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Lycopene cyclase paralog CruP protects against reactive oxygen species in oxygenic photosynthetic organisms

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Carotenoids are C40 compounds found in a wide variety of organisms, where they play important roles in photoprotection and light harvesting (1). In photosynthetic organisms, carotenoids with cyclic end groups are essential for light harvesting (2, 3). Lycopene, a linear carotenoid, is the major branch point for the formation of different cyclic carotenoids, such as α-carotene or β-carotene (4). The cyclization of the ends of lycopene is performed by a class of enzymes known as lycopene cyclases (5–8).

In photosynthetic organisms, carotenoids serve essential roles in photosynthesis and photoprotection. A previous report described CruP as a secondary lycopene cyclase involved in carotenoid biosynthesis (Maresca J, et al. (2007) Proc Natl Acad Sci USA 104:11784–11789). However, we found that cruP KO or cruP overexpression plants do not exhibit correspondingly reduced or increased production of cyclized carotenoids, which would be expected if CruP was a lycopene cyclase. Instead, we show that CruP aids in preventing accumulation of reactive oxygen species (ROS), thereby reducing accumulation of β-carotene-5,6-epoxide, a ROS-catalyzed autoxidation product, and inhibiting accumulation of anthocyanins, which are known chemical indicators of ROS. Plants with a nonfunctional cruP accumulate substantially higher levels of ROS and β-carotene-5,6-epoxide in green tissues. Plants overexpressing cruP showed reduced levels of ROS, β-carotene-5,6-epoxide, and anthocyanins. The observed up-regulation of cruP transcripts under photoinhibitory and lipid peroxidation-inducing conditions, such as high light stress, cold stress, anoxia, and low levels of CO2, fits with a role for CruP in mitigating the effects of ROS. Phylogenetic distribution of CruP in prokaryotes showed that the gene is only present in cyanobacteria that live in habitats characterized by large variation in temperature and inorganic carbon availability. Therefore, CruP represents a unique target for developing resilient plants and algae needed to supply food and biofuels in the face of global climate change.

Results

Functional Analysis of SynCruP. To confirm published reports that synCruP is a lycopene cyclase (8), we expressed both synCruP and cruA from Chlorobium phaeobacteroides (CpCruA) in E. coli BL21 containing pAC-CRT-EIB, which confers lycopene accumulation. In this system, a functional lycopene β-cyclase converts lycopene into γ-carotene or β-carotene. However, expression of pET16b synCruP in this lycopene-accumulating strain of E. coli revealed that despite high production of CruP protein (Fig. S1), there was no change in the pigments produced in comparison to a strain containing pAC-CRT-EIB and the empty pET16b vector (Fig. 1 A and B). In contrast, expression of p16-CPL1 containing synCruA led to conversion of all the lycopene into γ-carotene (Fig. 1 C). Therefore, only CruA, and not CruP, appeared to have lycopene cyclase activity in E. coli.

Zea mays CruP Protein Subcellular Localization. If CruP is a lycopene cyclase, it should be localized to chloroplasts, the site of carotenoid biosynthesis. We therefore tested the location of Zea mays CruP (zmCruP) in chloroplasts via in vitro import experiments. Incubation of zmCruP with isolated pea chloroplasts led to im-

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The authors declare no conflict of interest.

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and is absent from the soluble fraction (Fig. 2A, T), only the smaller protein remained, showing that the mature protein is completely inside the chloroplast and is protected by the outer membrane. Fractionation of chloroplasts not treated with thermolysin showed that ZmCruP is present in the membrane fraction (Fig. 2A, M) and is absent from the soluble fraction (Fig. 2A, S). After alkaline treatment (Fig. 2A, A), the membrane fraction was devoid of ZmCruP, showing that it is a peripherally membrane-bound chloroplast protein. Chloroplast localization of ZmCruP was further corroborated by transient expression in maize leaf protoplasts using a ZmCruP:GFP fusion driven by a 3S promoter. The GFP fluorescence of the fusion protein localized with chlorophyll fluorescence (Fig. 2B). Therefore, the import and transient expression experiments demonstrate chloroplast localization of ZmCruP. The precise suborganellar location is likely to be thylakoids, as suggested by proteomic surveys conducted in Arabidopsis (The Plant Proteome Database; http://ppdb.tc.cornell.edu/dbsearch/searchsample.aspx).

In Vivo Analysis of CruP from Higher Plants. Photosynthetic organisms that lack lycopene cyclase activity exhibit accumulation of lycopene along with aberrant chloroplast ultrastructure (19), which appears not to be the case for CruP mutants. Previous reports of phenotypes from cruP KOIs in cyanobacteria range from no phenotype (8) to descriptions of disordered thylakoid membranes (17). In both cases, no change in carotenoid pigment profile was observed. To test whether plant mutants of CruP might exhibit evidence of lycopene cyclase activity, we analyzed cruP KO and overexpressing Arabidopsis plants. RT-PCR confirmed an absence of cruP transcripts in the KO line (SALK_011725) (20) and overexpression of ZmCruP transcripts in the four 35S:ZmCruP transgenic lines (Fig. S2). We noted that the growth rate of the KO plants was significantly slower than that of the WT plants (Fig. 3). At 2 wk of growth on MS medium, WT plants had, on average, seven leaves, whereas KO plants had, on average, only four leaves. The pigment profile of the KO plants showed no change in levels of lutein (the hydroxylation product of α-carotene), suggesting CruP was not involved in the production of α-carotene, as had been previously suggested (8). The only consistent difference observed was that of a small peak barely noticeable in the WT, which was found to be present at levels roughly 10-fold higher in the cruP KO plants (Fig. 4).

To confirm that the presence of the unknown peak was not attributable to another random mutation within this KO line, a segregating population was obtained by crossing the KO line with the Columbia WT, followed by selling of the progeny. Eight homozygous KOIs obtained from this cross were analyzed by HPLC, and all were found to contain the unknown peak (Fig. S5C). In contrast, this peak was virtually absent in all seven of the analyzed WT plants generated from this cross (Fig. 5A). Three heterozygous plants were also analyzed and showed half as much of the unknown peak as the homozygous KO plants (Fig. 5B). We also crossed lines to produce plants that were heterozygous for the cruP KO and hemizygous for 35S:ZmCruP, the pigment profile of these plants was examined and showed complete absence of the unknown peak (Fig. 5D).

Table 1. Carotenoid gene transcripts up-regulated more than 1.5-fold by chilling stress

<table>
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<tr>
<th>Protein</th>
<th>Gene</th>
<th>20 °C, average</th>
<th>4 °C, average</th>
<th>Fold up-regulated at 4 °C</th>
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<td>NCED5</td>
<td>AT1G30100</td>
<td>3.79</td>
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<tr>
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<td>250.04</td>
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<td>HYD2</td>
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<td>88.97</td>
<td>166.32</td>
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<tr>
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<td>1,508.78</td>
<td>1.73</td>
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<td>25.13</td>
<td>39.42</td>
<td>1.57</td>
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Data obtained from published microarray data available via Genevestigator. CruP highlighted in green.
Table 2. Carotenoid gene transcripts up-regulated more than 1.5-fold by anoxia

<table>
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<th>Carotenoid</th>
<th>Protein</th>
<th>Gene</th>
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<th>No air, average</th>
<th>Fold up-regulated under dark anoxia</th>
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<td>407.7</td>
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<td>KO</td>
<td>37.6</td>
<td>57.75</td>
<td>1.54</td>
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</tbody>
</table>

Data obtained from published microarray data available via Genevestigator. CruP highlighted in green.

The retention time of the unknown peak was of a carotenoid that was more polar than α-carotene and β-carotene but less polar than α-cryptoxanthin and β-cryptoxanthin (monohydroxylated carotenones). The spectrum (Fig. 5E) suggested that this compound had fewer conjugated double bonds than β-carotene. A study of the literature for rare carotenones found in plants provided many possibilities (21–24), but none seemed more likely than β-carotene-5,6-epoxide, a carotenoid that has previously been observed in photosynthetic tissues (21). β-carotene-5,6-epoxide was synthesized (25) and subjected to HPLC analysis, where it eluted at the same time and with the same spectrum as the unknown carotenoid (Fig. 6). Additional liquid chromatography (LC)-MS analysis of the peak from the KO plant confirmed a major ion fragment with a mass of 553 [M+H]+, corresponding to the mass of β-carotene-5,6-epoxide (Fig. S3).

Because 35S:crup transcripts are up-regulated at low temperatures (Tables 1 and 2), 35S:crup KO plants were grown at 4 °C to observe the impact on levels of β-carotene-5,6-epoxide. Growth of both KO and WT plants at 4 °C for 10 d led to an increase in β-carotene-5,6-epoxide in both plants relative to plants grown at 21 °C (Fig. 7). A slight decrease in β-carotene was also observed in the 35S:crup KO plant in comparison to the WT plant (Fig. 7). This decrease in β-carotene was approximately equivalent to the increase in β-carotene-5,6-epoxide. At 4 °C, there was much more variation in β-carotene levels in both the 35S:crup KO and WT plants, but a decrease in β-carotene was still observed in the 35S:crup KO plants (Fig. 7).

Reactive Oxygen Species. Considering published reports of β-carotene-5,6-epoxide formation via reactive oxygen species (ROS)-mediated degradation of β-carotene in photosynthetic tissues exposed to high light stress (21), together with our own observations of β-carotene-5,6-epoxide accumulation in 35S:crup KO plants and up-regulation of cruP in ROS-producing conditions (cold stress and dark anoxic treatment), we considered that CruP may play a role in reducing the accumulation of ROS. Cotyledons from Columbia (WT), 35S:crup KO, and 35S:crup KO plants exposed to anoxic conditions for 1 wk were stained with nitrotetrazolium blue (NTB) to screen for levels of ROS (Fig. 8). WT plants showed partial staining, whereas 35S:crup KO plants showed staining throughout the entire cotyledon. 35S:crup KO lines showed minimal to no staining in comparison to the WT plants. These results clearly demonstrate that ROS levels in cotyledons are inversely correlated with CruP transcript levels. To investigate the connection between CruP and stress responses further, WT Columbia, 35S:crup KO, and three 35S:crup KO lines were grown for 1 mo under standard conditions at 21 °C before being transferred to 4 °C with continuous light (50 μmol·m⁻²·s⁻¹) for 2 mo. This cold stress treatment revealed a striking difference between plants that differed only in CruP levels. Columbia and 35S:crup KO plants developed deep anthocyanin staining throughout the entire plant, whereas the three overexpressor 35S:crup KO lines remained a deep green color with minimal or no anthocyanin development (Fig. 9). The anthocyanin response is consistent with increased ROS in the WT and KO plants and decreased ROS in the overexpression lines.

Coexpression Analysis of CruP. Coexpression analysis of genes encoding AtcruP and AtLCYE (Dataset S1) showed that the majority of coexpressed genes encode proteins involved in chlorophyll biosynthesis, photosystem repair, or other photosynthesis-related functions. Photosynthesis-related genes that were coexpressed with AtcruP but not AtLCYE included many genes involved in protection of PSII against oxidative damage as well as those involved in repair of PSII after damage by singlet oxygen. Such genes include those encoding thioredoxins; HCF156; the PSII reaction center D1 proteases DEG8 (26) and FtsH5 (also known as VAR1) (27); SVR3, which is important for chloroplast development in cold conditions, mutants of which suppress variegation in var2 mutants (28); and PPL1 involved in PSII repair (29). Other notable coexpressed genes include cch1-1 (gun5), which is involved in generating a putative plastid-to-nucleus signal in response to high light stress (30), as well as genes encoding dicarboxylate transporters DIT1 and DIT2, mutants of which require high CO₂ fixation (31); a ribose 5-phosphate isomerase involved in CO₂ fixation (32); and a YfhF homolog involved in inhibiting chloroplast division (33) (Dataset SL1A). Many of these genes were also coexpressed with the gene encoding CruP from Oryza sativa (Os cruP) (Dataset S1B). Chlorophyll-related genes that were coexpressed with the gene encoding AtLCYE but not AtcruP include four genes encoding proteins that are part of the NAD(P)H dehydrogenase complex that is involved in cyclic electron transfer around PSI (34, 35), as well as genes that encode proteins involved in chlorophyll synthesis (e.g., protorhodophtylide reductase) and chlorophyll binding proteins of PSI and PSIII (e.g., chlorophyll A-B binding protein) (Dataset S1C). Another gene of note is ftsZ, encoding a chloroplast division protein that functions antagonistically with the YfhF homolog mentioned above (33).

Phylogenetic Distribution. Previous reports of CruP showed distribution of the gene in higher plants and some cyanobacterial species. To determine how important CruP is for fitness in photosynthetic organisms, we performed a BLAST analysis to determine the distribution of CruP more thoroughly. A protein BLAST analysis of CruP from Synecococcus sp. PCC 7002 revealed that CruP orthologs are only found in oxygenic photosynthetic organisms. These organisms encompass various families, such as cyanobacteria, green and brown algae, diatoms, mosses, and higher plants, including both monocots and dicots. An analysis of CruA orthologs showed that as well as being found in oxygenic
cyanobacteria that lack CrtL type cyclases, CruA was present in nonoxygenic organisms, such as Chlorobi, Chloroflexi, and delta-proteobacteria. A protein BLAST analysis of CrtL from the cyanobacterium Synechococcus elongatus PCC 6301 showed that CrtL orthologs were scattered throughout various species and are by no means isolated to oxygenic photosynthetic organisms, as in the case of CruP (Table 3 and Dataset S2).

A phylogenetic tree (Fig. 10) was constructed using 16S rRNA sequences of fully sequenced cyanobacteria and mitochondrial 16S rRNA of Arabidopsis thaliana as an outlier. The tree revealed that those cyanobacteria that do not contain CruP belong to a distinct clade. Further BLAST analysis was undertaken using CsoS2 and CcmN, proteins involved in CO₂-concentrating mechanisms of distinct cyanobacterial groups, known as α-cyanobacteria and β-cyanobacteria, respectively (36). The results revealed that β-cyanobacteria contain CruP, whereas α-cyanobacteria do not. Two exceptions were noted, Thermosynechococcus elongatus BP-1 and cyanobacterium UCYN-A, which are β-cyanobacteria but do not contain CruP. The reason for this presence or absence of CruP in the separate groups is likely attributable to the different environments inhabited by these organisms. Hints as to the precise environmental factor(s) that influence the presence or absence of CruP might be gleaned from T. elongatus and cyanobacterium UCYN-A, two β-cyanobacteria that do not contain CruP (Discussion).

**Genes That Cluster with Cyanobacterial CruP.** In bacteria, genes involved in similar processes are often found clustered in the genome. We determined what genes tend to cluster with cruP in select cyanobacterial genomes to see if we could infer function of CruP. Analysis of genes that cluster with cruP revealed genes with functions similar to those of genes that are found to be coexpressed with AtcruP and OscruP. Examples include genes encoding proteins with roles related to PSII D1 degradation, such as YP_001733313, an FtsH5 homolog in Synechococcus sp. PCC 7002, and the ClpC

**Fig. 4.** HPLC analysis of carotenoids extracted from Arabidopsis plants. Columbia WT (A) and AtcruP KO (B), and absorbance spectra of the unique peak (*) identified in the AtcruP KO plants (C). AU, absorbance units.

**Fig. 5.** HPLC analysis of carotenoids extracted from a segregating population of Arabidopsis plants. Typical homozygous WT plant (A), typical heterozygous ΔcruP KO plant (B), typical homozygous ΔcruP KO plant (C), and typical heterozygous ΔcruP KO/hemizygous 35S::CruP plant (D). Absorbance spectra of the unique peak (*) identified in the ΔcruP KO plants (E), absorbance spectra of the unknown chlorophyll-like peak (F; Chl), and absorbance spectra of β-carotene (G; β). Unknown cis-carotenoids were also observed to elute between Chl and β. AU, absorbance units.
(NP_925429, YP_478720) and ClpB (YP_473669) proteases in Gloeobacter violaceus PCC 7421, Synechococcus sp. JA-2-3B′a (2-13), and Synechococcus sp. JA-3-3Ab (Fig. 11). The genes encoding the PSII reaction center proteins D2 (YP_399674) and CP43 (YP_399675) of Synechococcus elongatus PCC 7942 are also clustered near cruP, as are the genes encoding YP_001867515, a RuBisCO small subunit protein in Nostoc punctiforme PCC 73102, and YP_003721373, encoding a CO₂-concentrating mechanism protein known as CcmK in Nostoc azollae 0708 (Fig. 11). Orthologs of many of the genes that cluster with cruP in different cyanobacteria were found coexpressed with both _Ac_cruP and _Oe_cruP and are important in oxygenic photosynthetic organisms under cold stress and low CO₂, suggesting that CruP is involved in the same process in cyanobacteria as it is in plants.

Discussion

CruP Is a Chloroplast Protein but Does Not Exhibit Lycopene Cyclase Activity. Although Maresca et al. (8) observed lycopene cyclase activity from SynCruP in _E. coli_, we were not able to replicate their results despite obtaining ample expression levels of the SynCruP protein (Fig. S1). We were able to replicate cyclase activity of _Cp_CruA_. Although the differences observed between our results and those of Maresca et al. (8) could be attributable to _E. coli_ strain differences or differences in growth conditions, we did try multiple _E. coli_ strains and growth conditions, all with identical results (i.e., no cyclization of lycopene was observed). The finding that CruP is a peripheral thylakoid membrane protein in chloroplasts suggested the possibility of another chloroplast-localized role.

_CruP, β-Carotene-5,6-Epoxide, and ROS_. We identified β-carotene-5,6-epoxide in the pigment profile of Arabidopsis cruP KO plants at levels substantially higher than those found in WT or 35S:_Zm_cruP_ plants. The increase of β-carotene-5,6-epoxide in the _Ac_cruP KO plants coincided with an approximate equivalent decrease in β-carotene levels (Fig. 7). The level of β-carotene-5,6-epoxide was observed to increase in the _Ac_cruP KO plants in response to chilling conditions.

Fig. 6. Comparison of HPLC elution profiles of pigments extracted from a homozygous _A_cruP KO plant (A) to a synthesized β-carotene-5,6-epoxide standard (B). (C) Absorbance spectra of the unknown peak from the _A_cruP KO plants. (D) Absorbance spectra of the synthesized β-carotene-5,6-epoxide standard. (E) Chemical structure of β-carotene-5,6-epoxide. AU, absorbance units.

Fig. 7. Levels of β-carotene and β-carotene-5,6-epoxide displayed as a ratio of total chlorophyll from leaves of Arabidopsis plants (Columbia WT and _A_cruP KO) grown under different temperatures.

Fig. 8. ROS levels in cotyledons shown as a percent area of cotyledons stained by NTB. Lines used are Columbia WT (Col); CruP KO (K/O); and 35S:_Zm_cruP_ lines p1, pX1, pX2, and p5.
stress (Fig. 7), a condition known to up-regulate \( \text{AtCruP} \) transcripts (Table 1). \( \beta \)-carotene-5,6-epoxide has been identified in intact and isolated thylakoid membranes of spinach and \( T. \) elongatus (21). The level of \( \beta \)-carotene-5,6-epoxide in thylakoid membranes of spinach increased in proportion to light intensity. In a study of the protective role of \( \beta \)-carotene in photosystems (37), isolated bacteriochlorophyll and \( \beta \)-carotene dissolved in oxygenated acetone were exposed to light and chlorophyll molecules were observed to be protected at the expense of \( \beta \)-carotene. The first product formed in this reaction was \( \beta \)-carotene-5,6-epoxide, followed by progressively more oxygenated \( \beta \)-carotene products. Oxidation of carotenoids by singlet oxygen is an unavoidable consequence of oxygenic photosynthesis. This oxidation is especially true of \( \beta \)-carotene, which is the only carotenoid found in the core of PSII, the site of the water-splitting/oxygen-evolving complex (10, 38). The main role of \( \beta \)-carotene in the reaction center is quenching of singlet oxygen (10, 11, 39). Bleaching of this \( \beta \)-carotene by singlet oxygen has been proposed to trigger turnover of the D1 protein in the PSII reaction center (40). We showed that the absence of CruP was associated with increased ROS and increased \( \beta \)-carotene-5,6-epoxide, whereas the overexpression of CruP was associated with reduced ROS and reduced \( \beta \)-carotene-5,6-epoxide (Figs. 5 and 8). The impact of CruP overexpression on anthocyanin production, a known ROS response, in cold-treated plants was quite striking in comparison to WT and CruP KO plants (Fig. 9). WT and CruP KO plants both showed accumulation of large amounts of anthocyanins under this stress condition, whereas the overexpressors remained green and healthy. Anthocyanin accumulation is a well-characterized response of plants to increased ROS production, again showing the impact of CruP activity on ROS levels in plants treated under photoinhibitory conditions.

**CruP Transcripts Are Up-Regulated in Response to Photoinhibition.** *In silico* analysis revealed that *A. thaliana CruP* is up-regulated under chilling stress and dark anoxia (Tables 1 and 2). Cotyledons and pedicels, where CruP transcripts were shown to be elevated, have been shown to undergo photoinhibition stress and high singlet oxygen production under normal “nonphotoinhibitory” conditions, in comparison to true leaves (41, 42). Chilling stress in plants causes a range of physiological effects similar to those observed under high light stress (e.g., 43, 44). In addition, cold stress causes inhibition of the PSII D1 repair mechanism as a result of decreased membrane fluidity (45), leading to further increases of ROS. Dark anoxia treatment of plants leads to generation of nitric oxide and also, paradoxically, to increased levels of ROS, including super oxide anions and hydrogen peroxide (46, 47). Dark anoxia for prolonged periods causes peroxidation of lipid membranes (47). A recent analysis of the expression of all known carotenoid synthesis genes in *Synechococcus* sp. *PCC 7002* showed that despite the fact that “transcription levels for genes encoding enzymes producing \( \gamma \)- and \( \beta \)-carotene from geranylgeranyl-pyrophosphate were generally much lower under anoxic conditions,” cruP is, in fact, up-regulated greater than 10-fold under dark anoxic conditions and is up-regulated greater than threefold by low CO\(_2\) conditions, whereas *cruA* is down-regulated twofold under both of these photoheterotrophic conditions in comparison to true photosynthetic conditions and optimal growth conditions.

**Coexpression and Clustering of CruP with PSII-Related Protection Mechanisms.** Coexpression analysis of \( \text{AtCruP} \) revealed that \( \text{AtCruP} \) was coexpressed with genes that code for proteins that function in the protection or repair of PSII from oxidative damage (e.g., the D1 proteases FtsH5 and DEG8) or proteins involved in prevention of photoinhibition (e.g., those involved in inorganic carbon transport and fixation as well as chloroplast development in cold conditions). Cyanobacterial cruP genes were clustered with genes of the PSII reaction center and with genes involved in the repair of oxidatively damaged PSII reaction center proteins (e.g., FtsH5, ClpC, ClpB), as well as with those involved in carbon acquisition and fixation. This gene clustering pattern fits with the observation that cruP transcripts are up-regulated in both *Arabidopsis* and *Synechococcus* sp. *PCC 7002* under photoheterotrophic/ROS-producing conditions, such as low CO\(_2\) and chilling stress. In contrast, the gene encoding \( \text{AtLCYE} \) was coexpressed with genes involved in chlorophyll synthesis and photosystem assembly as well as *ftsZ*, a gene involved in chloroplast division that functions antagonistically with *yfhP* (a gene coexpressed with *cruP*) (Dataset S1). The observed coexpression

**Table 3.** Phylogenetic distribution of CruA, CrtY, and CrtL type cyclases and CruP protein

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<tr>
<th>Family</th>
<th>CrtY</th>
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<th>CruA</th>
<th>CruP</th>
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<td></td>
<td></td>
<td></td>
<td>Nonphotosynthetic</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Mostly</td>
<td>One</td>
<td>One</td>
<td>None</td>
<td>Nonphotosynthetic</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td></td>
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<tr>
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<td>Photosynthetic</td>
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</table>

Highlighted in green are oxygenic photosynthetic organisms (note that this covers the range of cruP containing organisms). Nonphotosynthetic photosynthetic organisms are highlighted in yellow.
pattern suggests differing roles for LCYE and CruP in growth and protection from oxidative damage, respectively.

**CruP Was Found Only in Oxygenic Photosynthetic Organisms.** Although all other lycopene cyclases are found in a wide variety of organisms, both oxygenic and nonoxygenic phototrophs as well as nonphototrophic organisms, CruP was found only in oxygenic phototrophs and only in conjunction with another lycopene β-cyclase. CruP was never found as the sole cyclase of any organism, whereas nonoxygenic phototrophs and nonphototrophs, as well as a few cyanobacteria, have only one lycopene cyclase (Dataset S2). This phylogenetic distribution of CruP, in comparison to other lycopene cyclases, suggests that either oxygenic photosynthesis has a requirement for more than one lycopene β-cyclase or that CruP has a function other than that of lycopene cyclization. The former hypothesis, that oxygenic photosynthetic organisms require more than one lycopene β-cyclase, seems unlikely because many cyanobacterial species have only one lycopene β-cyclase (either CrtL or CruA). Furthermore, this hypothesis does not explain the exclusion of CruP from all carotenoid-producing nonoxygenic organisms.

A phylogenetic tree was constructed based on 16S rRNA from fully sequenced cyanobacteria (Fig. 10). This tree identified evolutionarily distinct clades of cyanobacteria. One clade contained cyanobacteria with CruP, and another distinctly separate clade contained cyanobacteria that lacked CruP. The top clade (Fig. 10) was populated by open ocean cyanobacteria, encompassing all but two of the non-CruP-containing cyanobacteria. These open ocean cyanobacteria are also known as α-cyanobacteria, which are characterized by having a CO₂-concentrating mechanism involving a CsoS2 protein that is not found in β-cyanobacteria (36). Those cyanobacteria that contain CruP (bottom clade in Fig. 10) are from diverse ecological habitats, including fresh water, salt lakes, intertidal zones, hot springs, dry rocks, and symbiotic relationships, for example (36). These CruP-containing cyanobacteria, known as β-cyanobacteria, contain CO₂-concentrating mechanisms that use a CcmN protein not found in α-cyanobacteria (36). Cyanobacteria are able to exchange genetic material.
via conjugation, and would therefore retain cruP in the genome if it provided an evolutionary advantage. This distribution suggests that CruP provides increased fitness to most β-cyanobacteria but not to α-cyanobacteria. β-cyanobacteria are exposed to variety of environmental extremes; in particular, temperature fluctuations (including chilling stress) and inorganic carbon limitations are two environmental conditions that β-cyanobacteria have to deal with but α-cyanobacteria do not (36). Chilling stress and low inorganic carbon are both conditions that lead to photoinhibition and to the up-regulation of cruP transcripts. Two cyanobacteria were noted as exceptions to the β-cyanobacterial distribution of CruP: *T. elongatus* and *cyanobacterium UCYN-A* are both β-cyanobacteria that lack CruP. *T. elongatus* is a thermophilic cyanobacterium isolated from Beppu hot springs in Japan, and this cyanobacterium reportedly has a reduced set of inorganic carbon transporters in comparison to other fresh water cyanobacteria (36). It is likely that the waters at this hot spring contain high levels of inorganic carbon attributable to mixing of volcanic CO₂, as has been reported for nearby thermal springs (49). As such, this cyanobacterium would not experience cold stress or inorganic carbon limitations in its natural environment, explaining the absence of CruP in this organism. *Cyanobacterium UCYN-A* is an unusual cyanobacterium with a reduced genome and no genes encoding PSII complex proteins or carbon fixation enzymes (50, 51). BLAST analysis revealed no genes with homology to Crtl, CruA, or CruP in the complete genome of this organism (Fig. 10), suggesting that another class of lycopene cyclase may exist and adding further evidence that CruP is not required in the absence of PSII (i.e., in nonoxygenic photosynthetic organisms).

**Conclusions**

We showed that absence of CruP was associated with increased ROS and increased β-carotene-5,6-epoxide, whereas overexpression of CruP was associated with reduced ROS, reduced β-carotene-5,6-epoxide, and a significantly reduced anthocyanin response under cold stress. The above results suggest that the function of CruP is to reduce oxidative damage caused by singlet oxygen. The above conclusion would explain the presence of β-carotene-5,6-epoxide, the anthocyanin response (or lack thereof in overexpressors) observed in cold-treated plants (Fig. 9), and the slow growth of the *Arabidopsis* mutant as well as the disordered thylakoid structure of the *Synechocystis cruP* KO (17). ROS generated by high excitation pressure of the photosystems during early development can cause a failure of chloroplasts to assemble organized internal structures (52). The lack of an observable difference in the pigment profile of cyanobacterial cruP KOs (8, 17), combined with the lack of lycopene cyclase activity of *Synechocystis* CruP in our study and the limited phylogenetic distribution of CruP, strongly suggests that CruP has a function other than lycopene cyclization. Considering the consistently observed up-regulation of *cruP* transcripts to photoinhibitory ROS-producing conditions, the limited phylogenetic distribution of CruP, and the inverse association between *cruP* transcript levels and ROS levels (and chemical markers of ROS levels), it appears that CruP plays a role, directly or indirectly, in reducing ROS levels in oxygenic photosynthetic organisms under photoinhibitory stress. Thus, CruP represents a unique target for developing resilient plants and algae needed to supply food and biofuels in the face of global climate change. The up-regulation of *cruP* during cold and anoxic conditions, such as flooding, suggests also that *cruP* will be an important locus to consider in screening for cold and submergence (anoxia) tolerance in plants.

**Materials and Methods**

**Plasmids Used in This Study.** Full details on plasmid construction are provided in SI Materials and Methods. pUC35S:cruP:GFP, pTNT-<i>apoCruP</i>:StrepTag, and pRed-<i>apoCruP</i> contained the full-length 2<sup>–</sup>5<sup>–</sup>cruP transcript. p16-CPL1 and pET-<i>apoCruP</i> are pET16b-based vectors containing the *C. phaeobacteroides* <i>apoCruA</i> and *Synechococcus* sp. PCC 7002 cruP ORFs, respectively.

**Bacterial Complementation Studies.** *E. coli* BL21 (DE3) cells carrying plasmid pACCRT-EIB (12), which confers lycopene accumulation, were additionally transformed with either pET-<i>apoCruP</i>, p16-CPL1, or empty pET-16b as a negative control. Bacterial growth and extraction of carotenoids were performed as described previously (53) (SI Materials and Methods).

**atcruP KO and 35S:cruP Lines.** An *A. thaliana* cruP KO line (SALK_011725) carrying a T-DNA insert in the *cruP* gene in the Columbia background was obtained from The Arabidopsis Information Resource (20). Real-time PCR was performed to confirm the absence of *cruP* transcripts in the KO line as described below. For the generation of 35S:<i>cruP</i>-overexpression *A. thaliana* plants, *Agrobacterium tumefaciens* strain GV3101 (pMP90) was transformed with pRed-<i>cruP</i> using the freeze–thaw method (54) and selected using 50 μg/mL gentamicin and 50 μg/mL kanamycin. Floral dip transformation of *A. thaliana* was performed according to the method of Clough and Bent (55) (SI Materials and Methods). Transgenic seeds were selected using a pair of red-lens sunglasses (KO’s Dark Red). Seeds that glowred under a light of wavelength 550–560 nm were used in this study for overexpression experiments. Real-time PCR was performed to confirm overexpression as described previously (56). Primers 2617 and 2618 (Table S1) were used for amplification of actin cDNA, 1871 and 2190 (Table S1) for amplification of 2<sup>–</sup>5<sup>–</sup>cruP cDNA, and 2978 and 2979 (Table S1) for amplification of *apoCruP* cDNA (SI Materials and Methods).

**Fig. 11.** CruP genes (red arrow) of select cyanobacteria show clustering with genes, the products of which are involved in carbon acquisition (blue arrows), the PSII reaction center (green arrows), and PSII reaction center repair (purple arrows).
Standard Plant Growth Conditions. Unless otherwise stated, plants were grown in a Percival Scientific growth chamber under a 16-h day/8-h night cycle with a light intensity of 30 μmol m−2 s−1 and a constant temperature of 21 °C. Plants were watered every 4 to 7 d.

Pigment Extraction and Analysis. Epoxy-5,6-β-carotene was synthesized according to the method of Barua (57) (SI Materials and Methods). Plant carotenoids were extracted by grinding roughly 30 mg of 4-wk-old plant tissue in 500 μL of 60:40 acetonitrile/ethyl acetate; 400 μL of H2O was added before vortexing and centrifugation for 5 min at 1,700 × g. The upper ethyl acetate fraction was washed, spun at 17,000 × g for 5 min, and then transferred to a different tube and dried under nitrogen before resuspension in methanol for HPLC analysis.

Separation of carotenoid and chlorophyll pigments was carried out using a Waters HPLC system equipped with a 2695 Alliance separation module, a 996-photo diode array detector, a Develosil C30 RP-Aqueous (5 μm, 250 mm × 4.6 mm) column (Phenomenex), and a Nucleosil C30 (5 μm, 4 × 3 mm) guard column (Sorvall, TX). Solvent A consisted of acetonitrile/methanol/H2O (84:2-14). and solvent B consisted of methanol/ethyl acetate (68:32). Initial flow conditions consisted of 100% A at a flow rate of 0.6 mL/min. Using a linear gradient, flow was changed to 100% B by the 60-min mark; at this point, the flow rate was increased to 1.2 mL/min and held for an additional 5 min before being reequilibrated with A for 5 min. Column temperature was held at 30 °C, and 100 μL of each sample was injected for pigment analysis.

LC-MS was performed on a Waters 2695 HPLC machine equipped with a 2998 PDA detector coupled to a Waters LCT Premiere XE TOF MS system using electrospray ionization in positive ion mode.

Chloroplast Isolation and Protein Import. 2n-CruP was transcribed and translated in vitro from pNTY4-CruP-StrepTag using SP6 polymerase in a rabbit reticulocyte lysate system (Promega) in the presence of [15S]methionine (PerkinElmer). Psa (Pssum satsum var. Green Water; Jung Seed) plants were grown in a growth chamber, at 18–20 °C in a 14-h light/10-h dark cycle for 2 months. Plants were harvested and used for chloroplast isolation after 10–14 d as described previously (58) (SI Materials and Methods).

Protoplast Isolation and Transient Expression. Maize (Z. mays var. B73) mesophyll protoplasts were isolated from 10-d old second leaves and transfected with the pUC35S::Zm-CruP-GFP vector, encoding a Zm-CruP-GFP fusion protein, by PEG-mediated transformation (59, 60) (SI Materials and Methods).

Phylogenetic Silico Analysis. The Arabidopsis Coexpression Data Mining Tools Web site (61) was used for analysis of genes that were coexpressed with α-cruP (At2g32640) and the gene encoding α-LCYE (At5g57030). The Rice Oligonucleotide Array Database (62) Web site was used for analysis of genes that were coexpressed with α-CruP (Os09g32630). Genevestigator (63) was used for analyzing variation of α-CruP transcript levels under different conditions and in various tissues. The SEED database (64) was used for analysis of genes that clustered with cyanobacterial CruP genes.

The results of a protein BLAST search using the Synechococcus sp. PCC 7002 CruP protein sequence were compared with the results of a protein BLAST search of the Synechococcus sp. PCC 7002 CruA protein sequence. Only with an E-value greater than 0.005 (the standard BLAST cutoff score) were used. Those proteins that had a smaller E-value in the CruP set were considered CruP orthologs; the others were considered CruA orthologs.

165 RNA sequences from cyanobacteria with complete genomes were obtained from National Center for Biotechnology Information genomes (http://www.ncbi.nlm.nih.gov/genome) aligned using ClustalW2 (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/msa/clustalw2/). Alignments were then imported into MEGA 5.05 (65) for construction of a neighboring-tree with 1,000 replications for bootstrap values.

ROS Analysis. Seeds were sterilized in 1 mL of 70% ethanol for 5 min, followed by 5 min in 1 mL of 25% bleach solution containing 0.01% Tween 100. The bleach solution was removed, and seeds were rinsed five times with sterile milliQ H2O. The seeds were soaked under sterile milliQ H2O (to create a photoinnhibitory environment) and placed at 4 °C for 2 d before washing. After washing at a 21 °C, 14-h light/10-h dark cycle for 3 d, plants were stained with 2 mM NBT in 20 mM phosphate buffer (pH 6.1) for 15 min (66). Reactions were stopped by removing the NBT solution and flushing with sterile distilled water.

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