlings per genotype. C, Opened green siliques of Col-0 and mutant plants grown under LD (16-h light/8-h dark) conditions. Corresponding $F_{\psi}/F_m$ pictures illustrate $F_{\psi}/F_m$. Scale bars = 0.5 cm. D, Phenotypes (top) and corresponding $F_{\psi}/F_m$ pictures (two at bottom) of 3-d-old etiolated seedlings, which had been exposed to 12-h continuous light. Scale bars = 0.5 cm. E, Graph displaying $F_{\psi}/F_m$ values measured for 3-d-old etiolated seedlings, which had been exposed to 2-24-h continuous light. The data represent mean values ± SD of three independent experiments, each performed with at least 10 seedlings per genotype. F, Images of 4-week-old Col-0 and mutant plants grown under LD conditions (−120 μmol photons m$^{-2}$ s$^{-1}$ on leaf surfaces; 16-h light/8-h dark) together with their Imaging PAM profiles. $F_{\psi}/F_m$ values are indicated for selected leaves of the same age. Scale bars = 1 cm.
ven4-2 (Fig. 1, A and B), indicating that inactivation of VEN4 does have an impact – albeit a weaker one than the absence of PP7L – on photosynthesis in seedlings. Because chloroplast development could be delayed by differences in germination rates, wild-type and mutant seedlings were germinated on Murashige and Skoog (MS) plates and germination rates were scored after 24, 36, 48, and 72 h. Indeed, germination efficiency of the *pp7l-1* mutant was delayed especially in the early time points, whereas 100% germination was achieved after 72 h (Supplemental Fig. S1C). Germination rates of mutants lacking PHYB were slightly, but not significantly, reduced, and those of mutants lacking VEN4 were wild type like (Supplemental Fig. S1C). Accordingly, *pp7l-1* was the only mutant with perturbed chloroplast development in developing seeds as demonstrated by the measurement of *Fv/Fm* values in opened green siliques (Fig. 1C). To exclude that a developmental delay caused by retarded germination – at least in *pp7l-1* – was responsible for impaired chloroplast development in young seedlings, we investigated etiolated seedlings and their photosynthetic performance after onset of light. To this end, wild-type and mutant seedlings were grown for 3 d in darkness and were then transferred for 24 h into continuous light. The maximum quantum yield of PSII was recorded in 2- or 4-h intervals after the onset of light exposure. Under these conditions, all mutant seedlings behaved comparable with seedlings directly germinated in light with respect to photosynthetic performance. Reductions in *Fv/Fm* were observed in the *phyB-9OG, bnen, ven4-1*, and *pp7l-1* mutant lines during the whole time-course (Fig. 1, D and E), indicating again that whereas germination differences have an impact on photosynthetic development, a delay of such development occurs also when germination differences become irrelevant in young seedlings lacking either VEN4 or PP7L.

VEN4 Is a Nuclear Protein Involved in dNTP Metabolism

Notably, VEN4 is predicted to be localized to the nucleus and/or the cytosol (The Arabidopsis Information Resource; https://www.arabidopsis.org/servlets/TairObject?id=134598&type=locus; SUBA4; http://suba.live/factsheet.html?id=AT5G40270.1). To experimentally determine the subcellular localization of VEN4, Arabidopsis protoplasts were transformed with a 35S:VEN4-eGFP construct. Fluorescence imaging of eGFP in protoplasts confirmed that VEN4-eGFP, like PP7L-eGFP (Xu et al., 2019), was targeted to the nucleus (Fig. 2A). To test whether VEN4 and PP7L genetically interact, *pp7l-1 bnen* double mutants were selected from the F2 offspring of *pp7l-1 phyB-9OG* crosses that no longer harbored the *phyB-9* mutation. In 4-week-old plants, *Fv/Fm* was reduced to 0.6 in all mutant alleles lacking VEN4 and to 0.54 in *pp7l-1* mutants compared with the wild type under control conditions (Fig. 2B). Addition of either 2 mM dATP or 2 mM dGTP had detrimental effects: germination efficiencies of wild-type and *ven4* mutant lines were reduced and *Fv/Fm* values in all three genotypes were equally low. Addition of 2 mM dNTPs reduced germination efficiency, but had only a low impact on photosynthetic activity of the wild type. Intriguingly, under these conditions, photosynthetic activity of seedlings lacking VEN4 was recovered. Addition of either 2 mM dCTP or 2 mM dTTP to the medium did not alter wild-type properties with respect to germination and photosynthetic activity. However, *ven4* seedling behaved wild type-like in the presence of 2 mM dCTP, and performed worse, comparable with that under control conditions, in the presence of 2 mM dTTP. The positive effect of dCTP addition was specific for *ven4* and is not applicable to all mutants defective in chloroplast gene expression, because the *pp7l* photosynthesis phenotype did not recover by the addition of dCTP to the medium (Fig. 2C). These results suggest that Arabidopsis VEN4, like human SAMHD1, is indeed involved in dNTP catabolism, in ways that paradoxically lead to presumably a lower dCTP pool and higher dGTP, dTTP, and dATP pools in the absence of VEN4. Here, it has to be said that the maintenance of a balanced dNTP pool is complex. In the presence of all four dNTP substrates, the hydrolysis rates by SAMHD1 are in the order of dGTP > dCTP > dTTP > dATP, which is the opposite of that obtained in the single dNTP substrate experiments (dATP > dCTP > dTTP > dGTP; Ji et al., 2014). Excess dGTP on the other hand leads to a decrease of dCTP and dTTP, but increased dATP pools (Kunz, 1982). RNR is responsible for de novo dNTP synthesis, and this process is under allosteric regulation in which dATP is an inhibitor of RNR activity (Hofer et al., 2012).

Lack of VEN4 Impairs Chloroplast Translation

But why is photosynthetic activity reduced in *ven4* mutants? This is a consequence of reduced accumulation of photosynthetic proteins, because PsAD and PsBD
(D2), representative subunits of PSI and PSII, respectively, were less than half as abundant in phyB-9OG, bnen, ven4-1, and ven4-2 seedlings as in wild type (Fig. 3A). In addition, staining of Western blots with Coomassie Brilliant Blue indicated reduced amounts of RbcL. In contrast, levels of psaD, psbD, rbcL, and psbB transcripts were virtually unchanged or slightly elevated in phyB-9OG, bnen, ven4-1, and ven4-2 seedlings, as quantified on RNA gel blots and by reverse transcription quantitative PCR (RT-qPCR; Fig. 3, B and C). Although psbA (encoding D1) and psaA transcripts were less abundant in ven4-1 and ven4-2 (Fig. 3C), the reduced amounts of chloroplast-encoded proteins in the ven4 mutants cannot be attributed solely to a relative deficiency of their transcripts. To evaluate whether perturbation of VEN4 function also reduces chloroplast translational capacity, as seen in the pp7l-1 mutant (Xu et al., 2019), the synthesis of plastid-encoded thylakoid membrane proteins was studied by in vivo labeling. After pulse labeling for 30 min, de novo synthesis of the D1 (encoded by psbA) and D2 (encoded by psbD) proteins was found to be reduced to less than half of the wild-type levels in phyB-9OG, bnen, ven4-1, and ven4-2 seedlings (Fig. 3D), although psbD (encoding the D2 protein) transcript levels were slightly elevated. These findings suggest that chloroplast transcription is not primarily affected in ven4 seedlings, but hint at a perturbation of downstream processes in chloroplast gene expression, which would account for the reduction in synthesis of chloroplast proteins and the delay in chloroplast development in ven4 seedlings. Notably, accumulation of chloroplast-encoded proteins and transcripts and the translation capacity of phyB-9BC seedlings were either unchanged or enhanced compared to the wild type, again supporting the notion that a lack of phyB does not have a negative impact on chloroplast development (Fig. 3).

VEN4 and PP7L Are Required for Chloroplast Biogenesis under Various Stresses

Disturbances of organellar gene expression can result in altered stress responses. Examples include the reduced cold tolerance of plants lacking chloroplast ribonucleoprotein CP29A or CP31A (Kupsch et al., 2012),
Figure 3. The nuclear VEN4 protein functions in chloroplast gene expression. A, Immunoblot analysis of representative thylakoid proteins. Total protein extracts from wild-type (Col-0), phyB-9OG, phyB-9BC, bnen, ven4-1, and ven4-2 seedlings (78 h after imbibition) were fractionated by SDS-PAGE, and blots were probed with antibodies raised against PsaD and PsbD. Decreasing levels of wild-type proteins were loaded in the lanes marked Col-0 and 0.5x Col-0 (1x Col-0 = 5 μg). The membrane stained with Coomassie Brilliant Blue (C.B.B.) served as a loading control. Please note that samples were run on the same gel, and RbcL protein levels are decreased in seedlings bearing the ven4 mutation. The numbers represent mean values ± SD from three different plant pools. The results were normalized to Col-0, which was set to 100. B, Steady-state levels of transcripts of photosynthetic genes. Total RNA was isolated from Col-0 and mutant seedlings (78 h after imbibition), and 7-μg aliquots were fractionated in a formaldehyde-containing denaturing gel, transferred onto a nylon membrane, and probed with [α-32P]dCTP-labeled complementary DNA fragments specific for PsaD and psbD transcripts. rRNA was visualized by staining the membrane with methylene blue (M. B.) and served as a loading control. C, RT-qPCR analysis of wild-type (Col-0) and mutant plants. RT-qPCR was performed with primers specific for the transcripts of interest and for AT4G36800 (RCE1, encoding a RUB1-conjugating enzyme), which served as a control. Expression values are reported relative to the corresponding transcript levels in Col-0. The results were normalized with respect to the expression level of RCE1. Data are shown as mean values ± SD from three independent experiments. Statistically significant differences (Tukey’s test; P < 0.05) between wild-type and mutant samples are indicated by an asterisk. D, In vivo pulse-labeling of thylakoid membrane proteins with [35S]Met in the presence of cycloheximide indicates that translation occurs at reduced rates in chloroplasts of lines lacking functional VEN4, but at normal (wild-type) rates in the phyb-9 back-crossed line lacking functional phyB. Thylakoid proteins were loaded based on equivalent amounts of radioactivity (100,000 cpm) and were resolved by SDS-PAGE (SDS) after pulse-labeling for 30 min and visualized by autoradiography. Quantification of radioactivity was performed by ImageQuant with equal amounts of all samples loaded on a single gel. The radioactivity incorporated into D1/D2 proteins of Col-0 after the 30-min pulse was set to 100%. Note that the membrane stained with C.B.B. confirms that chloroplast protein levels are lower in the lines lacking functional VEN4 than in the wild type.
and the diminished tolerance to salt and high light intensities seen in plants lacking PP7L (Xu et al., 2019). Multiple lines of evidence support the notion that photoreceptors, especially phyB, are also involved in direct perception and/or modulation of stress responses to low and high temperatures (Kim et al., 2002; Franklin and Whitelam, 2007; Lee and Thomashow, 2012; Jung et al., 2016; Legris et al., 2016; Song et al., 2017). Although the phyb-9 mutant was used in some of these experiments (Kim et al., 2002; Lee and Thomashow, 2012; Legris et al., 2016), the role of phyB in thermomorphogenesis (temperature dependence of hypocotyl length) is securely supported by the usage of further phyB alleles and modeling approaches (Legris et al., 2016). To clarify whether VEN4 and/or phyB are involved in cold- (4°C, not freezing), heat-, or salt-stress tolerance, and whether PP7L is involved also in cold and heat tolerance, phyB-9 OG, phyB-9 BC, bnen, ven4-1, pp7l-1, and wild-type seedlings were germinated and grown for 6 weeks under cold conditions (4°C). Additionally, 18-d-old plants were shifted for 12 d to 4°C and 32°C, respectively, and 14-d-old plants were watered for 7 d with NaCl. After 6 weeks under cold conditions, the majority of wild-type seedlings had well-developed green cotyledons and, accordingly, \( F_v/F_m \) was detectable in wild-type seedlings, indicating the formation of functional PSII complexes (Fig. 4A). On the contrary, phyB-9 OG, phyB-9 BC, bnen, ven4-1, and pp7l-1 cotyledons accumulated anthocyanins, but did not turn green, and some cotyledons of phyB-9 BC only started to turn green (Fig. 4A). When adult plants were shifted to cold conditions, \( F_v/F_m \) values of wild type and all mutants were reduced after 1 d compared with control conditions (Fig. 4B; Supplemental Fig. S3A). All mutants except of phyB-9 BC did not show a noticeable difference in the trend of their \( F_v/F_m \) values during the next 12 d when compared with the wild type; phyB-9 BC, indeed, was more sensitive to cold treatment corroborating the involvement of phyB in temperature responses. \( F_v/F_m \) values remained more or less constant in wild type and phyB-9 BC under heat conditions (Fig. 4B; Supplemental Fig. S3A). However, heat helped all other genotypes to recover photosynthetic activity, especially in plants lacking VEN4 that recovered to wild-type-like \( F_v/F_m \) values after 5 d (Supplemental Fig. S3A). When plants were watered with NaCl, growth of all plants was retarded, especially that of the pp7l-1 mutant, and the reduction in \( F_v/F_m \) of plants lacking VEN4 was exacerbated (Fig. 4B). The enhanced sensitivity of ven4 mutants to NaCl was reproducible when seeds were germinated in the presence of NaCl; the \( F_v/F_m \) phenotype of bnen and ven4 seedlings became evident on salt-supplemented medium, but was not apparent under control conditions (Supplemental Fig. S3B). The enhanced sensitivity of the pp7l-1 mutant to NaCl was manifested by the substantially retarded growth of pp7l-1 seedlings (Supplemental Fig. S3B). This suggests that VEN4 and – to a lesser extent – PP7L are both necessary for optimal tolerance of salt stress.

Figure 4. Lack of either VEN4, PHYB, or PP7L affects tolerance responses to varying degrees. A, Images of wild-type (Col-0), phyB-9 OG, phyB-9 BC, bnen, ven4-1, and pp7l-1 seedlings grown for 6 weeks on MS under cold stress (4°C, 100 μmol photons m\(^{-2}\) s\(^{-1}\)). The Imaging PAM pictures show the \( F_v/F_m \). Scale bar = 1 cm. B, Images of wild-type (Col-0), phyB-9 OG, phyB-9 BC, bnen, ven4-1, and pp7l-1 plants grown for 18 d (cold and heat experiment) or 14 d (salt experiment) under long-day conditions (LD; 120 μmol photons m\(^{-2}\) s\(^{-1}\)) or 14 d (salt experiment) under long-day conditions (LD; 120 μmol photons m\(^{-2}\) s\(^{-1}\)) or 14 d (salt experiment) under long-day conditions (LD; 120 μmol photons m\(^{-2}\) s\(^{-1}\)). The plants have been subsequently shifted for the indicated time periods to cold (LD, 4°C and 35 μmol photons m\(^{-2}\) s\(^{-1}\) or heat (continuous light, 32°C) conditions, respectively, or were watered with salt (500 mM NaCl). The Imaging PAM pictures show the \( F_v/F_m \). Scale bar = 2 cm.
CONCLUSION

Overall, these data show that the nuclear proteins PP7L and VEN4 are independently required for physiologically optimal chloroplast development. PP7L acts mainly in cotyledons and emerging leaves, whereas VEN4 acts on chloroplast development in older leaves and has a weaker function in cotyledons. The negative impact on chloroplast development seen in phyB-9GC seedlings is entirely attributable to the bnen mutation, as the lack of PHYB alone (in phyB-9GC) can actually enhance chloroplast development in young seedlings. It has to be noted here that delayed chloroplast development in bnen or ven4 mutants can be partially promoted by simultaneous loss of PHYB and VEN4 in phyB-9GC plants (see Fig. 1; Supplemental Fig. S3), uncovering an epistatic relationship between phyB and ven4. In addition, biochemical activity of VEN4 is required for optimal responses to cold and salt stresses, because dNTP imbalances in ven4 mutants render plants more sensitive to these stresses. Therefore, our results indicate that finely tuned dNTP levels are necessary for chloroplast development and acclimation to adverse environmental conditions.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (Arabidopsis thaliana) plants used in this study are in the Col-0 ecotype background. The transfer DNA insertion lines SALK_023714 (ven4-1) and SALK_077401 (ven4-2) were obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/); phyb-9 (phyb-9GC) and pp7l-1 have been already described (Yoshida et al., 2018; Xu et al., 2019). Seeds of P35S:PHYB-YFP phyb-9GC were kindly provided by Ferenc Nagy (Medzihradszky et al., 2013). To obtain pp7l-1 bnen, pp7l-1 was crossed with phyb-9GC. phyb-9GC was crossed with Col-0 to obtain phyb-9GC and bnen. Primer sequences are listed in Supplemental Table S1. Unless specifically noted, wild-type and mutant seeds were sterilized with 20% (v/v) bleach and 0.01% (v/v) Triton X-100 and sown on half-strength MS medium, pH 5.8, containing 0.8% (w/v) agar (Sigma-Aldrich) and 1% (w/v) Suc, and placed in the dark at 4°C for 2 d to ensure synchronized germination. The maximum yield of chlorophyll fluorescence in the dark-adapted state (Fo), was used to calculate the Fv/Fm. The calculations of the parameters were performed using the ImagingWin software (Walz).

Protein Extraction and Immunoblotting

For immunoblot analysis, 100 mg cotyledon tissues from seedlings at 78 h after imbibition (HAI) were dissected and homogenized in 400 μL of sample buffer of 0.125 M Tris, 1% (v/v) SDS, 10% (v/v) glycerol, 0.05 M sodium meta-bisulfite containing 1 μl phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). The samples were cleared of insoluble debris by centrifugation at 16,000 g for 20 min at room temperature, and the supernatant was boiled with 5% loading dye [5% (w/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue, 30% (v/v) glycerol] at 95°C for 5 min. For each lane, equal amounts of total protein were resolved on a 10% (w/v) SDS-PAGE minigel and blotted 30 min onto a polyvinylidene difluoride membrane using the transblot turbo transfer system (Bio-Rad). After incubation with primary and secondary antibodies (Agrisera; catalog no. AS90 602), chemiluminescence was generated using the PierceECL western Blotting Kit (Thermo Scientific), and the blots were imaged with the Fusion FX7 system (Vilber Lourmat). Antibodies against PsaD (AS90 461, 1:1000) and PsbD (AS06 146, 1:5000) were purchased from Agrisera.

RNA Extraction and Northern Analysis

RNA extractions from 78-HAI-old seedlings (50–100 mg) using the Trizol RNA reagent (Invitrogen) and RNA gel blot analyses were performed as described by Xu et al. (2019). RT-PCR amplification products of PsaD and PsbD were used as probes for northern analyses. Primer sequences are listed in Supplemental Table S1.

Complementary DNA Synthesis and RT-qPCR Analysis

Total RNA was extracted from 78-HAI-old seedlings with the RNeasy Plant Mini Kit (Qiagen). Total RNA (1 μg) was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). Real-time qPCR was performed using the iQ5 real-time PCR instrument with the iQSYBR Green Supermix (Bio-Rad) with primers listed in Supplemental Table S1.

In Vivo Translocation Assay of Thylakoid Proteins

Chloroplast proteins were labeled in vivo using 78-HAI-old seedlings. In brief, seedlings were vacuum-infiltrated in 1 mL of labeling buffer containing 20 μg/mL cycloheximide; 10 mM Tris-HCl; 5 mM MgCl2; 20 mM KCl, pH 6.8; and 0.1% (v/v) Tween 20, and incubated for 30 min to block cytosolic translation. [35S]Met (1 μCi) was applied to the same solution, and the leaves were vacuum-infiltrated for 10 s. After a 30-min labeling pulse, thylakoid proteins were isolated with cold buffer: 300 mM Suc, 50 mM HEPES-KOH, pH 7.6, 5 mM MgCl2, 1 mM Na-EDTA, 1.25% bovine serum albumin (w/v), 22 mM ascorbate, and 10 mM NaF. Subsequently, thylakoid proteins were fractionated by 15% Tricine-SDS-PAGE gel. The gels were dried using a Geldryer (Bio-Rad) and exposed to an autoradiograph film.

Intracellular Localization of the VEN4-eGFP Fusion

The sequence of VEN4 (ATSG40270) without the stop codon was amplified from complementary DNA with primers listed in Supplemental Table S1. This amplified sequence was introduced into pDONR207 and subsequently into the destination vector pBFWG2 (35S promoter, eGFP) by the BP and LR clonase reactions (Invitrogen), respectively, generating 35S:VEN4-eGFP. Subcellular localization analysis of the fused protein was conducted in protoplasts isolated from Col-0 as described by Xu et al. (2019).

Statistical Analyses

Statistical analyses were performed with the SPSS 17.0 Statistics software.

SUPPLEMENTAL DATA

The following supplemental materials are available.

Chlorophyll Fluorescence Analysis

To measure chlorophyll fluorescence emission, the Imaging PAM (M-Series; Walz) was used according to the instructions provided by the manufacturer. Samples were dark adapted for 30 min before determination of PSII quantum efficiency (Fv/Fm) at room temperature. For cold tolerance experiments, dark acclimation was carried out at 4°C. The maximum yield of chlorophyll fluorescence in the dark-adapted state (F0) was induced by an 0.8-s pulse of saturating white light (2700 μmol photon m−2 s−1). Variable fluorescence Fv, which is defined as the difference of the maximum fluorescence (Fm) and the minimum
Supplemental Figure S1. Phenotypic characterization of the original phyB-9 and the ac-PHYB (phyB-9) lines.

Supplemental Figure S2. Identification of phyB-9 and vent mutants.

Supplemental Figure S3. VEN4 and PPTL function is required for salt tolerance.

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