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LIPID COMPOSITION IN LEAVES AND COTYLEDONS OF *CUCURBITA PEPO* L. (ZUCCHINI) DURING NATURAL AND INDUCED SENESCENCE

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Summary. Fatty acid composition analysis was carried out in primary leaves and intact cotyledons of *Cucurbita pepo* L. (zucchini) plants during age-dependent and induced senescence. The artificial senescence was induced either by transfer of young 8-days-old plants to darkness for two days or after treatment with methyl ester of jasmonic acid (MeJA). Age-dependent senescence in 25-days-old cotyledons resulted in about 2-fold increase in the content of short-chain (14:0) and the saturated fatty acids palmitic acid (16:0) and stearic acid (18:0) as well as in the content of monoenoic fatty acids (palmitoleic acid, 16:1 and oleic acid, 18:1). The strongest increase (about 10-fold) was observed in the content of long-chain (20:0, 22:0, 24:0) fatty acids during age-dependent senescence. On the other hand, senescence caused a 2-fold decrease in the content of the unsaturated linoleic acid (18:2) and α-linolenic acid (18:3) fatty acids. The same trend of changes was observed also after two-day dark stress as well as after MeJA treatment, the latter being a stronger inducer of senescence as compared to darkness. In addition, the comparative study of fatty acid composition revealed enhanced content of unsaturated α-linolenic acid in leaves and cotyledons (62% and 46%, respectively) and low content of linoleic acid both in leaves (10%) and cotyledons (24%).

Keywords: *Cucurbita pepo* L. (zucchini); cotyledons; primary leaves; dark stress; MeJA; main lipid classes; fatty acid composition.

Abbreviations: MeJA – methyl ester of jasmonic acid; FAME – fatty acid methyl esters; MGDG – monogalactosyldiacyl glycerol; DGDG – digalactosyldiacyl glycerol; SQDG – sulfoqunovosyldiacyl glycerol; PL – phospholipids; TAG – triacyl glycerol.

INTRODUCTION

Senescence is often described as the terminal phase of plant development (Nooden, 1988). Senescence can be induced by endogenous signals including age or plant growth regulators (Gan and Amasino, 1995; Ananieva et al., 2004a). It can also be triggered prematurely by exogenous stress factors including

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treatment with jasmonic acid (He et al. 2002; Ananieva et al., 2004b) or dark treatment (Weaver and Amasino, 2001; Ananieva et al., 2004a). The exogenous and endogenous signals act in coordination through a common signaling network including transcriptional activity and up- or down-regulation of genes (Buchanan-Wollaston et al., 2005; Hopkins et al., 2007). One of the earliest manifestations of senescence is the loss of selective permeability of membranes due to their molecular disassembly. The onset of membrane leakage is a result of changes in the composition and molecular organization of the lipid bilayers due mainly to de-esterification of the major membrane lipids: phospholipids in membrane envelopes and galactolipids in thylakoids. Chloroplast thylakoid membranes undergo degradation changes very early in the senescence cascade while deterioration of mitochondrial membrane functions occurs in later stages of senescence (Kolodzieujek et al., 2003; Keskitalo et al., 2005). Dismantling of thylakoids in senescing chloroplasts is accompanied by formation of plastoglobuli which contain lipids and their catabolites, including plastoquinone, α-tokopherol, triacylglycerol, carotenoids and free fatty acids (FFA) (Tevini and Steinmuller, 1985; Kaup et al., 2002). Except plastoglobuli, other lipid-protein particles enriched in FFA, steryl/wax esters and triacylglycerol (TGA) have also been identified (Hudak and Thompson, 1996). The synthesis of TAG from de-esterified FFA in parallel with dismantling of galactolipids is the most dramatic change observed in membrane lipid composition with progression of foliar senescence (Thompson et al., 1997; Kaup et al., 2002). Further on, TAG can be metabolized by β-oxidation to acetyl CoA, which is the final step of lipid catabolism in senescing tissues.

Most of the data on lipid degradation during senescence were obtained with true leaves. The results on senescence in cotyledons are quite scarce (Thompson et al., 1998). The changes in protein and RNA synthesis as well as in photosynthetic activity during age-dependent and stress-induced senescence have been studied in zucchini cotyledons (Ananieva et al., 2004; Mishev et al., 2005; Ananieva et al., 2007). In order to extend the previously obtained results, in the present work, we aimed at elucidating the effect of natural or stress-induced senescence on lipid composition in leaves and cotyledons of *Cucurbita pepo* L. (zucchini) plants. As exogenous stimuli of induced senescence we used 2-day dark treatment and treatment with MeJA.

MATERIALS AND METHODS

Growth conditions and treatments

Seeds of *Cucurbita pepo* L. (zucchini) were germinated on moistened filter paper in darkness at 28°C for 96h. The 4-day-old etiolated seedlings were grown further on a nutrient solution (Yamagishi and Yamamoto, 1994) in a growth chamber at a photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$, 26±2°C, relative humidity of 60% and a 12h/12h day/night cycle. Under these conditions cotyledons of the control plants reached their maximum photosynthetic activity at days 7-8 after the onset of germination (Mishev et al., 2005), started to yellow at the age of 23-25 d and died by days 27-28. Dark treatment was applied to whole plants for two days at the age of 8

days. Plants were induced to senesce also by spraying with an aqueous solution of MeJA applied at a concentration of 100 μM twice in two consecutive days in a 24-h interval (8-9 day). Measurements were done either on yellow cotyledons (natural senescence) or on young cotyledons at day 10 from the onset of germination (induced senescence). In addition, lipid composition was studied also in primary leaves of 10-days-old seedlings.

Isolation of total lipophilic extract

The fresh material was homogenized with methanol and refluxed for 10 min in order to inactivate the lipases. Total lipids were extracted according to Blight and Dyer (1959).

Isolation of the main lipid classes

Part of the total lipophilic extract was applied on 20 x 20 cm silica gel G (Merck) plates (layer thickness 0.5mm) for thin layer chromatography (TLC) using chloroform-methanol-acetone-acetic acid (70:14:24:0.4) as mobile phase. The spots of the main lipid classes were visualized under UV light, scrapped off with the silica gel layer and transferred to small vials with teflon screw caps. Five ml of 15% acetyl chloride in absolute methanol was added in each vial and the samples were heated for 4h at 55°C (Christie, 1989). After cooling, the samples were diluted with equal amounts of water and the obtained fatty acid methyl esters (FAME) were extracted twice with hexane (2 x 5ml). The FAME in the combined hexane extracts were purified by preparative TLC on 20 x 20 cm silica gel G plates with hexane–acetone (95:5 v/v). The spots of the FAME were visualized under UV light, scrapped off with the silica-gel layer and eluted with hexane. The amount of each sample was determined gravimetrically and calculated as lipid content using converting factors (Elenkov et al., 1993).

Analysis of the fatty acids

The FAME were analyzed by gas chromatography (GC) on Hewlett Packard 5890 (Hewlett Packard, Palo Alto, California, USA) equipped with FID and capillary column SP WAX 52CB (30m x 0.25mm). The temperature was programmed from 165 to 230°C at a rate of 4°C min^{-1} and a 10 min hold at 230°C. The temperature of the injector was 260°C, and of the detector - 280°C; the carrier gas was nitrogen (1.45 x 10^{-3} Pa).

RESULTS AND DISCUSSION

Lipid composition in primary leaves and intact cotyledons

The amount of lipid classes and their fatty acid profiles in primary leaves and intact zucchini cotyledons are presented in Table 1. The main lipids in primary leaves were the glycolipids (47%) and the digalactosyldiacyl glycerol (DGDG) predominated. The content of phospholipids (PL) comprised about a half of all lipid classes and the amount of TAG was 10%. In general, the lipid composition of primary zucchini leaves was typical for green plants. The composition of the main lipid classes in intact cotyledons differed from that of the true leaves. TAG and PL were the dominating classes. The main lipid class was TAG (44.3%), followed by phospholipids (PL) (29.7%). Monogalactosyldiacyl glycerol (MGDG) predominated among the glycolipids (17.4%), while DGDG and sulfoqunovosyldiacyl glycerol (SQDG)

Table 1. Amounts of the main lipid classes in primary leaves and cotyledons of 10-days-old *Cucurbita pepo* L. (zucchini) plants.

Sample	Lipid classes	Lipids		
		[mg]	[mg.g^{-1}DW]	% of total FA
Primary leaves	TAG	10.1 ± 0.8	3.2 ± 0.3	9.9
	MGDG	14 ± 1	4.4 ± 0.4	13.9
	DGDG	18 ± 1	5.6 ± 0.4	17.5
	SQDG	16 ± 1	5.0 ± 0.4	15.6
	PL	44 ± 4	14 ± 1	43.1
	total	102 ± 8	32 ± 3	100.0
Cotyledons	TAG	100 ± 8	24 ± 2	44.3
	MGDG	40± 3	9.8 ± 0.8	17.4
	DGDG	10.2 ± 0.8	2.5 ± 0.2	4.3
	SQDG	10.1 ± 0.8	2.5 ± 0.2	4.3
	PL	68 ± 5	17 ± 1	29.7
	total	230 ± 18	56 ± 4	100. 0

were present in much lower amounts (4.3%).

The fatty acid composition of the main lipid classes in primary leaves and intact cotyledons is presented in Table 2. In cotyledons, the TAG class consisted mainly of linoleic (18:2) and oleic (18:1) acids (46.1% and 23.4%, respectively), probably due to their role as precursors in the biosynthesis of α-linolenic (18:3) acid. Most of the fatty acids in MGDG and DGDG were presented by linolenic acid (85.6% and 55.9%, respectively). Among the fatty acids with a long chain, a relatively high amount of the long-chained fatty acid 20:0 in DGDG (3.6%) was found.

In the primary leaves, 18:2 and 18:3 acids predominated. The content of 18:2 was 29.4% of total fatty acids in TAG. Among GL and PL, 18:3 was the main fatty acid. A relatively high amount of 16:1 acid in TAG was found in primary leaves in comparison to cotyledon TAG lipids.

Our results showed that in cotyledons 14:0 acids were absent in all lipid classes with the exception of PL. On the other hand, the comparative analysis indicated that in cotyledons 16:1 acids were available only in TAG and PL whereas they were found in significant amounts in the main lipid classes of primary leaves (Table 2). A similar difference was observed by Whitaker (1986) in a comparative study on the fatty acid composition of polar lipids from fruit and leaf chloroplasts in Solanaceous and cucurbit species.

Changes in lipid composition during cotyledon senescence

The changes in the amount of the total lipid extracts and their FA composition in primary leaves and intact cotyledons during natural and artificially induced senescence triggered either by darkness

Table 2. Fatty acid composition of the main lipid classes in primary leaves and cotyledons of 10-days-old *Cucurbita pepo* L. (zucchini) plants.

FA	Primary leaves					Cotyledons				
	TAG	MGDG	DGDG	SQDG	PL	TAG	MGDG	DGDG	SQDG	PL
14:0	9.0	-	-	-	-	-	-	-	-	0.6
16:0	14.9	3.4	14.8	31.4	23.6	12.4	3.2	11.9	24.3	24.8
16:1	17.7	1.1	3.7	1.6	6.1	1.4	-	-	-	2.0
18:0	5.0	0.9	3.1	4.4	3.3	8.7	1.2	3.3	7.2	7.4
18:1	15.3	0.8	2.8	2.9	3.2	23.4	1.8	3.4	3.3	3.1
18:2	29.4	1.9	10.5	10.2	5.7	46.1	8.2	21.9	26.9	15.1
18:3	8.7	91.9	61.5	49.5	57.0	6.7	85.6	55.9	38.3	46.0
20:0	-	-	3.6	-	-	0.9	-	3.6	-	0.4
22:0	-	-	-	-	1.0	0.4	-	-	-	0.6

Values are obtained from three parallel measurements; the standard deviations (related to peak proportions of the chromatograms) are as follows: ± 0.3 for 18:3; ± 0.2 for 18:2 and 16:0; ± 0.1 for the others.

or MeJA treatment are presented in Table 3. Our results showed that the amount of total lipids was higher in the primary leaves and decreased in the cellular extracts during both types of senescence, which might be due to degradation of the membrane system of cotyledons. Cellular membranes do not degrade simultaneously during senescence. It is well known that dismantling of thylakoid membranes is the first change observed during senescence. It is followed by structural alterations in the internal mitochondrial membranes, and finally by dismantling of the chloroplast envelope (Kolodziejek et al., 2003). Catabolism of macromolecules is metabolically coupled to energy production and reallocation of carbon, nitrogen and mineral elements to growing parts of the plant (Matile, 1992). The first manifestation of leaf senescence is the loss of chlorophyll, which reflects dismantling of thylakoids. By contrast, deterioration of the inner membranes of mitochondria does not occur until late in the leaf senescence cascade (Kolodrizijek et al., 2003). Thus, the capacity for chloroplast energy production is eliminated during the early stages of senescence, whereas the mitochondrial energy production is preserved until late periods of senescence.

Our results showed that natural senescence of cotyledons was accompanied by certain changes in total lipid extracts. The amount of saturated palmitic (16:0) and stearic (18:0) acids increased 2-fold and the same extent of increase was observed also in the content of monoenoic (palmitoleic, 16:1 and oleic, 18:1) fatty acids (Table 3). The strongest increase was found in long-chain (20:0, 22:0, 24:0) fatty acids. At the same time, the amount of the unsaturated linoleic (18:2) and linolenic (18:3) acids decreased to the same extent. As these fatty acids are mainly parts of MGDG and DGDG, it can be suggested that re-esterification of these two important glycolipids occurred during

Table 3. Fatty acid composition of total lipid extracts from primary leaves and cotyledons of *Cucurbita pepo* L. (zucchini) plants after dark- or MeJA-induced senescence (% of total).

Fatty acids	Primary leaves	Cotyledons from control plants (10-days-old plants)	Cotyledons from dark-treated plants (8-10- days-old plants)	Cotyledons from MeJA-treated plants (8-10-days-old plants)	Cotyledons from senescing 25-days-old plants
14:0	0.2	0.5	0.9	0.8	1.3
16:0	18.6	13.6	19.3	21.2	26.0
16:1	2.9	1.3	4.7	8.4	11.1
18:0	1.9	4.9	6.6	7.4	5.6
18:1	3.8	9.4	6.8	5.9	5.2
18:2	9.7	23.5	18.6	13.3	10.6
18:3	62.2	46.1	39.6	38.1	31.9
20:0	-	0.4	0.5	0.7	2.0
22:0	0.3	0.2	0.9	1.2	2.8
24:0	0.4	0.1	2.1	3.0	3.5
Total lipid extract [mg.g^{-1}DW]	178.1	153.3	135.7	145.4	135.7

age-dependent senescence. The same trend of changes was observed also after 2-day dark stress as well as after MeJA treatment, the latter being a stronger inducer of senescence as compared to darkness.

Fatty acids of thylakoid membranes are the most abundant source of carbon in leaves. Therefore, the use of carbon during early stages of senescence, while much of the cell metabolic machinery is still intact, can serve as an useful tool for its conversion into a phloem-mobile sucrose, the latter transported to fruits and developing seeds (Wanner et al., 1982, 1991; Froman et al., 2000; Page et al., 2001; Cornah and Smith, 2002). Moreover, fatty acids originating from thylakoids can be also used as a substrate for mitochondrial ATP synthesis through β-oxidation (Buchanan–Wollaston, 1997; Charlton et al., 2005), thus leading to gluconeogenesis and the formation of sucrose (DeBellis et al., 1990).

It is known that de-esterified fatty acids from PL of senescing membranes are not immediately removed from the membrane lipid bilayer. This leads to an increase in the free fatty acids/esterified fatty acids ratio within membranes (Thompson et al., 1997). Despite some accumulation of FFA in senescing membranes, there is also a large increase in the levels of steryl esters and waxes, as well as TAG (Thompson et al., 1997). Thus, it would appear that senescing cells cope with the progressive increase of membrane FFA in part by converting them to steryl esters, waxes of TAG and molecular species that can be accommodated within the lipid bilayer with minimal structural perturbations.

Thylakoids are the most abundant

membranes in nature and hence a rich source of membrane fatty acid carbon used for energy production and carbon recycling during senescence (Lee, 2000). Moreover, there is a progressive accumulation of TAG coincident with the dismantling of thylakoids during foliar senescence, implicating TAG in the metabolism of thylakoid FA (Kaup et al., 2002). This means that the de-esterified FA derived from galactolipids are initially converted to TAG within the thylakoid membrane. Further on, TAG together with other lipids and possibly protein catabolites move laterally through the plane of the membrane to form discreet domains that are subsequently voided into the stroma, giving rise to plastoglobuli. The sequestering of the FFA derived from galactolipids into TAG constituents is an intermediate step in the conversion of thylakoid FA to energy and phloem-mobile sucrose in senescing leaves.

There is also evidence for the metabolic conversion of galactolipid FA to TAG in response to a variety of plant stresses. A decrease in MGDG accompanied by an increase in TAG has been observed in leaves of plants subjected to rust infection (Loesel and Lewis, 1974), cold hardening and freezing (Nordby and Yelenosky, 1984). The same was observed in some halophyte plants from the Bulgarian coast of Black Sea (Ivanova et al., 2005) or in bean plants after acid rain treatment (Velikova et al., 2002).

In conclusion, the comparative analysis of fatty acid composition showed a similar decreasing trend in the amount of the unsaturated linoleic (18:2) and α-linolenic (18:3) fatty acids observed during both age-dependent senescence and senescence triggered by darkness or MeJA treatment. The lowered proportion of the unsaturated fatty acids is indicative of decreased fluidity of the cell membranes that occur with progression of senescence. The stronger effect of MeJA was in agreement with our previously published results (Ananieva et al, 2007) showing that methyl jasomate was a more effective senescence-promoting factor when compared with darkness at the early stage of cotyledon senescence. In addition, the comparative study of fatty acid composition revealed enhanced content of unsaturated α-linolenic acid in leaves and cotyledons (62% and 46%, respectively) and low content of linoleic acid both in leaves (10%) and cotyledons (24%).

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