Effect of aluminum and the over expression of an *Arabidopsis thaliana* Δ^8-sphingolipid desaturase gene on the sphingoid base composition in maize (*Zea mays*, L.) roots

Dissertation

zur Erlangung des Doktorgrades
des Fachbereiches Biologie
der Universität Hamburg

vorgelegt von
Ana Lúcia Stival da Silva
aus Ijuí/Brasilien

Hamburg, 2003

Genehmigt vom
Fachbereich Biologie der
Universität Hamburg
auf Antrag von Herrn Professor Dr. H. LÖRZ
Weitere Gutachter der Dissertation:
Herr Professor Dr. E. HEINZ

Tag der Disputation: 23. Mai 2003

Hamburg, den 09. Mai 2003

Professor Dr. A. Frühwald
Dekan

"Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes"

Abbreviations

Chemicals

2-4,D	2,4–dichlorophenoxyacetic acid
BSA	bovine serum albumin
DEPC	diethyl pyrocarbonate
DNP	dinitrophenyl
DTNB	5,5´-dithiobis(2-nitrobenzoic acid)
EDDHA	ethylenediaminedi(*o*-hydroxyphenylacetic) acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid
EtBr	ethidium bromide
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
MOPS	3-(N-morpholino) propanesulfonic acid
NaOAc	sodium acetate
NaOCl	sodium hypochlorite
PMSF	phenyl methyl sulfonyl fluoride
PPT	phosphinothricin
PVP- 40	polyvinylpyrrolidone
SDS	sodiumdodecylsulfate
SSC	sodiumchloride -sodiumcitrate

Molecular biology

cDNA	complementary DNA
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
RNA	ribonucleic acid

Units

°C	degree Celsius
µm	micrometer
µmol	micromole
µg	microgram
µl	microliter
µM	micromolar
bp	base pair
Ci	Curie
cm	centimeter
cpm	counts per minute
g	gram
g	grav (acceleration unit)
h	hour
kb	kilobase
l	liter
m	meter
M	molar
mg	milligram
min	minute

mJ	millijoule
ml	milliliter
mm	millimeter
mM	millimolar
mol	mole
mOsm	milliosmole
nm	nanometer
nM	nanomolar
nmol	nanomole
Pa	Pascal
psi	pound per square inch
rpm	rotations per minute
s	second
V	volt
W	watt

Other abbreviations

d.w.	dry weight
d18:2^{4E,8E}	(4*E*,8*E*)-4,8-sphingadienine
d18:2^{4E,8Z}	(4*E*,8*Z*)–4,8–sphingadienine
f.w.	fresh weight
Fig.	figure
HPLC	high performance liquid chromatography
MS	mass spectrometry
n.s.	not significant
t18:0	phytosphinganine
t18:1^{8E}	(8*E*)-4-hydroxy-8-sphingenine
t18:1^{8Z}	(8*Z*)-4-hydroxy-8-sphingenine
Tab.	table
TLC	thin layer chromatography
UV	ultra violet

1 Introduction

1.1 Sphingolipids: structure, metabolism, occurrence and function

Cells from all living organisms tend to respond to membrane-perturbing environmental factors by altering membrane lipid composition, and such changes are thought to restore optimal physical properties. A large body of literature has documented changes in lipid composition in response to cold acclimation and development of freezing tolerance. However, studies on the effects of aluminum (Al) on lipid composition are less common.

Sphingolipids are present in all eukaryotic cells and a few prokaryotic organisms. They were first described in the second half of the nineteenth century in brain tissue by THUDICUM (1884). The first reports on the occurrence of sphingolipids in plant tissues were made by Carter and co-workers (CARTER *et al.*, 1961).

Sphingolipids are structurally different from the more prevalent glycerolipids, such as phospholipids, and the respective biosynthetic pathways of these two lipid classes apparently share no common intermediate other than acyl coenzyme A (CoA). In contrast to glycerolipids that have fatty acids esterified to glycerol, sphingolipids contain a ceramide backbone, which consists of a fatty acid attached to a sphingoid base (long chain base) (LYNCH, 1993). Sphingoid bases are usually C_{18} amino alcohols. The basic structure of sphinganine (d18:0) (**Fig. 1.1 A**) is often modified by hydroxylation and desaturation resulting in a great diversity of compounds. In mammals the sphingoid base moiety is mostly (*E*)-sphing-4-enine (sphingosine, d18:1^{4E}) (**Fig. 1.1 C**), whereas in the yeast *Saccharomyces cerevisiae* the predominant sphingoid base is 4-hydroxysphinganine (phytosphinganine, t18:0) (**Fig. 1.1 B**) formed by hydroxylation of sphinganine at C-4. In contrast, the sphingoid base composition of plants is more variable and includes an additional *cis* or *trans*-desaturation at C-8, leading to the major Δ^8-unsaturated sphingoid bases, phytosphingenines and sphingadienines (**Fig. 1.1 D** to **I**). The fatty acids of plant sphingolipids, on the other hand, are almost exclusively composed by 2-hydroxy fatty acids. Saturated and monounsaturated C_{16}-C_{24} acyl chains occur frequently, C_{14} and C_{26} acyl chains appear in a few species (CAHOON and LYNCH, 1991).

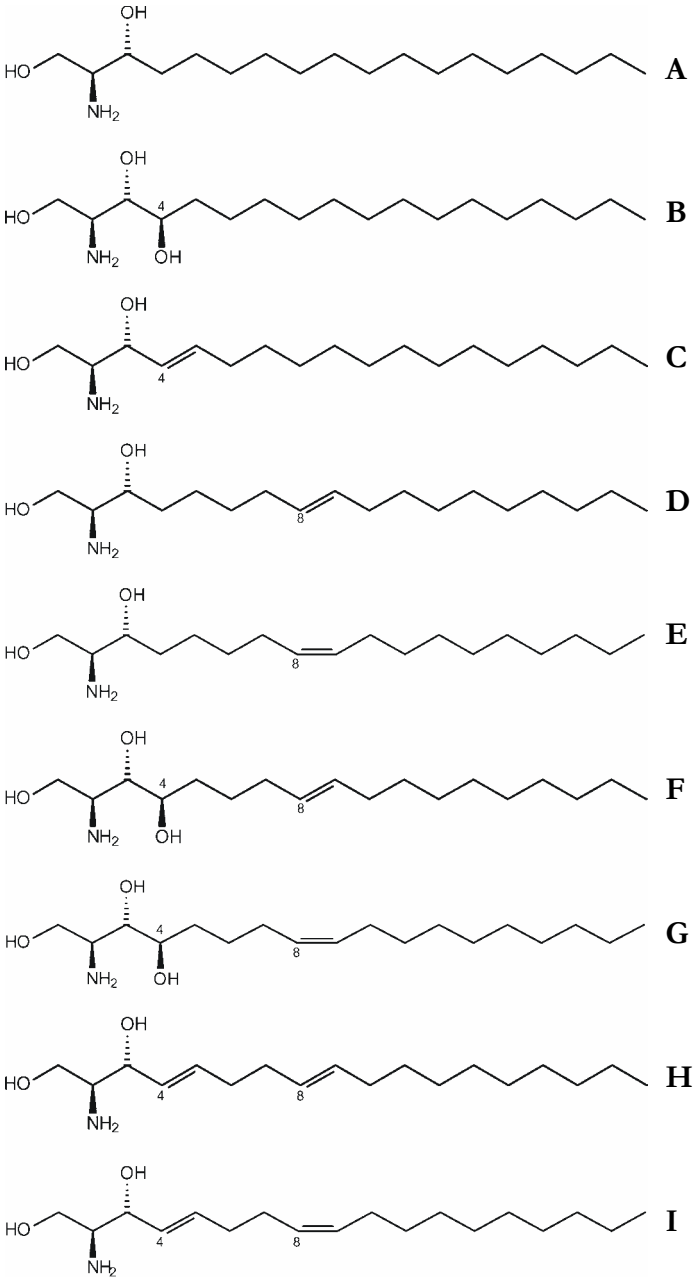

Fig. 1.1: Structure of important sphingoid bases. **A)** sphinganine (d18:0); **B)** 4-hydroxysphinganine (t18:0); **C)** sphingosine (d18:1^{4E}); **D**) (*E*)-8-sphingenine (d18:1^{8E}); **E)** (*Z*)-8-sphingenine (d18:1^{8Z}); **F)** (8*E*)-4-hydroxy-8-sphingenine (t18:1^{8E}); **G)** (8*Z*)-4-hydroxy-8-sphingenine (t18:1^{8Z}); **H)** (4*E*,8*E*)-4,8-sphingadienine (d18:2^{4E,8E}); **I)** (4*E*,8*Z*)-4,8-sphingadienine (d18:2^{4E,8Z}). The first letter of the abbreviation designates the number of hydroxyl groups present on the free base ("d" for dihydroxy, "t" for trihydroxy). The number before the colon refers to the number of carbon atoms in the alkyl chain, the number following the colon refers to the number of double bonds present, and the superscript designates the position of the double bond(s), which may be in the *trans (E)* or *cis (Z)* configuration (according to TERNES, 2000).

The ceramide backbone constitutes the hydrophobic core of a sphingolipid, which is immersed into membranes (**Fig. 1.2 A**). Free ceramide is present in the membranes as an independent lipid component only in minor amounts. In a complex sphingolipid, the 1-OH group of the ceramide is linked to a polar head group, which builds the hydrophilic moiety of the sphingolipids. Depending on the type of polar group, sphingolipids are classified into glycosphingolipids (glucocerebrosides) (**Fig. 1.2 B)**, in which the polar head group is formed by one or several sugar molecules, or phytoglycolipids (phosphosphingolipids), in which a 1-phosphoinositol is attached to the ceramide, which can be further glycosylated (**Fig. 1.2 C**). Glycosphingolipids appear in mammals, plants and most fungi (with the exception of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*). Phosphoinositol-containing sphingolipids are not found in mammals, which contain instead sphingomyelin, with phosphorylcholine as a polar head group.

Some of the genes coding for enzymes involved in the biosynthetic pathway of complex sphingolipids have been recently identified in yeast, animals and plants, but there are still several enzymes which have not been characterized. The first step in the synthesis of ceramide is the condensation of *L*-serine with palmitoyl-CoA, catalyzed by serine palmitoyltransferase (SPT) (**Fig. 1.3**) (NAGIEC *et al.*, 1994; IKUSHIRO *et al.*, 2001; TAMURA *et al.*, 2001). SPT is a rate-limiting enzyme in the sphingolipid synthetic pathway and is therefore recognized as a key enzyme for regulating cellular sphingolipid content. The existing data show that the catalytic mechanism and subcellular localization of SPT have been conserved among animals, fungi and plants (TAMURA *et al.*, 2001). The product of the condensation of *L*-serine with palmitoyl-CoA, 3-ketosphinganine, is immediately reduced to sphinganine by an NADPH-dependent reaction catalyzed by 3-ketosphinganine reductase. The synthesis of ceramide involves formation of an amide bond via condensation of a fatty acid with the amino group of sphinganine (GUILLAS *et al.*, 2001). In yeast sphinganine is probably hydroxylated to phytosphinganine before acylation (GRILLEY *et al.*, 1998). The desaturation of sphinganine to sphingosine, however, seems to use ceramide as a substrate (MICHEL *et al.*, 1997). Finally, complex sphingolipids, such as glucosylceramide, are produced by the addition of polar head groups to the ceramide unit (LEIPELT *et al.*, 2001).

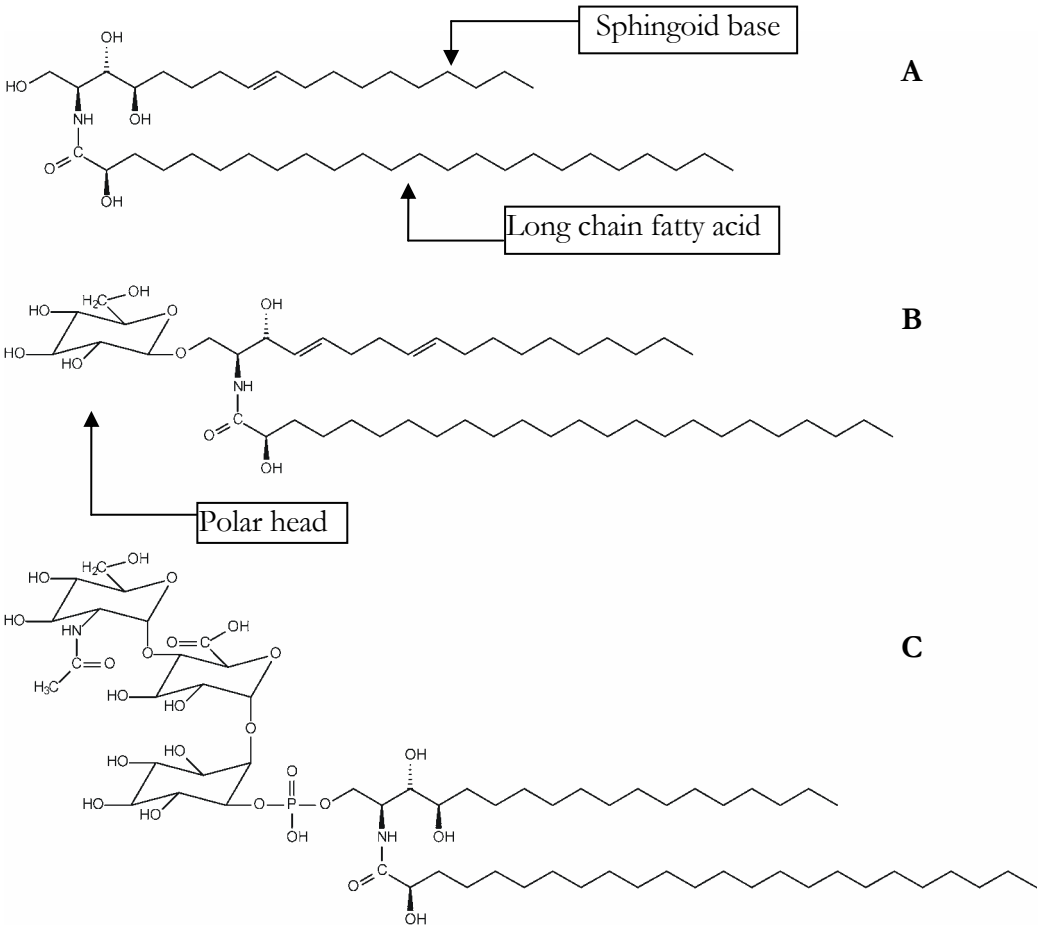

Fig. 1.2: Structure of some typical plant sphingolipids. **A)** Free ceramide with (8*E*)-4-hydroxy-8-sphingenine (t18:1^{8E}) as a sphingoid base and 2-hydroxylignoceric acid as a long-chain fatty acid. **B)** β-*D*-Glucosylceramide as an example of a glycosphingolipid with (4*E*, 8*E*)-4,8-sphingadienine (d18:2^{4E,8Z}) as a sphingoid base and 2-hydroxylignoceric acid as a long-chain fatty acid. **C)** *N*-acetyl-α-*D*-glucosamin-(1→4)-α-*D*-glucuronic acid-(1→2)-*D*-inositol-1-*O*-phosphoceramide as an example of a phytoglycolipid with 4-hydroxysphinganine (t18:0) as a sphingoid base and 2-hydroxylignoceric acid as a long-chain fatty acid (according to TERNES, 2000).

All the steps for *de novo* sphingoid base synthesis up to, and including, ceramide formation occur in the endoplasmic reticulum (ER) (**Fig. 1.4**). It is believed that most of the ceramide is subsequently transported to the Golgi apparatus, where the polar heads are incorporated into the ceramide molecule to form complex sphingolipids (LANNERT *et al.*, 1998), which are then transported to the plasma membrane by secretory vesicles. A different theory has been proposed for plants by CANTATORE *et al.* (2000), who found evidence for glucosylceramide formation on the apoplastic side of the plasma membrane, using sterylglucoside as a donor of glucose to ceramide. An accumulation of plasma membrane sphingolipids in the outer leaflet of the membrane exposed to the cell surface has been indeed reported in mammalian systems (VAN MEER and HOLTHUIS, 2000). There is some evidence in the literature that this asymmetric distribution of sphingolipids is

also adopted by plants (LYNCH and PHINNEY, 1995). In addition to *de novo* biosynthesis, ceramide and free sphingoid bases may be released after hydrolysis of complex sphingolipids in the lysosomes or the plasma membrane (KOLTER and SANDHOFF, 1999).

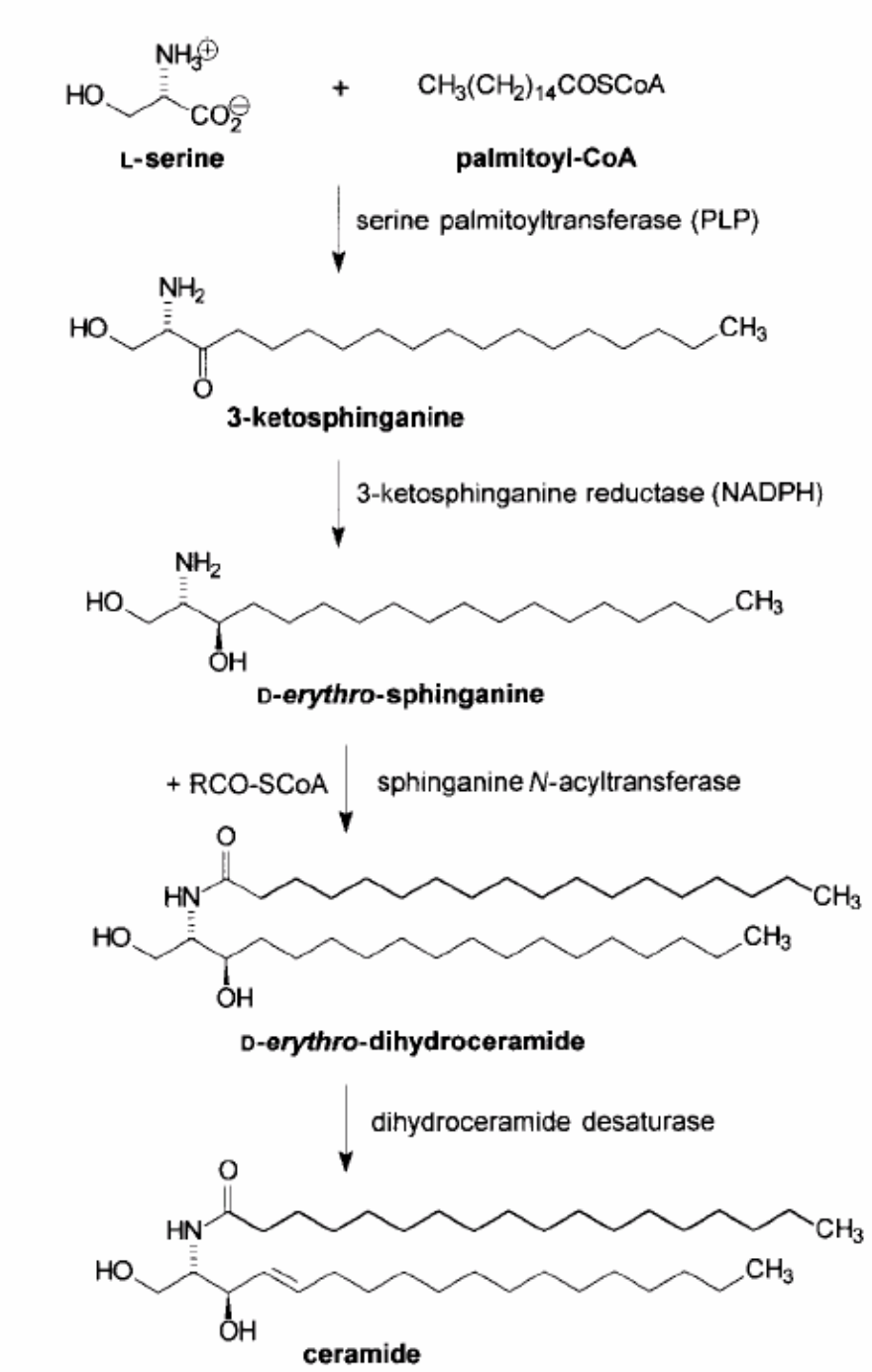

Fig. 1.3: Ceramide biosynthesis in mammals. The formation of ceramide occurs on the cytosolic face of the membrane of the endoplasmic reticulum (according to KOLTER and SANDHOFF, 1999).

The ubiquity of sphingolipids in eukaryotic cells and their subcellular localization suggest important biological roles for these lipids. Though typically accounting for less than 5 % of the total lipid extracted from plant tissues, cerebrosides make up as much as 15 to 30 mol% of the total

lipids in highly purified plant plasma membranes (UEMURA & STENPONKUS, 1994; BOHN *et al.*, 2001). Sphingolipids are not uniformly distributed in the plasma membrane of animal and plant cells but concentrated in microdomains called "lipid rafts" (PESKAN *et al.*, 2000; XU *et al.*, 2001). The content of sphingomyelin, a common sphingolipid in animal cells, is, for example, 30% higher in lipid rafts than in the bulk plasma membrane (PIKE *et al.*, in press). In addition to sphingolipids, lipid rafts are also enriched in sterols, but are often poor in glycerophospholipids. Due to the presence of sterols, together with a predominance of saturated fatty acids, lipid rafts impose an organization on the distribution of proteins in the lipid bilayer and act as platforms to co-localize the components of signaling cascades and facilitate their interaction (HOLTHUIS *et al.*, 2001). Heterotrimeric G-proteins and their receptors, for example, are often associated with such domains, suggesting that these structures are involved in G-protein-coupled signaling. In fact, it has been recently shown that lipid rafts are involved in cellular processes like membrane trafficking, cell morphogenesis and the activation of signaling cascades (MELKONIAN *et al.*, 1999; PRIESCHL and BAUMRUKER, 2000; XU *et al.*, 2001).

An increasing number of studies using mammalian and yeast cells show that sphingolipids play an important role as dynamic regulators of many cellular functions. Several stresses, such as ultraviolet light (FARRELL *et al.*, 1998), heat shock (MAO *et al.*, 1999; CHUNG *et al.*, 2000; JENKINS and HANNUN, 2001), DNA damage, chemotherapeutic agents (STOCK *et al.*, 2000; THEVISSEN *et al.*, 2000), low pH and osmotic stress (SAKAKI *et al.*, 2001) alter the concentrations of sphingolipid metabolites, like ceramide, sphingosine or sphingosine-1-phosphate, in the cells. A change in the concentration of these metabolites activates intra-cellular signal transduction pathways involved in a wide range of processes, like regulation of cell growth (KIM *et al.*, 2000), cell cycle progression (CORONEROS *et al.*, 1995; LEE *et al.*, 1998; BOURBON *et al.*, 2000; OGRETMEN *et al.*, 2001), apoptosis (SHIMABUKURO *et al.*, 1998; GHAFOURIFAR *et al.*, 1999; LEHTONEN *et al.*, 1999; HERGET *et al.*, 2000; PERRY *et al.*, 2000; KROESEN *et al.*, 2001), endocytosis (FRIANT *et al.*, 2001), cell migration (WANG *et al.*, 1999), senescence and transport/allocation of proteins to the plasma membrane (LEE *et al.*, 2002). Many of these processes have been shown to be mediated by the activation/inhibition of protein kinases (HANNUN *et al.*, 1986; MERRIL JR. *et al.*, 1986; WILSON *et al.*, 1986; FRIANT *et al.*, 2000; IGARASHI *et al.*, 2001) or by regulation of the calcium homeostasis in cells (SUGIYA and FURUYAMA, 1991; BREITTMAYER *et al.*, 1994; FATATIS and MILLER, 1996; SAKANO *et al.*, 1996; MATHES *et al.*, 1998; MELENDEZ *et al.*, 1998; BIRCHWOOD *et al.*, 2001), which in turn are regulated by the above mentioned sphingolipid metabolites.

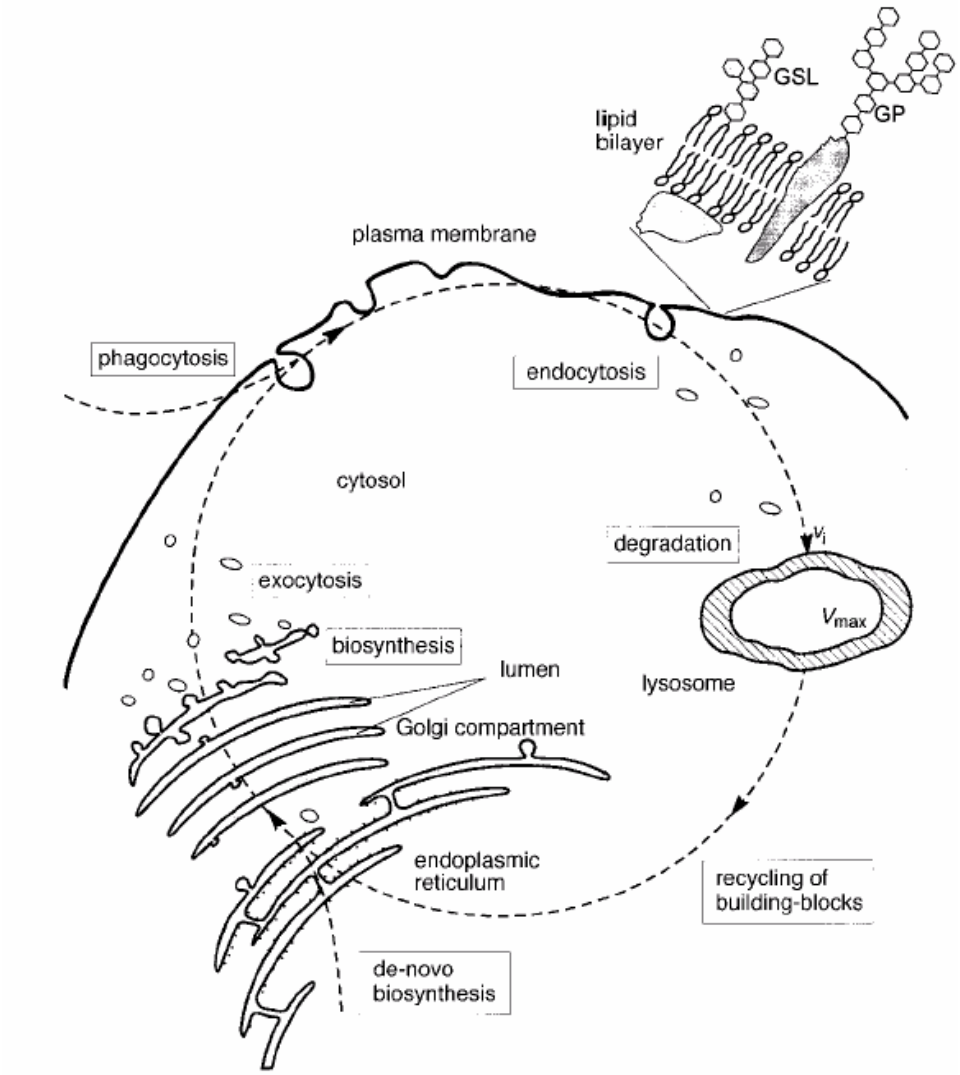

Fig. 1.4: Intracellular metabolic flux of sphingolipids. GSL: glycosphingolipid; GP: glycoprotein; v_i: the influx rate of the substrate into the lysosome (according to KOLTER and SANDHOFF, 1999).

The importance of sphingolipids in plants is becoming clearer as evidence accumulates for functions similar to those found in yeast and mammalian systems. Sphingosine-1-phosphate induces oscillations in cytosolic Ca^{+2} levels in stomata guard cells (NG *et al.*, 2001). KAWAGUCHI *et al.* (2000) proposed that high levels of $t18:1^{8Z}$ in cerebrosides from grapevine leaves are correlated with freezing tolerance in this species. Several studies have shown that some species of phytopathogenic fungi exert their toxic effects by inhibiting the sphingolipid metabolism (ABBAS *et al.*, 1994; ASAI *et al.*, 2000; BRANDWAGT *et al.*, 2000; SPASSIEVA *et al.*, 2002). Furthermore, the studies of KOGA *et al.* (1998) and UMEMURA *et al.* (2000 and 2002) have shown that cerebrosides have elicitor activity in plants.

1.2 Aluminum toxicity and resistance in higher plants

Aluminum (Al) is a light metal that makes up 7% of the earth's crust, where it is the third most abundant element after oxygen and silicon (MA *et al.*, 2001). Plant roots are therefore almost always exposed to Al. Though most of this Al occurs as oxides and aluminum silicates, Al is solubilized in the form of the phytotoxic trivalent cation, Al^{3+}, when soils become acidic as a result of natural processes or human activities, such as the excessive use of nitrogen fertilizer and acid rain. Micromolar concentrations of Al^{3+} can inhibit both primary root and root hair growth in many agriculturally important plant species, such as maize, wheat and barley (KOCHIAN, 1995). Therefore, Al toxicity has been recognized as a major limiting factor of plant productivity on acidic soils, which are especially prevalent in tropical regions and account for about 40 % of the earth's arable land (VON UEXKÜLL and MUTERT, 1995).

Because of the anticipated growing world population in the future, attention has been paid to improve the agricultural production in acidic soils by developing Al-resistant crops and pastures. For some species, such as wheat and maize, some progress has been achieved through traditional breeding. Nevertheless, genetic manipulation appears to be a promising, supplementary tool to traditional breeding, especially for species showing little natural variation for Al resistance, such as barley. Some of the bio-engineering approaches to improve Al resistance focused on the increase of organic acid efflux. Many studies have pointed to a central role for certain organic acids in detoxifying Al^{+3} by complexing these cations in the cytosol or at the root-soil interface. Over a dozen Al-resistant species are currently known to secrete organic acids from their roots in response to Al treatment (MA, 2000; RYAN *et al.*, 2001). Malate is released from the roots of wheat (*Triticum aestivum*, DELHAIZE *et al.*, 1993); citrate from snapbean (*Phaseolus vulgaris*, MIYASAKA *et al.*, 1991), maize (*Zea mays*, PELLET *et al.*, 1995) and *Cassia tora* (MA *et al.*, 1997); and oxalate from buckwheat (*Fagopyrum esculentum*; MA *et al.*, 1997) and taro (*Colocasia esculenta*; MA and MIYASAKA, 1998). The organic acids protect the root apex from the toxic Al^{3+} cations.

Transgenic tobacco lines expressing a bacterial citrate synthase gene showed increased internal citrate concentrations, increased citrate secretion and enhanced Al resistance (DE LA FUENTE *et al.*, 1997; LOPEZ-BUCIO *et al.*, 2000). However, another group that examined the same tobacco lines, as well as a set of additional transgenic lines expressing the bacterial gene at much higher levels, could not repeat these findings (DELHAIZE *et al.*, 2001). In a different study, over expression of the carrot mitochondrial citrate synthase in *Arabidopsis* resulted in increased citrate synthase activity, increased cytosolic citrate concentrations and a 60 % increase in citrate efflux. However, these changes were not associated with a significant enhancement in Al resistance

(KOYAMA *et al.*, 2000). These controversial results seem to indicate that increased organic acid biosynthesis might need to be coupled to an increased capacity to transport organic acid anions to the apoplast. In some cases, expression of a transporter (for example, an anion channel) may be sufficient to cause increased organic acid secretion. However, a gene encoding such a transporter has not been cloned from any organism so far.

One of the main difficulties for the conception of alternative strategies to improve Al resistance is the limited understanding about the cellular and physiological effects of Al. Actually, most of the Al associated with cells seems to be localized at the apoplastic side of the plasma membrane (PM) (YAMAMOTO *et al.*, 2001). Therefore, the cell wall and the outer surface of the PM seem to be major targets of Al. Several toxic effects of Al, like the blockage of Ca^{2+} channels, the depolarization of transmembrane electrical potentials (PAPERNIK and KOCHIAN, 1997; TAKABATAKE and SHIMMEN, 1997), the inhibition of the H_2O_2-stimulated increase of inositol 1,4,5-trisphosphate (JONES and KOCHIAN, 1995), and the enhancement of Fe(II or III)-mediated peroxidation of lipids (ONO *et al.*, 1995; YAMAMOTO *et al.*, 1997) seem to be related to the alteration of PM functions (RENGEL, 1996). Furthermore, the Al-induced callose production, which has been used as an indicator for Al sensitivity in plant roots, also seems to be related to the alteration of the PM function, since β-1,3-glucan synthase (callose synthase) is embedded in the PM and is activated by an increase in intracellular Ca^{2+} concentration (WAGATSUMA *et al.*, 1995).

Investigations of the effects of Al on roots have indicated that Al toxicity does not result from a significant damage or a loss of PM integrity (KINRAIDE, 1988; HUANG *et al.*, 1992; YAMAMOTO *et al.*, 2001). Therefore, Al interactions with the PM seem to be of a more specific nature. Several studies have reported that binding of Al to negatively charged phosphate groups of phospholipids induces a decrease of membrane fluidity (VIERSTRA and HAUG, 1978; DELEERS *et al.*, 1986; AKESON *et al.*, 1989; JONES and KOCHIAN, 1997). It has been proposed that these effects are caused by a stronger association of membrane phospholipids, reduced kink frequency of membrane fatty acids and a higher packing density of phospholipids (STAß and HORST, 1995). Furthermore, a rapid alteration of the PM permeability following temporal contact with Al ions seemed to correlate to Al sensitivity among a variety of plant species (ISHIKAWA and WAGATSUMA, 1998). ZHANG *et al.* (1997) detected no change in total phospholipid content after exposure of an Al-resistant wheat genotype to 20 μM Al for 3 days. Nevertheless, the proportion of phosphatidylcholine (PC) increased at the expense of other phospholipids. Furthermore, the concentration of free sterols decreased. In contrast, Al had no effect on phospholipids and sterols in an Al-sensitive variety. LINDBERG and GRIFFITHS (1993) found a similar increase in the content

of PC at the expense of phosphatidylethanolamine (PE) in the plasma membrane of Al-treated *Beta vulgaris* roots. The higher ratio of PC to PE, as well as lower free sterol contents, would lead to less ordered membranes and compensate the Al-induced decrease in membrane fluidity. Similarly, an increase in membrane fluidity was observed in an Al-resistant fungus, *Lactarius piperatus* (ZEL *et al.*, 1993a), whereas an Al-induced decrease in lipid fluidity was demonstrated in an Al-sensitive fungus, *Amanita muscaria* (ZEL *et al.*, 1993b). Although the relationship between specific changes in lipid composition and Al resistance could not be elucidated, these data suggest that genotype-specific changes in lipid composition could contribute to maintain root growth in the face of Al stress.

1.3 Scope of the thesis

As reported in the section 1.1, sphingolipids are main components of lipid rafts, with important structural and signaling functions in the PM. Though glucosylceramides and related sphingolipids are known to increase stability and decrease permeability of membranes as a consequence of intra- and intermolecular hydrogen bonding between amide and hydroxyl groups of the ceramide moiety (MASSEY, 2001), up to now sphingolipids have not been taken into consideration in studies dealing with Al-induced lipid changes. The aim of this study was to investigate whether Al affects the sphingoid base composition in root tips of maize genotypes with different Al sensitivities. In addition, over expression of a heterologous *A. thaliana* Δ^8-sphingolipid-desaturase should elucidate, whether Δ^8-unsaturation of sphingolipids influences Al sensitivity of transgenic maize.

2 Material and Methods

2.1 Chemicals and other materials

Analytical grade or pure quality chemicals were purchased from the companies: AppliChem (Darmstadt, Germany), Bayer (Leverkusen, Germany), Difco (Detroit, USA), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma (St. Louis, USA).

Deionized water (Milli Q Water System, Millipore, Bedford, USA) was used for preparation of all solutions and media. According to different requirements, solutions and media were autoclaved (20 min, 120 °C, 2 x 10^5 Pa) or filter sterilized with 0.2 µm filters (Nalgene, Rochester, USA).

2.2 Molecular work

2.2.1 General remarks on enzymes, kits and equipment

Restriction endonucleases, DNA modifying enzymes and oligonucleotides were purchased from Gibco BRL (Eggenstein, Germany). Kits and other molecular reagents were purchased from the companies: Amersham Pharmacia Biotech (Freiburg, Germany), Qiagen (Düsseldorf, Germany), and Stratagene (La Jolla, USA). DNA size marker (*λEco* 130I) was purchased from MBI-Fermentas (St. Leon-Rot, Germany) and RNA size marker (0.24–9.25 kb) from Gibco BRL.

2.2.2 Construction of the expression vector for plant transformation

For the expression of heterologous genes in plants a strategy based on the plasmid pUbi.cas was chosen (Appendix, **Tab. 7.1**). It contains a modified version of the ubiquitin promoter from maize, which allows a strong constitutive expression of genes in monocotyledonous plants.

The *Arabidopsis thaliana* Δ^8-sphingolipid desaturase gene was first identified and isolated by SPERLING *et al.* (1998). The plasmid pGEMTB5AT (Appendix, **Tab. 7.1**) containing the respective cDNA, was digested with *Asp* 718/*Eco* RI releasing a fragment of 1366 bp, corresponding to the *A. thaliana* Δ^8-sphingolipid desaturase cDNA, from now on named *B5AT*. After eluting the fragment from a 1 % EtBr-agarose gel (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech), sticky ends were filled in using the Large (*Klenow*) Fragment of DNA Polymerase I (Gibco, BRL). Vector pUbi.cas was digested with *Sma* I and subsequently dephosphorylated by Thermosensitive Alkaline Phosphatase (TsAP, Gibco BRL). After

photometrical determination of the DNA concentration, blunt insertion of the fragment *B5AT* into the *Sma* I cutting site of vector pUbi.cas was done with T4 DNA Ligase (Gibco, BRL). Reactions were performed according to producer's recommendations. Transformation of competent *Eschirichia coli* cells (DH5α™ strain, Gibco BRL) was conducted according to SAMBROOK *et al.* (1989). Transformed bacteria were selected on LB medium (LURIA *et al.*, 1960) plus 100 μg ml^{-1} ampicillin. Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen) and the right orientation of the insert was confirmed by restriction analysis. The resulting construct, called pUbiB5AT, was sequenced (SeqLab, Göttingen, Germany) and used for maize transformation.

2.2.3 Maize transformation

2.2.3.1 Growth conditions of donor plants

Maize plants from the lines A188 (GREEN and PHILLIPS, 1975) and H99 (D´HALLUIN *et al.*, 1992) were grown in a greenhouse under controlled light and temperature conditions (24/16 ºC day/night, 16 h light, 310 μmol m^{-2} s^{-1}) until flowering. The air humidity varied between 55-95 %. To prevent uncontrolled pollination, ears were covered before the appearance of the silks and flowering plants were manually pollinated. Both lines were used as male and female in the crosses to produce hybrid immature embryos.

2.2.3.2 Production of transgenic plants

Stably transformed plants were produced according to BRETTSCHNEIDER *et al.* (1997). Eleven to 13 days after pollination caryopses were collected and sterilized for 15 min in 1 % NaOCl, 0.5 % Mucasol®, followed by three washes with sterile distilled water. Immature embryos were isolated and cultivated with the scutellum side up for one day on the osmotic medium (700 mOsm) (Appendix, **Tab. 7.2**). Following this pre-treatment embryos were cultivated for 5 to 7 days on the induction medium at 26 °C in the dark. Four h before the bombardment the osmotic treatment was repeated. After the bombardment the embryos were left on the osmotic medium for another 20 h.

Exogenous DNA was introduced into plant cells using a particle gun device (PDS 1000/He, BioRad, Munich, Germany). Bombardment of scutellar cells was performed at 1350 psi according to BRETTSCHNEIDER *et al.* (1997). Gold particles with a diameter of 0.4-1.2 μm (Heraeus Feinchemikalien, Karlsruhe, Germany) were used as micro-carriers. Plasmid DNA (2.5 μg of each plasmid) was precipitated according to BECKER *et al.* (1994). The phosphinothricin acetyl transferase (*pat*) gene from *Streptomyces viridochromogenes* was introduced together with the gene of interest. This gene provides resistance against PPT, the active component of the herbicide Basta®

(AgrEvo, Berlin, Germany) and is used as a selectable marker gene selectable marker gene in plant transformation. The plasmid p35S-PAT (P. Eckes, Aventis, unpublished), containing the *pat* gene, is shown in **Fig. 2.1**.

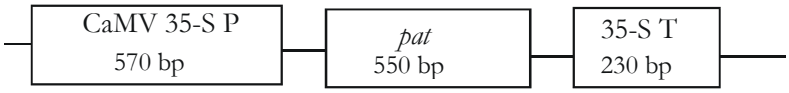

Fig. 2.1: Schematic model of the vector p35S-PAT. CaMV 35-S P: 35S RNA promoter from cauliflower mosaic virus (Gene Bank Accession V00141); *pat*: coding sequence for phosphinotricin acetyl transferase gene from *S. viridochromogenes* (Gene Bank Accession M22827); 35-S T: 35S RNA polyadenylation signal from cauliflower mosaic virus.

In vitro culture of the bombarded tissue was performed following a modified protocol from Dr. R. Brettschneider (University of Hamburg, personal communication). As shown in **Fig. 2.2**, it took approximately 3 months from the bombardment of the embryos until the transfer of regenerated plantlets to the greenhouse. Initially, the bombarded explants were cultured on induction medium (Appendix, **Tab. 7.2**) to induce formation of calli. After two weeks, selection of transformed tissue was initiated by transferring the calli to selection medium 1 (Appendix, **Tab. 7.2**). Subsequently, the calli were transferred to selection medium 2 (Appendix, **Tab. 7.3**). These two culture media differed in their macro and micro nutrients. In order to induce regeneration of plants, calli were transferred to a medium lacking 2,4-D (regeneration medium) (Appendix, **Tab. 7.3**) and placed in the light (37 μmol m^{-2} s^{-1}, 16 h light). Regenerated green buds were transferred to Magenta® vessels containing rooting medium (Appendix, **Tab. 7.3**). This medium was less concentrated (½ concentration of regeneration medium) and contained a lower amount of Basta® (3 mg l^{-1}). Green plantlets were kept on this medium until the root system was fully developed (up to 1 month). Finally, regenerated plants were transplanted to trays containing a mixture of pit and soil and placed in the greenhouse. To keep humidity high, plants remained covered with a plastic lid during the first week after transplantation. Thereafter, plants were sprayed twice within two weeks with 250 mg l^{-1} Basta® plus 0.1 % Tween® 20. Plants surviving the spraying were analyzed by Southern blot analysis and grown to maturity.

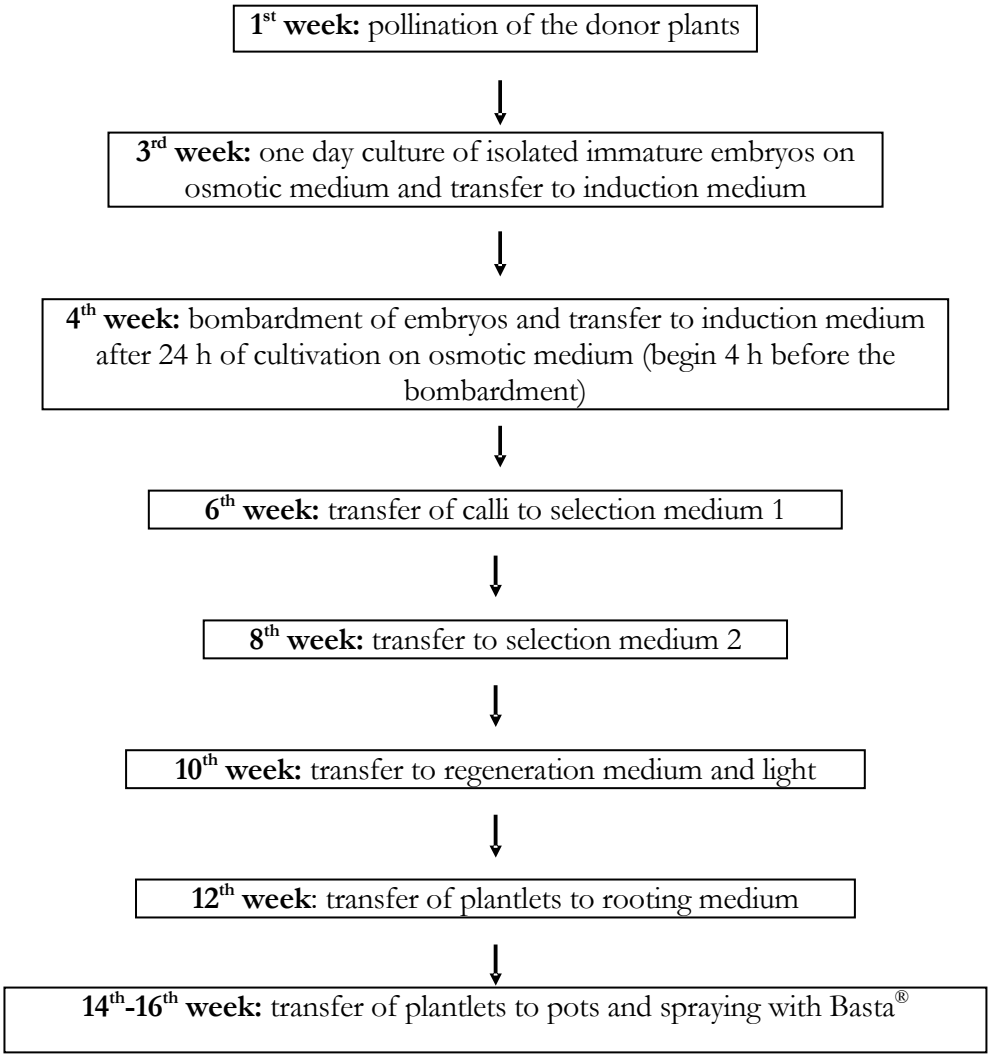

Fig. 2.2: Time schedule for the production of transgenic maize plants.

2.2.3.3 Production of the T_1 and T_2 progenies

In order to multiply the seeds and obtain a population of homozygous transgenic lines, where all the plants carry the same number of transgenic alleles, transgenic plants were grown to maturity and self-pollinated. In case the transgenic plants failed to develop female or male flowers, they were out-crossed with H99 or A188.

In order to identify lines in which the transgene was expressed, part of the obtained T_1 seed pool of each transgenic line was germinated and analyzed by Northern Blot analysis. Due to the absence of a positive signal on homozygous negative plants, it was possible to differentiate them from heterozygous and homozygous transgenic plants. In case of a single locus integration of the transgene a segregation of 1:2:1 (transgenic homozygous: heterozygous: wild-type homozygous) was expected. Plants expressing the transgene were grown up to maturity and self-pollinated again

generating a pool of T_2 seeds. If the pool of T_2 seeds originated from a homozygous transgenic plant no further segregation of the transgene should be observed. These homozygous T_2 lines were identified by Northern blot analysis and Basta® spraying of a representative part of the population.

2.2.4 Molecular analysis of transgenic plants

2.2.4.1 Extraction of genomic DNA and total RNA from leaf tissue

For extraction of genomic DNA 200-300 mg leaf tissue were collected into 2 ml test tubes and immediately frozen in liquid nitrogen. Frozen material was pulverized using a swing-mill (Mixer Mill MM 200, Retsch, Haan, Germany) at an intensity of 70 % for 3 min. Extraction buffer (100 mM Tris-HCL pH 8, 100 mM NaCl and 10 mM EDTA) was added, followed by a phenol/chloroform extraction. After centrifugation, the supernatant was precipitated with NaOAc (3 M, pH 5.2):isopropanol (0.1:1, v/v). Pellets were washed with 70 % ethanol. Dried pellets were re-suspended in deionized water plus 40 µg ml^{-1} RNAse A. The DNA concentration was photometrically assayed and the DNA was stored at 4 °C.

For extraction of total RNA leaf tissue was homogenized as described above. RNA extraction was performed using the TRIzol™ reagent (Gibco BRL) according to the manufacturer's instructions. RNA pellets were incubated at 65 °C for 5 min in 50 µl DEPC-water, the concentration was photometrically assayed and RNA was stored at –80 °C.

2.2.4.2 Radioactive labeling of DNA fragments

Plasmid DNA was digested with restriction enzymes and run in 0.8 % EtBr-agarose gels. The expected DNA-fragment (**Tab. 2.1**) was extracted from the gel (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech) and the DNA concentration was estimated by comparison of the intensity of the fragment in EtBr-agarose gels as well as by photometric measurements. Radioactive labeling was performed with the Prime-It® II Random Primer Labeling kit (Stratagene) according to producer's instructions, using α-^{32}P-dATP or –dCTP (6000 Ci mM^{-1}, Amersham Pharmacia Biotech). Separation of unincorporated nucleotides and oligonucleotides was performed with MicroSpin Columns S-300 HR (Amersham Pharmacia Biotech). Efficiency of the incorporation of radioactive nucleotides was assayed by scintillation counting. Only probes with at least 30 % incorporated radioactive labeled nucleotides were used.

Tab. 2.1: Radioactively labeled DNA fragment for filter hybridization.

Plasmid	Fragment	Size (bp)
pUbiB5AT	*Apa* I/*Kpn* I *Hinc* II	1806 1270

2.2.4.3 Southern blot analysis

In order to identify plants containing a fragment of the size corresponding to the inserted gene, and to detect the number of copies of the gene present in each transgenic plant, T_0 plants surviving the Basta® spraying were analyzed by Southern blot analysis.

Approximately 20 µg of genomic DNA from leaves of transgenic plants (**2.2.4.1**) were digested with endonucleases. DNA from the maize lines used for transformation (A188 and H99) was used as a negative control. Two different kinds of digestions were conducted. Initially, the genomic DNA was digested with endonucleases that cut the construct only once. In this case, the size of the signals obtained by hybridizing with a homologous probe depended on the distance between the cutting site and the next site available in the genomic regions flanking the inserted construct. Every transgenic plant will present a unique set of bands, reflecting the number of copies of the transgene integrated into the genome. Clonal individuals can be recognized by their identical set of bands.

A second digestion was performed to verify that the construct was fully integrated into the genome of the transgenic plants. If this was the case, a fragment corresponding to the whole expression cassette, including regulatory and coding sequences, was separated. As a control, the same digestion was done with the plasmid used for transformation. After hybridizing with a homologous probe, genomic and plasmidial fragments were compared. A difference in size indicated that, due to an incomplete integration of the vector into the genome of the transgenic plants, some part of the construct was missing.

Digested and undigested DNA were run in 0.8 % EtBr-agarose gels. Undigested DNA from the transgenic plants was loaded as a control of contamination of the genomic DNA with the plasmid used for transformation. DNA denaturizing and neutralization was performed according to SAMBROOK *et al.* (1989). DNA was transferred overnight to nylon membranes (Hybond™ N^+, Amersham Pharmacia Biotech) with 10 xSSC. Cross-linking was done by exposing membranes to 500 mJ in a UV crosslinker (Stratalinker® TM 1800, Stratagene). Pre-hybridization was performed at 65 °C for at least 2 h with hybridization buffer (1 % SDS, 1 M NaCl, 10 % dextransulfate, 50 µg ml^{-1} herring sperm DNA). DNA was hybridized overnight by adding 1-1.5 x 10^6 cpm of a

radioactive labeled homologous probe per ml of hybridization buffer (**2.2.4.2**). Filters were washed under stringent conditions: 2x10 min, (1 xSSC, 0.1 % SDS, 65 °C), 1 x10 min (0.5 xSSC, 0.1 % SDS, 65 °C) and 1 x10 min (0.2 xSSC, 0.1 % SDS, 65 °C). X-ray films (Hyperfilm MP, Amersham Pharmacia Biotech) were exposed at –80 °C for 3 to 7 days.

2.2.4.4 Northern blot analysis

Northern blot analysis was carried on from the T_0 to the T_2 generation. Ten µg of total RNA was precipitated at –20 °C with NaOAc (3 M, pH 5.2):95 % ethanol (0.1:2, v/v). Pellets were re-suspended in 2 µl 10 xMOPS, 3.5 µl formaldehyde (37 %), 10 µl formamide, 4.5 µl loading buffer:ethidium bromide (10:1, v/v) and denatured for 10 min at 65 °C. RNA was run in 1.4 % denaturing EtBr-agarose gels (15 % formaldehyde). RNA transfer, hybridization, washing and exposition of membranes were performed according to the procedure described above (**2.2.4.3**).

2.3 Physiological and biochemical work

2.3.1 Incubation of maize plantlets in nutrient solution

After germinating maize seeds for 4 days between layers of filter paper and foam soaked with 1 mM $CaSO_4$ ("sandwich" technique), morphologically uniform seedlings were transferred to pots containing 22 l of continuously aerated nutrient solution (Appendix, **Tab. 7.4**). The nutrient solution was exchanged every 3 days. Plantlets were kept under controlled environmental conditions (16/8 h day/night, 27/25 °C day/night, 75±5 % relative air humidity) with a photon flux density of 230 µmol m^{-2} s^{-1} photosynthetic active radiation (Sylvania Cool White, 195 W, Philips, Germany) as measured at mid plant height. Depending on the amount of material needed for subsequent analysis, the time of pre-culture in nutrient solution varied from 1 week to 15 days.

2.3.2 Treatment of maize plants with aluminum

Aluminum treatment was started by adding 25 µM of aluminum ($AlCl_3$ $6H_2O$) to the nutrient solution. Previous experiments had shown that a concentration of 25 µM of aluminum is optimal for assessing genotypic differences in aluminum resistance of maize on the basis of root growth inhibition and callose formation (HORST *et al.*, 1997). One day before adding aluminum, the pH of the nutrient solution was lowered stepwise to 4.3 and kept stable by the addition of HCl or KOH (0.1 M). After 12 h of aluminum treatment, root growth was measured and root tips (3 mm in length) were collected for biochemical analysis.

2.3.3 Determination of callose content in maize root tips

Production of callose [(1→3)β-D-glucan] was used as a parameter to measure aluminum toxicity. At harvest, plant roots were rinsed with deionized water, root tips (0.5 cm in length) were excised using a razor blade, placed in a 2 ml test tube and frozen immediately in liquid nitrogen. Three root tips were taken as one replicate. Before thawing, 1 ml of 1 M NaOH was added and samples were homogenized with a swing-mill (Mixer Mill MM 200, Retsch, Haan, Germany) for 3 min at maximum speed (30 cycles per sec). Homogenized samples were heated for 30 min at 80 °C in a water bath to dissolve callose. Samples were then centrifuged at 15,000 rpm for 15 min at room temperature and the supernatant was filtered (0.45 µm) to eliminate solid residues. Callose was determined according to KAUSS (1989). Two hundred µl from the filtrate were mixed with 1.25 ml aniline mix [0.5 M Glycin/NaOH-buffer pH 9.5; 0.2 M HCl and 0.03 % water blue (Fluka, w/v)]. Samples were incubated for 20 min at 50 °C. After the samples cooled to room temperature, callose was measured with a fluorescence spectrophotometer (F2000, Hitachi, Tokyo, Japan; excitation λ=394 nm, emission λ=484 nm, slit 10 nm, 700 V). Pachyman (Calbiochem, Deisenhofen, Germany) was used as a standard. The calibration curve ranged from 0 to 25 µg ml^{-1}. The callose content was expressed as µg Pachyman equivalents (PE) per root tip.

2.3.4 Determination of sphingoid bases in maize roots and root tips

Complete roots or root tips were used for the sphingoid base analysis, which was done according to SPERLING *et al.* (1998). Roots were harvested from 15 day-old plants growing in a mix of peat and soil, washed with tap water and dried with a paper towel. Three to four hundred mg (f.w.) of roots were cut into small pieces and immediately processed as described below. Root tips (3 mm in length) were collected from roots of plants growing in nutrient solution for 15 days, immediately frozen in liquid nitrogen and stored at –80 °C. To avoid thawing, the root tips were dried under vacuum at –20 °C and 20 mg (d.w.) were used for hydrolysis of sphingolipids. The plant tissues were submitted to strong alkaline hydrolysis with 10 % aqueous $Ba(OH)_2$/dioxane (1:1, v/v) at 110 °C for 24 h. The released sphingoid bases were extracted with chloroform/dioxane/water (6:1:5, v/ v/ v) and converted to dinitrophenyl (DNP) derivatives. The DNP derivatives were, then, extracted by phase separation using chloroform/methanol/water (8:4:3, v/v/v) and purified by TLC on silica gel plates in chloroform/methanol (90:10, v/v). The derivatized sphingoid bases were detected by their yellow color, eluted from the silica gel with methanol and analyzed by reversed-phase HPLC. Separation was achieved on a Multospher™ 100 RP 18-5 column (5 µm, 25 x 4.6 cm) with a concave-3-gradient at a flow rate of 0.8 ml min^{-1} from 84 %

methanol/acetonitrile/2-propanol (10:3:1, v/v/v) and 16 % water to 0 % water in 55 min. Elution was monitored at 350 nm. Identity of the peaks relied on standards provided by Dr. P. Sperling (Institute of General Botany, University of Hamburg) and on HPLC-mass spectrometry analysis of DNP bases carried out by Dr. S. Franke (Institute of Organic Chemistry, University of Hamburg), according to the method described by TERNES *et al.* (2002).

2.4 Statistical analysis

The program SAS was used for the statistical analysis. The analysis of variance was followed by mean comparison with Tukey's test. Unless remarked, statistical tests were conducted with $P \leq 0.05$.

3 Results

3.1 Production of transgenic maize plants

3.1.1 Construction of the expression plasmid

For cloning of the *A. thaliana* Δ^8-sphingolipid desaturase gene, a fragment (*B5AT*) was isolated by digesting the plasmid pGEMTB5AT with *Asp* 718/*Eco* RI. After filling in of its 3´-termini, the fragment was blunt ligated into the *Sma* I linearized pUbi.cas vector, between the promoter of the maize ubiquitin gene (UP) and the polyadenylation signal from the *A. tumefasciens* nopaline synthase gene (NT). The final construct (pUbiB5AT) containing the cDNA of the *A. thaliana* Δ^8-sphingolipid desaturase gene (*B5AT*) flanked by UP and NT is shown in **Fig. 3.1**.

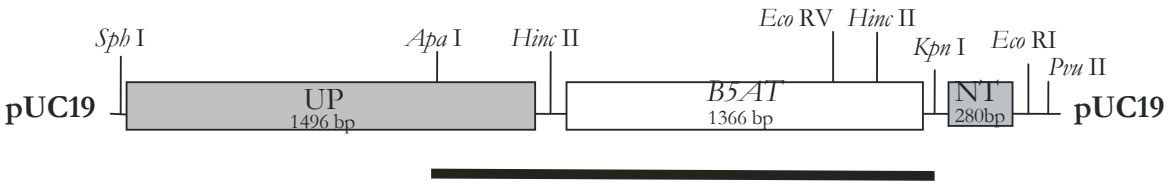

Fig. 3.1: Schematic model of the construct pUbiB5AT. UP: promoter from the *Zea mays* ubiquitin gene (Gene Bank Accession S94464); B5AT: coding sequence from *A. thaliana* Δ^8-sphingolipid desaturase gene (Gene Bank Accession AF428464); NT: polyadenylation signal from the *Agrobacterium tumefasciens* nopaline synthetase gene (Gene Bank Accession V00087). The construct was cloned into the pUC 19 vector. Cutting sites for some restriction enzymes are indicated. The black line represents the fragment used as a probe in Southern and Northern blot analysis.

3.1.2 Maize transformation

Eight independent biolistic transformation experiments were conducted, in which 530 immature embryos from reciprocal crosses between the maize inbred lines H99 and A188 were bombarded (**Tab. 3.1**). The construct pUbiB5AT, containing the Δ^8–sphingolipid desaturase cDNA, was introduced by co-transformation together with the construct p35S-PAT, which contained the *pat* marker gene. After treating with Basta® a total of 22 resistant plants could be selected.

The efficiency of the transformation (number of herbicide resistant plants obtained per 100 embryos) varied from 2 to 7.1 %, with an average of 4.2 %. The best results were achieved by

pollinating plants of the line H99 with pollen from the line A188. If just these crosses are considered, the average efficiency of the transformation rises to 5 %.

Tab. 3.1: Overview of the maize transformation with *A. thaliana* Δ^8-sphingolipid desaturase.

Nr. of Exp.	Cross ♀x♂	Nr. of Bomb. E.	Nr. of Reg. P.	Reg.P./Bomb.E (%)	Nr. of Res. P.	Efficiency of transformation (%)	
						Res.P./Reg.P.	Res.P./Bomb.E.
1	H99 x A188	18	9	50	1	11.1	5.5
2	A188 x H99	70	38	54.3	2	5.2	2.9
3	H99 x A188	50	13	26	2	15.4	4.0
4	H99 x A188	60	13	21.7	4	30.8	6.6
5	H99 x A188	70	48	68.6	5	10.4	7.1
6	A188 x H99	50	4	8	1	25	2
7	H99 x A188	150	42	28	5	11.9	3.3
8	H99 x A188	60	5	8.3	2	40	3.3
Total		**530**	**172**		**22**		
Mean				**33.1**		**12.8**	**4.2**

Exp. = experiment
Bomb. E. = bombarded embryos
Reg. P. = regenerated plants
Res. P. = resistant plants

3.1.3 Molecular characterization of transgenic lines

3.1.3.1 Southern blot analysis of T_0 plants

In order to investigate the integration and the number of copies of the transgene into the genome of the plants, transgenic plants which survived the herbicide selection were analyzed by Southern Blot analysis. Parts of the sequence can be lost during the process of integration and, therefore, it is necessary to verify that at least one single copy of the whole construct, including the promoter and terminator, is found in the transgenic plants. For that purpose, a digestion of the genomic DNA was done with a pair of endonucleases that cut on the 5´-end of the ubiquitin promoter (*Sph* I) and on the 3´-end of the terminator (*Pvu* II). The DNA was probed against a fragment of the plasmid pUbiB5AT (*Apa* I/*Kpn* I) containing the *A. thaliana* Δ^8-sphingolipid desaturase cDNA. A preliminary analysis showed that several plants (19/1, 20/2, 21/7, 27/20, 27/34 and 28/1) contained only incomplete fragments of the construct, while two of the plants (18/6 and 26/4) did not show any positive hybridizing signal (data not shown). An integration of the whole construct was confirmed for 9 out of 22 herbicide resistant plants, which presented a fragment of the expected size (3 Kb), corresponding to the *Sph* I/*Pvu* II fragment of the plasmid pUbiB5AT (**Fig. 3.2**).

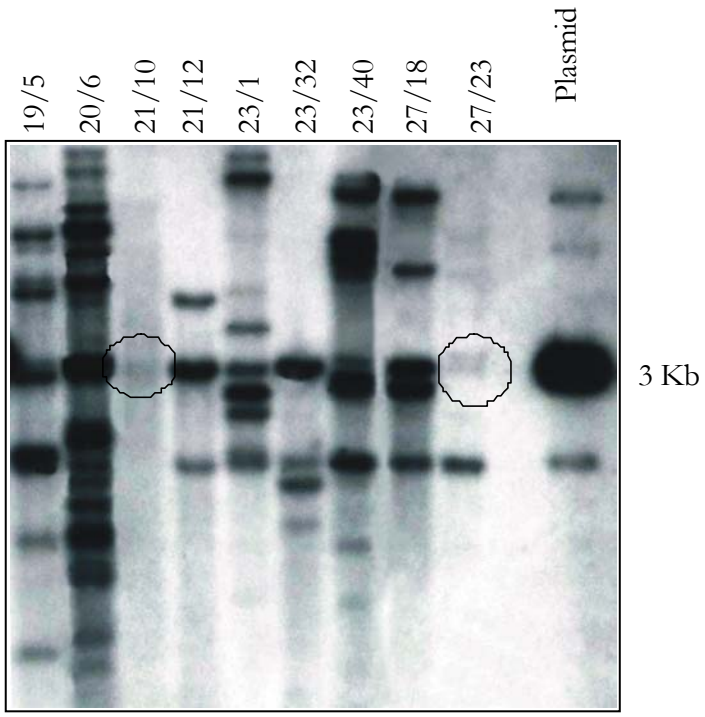

Fig. 3.2: Southern blot analysis of T_0 maize plants. Genomic DNA (20 µg) from leaves of transgenic maize was run on a 0.8 % agarose gel. DNA was digested with *Sph* I/*Pvu* II. Following its transfer, DNA was hybridized against a radioactively labeled probe made up of an *Apa* I/*Kpn* I fragment from the plasmid pUbiB5AT. Very faint signals are indicated by a circle.

In addition to the expected fragment some of the plants showed a different number of extra, greater and smaller, hybridizing fragments. These extra signals are probably caused by the integration of various copies of the construct into the genome, with partial deletions of various sizes and/or methylation of the restriction sites. In order to find out how many copies of the transgene were integrated in the genome, plants with a fragment of the expected size in the previous analysis were submitted to a second set of digestions with *Eco* RI. This endonuclease cuts the plasmid pUbiB5AT on a single site, and the size of the fragments obtained after digestion depends on the distance to the next cutting site in the genome. The number of signals obtained is equal to the number of copies of the transgene integrated into the genome of the transgenic plant. The results of this digestion can be seen in **Fig. 3.3**. The copy number of the transgene varied from 1 to 9. Single copy integration was a relatively rare event.

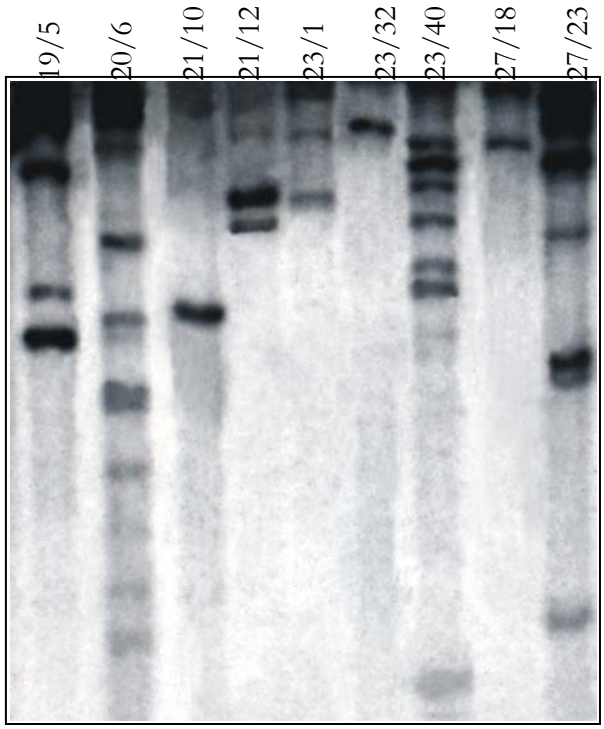

Fig. 3.3: Southern blot analysis of T_0 maize plants. Genomic DNA (20 µg) from leaves of transgenic maize was run on a 0.8 % agarose gel. DNA was digested with *Eco* RI. Following its transfer, DNA was hybridized against a radioactively labeled probe made up of an *Apa* I/*Kpn* I fragment from the plasmid pUbiB5AT.

An overview of the Southern blot analysis is shown in **Tab. 3.2**. In addition to the plants in which the integration of the whole construct was confirmed (indicated by shadowed lines), several plants contained positive hybridizing signals of different sizes and 5 plants were not analyzed (21/11, 23/18, 23/22, 27/19 and 28/2).

Tab. 3.2: Overview of the Southern blot analysis of T_0 maize plants. Plants that contained a fragment of the right size are indicated by shadowed lines.

T_0 plant	Southern blot
18/6	No integration event.
19/1	Integration of incomplete fragments.
19/5	Integration of the complete construct was confirmed. Between 4 and 5 integration events.
20/2	Integration of incomplete fragments.
20/6	Integration of the complete construct was confirmed. Between 8 and 10 integration events.
21/7	Integration of incomplete fragments.
21/10	Integration of the complete construct was confirmed. Between 3 and 4 integration events.
21/11	Integration of the complete construct was not determined.
21/12	Integration of the complete construct was confirmed. Between 3 and 4 integration events.
23/1	Integration of the complete construct was confirmed. Three integration events.
23/18	Integration of the complete construct was not determined.
23/22	Integration of the complete construct was not determined.
23/32	Integration of the complete construct was confirmed. One integration event.
23/40	Integration of the complete construct was confirmed. Between 8 and 9 integration events.
26/4	No integration event
27/18	Integration of the complete construct was confirmed. Two integration events.
27/19	Integration of the complete construct was not determined.
27/20	Integration of incomplete fragments
27/23	Integration of the complete construct was confirmed. Between 5 and 6 integration events.
27/34	Integration of incomplete fragments
28/1	Integration of incomplete fragments
28/2	Integration of the complete construct was not determined.

3.1.3.2 Northern blot analysis of T_0 plants

Transgenic plants were submitted to Northern blot analysis in order to investigate the transcription of the gene coding for the *A. thaliana* Δ^8-sphingolipid desaturase. The transferred RNA was hybridized against a radioactively labeled probe made from the *Apa* I/K*pn* I fragment of the plasmid pUbiB5AT. A good correlation between integration and transcription of the transgene was present. A fragment of 1.3 kb, corresponding to the transcript of the *A. thaliana* Δ^8-sphingolipid desaturase, was detected in the plants 19/5, 20/6, 21/10, 21/12, 23/32, 27/18 and 27/23, in which an integration of the complete construct has been previously confirmed by Southern blot analysis (**Fig. 3.4**). The control plants A188 and H99 showed no positive hybridizing signal. Furthermore,

no transcript was observed in plants with no integration of the transgene or with incomplete copies of the construct (plants 20/2, 21/7, 26/4, 27/34 and 28/1).

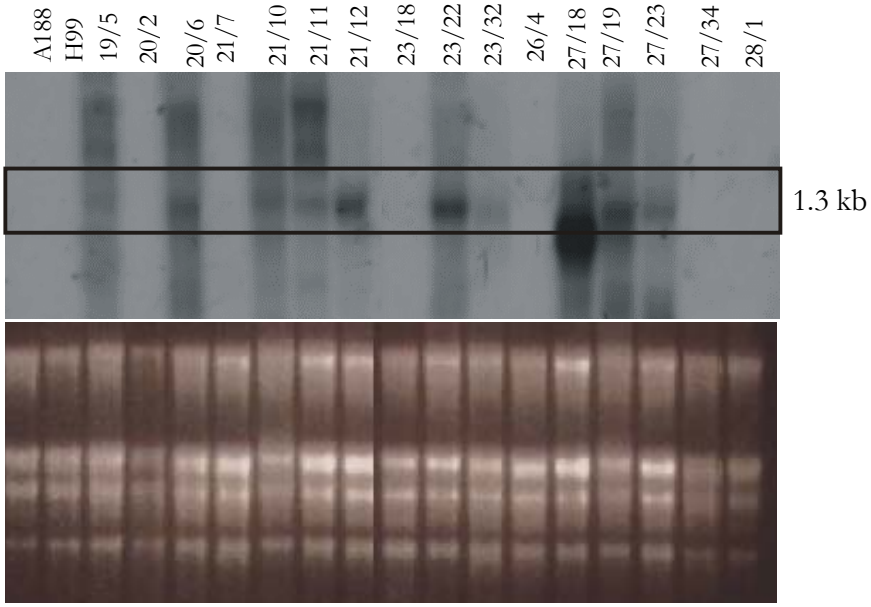

Fig. 3.4: Northern blot analysis of T_0 maize plants. Total RNA (10 µg) from leaves of transgenic maize plants was separated on a 1.4 % agarose denaturing gel. RNA was hybridized against an *Apa* I/*Kpn* I fragment from the plasmid pUbiB5AT. The fragment corresponding to the transcript of the *A. thaliana* Δ^8–sphingolipid desaturase is indicated by a black line.

3.1.3.3 Crossing and segregation of the transgene in the T_1 and T_2 progenies

Seven transgenic T_0 plants, in which an integration and expression of the transgene was confirmed, were crossed to produce the T_1 progeny. A table with the crosses is shown in the Appendix (**Tab. 7.5**). Five of the T_0 plants were self-pollinated (19/5, 20/6, 21/12, 23/32 and 27/18) and 2 of them (21/10 and 27/23) had to be outcrossed, due to sterility of one of the reproductive organs. In this case, pollen from the transgenic plants was used to pollinate plants from the variety H99.

A T_1 progeny was obtained from all crosses, though in some cases (plants 21/10 and 27/23) only a few kernels were produced. Fertility problems were more common during the winter season, when the high light requirement of maize could not be entirely satisfied by artificial illumination. The segregation of the gene of interest in the T_1 progeny from line 21/12 was investigated by Northern blot analysis. As shown in **Fig. 3.5**, from 21 analyzed plants, 12 expressed the transgene, giving a segregation rate of 1.5:1.

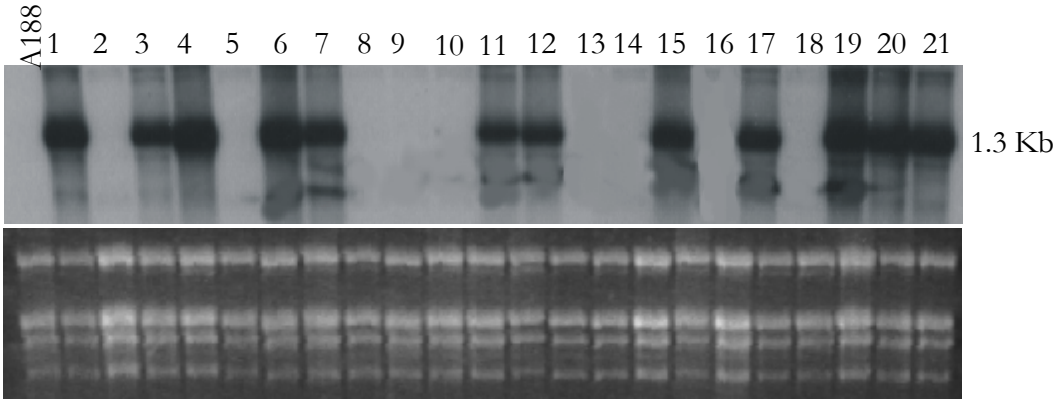

Fig. 3.5: Northern blot analysis of the T_1 progeny obtained after selfing of the transgenic line 21/12. Total RNA (10 µg) from leaves of transgenic maize plants was run on a 1.4 % agarose denaturing gel. RNA was hybridized against a radioactive labeled *Apa* I/*Kpn* I fragment from the plasmid pUbiB5AT.

The T_1 progenies obtained from the T_0 plants were crossed to produce T_2 progenies. A table with the crosses is shown in the Appendix (**Tab. 7.6**). Due to sterility of one of the reproductive organs, in many cases it was not possible to self-pollinate the T_1 plants and they were outcrossed with wild type plants (A188 or H99). Some crosses produced only a few seeds. Nine different selfed plants (indicated by shadowing in **Tab. 7.6**), originating from the transgenic lines 23/32 and 27/18, produced enough seeds and were then chosen to perform genetic and phenotypic analyses in order to find homozygous T_2 transgenic lines.

To verify that the expression of the gene of interest was correlated with the marker gene, plants were individually tested by Basta® spraying (250 mg l^{-1}) and Northern blot analysis (**Fig. 3.6** and **Tab. 3.3**). A complete correlation between the Basta® resistance and the expression of the *A. thaliana* Δ^8-sphingolipid desaturase was found for all the transgenic lines tested (**Fig. 3.6**). From this point on, spraying with Basta® was used to identify homozygous transgenic lines.

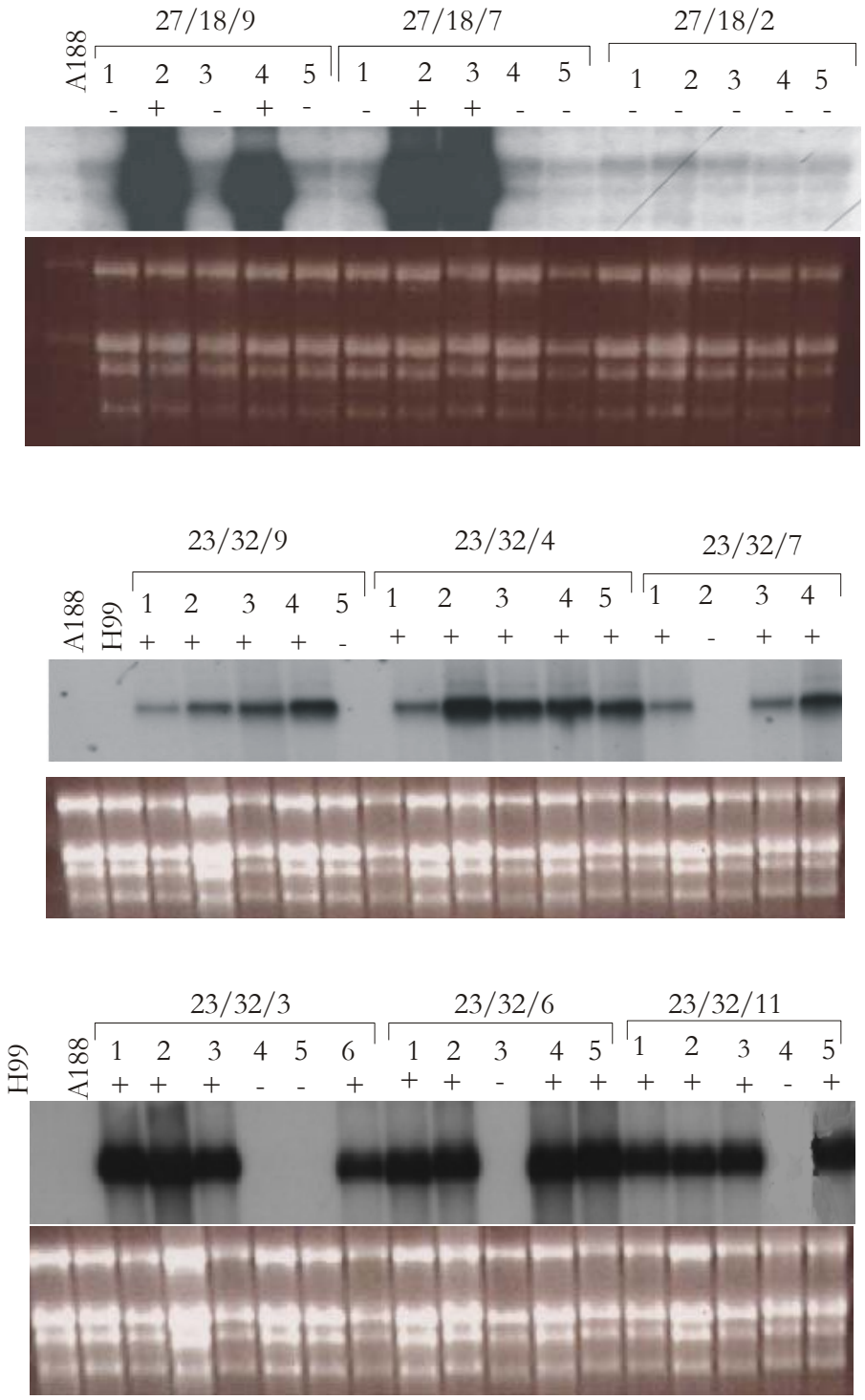

Fig. 3.6: Expression of the *A. thaliana* Δ^8-sphingolipid desaturase gene in the T_2 progenies of maize transgenic lines. Northern blot analysis was performed with 10 µg total RNA extracted from leaves of 4 to 6 plants from each transgenic line. RNA was hybridized to the fragment *Apa* I/*Kpn* I from the plasmid pUbiB5AT. Under each lane tolerance (+) or sensitivity (-) to Basta® (250 mg l^{-1}) is indicated.

The segregation rate for the Basta® resistance of the nine transgenic lines is shown in **Tab. 3.3**. Lines 23/32/3, 23/32/6, 23/32/7, 23/32/9 and 23/32/11 showed a segregation rate close to the expected rate of 3:1. On the other hand, line 27/18/2 was completely killed by the Basta® spraying and the other lines originating from the T_0 plant 27/18 (27/18/7 and 27/18/9) showed a segregation rate close to 1:1 rather than the expected rate of 3:1. This may be explained by an accidental pollination of these lines with pollen from wild-type plants. Only the line 23/32/4 showed complete resistance to the herbicide Basta® (**Fig. 3.7**). It was considered as a homozygous transgenic line and was subsequently used in physiological and biochemical studies.

Tab. 3.3: Segregation of the marker gene in the T_2 generation of transgenic plants transformed with the *A. thaliana* Δ^8-sphingolipid desaturase.

	Basta® spraying		
T_2 transgenic line	**Nr. of treated plants**	**Nr. of resistant plants**	**Segregation rate (resistant:sensitive)**
23/32/3	18	13	2.6:1
23/32/4	18	18	None (homozygous transgenic line)
23/32/6	17	13	3.25:1
23/32/7	8	5	1.67:1
23/32/9	9	7	3.5:1
23/32/11	18	12	2:1
27/18/2	18	0	None (homozygous wild - type line)
27/18/7	16	8	1:1
27/18/9	7	4	1.33:1

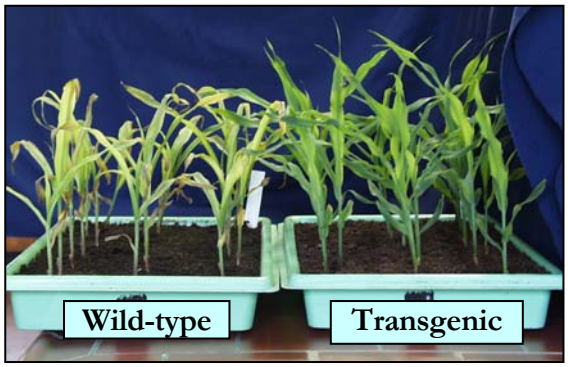

Fig. 3.7: Basta® treatment (250 mg l^{-1}) of the T_2 transgenic line 23/32/4. Plants were sprayed twice within two weeks.

3.2 Biochemical and physiological analysis

3.2.1 Sphingoid base composition in roots of transgenic *vs.* wild-type maize

The sphingoid bases were obtained from adventitious and main roots of 15 day-old maize plants and their DNP derivatives were analyzed by HPLC. Characteristic chromatograms obtained for wild-type and transgenic plants are shown in **Fig. 3.8.** The peaks were identified by MS analysis as the following sphingoid bases: (8*Z*)-4-hydroxy-8-sphingenine (t18:1^{8Z}), (8*E*)-4-hydroxy-8-sphingenine (t18:1^{8E}), (4*E*,8*Z*)-4,8-sphingadienine (d18:2^{4E,8Z}), phytosphinganine (t18:0) and (4*E*,8*E*)-4,8-sphingadienine (d18:2^{4E,8E}). The last two sphingoid bases co-elute and appear as a single peak in the chromatogram.

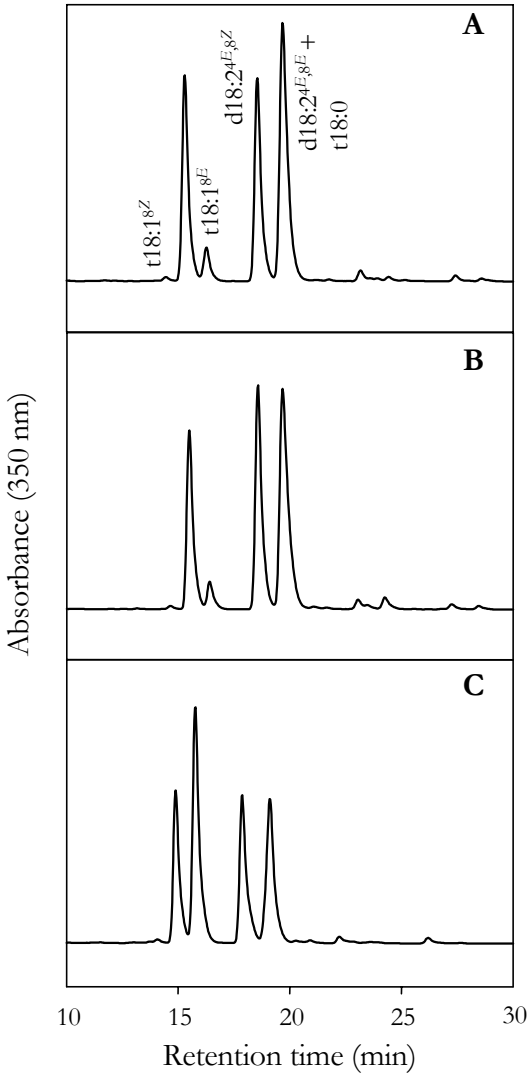

Fig. 3.8: HPLC analysis of DNP derivatives of sphingoid bases released from maize roots. **A)** Wild-type maize (line A188), **B)** wild-type maize (line H99), **C)** transgenic maize (line 21/10).

The sphingoid bases were quantified as percentages of the total peak area and expressed as mol % of total bases. The sphingoid base pattern in roots of wild-type maize was determined using single plants from the parental lines, H99 and A188, and wild-type plants from 3 different T_1 progenies originated by self-pollination of the transgenic lines 21/10, 21/12 and 27/18. Northern blot analysis was conducted in the segregating T_1 progenies in order to distinguish transgenic from wild-type plants. An example of this analysis for line 21/10 is shown in **Fig. 3.5**. Plants not expressing the *A. thaliana* Δ^8-sphingolipid desaturase were considered as wild-type maize. No significant differences in the sphingoid base composition of the lines A188 and H99 as well as the wild-type plants from the 3 tested T_1 progenies were found (**Fig. 3.9**). In roots of wild-type maize the peak corresponding to t18:0 plus d18:2^{4E,8E} represented 40 to 50 mol % of the total area. The Δ^8-*cis*-unsaturated sphingoid bases, t18:1^{8Z} and d18:2^{4E,8Z}, represented 20 to 30 mol %, while the Δ^8-*trans*-unsaturated isomer, t18:1^{8E}, accounted for less than 5 mol % the total sphingoid bases.

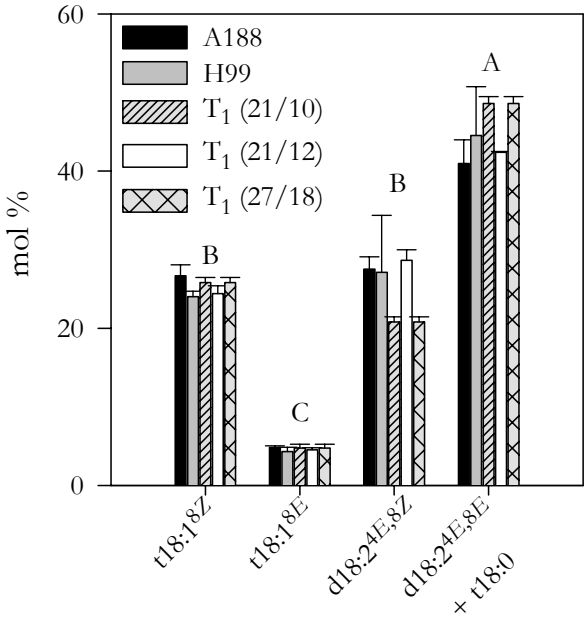

Fig. 3.9: Sphingoid base composition in roots of 15-day old wild-type maize. The following genotypes were used for the HPLC analysis: two maize lines, A188 and H99, and wild-type plants from 3 independent T_1 progenies obtained after selfing of the transgenic lines 21/10, 21/12 and 27/18 (n=2-5). Wild-type plants were selected from the segregating T_1 progenies by Northern blot analysis. Sphingoid base composition is expressed as mol % of total bases. No significant statistical difference between the genotypes was found. Significant statistical differences between the individual sphingoid bases are indicated by different capital letters (5 %, Tukey).

The effect of over-expressing an *A. thaliana* Δ^8-sphingolipid desaturase gene on the sphingoid base composition in roots of transgenic maize plants was investigated with 3 independent transgenic lines (21/10, 21/12 and 27/18). Analyses were done with segregating T_1 progenies obtained after self-pollination of each transgenic line. Wild-type and transgenic plants were distinguished by Northern blot analysis. The wild-type population was represented by plants not expressing the *A. thaliana* sphingolipid desaturase, whereas the transgenic population was composed of plants expressing the gene. All three transgenic lines showed a similar sphingoid base composition (**Fig. 3.10**). In contrast to wild-type plants, transgenic plants expressing the *A. thaliana* Δ^8-sphingolipid desaturase had a 10-fold increase in the amount of t18:1^{8E}, which became the most abundant sphingoid base (40 to 50 mol %) at the expense of t18:0, which was approximately reduced by half, representing 20 to 30 mol % in roots of transgenic plants. The proportions of (8*Z*)-4-Hydroxy-8-sphingenine (t18:1^{8Z}) and (4*E*, 8*Z*)-4,8-sphingadienine (d18:2^{4E,8Z}) were also reduced, but to a lesser extent.

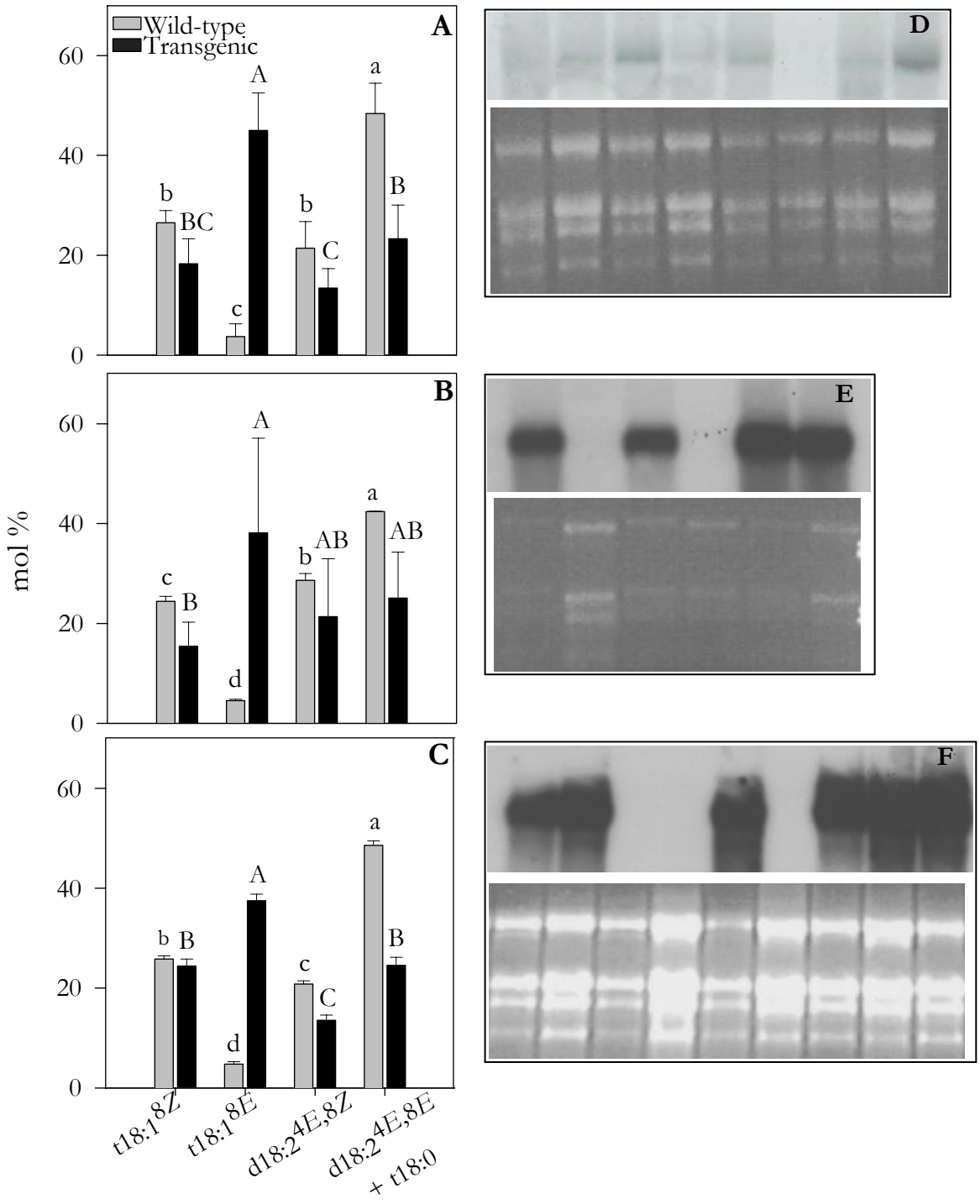

Fig. 3.10: Sphingoid base composition in roots of 15 day-old maize plants expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene. Three independent transgenic lines were tested: Line 21/10 **(A)**, line 21/12 **(B)** and line 27/18 **(C)**. HPLC analysis was done with segregating T_1 progenies obtained after selfing of each transgenic line (n=2-9). Segregation of wild-type and transgenic plants in the T_1 progenies of lines 21/10, 21/12 and 27/18 is shown in the Northern blot analysis (**D**, **E** and **F**, respectively). Sphingoid base composition is expressed in mol % of total bases. Significant statistical differences between wild-type and transgenic were found for all sphingoid bases, except d18:2^{4E,8Z} (**B**) and t18:1^{8Z} (**C**) (10 %, Tukey). Significant statistical differences between the levels of individual sphingoid bases in the transgenic or wild-type populations are indicated by different capital or small letters, respectively.

A regression analysis was conducted in order to find out if relationships between the levels of different sphingoid bases exist. A negative correlation stays for an increase in the content of a certain sphingoid base followed by a decrease in the content of another and vice-versa. A positive correlation implies a concomitant increase or decrease in the concentrations of two sphingoid bases. In wild–type plants there were highly significant negative correlations between the levels of d18:2^{4E,8Z} and the levels of the trihydroxy sphingoid bases: t18:0 (**Fig. 3.11 D)**, t18:1^{8Z} (**Fig. 3.11 C**) and t18:1^{8E} (**Fig. 3.11 F**). Positive correlations existed in wild-type plants between the levels of the trihydroxy bases themselves. This correlation was highly significant between the two Δ^8-unsaturated sphingoid bases t18:1^{8Z} and t18:1^{8E} (**Fig. 3.11 A**), and less or not significant between both unsaturated trihydroxy bases and t18:0 (**Fig. 3.11 B** and **E**).

In transgenic plants over–expressing the *A. thaliana* Δ^8-sphingolipid desaturase the relationships between the sphingoid bases were modified. Highly significant negative correlations appeared between t18:1^{8E} and the remaining sphingoid bases: t18:1^{8Z} (**Fig. 3.11 A**), t18:0 (**Fig. 3.11 E**) and d18:2^{4E,8Z} (**Fig. 3.11 F**). In contrast to wild-type plants, in the roots of transgenic plants there were positive correlations between d18:2^{4E,8Z} and the trihydroxy sphingoid bases, t18:0 (**Fig. 3.11 D**) and t18:1^{8Z} (**Fig. 3.11 C**). Additionally, in transgenic plants the correlation between t18:0 and t18:1^{8Z} was more significant than in wild-type plants (**Fig. 3.11 B**).

In conclusion, the pattern of sphingoid bases present in roots of wild-type maize is characterized by high levels of Δ^8-*cis*-unsaturated sphingoid bases. In roots of transgenic maize expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene a significant increase in the proportion of *trans*-double bonds at the Δ^8 position was observed. Additionally, it was demonstrated that in roots of wild–type plants there is a competition between d18:2^{4E,8Z} and the trihydroxy bases, which are, on their side, positively correlated to each other. The over expression of an additional Δ^8-desaturase causes an increase in the level of t18:1^{8E} and a decrease in the proportion of the remaining sphingoid bases, especially of t18:0.

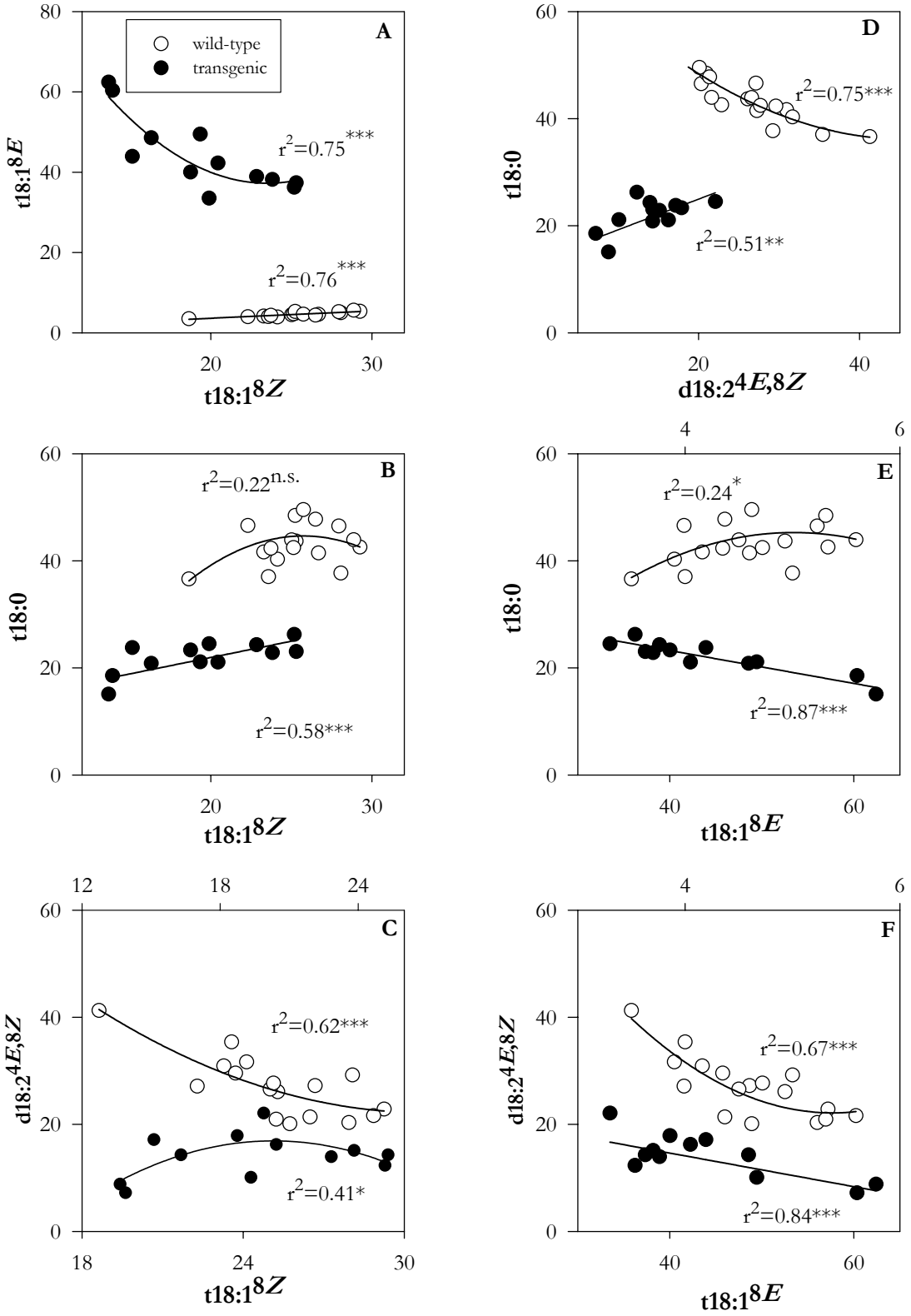

Fig. 3.11: Correlations between the proportions of different sphingoid bases (mol %) in roots of 15 day-old wild-type maize plants and maize plants expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene. The wild-type population (n=17) was formed by the lines H99 and A188, and wild-type plants from the segregating T_1 progenies obtained after self-pollination of 3 independent transgenic lines (21/10, 21/12 and 27/18). The transgenic population (n=12) was composed of transgenic plants from the lines 21/10, 21/12 and 27/18. Transgenic and wild-type plants were distinguished by Northern blot analysis of the T_1 progenies. Regression coefficients (r^2) and degrees of significance (n.s. = not significant, *=10%, **=5% and ***=1%) are indicated.

3.2.2 Effect of aluminum on the sphingoid base composition in root tips of maize

3.2.2.1 Analysis of transgenic *vs.* wild-type maize

In order to verify how aluminum (Al) affects the composition of the sphingoid bases in root tips of transgenic and wild-type maize, plants from the parental lines, H99 and A188, and the homozygous transgenic line 23/32/4 were germinated and cultivated in low ionic strength nutrient solution for 15 days. Twenty-four hours before applying Al, plants were transferred to fresh nutrient solution. The pH of half of the pots was kept at the initial level (pH 5.6), while for the remaining pots it was lowered stepwise to pH 4.3 within 24 h. Subsequently, Al (25 μM) was added to half of the pots at pH 4.3.

After 12 h of Al treatment, root tips (3 mm in length) were harvested from the main and adventitious roots and immediately frozen in liquid nitrogen. After lyophilization, 20 mg (d. w.) were weighed and used for the strong alkaline hydrolysis to release the sphingoid bases. Due to the limited number of seeds and high amount of material necessary for analysis (about 100 root tips), it was not possible to repeat the experiments and to perform a statistical analysis. The liberated sphingoid bases were converted into their DNP derivatives and analyzed by HPLC. Their identities were clarified by mass spectrometry and their quantification was based on the recovery of an internal standard (C14-sphingosine) not present in plant sphingoid bases.

The sphingoid base composition in root tips of wild-type maize was comparable to that present in complete roots. Low proportions of Δ^8-*trans*-unsaturated sphingoid bases, t18:1^{8E} (5 to 8 mol %) and d18:2^{4E,8E} (1.8 to 4.7 mol %) were found, independently of pH or Al treatment (**Fig. 3.12 D** and **E**). At pH 5.6, Δ^8-*cis*-unsaturated bases were the most abundant sphingoid bases found in root tips of wild-type maize. Though the recognition of more subtle differences is hampered by the lack of repetitions, H99 (**Fig. 3.12 A** and **D**) seemed to be richer in sphingadienines (d18:2^{4E,8Z} and d18:2^{4E,8E}) than A188 (**Fig. 3.12 B** and **E**), which comprised 41.5 mol % and 32.3 mol % of the total sphingoid bases of H99 and A188, respectively.

Though the proportion of t18:1^{8E} was not as large as that reported for complete roots, root tips of transgenic plants expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene also had higher contents of Δ^8-*trans* unsaturated sphingoid bases, especially of t18:1^{8E} (11.6 mol %), independently of pH or Al treatment (**Fig. 3.12 C** and **F**). Like in complete roots, the increase in the level of t18:1^{8E} was accompanied by a reduction in t18:0. At pH 5.6, the total sphingoid base content in root tips of transgenic plants (5 to 6 nmol mg^{-1}) was comparable to that of both parental lines.

Lowering the pH from 5.6 to 4.3 caused a slight reduction in the total sphingoid bases content in the root tips of H99 and A188, mainly caused by a reduction in the content of d18:2^{4E,8Z} (**Fig. 3.12 A** and **B**). In contrast, in root tips of the transgenic plants (**Fig. 3.12 C**), an increase in the total sphingoid base content was observed (from 5.5 to 6.9 nmol mg^{-1}) caused mainly by higher contents of t18:1^{8E} and t18:1^{8Z}.

Aluminum had a different effect on each of the parental lines. While the total amount of sphingoid bases in root tips of A188 plants was not affected by Al (**Fig. 3.12 B** and **E**), it caused a 50 % decrease in the total content of sphingoid bases in root tips of H99 plants (**Fig. 3.12 A**). The mainly affected sphingoid bases were d18:2^{4E,8Z} and t18:1^{8Z}, which decreased from 1.7 to 0.3 nmol mg^{-1} and from 1.5 to 0.5 nmol mg^{-1}, respectively. The concentration of t18:0 was not changed in both parental lines (**Fig. 3.12 A** and **B**). As a consequence of the significant decrease in *cis*-unsaturated sphingoid bases, t18:0 increased up to 60 mol % of total sphingoid bases in root tips of Al-treated H99 plants (**Fig. 3.12 D**).

Like in H99, in transgenic plants a similar trend of reduction of *cis*-unsaturated sphingoid bases following the Al treatment was observed (**Fig. 3.12 C**). On the other hand, since the content of t18:1^{8E} increased, no severe reduction in the total sphingoid base content was observed in the transgenic line.

These results suggest that changes in the total level of sphingoid bases in root tips of maize plants treated with aluminum are mostly due to a reduction of the Δ^8-*cis*-unsaturated bases t18:1^{8Z} and d18:2^{4E,8Z}. Due to the activity of the heterologously expressed Δ^8-sphingolipid desaturase, transgenic plants can compensate this reduction of Δ^8-*cis*-unsaturated bases observed in Al-treated root tips by increasing the synthesis of t18:1^{8t} and, to a lower extent, of t18:1^{8c}.

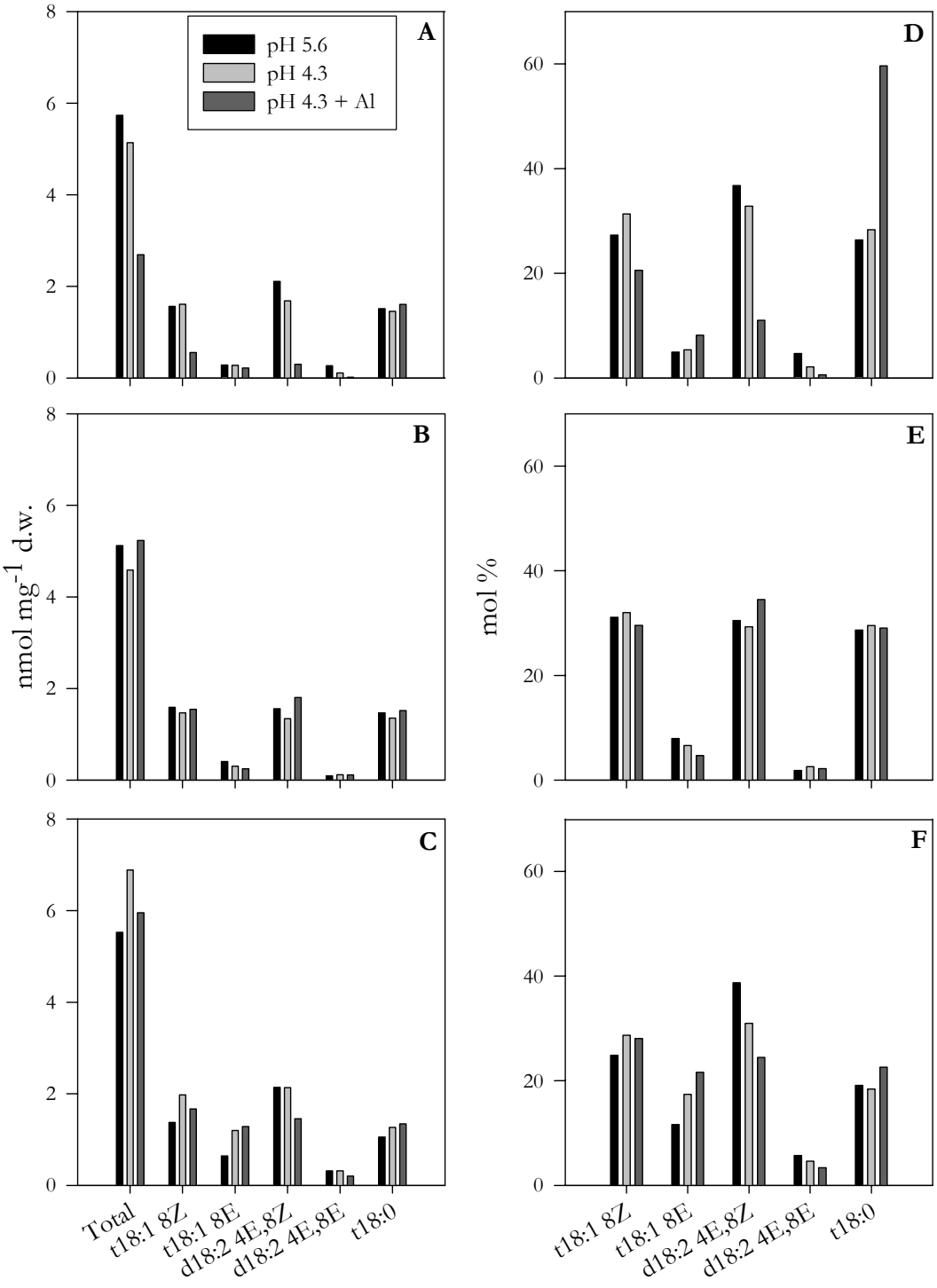

Fig. 3.12: Sphingoid base composition in 3-mm root tips of 1-week old maize plants cultivated in nutrient solution at different pHs with and without 25 μM Al, respectively. **A)** and **D)** wild-type, line H99; **B)** and **E**) wild-type, line A188; **C)** and **F**) transgenic, line 23/32/4. Sphingoid base composition is shown in nmol mg^{-1} d.w. (**A-C**) or in mol % of total bases (**D-F**).

3.2.2.2 Analysis of aluminum-resistant *vs.* –sensitive varieties

To investigate the effect of aluminum (Al) on the sphingoid base compositions in root tips of two maize varieties differing in their Al resistance, plants from ATP-Y (Al-resistant) and Lixis (Al-sensitive) (HORST *et al.*, 1997) were germinated and subjected to different pH values and 25 μM Al as described above.

At pH 5.6 both varieties showed similar total sphingoid base contents (**Fig. 3.13 A** and **C**). However, differences in the sphingoid base composition were found. ATP-Y was richer in the unsaturated sphingadienine, d18:2^{4E,8Z} (**Fig. 3.13 D**), while Lixis had more t18:0 (**Fig. 3.13 B**). A reduction of the pH to 4.3 did not significantly alter the sphingoid base composition of both varieties (**Fig. 3.13 B** and **D**). A marked change in the sphingoid base composition was observed, however, when the root tips of both varieties were treated with Al. In Lixis an increase from 4.1 to 6.3 nmol mg^{-1} in the total sphingoid base content was caused exclusively by a 2-fold increase in the content of d18:2^{4E,8Z} since no other significant changes in the sphingoid base composition were observed (**Fig. 3.13 A**). As shown in **Fig. 3.13 B**, in the presence of Al the proportion of unsaturated sphingoid bases in root tips of Lixis increased, while the proportion of t18:0 decreased.

In ATP-Y the very high standard deviations observed for the aluminum-treated root tips did not allow to draw any conclusions. However, in accordance to the results for H99 shown in 3.2.2.1 and in contrast to Lixis, a tendency to reduce d18:2^{4E,8Z} and to increase t18:0 is observed (**Fig. 3.13 C** and **D**). Following Al treatment there was a minor increase in the total sphingoid base content in roots of ATP-Y, which was exclusively due to the increase of t18:0 (**Fig. 3.13 C**).

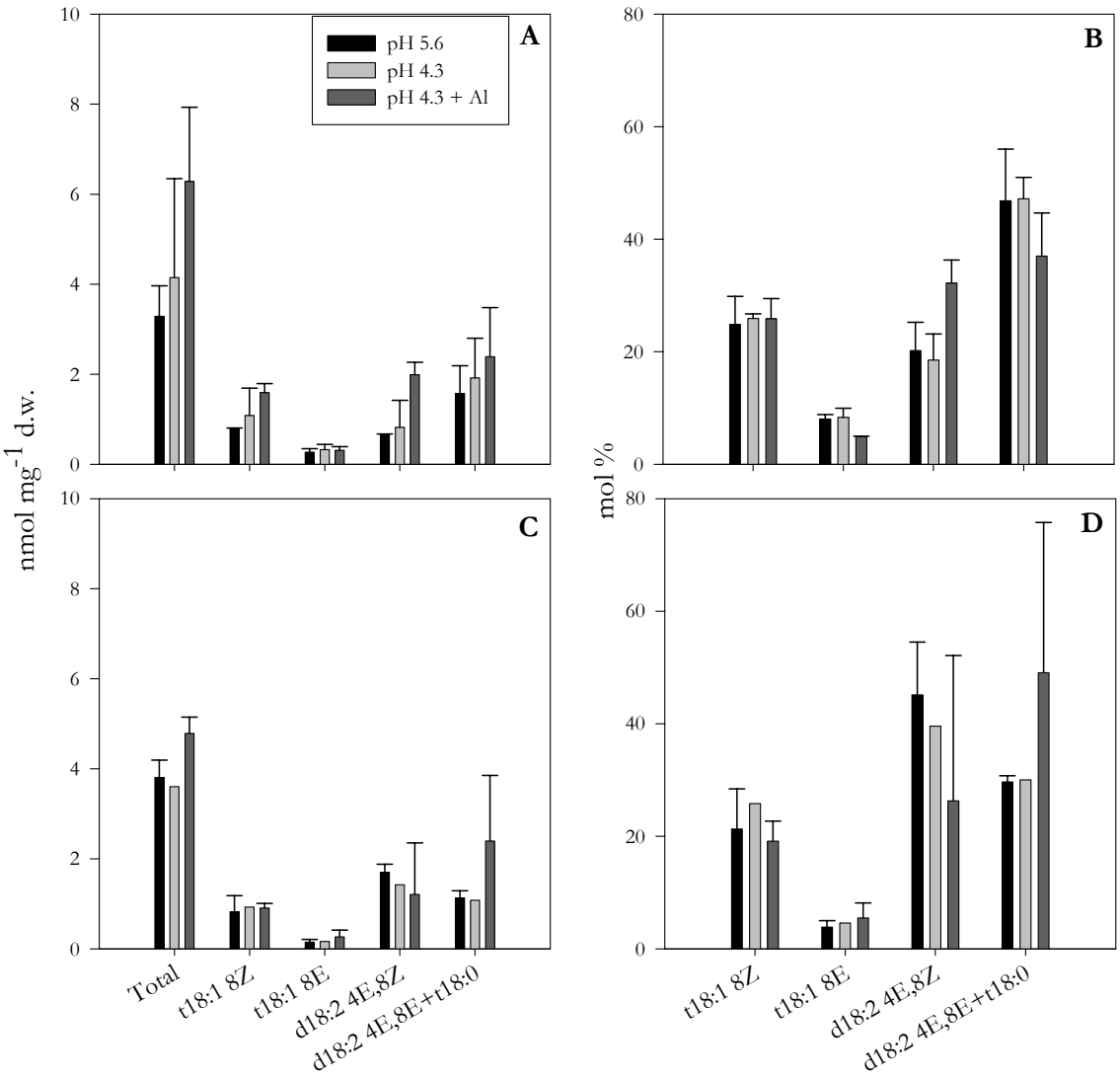

Fig. 3.13: Sphingoid base composition in 3-mm root tips of 1-week old maize plants from the Al-sensitive variety Lixis (**A** and **B**) and the Al-tolerant line ATP-Y (**C** and **D**). Plants were cultivated for 12 h in nutrient solution at two pH values, with or without Al (25 µM). Sphingoid base compositions are shown in nmol mg^{-1} d.w. (**A** and **C**) or in mol % of total bases (**B** and **D**). Except for ATP-Y pH 4.3 (n=1), the average of 2 repetitions is shown.

3.2.3 Analysis of the aluminum resistance of transgenic lines

The induction of callose formation in the root apices has been correlated to aluminum (Al) toxicity in maize (HORST *et al.*, 1997). In the present work no differences in the callose content in the root tips of the tested genotypes were found without Al. Decreasing the pH had no effect on callose formation.

The addition of Al caused an up to 10-fold increase in callose content (**Fig. 3.14**). Clear differences between the callose content of different genotypes appeared. The previous

characterization of ATP-Y as Al-resistant/low callose and Lixis as Al-sensitive/high callose was confirmed.

The callose content in root tips of the transgenic line 23/32/4 was similar to the one of Lixis and higher than in both parental lines, H99 and A188. Due to high standard deviations, a significant difference was only found in comparison to H99, which had similar callose content to ATP-Y (**Fig. 3.14**). Based on the induction of callose formation upon aluminum treatment, it seems that the expression of the *A. thaliana* Δ^8-sphingolipid desaturase gene increases the Al sensitivity in maize.

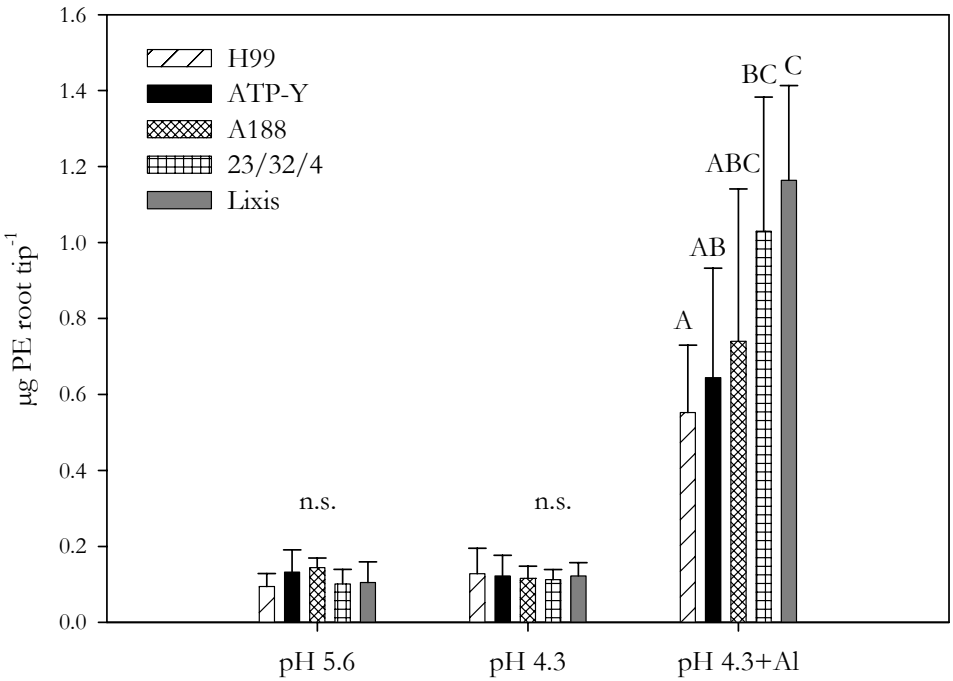

Fig. 3.14: Callose content in 5-mm root tips of 1-week old maize plants exposed for 12 h to two pH values, with or without Al (25 µM). Five different genotypes (n=2-9) were tested: 23/32/4, a transgenic line expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene; H99 and A188, the parents of the transgenic line; ATP-Y, an Al-resistant line; and Lixis, an Al-sensitive line. Callose content is expressed as µg PE root tip^{-1}. At pH 5.6 and pH 4.3 no significant differences between the genotypes were found (n.s. =not significant). At pH 4.3+Al significant differences between the genotypes (5 %, Tukey) are indicated by different capital letters.

4 Discussion

4.1 The sphingoid base composition in maize roots

At first, a preliminary analysis of the sphingoid base composition was conducted in order to determine the proportions of different sphingoid bases in roots of wild-type maize. In agreement with previous studies on plant cerebrosides (LYNCH and STEPONKUS, 1987; CAHOON and LYNCH, 1991; LYNCH, 1993; IMAI *et al.*, 1997; KAWAGUCHI *et al.*, 2000; IMAI *et al.*, 2000), di- and trihydroxy unsaturated sphingoid bases (t18:1$^{8Z/E}$ and d18:2$^{4E,8Z/E}$) represented 60 mol % of the total sphingoid bases in roots of wild-type maize.

Interestingly, in 3-mm root tips the relative proportion of unsaturated sphingoid bases was in average 10 mol % higher than in complete roots, indicating that the differences in the ratios of Δ^8-desaturation may be related to the developmental stages of the root tissues. Accordingly, a sphingolipid Δ^8-desaturase from *Borago officinalis* was primarily expressed in young leaves (SPERLING *et al.*, 2001b). No expression was detected in old leaves or roots, suggesting that rapidly expanding tissues exhibit higher rates of desaturation and, consequently, of sphingolipid synthesis.

Sphingoid bases can be unsaturated at their Δ^4- and/or Δ^8-positions. While Δ^4-desaturases exclusively synthesize *trans*-double bonds, Δ^8-sphingolipid desaturases from plants do not show absolute stereo-selectivity, synthesizing both stereo-isomers (SPERLING *et al.,* 1998 and 2001b). The occurrence of both Δ^8-*cis*- and *trans*-isomers seems to be restricted to plant sphingolipids. In fact, different plant families are characterized by specific ratios of *trans:cis* stereo-isomers. IMAI *et al.* (1997 and 2000) demonstrated that families like *Cucurbitaceae*, *Solanaceae* and *Brassicaceae* have considerable amounts of Δ^8-*trans*-unsaturated sphingoid bases, whereas in *Gramineae cis*-double bonds predominate. In agreement with these data, the majority of the Δ^8-unsaturated sphingoid bases in maize has *cis*-double bonds, with an average *trans:cis* ratio of 1:5. This ratio was similar in di- and trihydroxybases, suggesting that in maize the same Δ^8-desaturase introduces double-bonds in both types of sphingoid bases.

Surprisingly, in maize roots relatively high proportions of phytosphinganine (t18:0, 20 to 50 mol %) were present. Though it is a main component of baker's yeast sphingolipids (DICKSON and LESTER, 1999), only trace amounts of t18:0 have been reported in leaf cerebrosides (IMAI *et al.*, 1997; KAWAGUCHI *et al.*, 2000; IMAI *et al.*, 2000). It has been demonstrated that in several fungi (JENNEMANN *et al.*, 2001) and in plants (CARTER *et al.*, 1969) t18:0 is mainly present in phytoglycolipids (glycosyl inositol

phosphorylceramides, GIPC). Though at present no exact data on the relative proportions of cerebrosides and phytoglycolipids in plants are available, the use of direct alkaline hydrolysis, which generates a complete pool of sphingoid bases, has brought some evidence that phytoglycolipids are much more abundant than cerebrosides (SPERLING and HEINZ, in press). The amount of t18:0 may also vary with the organ or tissue analyzed. Furthermore, divergences in the sphingoid base composition of *A. thaliana* leaves obtained by direct alkaline hydrolysis (SPERLING *et al.*, 1998) or lipid extraction of cerebrosides with organic solvents (IMAI *et al.*, 2000) indicate a channeling of different sphingoid bases to different classes of sphingolipids. Therefore, it is possible that in roots of maize, as for some fungi and plants, t18:0 may be channeled to phytoglycolipids, while unsaturated bases are channeled to cerebrosides. If this is also true for other plant species, the amount of t18:0 has possibly been underestimated at least in some tissues, since GIPCs are hardly extractable in the organic solvents, which have been used for the extraction of plant cerebrosides (IMAI *et al.*, 1997 and 2000; KAWAGUCHI *et al.*, 2000).

A regression analysis was conducted in order to determine whether significant relationships between the level of different sphingoid bases existed, and how a change in the level of a certain sphingoid base influenced the level of others. This analysis should give some insight into the metabolic routes responsible for the synthesis of different sphingoid bases. In complete roots of maize a clear negative correlation between the levels of tri- (t18:0 and $t18:1^{8E/Z}$) and dihydroxy ($d18:2^{4E,8Z}$) bases was observed, indicating that the latter is increased at the expense of the former and vice-versa. Their metabolic pathways seem to compete for the same substrate pool. On the other hand, a strong positive correlation between the levels of the Δ^8-*cis- and trans*-stereo-isomers of t18:1 was observed, confirming that the concentrations of both stereo isomers in maize roots are also controlled by the activity of a bifunctional Δ^8-sphingolipid desaturase.

Based on the sphingoid base composition, the regression analysis and the results from previous studies (SPERLING *et. al.*, 2000 and 2001a; MICHAELSON *et al.*, 2002; TERNES *et al.*, 2002), a tentative pathway for the formation of unsaturated sphingoid bases in maize roots has been developed. The routes probably leading to the synthesis of tri- and dihydroxybases diverge at the initial step following d18:0 hydroxylation or desaturation. The occurrence of route C (**Fig. 4.1**) in plants could be experimentally demonstrated by SPERLING *et al.* (2000 and 2001a). C4-hydroxylation of d18:0 yielding t18:0 has been confirmed by expressing a C4-hydroxylase from *A. thaliana* in the *S. cerevisiae* mutant *sur2Δ*, which lacks C4-hydroxylation. The next step in t18:1 biosynthesis, the Δ^8-desaturation of t18:0, was confirmed by formation of $t18:1^{8Z/E}$ after feeding t18:0 to a yeast *sur2Δ* mutant expressing a plant Δ^8-sphingolipid desaturase.

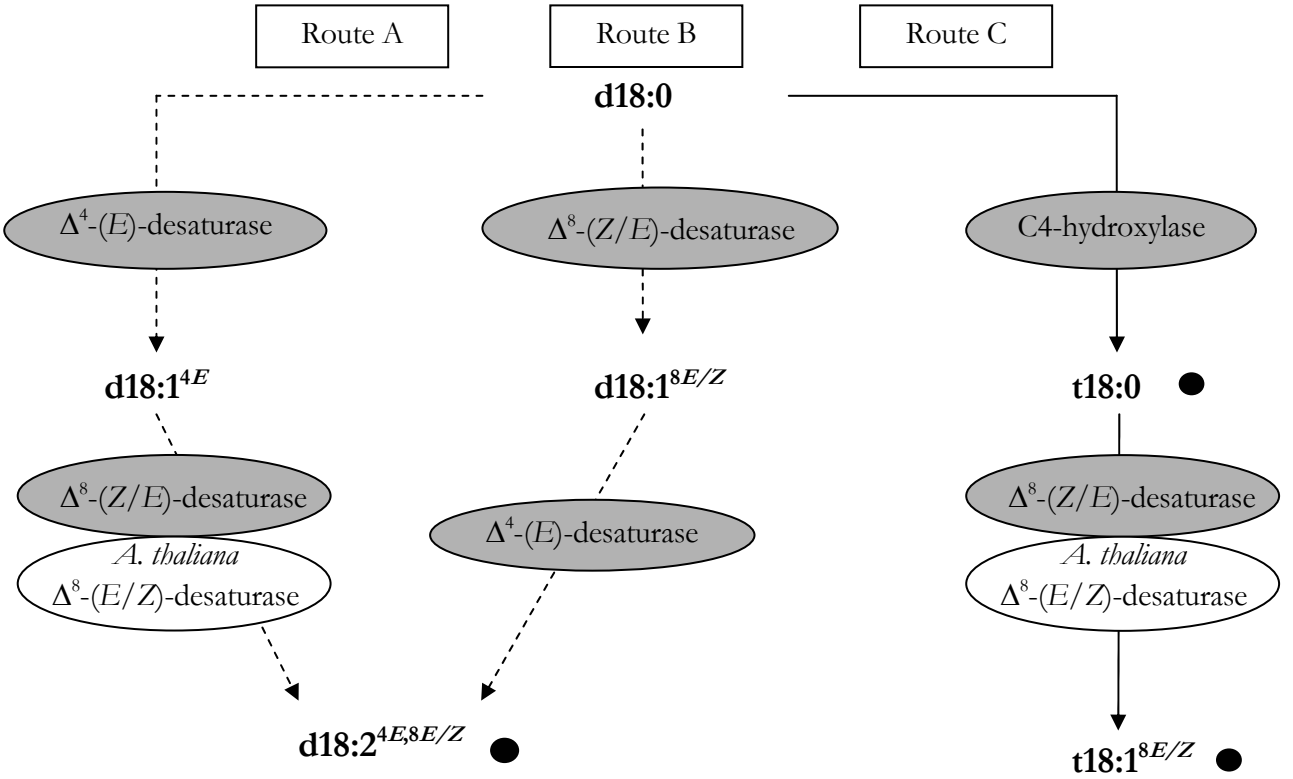

Fig. 4.1: Hypothetical pathways for the formation of unsaturated tri- and dihydroxy sphingoid bases in maize. The dotted lines represent tentative pathways. Endogenous enzymes are shown on a grey background, while the heterologous *A. thaliana* Δ^8-(*E*/*Z*)-desaturase is shown on a white background. The prevalent stereo-isomer formed by the bi-functional Δ^8-desaturases is indicated by the first letter in the parenthesis. A black dot indicates that the accompanying sphingoid base was detected in the HPLC and MS analyses from maize roots.

Up to now, the correct sequence of d18:0 desaturation has not been clarified. The presence of significant proportions of Δ^8-*cis*/*trans*-sphingenine (d18:1$^{8Z/E}$) in leaf cerebrosides (IMAI *et al.*, 1997) suggests that, at least in some plant species, d18:0 can act as a substrate for Δ^8-desaturation, as proposed in route B (**Fig. 4.1**). The expression of plant Δ^8-sphingolipid desaturases in the baker's yeast mutant *sur2Δ* has brought controversial results, with the formation of Δ^8-sphingenine (d18:1^8) in the case of *Aquilegia vulgaris* (MICHAELSON *et al.*, 2002), but not for *A. thaliana* and *Brassica napus* (SPERLING *et. al.*, 2000). In maize roots no significant proportion of d18:1$^{8Z/E}$ was detected, indicating that if Δ^8-desaturation really occurs first, then Δ^4-desaturation must follow very quickly, so that d18:1$^{8Z/E}$ does not accumulate.

The formation of (*E*)-sphing-4-enines (d18:1^{4E}) following the expression of Δ^4-desaturases from different organisms (*Mus musculus*, *Homo sapiens*, *Drosophila melanogaster* and *Candida albicans*) in the *S. cerevisiae* mutant *sur2Δ* showed that, at least in animals and yeasts, d18:0 can serve as a substrate for Δ^4-

long chain base desaturases (TERNES *et al.*, 2002), as anticipated in route A (**Fig. 4.1**). A final characterization of plant Δ^4-desaturases is still missing. In a first study, the functional expression of a putative *A. thaliana* Δ^4-desaturase in *S. cerevisiae* did not result in the formation of d18:1^{4E} (P. TERNES, personal communication). However, as already mentioned in the previous paragraph, the expression of an *A. thaliana* Δ^8-desaturase in the baker's yeast *sur2Δ* mutant did not lead to the production of d18:1$^{8E/Z}$ as well (SPERLING *et. al.*, 2000). Because the main sphingoid base present in yeast is t18:0, the absence of d18:1$^{4/8}$ in yeast expressing *A. thaliana* desaturases might be due to reduced amounts of d18:0, rather than incapacity of the plant enzymes to use d18:0 as a substrate. The Δ^8-desaturation of Δ^4-sphingenine (d18:1^{4E}), as suggested in route A, has not been demonstrated yet, due to an inefficient incorporation of d18:1^{4E} into yeast (P. SPERLING, personal communication).

4.2 Changes in sphingoid base composition in roots of transgenic plants

The HPLC analyses of sphingoid bases present in roots and root tips of transgenic maize expressing an *A. thaliana* Δ^8-(*E*/*Z*)-sphingolipid desaturase gene showed that the pattern of sphingoid bases was changed in comparison to wild-type maize as described in the following paragraphs.

It has been shown that transgenic yeast expressing the bi-functional *A. thaliana* Δ^8-sphingolipid desaturase had a *cis:trans* ratio of 1:3, indicating that this plant Δ^8-desaturase preferentially introduces *trans*-double bonds (SPERLING *et al.*, 1998). This is in agreement with the 10-fold increase observed in transgenic maize roots in the proportion of t18:1^{8E}, which never corresponded to more than 5 mol % of total bases in roots of wild-type maize. In contrast to wild-type roots, in which the most abundant sphingoid base is t18:0, in transgenic roots t18:1^{8E} represents the most abundant sphingoid base, accounting for 35 to 55 mol % of total bases.

A regression analysis has shown that the increase in *trans*-unsaturated sphingoid bases in roots of maize expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene is followed by a remarkable decrease in t18:0 (**Fig. 3.11**). This indicates that t18:0 probably serves as a substrate for the heterologous Δ^8-desaturase. A similar decrease of t18:0 was observed in the yeast expressing the *A. thaliana* Δ^8-sphingolipid desaturase (SPERLING *et al.*, 1998). Moreover, the relative proportion of Δ^8-*cis*- to Δ^8-*trans*-unsaturated trihydroxy bases has also decreased, though in a less pronounced manner (see below).

In contrast to a 10-fold increase in complete roots, in root tips of transgenic maize there was only a 2.5 -fold increase in t18:1^{8E}. There are no indications in the literature that the ubiquitin promoter

controlling the expression of the *A. thaliana* Δ^8-sphingolipid desaturase gene is less active in root tips than in other parts of the roots. Assuming a similar gene expression in all root tissues, different *cis* to *trans* ratios in different root tissues might be caused by varying activities of the endogenous maize Δ^8-sphingolipid desaturase. Indeed, in root tips the relative proportion of Δ^8-*cis*-unsaturated sphingoid bases is higher than in mature roots, denoting a higher activity of the endogenous Δ^8-desaturase in this part of the roots. These higher levels of Δ^8-*cis*-unsaturated sphingoid bases are still present in transgenic root tips, indicating that the activity of the endogenous desaturase is not influenced by the heterologous expression of the *A. thaliana* Δ^8-desaturase. Higher levels of desaturation in rapidly expanding tissues have been previously demonstrated by SPERLING *et al.* (2001b). Therefore, it can be concluded that lower levels of t18:1^{8E} in transgenic root tips might be due to a competition of the heterologous and the endogenous Δ^8-sphingolipid desaturase for the same substrate (t18:0).

Furthermore, a small increase in d18:2^{4E,8E}, which has been observed in root tips of transgenic maize, indicates that the *A. thaliana* Δ^8-sphingolipid desaturase is also capable of introducing Δ^8-double bonds into dihydroxy bases. However, d18:2^{4E,8Z} remains the most abundant sphingoid base in transgenic root tips, suggesting that the maize endogenous Δ^8-desaturase has a higher affinity for d18:1^{4E} or d18:0, respectively, than the heterologous Δ^8-desaturase, which, at least in *A. thaliana*, preferentially synthesizes t18:1 but no d18:2 (SPERLING *et al*, 1998; IMAI *et al*., 2000).

The over expression of a heterologous Δ^8-desaturase did not increase the total sphingoid bases content in transgenic root tips. It can be concluded that sphingolipid desaturases and hydroxylases compete for the same substrate pool derived from d18:0 (**Fig. 4.1**). This substrate pool is strictly dependent on the activity of the serine palmitoyltransferase (SPT), which represents a rate-limiting step in ceramide biosynthesis (HANADA *et al*., 2000). Without an accompanying increase in *de novo* synthesis of d18:0, Δ^8-*trans*-unsaturated sphingoid bases are increased in transgenic roots at the expense of other sphingoid base species.

4.3 pH- or aluminum-induced changes in sphingoid base composition

Maize is not especially susceptible to low pH values. Accordingly, the changes in the sphingoid base pattern of wild-type plants observed at acidic pH were negligible. Furthermore, since the pH has been gradually reduced over a period of 24 h before the addition of Al, at the time of harvest the root tips were already adapted to the low pH. Nevertheless, at pH 4.3 a modest but significant decrease in the proportions of d18:2 and a small increase in the proportions of t18:1 was observed in the root tips of all genotypes, indicating a specific inhibition of the Δ^4-desaturase by acidic pH. The opposing changes of t18:1 and d18:2 suggest that, during the biosynthesis of d18:2, Δ^4-desaturation precedes Δ^8-desaturation as shown in route A (**Fig. 4.1**). On the other hand, the two–fold increase in the amount of t18:1^{8E} in root tips of transgenic plants and the smaller increase in the amount of t18:1^{8Z} at acidic pH indicates that the heterologous *A. thaliana* Δ^8-desaturase is not inhibited by the acidic pH. These data support the hypothesis that the inhibition of the Δ^4-desaturase allows d18:0 to accumulate, which is then used by the C4-hydroxylase for t18:0 synthesis and, subsequently, by the heterologous *A. thaliana* Δ^8-desaturase to produce mainly higher amounts of t18:1^{8E}. Root tips of transgenic plants at low pH had a higher total sphingoid base content suggesting that serine palmitoyl transferase (SPT) is stimulated under acidic conditions.

Different modifications on the sphingoid base pattern were observed in Al-treated root tips of different genotypes. Due to the high standard deviations and the lack of a large number of repetitions, any conclusion must be taken with caution. The following conclusions drawn for wild-type maize refer only to the 3 major sphingoid bases: t18:1^{8Z}, d18:2^{4E,8Z} and t18:0+d18:2^{4E,8E}. The MS data showed that d18:2^{4E,8E} co-eluting with t18:0 was minimal. Since t18:1^{8E} is abundant in transgenic plants, it will be included in the discussion involving these plants. Due to their high production of callose in response to Al, Lixis and A188 were considered to be Al-sensitive. In these genotypes there was an increase in the total sphingoid base content, mainly due to a large increase in d18:2^{4E,8Z}. Furthermore, in Lixis, these changes lead to an increase in the ratio of unsaturated:saturated bases. H99 and ATP-Y produced very low amounts of callose and were considered to be Al-resistant genotypes. In contrast to Lixis and A188, these genotypes showed a significant decrease in the amount of d18:2^{4E,8Z}. The total sphingoid base content was decreased for H99, while it remained unchanged for ATP-Y.

Similarly to the Al-resistant genotypes, in the transgenic line a reduction in the level of d18:2 was observed. However, the level of t18:1, especially t18:1^{8E}, was maintained, suggesting that the activity of the heterologous Δ^8-desaturase was not affected by the Al treatment. These results show that in root tips

of maize Al changes the amount of unsaturated sphingoid bases, especially d18:2. An increase or decrease in the amounts of this sphingoid base seems to depend on the sphingoid base composition present in the corresponding genotypes before the addition of Al. If the previously existing proportion of unsaturated sphingoid bases and total sphingoid base content is high, as for the Al-resistant genotype H99, Al seems to decrease d18:2. On the other hand, if the total sphingoid base content and the proportion of unsaturated sphingoid bases previous to the addition of Al is low, as in the Al-sensitive genotype Lixis, Al increases d18:2 and total sphingoid base content.

4.4 Relationships between Al-induced changes in sphingoid base composition and Al resistance/toxicity mechanisms

Since the physiological and molecular basis of Al toxicity as well as the role of sphingolipids in plant cells are not fully understood, any proposed explanation for the observed Al-activated changes on the sphingoid base patterns in Al-treated root tips from Al-resistant/sensitive genotypes will be highly speculative.

The major site of Al localization in plant cells has been reported to be the apoplast (HORST, 1995; RENGEL, 1996; JONES and KOCHIAN, 1997). Though Al has been shown to enter the cytosol rapidly, it is likely that, at the cytoplasmic pH values (pH 7.0-7.5), most of the Al in the cytoplasm will be complexed (MARTIN, 1986). Therefore, the plasma membrane (PM) is believed to be a primary target of Al toxicity. Recent studies have shown that the PM is not a random ocean of lipids. Rather, different domains with different degrees of fluidity coexist as shown in **Fig. 4.2**. Liquid ordered domains, called "lipid rafts", which show less fluidity than the surrounding PM, are formed due to a closer packing of abundant saturated acyl chains mainly present in sphingolipids (BROWN and LONDON, 2000). In addition to sphingolipids, cholesterol in mammals, as well as sterols in plants (PESKAN *et al.*, 2000; XU *et al.*, 2001), are another important component of lipid rafts. Sterols increase even more the rigidity of the raft by tightly packing with sphingolipids having saturated acyl chains. It has been shown that lipid rafts may be quite abundant, representing up to 50% of the plasma membrane surface area, depending on the cell type (HAO *et al.*, 2001). Al-induced changes in the physical properties of the PM, like rigidification and phase separation, match the characteristics of lipid rafts. Therefore, it is possible that changes in sphingolipid content and/or composition might be more relevant for Al-induced changes in the physical properties of the PM than changes in the phospholipid composition.

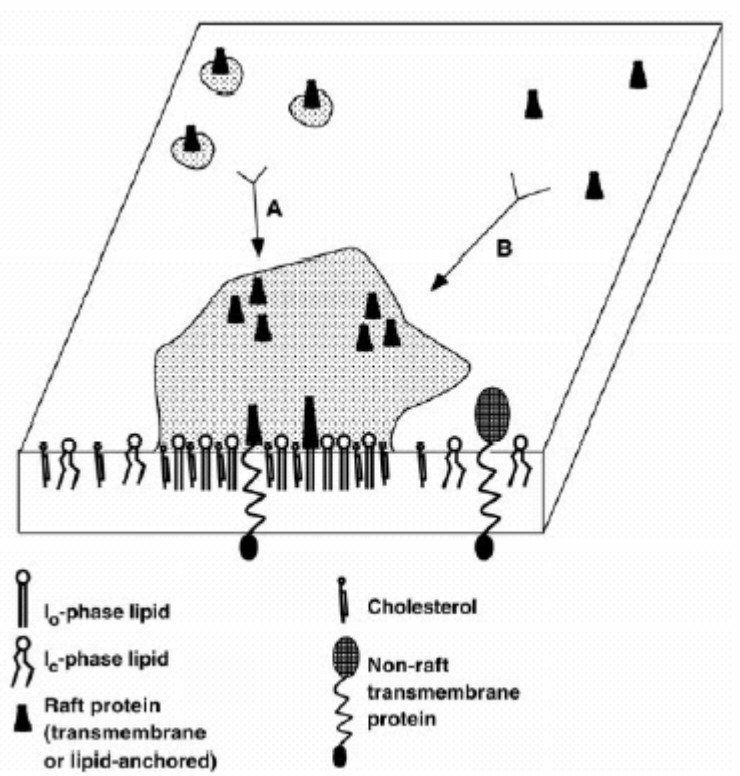

Fig. 4.2: Model of raft organization. Tightly packed sphingolipids are enriched in liquid ordered phase rafts (light gray) in the cholesterol-rich plasma membrane. Clustering of a protein that has an affinity for rafts (indicated by arrows) could either cause small, dispersed rafts containing the protein to coalesce into larger rafts (A), or increase the overall raft affinity of the protein cluster enough to recruit it to rafts (B). Clustering of proteins could even induce raft formation (according to BROWN and LONDON, 2000).

In the present work a 12-h exposure to 25 μM Al induced a remarkable decrease in unsaturated sphingoid bases, especially d18:2^{4E,8Z}, in root tips of H99, a low callose-producing Al-resistant genotype. In contrast, in root tips of Lixis, an Al-sensitive variety, there was an increase in unsaturated sphingoid bases. Further evidence that the synthesis of unsaturated bases leads to increased Al sensitivity is confirmed by the fact that the transgenic line, which has higher amounts of t18:1^{8E}, produced more Al-induced callose than the control lines. No significant modification in the content of t18:0 was observed in any of the investigated genotypes. As already discussed in section 4.1, different sphingoid bases might be channeled to different classes of sphingolipids. Therefore, t18:0 seems to be mainly incorporated into phytoglycolipids (phosphosphingolipids) (CARTER *et al.*, 1969; JENNEMANN *et al.*, 2001), whereas unsaturated bases are channeled to cerebrosides (glycosphingolipids) (IMAI *et al.,* 2000). If the high content of t18:0 present in Lixis before the addition of Al is indeed correlated to a higher content of phosphosphingolipids, phosphate groups present in these phosphosphingolipids may strongly attract Al^{3+} ions upon exposure to Al, just as has been demonstrated for phospholipids (VIERSTRA and HAUG, 1978; DELEERS *et al.*, 1986; AKESON *et al.*, 1989; JONES and KOCHIAN, 1997). The binding of Al to phosphosphingolipids could modify protein/lipid interactions in the lipid rafts and induce *de novo*

synthesis of unsaturated sphingoid bases, especially d18:2^{4E,8Z}, observed in Al-treated root tips of Lixis. In fact, increased ceramide synthesis upon activation of PM receptors has been demonstrated in several studies. GHAFOURIFAR *et al.* (1999) showed *de novo* ceramide synthesis upon TNF-α receptor stimulation. KROESEN *et al.* (2001) demonstrated that upon activation of a receptor (BcR) at the cell surface of B cells (Burkitt's lymphoma Ramos cells) there was an early rise in ceramide, which was exclusively due to *de novo* ceramide synthesis and could be inhibited by fumosin B1, a specific inhibitor of ceramide synthase. As suggested above, *de novo* synthesized unsaturated sphingoid bases might be channeled to cerebrosides. SAKAKI *et al.* (2001) showed that abiotic stresses, such as heat shock and ethanol stress induce a similar accumulation of sterols and cerebrosides in several fungal species, probably affecting the structure of lipid rafts. An increase in the concentration of cerebrosides in chilling-sensitive rice cells submitted to chilling caused a reduction of the H^+-ATPase activity and decreased membrane fluidity of tonoplast vesicles (KASAMO *et al.*, 2000). In fact, a small amount of ceramide (3 mol %) has been shown to stabilize domain/raft formation significantly (XU *et al.*, 2001). Furthermore, ceramide can promote phase separation when mixed with phospholipids (MASSEY, 2001). On the other hand, the decrease of unsaturated sphingoid bases, observed in the Al-resistant genotype H99 could represent an attempt to improve membrane stability under Al stress. LYNCH and STEPONKUS (1987) showed that cold acclimation of winter rye seedlings involved a pronounced decrease in the cerebroside content of the plasma membrane. The decrease in glucocerebrosides and the increase in free sterols and phospholipids following acclimation were expected to increase membrane stability by preventing the formation of separate phases or domains within the membrane.

As main components of lipid rafts, sphingolipids are known to have both structural and signaling roles. It is possible that, under normal conditions, phosphosphingolipids, similar to phospholipids, represent a pool of relatively stable lipids, which remain unchanged upon a range of environmental conditions in order to maintain the integrity of the PM. This hypothesis is supported by the fact that the concentration of t18:0, probably the prevalent sphingoid base in phosphosphingolipids, was not significantly affected by the Al treatment. On the other hand, glycosphingolipids, mainly composed of unsaturated bases, might represent a more flexible pool of sphingolipids, which participate in signaling. In fact, it has been demonstrated that the presence of unsaturated sphingoid bases, such as sphingosine (d18:1^{4E}) in animal cells, is essential for the biological activity of important signaling sphingolipid metabolites, like ceramide and sphingosine-1-phosphate. Whether plant-specific unsaturated sphingoid bases, such as t18:1$^{8Z/E}$, d18:2$^{4E,8Z/E}$ or/and d18:1$^{8E/Z}$, may have similar signaling functions has not been investigated so far. Interestingly, in the present work high levels of t18:0 were mainly found in the mature parts of maize roots, whereas in the root apices, which are zones of high cell division and membrane

formation, the content of t18:0 decreased and the content of unsaturated sphingoid bases increased significantly, thus confirming possible roles as structural and signaling molecules, respectively.

Though, at present, the relationship between sphingolipids and Al toxicity has not been investigated, some interesting parallels between Al- and sphingolipid metabolites-triggered events will be discussed in the following. Due to the production of reactive oxygen species (BOSCOLO *et al.*, 2003) and callose deposition, Al has been proposed to act as an elicitor of pathogenic response (HAMEL *et al.*, 1998). KOGA *et al.* (1998) were the first to show that sphingolipids have elicitor activity in plants. Treatment of rice leaves with cerebrosides induced hypersensitive reaction and cell death. The main sphingoid base with elicitor activity was 9-methyl-d18:2^{4E,8E}. Hydrogenation of the Δ^4-double bond showed that this unsaturation is essential for activity. Similarly, an increase in the content of d18:2^{4E,8Z} could mediate Al toxicity by triggering a hypersensitive reaction and cell death in Al-treated root tips of the Al-sensitive genotype Lixis. In fact, recent studies showed that Al treatment induces hypersensitive reaction and programmed cell death. DELISLE *et al.* (2001) found a correlation between Al toxicity and hypersensitive reaction by detecting significant cell death after a short time of exposure to Al (8 h). Furthermore, DNA fragmentation has been detected after exposure to Al (YAMAGUCHI *et al.*, 1999). EZAKI *et al.* (2001) observed a conformational change of nuclei and small DAPI-specific particles in Al-treated roots of *Arabidopsis,* which were treated with 50 μM Al for 24 h. More recently, BOSCOLO *et al.* (2003) investigated the induction of cell death in two maize lines differing in Al resistance. After 48 h of exposure to 36 μM Al, almost all nuclei were vital in Al-resistant maize, whereas in sensitive maize high chromatin fragmentation was observed, indicating the occurrence of cell death. The authors suggested that a mechanism is activated to destroy the root tip cells of roots submitted to toxic levels of Al and break the apical dominance, inducing the formation of secondary roots to explore other soil portions with lower Al concentrations.

4.5 Future prospects

This is the first study showing Al-induced changes in plant sphingoid bases. The differences observed between Al-resistant and –sensitive varieties suggest that sphingolipids may play a role in Al toxicity. However, several additional experiments must be conducted in order to clarify this role. First of all, it is necessary to differentiate between different types of sphingolipids, i.e. phosphosphingolipids, cerebrosides and sphingolipid metabolites, such as sphingosine-1-phosphate and ceramide, which obviously have different functions in the cells. Therefore, the cellular concentrations of these metabolites in Al-treated root tips of genotypes differing in Al-resistance should be determined. Because it has been shown that a coordinated regulation of biosynthetic pathways of different lipid species exists (HARTMANN *et al.,* 2002), it would also be interesting to investigate the concentrations of different phospholipids, such as phosphatidylcholine and phosphatidylserine, and free sterol contents in the cells. The amount, size and composition of lipid rafts have to be determined in order to verify if Al affects the physical properties of lipid rafts, where the majority of sphingolipids may be located. In order to confirm if ceramide is really mediating Al toxicity, the transcription of serine palmitoyl transferase, a key enzyme in *de novo* ceramide synthesis, should be investigated in Al-stressed cells. Another way to get information about the role of ceramide is to prove if inhibitors of *de novo* ceramide biosynthesis can improve Al resistance.

A better understanding of the role of sphingolipids in Al toxicity will be a pre-requisite for the use of genetic engineering of sphingolipids to improve Al resistance in crops. The over expression of the *A. thaliana* Δ^8-sphingolipid desaturase, which leads to a higher content of mainly *trans*-unsaturated sphingoid bases, rendered the maize plants more sensitive to Al. Because Al resistance seems to be correlated to a decrease rather than to an increase of unsaturated sphingoid bases, a better Al resistance may be achieved by knocking-out the endogenous maize Δ^4- and/or Δ^8-sphingolipid desaturases in an Al-sensitive maize line, thereby mimicking the changes observed in the Al-resistant genotypes.

5 Summary

Aluminum (Al) is a light metal that makes up 7% of the earth's crust, where it is the third most abundant element after oxygen and silicon. When soils become acidic as a result of natural processes or human activities, Al is solubilized into the phytotoxic trivalent cation, Al^{3+}, which can inhibit root growth in many agriculturally important plant species, such as maize, wheat and barley. Cell wall and the apoplastic surface of the plasma membrane seem to be major targets of Al. Binding of Al to negatively charged phosphate groups of phospholipids induces a decrease of membrane fluidity and permeability. Though glucosylceramides and related sphingolipid metabolites are known to increase stability and decrease permeability of membranes, up to now sphingolipids have not been taken into consideration in studies dealing with Al-induced lipid changes. Sphingolipids contain a ceramide backbone, which consists of a fatty acid attached to an amino alcohol, the sphingoid base. The aim of this study was to investigate whether Al affects the sphingoid base composition in roots of maize genotypes with different Al sensitivities. In addition, over expression of a heterologous *Arabidopsis thaliana* Δ^8-sphingolipid desaturase should elucidate, whether Δ^8-unsaturation of sphingoid bases influences Al sensitivity.

In agreement to the sphingoid base pattern found in other *Gramineae*, in roots of wild-type maize high levels of Δ^8-*cis*-unsaturated sphingoid bases were present. In transgenic maize expressing the *A. thaliana* Δ^8-sphingolipid desaturase gene there was a significant increase in the proportion of Δ^8-*trans*-unsaturated sphingoid bases, especially of (8*E*)-4-hydroxy-8-sphingenine (t18:1^{8E}), confirming that this desaturase preferentially introduces *trans*-double bonds. The over expression of the heterologous Δ^8-desaturase did not increase the total sphingoid base content. A regression analysis showed that the increase in Δ^8-*trans*-unsaturated sphingoid bases occurred rather at the expense of the remaining sphingoid bases, especially of phytosphinganine (t18:0).

The analysis of the effect of Al on the sphingoid base composition showed that the Al treatment decreased the content of unsaturated sphingoid bases, mainly of (4*E*,8*Z*)-4,8-sphingadienine (d18:$2^{4E,8Z}$), and the total sphingoid base content in an Al-resistant genotype. In contrast, in an Al-sensitive genotype, the content of d18:$2^{4E,8Z}$ and the total sphingoid base content increased. The level of unsaturated sphingoid bases, especially of t18:1^{8E}, was maintained in the root tips of Al-treated transgenic plants, suggesting that the activity of the heterologous Δ^8-sphingolipid desaturase was not affected by Al. Based on Al-induced callose production, the transgenic plants were more sensitive to Al than the control wild-type lines, suggesting that the loss of the ability to down regulate the content of unsaturated sphingoid bases increased Al toxicity in the transgenic line.

This is the first study showing Al-induced changes in plant sphingoid bases. The differences observed between Al-resistant and –sensitive varieties suggest that sphingolipids may play a role in Al toxicity. A better understanding of this role will be a pre-requisite for the use of genetic engineering of sphingolipids to improve Al resistance in crops. The over expression of the *A. thaliana* Δ^8-sphingolipid desaturase, which leads to a higher content of mainly *trans*-unsaturated sphingoid bases, rendered the maize plants more sensitive to Al. Because Al resistance seems to be correlated to a decrease rather than to an increase of unsaturated sphingoid bases, a better Al resistance may be achieved by knocking-out the endogenous maize Δ^4- and/or Δ^8-sphingolipid desaturases in an Al-sensitive maize line, mimicking the changes observed in the Al-resistant genotypes.

6 Zusammenfassung

Das Leichtmetall Aluminium (Al) ist, mit einem Anteil von 7%, nach Sauerstoff und Silizium das dritthäufigste Element der Erdkruste. Wenn Böden als Konsequenz natürlicher Prozesse oder menschlicher Aktivitäten versauern, geht Al als phytotoxisches Kation Al^{3+} in Lösung. Dieses Kation verursacht bei vielen, für die Landwirtschaft wichtigen Pflanzenarten, wie Mais, Weizen und Gerste eine Wachstumshemmung der Wurzeln. Die Zellwand und die apoplastische Seite der Plasmamembran sind wichtige Wirkorte der Aluminiumionen. Die Bindung von Aluminium an die negativ geladenen Phosphatgruppen der Phospholipide verursacht eine Abnahme der Membranfluidität und Permeabilität. Obwohl bekannt ist, dass verschiedene Sphingolipidmetabolite, u. a. Glycosylceramid, einen Einfluss auf die o. g. Parameter haben, wurde diese Klasse der Lipide in Untersuchungen zu Al-induzierten Veränderungen der Lipidzusammensetzung bisher nicht in Betracht gezogen. Alle Sphingolipide weisen ein Ceramidgrundgerüst auf, das aus einer, an einen Aminoalkohol (Sphingobase) gebundene, Fettsäure besteht. Das Ziel dieser Studie war es, die Effekte von Aluminium auf die Zusammensetzung der Sphingobasen in Wurzeln von Mais-Genotypen mit unterschiedlicher Al-Sensitivität zu untersuchen. Darüber hinaus, sollte durch die Überexpression einer heterologen Δ^8-Sphingolipiddesaturase aus *Arabidopsis thaliana* untersucht werden, ob die Δ^8-Desaturierung der Sphingobasen die Al-Sensitivität beeinflusst.

In Übereinstimmung mit dem, für die *Gramineae* üblichen Sphingobasenmuster, wurden in den Wurzeln des Wildtypmais hauptsächlich Δ^8-*cis*-ungesättigte Sphingobasen gefunden. In transgenem Mais, der die Δ^8-Sphingolipiddesaturase überexprimiert, wurde eine signifikante Zunahme an Δ^8-*trans*-ungesättigten Sphingobasen, insbesondere an (8*E*)-4-Hydroxy-8-sphingenin (t18:1^{8E}), beobachtet. Diese Ergebnisse bestätigen, dass diese Desaturase aus *A. thaliana* eine Präferenz für *trans*-Doppelbindungen aufweist. Die Überexpression der heterologen Δ^8-Desaturase verursachte keine Zunahme im Gesamtgehalt der Sphingobasen. Durch eine Regressionsanalyse konnte gezeigt werden, dass die Zunahme an Δ^8-*trans*-ungesättigten Sphingobasen auf Kosten der anderen Sphingobasen, insbesondere durch eine Abnahme an Phytosphinganin (t18:0), erreicht wurde.

Durch die Analyse der Effekte von Aluminium auf die Sphingobasenzusammensetzung in den Wurzelspitzen eines Al-resistenten Genotyps konnte gezeigt werden, dass die Al-Behandlung den Gehalt an ungesättigten Sphingobasen, hauptsächlich an (4*E*,8*Z*)-4,8-Sphingadienin (d18:$2^{4E,8Z}$), und auch den Gesamtgehalt an Sphingobasen reduzierte. Im Gegensatz dazu, wurde der Gehalt an d18:$2^{4E,8Z}$ und der gesamte Sphingobasengehalt eines Al-empfindlichen Genotyps erhöht. Die Menge an ungesättigten Sphingobasen, insbesondere an t18:1^{8E}, blieb in den Al-behandelten Wurzelspitzen transgener Pflanzen

unverändert. Dies deutet darauf hin, dass die Aktivität der heterologen Δ^8-Sphingolipiddesaturase durch Al nicht beeinflusst wird. Durch Messung der Kallosebildung und Vergleich mit untransformierten Kontrollpflanzen wurden die transgenen Pflanzen als Al-empfindlich eingestuft. Die erhöhte Al-Sensitivität der transgenen Pflanzen weist darauf hin, dass die Fähigkeit der Pflanzen, den Gehalt ungesättigter Sphingobasen herunterzuregulieren, von Bedeutung für die Al-Resistenz ist.

Dies ist die erste Untersuchung über Al-induzierte Veränderungen der Sphingobasenzusammensetzung in Pflanzen. Die unterschiedlichen Veränderungen, die in Al-resistenten und Al-empfindlichen Genotypen beobachtet wurden, deuten darauf hin, dass Sphingolipide eine wichtige Rolle in der Al-Toxizität spielen. Ein besseres Verständnis der Zusammenhänge zwischen Al-Toxizität und Sphingolipide ist eine Voraussetzung für zukünftige gentechnische Veränderungen der Sphingolipidzusammensetzung zur Verbesserung der Al-Resistenz verschiedener Kulturpflanzen. Die Überexpression einer *A. thaliana* Δ^8-Sphingolipid Desaturase führt zu einer erhöhten Menge *trans*-ungesättigter Sphingobasen und zu einer Erhöhung der Al-Sensitivität von Mais. Um eine verbesserte Al-Resistenz zu erreichen, wäre es daher aussichtsreicher, das Verhalten des Al-resistenten Genotyps zu simulieren und eine Abnahme an ungesättigten Sphingobasen anzustreben. Dies könnte durch das Ausschalten der endogenen Δ^4- und/oder Δ^8-Sphingolipiddesaturasen in einer Al-empfindlichen Maissorte erreicht werden.

7 Appendix

Tab. 7.1: Plasmids used in this work as a vector or a source of genes for cloning.

Plasmid	Source	General Features
pUbi.cas	D. Becker, University of Hamburg, unpublished	This is a vector for expression of exogenous genes in monocots. The promoter from the *Zea mays* ubiquitin gene was cloned between the *Pst* I/*Hinc* II restriction sites, and the polyadenylation signal from the *Agrobacterium tumefasciens* nopaline synthetase gene was cloned between the *Sac* I/*Eco* RI cutting sites on pUC-19 vector (YANISCH-PERRON *et al.*, 1985).
pGEMTB5AT	P. Sperling, University of Hamburg, unpublished	The cDNA for the *Arabidopsis thaliana* Δ8-sphingolipid desaturase gene was inserted between the *Sac* II and *Spe* I cutting sites on pGEM-T vector (Promega).

Tab. 7.2: Composition of media for tissue culture of maize (mg l^{-1}).

	Osmotic medium*	**Induction medium***	**Selection medium 1***
KNO_3	2830	2830	2830
$(NH_4)_2SO_4$	463	463	463
$MgSO_4.7H_2O$	185	185	185
$CaCl_2.2H_2O$	166	166	166
KH_2PO_4	400	400	400
$MnSO_4.H_2O$	3.9	3.9	3.9
$ZnSO_4.7H_2O$	1.5	1.5	1.5
H_3BO_4	1.6	1.6	1.6
KI	0.8	0.8	0.8
NaFeEDTA	27.5	27.5	27.5
$AgNO_3$		1.7	
Glycine	2	2	2
Pyridoxine-HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Thiamine-HCl	1	1	1
Myo-inositol	100	100	100
Casaminoacids		100	
Prolin		2,900	
2,4-D	1	1	1
PPT			5
Sucrose	400,000	40,000	40,000
Phytagel	3,000	3,000	3,000
pH	5.8	5.8	5.8

*These media are modifications of the basal N6 medium (CHU, 1978).

Tab. 7.3: Composition of media for tissue culture of maize (mg l^{-1}).

	Selection medium 2*	**Regeneration medium***	**Rooting medium***
KNO_3	1,900	1,900	950
NH_4NO_3	1,650	1,650	825
$MgSO_4.7H_2O$	370	370	185
$CaCl_2.2H_2O$	440	440	220
KH_2PO_4	170	170	85
$MnSO_4.H_2O$	11.2	11.2	5.6
$ZnSO_4.7H_2O$	5.8	5.8	2.9
H_3BO_4	6.2	6.2	3.1
KI	0.8	0.8	0.4
$Na_2MoO_4.2H_2O$	0.25	0.25	0.125
$CuSO_4.5H_2O$	0.025	0.025	0.0125
$CoCl_2.6H_2O$	0.025	0.025	0.0125
NaFeEDTA	27.5	27.5	13.8
Glycine	2	2	
Pyridoxine-HCl	0.5	0.5	
Nicotinic acid	0.5	0.5	
Thiamine-HCl	0.1	0.1	
Myo-inositol	100	100	
2,4-D	1		
PPT	5	5	3
Sucrose	40,000	40,000	20,000
Phytagel	3,000	3,000	3,000
pH	5.8	5.8	5.8

*These media are modifications of the basal MS medium (MURASHIGE and SKOOG, 1962).

Tab. 7.4: Composition of the nutrient solution used for incubation of maize plants.

Nutrient	**Concentration (µM)**
KNO_3	400
$CaSO_4$	250
NH_4NO_3	200
$MgSO_4$	100
Fe-EDDHA	20
Fe-EDTA	40
KH_2PO_4	10
H_3BO_3	8
$MnSO_4$	1
$ZnSO_4$	0.2
$CuSO_4$	0.2
$(NH_4)_6Mo_7O_{24}$	0.1

Tab. 7.5: Summary of the crosses of T_0 transgenic plants transformed with the *A. thaliana* Δ^8-sphingolipid desaturase.

T_0 plant	**Cross ♀x♂**	**Nr. of kernels in the T_1 progeny**
19/5	19/5 x 19/5	50
20/6	20/6 x 20/6	70
21/10	H99 x 21/10	20
21/12	21/12 x 21/12	70
23/32	23/32 x 23/32	100
27/18	27/18 x 27/18	70
27/23	H99 x 27/23	12

Tab. 7.6: Summary of the crosses in the T_1 progeny of transgenic plants transformed with the *A. thaliana* Δ^8-sphingolipid desaturase. Shadowed lines indicate the T_2 populations which were treated with Basta® and analyzed by Northern blot analysis.

T_1 plant	**Cross ♀x♂**	**Nr. of kernels in the T_2 progeny**
19/5/1	19/5/1 x 19/5/1	15
19/5/2	19/5/2 x 19/5/2	18
19/5/9	19/5/9 x 19/5/9	13
20/6/1	20/6/1 x A188	10
20/6/2	20/6/2 x A188	10
20/6/3	20/6/3 x H99	15
20/6/4	20/6/4 x H99	15
21/10/1	21/10/1 x A188	4
21/10/2	H99 x 21/10/2	90
21/10/4	H99 x 21/10/4	100
21/10/5	21/10/5 x A188	25
21/10/6	21/10/6 x A188	20
21/10/7	H99 x 21/10/7	120
21/12/1	21/12/1 x A188	7
21/12/3	A188 x 21/12/3	130
21/12/5	21/12/5 x A188	30
21/12/8	A188 x 21/12/8	30
21/12/9	A188 x 21/12/9	90
21/12/10	A188 x 21/12/10	150
23/32/1	23/32/1 x A188	55
23/32/2	23/32/2 x H99	100
23/32/3	23/32/3 x 23/32/3	150
23/32/4	23/32/4 x 23/32/4	200
23/32/5	23/32/5 x A188	100
23/32/6	23/32/6 x 23/32/6	90
23/32/7	23/32/7 x 23/32/7	130
23/32/8	23/32/8 x H99	60
23/32/9	23/32/9 x 23/32/9	100
23/32/10	H99 x 23/32/10	38
23/32/11	23/32/11 x 23/32/11	58
27/18/2	27/18/2 x 27/18/2	110
27/18/5	27/18/5 x H99	80
27/18/7	27/18/7 x 27/18/7	170
27/18/8	27/18/8 x 27/18/8	30
27/18/9	27/18/9 x 27/18/9	170
27/18/10	27/18/10 x H99	43
27/18/11	27/18/11 x H99	140
27/18/12	27/18/12 x H99	70
27/23/1	27/23/1 x 27/23/1	15
27/23/2	27/23/2 x 27/23/2	15
27/23/5	27/23/5 x H99	40
27/23/6	27/23/6 x 27/23/6	100

8 References

Abbas HK, Tanaka T, Duke SO, Porter JK, Wray EM, Hodges L, Sessions AE, Wang E, Merril Jr. AH, Riley RT (1994) Fumosin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. Plant Physiol 106: 1085-1093.

Akeson MA, Munns DN (1989) Lipid bilayer permeation by neutral aluminum citrate and by three α–hydroxy carboxylic acids. Biochim Biophys Acta 984: 200-206.

Asai T, Stone JM, Heard JE, Kovtun Y, Yorgey P, Sheen J, Ausubel FM (2000) Fumosin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell 12: 1823-1835.

Becker D, Brettschneider R, Lörz H (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J 5: 299-307.

Birchwood C, Saba JD, Dickson RC, Cunningham KW (2001) Calcium influx and signaling in yeast stimulated by intracellular sphingosine 1-phosphate accumulation. J Biol Chem 276: 11712-11718.

Bohn M, Heinz E, Lüthje S (2001) Lipid composition and fluidity of plasma membranes isolated from corn (*Zea mays* L.) roots. Arch Biochem Biophys 387: 35-40.

Boscolo PRS, Menossi M, Jorge RA (2003) Aluminum-induced oxidative stress in maize. Phytochem 62: 181-189.

Bourbon N, Yun J, Kester M (2000) Ceramide directly activates protein kinase C ζ to regulate a stress-activated protein kinase signaling complex. J Biol Chem 275: 35617-35623.

Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumosin B_1. Proc Natl Acad Sci USA 97: 4961-4966.

Breittmayer J, Bernard A, Aussel C (1994) Regulation by sphingomyelinase and sphingosine of Ca^{2+} signals elicited by CD3 monoclonal antibody, thapsigargin, or ionomycin in the Jurkat T cell line. J Biol Chem 269: 5054-5058.

Brettschneider R, Becker D, Lörz H (1997) Efficient transformation of scutellar tissue of immature maize embryos. Theor Appl Genet 94: 737-748.

Brodersen P, Petersen M, Pike HM, Olszak B, Skov S, Odum N, Jørgensen LB, Brown RE, Mundy J (2002) Knockout of *Arabidopsis ACCELERATED-CELL-DEATH11* encoding a sphingosine transfer protein causes activation of programmed cell death and defense. Genes & Develop 16: 490-502.

Brown DA, London E (2002) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem 275: 17221-17224.

Cahoon EB, Lynch DV (1991) Analysis of glucocerebrosides of rye (*Secale cereale* L. cv Puma) leaf and plasma membrane. Plant Physiol 95: 58-68.

Cantatore JL, Murphy SM, Lynch DV (2000) Compartmentation and topology of glucosylceramide synthesis. Biochem Soc Trans 28: 748-750.

Carter HE, Hendry RA, Nojima S, Stanacev NZ, Ohno K (1961) Biochemistry of the sphingolipids. XIII. Determination of the structure of cerebrosides from wheat flours. J Biol Chem 236: 1912.

Carter HE, Strobach DR, Hawthorne JN (1969) Biochemistry of the sphingolipids. 18. Complete structure of tetrasaccharide phytoglycolipid. Biochem 8: 383-388.

Chu, C C (1978) The N6 medium and its applications to anther culture of cereal crops. Proc Symp Plant Tissue Culture, Beijing 1978. Pitman, Boston, pp. 43-50.

Chung N, Jenkins GM, Hannun YA, Heitman J, Obeid L (2000) Sphingolipids signal heat stress-induced ubiquitin-dependent proteolysis. J Biol Chem 275: 17229-17232.

Coroneros E, Martinez M, McKenna S, Kester M (1995) Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. Implications for cellular proliferation and differentiation. J Biol Chem 270: 23305-23309.

D'Halluin K, Bonne E, Bossut M, De Beuckeleer M, Leemans J (1992) Transgenic maize plants by tissue electroporation. Plant Cell 4: 1495-1505.

de la Fuente JM, Ramírez-Rodríguez V, Cabrera-Ponce JL, Herrera-Estrella L (1997) Aluminum tolerance in transgenic plants by alteration of citrate synthesis. Science 276: 1566-1568.

Deleers M, Servais JP, Wülfert E (1986) Neurotoxic cations induce membrane rigidification and membrane fusion at micromolar concentrations. Biochim Biophys Acta 855, 271-276.

Delhaize E, Ryan PR, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol 103: 695-702.

Delisle G, Champoux M, Houde M (2001) Characterization of oxalate oxidase and cell death in Al-sensitive and tolerant wheat roots. Plant Cell Physiol 42: 324-333.

Dickson RC, Lester RL (1999) Yeast sphingolipids. Biochim Biophys Acta 1426: 347-357.

Ezaki B, Katsuhara M, Kawamura M, Matsumoto H (2001) Different mechanisms of four aluminum (Al)-resistant transgenes for Al toxicity in *Arabidopsis*. Plant Physiol 127: 918-927.

Farrell AM, Uchida Y, Nagiec MM, Harris IR, Dickson RC, Elias PM, Holleran WM (1998) UVB irradiation up-regulates serine palmitoyltransferase in cultured human keratinocytes. J Lipid Res 39: 2031-2038.

Fatatis A, Miller RJ (1996) Sphingosine and sphingosine 1-phosphate differentially modulate platelet-derived growth factor-BB-induced Ca^{2+} signaling in transformed oligodendrocytes. J Biol Chem 271: 295-301.

Friant S, Zanolari B, Riezman H (2000) Increased protein kinase or decreased PP2A activity bypasses sphingoid base requirement in endocytosis. EMBO J 19: 2834-2844.

Friant S, Lombardi R, Schmelzle T, Hall MN, Riezman H (2001) Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. EMBO J 20: 6783-6792.

Ghafourifar P, Klein SD, Schucht O, Schenk U, Pruschy M, Rocha S, Richter C (1999) Ceramide induces cytochrome *c* release from isolated mitochondria. Importance of mitochondrial redox state. J Biol Chem 274: 6080-6084.

Green CE, Phillips RL (1975) Plant regeneration from tissue cultures of maize. Crop Sci 15: 417-421.

Grilley MM, Stock SD, Dickson RC, Lester RL, Takemoto JY (1998) Syringomycin action gene *SYR2* is essential for sphingolipid 4-hydroxylation in *Saccharomyces cerevisiae*. J Biol Chem 273: 11062-11068.

Guillas I, Kirchman PA, Chuard R, Pfefferli M, Jiang JC, Jazwinski SM, Conzelmann A (2001) C26-CoA-dependent ceramide synthesis of *Saccharomyces cerevisiae* is operated by Lag1p and Lac1p. EMBO J 20: 2655-2665.

Hamel F, Breton C, Houde M (1998) Isolation and characterization of wheat aluminum-regulated genes: possible involvement of aluminum as a pathogenesis response elicitor. Planta 205: 531-538.

Hanada K, Hara T, Nishijima M (2000) Purification of the serine palmitoyltransferase complex responsible for sphingoid base synthesis by using affinity peptide chromatography techniques. J Biol Chem 275: 8409-8415.

Hannun YA, Loomis CR, Merril Jr. AH, Bell RM (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. J Biol Chem 261: 12604-12609.

Hao M, Mukherjee S, Maxfield FR (2001) Cholesterol depletion induces large scale domain segregation in living cell membranes. Proc Natl Acad Sci USA 98: 13072-13077.

Hartmann M, Perret A, Carde J, Cassagne C, Moreau P (2002) Inhibition of the sterol pathway in leek seedlings impairs phosphatidylserine and glucosylceramide synthesis but triggers an accumulation of triacylglycerols. Biochim Biophys Acta 1583: 285-296.

Herget T, Esdar C, Oehrlein SA, Heinrich M, Schütze S, Maelicke A, van Echten-Deckert G (2000) Production of ceramides causes apoptosis during early neural differentiation *in vitro*. J Biol Chem 275: 30344-30354.

Holthuis JCM, Pomorski T, Raggers RJ, Sprong H, van Meer G (2001) The organizing potential of sphingolipids in intracellular membrane transport. Physiol Rev 81: 1689-1723.

Horst WJ (1995) The role of the apoplast in aluminium toxicity and resistance of higher plants: a review. Z Pflanzenernähr Bodenk 158: 419-428.

Horst WJ, Püschel A-K, Schmohl N (1997) Induction of callose formation is a sensitive marker for genotypic aluminium sensitivity in maize. Plant Soil 192: 23-30.

Huang JW, Grunes DL, Kochian LV (1992) Aluminum effects on the kinetics of calcium uptake into cells of the wheat root apex. Quantification of calcium fluxes using a calcium-selective vibrating microeletrode. Planta 188: 414-421.

Igarashi J, Bernier SG, Michel T (2001) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. Differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. J Biol Chem 276: 12420-12426.

Ikushiro H, Hayashi H, Kagamiyama H (2001) A water-soluble homodimeric serine palmitoyltransferase from *Sphingomonas paucimobilis* EY2395^T strain. Purification, characterization, cloning, and overproduction. J Biol Chem 276: 18249-18256.

Imai H, Ohnishi M, Hotsubo K, Kojima M, Ito S (1997) Sphingoid base composition of cerebrosides from plant leaves. Biosci Biotechnol Biochem 61: 351-353.

Imai H, Morimoto Y, Tamura K (2000) Sphingoid base composition of monoglucosylceramide in Brassicaceae. J Plant Physiol 157: 453-456.

Ishikawa S, Wagatsuma T (1998) Plasma membrane permeability of root-tip cells following temporary exposure to Al ions is a rapid measure of Al tolerance among plant species. Plant Cell Physiol 39: 516-525.

Jenkins GM, Hannun YA (2001) Role for *de novo* sphingoid base biosynthesis in the heat-induced transient cell cycle arrest of *Saccharomyces cerevisiae*. J Biol Chem 276: 8574-8581.

Jennemann R, Geyer R, Sandhoff R, Gschwind RM, Levery SB, Gröne H, Wiegandt H (2001) Glycoinositolphosphosphingolipids (basidiolipids) of higher mushrooms. Eur J Biochem 268: 1190-1205.

Jones DL, Kochian LV (1995) Aluminum inhibition of the inositol 1,4,5-trisphosphate signal transduction pathway in wheat roots: a role in aluminum toxicity? Plant Cell 7: 1913-1922.

Jones DL, Kochian LV (1997) Aluminum interaction with plasma membrane lipids and enzyme metal binding sites and its potential role in Al cytotoxicity. FEBS Letters 400: 51-57.

Kasamo K, Yamaguchi M, Nakamura Y (2000) Mechanism of the chilling-induced decrease in proton pumping across the tonoplast of rice cells. Plant Cell Physiol 41: 840-849.

Kauss H (1989) Fluorometric measurement of callose and other 1,3-β-glucans. In Plant fibers. Springer-Verlag, Berlin, pp 127-137.

Kawaguchi M, Imai H, Naoe M, Yasui Y, Ohnishi M (2000) Cerebrosides in grapevine leaves: distinct composition of sphingoid bases among the grapevine species having different tolerances to freezing temperature. Biosci Biotechnol Biochem 64: 1271-1273.

Kim S, Fyrst H, Saba J (2000) Accumulation of phosphorylated sphingoid long chain bases results in cell growth inhibition in *Saccharomyces cerevisiae*. Genetics 156: 1519-1529.

Kinraide TB (1988) Proton extrusion by wheat roots exhibiting severe aluminum toxicity symptoms. Plant Physiol 88: 418-423.

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46: 237-260.

Koga J, Yamauchi T, Shimura M, Ogawa N, Oshima K, Umemura K, Kikuchi M, Ogasawara N (1998) Cerebrosides A and C, sphingolipid elicitors of hypersensitive cell death and phytoalexin accumulation in rice plants. J Biol Chem 273: 31985-31991.

Kolter T, Sandhoff K (1999) Sphingolipids-their metabolic pathways and the pathobiochemistry of neurodegenerative diseases. Angew Chem Int Ed 38: 1532-1568.

Koyama H, Kawamura A, Kihara T, Hara T, Takita E, Shibata D (2000) Overexpression of mitochondrial citrate synthase in *Arabidopsis thaliana* improved growth on a phosphorus-limited soil. Plant Cell Physiol 41: 1030-1037.

Kroesen B, Pettus B, Luberto C, Busman M, Sietsma H, de Leij L, Hannun YA (2001) Induction of apoptosis through B-cell receptor cross-linking occurs via *de novo* generated C16-ceramide and involves mitochondria. J Biol Chem 276: 13606-13614.

Lannert H, Gorgas K, Meißner I, Wieland FT, Jeckel D (1998) Functional organization of the Golgi apparatus in glycosphingolipid biosynthesis. Lactosylceramide and subsequent glycosphingolipids are formed in the lumen of the late Golgi. J Biol Chem 273: 2939-2946.

Lee JY, Leonhardt LG, Obeid L (1998) Cell-cycle-dependent changes in ceramide levels preceding retinoblastoma protein dephosphorylation in $G_{2/M}$. Biochem J 334: 457-461.

Lee MCS, Hamamoto S, Schekman R (2002) Ceramide biosynthesis is required for the formation of the oligomeric H^+-ATPase Pma1p in the yeast endoplasmic reticulum. J Biol Chem 277: 22395-22401.

Lehtonen JYA, Horiuchi M, Daviet L, Akishita M, Dzau VJ (1999) Activation of the *de novo* biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis. J Biol Chem 274: 16901-16906.

Leipelt M, Warnecke DC, Zähringer U, Ott C, Müller F, Hube B, Heinz E (2001) Glucosylceramide synthases, a gene family responsible for the biosynthesis of glucosphingolipids in animals, plants, and fungi. J Biol Chem 276: 33621-33629.

Lindberg S, Griffiths G (1993) Aluminium effects on ATPase activity and lipid composition of plasma membranes in sugar beet roots. J Exp Bot 44: 1543-1550.

López-Bucio J, de la Vega OM, Guevara-García A, Herrera-Estrella L (2000) Enhanced phosphorus uptake in transgenic tobacco plants that overproduce citrate. Nature Biotechnol 28: 450-453.

Luria SE, Adam JN, Teng RC (1960) Transduction of lactose utilizing ability among strains of *Escherichia coli* and *Shigella dysenteriae* and the properties of the transducing phage particles. Virology 12: 348-390.

Lynch DV, Steponkus PL (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol 83: 761-767.

Lynch DV (1993) Sphingolipids. In TS Moore, ed Lipid metabolism in plants. CRC Press, Boca Raton, pp 285-308.

Lynch DV, Phinney AJ (1995) The transbilayer distribution of glucosylceramide in plant plasma membrane. In J Kader, P Mazliak, eds Plant lipid metabolism. Kluwer Academic Publishers, Dordrecht, pp 239-241.

Ma JF, Zheng SJ, Matsumoto H (1997) Secretion of citric acid as an aluminium-resistant mechanism in *Cassia tora* L. In T Ando, ed Plant nutrition- for sustainable food production and environment. Kluwer Academic Publishers, Dordrecht, pp 449-450.

Ma JF (2000) Role of organic acids in detoxification of aluminum in higher plants. Plant Cell Physiol 41[4]: 383-390.

Ma JF, Ryan PR, Delhaize E (2001) Aluminium tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6: 273-278.

Ma Z, Miyasaka SC (1998) Oxalate exudation by taro in response to Al. Plant Physiol 118: 861-865.

Mao C, Saba J, Obeid L (1999) The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses. Biochem J 342: 667-675.

Martin RB (1986) The chemistry of aluminum as related to biology and medicine. Clin Chem 32: 1797-1806.

Massey JB (2001) Interaction of ceramides with phosphatidylcholine, sphingomyelin and sphingomyelin/cholesterol bilayers. Biochim Biophys Acta 1510: 167-184.

Mathes C, Fleig A, Penner R (1998) Calcium release-activated calcium current (I_{CRAC}) is a direct target for sphingosine. J Biol Chem **273**: 25020-25030.

Melendez A, Floto RA, Gillooly DJ, Harnett MM, Allen JM (1998) FcγRI coupling to phospholipase D initiates sphingosine kinase-mediated calcium mobilization and vesicular trafficking. J Biol Chem 273: 9393-9402.

Melkonian KA, Ostermeyer AG, Chen J, Roth MG, Brown DA (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. J Biol Chem 274: 3910-3917.

Merril Jr. AH, Sereni AM, Stevens VL, Hannun YA, Bell RM, Kinkade Jr. JM (1986) Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. J Biol Chem 261: 12610-12615.

Michaelson LV, Longman AJ, Sayanova O, Stobart AK, Napier JA (2002) Isolation and characterization of a cDNA encoding a Δ^8 sphingolipid desaturase from *Aquilegia vulgaris*. Biochem Soc Trans 30: 1073-1075.

Michel C, van Echten-Deckert G, Rother J, Sandhoff K, Wang E, Merril Jr. AH (1997) Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-*trans*-double bond of sphingosine at the level of dihydroceramide. J Biol Chem 272: 22432-22437.

Miyasaka SC, Buta JG, Howell RK, Foy CD (1991) Mechanism of aluminum tolerance in snapbeans. Root exudation of citric acid. Plant Physiol 96: 737-743.

Murashige T, Skoog, F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nagiec MM, Baltisberger JA, Wells GB, Lester RL, Dickson RC (1994) The LCB2 gene of *Saccharomyces* and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. Proc Natl Acad Sci USA 91: 7899-7902.

Ng CK, Carr K, McAinsh MR, Powell B, Hetherington AM (2001) Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. Nature 410: 596-599.

Ogretmen B, Kraveka JM, Schady D, Usta J, Hannun YA, Obeid L (2001) Molecular mechanisms of ceramide-mediated telomerase inhibition in the A549 human lung adenocarcinoma cell line. J Biol Chem 276: 32506-32514.

Ono K, Yamamoto Y, Hachiya A, Matsumoto H (1995) Synergistic inhibition of growth by aluminum and iron of tobacco (*Nicotiana tabacum* L.) cells in suspension culture. Plant Cell Physiol 36: 115-125.

Papernik LA, Kochian LV (1997) Possible involvement of Al-induced electrical signals in Al tolerance in wheat. Plant Physiol 115: 657-667.

Pellet DM, Grunes DL, Kochian LV (1995) Organic acid exudation as an aluminum-tolerance mechanism in maize (*Zea mays* L.). Planta 196: 788-795.

Perry DK, Carton J, Shah AK, Meredith FI, Uhlinger DJ, Hannun YA (2000) Serine palmitoyltransferase regulates *de novo* ceramide generation during etoposide-induced apoptosis. J Biol Chem 275: 9078-9084.

Peskan T, Westermann M, Oelmüller R (2000) Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants. Eur J Biochem 267: 6989-6995.

Pike LJ (2003) Lipid rafts: bringing order to chaos. J Lipid Res, in press.

Prieschl EE, Baumruker T (2000) Sphingolipids: second messengers, mediators and raft constituents in signaling. Immunol Today 21: 555-560.

Rengel Z (1996) Uptake of aluminium by plant cells. New Phytol 134: 389-406.

Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52: 527-560.

Sakaki T, Zähringer U, Warnecke DC, Fahl A, Knogge W, Heinz E (2001) Sterol glycosides and cerebrosides accumulate in *Pichia pastoris*, *Rhynchosporium secalis* and other fungi under normal conditions or under heat shock and ethanol stress. Yeast 18: 679-695.

Sakano S, Takemura H, Yamada K, Imoto K, Kaneko M, Ohshika H (1996) Ca^{2+} mobilizing action of sphingosine in Jurkat human leukemia T cells. Evidence that sphingosine releases Ca^{2+} from inositol trisphosphate and phosphatidic acid sensitive intracellular stores through a mechanism independent of inositol trisphosphate. J Biol Chem 271: 11148-11155.

Sambrook SG, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2^{nd} ed.) Cold Spring Harbour Press, New York.

Shimabukuro M, Higa M, Zhou Y, Wang M, Newgard CB, Unger RH (1998) Lipoapoptosis in beta-cells of obese prediabetic *fa/fa* rats. Role of serine palmitoyltransferase overexpression. J Biol Chem 273: 32487-32490.

Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene *Asc-1* prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J 32: 561-572.

Sperling P, Zähringer U, Heinz E (1998) A sphingolipid desaturase from higher plants. Identification of a new cytochrome b_5 fusion protein. J Biol Chem 273: 28590-28596.

Sperling P, Blume A., Zähringer U, Heinz E (2000) Further characterization of Δ^8-sphingolipid desaturase from higher plants. Biochem Soc Trans 28: 638-641.

Sperling P, Ternes P, Moll H, Franke S, Zähringer U, Heinz E (2001a) Functional characterization of sphingolipid C4-hydroxylase genes from *Arabidopsis thaliana*. FEBS Letters 494: 90-94.

Sperling P, Libisch B, Zähringer U, Napier JA, Heinz E (2001b) Functional identification of a Δ^8-sphingolipid desaturase from *Borago officinalis*. Arch Biochem Biophys 388: 293-298.

Sperling P, Heinz E (2003) Plant sphingolipids: structural diversity, biosynthesis, first genes and functions. Biochim Biophys Acta, in press.

Staß A, Horst W (1995) Effect of aluminium on membrane properties of soybean (*Glycine max*) cells in suspension culture. In RA Date, ed Plant soil interactions at low pH. Kluwer Academic Publishers, Dordrecht, pp 279-284.

Stock SD, Hama H, Radding JA, Young DA, Takemoto JY (2000) Syringomycin E inhibition of *Saccharomyces cerevisiae*: requirement for biosynthesis of sphingolipids with very-long-chain fatty acids and mannose- and phosphoinositol-containing head groups. Antimicr Agents Chemotherapy 44: 1174-1180.

Sugiya H, Furuyama S (1991) Sphingosine stimulates calcium mobilization in rat parotid acinar cells. FEBS Letters 286: 113-116.

Takabatake R, Shimmen T (1997) Inhibition of electrogenesis by aluminum in characean cells. Plant Cell Physiol 38[11]: 1264-1271.

Tamura K, Mitsuhashi N, Hara-Nishimura I, Imai H (2001) Characterization of an *Arabidopsis* cDNA encoding a subunit of serine palmitoyltransferase, the initial enzyme in sphingolipid biosynthesis. Plant Cell Physiol **42**: 1274-1281.

Ternes P (2000) Klonierung zweier cDNAs aus *Arabidopsis thaliana* (L.) Heynh. und ihre funktionale Charakterisierung als Sphinganin-C-4-Hydroxylasen. Dipl. University of Hamburg. pp 1-85.

Ternes P, Franke S, Zähringer U, Sperling P, Heinz E (2002) Identification and characterization of a sphingolipid Δ4-desaturase family. J Biol Chem 277: 25512-25518.

Thevissen K, Cammue BPA, Lemaire K, Winderickx J, Dickson RC, Lester RL, Ferket KKA, Van Even F, Parret AHA, Broekaert WF (2000) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (*Dahlia merckii*). Proc Natl Acad Sci USA 97: 9531-9536.

Tudichum JLW (1884) The chemical constitution of the brain. Bailliere, Tindal & Cox, London.

Uemura M, Steponkus PL (1994) Contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. Plant Physiol 104: 479-496.

Umemura K, Ogawa N, Yamauchi T, Iwata M, Shimura M, Koga J (2000) Cerebroside elicitors found in diverse phytopathogens activate defense response in rice plants. Plant Cell Physiol 41: 676-683.

Umemura K, Ogawa N, Koga J, Iwata M, Usami H (2002) Elicitor activity of cerebroside, a sphingolipid elicitor, in cell suspension cultures of rice. Plant Cell Physiol 43: 778-784.

van Meer G, Holthuis JCM (2000) Sphingolipid transport in eukaryotic cells. Biochim Biophys Acta 1486: 145-170.

Vierstra R, Haug A (1978) The effect of Al^{3+} on the physical properties of membrane lipids in *Thermoplasma acidophilum*$^{+}$. Biochem Biophys Res Comm 84: 138-143.

von Uexküll HR, Mutert E (1995) Global extent, development and economic impact of acid soils. Plant Soil 171: 1-15.

Wagatsuma T, Ishikawa S, Obata H, Tawaraya K, Katohda S (1995) Plasma membrane of younger and outer cells is the primary specific site for aluminium toxicity in roots. Plant Soil 171: 105-112.

Wang F, Van Brocklyn JR, Hobson JP, Movafagh S, Zukowska-Grojec Z, Milstien S, Spiegel S (1999) Sphingosine 1-phosphate stimulates cell migration through a G_i-coupled cell surface receptor. Potential involvement in angiogenesis. J Biol Chem 274: 35343-35350.

Wang H, Li J, Bostock RM, Gilchrist DG (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell 8: 375-391.

Wilson E, Olcott MC, Bell RM, Merril Jr. AH, Lambeth JD (1986) Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. Role of protein kinase C in activation of the burst. J Biol Chem 261: 12616-12623.

Xu X, Bittman R, Duportail G, Heissler D, Vilcheze C, London E (2001) Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. J Biol Chem 276: 33540-33546.

Yamaguchi Y, Yamamoto Y, Ikegawa H, Matsumoto H (1999) Protective effect of glutathione on the cytotoxicity caused by a combination of aluminum and iron in suspension-cultured tobacco cells. Physiol Plant 105: 417-422.

Yamamoto Y, Hachiya A, Matsumoto H (1997) Oxidative damage to membranes by a combination of aluminum and iron in suspension-cultured tobacco cells. Plant Cell Physiol 38[12]: 1333-1339.

Yamamoto Y, Kobayashi Y, Matsumoto H (2001) Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation in inhibition in pea roots. Plant Physiol 125: 199-208.

Yanisch-Perron C, Vieira J, Messing J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

Zel J, Schara M, Svetek J, Nemec M, Gogala N (1993) Influence of aluminum on the membranes of mycorrhizal fungi. Water, Air, Soil Poll 71: 101-109.

Zel J, Crne H, Schara M (1993) Effects of aluminum on membrane fluidity of the mycorrhizal fungus *Amanita muscaria*. Physiol Plant 89: 172-176.

Zhang G, Slaski JJ, Archambault DJ, Taylor GJ (1997) Alteration of plasma membrane lipids in aluminum-resistant and aluminum-sensitive wheat genotypes in response to aluminum stress. Physiol Plant 99: 302-308.

Acknowledgements

To Prof. Dr. Horst Lörz for accepting me as a Ph.D. student and for providing laboratory and research facilities.

To Prof. Dr. Walter Horst for the opportunity of doing part of my Ph.D. in his institute and for financing the last year of my studies. An extra special thanks for the evaluation of this thesis.

To Prof. Dr. Ernst Heinz for allowing me to conduct experiments in his laboratory.

To Dr. Angelika Staß, Dr. Dirk Becker and Dr. Petra Sperling for supervising me at different stages of my work. A special thanks to Dr. Sperling for her careful reading and correction of this thesis.

To Dr. Thomas Dresselhaus and Dr. Stefan Scholten for valuable suggestions.

To Dr. Stephan Franke for conducting the mass spectrometry analysis.

To Dr. Manfred Gahrtz for correcting this thesis and for interesting and encouraging discussions.

To Dr. Oswaldo da Costa e Silva for corrections of the English language.

To DAAD (Deutscher Akademischer Austauschdienst) for a Ph.D. fellowship.

To my friends Ana, Dani, Guillaume, Hans, Micky and Suseno, who have been working at AMPII, for sharing their friendship with me.

To all the colleagues at the Institute of Plant Nutrition in Hanover for a nice working atmosphere.

To my husband, Jouber, and our son, for supporting me with love and patience.

Curriculum vitae

Personal data:

Name:	Ana Lúcia Stival da Silva
Date of birth:	25.03.1969
Place of origin:	Ijuí/Brazil
Marital status:	married

Academic grades:

1983-1985:	Secondary school. Colégio Nossa Senhora do Rosário, Porto Alegre, Brazil
1987-1992:	B.Sc. in Biology. Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
1993-1995:	M.Sc. in Botany. Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. Thesis: Physiological, anatomical and agronomical aspects of the induction and regeneration of androgenetic double-haploids from Brazilian barley genotypes (*Hordeum vulgare* L.). Note A = excellent.
1995-1998:	Scientific co-worker in the project „Development of molecular and cellular techniques for cereals". Federal Center for Wheat Research (EMBRAPA), Passo Fundo, Brazil.
1998-2001:	Ph.D. project at AMPII, Institute of General Botany, University of Hamburg.
2001-2003:	Continuation of the Ph.D. project at the Institute for Plant Nutrition, University of Hanover.