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γ-Tocopherol dominates in young leaves of runner bean (*Phaseolus coccineus*) under a variety of growing conditions: The possible functions of γ-tocopherol

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ABSTRACT

It has been shown that young leaves of runner bean (*Phaseolus coccineus*) plants grown under natural conditions have an unusually high content of γ-tocopherol, accounting for up to 90% of all tocopherols and 50% of the chlorophyll content. The level of γ-tocopherol gradually decreased during the first two weeks of leaf development. The high content of γ-tocopherol in young leaves was not significantly influenced by growth conditions. In contrast to seeds, γ-tocopherol was also the main tocopherol found in light-grown and etiolated primary leaves of runner bean. The obtained results suggest that γ-tocopherol decline during leaf development is not only due to conversion of γ- to α-tocopherol but probably also due to degradation of γ-tocopherol to non-tocochromanol compounds. We have also shown that γ-tocopherol found in young leaves is mainly localized in thylakoid membranes within chloroplast. In the primary leaves subjected to different abiotic stresses, only during simultaneous drought and light stress, γ-tocopherolquinone, an oxidation product of γ-tocopherol, was preferentially accumulated. Since one of the other possible functions of γ-tocopherol could be its action as a nitric oxide scavenger, young leaves were analyzed for the presence of nitro-γ-tocopherol. However, despite the use of a sensitive detection method, it was not found. The possible physiological function of the increased level of γ-tocopherol in the young leaves was discussed.

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1. Introduction

Tocopherols are a group of four (α, β, γ, δ) lipophilic antioxidants synthesized by photosynthetic organisms, occurring mainly in leaves and seeds (Munné-Bosch and Alegre, 2002). Their antioxidant function is attributed to the inhibition of membrane lipid peroxidation and the scavenging of reactive oxygen species (Munné-Bosch and Alegre, 2002; Trebst et al., 2002; Kruk et al., 2005; Kruk and Trebst, 2008) but other functions have also been shown in plant metabolism such as role in sugar export from leaves to phloem (Porfirova et al., 2002; Hofius et al., 2004). The literature data on tocopherol content and composition are mainly devoted to seed oils because of the nutritional importance of vitamin E. With respect to tocopherol composition, seed oils are usually dominated by γ-tocopherol (Ong, 1993). In contrast to seeds, the most abundant form in leaves is α-tocopherol (DellaPenna, 2005; Lichtenthaler, 2007; Szymańska and Kruk, 2008a) with only some exceptions reported for lettuce (DellaPenna, 2005; Szymańska and Kruk, 2008a) or parasitic dodder shoots (van der Kooij et al., 2005; Szymańska and Kruk, 2008a) where the highest levels of γ- or δ-tocopherol can be found. The significance of these differences is presently unknown. Comparing the antioxidant activity of tocopherols *in vitro*, α-tocopherol has been shown to be more active than the γ homologue both in the inhibition of lipid peroxidation (Fryer, 1992) and singlet oxygen (Neely et al., 1988) scavenging activity. However, their relative antioxidant activity in vivo may depend on their localization within chloroplasts and membranes. In the γ-tocopherol methyltransferase-deficient plants of *Arabidopsis* and tobacco, which lack α-tocopherol, the γ homologue accumulates to amounts similar to those of α-tocopherol in the wild-type plants (Bergmuller et al., 2003; Maeda et al., 2006; Abbasi et al., 2007). Based on inhibitor studies, it has been shown that α-tocopherol acts as a singlet oxygen scavenger in the photosystem II of *Chlamydomonas reinhardtii* (Trebst et al., 2002; Kruk et al., 2005). The inhibitory effect can be overcome by the addition of membrane-permeable short chain α- and γ-tocopherol derivatives. This observation suggests that α-tocopherol can be substituted by γ-tocopherol in leaves in the protection against singlet oxygen. A similar conclusion was drawn from the biochemical analysis of the *Arabidopsis* γ-tocopherol methyltransferase-deficient mutant (Bergmuller et al., 2003). The photosynthetic parameters of this mutant were indistinguishable from those of the wild-type under a variety of stress conditions. These experiments indicate that γ-tocopherol takes over the function of

Abbreviations: chl, chlorophyll; Toc, tocopherol; TQ, tocopherolquinone.

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α-tocopherol in the antioxidant function. However, it has recently been shown that γ-tocopherol is more potent than α-tocopherol in transgenic tobacco in mediating osmoprotection in vivo (Abbasi et al., 2007). Based on these studies, it was concluded that γ-tocopherol is not only indispensable for seed desiccation tolerance, but that it can also increase desiccation tolerance in leaves.

In the present study we have found that γ-tocopherol is the main tocopherol in the young leaves of adult runner bean plant under a variety of growing conditions, as well as in the young leaves of seedlings and that its level gradually decreases during leaf development. γ-Tocopherol is also the main homologue in the primary leaves of light-grown and etiolated seedlings. In order to find some possible significance in this unusual tocopherol composition in young leaves, we have investigated whether γ-tocopherol could be a nitric oxide scavenger in young leaves which have previously been shown to be metabolically active in the release of this endogenous signaling molecule (Desel and Krupinska, 2005; Qu et al., 2006; Zhang et al., 2006). In contrast to α-tocopherol, γ-tocopherol is a potent nitric oxide scavenger (Cooney et al., 1993, 1995) and such a reaction has been shown in animal metabolism (Hensley et al., 2000) and there are also indications of this reaction in early seedling development (Desel and Krupinska, 2005) and in germinating seeds (Desel et al., 2007). Moreover, we have investigated a possible specific role of γ-tocopherol in drought stress resistance in young leaves.

2. Results

An analysis of tocopherol composition in leaves of different age from plants grown under natural conditions (Fig. 1) revealed that γ-tocopherol dominates in young leaves and its level gradually decreases with leaf development, reaching the same level as that of α-tocopherol in 7-days old leaves. In the youngest leaves, γ-tocopherol level was as high as 30% of the chlorophyll level and the γ- to α-tocopherol ratio was 6–7 (Table 1). For different plants analyzed from the same growth conditions, γ-tocopherol level in the youngest leaves varied between 75 and 150 μg/g FW, the γ- to α-tocopherol ratio varied between 5 and 10 and the γ-tocopherol level reached as much as 50% of the chlorophyll level (data not shown). During leaf development, the α-tocopherol level increased slowly for the first 3–4 weeks and only afterwards did its level increase significantly, reaching 80% of the chlorophyll level in the oldest leaves examined. The chlorophyll content was relatively low in the youngest leaves and increased gradually with their age and decreased finally during senescence (Table 1). The content of δ-tocopherol was very low in young leaves and its content increased slightly with leaf age (Fig. 1).

Table 1
Proportion of tocopherol isomers to chlorophyll and chlorophyll content in leaves of different age of 50-day old runner bean (*Phaseolus coccineus*) plant (Fig. 1) growing under natural conditions in summer (analyzed September 13/14)

Leaf age (days)	Leaf length (cm)	α-Toc/chl (mol/100 mol)	γ-Toc/chl (mol/100 mol)	δ-Toc/chl (mol/100 mol)	mg chl/g FW
1	0.5–0.8	3.8	29.3	0.90	0.70
2	1.0–1.5	4.9	26.7	0.90	0.59
8	3.5–4.0	4.2	4.4	0.15	1.30
13	5.0–5.5	5.3	1.2	0.06	1.30
19	6.5–7.5	6.2	1.4	0.05	1.60
24	9.0–9.5	9.4	1.8	0.05	1.82
29	9.0–10.0	12.9	0.7	0.08	1.57
31	9.5–11.0	21.7	1.3	0.11	1.28
39	12.0–14.0	81.3	3.0	0.20	0.82

The SE ($n = 3$) was not higher than 10%, 17% and 35% of the average values for α-, γ- and δ-tocopherols, respectively. The SE ($n = 3$) was $\leqslant$6% for the chl content.

In order to investigate if the high γ-tocopherol level in young leaves is influenced by light intensity during growth, an analysis of tocopherol homologues was performed on plants grown outdoors under limited incident light intensity (Fig. 2). The age-dependent tocopherol content in these plants was similar to that in plants grown under natural light conditions. In older, senescing leaves, when the chlorophyll content decreased (Table 2), the α-tocopherol level also strongly decreased (Fig. 2). In shade-grown plants, the γ-tocopherol content in the youngest leaves (varied between 50 and 100 μg/g FW) was lower than that in the former case but its proportion to α-tocopherol was similarly high. As compared to the plants shown in Fig. 1, the plants grown under shade were not exposed to direct sunlight (maximum light intensity of 2000 μmol/m^2/s) and grew under lower light intensity throughout the time of the experiment (maximum light intensity of 130 μmol/

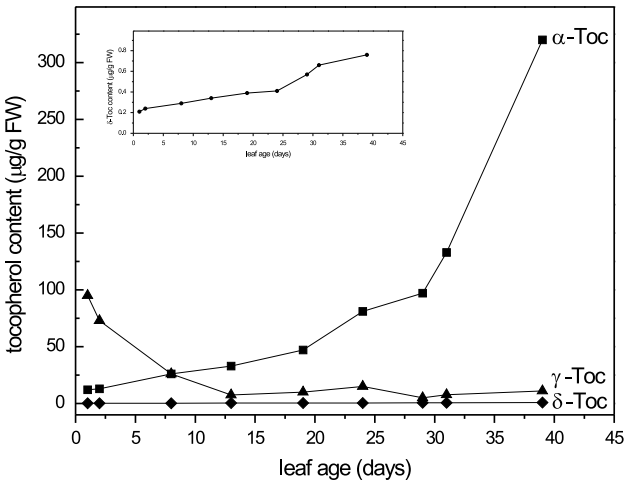

Fig. 1. Tocopherol content in leaves of different ages collected from a 50-day old runner bean (*Phaseolus coccineus*) plant grown under natural conditions in summer. Individual tocopherol to chlorophyll ratio and chlorophyll content in leaves is given in Table 1. The shown values are the mean from 3 samples taken from 1 to 2 leaves of the same age. The SE was not higher than 7%, 14% and 25% of the average values for α-, γ- and δ-tocopherols, respectively. Other details are given in Section 4.

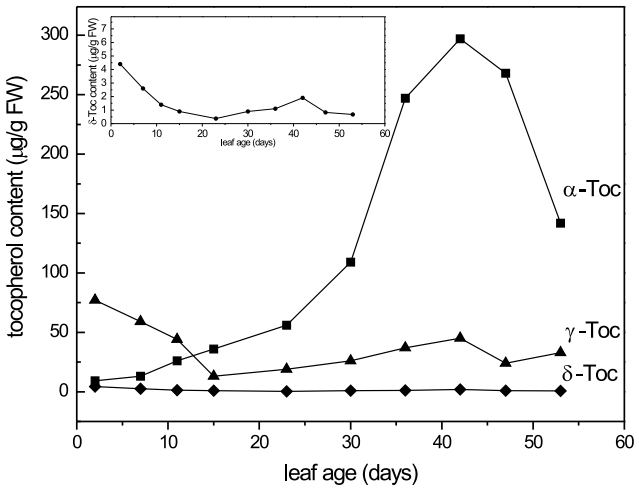

Fig. 2. Tocopherol content in leaves of different ages collected from a 58-day old runner bean (*Phaseolus coccineus*) plant grown under natural conditions in summer under shade. Individual tocopherol to chlorophyll ratio and chlorophyll content in leaves in given in Table 2. The SE was not higher than 7%, 11% and 30% of the average values for α-, γ- and δ-tocopherols, respectively. Other details are given in Section 4.

Table 2
Proportion of tocopherol isomers to chlorophyll and chlorophyll content in leaves of different age of 58-day old runner bean (*Phaseolus coccineus*) plant (Fig. 2) growing outdoors under shade (analyzed September 22/23)

Leaf age (days)	Leaf length (cm)	α-Toc/chl (mol/100 mol)	γ-Toc/chl (mol/100 mol)	δ-Toc/chl (mol/100 mol)	mg chl/g FW
2	0.9–1.3	5.2	44.7	2.54	0.37
7	2.0–2.2	5.7	27.0	1.12	0.47
11	4.0–4.5	5.5	9.5	0.29	1.01
15	8.5–9.0	5.0	1.9	0.13	1.49
23	8.0–8.7	7.4	2.9	0.05	1.47
30	8.5–9.5	15.9	4.3	0.13	1.44
36	11.5–12.5	29.3	4.5	0.12	1.76
42	12.0–13.0	37.3	5.9	0.24	1.66
47	11.5–13.0	39.3	3.6	0.12	1.43
53	13.0–14.0	27.3	6.5	0.13	1.09

The SE (n = 3) was not higher than 12%, 13% and 32% of the average values for α-, γ- and δ-tocopherols, respectively. The SE (n = 3) was ⩽6% for the chl content.

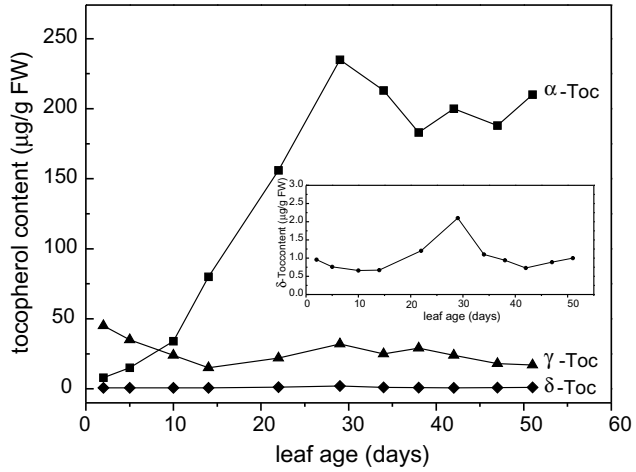

Fig. 3. Tocopherol content in leaves of different ages collected from a 57-day old runner bean (*Phaseolus coccineus*) plant grown under controlled, short-day conditions. Individual tocopherol to chlorophyll ratio and chlorophyll content in leaves in given in Table 3. The SE was not higher than 8%, 11% and 27% of the average values for α-, γ- and δ-tocopherols, respectively. Other details are given in Section 4.

m^2/s). Moreover, the plants facing direct sunlight were also exposed to higher temperatures during that time.

The plants grown in climate chambers, under controlled low-light and short-day conditions (Fig. 3, Table 3), showed a lower γ-tocopherol content in the youngest leaves than those grown under natural conditions, although it was still higher than that of α-tocopherol. The content of γ-tocopherol in the youngest leaves varied between 50 and 70 μg/g FW among individual plants. The leaves of these plants, both younger and older, accumulated higher chlorophyll amounts than those grown under natural conditions and reached up to 3 mg chl per gram of fresh weight (Table 3). Such a high chlorophyll content is typical of low-light-grown plants. The tocopherol to chlorophyll ratio was lower than in the case of plants grown under natural conditions. α-Tocopherol content increased linearly with the leaf age of chamber-grown plants and after reaching the maximum level, it did not change significantly (Fig. 3).

We have also examined the primary leaves of low-light-grown and etiolated seedlings for tocopherol composition (Fig. 4A). Both in light-grown and etiolated seedlings, γ-tocopherol was the main tocopherol. In seeds, as opposed to primary leaves, α-tocopherol accounted for over 97% of all tocopherols (Fig. 4A). These data indicate that γ-tocopherol in young leaves is synthesized *de novo* and its synthesis is not light-dependent. When different parts of the etiolated hypocotyls were examined (Fig. 4B), it turned out that

Table 3
Proportion of tocopherol isomers to chlorophyll and chlorophyll content in leaves of different age of 57-day old runner bean (*Phaseolus coccineus*) plant (Fig. 3) growing under controlled, short-day conditions (10 h day/14 h night, light intensity – 100 μmol/m^2/s, 23 ± 2 °C) (analyzed September 20/21)

Leaf age (days)	Leaf length (cm)	α-Toc /chl (mol/100 mol)	γ-Toc/chl (mol/100 mol)	δ-Toc/chl (mol/100 mol)	mg chl/g FW
2	0.6–0.8	2.0	11.6	0.25	0.83
5	1.0–1.5	2.2	5.4	0.11	1.40
10	2.0–2.7	4.2	3.1	0.08	1.66
14	3.0–4.0	8.9	1.7	0.08	1.90
22	4.5–5.4	16.7	2.4	0.13	1.95
29	5.5–6.5	16.2	2.2	0.15	2.24
34	5.8–6.9	14.9	1.8	0.08	2.98
38	9.0–9.5	13.1	2.2	0.07	2.86
42	10.0–11.0	14.7	1.8	0.05	2.88
47	12.5–13.5	17.1	1.7	0.08	2.20
51	15.0–17.0	27.1	2.3	0.13	1.61

The SE (n = 3) was not higher than 11%, 19% and 30% of the average values for α-, γ- and δ-tocopherols, respectively. The SE (n = 3) was ⩽5% for the chl content.

the youngest part of the hypocotyls, located most closely to the primary leaves, showed the highest γ-tocopherol content and its level decreased with hypocotyl age in contrast to α-tocopherol. These data show that γ- to α-tocopherol conversion is not light-dependent and suggests that part of γ-tocopherol could be degraded into non-chromanol products since the decrease of γ-tocopherol level is considerably more pronounced than the α-tocopherol synthesis.

To examine the intrachloroplast distribution of tocopherol homologues in young runner bean leaves, isolated thylakoids were treated by two methods that are known to release the non-specifically bound prenyllipids from thylakoids, namely sonication (Lichtenthaler and Sprey, 1966) and French press treatment (Chapman and Barber, 1996). For these experiments, 13-day-old leaves were analyzed that contained comparable proportions of α- and γ-tocopherols, which makes the analysis of their distribution more precise. Plastoglobuli-free thylakoids were significantly enriched in γ-tocopherol as compared to the whole chloroplast, while plastoglobuli were the main site of α-tocopherol accumulation (Fig. 4C). This indicates that γ-tocopherol dominates in the thylakoids of young leaves and α-tocopherol is predominantly localized outside thylakoids in plastoglobuli. However, thylakoid fraction obtained after sucrose gradient centrifugation showed only slight enrichment in γ-tocopherol as compared to chloroplasts, while plastoglobuli were mainly composed of α-tocopherol (Fig. 4C). This is probably due to the poor efficiency of this method for the release of plastoglobuli from thylakoids. This conclusion was also supported by the low proportion of total tocopherols in the plastoglobuli fraction as compared to the thylakoid fraction.

In order to investigate the function of γ-tocopherol as a potential scavenger of reactive oxygen species formed during stress, we analyzed the level of tocopherolquinones, the oxidation products of tocopherols, under a variety of stress conditions applied to primary bean leaves that contain high levels of γ-tocopherol. As can be seen in Fig. 5, high light stress on its own caused only a slight increase in the level of both tocopherolquinones, while high light in combination with drought stress stimulated clearly an increase in both tocopherolquinones with a preferential increase in the concentration of γ-tocopherolquinone. Its level increased nearly fivefold more than in the control leaves, while during the same time of the experiment the level of α-tocopherolquinone increased 2.5-fold. In contrast to drought stress, cold stress followed by high light illumination caused a considerable increase only in the level of α-tocopherolquinone.

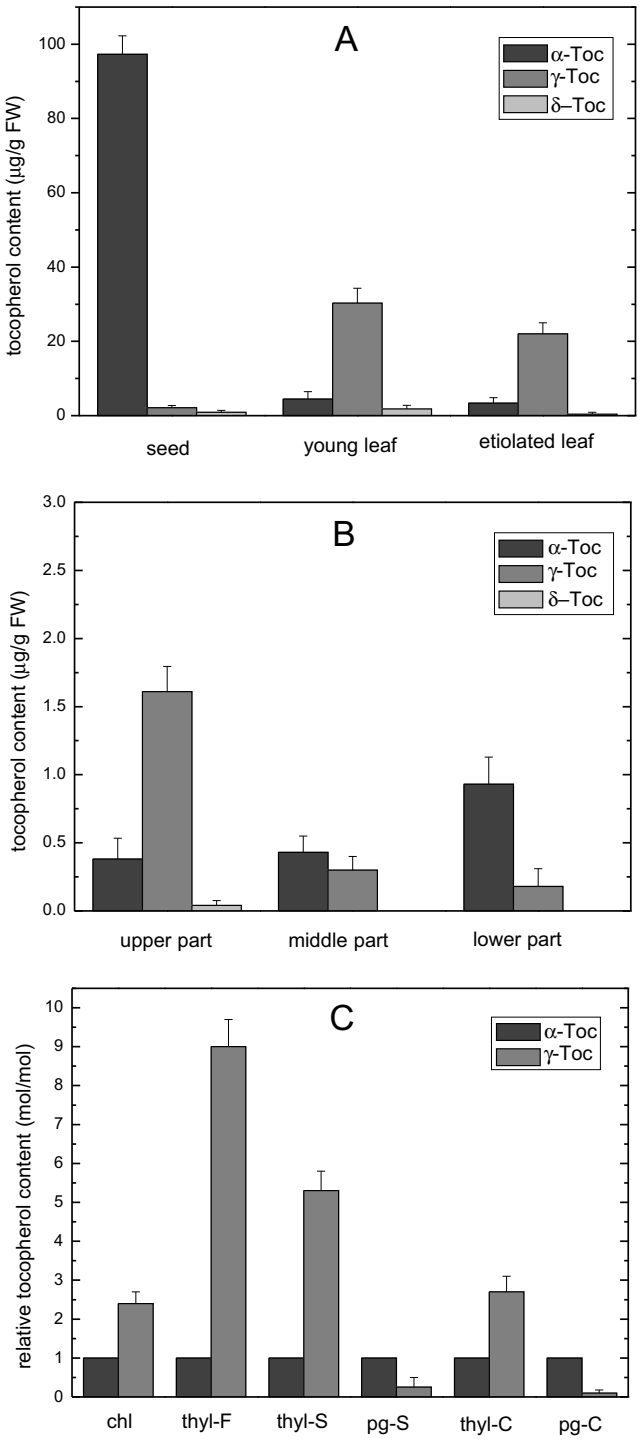

Fig. 4. (A) Tocopherol content in runner bean (*Phaseolus coccineus*) seeds and the primary leaves of climate chamber-grown plants (7-days old) under light (10 h day/14 h night, light intensity – 100 μmol/m^2/s, 23 ± 2 °C) and dark (etiolated) conditions; (B) tocopherol content in different parts of 7-day old etiolated runner bean (*Phaseolus coccineus*) hypocotyls; (C) the relative tocopherol composition of chloroplasts (chl), French press-treated thylakoids (thyl-F), sonicated thylakoids (thyl-S), plastoglobuli released from sonicated thylakoids (pg-S), sucrose gradient-purified thylakoids (thyl-C) and sucrose gradient-purified plastoglobuli (pg-C). The fractions were isolated from the primary leaves of 13-day old runner bean (*Phaseolus coccineus*) plants grown under controlled conditions ((10 h day/14 h night, 100 μmol/m^2/s, 23 ± 2 °C).

In order to find an additional possible function of the unusually high γ-tocopherol level in young leaves, we investigated whether γ-tocopherol could act as a nitric oxide scavenger and

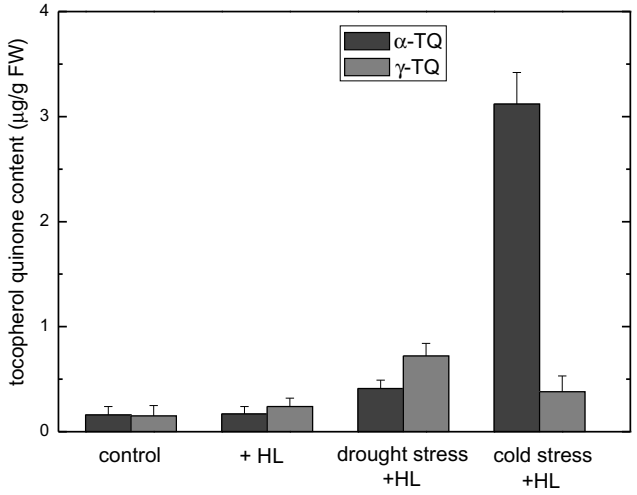

Fig. 5. Effect of high light (HL), drought stress and high light, and cold stress followed by high light illumination on the level of tocopherolquinones in the primary leaves of climate chamber-grown plants (7-days old) under light (10 h day/14 h night, light intensity – 100 μmol/m^2/s, 23 ± 2 °C). High light of 750 μmol/m^2/s intensity was applied for 3 h alone, during drought stress, or after a 12 h of cold and dark incubation of leaves.

whether nitro-γ-tocopherol is formed as a result of this reaction. Using a sensitive and selective electrochemical detection method of nitro-γ-tocopherol (Hensley et al., 2000) we measured its content in extracts from young bean leaves. In order to avoid the interference of photosynthetic pigments, we used hexane extracts of lyophilized leaves for the analysis. However, in spite of the high sensitivity of the applied method we were not able to detect nitro-γ-tocopherol in the extracts. Based on the recovery of the added nitro-γ-tocopherol standard to the leaf extracts, the estimated detection limit was one nitro-γ-tocopherol per 5000 chl molecules originally present in leaves (Fig. 6).

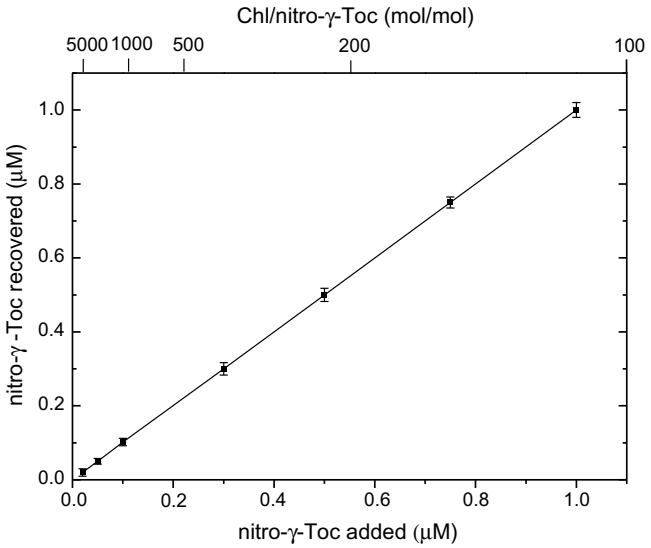

Fig. 6. Response of the electrochemical HPLC detector for nitro-γ-tocopherol peak in the leaf extract as a function of the added nitro-γ-tocopherol standard. The calculated molar proportion of chlorophyll originally present in leaves to the detected nitro-γ-tocopherol in the extract is also shown.

3. Discussion

The unusually high γ-tocopherol level in young leaves of runner bean plants found in the present study has not been reported yet for any other plant species. It should be noted that we have also observed a similarly high γ-tocopherol level in leaves of common bean (*Phaseolus vulgaris*), pea (*Piscum sativum*) or broad bean (*Vicia faba*) seedlings (data not shown). Moreover, it has been recently shown (Szymańska and Kruk, 2008a) that in developing maple leaves, the level of γ-tocopherol also exceeded that of α-tocopherol. This indicates that the dominancy of γ-tocopherol in young leaves might be more widespread and points to a special function of γ-tocopherol during leaf development. The decline in γ-tocopherol level is only partially associated with an increase in α-tocopherol level, suggesting that γ-tocopherol is not only converted to α-tocopherol but also to some non-chromanol products. To date, no enzymes responsible for specific tocopherol degradation have been isolated, although the existence of tocopherol oxidase in seeds and the etiolated shoots of pea plants has been suggested (Gaunt and Barlow, 1971).

It has been reported that the γ-tocopherol level increases in senescing leaves of *Arabidopsis* wild-type (Holländer-Czytko et al., 2005; Mishina et al., 2007), transgenic *Arabidopsis* plants with overexpressed nitric oxide dioxygenase (Mishina et al., 2007) and others (Chrost et al., 1999), but such a correlation was not observed in the present study for runner bean, nor for tobacco (Abbasi et al., 2007).

The low γ-tocopherol content in runner bean seeds, together with its relatively high level as compared to α-tocopherol in the primary leaves of light-grown and etiolated seedlings suggest that γ-tocopherol is intensively synthesized in young leaves and its synthesis is not light-dependent. The age-related decrease in γ-tocopherol content in etiolated hypocotyls, associated with the considerably slower increase in α-tocopherol content, indicates that γ-tocopherol degradation is age-related, genetically determined and is not light-dependent.

The first specific steps in tocopherol biosynthesis, the prenylation of homogentisic acid and the subsequent methylation of the ring take place in the inner chloroplast envelope (Munné-Bosch and Falk, 2004) while ring cyclisation by the tocopherol cyclase proceeds in plastoglobuli (Vidi et al., 2006). The site of the last step of α-tocopherol synthesis, the methylation of γ-tocopherol by a specific methyltransferase, was not identified within chloroplast, and neither has this enzyme been found in plastoglobuli (Vidi et al., 2006). Therefore, the localization within chloroplasts of the high amount of γ-tocopherol remains an open question. Could it be in thylakoid membranes, or outside thylakoids in plastoglobuli, which are known for the high content of prenyllipids (Lichtenthaler et al., 1981). The obtained results showed that γ-tocopherol is preferentially localized in thylakoid membranes, which suggest a specific function for it there.

We found that γ-tocopherolquinone is accumulated with some preference during drought stress in the high light-treated young leaves of seedlings, while α-tocopherolquinone is mainly formed during cold stress followed by high light illumination. This indicates different mechanisms of oxidation of both tocopherolquinones under the applied conditions. Tocopherolquinones have been found as oxidation products of tocopherols both *in vitro* and in vivo (Michalski and Kaniuga, 1981; Kruk and Trebst, 2008) as a result of the scavenging of reactive oxygen species during oxidative stress. One of the main reactive oxygen forms generated under different stress conditions (Mittler, 2002), including drought stress (Smirnoff, 1993) is a superoxide radical which is formed by photosystem I both within (Kruk et al., 2003) and outside of this complex in stroma (Asada, 2000). The superoxide radical is scavenged in stroma by superoxide dismutase (Asada, 2000) which is deactivated by cold stress (Michalski and Kaniuga, 1982). The preferential accumulation of α-tocopherolquinone under cold stress suggests that α-tocopherol is mainly oxidized in a chloroplast compartment where superoxide dismutase is located, i.e. in the stroma. This would be consistent with the enrichment of plastoglobuli in α-tocopherol. On the other hand, during drought stress, the production of reactive oxygen species within thylakoid membranes, where γ-tocopherol resides, is the main reason for tocopherol oxidation.

One of the other possible functions of γ-tocopherol could be a nitric oxide scavenging reaction in thylakoid membranes. Nitric oxide has been shown to be formed in relatively high amounts in young developing leaves (Desel and Krupinska, 2005; Qu et al., 2006; Zhang et al., 2006) and to be formed in chloroplasts (Jasid et al., 2006). Moreover, it has been shown that the reaction of NO with molecular oxygen to nitric dioxide is especially fast within the hydrophobic interior of the membrane (Liu et al., 1998). NO could also react at a high rate with superoxide (Hensley et al., 2000), which is formed mainly within the hydrophobic thylakoid membrane interior (Kruk et al., 2003). However, we were not able to detect it in extracts of young leaves. This indicates that nitro-γ-tocopherol is not formed in young leaves in detectable amounts or that it is quickly degraded or metabolized, although this compound is highly stable *in vitro*.

The question arises as to the function of the high γ-tocopherol level in young runner bean leaves. One of the possibilities is that it has no special function and its accumulation is only a result of the delayed synthesis of γ-tocopherol methyltransferase in developing leaves. However, such a possibility hardly seems probable. The literature data indicate that leaves at the early stage of development are more resistant to high light and drought stress than mature leaves (Jung, 2004; Jiang et al., 2005). This enhanced protection is provided by increased levels of xanthophyll cycle carotenoids and antioxidant enzymes in developing leaves. On the other hand, it is known that the level of membrane-localized prenyllipid antioxidants, such as α-tocopherol or plastoquinol is low in young leaves and increases with leaf age (Lichtenthaler, 2007; Szymańska and Kruk, 2008a,b). Therefore, it is probable that the high level of γ-tocopherol in young leaves replaces other lipophilic antioxidants present in higher concentrations in mature leaves and contributes to the overall protection of young leaves against oxidative stress.

Water deficit is known to cause oxidative stress in plants (Smirnoff, 1993; Moran et al., 1994; Jiang and Zhang, 2002) and it affects α-tocopherol levels in a number of plant species. It has been reported that its level increases remarkably in response to water deficit both in spinach and pea leaves (Munné-Bosch and Alegre, 2002), as well as in tobacco (Munné-Bosch et al., 2005). Drought stress induces also an accumulation of α-tocopherolquinone (Munné-Bosch and Alegre, 2003; Munné-Bosch et al., 2005; Müller et al., 2006). However, neither young leaves nor γ-tocopherolquinone level were investigated in these studies.

The preferential accumulation of γ-tocopherolquinone in drought stressed leaves observed in our study might suggest some involvement of γ-tocopherol in drought stress protection. Recently, the specific function of γ-tocopherol in osmoprotection of transgenic tobacco plants has been shown (Abbasi et al., 2007). It has been concluded from these studies that γ-tocopherol is more potent than α-tocopherol in conferring desiccation tolerance in vivo both for seeds and in leaves.

Interestingly, the trigger activating the enhanced level of antioxidant enzymes in young soybean leaves was suggested to be the low leaf turgor of developing leaves, even though the plants were well watered (Jiang et al., 2005). Maybe, this is also a signal for the increased γ-tocopherol synthesis in young leaves.

4. Experimental

4.1. Plant material and growing conditions

Throughout the present study, runner bean (*Phaseolus coccineus* cv. Piękny Jaś) plants were used. The plants were sown at the end of July and grown for the time shown in the description of Tables under three different conditions: under natural weather conditions in Krakow, under natural conditions but under shade (maximal light intensity of 100-130 μmol/m^2/s) and under controlled, climate chamber conditions (10 h day/14 h night, 100 μmol/m^2/s, 23 ± 2 °C). After that time, all the leaves were analyzed within 1–2 days. In the case of young leaves, the whole leaves were taken for analysis, while for older leaves, only the tip parts were analyzed. All the leaves were collected for analysis at noon time from outdoor grown plants or 4 hours after the onset of illumination for chamber-grown plants in order to avoid the possible circadian changes in the tocopherols level. For some experiments, seeds or leaves of young, light-grown or etiolated plants grown under climate chamber conditions were used.

4.2. Extraction and HPLC analysis of tocopherols

For tocopherol analysis, 10–150 mg of leaves (depending on the leaf size) were ground thoroughly in a mortar with 1.5 ml of cold HPLC solvent (acetonitrile/methanol/water, 72/8/1, v/v/v). The extract was transferred to an Eppendorf tube, briefly centrifuged (60 s) on a benchtop centrifuge (10,000 *g*) and analyzed by HPLC.

The HPLC measurements were performed using 100 μl loop, Jasco PU-980 pump and UV–VIS detector system UV-970, Shimadzu RF10-AXL fluorescence detector (excitation/emission detection at 290/330 nm), Teknokroma (Barcelona, Spain) C_{18} reverse-phase column (Nucleosil 100, 250 × 4 mm, 5 μm), isocratic solvent system – acetonitrile/methanol/water (72/8/1, v/v/v) at a flow rate of 1.5 ml/min. Tocopherol homologues of HPLC grade (⩾99.5%) were purchased from Merck. Tocopherols have been detected taking advantage of their native fluorescence in the ultraviolet range (Kruk et al. 2006).

Tocopherolquinones were measured in primary 7-day old bean leaves using zinc post column reduction and fluorescence detection as previously described (Kruk and Trebst, 2008; Kruk et al., 2008). The detached leaves were illuminated for 3 h with high light (750 μmol/m^2/s) at room temperature. In the case of cold and light stress, the leaves were stored for 12 h on ice in the dark before illumination. With the exception of drought stress, the leaves were illuminated in Petri dishes filled with water.

4.3. Chlorophyll content analysis

Chlorophyll content was determined spectrophotometrically in acetone extracts of leaves according to the method of Lichtenthaler (1987).

4.4. Analysis of tocopherols in subchloroplast fractions

Tocopherol distribution within the chloroplasts of runner bean was investigated using 13-day old leaves grown under short-day conditions (10 h day/14 h night, 100 μmol/m^2/s, 23 ± 2 °C) showing comparable levels of both α- and γ-tocopherols. The leaves were homogenized for 15 s in a 0.8 M sucrose/ 50 mM Hepes buffer containing 20 mM NaCl and 5 mM $MgCl_2$ (pH 7.5), filtered through nylon cloth, and centrifuged at 3000 *g* × 5 min. Part of the chloroplast pellet was taken for tocopherol analysis and the remaining part was osmotically shocked by suspension in the buffer for 5 min. The mixture was then centrifuged as described above to give thylakoids preparation. In order to remove tocopherols bound non-specifically to thylakoids in the form of plastoglobuli, two methods were applied which are known to remove plastoglobuli from thylakoids (Lichtenthaler and Sprey, 1966; Chapman and Barber, 1996). For this purpose, thylakoids were suspended in the buffer and divided into two parts. One part was sonicated for 3 min on ice with a 50% 'duty cycle' (Cole Parmer Instruments, model 4710, IL, USA) and microtip at full power. The second part was treated twice with French Press (Aminco, IL, USA) at 500 PSI. Subsequently, both suspensions were centrifuged at 100,000 *g* for 100 min using a Beckman L70 ultracentrifuge. The supernatants containing the plastoglobuli fraction on the top were extracted with hexane and the sediment of plastoglobuli-free thylakoids was extracted with an HPLC solvent (acetonitrile/methanol/water, 72/8/1, v/v/v) and the fractions were analyzed for tocopherol composition. Additionally, chloroplast were fractionated into thylakoids and plastoglobuli using sucrose gradient centrifugation as described by Vidi et al. (2006). The collected fractions were extracted with methanol and analyzed using HPLC as described above.

4.5. Analysis of nitro-γ-tocopherol content in young leaf extracts

5-Nitro-γ-tocopherol was obtained by a modified procedure described by (Cooney et al., 1995). Two ml of 10 mM γ-tocopherol in hexane was shaken with 1 ml portions of gaseous nitrogen dioxide and the progress of the reaction was followed using HPLC on C_{18} reverse-phase column in methanol as a mobile phase, at a flow rate of 1.5 ml/min and absorption detection at 300 nm. Under such conditions, the retention times of γ-tocopherol and nitro-γ-tocopherol were 5.0 and 8.3 min, respectively. Nitrogen dioxide was obtained in a test tube by adding drops of saturated $NaNO_2$ solution to a saturated $FeSO_4$ solution acidified previously with H_2SO_4. When the reaction was completed, the formed nitro-γ-tocopherol was purified by TLC on silicagel plates developed in chloroform/methanol (100/0.5, vol/vol). Nitro-γ-tocopherol was identified as the only yellow band with the highest R_f. The identity of the purified nitro-γ-tocopherol was confirmed by comparing its absorption spectra in acetonitrile with the literature data (Cooney et al., 1995). The determined molar extinction coefficients for this compound in acetonitrile were 5800 at 302 nm and 1000 at 424 nm.

The analysis of the nitro-γ-tocopherol content was performed on 7-day old leaves of runner bean seedlings grown under short-day conditions (10 h day/14 h night, 100 μmol/m^2/s, 23 ± 2 °C). The leaves were lyophilized overnight in 15-ml Falcon tubes, ground with a glass rod and shaken overnight with hexane in the dark. Afterwards, the extract was filtered, evaporated to dryness under a stream of nitrogen, dissolved in a small volume of methanol and applied to the HPLC system. The nitro-γ-tocopherol was analyzed using a YMC (Europe GmbH, Germany) C_{30} reverse-phase column (250 × 4.6 mm, 3 μm) and Decade electrochemical detector (Antec, Leyden, The Netherlands) equipped with a VT-03 flow cell. The isocratic mobile phase was methanol containing 25 mM sodium chlorate and 2 mM $HClO_4$, the flow rate – 1.5 ml/min and the applied oxidizing potential of + 1.1 V. Under the described conditions, the nitro-γ-tocopherol standard showed a retention time of 11.8 min.

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