Phyton (Austria) Special issue: "Free Radicals"	**Vol. 37**	Fasc. 3	(169)-(174)	1. 7. 1997

Consequences of Photo-Oxidation in Transgenic Tobacco with Co-Suppression of Coproporphyrinogen Oxidase

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Key words: Chlorophyll synthesis, tetrapyrrole, transgenic plants, reactive oxygen species.

Summary

MOCK H.-P., LERMONTOVA I., KEETMAN U. & GRIMM B. 1997. Consequences of photo-oxidation in transgenic tobacco with co-suppression of coproporphyrinogen oxidase. - Phyton (Horn, Austria) 37 (3): (169) - (174).

Introduction of additional copies of genes encoding coproporphyrinogen oxidase (CPO) caused in some transgenic lines necrotic leaf lesions. As a result of mutual silencing of transgenes and endogenous genes CPO activities were reduced and photosensitive coproporphyrin(ogen) accumulated. Photo-oxidation of these porphyrins generated reactive oxygen species which induced the protection mechanism against oxidative stress. Activities of ascorbate peroxidase, glutathione reductase and superoxide dismutase were increased in these transgenic lines. We conclude that these plants accumulating porphyrin are appropriate for studies on the induction mechanisms of the antioxidative defense system.

Introduction

Natural occurring tetrapyrroles have been implicated in light absorbing or electron transfer processes. In higher plants they are synthesized in plastids (BEALE & WEINSTEIN 1990). Their endproducts such as chlorophyll and heme are subsequently assembled with pigment binding proteins or cytochromes, catalases or peroxidases. Free porphyrins and their metal complexes are known for

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sensitizing photo-oxidative processes. These processes are extensively described for the porphyrias, which compose a group of hereditary disorders of the tetrapyrrole metabolism (MOORE 1990), or for the use of tetrapyrrole dependent photodynamic herbicides, which are mainly targeted against protoporphyrinogen IX oxidase (DUKE & REBEIZ 1994). Furthermore, the destructive effects of accumulating porphyrins are used in the photodynamic therapy which is an experimental cancer treatment using photosensitizers that produce tissue destruction upon absorbing light of an appropriate wavelength and dose. These photodynamic effects, independently from being induced or inherited in man or plants involve reactive oxygen species which are generated during the light dependent oxidation of the photolabile porphyrins.

We recently cloned and sequenced cDNA sequences from tobacco and barley coproporphyrinogen oxidase (CPO), an enzyme of the porphyrin synthesizing pathway (KRUSE & al. 1995a). This enzyme catalyzes oxidative decarboxylation of two propionate side chains of tetrapyrrole ring A and B into vinyl groups to form protoporphyrinogen IX, the substrate for protoporphyrinogen oxidase. We began studies on the significance of CPO for the control of the metabolite flow in tetrapyrrole biosynthesis. We used transgenic plants expressing antisense RNA for CPO to selectively reduce its gene product level and to permit analysis of the cellular effects caused by its deficiency (KRUSE & al. 1995b). The transgenic plants showed necrotic leaf lesions as a result of accumulating photosensitive coproporphyrins. This phenotype mimics processes of cell death induced by exposure to toxic ozone concentration or resembles the hypersensitivity reaction after pathogen attack.

In this communication we address the question how additional copies of the gene encoding CPO can deregulate tetrapyrrole biosynthesis, and examine the cellular antioxidative response against reactive oxygen species generated as consequence of accumulating porphyrins.

Material and Methods

Generation of transgenic plants. A 1582 bp cDNA fragment representing a full-length sequence encoding tobacco CPO was cut with Sal I/Sma I. Ligation into the Sal I/Sma I digested binary vector BinAR containing the 35SCaMV promotor as well as tobacco (*Nicotiana tabacum* L. c.v. SNN) leaf disc transformation with *Agrobacterium tumefaciens* followed the protocols given in KRUSE & al. 1995b.

Plant growth. A range of primary transformants (n = 50) was grown in the greenhouse (KRUSE & al. 1995b) and inspected for morphological differences in comparison to wild-type. Three selected primary transformants as well as T1 progenies were used for biochemical characterization. Eight week old plants were harvested for their biochemical analysis.

Enzyme assays. CPO was determined as described (KRUSE & al. 1995a). Extracts for determination of ascorbate peroxidase (APX) and glutathione reductase (GR) were prepared as given by AONO & al. 1995. GR and APX were assayed as described by AONO & al. 1991 and NAKANO & ASADA 1981, respectively.

Miscellaneous. Chlorophyll and Western blot analysis were performed as described by KRUSE & al. 1995b and accumulated porphyrins were determined according to MOCK & al. 1995. Protein was quantitated according to BRADFORD 1976 and ascorbate according to LAW & al. 1983.

Statistics. Biochemical analysis was carried out for three independent extractions and several replicates (n = 3-5) were performed for each assay.

Results and Discussion

A full-length cDNA sequence encoding CPO was inserted behind a CaMV 35S promoter and introduced into the tobacco genome. Fifty transgenic plants were primarily cultivated in the greenhouse. Many of these plants grew slower than control plants and exhibited necrotic leaf lesions similar to those of the CPO antisense plants (KRUSE & al. 1995b) which showed correlation of intensity of transgenic phenotype and reduction in CPO activity.

Molecular analysis of many different transformants revealed that wild-type like transformants contained mainly excess of CPO mRNA and protein. These plants showed increasing CPO activity, but no accumulation of coproporphyrin(ogen) or following intermediates in chlorophyll biosynthesis (data not shown). For the present we conclude that CPO overexpression does not significantly effect the substrate flow in the pathway indicting that CPO is not rate limiting for chlorophyll synthesis as it was proposed earlier for porphyrin synthesizing enzymes (BEALE & WEINSTEIN 1990).

We focused our analysis on three transgenic lines designated 8, 18 and 38 having necrotic lesions with increasing severity in the order 38, 8, and 18. Quantification of the steady state level of CPO protein in these transgenic and control plants was carried out for leaf 3, 5 and 7 (counted from the top of the plants). While the CPO level was constant in control leaves, CPO showed a reduced occurrence in all transformants (Fig. 1). Introduction of additional copies of CPO genes resulted in all three transformants in silencing of the transgenes and most likely the endogenous gene. This phenomenon called co-supression appears to be associated with post-transcriptional events and can not be achieved by transcriptional gene inactivation (MEYER & SAEDLER 1996).

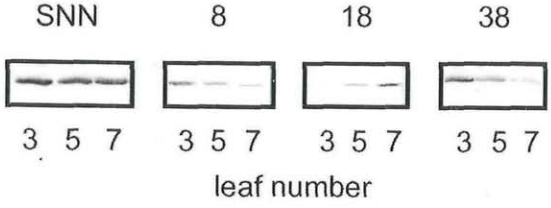

Fig. 1. Western blot analysis of wild-type tobacco (SNN) and selected transformants with additional copies for CPO. Extracts were separated by SDS-PAGE and blotted onto nitro-cellulose filter. Blots were decorated with antiserum against tobacco CPO (KRUSE & al. 1995a). A typical result from one series of experiments is shown.

CPO activity was assessed in leaf extracts to monitor the consequences of reduced levels of corresponding mRNA in the transgenic lines. Protein extracts of the six youngest leaves of 8-week-old plants were mixed and assayed for CPO (Table 1). In vitro CPO activity was reduced to a minimum in line 18 and to lesser extent in line 38 indicating that reduced CPO protein levels are paralleled by lower enzyme activity.

CPO expression seems to usually provide enzyme activity which ensures sufficient substrate flow through the pathway preventing accumulation of photosensitive tetrapyrrole intermediates. Coproporphyrin(ogen) content was measured in primary transformants and wild-type (Fig. 2). Coproporphyrin(ogen) III levels raised up to several hundredfold in transformants relative to controls and

Table 1. Specific activities of CPO in wild-type tobacco plants (SNN) and transgenic plants 8, 18 and 38 transformed with CPO sense genes. Activity of CPO was monitored by HPLC with fluorescence detection as described (KRUSE & al. 1995a). Activity of wild-type (100 %) corresponds to 26.9 pkat g^{-1} protein. Values represent means ± SD.

plant	SNN	8	18	38
activity (%)	100 ± 5	28 ± 3	16 ± 4	70 ± 10

correlated with reduced CPO activity. Additional accumulation of coproporphyrin(ogen) I could be explained with impairment of earlier steps in tetrapyrrole biosynthesis. Chemically formed uroporphyrinogen I could be converted to coproporphyrinogen I, which is no substrate for CPO.

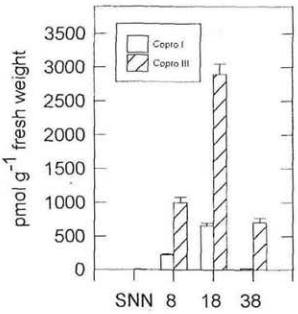

Fig. 2. Accumulation of coproporphyrin(ogen) I (copro I) and coproporphyrin(ogen) III (copro III) in wild-type (SNN) and transformants 8, 18, and 38 with additional copies for CPO. Accumulated porphyrin(ogen)s were analysed in the oxidised form as described (MOCK & al. 1995). Data represent mean ± SD from n=3 independent experiments.

The necrotic phenotype of the transformants with additional CPO genes most likely results from auto-oxidation of accumulated coproporphyrin(ogen). This reaction generates reactive oxygen species which are normally detoxified by

enzymatic and low molecular weight antioxidants that are present in all compartments, especially in photosynthetic organelles (FOYER & al. 1994). Total activity of superoxide dismutase (SOD) was increased in leaves of the transgenic lines investigated (data not shown). Activities of APX and GR weremeasured in transgenic lines 8, 18 and 38 and control plants (Fig. 3). Increased activities of APX, GR (and SOD, data not shown) in transformants indicate a general induction of the oxidative stress defense system. The reduced content of ascorbate and the decreased percentage of reduced ascorbate (Fig. 4) in leaves accumulating huge amounts of coproporpyhrin(ogen) indicate probably the limitation of the enhanced capacity of the oxidative stress defense system to prevent oxidative damage. These results resembled the antioxidative protection response in CPO antisense plants (KRUSE & al. 1995b).

We suggest a sequence of events leading to the necrotic phenotype. Porphyrins initially accumulated in plastids or leaked out into the cytoplasm or other compartments cause phototoxic processes. Increased SOD activity will probably cause a rise in the concentration of intracellular hydrogen peroxide

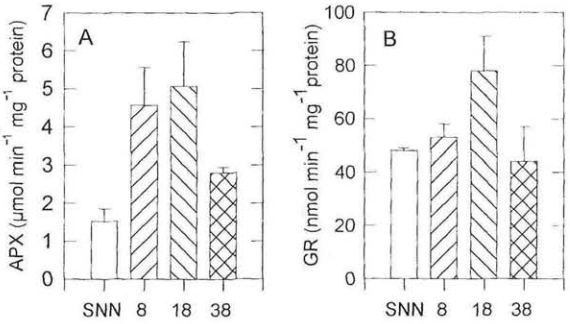

Fig. 3. Activity of (A) ascorbate peroxidase (APX) and (B) glutathione reductase (GR) in wild-type (SNN) and transformants harbouring additional copies of CPO. Data represent mean ± SD from n = 3 independent experiments.

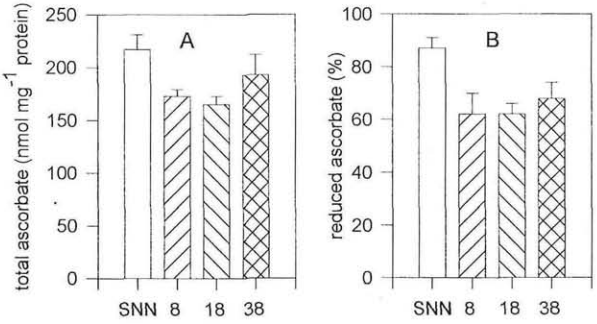

Fig. 4. Content of ascorbate (A) and percentage of reduced ascorbate (B) in wild-type (SNN) and transformants with additional copies for CPO. Data represent mean ± SD from n = 3 independent experiments.

encountered by an increase of the H_2O_2 detoxifying and ascorbate consuming activity of APX. Higher GR activity might reflect enhanced use of glutathione required for elevated enzymatic or non-enzymatic regeneration of ascorbate.

We concluded that coproporphyrin(ogen) accumulating transgenic plants seemed to be useful to examine the molecular induction mechanisms for the defense system against endogenous and environmental oxidative stress.

Acknowledgments

Financial support to B.G. by the Deutsche Forschungsgemeinschaft (Gr936/3-1 & INK20/A1-1) is gratefully acknowledged. We thank E. BARTHEL for excellent technical assistance.

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