Mg Protoporphyrin Monomethylester Cyclase Deficiency and Effects on Tetrpyrrole Metabolism in Different Light Conditions

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Mg protoporphyrin monomethylester (MgProtoME) cyclase catalyzes isocyclic ring formation to form divinyl protoporphyrin. The CHL27 protein is part of the cyclase complex. Deficiency of CHL27 has been previously reported to compromise photosynthesis and nuclear gene expression. In a comprehensive analysis of different CHL27 antisense tobacco lines grown under different light conditions, the physiological consequences of gradually reduced CHL27 expression on the tetrapyrrole biosynthetic pathway were explored. Excessive amounts of MgProtoME, the substrate of the cyclase reaction, accumulated in response to the reduced CHL27 content. Moreover, 5-aminolevulinic acid (ALA) synthesis, Mg chelatase and Mg protoporphyrin methyltransferase activities were reduced in transgenic plants. Compared with growth under continuous light exposure, the CHL27-deficient plants showed a stronger reduction in Chl content, cell death and leaf necrosis during diurnal light/dark cycles. This photooxidative phenotype correlated with a rapidly increasing MgProtoME steady-state level at the beginning of each light period. In contrast, the same transformants grown under continuous light exposure possessed a permanently elevated amount of MgProtoME. Its lower phototoxicity correlated with increased activities of ascorbate peroxidase and catalase, and a higher amount of reduced ascorbate. It is proposed that improved stress acclimation during continuous light in comparison with light–dark growth increases the capacity to prevent photo-oxidation by excess tetrapyrrole precursors and lowers the susceptibility to secondary photodynamic damage.

Keywords: Chlorophyll • Mg protoporphyrin monomethylester cyclase • Photooxidation • Photosensitization • Tetrapyrrole biosynthesis • Tobacco.

Abbreviations: ALA, 5-aminolevulinic acid; APX, ascorbate peroxidase; CaMV, cauliflower mosaic virus; CL, continuous light; 2-CP, cysteine peroxiredoxin; DHAR, dehydroascorbate reductase; DTT, dithiothreitol; GluTR, glutamyl-tRNA reductase; GR, glutathione reductase; L/D, light/dark; LHC, light-harvesting complex; MDHAR, monodehydroascorbate reductase; MgProto, magnesium protoporphyrin; MgProtoME, magnesium protoporphyrin monomethylester; PCHlide, protoporphyrin浔ide; POR, protochlorophyllide oxidoreductase; Proto, protoporphyrin IX; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR.

Introduction

The formation of a fifth ring in the tetrapyrrole macrocycle distinguishes Chls from other tetrapyrrole end-products. Its oxidative formation is catalyzed by Mg protoporphyrin monomethylester (MgProtoME) (oxidative) cyclase (Bollivar and Beale 1996). Although the cyclase reaction is basically understood (Walker et al. 1988, Porra et al. 1995), the entire enzymatic mechanism of the oxidative ring closure remains to be elucidated. A breakthrough was the characterization of the acsf mutant (aerobic cyclization system Fe-containing protein) of the purple bacterium Rubrivivax gelatiniens which accumulates MgProtoME. The mutated gene encodes a protein involved in the oxidative cyclization step (Pinta et al. 2002).

Genes with sequences homologous to acsf were found in other aerobically grown and photosynthetically active pro- and eukaryotes. Photosynthetic unicellular organisms, e.g. algae and cyanobacteria, contain two or more cyclose-encoding genes, which are apparently induced in response to varying environmental conditions. Chlamydomonas reinhardtii possesses two homologous genes, crd1 and cth1, which have a distinct expression profile under copper and oxygen deficiency (Moseley et al. 2000, Moseley et al. 2002). The two Synechocystis PCC6803 genes chlA and chlAα function differently under ambient and...
low oxygen conditions (Minamizaki et al. 2008, Peter et al. 2009). In higher plants, acsf homologs were also found in Arabidopsis thaliana, Nicotiana tabacum, Oryza sativa and Pharbitis nil (Zheng et al. 1998, Tottey et al. 2003, Liu et al. 2004, Rzeznicka et al. 2005). However, the sole encoded protein is obviously insufficient to support a continuous cyclase reaction in vitro. In higher plants, at least a soluble and a membrane-bound protein fraction are required (Walker et al. 1991), including an NADPH-regenerating system for the cyclization reaction (Stenbaek et al. 2008).

The cyclase has been reported to function as a tightly regulated pacemaker in Chl synthesis, since partial inactivation of cyclase expression led to severe phenotypes in various species (Pinta et al. 2002, Tottey et al. 2003, Bang et al. 2008, Minamizaki et al. 2008, Peter et al. 2009). The Chlamydomonas crdi and chl mutants and the Arabidopsis chl27 mutant have malfunctions in the expression of acsf homologs and show pleiotropic phenotypes, such as low photosynthetic activity, PSI instability, reduced nuclear gene expression and modifications of other metabolic processes (Moseley et al. 2000, Tottey et al. 2003, Bang et al. 2008). However, it is not excluded that CHL27 deficiency and the resulting accumulation of free and potentially very destructive light-absorbing MgProtoME also compromises the tetrapyrrole biosynthetic pathway. Likewise, accumulation of protoporphyrin IX (Proto) has been observed to cause leaf necrosis, when either herbicides inhibit protoporphyrinogen oxidase or antisense RNA synthesis silences expression of ferrochelatase (Lee et al. 1989, Papenbrock et al. 2001). Similarly, the flu mutant lacking the negative regulator FLU does not suppress 5-aminolevulinic acid (ALA) synthesis and accumulates protochlorophyllide (PChlide) in the dark, resulting in photodynamic damage of leaf cells upon subsequent light exposure (Meskauskiene et al. 2000).

Thus, as a consequence of accumulating free Chl precursors, in light the excited porphyrins and Mg porphyrins can transfer the excitation energy to oxygen, leading to the generation of reactive oxygen species (ROS), mainly singlet oxygen (‘O2) (Liszky et al. 2004, Apel and Hirt 2004, Kim et al. 2008, Triantaphylidès and Havaux 2009). Excessive generation of ROS causes enzymatic oxidation of cellular compounds, often leading to cell death. However, apart from the direct tetrapyrrole-mediated photooxidative damage, ROS also activate signal transduction pathways triggering cellular processes that range from acclimation and altered photosynthetic function up to cell death (op den Camp et al. 2003, Laloi et al. 2007, Kim et al. 2008).

Because of the risk of photooxidative damage, photosynthetic organisms have to prevent accumulation of non-metabolized Chl precursors. In previous studies, transgenic plants with inactivated genes for the enzymes preceding the cyclase, Mg chelatase and MgProto methyltransferase, show no or hardly any accumulation of the respective substrates, Proto and MgProto. These experiments indicate a feedback control of the Mg branch on ALA synthesis to restrict the accumulation of metabolic intermediates (Papenbrock et al. 2000a, Papenbrock et al. 2000b, Alawady and Grimm 2005, Peter and Grimm 2009). Although CHL27 expression is tightly regulated (Matsumoto et al. 2004), it is not clear whether CHL27 also contributes to the feedback control originating from the Mg branch of tetrapyrrole biosynthesis.

Since all reports on CHL27 function in plants have hardly addressed the metabolic and regulatory consequences of reduced cyclase activity on plant tetrapyrrole biosynthesis, we examined the activities of the metabolic pathway in response to down-regulated CHL27 expression in continuous light (CL) and light/dark (L/D) conditions in CHL27 antisense lines of tobacco (N. tabacum). The steady-state levels of porphyrin and Mg porphyrin intermediates under CL and L/D conditions correlated with changes in the tetrapyrrole biosynthetic pathway. As result of photooxidative damage the leaves show a necrotic phenotype during L/D growth. The content of antioxidants and the activity of protective enzymes were determined and related to accumulation of photoreactive tetrapyrrole intermediates, cell death and leaf necrosis.

### Results

#### Generation of transgenic tobacco lines expressing antisense RNA from the CHL27 gene

A tobacco full-length cDNA encoding the cyclase protein was identified and sequenced (accession No. AY221168; Liu et al. 2004). The previous nomenclature of the homologous A. thaliana CHL27 gene was adopted for the tobacco homolog. Among the homologous protein sequences, the highest structural similarity of tobacco CHL27 was found to P. nil PnZIP with 91% (AA819120.1; Zheng et al. 1998), followed by 86% to O. sativa OsCHL27 (BAA87823), 85% to A. thaliana CHL27 (Q9M591; Tottey et al. 2003), 83% to Xantha-l of Hordeum vulgare (AAW80518, Rzeznicka et al. 2005), 62% to C. reinhardtii CRD1 (Q9LD46; Moseley et al. 2000) and 59% to Synechocystis PCC6803 chla, (Minamizaki et al. 2008). In comparison with the bacterial cyclase proteins, tobacco CHL27 contains an N-terminal extension, which is likely to be the chloroplast transit sequence.

To study the effects of CHL27 antisense RNA inactivation on the cyclase reaction and the entire tetrapyrrole biosynthesis, a 1,080 bp fragment of the NtCHL27 cDNA sequence was inserted in reverse orientation behind the strong and constitutive cauliflower mosaic virus (CaMV) 35S promoter of the binary vector pBinAR and introduced into the tobacco genome by Agrobacterium tumefaciens-mediated gene transfer. A large number of primary transformants was generated and confirmed by PCR of genomic DNA using primers for the 35S CaMV promoter and the NtCHL27 cDNA. Many lines showed growth retardation and reduced Chl content; some lines were characterized by white bleached and/or transparent necrotic leaf areas. Almost all transgenic lines contained reduced transcript levels for CHL27 and increased steady-state...
levels of MgProtoME, indicating that CHL27 antisense inactivation compromised the cyclase reaction. For further analysis, representative lines among the transformants were selected because of their stable and distinct chlorotic and/or necrotic leaf phenotype (Fig. 1A). Leaves of lines #6 and #15 exhibited weak and extensive necrosis, respectively (Fig. 1B). In response to antisense RNA synthesis, the transgenic lines contained reduced CHL27 protein levels as indicated by immunodetection (Fig. 1C).

**Decreased CHL27 expression hampers conversion of MgProtoME**

The impact of decreased CHL27 content on tetrapyrrole biosynthesis was monitored in plants of the T3 generation of the three transgenic lines #5, #6 and #15. When grown at the same photon flux density (PFD) of 140 µmol photons m⁻² s⁻¹, a less severe phenotype of these three antisense lines was observed in CL as compared with L/D. The CL-grown CHL27 antisense plants still showed reduced green pigmentation but hardly any necrotic leaf spots in comparison with those grown in L/D conditions (Fig. 1A). The Chl content was gradually reduced in leaves of the transgenic lines and correlated with the reduction of CHL27 protein (Fig. 2A). Except for line #15, the leaves of antisense lines and the wild type contained slightly higher Chl contents under CL than under L/D conditions. Additionally, the heme content was not altered in L/D-grown control and transgenic lines, but CL conditions led to a gradual reduction of heme levels in transgenic plants (Fig. 2B).

MgProtoME accumulated up to 10-fold under L/D conditions and up to 15-fold under CL in transgenic lines relative to wild-type plants. However, considering the lower steady-state levels in the wild type, the antisense plants accumulated less or even similar amounts of MgProtoME under CL (Fig. 3A). In contrast to accumulating MgProtoME, the steady-state contents of Proto and MgProto were found to be lower or similar in leaves of L/D-grown lines #6 and #15 compared with wild-type leaves (Fig. 3B, C), probably reflecting reduced enzyme activities at other steps of the pathway.

Furthermore, with the exception of line #15, NtCHL27 inactivation hardly affected PChlide accumulation in darkness (Fig. 3D). The different tetrapyrrole metabolite levels in leaves of lines #5, #6 and #15 are the result of the different intensities of the transgenic phenotype. The dramatic reduction of Proto, MgProto and PChlide contents in line #15 compared with the wild type in parallel to the highest accumulation of MgProtoME corroborates the adverse effects of strongly silenced CHL27 expression.

**Protein accumulation in CHL27 antisense plants**

As stated above, the reduced CHL27 accumulation impedes Chl biosynthesis. For different transgenic plants with impaired Mg porphyrin synthesis, a down-regulation of RNA expression and, consequently, protein abundance and activity of key enzymes of the pathway was reported (Papenbrock et al. 2000a, Papenbrock et al. 2000b, Alawady and Grimm 2005). Besides CHL27, contents of glutamyl-tRNA reductase (GluTR), Mg chelatase subunit CHLH, Pchlide oxidoreductase (POR) and proteins of the light-harvesting complexes (LHCs) of PSI and PSII were assessed in transgenic and wild-type plants. As expected in response to the antisense RNA inactivation, the CHL27 content was gradually reduced under both light regimes (Fig. 4), but was similar in each transgenic line grown under CL or L/D conditions. CHL27 was hardly detectable in

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**Fig. 1** Phenotype and initial analysis of CHL27 antisense plants. (A) Leaves of the same developmental stage from tobacco wild-type plants and representative lines, which were grown under 12 h/12 h light/dark (L/D) or continuous light (CL) conditions with an intensity of 140 µmol photons m⁻² s⁻¹ in growth chambers at 24°C. (B) Enlarged leaf segments of L/D-grown plants of lines #6 and #15 displaying different degrees of damage. (C) Western blot analysis shows the CHL27 content of a leaf of similar age from the wild type and the transformants #5, #6 and #15.
line #15, indicating that a minimal amount of the protein still ensures metabolic activity and a flow of Mg porphyrins resulting in detectable Chl accumulation.

Lines #5 and #6 did not exhibit considerable changes in the abundance of GluTR and POR. Only the CHLH content was reproducibly increased in both lines compared with the wild type (Fig. 4). This observation is consistent with the increased CHLH content of the Arabidopsis chlM knockout mutant (Pontier et al. 2007) and may explain the slightly increased MgProto levels in CL-grown plants of these lines (Fig. 3C). In contrast to lines #5 and #6, the contents of GluTR and POR were reduced in line #15. These differences can be attributed to the more severe phenotype of this line. Additionally, the gradual decrease of antennae proteins of both photosystems (Fig. 4) correlated with the measurable Chl deficiency (Fig. 2A).

**Enzyme activities in the tetrapyrrole biosynthetic pathway**

Key steps of tetrapyrrole metabolism were investigated against the background of inactivated NtCHL27. The leaves

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**Fig. 2** The two end-products of the tetrapyrrole biosynthetic pathway, Chl and heme, in wild-type and transgenic plants grown under L/D and CL conditions. (A) CHL27 antisense plants showed gradually decreasing Chl accumulation. (B) The heme content was not clearly affected by antisense RNA inactivation under L/D conditions, but was significantly decreased in line #15 under CL. The values reflect the contents of leaf No. 4 counting from the top and represent data from three different biological replicates.

**Fig. 3** Steady-state levels of Chl precursors in the wild type and transformants grown in L/D and CL conditions. (A) Mg protoporphyrin monomethylester (MgProtoME), (B) protoporphyrin IX (ProtoIX), (C) Mg protoporphyrin IX (MgProto). All three tetrapyrrole intermediates were measured from leaf samples of the same age, which were harvested 2 h after onset of light under L/D and at the same time of the day for CL-grown plants. (D) Protochlorophyllide (PChlide) measured in leaf samples harvested after 10h darkness.
from L/D-grown plants were harvested 2 h after dark to light shift and from CL-adapted plants at the same time of the day. The leaf extracts were immediately assayed. ALA synthesis gradually decreased in parallel to a progressive decrease in Chl content in the NtCHL27 antisense lines, in both L/D and CL conditions (Fig. 5A). Particularly in line #15, the reduced ALA synthesis rate correlated with the weak immunoreactive band of GluTR (Fig. 4).

In addition, the activities of Mg chelatase and MgProto methyltransferase were lower in L/D-grown lines #6 and #15 (Fig. 5B, C), while the ferrochelatase activities of lines #5 and #6 were slightly elevated during L/D growth in comparison with wild-type activity. The ferrochelatase activity of the leaf extracts of all transgenic and control plants was relatively higher under CL than under L/D conditions (Fig. 5D). The increased ferrochelatase activities reflect an altered allocation of intermediates due to a different demand for heme, which can be attributed to an increased requirement for heme-containing proteins involved in ROS scavenging or photorespiration. Furthermore, activities of both Mg chelatase and ferrochelatase normally oscillate inversely under L/D conditions (Papenbrock et al. 1999), while the enzyme activities do not follow the diurnal rhythm during CL growth. During photoperiodic growth, enhanced Mg chelatase activity is observed at the beginning of the light phase and elevated activity of ferrochelatase at the beginning of the dark period. It is assumed that the lower Mg chelatase activity of CL-grown plants is the result of an acclimation process to ensure a lower but enduring constant supply of newly synthesized Chl molecules.
Different kinetics of MgProtoME accumulation during L/D and CL growth

Unlike all other characterized mutants and transgenic plants with impaired Mg porphyrin synthesis, the transgenic lines with stronger NtChl27 antisense inactivation displayed leaf necrosis (as seen to different extent in lines #6 and #15, Fig. 1A, B) indicating insufficient feedback control of early steps of the pathway. In addition, leaf necrosis was largely prevented in the CL-grown lines, even though these lines accumulated similar amounts of MgProtoME under CL conditions and during L/D cycles. These observations indicate that L/D conditions appeared to be more harmful for CHL27 antisense lines, consequently causing photodynamic cell death.

It is widely accepted that accumulating Chl precursors act as photosensitizers (Mock et al. 2002). Therefore, it is not surprising that the greatly increased levels of MgProtoME cause oxidative stress and finally cell death. The first indication of a general response to ROS was the increase of peroxidase activity in L/D-grown transgenic plants (Fig. 6A). The elevated catalase abundance in lines #6 and #15 under L/D conditions suggests that the ROS stress not only affected plastids but also spread throughout the whole cell. In contrast, CL-grown plants did not show a significant increase in catalase levels (Fig. 6B).

Despite a similar accumulation of vast amounts of MgProtoME in both L/D- and CL-grown transgenic plants, the Mg porphyrin accumulation was less disadvantageous under the latter condition. Considering the levels of intermediates of tetrapyrrrole biosynthesis (Fig. 3) and the Mg chelatase activities (Fig. 5B), an altered metabolite flow through the pathway was suggested during a 24h period under both light conditions. A detailed analysis of the diurnal MgProtoME accumulation under CL and L/D conditions was conducted with wild-type plants and line #6 as a representative CHL27 antisense transformant with a medium transgenic phenotype showing weak leaf necrosis. Leaf samples were harvested prior to darkness, after 0.5, 8.5 and 11.5 h in darkness and after 0.5, 2.5 and 4.5 h in the light, and assayed for the contents of Chl precursors (Fig. 6C, D).

During the dark period MgProtoME contents of line #6 dropped slowly, but substantially. However, at the beginning of the light period the MgProtoME level in leaves of line #6 increased >18-fold within the first 30 min of re-illumination, leading to an approximately 20-fold higher steady-state level than in the corresponding wild type. In parallel, the wild-type leaves harvested 30 min before and after the dark to light transition revealed only a 5-fold increase in MgProtoME (Fig. 6C).
The rapid burst of MgProtoME accumulation is explained by the diurnal light-induced stimulation of ALA and Mg porphyrin synthesis (Pöpperl et al. 1998, Pappenbrock et al. 1999) and, consequently, turns out to be stronger in the background of an insufficient conversion of MgProtoME due to CHL27 antisense inactivation.

Transgenic plants acclimated to continuous light maintained high Mg porphyrin levels during the whole day (Fig. 6D). However, CL-grown plants did not experience the periodical activation of Chl synthesis after the dark to light transition and, therefore, they did not encounter rapidly evoking oxidative stress caused by the suddenly emerging high levels of MgProtoME. This daily oscillating accumulation of excessive MgProtoME is proposed to be the reason for the photo-destructive phenotype of the transgenic lines. Oxidative stress emerging at the transition from darkness to light is certainly more challenging, because plants are not well prepared for photooxidative stress at this time and have to re-adjust their defense system.

**Response to accumulating MgProtoME**

Photosensitizing effects of porphyrins include the formation of singlet oxygen and peroxides (Mock et al. 2002, Apel and Hirt 2004, Liszkay et al. 2004, Kim et al. 2008, Triantaphylides and Havaux 2009). The latter are efficiently scavenged by an ascorbate-dependent system (Nakano and Asada 1981). Thus, reduced ascorbate is an important low molecular weight component of the ROS-scavenging system. Despite the fact that the total amounts of ascorbate were similar in wild-type and transgenic line #6, leaf extracts from transgenic plants harvested shortly after the dark to light transition contained significantly less reduced ascorbate than the corresponding wild type. In L/D-grown line #6 only one-third of the total ascorbate was reduced, while the same transgenic line grown under CL had >60% reduced ascorbate (Fig. 7A). Presumably, an increase of reduced ascorbate contributes to increased stress tolerance enabling higher activities of ascorbate-consuming enzymes and, in turn, the maintenance of the reduced ascorbate pool is ensured by the activity of regenerating enzymes.

We performed enzyme assays for ascorbate peroxidase (APX) and glutathione reductase (GR), two main components of the ascorbate-regenerating system (DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase) were present in slightly increased amounts. CL growth possibly enabled acclimation of transgenic plants to porphyrin-induced stress. An induction of 2-cysteine peroxiredoxin (2-CP) provides additional capacity to detoxify ROS and relieves the burden from the ascorbate-dependent water–water cycle. Samples of L/D-grown plants were harvested 2 h after the onset of light.
of the system that detoxifies peroxides. First the activities of both enzymes were higher under CL than under L/D (shortly after the dark to light transition), implying that CL-adapted plants have a generally more active scavenging system available (Fig. 7B, C). Secondly, in contrast to the corresponding wild type, the transgenic plants exhibited elevated activities of both assayed enzymes, which cannot be explained by higher protein levels as indicated by immunodetection (Fig. 7D). Additionally, the levels of the ascorbate-regenerating enzymes monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) were hardly changed. Only an increase of MDHAR under L/D conditions was observed. However, this up-regulation was obviously not sufficient to keep the ascorbate pool in a more reduced state (Fig. 7A). Interestingly, the induction of an alternative hydrogen peroxide detoxification system was obtained from leaves of CL-grown transgenic plants. The 2-cysteine peroxiredoxin (2-CP)-dependent water–water cycle supports the ascorbate-dependent antioxidative system under normal and, in particular, under stress conditions (Dietz et al. 2006). Immunodetection revealed that the abundance of the chloroplast-localized 2-CP A/B was increased under CL in CHL27 antisense plants, while no differences between wild-type and transgenic plants were observed under L/D conditions (Fig. 7D). This difference is also proposed to contribute to a less severe phenotype of CHL27 deficiency.

Discussion

Deregulation of CHL27 affects the metabolic activities of plant Chl biosynthesis

We examined in transgenic CHL27 antisense tobacco plants the possible contribution of the cyclase to the metabolic feedback control of the Mg branch on ALA biosynthesis. A chl27 knockout mutation is lethal and cannot be used to analyze the physiological consequences of deregulated cyclase. Thus, gradually increased inactivation of tobacco CHL27 is more suitable to monitor the physiological consequences of deregulation. A reduced CHL27 abundance led to progressive reduction of Chl content (Fig. 2) and growth rate. Necrotic leaf lesions were formed in transgenic plants with stronger reduction of CHL27 content (Fig. 1C) and correlated with excessive levels of MgProtoME (Figs. 1B, C, 3A). The ALA-synthesizing capacity as well as the Mg chelatase and MgProto methyltransferase activities progressively decreased in the transgenic lines, while the heme-synthesizing branch with ferrochelatase activity was hardly affected (Fig. 5; with the exception of line #15, see below). Lower Proto and MgProto levels in the CHL27 antisense plants are likely to be the result of attenuated ALA synthesis. MgProtoME accumulated in leaves of the transgenic seedlings due to the severely compromised CHL27 expression, although gradually reduced ALA synthesis would direct fewer metabolites into the Mg branch. In contrast to previous reports indicating that enzymes in the Mg branch exert feedback control on early steps of Chl biosynthesis (Papenbrock et al. 2000a, Papenbrock et al. 2000b, Alawady and Grimm 2005) it is concluded that CHL27 does not contribute to feedback control of ALA synthesis. As a result of our experiments with transgenic plants, which possess deregulated expression of enzymes in the Mg branch, it is suggested that the enzymatic steps of Mg chelatase and methyltransferase are more efficiently involved in fine-tuning the metabolic activities of the pathway than the cyclase. Instead, it is proposed that reduction of ALA synthesis and altered activity of both chelatases and MgProto methyltransferase in CHL27 antisense plants is the result of oxidative stress induced by accumulating MgProtoME.

Antioxidants and reducing power contribute to the protection against Mg porphyrin-induced photooxidation

MgProto has been suggested to function as a regulating intermediate in a plastid to nucleus signal transduction pathway to modulate the nuclear transcriptional activities in response to plastid activities (Strand et al. 2003, Beck 2005, Nott et al. 2006). However, a strict correlation between MgProto levels and Lhcb expression could not be confirmed (Moulin et al. 2008, Mochizuki et al. 2008). These reports prompted us to scrutinize additional functions of accumulating MgProtoME.

Tetrapyrrole intermediates of Chl biosynthesis can be excited by light. The dissipation of the excitation energy may lead to the formation of ROS, particularly singlet oxygen. Unless these ROS are scavenged by protective enzymes, they can further react with cellular components, such as lipids and proteins. Therefore, the accumulating porphyrins are potentially harmful (Moc et al. 2002). Presumably, the accumulation of huge amounts of MgProtoME in the transgenic lines #6 and #15 provoked the generation of ROS and, thus, led to cell death and leaf necrosis. It is suggested that the alterations of the steady-state levels of Proto and Mg porphyrins in light-exposed seedlings may reflect the disturbances of the metabolic flow through the pathway in response to up- and down-regulated enzymatic activities, and subsequent changes in nuclear gene expression may be triggered by intracellular processes of photosensitization and generation of ROS.

The ROS-mediated stress response includes the activation of genes and proteins involved in detoxification processes, such as peroxidases and catalases (Fig. 7; Mittler et al. 2004). It was remarkable to observe that the CL-grown transgenic lines were able better to tolerate reduced CHL27 expression and high accumulation of MgProtoME than the progeny of the same lines grown under L/D conditions (Figs. 1A, 6). First, a rapid daily increase in light-absorbing MgProtoME at the beginning of each light period causes a rapid production of ROS, while the expression of a less disadvantageous phenotype of CL-grown plants indicates that they are presumably adapted to continuously elevated Mg porphyrin levels (Fig. 6). Secondly, several reports emphasize the light-dependent activation of the cellular scavenging capacity for ROS (Zhong and McClung 1996, Kioya et al. 2006). Our observations are supported by these
Consequences of impaired CHL27 expression

reports, since the tobacco wild-type and transgenic plants exhibit higher activities of ROS-detoxifying enzymes when they were grown under CL (Fig. 7B, C). The protection against MgProtoME-dependent ROS generation is proposed to be less efficient during the daily dark to light shift. It is assumed that the rapid periodical transitions from dark to light are critical for a competent stress response.

In this context it is worth mentioning that the differences in sensitivity towards accumulating MgProtoME in CHL27 antisense plants under different light regimes mimic the phenotype of Arabidopsis NTRC mutants (Lepistö et al. 2009). NTRC is a chloroplast-localized, NADPH-dependent thioredoxin reductase. Besides its role as part of the thioredoxin system that controls important metabolic and regulatory pathways, NTRC was shown to affect cyclase activity (Stenbaek et al. 2008). As demonstrated for the CHL27 antisense plants, the length of the dark period was crucial for the escalating negative phenotype of these NTRC mutants. Growth under short-day conditions severely impaired leaf and chloroplast development in NTRC mutants, in contrast to mutant plants grown under long-day conditions or CL. Hence, it was proposed that NTRC is involved in the regulation of several diurnal light-dependent processes, including Chl biosynthesis (Lepistö et al. 2009). In agreement with this, a similar regulation of CHLH in NTRC mutants and CHL27 antisense plants (Fig. 4) was observed. In addition to this observation, a possible direct impact of NTRC on Chl biosynthesis is proposed for the cyclase reaction (Lepistö et al. 2009). NTRC and 2-CPs stimulate cyclase activity in vitro, and NTRC mutants of Arabidopsis accumulate MgProtoME upon ALA feeding (Stenbaek et al. 2008). Thus, a mutual dependency of NTRC and cyclase is proposed, and a dysfunctional cyclase may also contribute to impaired NTRC-dependent regulation of developmental processes. Since these regulatory functions of NTRC are mainly associated with photoperiodic growth (Lepistö et al., 2009), CL conditions are suggested to be less hazardous for cyclase-deficient plants.

Nevertheless, the enhanced photosensitivity of L/D-grown transgenic lines in comparison with the CL-grown plants also correlates with distinct diurnal variations of the Mg porphyrin levels, in particular those of MgProtoME. Due to the oscillating activities in tetrapyrrole biosynthesis in L/D-grown wild-type plants, the Mg porphyrin contents varied within a 24 h cycle (Papenbrock et al. 1999). In CHL27 antisense plants the lower cyclase activity led to excessive amounts of MgProtoME in the light, which were slowly converted to PChlide in the dark period. At the end of the dark period, lines #5 and #6 accumulated wild-type-like or slightly increased PChlide levels, while line #15, as a representative of the severely inhibited CHL27 antisense lines, failed to convert MgProtoME sufficiently to accumulate a wild-type-like PChlide content and still had elevated amounts of MgProtoME at the end of the dark period (Fig. 3D). Moreover, it cannot be excluded that, as result of lower POR abundance (Fig. 4), free non-photoconvertible PChlide accumulates, which in combination with accumulated MgProtoME can also contribute to phototoxic stress in the transgenic line #15 after daily transition from dark to light. Therefore, intracellular changes in activities and contents of proteins for oxidative stress defense and tetrapyrrole biosynthesis and the severe necrotic leaf phenotype are most probably explained by the generation of ROS as a consequence of porphyrin accumulation.

Apart from the PChlide accumulation during darkness as a result of slow conversion of accumulated MgProtoME, the immediate light-induced stimulation of ALA and Mg porphyrin biosynthesis at the beginning of the light phase is challenging for the CHL27 antisense lines. Excessive amounts of MgProtoME accumulated in CHL27-deficient plants within 30 min after transfer to light and accumulation further continued until the middle of the light period. The rapid burst of excessive MgProtoME at the beginning of the light period instantaneously provoked phototoxic stress, but could not be scavenged sufficiently by the antioxidant defense system. Derived from the leaf phenotype, the rapid accumulation of Mg porphyrins in L/D-grown CHL27 antisense lines is more harmful than a constant high level under CL conditions. It is suggested that the diurnal light–dark cycles impair the balance between ROS formation and scavenging. The L/D-grown transgenic plants periodically have to build up an adequate light-dependent ROS-scavenging system that is able to detoxify the excessively accumulating ROS generated in response to the rapid MgProtoME accumulation after dark to light transition. This hypothesis is supported by the observation that ROS-scavenging enzymes are more active under CL than under L/D. APX1 and CAT (Kubo et al. 1995, McClung 1997) are apparently less efficient during diurnal growth, when excessive fluctuation of MgProtoME levels may require a re-induction of the ROS-scavenging system. In contrast, these antioxidative activities were already elevated in tobacco CL-grown wild-type plants (Fig. 7B, C), suggesting a correlation between antioxidant levels and improved photoprotection. The CL-grown transgenic plants, which accumulate similar amounts of MgProtoME, are apparently long-term acclimated against Mg porphyrin-triggered elevated ROS levels.

Although the photodynamic stress originates from plastids, the effects of excessive ROS formation will rapidly spread throughout the entire cell. Modified catalase contents have been described as a marker for porphyrin-induced photodynamic stress, indicating the spread of either Mg porphyrins, ROS or lipophilic compounds into the cytoplasm (Kruse et al. 1995, Hu et al. 1998, Nishihara et al. 2003) or the activation of redox imbalance-induced signaling cascades that modify nuclear gene expression (Heiber et al. 2007). We can currently neither distinguish between direct ROS-dependent destruction and ROS-induced activation of cellular responses to photodynamic stress nor quantify the different ROS species generated during MgProtoME-triggered phototoxicity. However, different transgenic tobacco lines, which accumulate excessive levels of almost all kinds of Chl precursors, are available. Finally, these analyses in Arabidopsis wild-type and mutant seedlings are hampered due to low metabolic activities of Chl biosynthesis and low steady-state levels of metabolic intermediates.
Hence, an accumulation of intermediates of the tetrapyrrole biosynthetic pathway was only observed upon ALA feeding (Tottey et al. 2003). Conversely, the high capacity and sustained Chl metabolism of tobacco plants enable a detailed analysis of Chl precursors and the exploration of their phototoxic effects. Future time courses will elucidate how and which kind of ROS directly contribute to photooxidation and how photoprotective mechanisms will be induced to prevent, alleviate or reverse photooxidation and photodamage.

In conclusion, we propose that transgenic plants with compromised CHL27 expression and accumulation of MgProtoME show diverse adaptive responses to photooxidative stress and photodynamic damage dependent on different light regimes. The differential response of CL- and L/D-grown lines with minor changes of CHL27 abundance strongly affect Chl formation is unknown processes. In addition, the observation that metabolic feedback control, photooxidation by ROS affects the regulation of the pathway directly or indirectly by still unknown processes. In addition, the observation that minor changes of CHL27 abundance strongly affect Chl formation is important. Thus, the cyclization step in Chl synthesis might be an additional bottleneck. It is likely that CHL27 expression and cyclase activity are controlled in a manner dependent on photosynthesis (Moseley et al. 2000, Moseley et al. 2002, Tottey et al. 2003) and, consequently, by redox-dependent mechanisms (Stenbaek et al. 2008, Lepistö et al. 2009). It remains tempting to study the post-translational control of enzyme activities and long-term acclimation by control of nuclear gene expression in response to metabolic imbalances in tetrapyrrole biosynthesis.

**Materials and Methods**

**Growth of tobacco plants**

Tobacco plants were grown on soil under light/dark (12 h/12 h) cycles or continuous white light with an intensity of 140 µmol photons m$^{-2}$ s$^{-1}$ in growth chambers at 24°C.

**Generation of transgenic plants**

A 1,100 bp DNA fragment was amplified from total RNA of green tobacco leaves by reverse transcription–PCR (RT–PCR) with the two primers NtChl27 F1 5′-TATGTGAGACAGATGTGC AGCGCCACAC-3′ and NtChl27 R1 5′-ACGATTGTTGGTTC AACACTGCA-3′, subcloned into the pGEM-T vector and excised using the Sall site introduced by primer NtChl27 F1, and a BamHI site, which is close to the 3′ end of the cDNA. The 1,080 bp Sall–BamHI fragment was cloned in antisense orientation into the binary vector pBinAR (Höfgen and Willmitzer 1992). The construct was introduced into tobacco plants by A. tumefaciens (strain GV2260)-mediated gene transfer (Horsch et al. 1985). Transgenic plants were regenerated from leaf discs on kanamycin-containing MG medium. The presence of the transgene in the genome of transgenic lines was validated by PCR using specific primers.

**Determination of porphyrins, Mg porphyrins, PChlide, chlorophyll and heme**

Porphyrins were extracted and analysed by HPLC as described previously (Shalygo et al. 2009). Porphyrins were identified and quantified by fluorescence detection using authentic standards (Frontier Scientific).

Quantitative analysis of PChlide, Chl and carotenoids was performed as described in Shalygo et al. (2009).

The content of non-covalently bound heme was determined after removal of Chl from ground plant material by ice-cold alkaline acetone containing 0.1 N NH$_4$OH (9 : 1; v/v) (Weinstein and Beale 1984). Heme was then extracted with acidic acetone containing 5% HCl, transferred to diethyl ether, concentrated, and washed on a DEAE–Sephrose column. Absorbance was monitored at 398 nm using a millimolar extinction coefficient of 144 (Castelfranco and Jones 1975).

**Enzyme assays for Mg chelatase, MgProto methyltransferase and ferrochelatase**

Ten grams aliquot of fresh leaf material of wild-type and transgenic plants was harvested and homogenized in 50 ml of cold buffer [500 mM sorbitol, 1 mM dithiothreitil (DTT), 0.1% bovine seum albumin (BSA) and 0.1 M Tris–HCl pH 7.5] using a Warring blender. The homogenate was filtered through two layers of Miracloth and subsequently centrifuged at 5,000 × g for 3 min.

For determination of Mg chelatase activity the pellet was carefully resuspended in 2–3 ml of the same buffer. The crude plastid preparation was subdivided and 50 µl aliquots were pre-warmed at 30°C for 5 min and subsequently mixed with an equal volume of freshly prepared assay mix (3 mM Protot, 30 mg ml$^{-1}$ phosphocreatine, 8 µM creatine phosphokinase, 8 mM ATP/Mg$^{2+}$, 10 mM Tricine, pH 7.8) to start the reaction. Reaction mixtures were incubated at 30°C in darkness and the reaction was stopped after 0, 5, 10, 20 and 30 min by addition of 400 µl of ice-cold acetone. The extract was cleared by centrifugation and Chls were removed by hexane extraction. Finally, the volume of the hexane-extracted acetone residue was adjusted to 1 ml with alkaline 80% acetone. Mg porphyrins were quantified by HPLC as described above.

For determination of ferrochelatase activity the crude chloroplast pellets were resuspended in 2–3 ml of resuspension buffer [1 mM DTT, 0.25% (v/v) Triton X-100, 100 mM Tricine, pH 7.7]. Samples were adjusted to equal Chl concentrations. To start the reaction 150–200 µl of protein were mixed with 2 ml of pre-warmed assay buffer [4 µM ZnSO$_4$, 5 µM Proto, 0.03% (v/v) Tween-80 and 0.3 M Tris, pH 8.0]. Samples were incubated at 34°C and ferrochelatase activity was monitored by online recording of Zn Proto fluorescence using a Hitachi F4500 fluorometer. Zn Proto formed during incubation was quantified using a calibration curve.
For determination of MgProto methyltransferase activity the chloroplast pellets were resuspended in 2–3 ml of reaction buffer (0.5 M sorbitol, 1 mM DTT, 5 mM EGTA, 50 mM Tris, pH 8.4). Aliquots of 200 µl were transferred to reaction tubes and mixed with 388 µl of reaction buffer, 6 µl of 100 mM 5-adenosylmethylione (SAM) and 6 µl of 2 mM MgProto to start the reaction. Mixtures were incubated at 33°C for 0, 5, 10 or 15 min. The reaction was stopped by freezing the samples in liquid nitrogen. Porphyrins were extracted as described above and quantified by HPLC.

**ALA-synthesizing capacity**

Tobacco leaf discs were incubated under growth conditions in 30 mM Tris–HCl buffer (pH 7.2) containing 40 mM leucolactic acid for 2 h and subsequently homogenized in 1 ml of 20 mM K-phosphate buffer (pH 6.8). After centrifugation, 400 µl of supernatant was mixed with 100 µl of ethylacetocetate and boiled for 10 min. Finally an equal volume of Ehrlich’s reagent was added and ALA derivatives were quantified at λ = 553 nm.

**Western blots**

Leaf discs (100 mg) were pulverized in liquid nitrogen, resuspended in protein extraction buffer (56 mM Na₂CO₃, 56 mM 30 mM Tris–HCl buffer (pH 7.2) containing 40 mM levulinic acid for 2 h and subsequently homogenized in 1 ml of 20 mM K-phosphate buffer (pH 6.8). After centrifugation, 400 µl of supernatant was mixed with 100 µl of ethylacetocetate and boiled for 10 min. Finally an equal volume of Ehrlich’s reagent was added and ALA derivatives were quantified at λ = 553 nm.

**Assay for peroxidase activity**

Approximately 200 mg of tobacco leaf material was ground in ice-cold buffer (10 mM Tris, 0.1 % Tween-80, pH 6.8). The homogenate was transferred to an Eppendorf tube and incubated at room temperature for 10 min followed by 10 min centrifugation in a table-top centrifuge at maximum speed. The cleared supernatant was transferred to a new tube and stored on ice. Enzymatic reactions were performed at room temperature.

For measurements of APX activity, 950 µl of reaction buffer (50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA) were mixed with 50 µl of cleared homogenate and the reaction was started by adding 20 µl of 50 mM hydrogen peroxide. The decrease in absorption at 290 nm due to ascorbate consumption was monitored photometrically for 30 s. The activity of APX was calculated using an absorbance coefficient of 2.8 mM⁻¹ cm⁻¹ (Nakano and Asada 1981).

A typical assay for GR consisted of 2.1 ml of 100 mM potassium phosphate buffer, pH 7.8, 75 µl of 5 mM NADPH and 200 µl of plant homogenate. The reaction was started by addition of 100 µl of 5 mM oxidized glutathione. NADPH consumption was measured by monitoring changes in the absorption at 340 nm for 10 min. The GR activity was calculated using an absorbance coefficient of 6.2 mM⁻¹ cm⁻¹ for NADPH.

**Determination of ascorbate content of tobacco leaves**

The determination of reduced and total ascorbate was performed according to Law et al. (1983).

**Miscellaneous**

All experiments comprised 3–6 independent replicates. In order to test significant differences between calculated values, equality of variances was tested by an F-test followed by Student’s t-test using a P-value of <0.05.

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**References**


Consequences of impaired CHL27 expression


