Possible Involvement of Lipid Peroxidation in Cooled Tulip Bulbs

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Abstract

Lipid peroxides and their conversion products are active in various physiological processes in growth and development. Physiological role of lipid metabolism in dormancy and dormancy release in tulip bulbs is unknown. We found in tulip leaves high level of free and bound C18:3 and C18:2 acids and small amounts of C18:1, C18:0, C16:1, C16:0, C14:0, C12:0 acids. In our studies the level of lipid peroxides was determined in leaves, anthers and basal plate of uncooled and cooled tulip bulbs 'Apeldoorn' as a concentration of MDA, using a modified thiobarbituric acid–malondialdehyde (TBA–MDA) assay. The MDA level was lower in anthers than in leaves and basal plate. During 8 weeks of bulbs storage no changes in lipid peroxidation levels were found, with the exception of 3rd leaf at which a decrease was noted. After 8 weeks of bulbs storage, lipid peroxidation increased in all samples. However, there were no differences in MDA levels between samples from uncooled and cooled bulbs. It seems that in tulip bulbs lipid peroxidation was independent to temperature of bulbs storage. These results indicated that lipid peroxidation is probable not involved in dormancy and dormancy release in tulip bulbs.

INTRODUCTION

Lipoxygenases catalyze the hydroperoxidation of polyunsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene site. In higher plants, the natural substances for these enzymes are linolenic acid (C18:3) and linoleic acid (C18:2) (Siedow, 1991; Conconi et al., 1996). Lipid peroxides and their conversion products are active in various physiological processes in plant growth and development, for example during senescence and fruit maturation (Leverentz et al., 2002; Porta and Rocha–Sosa, 2002), with responses to wounding (Farmer and Ryan, 1992; Saravitz and Siedow, 1996), pathogen attack (Veronesi et al., 1996; Rance et al., 1998) and in the biosynthesis of signaling molecules, such as jasmonic acid (Creelman and Mullet, 1997). The lipoxygenase has been associated with the mobilization of storage lipids during germination (Feussner et al., 2001) and vegetative growth (Fischer et al., 1999). Many lipoxygenase genes are regulated differentially during *Arabidopsis* seedling development (Melan et al., 1994), tomato fruit ripening (Kausch and Handa, 1997), potato tuber development (Bachem et al., 1996; Kolomiets et al., 2001). In tulip bulbs there were isolated and characterized 5-lipoxygenase (Reddanna et al., 1988) and 9-lipoxygenase (Grechkin et al., 2000). Many studies concerning metabolic processes and changes in levels of some substances possibly associated with cooled tulip bulbs have been published, including changes in polyamine contents (Kollöffel et al., 1992), redistribution of organic nitrogen and carbon (Ohyama et al., 1988; Lambrechts et al., 1992), changes in the content of amino acids in anthers from tulip bulbs (Lukaszewska et al., 1989; Tonecki and Gorin, 1990), starch content and α-amylase activity (Gorin and Heidema, 1985; Heidema et al., 1986), chalcones content (Franssen and Kersten, 1992), carbohydrate content (Lambrechts et al., 1994) and invertase activity (Lambrechts and Kollöffel, 1993). Physiological role of lipid metabolism in dormancy and dormancy release in tulip bulbs is unknown. The present study aimed to investigate the level of fatty acid in tulip stem and leaves, and the level of lipid peroxides in some organs of uncooled and cooled bulbs.

MATERIALS AND METHODS

Plant Material

'Apeldoorn' tulips from commercial stocks were used in experiments. After lifting, bulbs with circumference of 12 cm were stored at 17-20°C until October 14. Then one group of the bulbs was kept at 17°C (uncooled bulbs), and another group was transferred to 5°C for dry cooling (cooled bulbs).

Assay of Free, Bound and Total Fatty Acid in Stem and Leaves

After 12 weeks of cooled storage, tunics were removed and bulbs were individually planted into pots and cultured at 18-20°C in natural light. Three to four days before flowering the first (basal) internode of tulip stem and first leaf were taken for determination of free and bound fatty acids. Tissue samples were weighed and blended with 40 mL of 0.005 N NaOH and a few drops of silicon antifoam solution in an Ultra-Turax tissue grinder at 13,500 rpm. The slurry was filtered with 1g of Celite-545 as a filter aid through Whatman No. 1 paper under reduced pressure. The filtrate was acidified with 1 mL of 6N HCl. After adding 50 µg internal standard (margarinic acid), free fatty acids were extracted twice with 50 mL of hexane. Combined hexane extracts were dried by passing through a 5 g of anhydrous sulphate layer, and then evaporated to dryness on a rotary evaporator. Methyl esters of fatty acids were prepared with boron trifluoride-methanol reagent (Metcalfe et al., 1966). Quantitative and qualitative determinations of fatty acid methyl esters were performed by GLC using a Pye Unicam 204 gas chromatograph equipped with a 200x0.2 cm column (packed with 10% Silar 10C on Chromosorb W, 80/100 mesh) and a flame ionization detector. The column temperature was programmed to increase from 121°C to 210°C at 6°C min^{-1}. The amounts of individual fatty acids were calculated from standard curves of appropriate acid esters. The total amounts of individual fatty acids were determined after lipid hydrolysis (3 h at 80°C) using 2 mL of 5% KOH in methanol. After cooling and adding 2 mL of 10% NaCl solution, the mixture was extracted twice with 2 mL of isooctane. The water fraction contained fatty acid potassium salts while isooctane layer contained sterols. The total amounts of individual fatty acids were then analyzed (after acidification of the water fraction with 6N HCL to pH=1) according to the procedure for free fatty acids, as ascribed above. Bound fatty acids contents were calculated from the total and free fatty acids contents by subtraction.

Assay of Lipid Peroxidation

The level of lipid peroxidation was determined in leaves, anthers and basal plate of uncooled and cooled tulip bulbs. Lipid peroxidation was measured after 0, 6, 8, 10 and 13 weeks of bulbs storage at 17°C (uncooled) and 5°C (cooled) as a concentration of MDA, using a modified thiobarbituric acid–malondialdehyde (TBA–MDA) assay (Hodges et al., 1999). Samples were homogenized with inert sand in 1:25 (g FW:mL) 80:20 (v/v) ethanol:water, followed by centrifugation at 3000 *g* for 10 min. One milliliter of the sample was added to a test tube with 1 mL of either 1) –TBA solution comprised of 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene or 2) +TBA solution containing the above and 0.65% (w/v) thiobarbituric acid. Samples were then mixed vigorously, heated at 95°C for 25 min, cooled and centrifuged at 3000 *g* for 10 min. Absorbance values were recorded at 440 nm, 532 nm and 600 nm. Malondialdehyde equivalents were calculated in the following manner:

1. $[(Abs532_{+TBA}\text{-}Abs600_{+TBA}) - (Abs532_{-TBA}\text{-} Abs600_{-TBA})] = A$
2. $[(Abs440_{+TBA}\text{-}Abs600_{+TBA})0.0571] = B$
3. MDA equivalents (nmol · mL^{-1}) = $(A\text{-}B/157000)10^{6}$

Results were expressed as MDA equivalents (nmol · g^{-1}FW) and represent the mean of five samples.

RESULTS AND DISCUSSION

Plant lipoxygenases are dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acid such as linolenic and linoleic acid (Kolomiets et al., 2001). In vitro, most lipoxygenases prefer free fatty acid, thought it has been shown that esterified fatty acid are also substrates for lipoxygenase in vivo (Feussner et al., 2001). In this study we measured the contents of some free and bound fatty acids in leaves and stem of tulips. We found: linoleic (C18:2), linolenic (C18:3), palmitic (C16:0), oleic (C18:1), myristic (C14:0), palmitoleic (C16:1), lauric (C12:0) and stearic (C18:0) acids. In leaves and stem were the highest levels of linolenic, linoleic and palmitic acids, both in free and bound forms. The contents of bound forms were higher than free forms of these fatty acids. The amounts of other unsaturated fatty acids were low, below 10% of contents of all determined fatty acids. The lauric acid as free form was found neither in leaves nor in stem (Table 1).

Lipid peroxidation is generally monitored by measuring some stable secondary end products of polyunsaturated fatty acid oxidation as a concentration of malonyldialdehyde (MDA). A common method for measuring MDA, referred to as the thiobarbituric acid-reactive substances (TBARS) assay, is to react it with thiobarbituric acid and record the absorbance. We studied the lipid peroxidation in leaves, anthers and basal plate of uncooled and cooled tulip bulbs (Fig. 1). The MDA level was low in anthers (below 20 nmol g^{-1}FW), independently to temperature of tulip bulbs storage. Lipid peroxidation levels in leaves and basal plate were higher than in anthers. During 8 weeks of bulbs storage lipid peroxidation levels remained almost constant in basal plate, 1st and 2nd leaves. In 3rd leaf it was higher at day 0 when bulbs were placed at 17°C and 5°C and decreased during 8 (uncooled) and 6 (cooled) weeks of bulbs storage. After 8 weeks of bulbs storage, lipid peroxidation increased in all samples. However, there were no differences in MDA levels between samples from uncooled and cooled bulbs (Fig. 1).

Kanneworff and van der Plas (1992) showed in the mitochondrial membranes, isolated from tulip bulbs scales, that the fatty acid composition did not alter significantly after cooling. The relative amounts of palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) for both 5°C bulbs and 17°C bulbs were equal. Of these fatty acids, linoleic acid was the most abundant followed by palmitic acid. Linolenic acid and oleic acid contributed for only minor percentages (Kanneworff and van der Plas, 1992).

It seems that in tulip bulbs lipid peroxidation was independent to temperature of bulbs storage. These results indicated that lipid peroxidation is probable not involved in dormancy and dormancy release in tulip bulbs. Nevertheless, MDA can only be formed from fatty acids with three or more double bonds (Halliwell and Gutteridge, 1989) and because tulip tissues contain high level of 18:2 (Table 1), the TBA assay may underestimate the actual extent of peroxidation. To estimate more accurately the extent of peroxidation further study are needed.

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Tables

Table 1. The content of some free and bound fatty acid (in μg g^{-1} FM) in stem and leaves of tulip.

Fatty acid	Stem		Leaves	
	Free fatty acid	Bound fatty acid	Free fatty acid	Bound fatty acid
Lauric acid (C 12:0)	0.0	10.7	0.0	15.0
Myristic acid (C 14:0)	3.3	14.2	11.0	31.6
Palmitic acid (C 16:0)	99.6	225.0	79.9	468.0
Palmitoleic acid (C 16:1)	2.6	10.5	16.7	21.4
Stearic acid (C 18:0)	3.7	4.6	6.1	6.0
Oleic acid (C 18:1)	7.4	24.5	20.9	43.4
Linoleic acid (C 18:2)	143.0	604.0	64.1	976.0
Linolenic acid (C 18:3)	40.8	141.0	84.9	1256.0

Figures

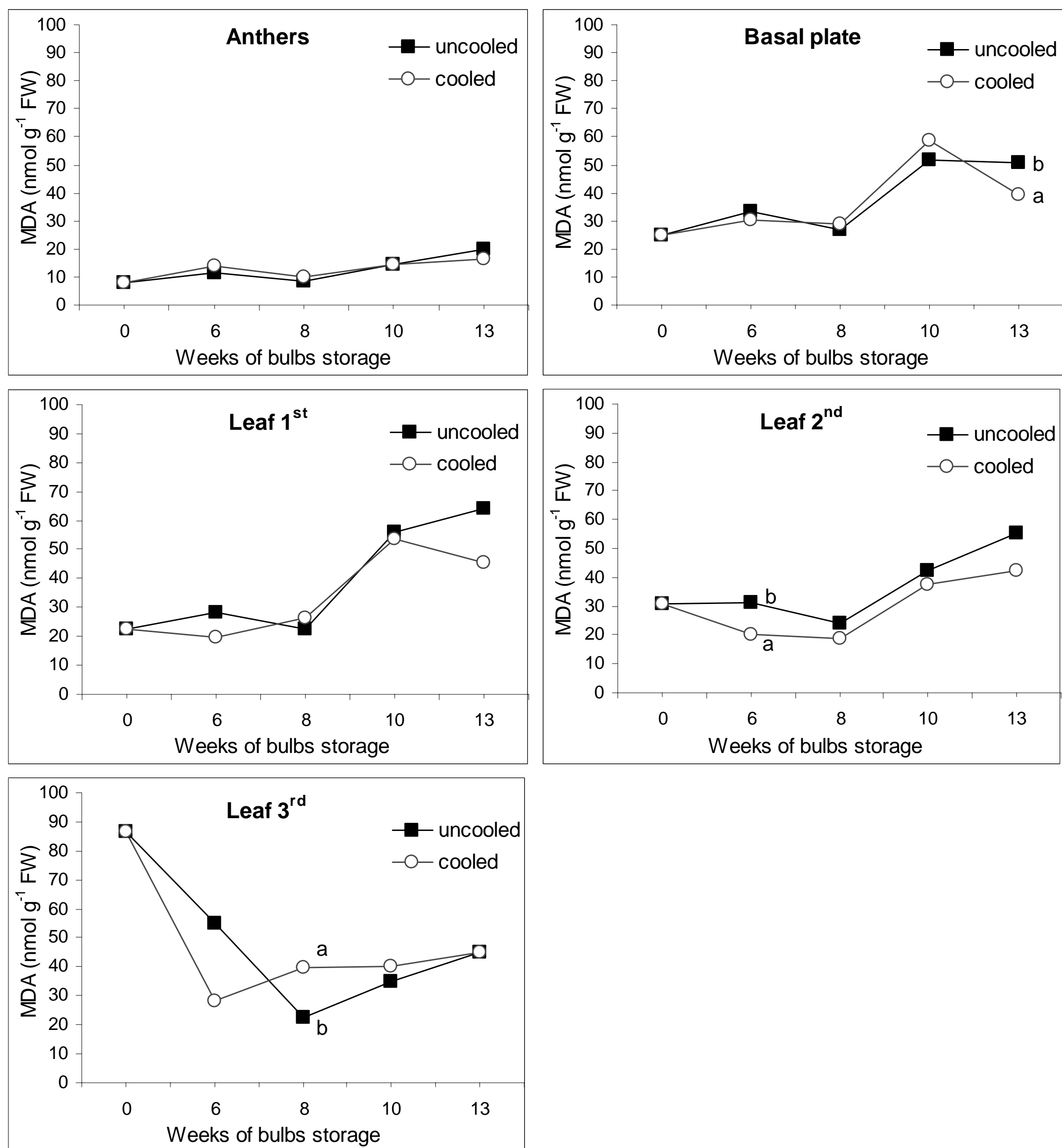

Fig. 1. MDA equivalent levels of anthers, basal plate and leaves during tulip bulbs storage at 17°C (uncooled) and 5°C (cooled). Different letters indicate significant differences according to Duncan's t-test (P=0.05), values are calculated separately for each week of measurements. Means with no letters not differ significantly.