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Biochemical characterization of elongase activity in corn (*Zea mays* L.) roots

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Abstract

Chemical analysis of 4-day-old corn (*Zea mays* L.) root cell walls revealed that the lipophilic biopolymer suberin forms an important constituent of rhizodermal and hypodermal cell walls. Identified aliphatic monomers had chain lengths ranging from C_{16} to C_{26} and they belonged to 5 substance classes (ω-hydroxycarboxylic acids, 1,ω-dicarboxylic acids, 2-hydroxycarboxylic acids, carboxylic acids and alcohols) by which suberin is characterized. Biochemical experiments proved the occurrence of elongase activities in corn roots. Highest enzymatic activities were found in corn root microsomes, and major products synthesized by root elongases were elongated fatty acids with chain lengths ranging from C_{20} to C_{24}. Preferred substrates of root elongases were acyl-CoAs of the chain length C_{18} and C_{20}, whereas monounsaturated acyl-CoAs (C_{16}:1 and C_{18}:1) and acyl-CoAs of lower (C_{12}–C_{16}) and higher chain lengths (C_{22}–C_{24}) were rarely elongated. Elongase activities significantly decreased over the length (40 cm) of 10-day-old corn roots going from the young tip to the older base of the root. Thus, results presented here show the presence and activity of elongases in roots of plants.

Keywords: *Zea mays* L.; Poaceae; Apoplast; Biopolymer; Cell wall; Elongase; Root; Suberin

1. Introduction

Leaves and stems, as aerial organs of land-living plants, are covered by the lipophilic biopolymer cutin that forms the leaf/environment interface. The main function of the cuticle is the protection of the living shoot tissue from desiccation (Schönherr, 1982). In addition, leaching of inorganic and organic substances from the apoplast is reduced (Tyree et al., 1991) and the cuticle forms a mechanical barrier protecting plants from microbial infections (Kerstiens, 1996a). In the past much work has been done analyzing functional properties (Kerstiens, 1996b; Riederer and Schreiber, 2001; Schönherr and Riederer, 1989), chemical structure (Kolattukudy, 1980; Walton, 1990; Bianchi, 1995), biosynthesis (Kolattukudy, 1981; Cassagne et al., 1994) and genetics (Kunst and Samuels, 2003; Lemieux, 1996; Post-Beittenmiller, 1996) of the leaf/environment interface of plants. However, significantly less information is available about the plant/soil interface formed by roots.

Roots are designed to take up water and dissolved nutrients efficiently from the soil solution (Hose et al., 2001; Steudle and Peterson, 1998). At the same time roots must avoid absorbing harmful substances, prevent excessive leaching of substances from the roots

Abbreviations: ECW, endodermal cell walls; FAE, fatty acid elongase; RHCW, rhizodermal and hypodermal cell walls.

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to the soil and protect themselves from microbial infections (Agrios, 1997). These demands are supported by apoplastic barriers in roots, in order to establish root selectivity and resistance (Marschner, 1995). There are two distinct root layers involved in the formation of apoplastic barriers in roots: (i) endodermal cell walls (ECW) with Casparian bands separating the central cylinder from the root cortex and (ii) rhizo- and hypodermal cell walls (RHCW) forming the interface between the root cortex and the soil environment (Esau, 1977). Casparian bands also occur in hypodermal cell walls in many species, in which case the layer is called an exodermis (Peterson and Perumalla, 1990).

Compared to normal cortex cell walls, apoplastic cell wall spaces of endodermal and rhizo- and hypodermal tissues are chemically modified by the incorporation of aromatic and aliphatic biopolymers (Wilson and Peterson, 1983). Analysis of the chemical composition of these cell walls revealed that lignin and suberin form important constituents of apoplastic barriers in roots (Hose et al., 2001; Schreiber et al., 1999). Lignin as an aromatic biopolymer will be more important mechanically reinforcing these cell walls making them more resistant to mechanical stress and microbial degradation (Nicholson and Hammerschmidt, 1992). Suberin is a biopolymer with a very complex composition. It consists of an aromatic domain (Bernards and Lewis, 1998; Kolattukudy, 1981), which clearly differs from lignin, and of an aliphatic domain composed of linear long-chain aliphatic compounds. This aliphatic domain of suberin significantly contributes to the barrier properties in terms of water and solute transport (Freundl et al., 2000; Vogt et al., 1983; Zimmermann and Steudle, 1998; Zimmermann et al., 2000).

Thus, it must be stated that these apoplastic barriers forming the root/soil interfaces have important physiological and ecological functions regulating exchange processes between roots and the soil environment. In view of their importance, surprisingly little information is available about the biosynthesis of their wall-modifying substances. In a first very short communication (Schreiber et al., 2000) it was recently reported that elongases are in fact expressed and active in corn roots. Here we present results from a much more detailed study, designed to get more information about biosynthesis of the aliphatic suberin domain in roots, which will in future help us to better understand the function of apoplastic barriers in roots. Since this aliphatic domain of suberin is composed of linear, long-chain, aliphatic compounds, elongases must be involved in the biosynthesis. Therefore, we chose a biochemical approach to test whether elongase activities in corn roots can be detected and measured.

2. Results

Looking at a cross-section of a 4-day-old corn root a pronounced autofluorescence of the rhizo- and hypodermal root cell walls was visible (Fig. 1). In addition, a strong whiteblue autofluorescence of the central cylinder with xylem vessels and a more yellow autofluorescence of the tiny spots forming the Casparian bands in the endodermis was detectable (Fig. 1). Suberin monomers having chain lengths between C_{16} and C_{26} belonging to different substance classes (ω-hydroxyfatty acids, diacids, carboxylic acids, 2-hydroxyfatty acids and primary alcohols) were released from isolated rhizo- and hypodermal root cell walls after transesterification (Fig. 2).

Elongase activity of corn roots was linear with time for 1 h (Fig. 3(a)) and increased linearly with increasing protein amounts up to 30 μg (Fig. 3(b)). Elongase activity was saturated with malonyl-CoA concentrations higher than 100 μM (Fig. 3(c)), whereas it was already saturated with C_{18}-stearoyl concentration higher than 10 μM (Fig. 3(d)). Highest elongase activities were obtained with the microsomal fraction M and with the crude extract CE (Fig. 4). Fractions containing the cell wall fragments and cell organelles P and the cytosol S had elongase activities which were only slightly higher than the background (Fig. 4). Radiolabelled elongated fatty acids obtained from the microsomal fraction were dominated by C_{20}, C_{22} and C_{24}, whereas only traces of C_{18} could be detected (Fig. 5).

Relevant cofactors for the elongase activity in roots were NADPH, NADH, DTT and acyl-CoAs (Fig. 6). Replacement of acyl-CoA by ATP resulted in elongase activities, that were 50% higher than those of the standard incubation (Fig. 6). Investigating elongase activi-

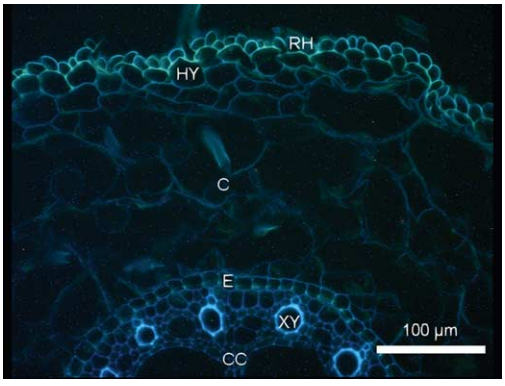

Fig. 1. Cross-section taken at 5 cm above the root tip of a 4-day-old corn (*Zea mays* L.) root viewed with an epifluorescence microscope (excitation $\lambda = 395$ nm). A strong and bright autofluorescence of the rhizodermal (RH) and hypodermal cell walls (HY) is visible, whereas the cortex cell walls (C) are faintly fluorescent. Casparian bands can be seen in the endodermal cell layer (E) as small autofluorescent dots, and the central cylinder (CC) of the root containing lignified xylem vessels (XY) shows a blue autofluorescence. The white bar corresponds to 100 μm.

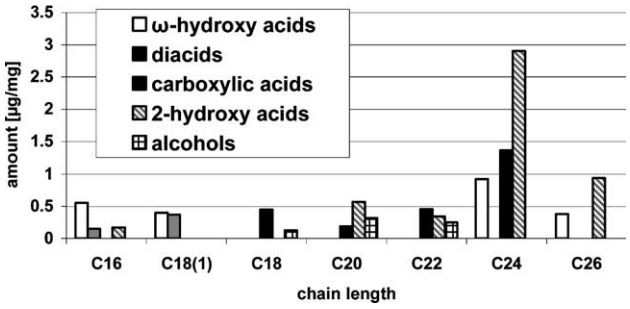

Fig. 2. Suberin monomers (µg mg^{-1}) released from enzymatically isolated rhizodermal and hypodermal cell walls of 4-day-old corn (*Zea mays* L.) roots after transesterification using BF_3MeOH. Monomers belonged to 5 substance classes, and chain lengths ranged from C_{16} to C_{26}.

ties over the length of 10 day-old roots showed a significant decrease going from the young (zone 1) to the old (zone 5) root sections (Fig. 7).

3. Discussion

Fatty acid elongases (FAEs) in plants catalyzing the synthesis of fatty acids longer than C_{16} and C_{18} have been described in plants. They synthesize very long chain fatty acids (VLCFA) occurring in sphingolipids and GPI-anchored proteins in plant cells (Lynch and Dunn, 2004). In the leaf epidermis such FAEs are involved in the biosynthesis of cuticular waxes (Hooker et al., 2002; Kunst and Samuels, 2003; Rhee et al., 1998: Xu et al., 2002), that are responsible for the water impermeability of leaf surfaces (Kerstiens, 1996a). In seeds of Brassicaceae FAEs are involved in the synthesis of seed oils (Kunst et al., 1992; Rossak et al., 2001), that serve as nutrients for developing seedlings. Furthermore, biosynthesis of seed oil in important crop plants like *Brassica* has been intensively investigated for biotechnological purposes (Barret et al., 1998; Wilmer et al., 1998).

As shown previously (Schreiber et al., 2000), the data presented here show in more detail that elongase activities can be measured in plant roots. Since the soil/root interface is characterized by specific cell wall modifications in ECW and RHCW (Fig. 1), it must be postulated that elongases are involved in the synthesis of suberin monomers with chain lengths up to C_{26} (Fig. 2) and even longer in older roots (Schreiber et al., 1999). Although not all details of the biosynthesis of elongated fatty acids

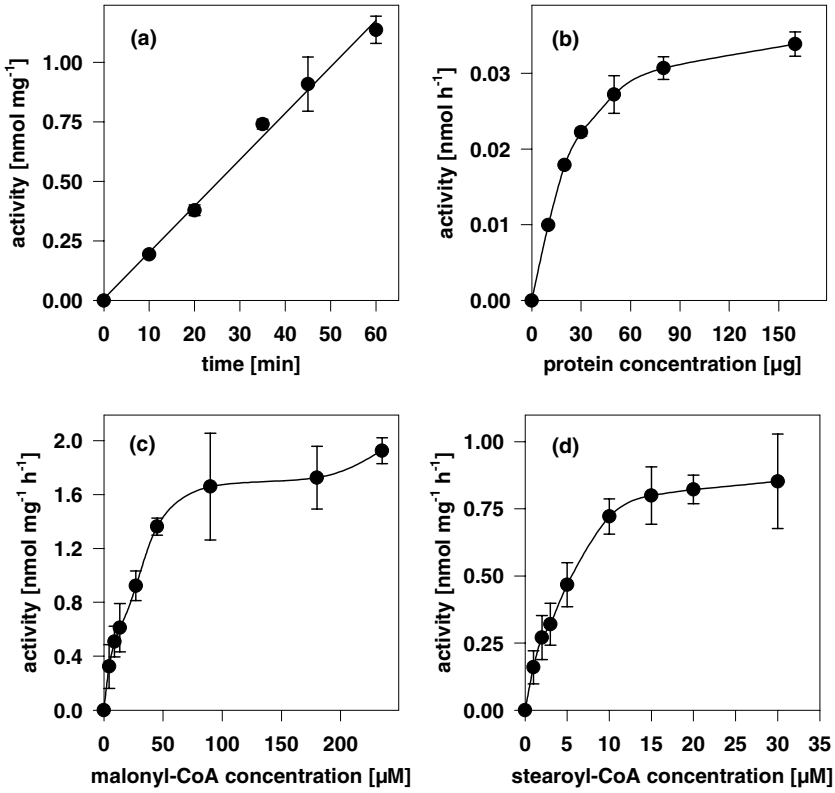

Fig. 3. Dependence of elongase activities in the microsomal fraction of 4-day-old corn (*Zea mays* L.) roots on various experimental boundary conditions using stearoyl-CoA as substrate. (a) Elongase activities (nmol mg^{-1}) were linearly correlated with incubation time up to 1 h. (b) Elongase activities (nmol h^{-1}) were linearly correlated with protein concentration up to 30 µg. (c) Elongase activities (nmol mg^{-1} h^{-1}) were linearly correlated with malonyl-CoA concentration up to 50 µM. (d) Elongase activities (nmol mg^{-1} h^{-1}) were linearly correlated with stearoyl-CoA concentration up to 10 µM.

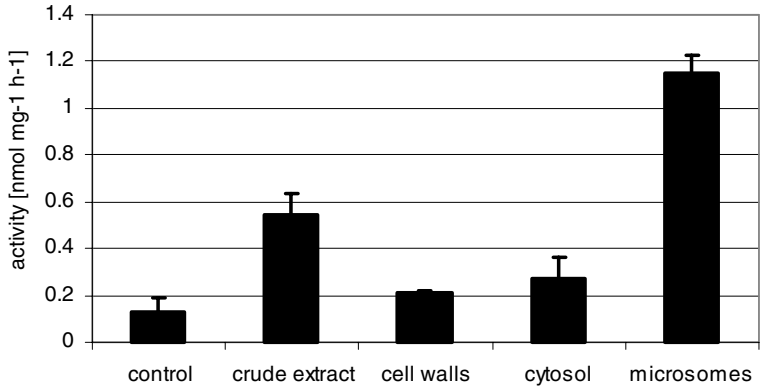

Fig. 4. Localization of elongase activities (nmol mg^{-1} h^{-1}) in 4-day-old corn (*Zea mays* L.) roots in different root compartments using stearoyl-CoA as substrate. Highest activities were measured in the microsomal fraction (M) and the crude extract (CE), whereas elongase activities in the cell wall fraction (P) and the cytosol (S) were only slightly higher than the control. Samples, where protein had been replaced by HEPES buffer, served as control.

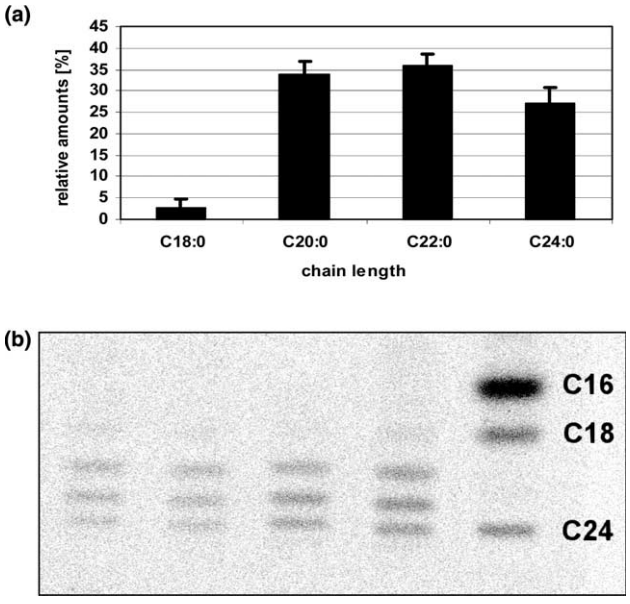

Fig. 5. Products of elongase activities in the microsomal fraction of 4-day-old corn (*Zea mays* L.) roots using stearoyl-CoA as substrate. (a) Chain lengths of C_{20}, C_{22} and C_{24} were the most prominent products of corn root elongase activities. Minor amounts of C_{18} are an indication that microsomes were probably contaminated with fatty acid synthetase activity. (b) Visualization of the chain length distribution of 4 independent experiments with increasing protein concentrations (10–100 μg) by phosphor imaging after separation of the products by reverse phase TLC (thin layer chromatography). Spots on the right side represent carboxylic acids of C_{16}, C_{18} and C_{24} chain length serving as radioactive standards.

in plants are known, it is well accepted that elongation takes place in the endoplasmatic reticulum (Cassagne et al., 1994). This is confirmed for corn roots by the experiments in which elongase activity in different root compartments was measured. Highest activities were in fact obtained in the microsomal fraction (Fig. 4). Although, it cannot completely excluded that this fraction contains to a certain extent microsomes from the plasmamembrane, it largely contains vesicles originating from the ER (Cassagne et al., 1994).

Monomers with a chain length of C_{24} were the most abundant compounds in isolated and transesterified RHCW of 4 day-old corn seedlings (Fig. 2). This pattern of chain length distribution was confirmed by the analysis of the products of the elongated fatty acids in roots ranging from C_{20} over C_{22} to C_{24} (Fig. 5). Only minor amounts of C_{18} were obtained, an indication that the microsomal fraction was contaminated to a small degree with fatty acid synthetase activity. Most preferred substrates for root elongases were C_{18} and C_{20} fatty acids, whereas those with lower or higher chain lengths were rarely elongated (Schreiber et al., 2000). Unlike the FAEs that synthesize fatty acids of seed oil in Brassicaceae (Kunst et al., 1992), root elongases did not use unsaturated fatty acids such as C16:1 or C18:1 (Schreiber et al., 2000). This can be predicted from suberin composition, since unsaturated fatty acids longer than C_{18} are not found as monomers in corn root suberin (Fig. 2) and in root suberin of all other species investigated to date (Schreiber et al., 1999).

Looking at the elongase activity over the length of the developing corn root of a 10 day-old seedling a clear gradient was visible. Highest elongase activities were observed in the youngest root zones and activities continuously decreased towards the older parts of the roots (Fig. 7). This corresponds to the results of an analytical study of the chemical composition of suberin in ECW and RHCW over the length of a growing corn root (Zeier et al., 1999). Amounts of suberin deposited in ECW and RHCW strongly increased going from root zone 1 over zone 2 to zone 3, indicating highest rates of suberin biosynthesis in these younger root zones. In root zones 4 and 5, however, further deposition of suberin ceased, which corresponds to the decrease of the measured elongase activities in these two root zones (Fig. 7).

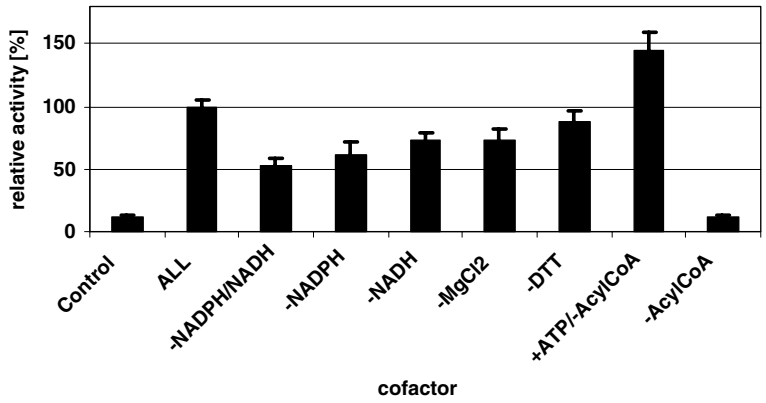

Fig. 6. Dependence of elongase activities in the microsomal fraction of 4-day-old corn (*Zea mays* L.) roots on different cofactors using stearoyl-CoA as substrate. Relative decrease or increase of elongase activities compared to the standard incubation (ALL) containing all cofactors are given. Depending on the respective cofactor, elongase activities decreased between 10% and 50%. Replacing the substrate stearoyl-CoA by ATP (1 mM) lead to an increase of elongase activities by 50%. An omission of the substrate stearoyl-CoA resulted in values similar to the control. Samples, where protein had been replaced by HEPES buffer, served as control.

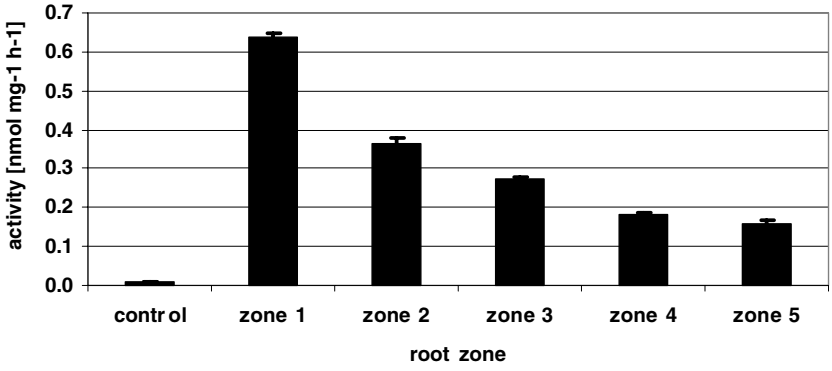

Fig. 7. Microsomal elongase activities (nmol mg^{-1} h^{-1}) in 5 different zones of a 10-day-old corn (*Zea mays* L.) root using stearoyl-CoA as substrate. Elongase activities decreased going from the young root tip to the older root base. Total root length was between 40 and 50 cm. Each zone had a length between 8 and 10 cm. Zone 1 corresponded to the youngest root zone at the root tip whereas zone 5 corresponded to the oldest root zone at the root base. Samples, where protein had been replaced by HEPES buffer, served as control.

Synthesis of elongated fatty acids using microsomal preparations has been measured in the past using coleoptiles of corn (Lessire et al., 1982) and activities of 12.1 nmol mg^{-1} h^{-1} were obtained. Maximum elongase activities in corn roots in this study were about 0.75 (Fig. 3(d)) to 1.75 (Fig. 3(c)) nmol mg^{-1} h^{-1}. Thus, elongase activities in roots contributing to root suberin biosynthesis were on average one order of magnitude lower than those in coleoptiles contributing to wax biosynthesis. The apparent K_m-value for stearoyl-CoA as a substrate estimated from Fig. 3(d) was 5.4 μM. This value is in good agreement with the K_m-value of 1.7 μM of an acyl-CoA elongase isolated from leek (*Allium porrum* L.; Bessoule et al., 1989).

A detailed study of cofactors needed for elongase activities in corn roots revealed that omission of most resulted in a reduced activity (Fig. 6). Most interestingly elongase activity completely ceased when the activated fatty acid stearoyl-CoA was omitted, whereas addition of ATP in the absence of stearoyl-CoA resulted in a 50% increase of elongation activity compared to the standard incubation. Eventually, ATP can be used to synthesize acyl-CoAs from the internal pool of fatty acids present in microsomes serving as substrates for the elongases as it was also discussed by Hlousek-Radojcic et al. (1998).

Up to now, biosynthesis of suberin in roots forming apoplastic transport barriers still is poorly understood. From the analysis of the chemical composition of root suberin, it can be postulated that 2 main types of enzymatic processes must be involved in its synthesis: elongases (FAEs) forming the linear long-chain aliphatic compounds, and cytochrome P450 monoxygenases (P450s) leading to the hydroxylation of the monomers in the omega-position. In *Arabidopsis*, genes for 21 putative FAEs (Kunst and Samuels, 2003) and 272 putative P450s (Schuler and Werck-Reichhart, 2003) can be found. Thus, our current approaches concentrate on

the identification of root-specific FAES and P450s in *Arabidopsis*, in order to find out which of these genes are involved in root suberin biosynthesis. Only on the basis of this information will a better understanding of root suberin biosynthesis will be obtained. The knowledge available for *Arabidopsis*, will facilitate investigations of suberin biosynthesis in roots of important crop plants. As a future aim, an improved knowledge of suberin biosynthesis in roots leading to the formation of apoplastic transport barriers might help to breed crop plants with altered apoplastic transport barriers in roots, rendering them more tolerant towards drought, salt stress and/or soil pathogens.

4. Experimental

4.1. Plant material

In standard experiments, corn kernels (*Zea mays* L. cv Helix; Kleinwanzlebener Saatzucht AG, Kleinwanzleben, Germany) were germinated in Petri dishes on wet filter paper in the dark at 22 °C. After 4 days, roots had an average length between 5 and 10 cm. One to two grams of primary roots was harvested from 30 to 50 seeds and used immediately for biochemical and analytical experiments. In some experiments cultivation of germinated seedlings was continued for a further 6 days in growth chambers (light period 16 h, 22 °C, 70% RH and 600 μmol m^{-2} s^{-1} PAR; dark period 8 h, 18 °C and 90% RH) in hydroponic solution (macronutrients: 1500 μM $Ca(NO_3)_2$, 1000 μM KCl, 1250 μM KNO_3, 750 μM $MgSO_4$, 375 μM KH_2PO_4, 126 μM NaFeEDTA; micronutrients: 68.9 μM H_3BO_4, 13.7 μM $MnCl_2$, 1.5 μM $ZnCl_2$, 1.5 μM Na_2MoO_4, 0.5 μM $CuCl_2$) having one fourth of the concentrations recommended by Hoagland and Arnon (1938). After 10 days 40–50-cm long roots were harvested, sectioned in 5 zones of 8–10 cm length and each section was immediately used in the further experiments.

4.2. Light microscopy

Roots were fixed for 48 h in a PBS-buffered solution (pH 7.4) of formaldehyde (3.7%; w/w) and sections 20 μm thick were cut at −25 °C using a cryo-microtome (Cryostat H 500 M, Microm). Sections were transferred to glass slides, mounted in glycerol/water (1:1; v/v) and examined with an Axioplan microscope (Zeiss, Jena, Germany) using fluorescence excitation at 395 nm (Zeiss filter set number 05).

4.3. Analysis of the chemical composition of root suberin

Isolation, transesterification and identification of root suberin in 4-day-old roots was carried out as previously described (Schreiber, 1996; Zeier and Schreiber, 1997). Suberized RHCW adhering together were isolated after an enzymatic treatment of the roots with cellulase (Onozuka R-10, Serva, Heidelberg, Germany) and pectinase (Macerozyme R-10, Serva). Transesterification of purified RHCW, releasing suberin monomers, was carried out according to Kolattukudy and Agrawal (1974). Gas chromatographic analysis and mass spectrometric identification of the derivatized degradation products were performed as previously described in detail by Zeier and Schreiber (1997, 1998).

4.4. Measurement of elongase activity

All steps were carried out on ice basically following a protocol by Lessire et al. (1982) with minor modifications. Freshly harvested corn roots were ground for 20 s in HEPES buffer (3 ml, 0.08 M, pH 7.2, 0.32 M sucrose and 10 μM β-mercaptoethanol) and filtered through miracloth (Calbiochem). Grinding was repeated two more times. The three fractions (9 ml), which were called crude extract (CE), were combined and centrifuged at 10,000 *g* for 5 min (CS 100, Hitachi, Tokyo, Japan). The pellet (P) containing larger cell wall fractions and cell organelles was separated from the supernatant and resuspended in 2 ml HEPES buffer. The supernatant was centrifuged at 100,000 *g* for 15 min. The obtained pellet, which was separated from the supernatant (S) representing the cytosol, was resuspended again in HEPES buffer without sucrose and centrifuged for a second time at 100,000 *g* for 15 min. The final pellet containing the microsomes (M) was resuspended in 1 ml HEPES buffer without sucrose. Protein concentrations in the four samples CE, P, S and M were determined according to Bradford (1976).

The four samples CE, P, S and M were used in a first set of experiments for the localization of the enzyme activity in roots. All further experiments were carried out using the microsomal fraction exclusively. In a second set of experiments, dependence of elongase activity on incubation time, protein concentration and substrate concentration was measured. Malonyl-CoA concentrations were increased mixing cold malonyl-CoA with ^{14}C-labelled malonyl-CoA.

Finally, in all further experiments, standard incubation conditions were used. A total volume of 100 μl containing 30 μg protein, 500 μM NADPH, 500 μM NADH, 1 mM $MgCl_2$, 2 mM DTT, 10 μM stearoyl-CoA (all biochemicals were from Sigma) and 20 μM ^{14}C-malonyl-CoA (specific activity 60 mCi $mmol^{-1}$; NEN) was incubated 1 h at 30 °C in a thermostat-regulated water bath shaking at 130 min^{-1}. Dependence of elongase activities on acyl-CoA chain lengths and saturation was investigated using different acyl-CoAs ranging from C_{12} to C_{24} (all substances were from Sigma).

As controls 2 additional samples were regularly run in parallel, where protein had been replaced by HEPES buffer.

Enzymatic reaction was stopped by adding 100 μl of a solution consisting of 5 N KOH and 10% methanol. Samples were saponified in closed reaction tubes at 70 °C for 1 h in order to release all fatty acids. After having cooled to room temperature, non converted radiolabelled ^{14}C-malonic acid had to be separated from newly synthesized ^{14}C-labelled fatty acid. Reaction mixtures were acidified with 100 μl of 10 N H_2SO_4 containing 10% malonic acid (w/v) and extracted with 2 ml $CHCl_3$. Chloroform extracts were washed three times using 2 ml water. The organic phase was mixed with scintillation cocktail and counted in a scintillation counter (TriCarb 2000, Canberra Packard).

In some experiments only one half (1 ml) of the organic phase was counted and the other half was used for the determination of the products of the enzymatic reaction. After evaporation of the chloroform, free fatty acids were converted to their methylesters by heating the samples for 1 h in 1 ml of H_2SO_4/methanol (1:40; v:v) to 80 °C. After cooling down to room temperature, 1ml water with 2.1% NaCl was added to the samples and they were extracted with 2 ml hexane. Hexane volumes were then reduced to 20 μl, and the samples were spotted on TLC plates (RP-18, Merck) together with a radiolabelled standard containing ^{14}C-labelled C_{16}, C_{18} and C_{24} methylesters of fatty acids. TLC plates were developed using butanol/acetic acid/water (5/2/3) and separated bands containing radiolabelled fatty acids were visualized using a phosphorimager.

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