LeAMT1;2, a molecular target to investigate ammonium transport in tomato roots and N-supply by diazotrophic bacteria

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Abstract

Ammonium (NH_4^+) transport into roots of tomato is mediated by high- and low-affinity uptake carriers, among which constitutive as well as inducible components are found. The *LeAMT1;2*-gene that was previously identified to encode for an inducible high-affinity NH_4^+-transport system has been used to elucidate the nitrogen supply of tomato plants by associated diazotrophic bacteria.

By means of northern blot analyses, numerous factors have been found to affect the transcriptional response of *LeAMT1;2*, and revealed that its expression is driven by both nitrogen demand and -availability. In roots of hydroponically grown plants, transcript-levels of *LeAMT1;2* have increased strongly within one hour upon first exposure to ammonium. Emphasizing its role in high-affinity intake, *LeAMT1;2*-expression peaked already at external NH_4^+-concentrations of 2 µM but was not induced under conditions of N-starvation, indicating that transcription of *LeAMT1;2* requires the presence of NH_4^+. This hypothesis was corroborated by physiological studies on the intake of NH_4^+ from the medium.

LeAMT1;2 was also induced by nitrate (NO_3^-), whereas nitrite (NO_2^-) strongly repressed *LeAMT1;2*-expression. Treatment of roots with glutamine, γ-butyric acid, or the glutamine synthetase inhibitor MSX likewise down-regulated *LeAMT1;2*-expression. Parallel analyses of root NH_4^+-contents in similar treated plants, however, revealed that *LeAMT1;2*-expression is likely regulated by the intracellular NH_4^+-pool indicating that LeAMT1;2 primary functions as a retention system for NH_4^+ that is subject to efflux.

The nitrogen status of the plant has been identified as a factor of overriding importance, as *LeAMT1;2*-transcripts have never been detected in nitrogen-repleted plants. This effect was partly due to the C-deficiency in roots, as the addition of sugars to the medium could slightly restore the NH_4^+-inducibility.

The involvement of NH_4^+-transporter, LeAMT1;2, in the well-known antagonsitic effect of potassium (K^+) to NH_4^+ was revealed by the strong down-regulation of its transcript-level in a high K^+-regime. Furthermore, the inhibition of K^+-channels by cesium (Cs^+) increased the *LeAMT1;2*-mRNA-level as well.

The solution's pH has been shown to affect *LeAMT1;2*-expression even in the absence of NH_4^+. A decrease in pH to 4.7 resulted in a rise of the *LeAMT1;2*-transcript-level, whereas shifting of plant roots to alkaline conditions had no effect. The pH-dependent response, however, was neutralized in nitrogen-repleted plants.

When plants were exposed to different plant growth regulators, it turned out that gibberellic acid (GA_3) and 2-naphtaleneacetic acid (2-NAA) induced *LeAMT1;2*, irrespective of whether NH_4^+ was present or not. Indole-3-acetic acid (IAA), abscisic acid (ABA) and the cytokinin kinetin (kin) revealed a concentration-dependent effect showing down-regulation of *LeAMT1;2* at higher levels. The *LeAMT1;2*-response to 1-naphtaleneacetic acid (1-NAA) was regulated inversely.

When *LeAMT1;2*-expression was followed upon a ten-hour inoculation of tomato roots with diazotrophic bacteria, the wild types of *Azospirillum brasilense*, *Azoarcus* Sp BH72 and *Herbaspirillum seropedicae* were capable of increasing the transcript-level of *LeAMT1;2*, while *Azotobacter vinelandii*, *Acetobacter diazotrophicus*, and the putative phytopathogen *Herbaspirillum rubrisubalbicans* had no effect.

To further elucidate the nature of this response, different mutants of *Azospirillum brasilense*, affected in N_2-fixation, nitrogen metabolism, and colonization properties as well as filtersterilized supernatants of these strains, previously grown in nitrogen-free, semisolid medium, were employed. Thereupon considerable evidence was provided that N_2-fixation and NH_4^+-excretion were involved in the transcriptional response of *LeAMT1;2* to these bacteria. However, there was also indication that factors other than NH_4^+ have a share in the regulation of *LeAMT1;2*.

Induction of *LeAMT1;2* by wild types of *Azospirillum brasilense* and *Azoarcus* Sp BH72 varied with inoculum concentration showing peak expression when cells of both strains were adjusted to 10^7 cfu/ml and reduced levels at 10^6 and 10^8 cfu/ml, respectively.

Inoculation experiments with the wild types of *Azospirillum brasilense* and *Azoarcus* Sp BH72 over a period of three days revealed the short-term nature of *LeAMT1;2*-induction. While expression was highest after 10 hrs of inoculation, the transcript-levels markedly decreased within the next two days to a level that was slightly above the control. In line with this observation, associative N_2-fixation could not be detected before day 11 of inoculation.

Both *Azospirillum brasilense* and *Azoarcus* Sp BH72 were capable of colonizing tomato roots within 48 hrs. *Azospirillum brasilense* was detected mainly at the sites of lateral root emergence, on root tips as well as on root hairs and had a pronounced influence on the root morphology. Mutants of *Azospirillum brasilense* affected in IAA-production revealed that auxins play a major role in this effect.

Key words: *Lycopersicon esculentum*,
ammonium transport,
biological nitrogen fixation

Zusammenfassung

Die Aufnahme von Ammonium (NH_4^+) in die Wurzel von Tomaten wird durch hoch- und niedrigaffine Transporter vermittelt, die sowohl konstitutiv vorliegen als auch induzierbar sind. Das *LeAMT1;2*-Gen repräsentiert ein induzierbares, hochaffines NH_4^+-Transportsystem, welches in dieser Studie verwendet wurde, um den Stickstoffeintrag durch assoziierte N_2-fixierende Bakterien zu beurteilen.

Experimente zur Expression von *LeAMT1;2* konnten zahlreiche Faktoren identifizieren, die an dessen Regulation beteiligt sind und belegten, daß sowohl der Stickstoffbedarf als auch die NH_4^+-Verfügbarkeit im Medium dabei eine wichtige Rolle spielen. Setzt man hydroponisch gewachsene Tomatenpflanzen erstmals NH_4^+ aus, kommt es binnen einer Stunde zu einer starken Akkumulation von *LeAMT1;2*-Transkripten in der Wurzel. Die stärkste Expression war bereits in Gegenwart von 2 µM NH_4^+ zu verzeichnen, wohingegen Stickstoffstreß in der Pflanze kein Signal für die Induktion darstellte. Dieses Muster charakterisiert LeAMT1;2 als hochaffinen NH_4^+-transporter, dessen Induktion die Gegenwart von NH_4^+ erfordert. Physiologische Studien der NH_4^+-Aufnahme untermauerten diese Hypothese.

LeAMT1;2 wurde auch durch Nitrat (NO_3^-) induziert, während Nitrit (NO_2^-) die Expression stark inhibierte. Auch Glutamin, γ-Aminobuttersäure und MSX, ein Inhibitor der Glutamin Synthetase, bewirkten eine Repression des Gens. Die Analyse der NH_4^+-Gehälter in ähnlich behandelten Wurzeln deutet jedoch eher darauf hin, daß der intrazelluläre NH_4^+-Gehalt der entscheidende Faktor für die Regulation von *LeAMT1;2* ist. Demnach ist LeAMT1;2 primär ein ‚Retrieval'-System, das ausleckendes NH_4^+ wieder aufnimmt.

Der Stickstoffgehalt der Pflanze stellt offenbar ein übergeordnetes Signal für die Regulation von *LeAMT1;2* dar, da ein hoher Stickstoffstatus die Expression von *LeAMT1;2* generell reprimierte. Dieser Effekt war zumindest zum Teil auf den Mangel an Kohlenhydraten in der Wurzel zurückführbar, denn die externe Zufuhr von Zuckerverbindungen konnte die Induzierbarkeit durch NH_4^+ wiederherstellen, wenn auch in abgeschwächtem Maß.

An der Regulation von *LeAMT1;2* ist offenbar auch Kalium (K^+) beteiligt und unterstreicht dessen Rolle als Antagonist von NH_4^+. Eine hohe K^+-Dosis bewirkte eine Abnahme der *LeAMT1;2*-Transkripte in der Wurzel, während die Inhibition der K^+-Kanäle durch Cäsium (Cs^+) für eine stärkere Akkumulation in der Zelle sorgte.

Der pH-Wert der Lösung muß als weiterer induzierender Faktor in Betracht gezogen werden, da die Ansäuerung des Mediums (pH 4.7) in einer Zunahme von *LeAMT1;2*-Transkripten resultierte, auch wenn NH_4^+ als Substrat nicht vorlag. pH-Werte im alkalischen Bereich hatten dagegen keinerlei Auswirkung auf die *LeAMT1;2*-Transkription.

Die Expression von *LeAMT1;2* wurde auch durch verschiedene Phytohormone beeinflußt. Gibberellinsäure und 2-Naphtylessigsäure (2-NAA) wirkten als einzige Substanzen auch ohne NH_4^+ induzierend, während Abscisinsäure (ABA),

Indolessigsäure (IAA) und Kinetin (Kin) in höheren Konzentrationen reprimierend wirkten. Bei 1-Naphtylessigsäure (1-NAA) wirkte eine höhere Konzentration dagegen induzierend.

Eine Induktion von *LeAMT1;2* konnte auch durch eine zehnstündige Inokulation mit verschiedenen diazotrophen Wildtypen erreicht werden. Während *Azospirillum brasilense*, *Azoarcus* Sp BH72 und *Herbaspirillum seropedicae* die *LeAMT1;2*-Expression induzierten, war in Gegenwart von *Azotobacter vinelandii*, *Acetobacter diazotrophicus* und dem vermeintlichen Pflanzenpathogen *Herbaspirillum rubrisubalbicans* kein Effekt sichtbar.

Hinweise, daß dabei die Expression von *LeAMT1;2* möglicherweise eine Folge der biologischen N_2-Fixierung und der Ausscheidung von NH_4^+ war, ergaben sich nicht nur durch den Einsatz von verschiedenen Mutanten von *Azospirillum* sondern auch durch die Wirkung sterilfiltrierter Überstände der in halbfesten Medien gewachsenen Stämme. Diese Mutanten wiesen Defekte in der N_2-Fixierung, dem Stickstoffmetabolismus oder der Kolonisierungsfähigkeit auf. Die Ergebnisse deuten jedoch darauf hin, daß auch noch andere ‚bakterielle Faktoren' die Expression von *LeAMT1;2* beeinflussen.

Die Expression von *LeAMT1;2* erwies sich ferner als Funktion der Inokulationsdichte von *Azospirillum brasilense* und *Azoarcus* Sp BH72. Bei beiden Bakterien waren die stärksten Signale in Gegenwart von 10^7 cfu/ml zu verzeichnen, während Zelldichten von 10^6 bzw. 10^8 cfu/ml eine schwächere Expression bewirkten.

Verfolgte man die Expression von *LeAMT1;2* in Gegenwart der Wildtypen von *Azospirillum brasilense* und *Azoarcus* Sp BH72 über einen Zeitraum von 3 Tagen, so erwies sich die anfangs hohe Expression als ein Effekt von kurzfristiger Natur, denn in beiden Fällen nahm die Signalstärke bis zum dritten Tag deutlich ab. Parallel dazu konnte biologische Stickstoffixierung in der Assoziation erst nach 11 Tagen Inokulation gemessen werden.

Die Kolonisierung von Tomatenwurzeln durch *Azospirillum brasilense* und *Azoarcus* Sp BH72 erfolgte innerhalb von 48 Stunden. *Azospirillum brasilense* war dabei primär an den Ausbruchstellen von Seitenwurzeln, den Wurzelspitzen und den Wurzelhaaren sichtbar und hatte einen ausgeprägten Effekt auf die Wurzelmorphologie. Mutanten, die in der Synthese von IAA betroffen waren, belegten die wichtige Rolle bakterieller Auxine bei diesem Prozeß.

Stichwörter: *Lycopersicon esculentum*,
Ammoniumtransport,
biologische Stickstoffixierung

Contents

Abbreviations and gene symbols

ABA	abscisic acid
ABP	auxin binding protein
ARA	acetylene reduction assay
AS	asparagine synthetase
Asp	aspartate
AspAT	aspartate aminotransferase
AtAMT1	*Arabidopsis thaliana* ammonium transporter 1
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCCM	Belgian Coordinated Collections of Microorganisms
BSA	bovine serum albumine
cfu	colony forming units
cv	cultivar
DEPC	diethyl pyrocarbonate
DMA	dimethylarsinic acid
DNA	desoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
GA_3	gibberellic acid
GABA	γ-aminobutyrate
GDH	glutamate dehydrogenase
Gln	glutamine
Glu	glutamate
GOGAT	glutamine-oxoglutarate aminotransferase
GS	glutamine synthetase
GUS	β-glucuronidase
hr	hour
hrs	hours
IAA	indole-3-acetic acid
IPyA	indole-3-pyruvic acid
Kin	kinetin
K_m	Michaelis constant
LB	Luria-Bertani
LeAMT1;2	*Lycopersicon esculentum* ammonium transporter 1;2
LMG	Laboratorium voor Microbiologie
MCS	multiple cloning site
MOPS	3-[N-Morpholino]propanesulfonic acid
MSX	DL-Methionine DL-sulfoximine
N	nitrogen
NAA	naphtaleneacetic acid
NAD^+	nicotinamide adenine dinucleotide (oxidized form)

$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide (reduced form)
NiR	nitrite reductase
NR	nitrate reductase
OD	optical density
PBS	phosphate buffered saline
PGR	plant growth regulator
PGPR	plant growth promoting rhizobacteria
RNA	ribonucleic acid
SEM	scanning electron microscope
Sp	species (singular)
spp.	species (plural)
TCC	total cell count
Tn	transposon
U	unit(s)
VK	Vorkultur (= overnight culture)
WT	wild type
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Azospirillum gene symbols relevant to this study

Gene	Product	Putative function
nifDK	Dinitrogenase	Structural genes of nitrogenase
glnA	Glutamine synthetase	Fixes NH_4^+ into glutamate to form glutamine
glnB	P_{II}	Signal transduction protein that activates NifA and glutamine synthetase
glnZ	P_{II}-like protein	unknown
ipdc	indole-3-pyruvate decarboxylase	Mediates IAA-biosynthesis in the IPyA pathway

1 Introduction

1.1 Back to the (plant) roots

In view of the impact excessive use of nitrogen (N) fertilizers has on environmental pollution, there is a growing concern to improve the efficiency of N-use in agricultural soils. In many circumstances, nitrogen is a rate-limiting element in plant growth (Jarvis, 1996).
In this context, inoculation of plants with diazotrophic bacteria, thereby being capable of fixing atmospheric dinitrogen (N_2), represents a promising method in meeting the actual nitrogen requirements of an ecosystem. N_2-fixation by the legume-*Rhizobium* symbiosis, for example, leads to considerable increases in combined nitrogen in the soil and is of agricultural importance. In non-leguminous plants, however, N_2-fixing bacteria have not been introduced successfully on a large scale, as the results are for the most part unpredictable and inconsistent (Bashan and Holguin, 1997). A molecular insight into the processes occurring at the plant-bacteria-interface may lead to a better exploitation of N_2-fixing bacteria with respect to the nitrogen supply and growth promotion of a host plant.
Within the scope of this thesis, the ammonium (NH_4^+) transport into tomato roots is investigated and the potential of a plant gene, encoding a high-affinity-NH_4^+-transport-system, to serve as a reporter system for the supply of bacterial-derived NH_4^+ is evaluated.

1.2 Nitrogen

The following sections provide an overview of the importance of nitrogen, the physiological and genetic processes involved in its uptake and assimilation, as well as the consequences of ammonium nutrition in higher plants.
As a macronutrient, nitrogen is required in large amounts during a plant´s life cycle (von Wirén *et al.*, 1997; Crawford, 1995). Since it is one of the major constituents of nucleic acids, proteins, porphyrins, cytochromes, and coenzymes (Sitte *et al.*, 1991; Ullrich, 1992), N-starved plants are severely restricted in the synthesis and activity of these molecules. N-deficient plants usually show reduced rates of photosynthesis, leaf production, tillering, cell expansion and cell division (Clarkson and Hanson, 1980). Furthermore, as a general sign of stress, the abscisic acid level in plant leaves increases (Chapin *et al.*, 1988b, Daie *et al.*, 1979), and the stem stains purplish blue due to the accumulation of anthocyanins (Nightingale and Mitchell, 1934). In tomato, a continuous lack of nitrogen in the plant medium leads to a rapid decline of the vacuolar nitrate (NO_3^-) –pool, and at later stages, organic N is mobilized by degradation in older plant parts (Chapin *et al.*, 1988b, Smart and Bloom, 1993). With regard to plant morphology, root growth is initially promoted upon N-starvation but finally stops after a prolonged period of N-removal (Chapin *et al.*, 1988a).

1.2.1 Available forms and uptake of nitrogen

Plants are able to use almost all forms of N, including NH_4^+, NO_3^-, nitrite (NO_2^-), organic N (amino acids, peptides etc.), volatile ammonia (NH_3), nitrogen oxides (NO_x) and even molecular N_2 (von Wirén *et al.*, 1997). Despite its abundance in the atmosphere, however, the use of the latter compound is restricted to plants living in symbiosis with diazotrophic bacteria (Section 1.3). This is due mainly to the high energy bond of the diatomic molecule (Salsac *et al.*, 1987).
In most cases, the nitrogen requirements of plants have to be covered by the uptake of NO_3^- and NH_4^+ from the soil (Kirkby, 1968). The predominant source is mainly dependent on environmental conditions like temperature, aeration, salinity, and soil-acidity (Peuke and Kaiser, 1996; Botella *et al.*, 1994; Clarkson and Warner, 1979; Bloom *et al.*, 1998). Most typical for the temperate zone are well-aerated soils of high fertility where the majority of nitrogen is present as NO_3^-, a result from the activity of nitrifying bacteria (Clarkson *et al.*, 1986; von Wirén *et al.*, 1997). In contrast, nitrification is strongly suppressed in waterlogged soils as well as in habitats with a low average temperature or pH (Clarkson *et al.*, 1986). Under such conditions, NH_4^+ may occur as the dominant nitrogen source (Smart and Bloom, 1988; Schenk, 1996). Besides its role in nitrogen nutrition, NO_3^- is able to serve as an osmoticum in cell vacuoles (Schenk, 1996), a function for which NH_4^+ is unsuitable due to its cytotoxic effects (Salsac *et al.*, 1987).
Bearing in mind that the usually low nitrogen content in soil is in addition subject to different abiotic and biotic processes such as erosion, leaching, and microbial consumption, it is not surprising that plants have evolved several mechanisms to efficiently scavenge nitrogen from their environment (Crawford, 1995). As the primary organ of nutrient uptake, the plant root is able to spaciously penetrate the soil through apical growth and extensive branching. The absorbing surface of the root is increased severalfold by a dense evolvement of epidermally-derived root hairs (Gazzarrini *et al.*, 1999) that are assumed to play a major role in the uptake of nutrients and water (Peterson and Farquhar, 1996; Röhm and Werner, 1987). Furthermore, root growth rate is strongly promoted in case of nutrient deficiencies, enabling the plant to intercept fresh sources in the soil (Kronzucker *et al.*, 1995).
Upon first exposure, nutrient transportation may occur via the apoplast, that is within the intercellular space in the root cortex. A further transfer into the stele, however, is blocked by the lipophilic casparian stripe within the endodermis, which is why at least the uptake of charged molecules and ions is finally dependent on the mediation by integral membrane proteins (Frommer *et al.*, 1994). These proteins consist of high- and low-affinity components (Epstein, 1976) and enable the plant to tightly control the uptake of ions both on the physiological and transcriptional level. For most of the essential ions including NO_3^- and NH_4^+ there are multiple, differentially regulated uptake carriers, indicating an involvement in diverse functions during plant growth (Gazzarrini *et al.*, 1999).
The following sections deal with the characteristics of NH_4^+-uptake and -nutrition in higher plants with short references to the transport of NO_3^-. Most of the information is based on physiological studies on the influx of these ions from the nutrient solution. In

these studies, the uptake process is monitored by measuring the removal of NH_4^+ from the medium or by following the accumulation of a ^{13}N- or ^{15}N-labelled tracer-molecule (Peuke and Kaiser, 1996). Alternatively, ammonium uptake is often determined by means of the ^{14}C-labelled NH_4^+-transport analogue, methylamine. Until recently, studies on the transcriptional regulation of nitrogen-uptake-carriers were hampered due to a lack of molecular characterization of these systems. Over the past few years, however, several ammonium transporters from different plants have been isolated and functionally identified (Section 1.2.1.3).

1.2.1.1 Characteristics of ammonium uptake

NH_4^+ is considered an immobile ion (Smart and Bloom, 1988), and its soil concentration in temperate zones is usually up to 1000 times lower than that of NO_3^- (Marschner, 1995). In several plants, at least two kinetically distinct transport systems could be identified (Ullrich *et al.*, 1984; Mäck and Tischner, 1994; Kronzucker *et al.*, 1996). This multiphasic uptake pattern is attributed to the presence of a high-affinity (HATS) and a low-affinity (LATS) transport system (Wang *et al.*, 1993b). The HATS is assumed to actively mediate NH_4^+-uptake in the micromolar range and shows saturable transport kinetics coupled with a low transport capacity. In tomato plants, the K_m value for the HATS is reported to range between 5 and 20 µM (Smart and Bloom, 1993; Kosola and Bloom, 1994), thus exhibiting a higher affinity than the analogous HATS for NO_3^- (40 to 100 µM, as reported by Smart and Bloom, 1993). Thereby the higher abundance of NO_3^- in soils of the temperate zone is reflected. The LATS for NH_4^+ reveals non-saturable uptake kinetics and a high transport capacity with an apparent K_m of > 1 mM (Wang *et al.*, 1993b; Kronzucker *et al.*, 1996). Hence, NH_4^+-uptake via the LATS may become significant at higher external NH_4^+-concentrations. It is remarkable that the LATS is not down-regulated in the presence of high NH_4^+-concentrations, that is NH_4^+-uptake increases with the supplied concentration (Rawat *et al.*, 1999). Under such conditions, NH_4^+ may also be transported by means of potassium-channels, particularly when favoured by a high H^+-ATPase-activity (Venegoni *et al.*, 1997; Cao *et al.*, 1993; Ninnemann *et al.*, 1994).

It has been reported that NH_4^+-uptake by the HATS is suppressed in plants of high nitrogen status, whereas suppression is relieved as plants undergo nitrogen deprivation (Morgan and Jackson, 1988a; Clarkson and Lüttge, 1991; Lee *et al.*, 1992). This feedback is caused either by an assimilation product of NH_4^+ (Clarkson and Lüttge, 1991; Wang *et al.*, 1993b) or by the ion itself (Jackson *et al.*, 1993; Kosola and Bloom, 1994). In several organisms, glutamine appears the negative effector, as it may reflect the N status on the whole-plant-level (Lee *et al.*, 1992; Rawat *et al.*, 1999). The uptake-activity in regard to NO_3^- has also been shown to be suppressed in N-repleted plants (Clarkson and Lüttge, 1991).

N-deficient plants have frequently been shown to have high initial NH_4^+-uptake rates upon first exposure to this ion, followed by an immediate decline to a more moderate level (Goyal and Huffaker, 1986). This observation is attributed to either the filling of

the ‚Apparent Free Space' within the root cell wall or an increasing efflux of NH_4^+ with the time of exposure, thus presenting a deceptive decline in the actual uptake rate (Mäck and Tischner, 1994). Indeed, considerable efflux of ammonium has been shown to occur in many plants, even when grown with NO_3^- as the sole source of nitrogen (Morgan and Jackson, 1989; Jackson *et al.*, 1993; Wang *et al.*, 1993b). With disregard of the initial phase, NH_4^+-uptake rates appear to increase with the time of exposure and a steady-state of NH_4^+ uptake is reached within a specific time-period (Mäck and Tischner, 1994). This time-period is assumed to be necessary for *de novo*-synthesis of transporter proteins. In conclusion, the carrier-mediated NH_4^+-uptake comprises both constitutive and inducible components.

The uptake of ammonium via the HATS is believed to be driven by the membrane potential showing depolarization upon NH_4^+-exposure (Henriksen *et al.*, 1992). The exact mechanism, however, is not yet clearly understood (Ninnemann *et al.*, 1994). Charge balance is maintained by the sum of potassium ions (K^+) and protons (H^+) extruded by the roots (Clarkson and Hanson, 1980; Morgan and Jackson, 1989). In this connection, a decrease in ambient acidity has frequently been observed, as the plants were exposed to NH_4^+ as the sole source of nitrogen (Clarkson and Warner, 1979; Marschner and Römheld, 1983). Obviously, the acidity drops to a degree that corresponds with the amount of NH_4^+ supplied. In contrast, the transportation of NO_3^- is believed to occur via 2 H^+ : 1 NO_3^- (Glass *et al.*, 1992), bringing the external pH to rise (Kafkafi *et al.*, 1971; Marschner and Römheld, 1983).

The addition of NH_4^+ to a NO_3^--containing medium turned out to either rapidly inhibit, barely influence, or even slightly stimulate the uptake of NO_3^- (Bloom and Finazzo, 1986; Peuke and Kaiser, 1996; Vessey *et al.*, 1990; Smart and Bloom, 1998). There are many different explanations for this discrepancy, the effect of which is presumed to be influenced by the ion composition of the nutrient solution, the pH, the nutrient status of the plant, the genotype, and the rapid depolarisation of the membrane potential upon NH_4^+-exposure (Bloom and Finazzo, 1986; Smart and Bloom, 1988; Deanne-Drummond and Glass, 1983; Ingemarsson *et al.*, 1987; Ayling, 1993). Recent results regarding tomato plants, suggest that the influence of NH_4^+ on NO_3^--absorption is a matter of the applied NH_4^+-concentration (Smart and Bloom, 1998). According to this particular study, NO_3^--absorption is enhanced if the NH_4^+-level is maintained at a low concentration. Research on barley showed that NO_3^--absorption is also stimulated in the presence of 5 µM NH_4^+ (Lee and Drew, 1989).

The activity of the HATS is strongly dependent on the pH of the nutrient solution, whereby optimum uptake rates are shown in the neutral range (Jungk, 1970; Ninnemann *et al.*, 1994). In acid solutions, the influx of NH_4^+ is severely inhibited (Raper *et al.*, 1991) and if the pH is dropped to 4.0 completely stopped (Tolley-Henry and Raper, 1986). This is mainly explained by the unfavourable conditions for the antiport of protons during the uptake of NH_4^+ (Tolley-Henry and Raper, 1986; Vessey *et al.*, 1990). In addition, a decreased affinity of the HATS to NH_4^+ was observed in acid media (Dyhr-Jensen and Brix, 1996). In the alkaline range, the uncharged NH_3-molecule may be able to cross the membrane passively (Rosen and Carlson, 1984). There is, however, no consensus to the significance of this process with respect to the

N-nutrition of plants (Wang *et al.*, 1993b). Consistent with the putative 2 H^+-symport-mechanism (Sivasankar and Oaks, 1996; Tsay *et al.*, 1993), the uptake of NO_3^- reveals to be optimum in acid solutions, thus contrasting with NH_4^+-transport (Marcus-Wyner, 1983; Jeong and Lee, 1996).
In several plants, the uptake of NH_4^+ from constant concentrations fluctuates diurnally, showing highest activities during the day- and lowest activities during the night-period (Jackson *et al.*, 1976; Ourry *et al.*, 1996; Macduff *et al.*, 1997; Gazzarrini *et al.*, 1999). The primary determinant of this phenomenon is the fluctuating ‚sink-strength' that is associated with nutrient utilization during the synthesis of new tissue. Under conditions of physiological stress, however, the uptake kinetics may be unrelated to nutrient demand. For NO_3^-, a similar diurnal uptake pattern has been observed (Cárdenas-Navarro *et al.*, 1998).
Above all, high-affinity transport systems for NH_4^+ are assumed to be involved in two functions: firstly, scavenging and sensing the environment efficiently for traces of NH_4^+ and secondly, serving as a retrieval system for NH_4^+ that leaks out of the root cells during uptake or assimilation (Frommer *et al.*, 1994; Ninnemann *et al.*, 1994; Gazzarrini *et al.*, 1999).

1.2.1.2 Consequences of ammonium nutrition

Although less energy is expended on the assimilation of NH_4^+ than on that of NO_3^- (Salsac *et al.*, 1987; Bloom *et al.*, 1992), most plants suffer severely from ammonium nutrition (Hoff *et al.*, 1974; Peuke and Kaiser, 1996). Supplying plants with large amounts of NH_4^+ often results in a rapid accumulation inside the root. Unlike NO_3^-, NH_4^+ is usually neither stored in vacuoles nor translocated to the shoot but has to be assimilated immediately (Rosen and Carlson, 1984; Salsac *et al.*, 1987). However, in rice and spruce the vacuole has been shown to serve as a major NH_4^+-pool (Wang *et al.*, 1993b; Kronzucker *et al.*, 1995). As the assimilation of NH_4^+ is dependent on the presence of carbon skeletons, the root C-content is quickly exhausted (Tiedjens, 1934; Kirkby, 1968), and in case of a high supply, the assimilation process is unable to maintain pace with the uptake of ammonium. A consequence most likely of C-starvation is that the roots stop growing (Cox and Reisenauer, 1973), and despite a considerable reflux of NH_4^+ to the medium, it is partly translocated to the shoot before assimilation can occur (Peet *et al.*, 1985; Rufty *et al.*, 1982). In *Arabidopsis*, the growth inhibition of roots has been shown to be caused by changes in phytohormonal levels (Cao *et al.*, 1993). In leaves, NH_4^+ is assumed to uncouple the non-cyclic photophosphorylation, resulting in reductions of photosynthetic capacity and carboxylation efficiency (Cox and Reisenauer, 1973; Barker *et al.*, 1966; Maynard *et al.*, 1966). To compensate for C-deficiency, organic molecules in the shoot are degraded, leading NH_4^+ to also being released endogenously (Barker *et al.*, 1966; Puritch and Barker, 1967; Tolley-Henry and Raper, 1989). The continuous uptake of ammonium reduces the uptake of other essential cations, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), and K^+ (Rosen and Carlson, 1984; Alarcon *et al.*, 1997). While Ca^{2+}-deficiency affects the integrity of cell membranes (Wilcox *et al.*, 1973), K^+

is known to be crucial for the activity of numerous enzymes, including those, responsible for NH_4^+-assimilation (Barker *et al.*, 1967). Furthermore, the external solution acidifies as a result of heavy proton-extrusion (Section 1.2.1.1) (Pill and Lambeth, 1977; Findenegg, 1987), which in turn leads to a reduced uptake of NH_4^+ (Chaillou *et al.*, 1991; Vessey *et al.*, 1990; Tolley-Henry and Raper, 1989). The implied N-starvation enhances the degradation of internal N-sources (Tolley-Henry and Raper, 1986), hence, there are high levels of amino acids and soluble carbohydrates but only low levels of organic acids in the plant sap (Chaillou *et al.*, 1991; Jungk, 1970; Barker, 1999). Furthermore, as a result of a reduced water uptake (Pill and Lambeth, 1977; Quebedeaux and Ozbun, 1973), the turgor pressure in the leaves declines (Bennett and Adams, 1970). Visible symptoms of the so-called ‚ammonium-toxicity-syndrome' are reduced plant growth and dark green leaves (Claassen and Wilcox, 1974), typically rolled upward (Magalhaes and Wilcox, 1983). Subsequent observations are severe necrotic stem lesions (Barker *et al.*, 1967) and chlorosis of plant leaves (Puritch and Barker, 1967; Maynard and Barker, 1969).
The development of this syndrome may be delayed or even prevented, most successfully by taking one or more of the following measures: firstly, the pH of the medium should be frequently adjusted to the neutral range in order to avoid acidification around the roots (Chaillou *et al.*, 1991; Peet *et al.*, 1985; Tolley-Henry and Raper, 1989). This should lead to a more ready assimilation of NH_4^+ (Barker *et al.*, 1966). Secondly, many authors report an increasing tolerance towards NH_4^+, if the K^+-concentration of the medium is raised (Warren, 1962; Claassen and Wilcox, 1974). Potassium stabilizes the conformation of many enzymes (Clarkson and Hanson, 1980; Wilcox, 1964) and protects proteins from the degrading effect of NH_4^+ (Maynard *et al.*, 1966; Barker *et al.*, 1967). K^+-ions have also been reported to inhibit NH_4^+-uptake (Peuke and Kaiser, 1996) and promote N-assimilation (Cao *et al.*, 1993; Ajayi et al, 1970). Thirdly, as the continuous supply of newly synthesized C-sources is a crucial step in the detoxification process, plants should be grown under adequate illumination and sufficient air supply (Givan, 1979). Finally, the supplied NH_4^+-concentration should not exceed that of the K_m of the HATS, as plant growth in this circumstance appears to be normal (Warren, 1962; Findenegg, 1987; Jeong and Lee, 1996).

1.2.1.3 *Lycopersicon esculentum* and the *AMT1* gene family

The tomato plant (*Lycopersicon esculentum*), of the group Solanaceae, was used in this study, due to several characteristics making it a suitable candidate in the investigation of ammonium transport.
Tomato plants and seeds are not able to store high amounts of nitrogen (Smart and Bloom, 1993). They have, however, a high N-requirement for growth (Smart and Bloom, 1988; Chapin *et al.*, 1988b), and hence are much more dependent on a continuous nitrogen supply from the environment. In addition, they are considered to be particularly sensitive to NH_4^+-nutrition (Borys *et al.*, 1970; Marcus-Wyner, 1983; Ganmore-Neumann and Kafkafi, 1980), and tend to develop symptoms of NH_4^+-

toxicity rapidly (Smart and Bloom, 1993). A tight control of pH and potassium levels, however, has also been shown to increase the plants' tolerance towards NH_4^+ (Claassen and Wilcox, 1974; Barker *et al.*, 1967; Vessey *et al.*, 1990). According to the low K_m (Section 1.2.1.1), the HATS reveals a high efficiency with respect to NH_4^+-uptake from low concentrations.

Knowledge of the regulation of ammonium transport systems on the level of transcription is up to date, and data is currently available on bacteria (Jayakumar *et al.*, 1986; Siewe *et al.* 1996) and yeasts (Marini *et al.*, 1997), as well as higher plants including tomato (Ninnemann *et al.*, 1994; Lauter *et al.*, 1996; Schulze *et al.*, 1999; von Wirén *et al.*, 2000). Three ammonium transporter genes have been isolated from a cDNA library of root hairs or leaves of tomato, referred to as *LeAMT1;1-3*. All three genes have been functionally identified by complementation of yeast mutants, defective in NH_4^+-uptake. The *LeAMT1;1-3* genes are members of the *AMT1* multigene family in plants (Gazzarrini *et al.*, 1999). They encode functional NH_4^+-transporters, are targeted to the plasma membrane, and are biochemically characterized as energy- and pH-dependent as well as being sensitive to potassium.

First studies on the expression of these genes reveal different patterns of transcriptional regulation with respect to localization, N-nutritional status, N-supply and light (von Wirén *et al.*, 2000). While *LeAMT1;1* is induced in roots of N-deficient plants, the *LeAMT1;2* gene is expressed in the presence of NO_3^- and NH_4^+. Within the root fraction, both genes are preferentially expressed in root hairs. *LeAMT1;2* is also transcribed in plant leaves, revealing a diurnal pattern with peak expression after the onset of light. On the contrary, *LeAMT1;3*-transcripts, which are not detectable in roots, show a reciprocal diurnal regulation in leaves, the highest levels occurring during darkness. Furthermore, the expression of both transporters in leaves is reduced upon exposure to elevated CO_2-levels.

The *LeAMT1;2*-cDNA has been used in this study to follow the expression pattern in tomato plants exposed to various environmental conditions including microbial inoculation. Studies on the regulation of *LeAMT1;2* have already been conducted within the scope of a diplomathesis (Stanke, 1996). The preliminary results, therein obtained, characterize *LeAMT1;2* as a gene expressed preferentially in roots and gaining highest transcript-levels after exposure of N-starved plants to 5 µM NH_4^+ (pH 6.0). A drop in the pH to 4.7 reveals a general increase in *LeAMT1;2*-expression and shifts the peak-level to a 10-fold NH_4^+-concentration (50 µM). N-repleted plants show no concentration-dependent expression pattern but strongly repress *LeAMT1;2*. High external NH_4^+-concentrations also repress the *LeAMT1;2*-gene expression unless the pH was lowered to 4.7. According to these results, *LeAMT1;2*-expression is regulated by the supplied NH_4^+-concentration, the plants' N-status as well as by the pH of the solution, hence properties, which favour its usage as a reporter gene for NH_4^+-supply (Section 1.4).

1.2.2 Assimilation of nitrogen

Nitrogen assimilation is the process, in which inorganic nitrogen is assimilated into the amino acids glutamine, glutamate, asparagine, and aspartate (reviewed by Lam *et al.*, 1996). These four amino acids are the predominant compounds of the entire free amino acid pool in plants, cycling between roots and shoots (Cooper and Clarkson, 1989; Peuke and Kaiser, 1996; Larsson *et al.*, 1991). The genes involved in this process are subject to tight regulation of factors such as light, metabolites, CO_2-concentration, circadian rhythms, plant growth regulators, and tissue type (Oliveira *et al.*, 1997; Crawford, 1995; Redinbaugh and Campbell, 1991; Kreps and Kay, 1997; Ono *et al.*, 2000; Chanda *et al.*, 1998). A simplified diagram reflecting the biochemical reactions of nitrogen assimilation is shown in Figure 1.1.

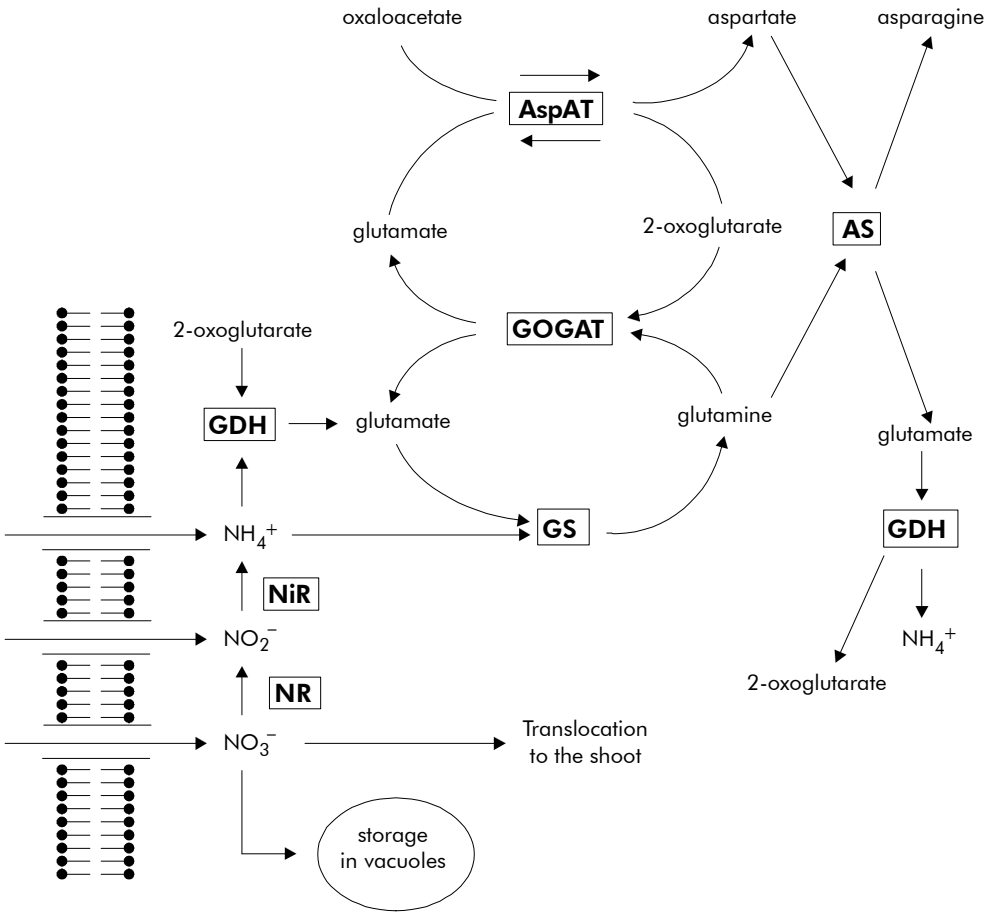


Figure 1.1 Assimilation of nitrogen into the four most abundant amino acids in plant roots. Participation of cosubstrates, sites of reactions, and efflux of ions are not considered. Further explanations are given in the text. Note that reactions are catalyzed by multiple isoenzymes, which are located in different subcellular compartments. Abbreviations: NR = nitrate reductase; NiR = nitrite reductase; GS = glutamine synthetase; GOGAT = glutamine-oxoglutarate aminotransferase; GDH = glutamate dehydrogenase; AspAT = aspartate aminotransferase; AS = asparagine synthetase.

Mineral nitrogen is taken up as NO_3^-, NO_2^- or NH_4^+. NO_3^- is either stored in vacuoles, translocated to the shoot, or reduced (Redinbaugh and Campbell, 1991; Frommer *et al.*, 1994; Andrews, 1986). Translocated NO_3^- can also be stored or reduced in the shoot (Padgett and Leonard, 1993). The assimilation of nitrate is a two-step process (Crawford, 1995). In the first step, the NADH-dependent reduction of nitrate to nitrite is catalyzed by the cytosolic enzyme nitrate reductase (NR). In the second step, the plastidic enzyme nitrite reductase mediates the reduction of nitrite to ammonium, using reduced ferredoxin as the electron donor. The reverse reaction probably does not occur in higher plants (Padgett and Leonard, 1993). The co-transcription of both enzymes is induced within minutes by NO_3^- (Crawford, 1995; Tsay *et al.*, 1993) and furthermore is subject to a diurnal rhythm, showing highest activity at the start of the day period (Galangau *et al.*, 1988). In addition, a negative feedback on the activity is assumed to be caused by an assimilation product of NO_3^- (Crawford, 1995). Nitrite is neither stored nor translocated as due to its toxic effects accumulation must be avoided (Crawford, 1995; Peuke and Kaiser, 1996; Aslam *et al.*, 1979).

NH_4^+, primarily derived from the uptake- or the NO_3^--reduction-process, is a central intermediate in the metabolism of nitrogen in plants (Chanda *et al.*, 1998). In addition, it is released from secondary sources during photorespiration, protein degradation, and amino acid catabolism (Oliveira *et al.*, 1997). Under normal circumstances, NH_4^+ is assimilated immediately in the root (Lewis *et al.*, 1982) by the concerted actions of glutamine synthetase (GS), glutamine-oxoglutarate aminotransferase (GOGAT), and glutamate dehydrogenase (GDH). The most important pathway for the assimilation of NH_4^+ in plants is via GS and GOGAT (Lewis *et al.*, 1983; Peuke and Kaiser, 1996). GS is responsible for the production of glutamine, condensing glutamate and ammonium in an amidation reaction. The GOGAT enzyme transfers the amide group of glutamine to 2-oxoglutarate, the result being two molecules of glutamate. GDH aminates 2-oxoglutarate into glutamate using NH_4^+. The assimilation of NH_4^+ via GDH represents a pathway of minor importance and is assumed to participate in the assimilation under N-excess (Miflin and Lea, 1976; Oaks and Hirel, 1985), as GDH exhibits only a low affinity to NH_4^+ (Givan, 1979; Lam *et al.*, 1996). In tomato plants, it has been shown that GDH is involved in NH_4^+-assimilation if the GS/GOGAT cycle is inhibited by methionine sulfoximine (MSX), a glutamate analogue (Magalhaes, 1991). Additionally, GDH plays an important role in amino acid catabolism by catalyzing the reverse reaction (endogenous NH_4^+-generation). In further reactions, glutamine and glutamate are channelled into aspartate and asparagine by aspartate aminotransferase (AspAT) and asparagin synthetase (AS). AspAT reversibly mediates the transfer of an amino group from glutamate to oxaloacetate to generate 2-oxoglutarate and aspartate. Asparagine synthetase catalyzes the ATP-dependent transfer of an amino group of glutamine to a molecule of aspartate to generate a molecule of glutamate and asparagine. Oxaloacetate and 2-oxoglutarate are derived from the citric acid cycle. Glutamate and aspartate are parent molecules of two amino acid families in nitrogen metabolism, that is the synthesis of many other amino acids begins from these molecules.

1.3 Biological nitrogen fixation

For certain plants, molecular nitrogen can also be considered a primary source of NH_4^+. This is solely for plants living in symbiosis or close association with diazotrophic bacteria, capable of fixing atmospheric dinitrogen and supplying it in the form of NH_4^+ or an assimilation product of NH_4^+ to their host plants. The reduction from N_2 to NH_4^+ is catalyzed by the enzyme complex nitrogenase, which is existent only in certain bacteria. Biological nitrogen fixation, however, is a very energy-demanding process. For this reason, it is tightly regulated by several factors at both the transcriptional and posttranslational level (Rudnick *et al.*, 1997). Nitrogenase activity for instance, is rapidly inactivated by O_2 (Burris, 1991; Hartmann and Burris, 1987) and externally supplied NH_4^+ (Zhang *et al.*, 1993; Hartmann *et al.*, 1986).
The most efficient plant-bacterial interaction is that between leguminous plants and bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. A corresponding infection of the plant leads to the formation of root nodules, which contain differentiated rhizobial cells, called bacteroids. Within mature root nodules, bacteroids fix N_2 efficiently and supply their host plants with significant amounts of N in exchange for C-sources. NH_4^+-transport from the nodule to the plant is mediated by voltage-driven channels operating at high activity in the symbiosome membrane (Mouritzen and Rosendahl, 1997; Tyerman *et al.*, 1995; Schroeder, 1995; Kaiser *et al.*, 1998).
Apart from the root nodule bacteria there are several other diazotrophs living either freely in the soil or in close association with higher plants of economic importance, such as cereals. These bacteria are found in large numbers within the plants' rhizosphere and efficiently colonize the root surface without causing the development of a symbiotic structure such as a root nodule. A specific form of this association occurs with bacteria capable of colonizing the interior of the plant, referred to as endophytic bacteria. The scope of this thesis uses bacteria of both associative groups. In the following sections, the two most frequently used bacteria namely *Azospirillum brasilense* spp. and *Azoarcus* Sp BH72, are described in more detail.

1.3.1 *Azospirillum brasilense*

Azospirillum brasilense is probably one of the best studied organisms in the field of associative nitrogen fixation. *Azospirillum* bacteria are Gram negative, aerobic chemoorganotrophs and belong to the α-subgroup of the proteobacteria. They have attracted research efforts due to their N_2-fixing and root-colonizing abilities. In addition, *Azospirillum* spp. are very versatile and possess a wide array of metabolic pathways, enabling them to obtain energy and intermediates from organic acids, sugars, and amino acids (Okon, 1985). Some of these compounds are released as root exudates into the surrounding medium through healthy and intact plant roots (Rovira, 1969; Pate, 1973). Due to their chemotactic properties (Bashan and Holguin, 1994; Reinhold *et al.*, 1985; Michiels *et al.*, 1989), *Azospirillum* bacteria are

attracted by a number of these substances, which is why they competitively enrich within the rhizosphere and even on the root surface of a wide variety of plants (Michiels *et al.*, 1989; Michiels *et al.*, 1991; Okon and Kapulnik, 1986; Bashan and Holguin, 1997). Bacterial adsorption on plant roots is assumed an essential step for plant growth promotion (Michiels *et al.*, 1991; Bashan and Holguin, 1997). It has often been observed that when *Azospirillum* spp. colonize roots, they increase yields by improving root development, increasing the rate of water and mineral uptake, and providing biological fixed nitrogen (Hadas and Okon, 1987; Bashan *et al.*, 1989; Bashan and Levanony, 1990; Baldani *et al.*, 1983; Fulchieri and Frioni, 1994; Dubrovsky *et al.*, 1994). For this reason, they are usually referred to as plant growth promoting rhizobacteria or PGPR (Glick, 1995). The lack of consistency, however, with regard to yielding responses, reflects the sensibility of the interaction and the multitude of factors influencing it (Quispel, 1991; Bashan and Levanony, 1990). In particular, the N-contribution of *Azospirillum* to plant growth via N_2-fixation is generally regarded to be of little significance (Kapulnik and Okon, 1983; Christiansen-Weniger and van Veen, 1991; Kapulnik *et al.*, 1985), whereby contrary reports exist (Kennedy and Tchan, 1992). The beneficial effect of *Azospirillum* inoculation on tomato plants has been described several times (Bashan and Levanony, 1990; Michiels *et al.*, 1989; Bashan, 1998; Hadas and Okon, 1987), however, positive yielding responses were not attributed to N_2-fixation (Bashan *et al.*, 1989). Fixed nitrogen is usually not excreted by *Azospirillum brasilense*, due mainly to the presence of a high-affinity-NH_4^+-transporter acting as a retrieval system for leaking NH_4^+ (Hartmann and Kleiner, 1982; Wiegel and Kleiner, 1982; Kleiner, 1984; van Dommelen *et al.*, 1998).

The mechanisms, by which *Azsopirillum* bacteria and other PGPR promote plant growth are partly understood (reviewed by Glick, 1995). There is considerable evidence that the observed effects are caused by excreted phytoeffective metabolites (Rudnick *et al.*, 1997). Among these are types of the plant growth regulators auxin, gibberellic acid, and cytokinin, whereby pronounced effects on plant development are known (Brown *et al.*, 1968; Bashan and Levanony, 1990; Costacurta and Vanderleyden, 1995; Jackson *et al.*, 1964; Dubrovsky *et al.*, 1994). Other mechanisms of PGPR include the secretion of iron-binding siderophores and solubilization of phosphorus in the soil (Höflich *et al.*, 1994), both of which are then available for the plant. Furthermore, *Azospirillum* bacteria are supposed to protect plants from the deleterious effects of phytopathogens due to their strong colonizing abilities and the excretion of antibiotics (Oliveira and Drozdowicz, 1981; Bashan, 1998).

As transposon mutagenesis has frequently been used as a powerful tool to create mutants of *Azospirillum brasilense* (Vanstockem *et al.*, 1987), numerous strains are presently available. These strains have substantially contributed to the understanding of the regulation of nitrogen fixation. A short reference to the function of the genes involved in N_2-fixation can be found in the Section ‚Abbreviations' of this thesis.

1.3.2 *Azoarcus* Sp BH72

Azoarcus Sp strain BH72 is an aerobic diazotrophic bacterium of the β-subclass (Hurek *et al.*, 1997), originally isolated from Kallar gras (Leptochloa fusca, cv. Kunth). It is able to live either freely in the soil or penetrate the root interior by means of cellulases and pectinases (Reinhold-Hurek *et al.*, 1993b). Using the vascular tissue, they may subsequently spread through the entire vegetative body. For this reason, the members of the genus *Azoarcus* are referred to as endophytic associative bacteria. Endophytes are assumed to have an enormous potential to enhance the biomass of plants (Triplett, 1996). Besides physical protection from other rhizosphere bacteria, they have direct access to photosynthates. N-compounds, on the other hand, released possibly after N_2-fixation, are immediately available for the plant (Quispel, 1991). *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*, which have also been used in this study, are further examples of endophytically living bacteria (Boddey and Döbereiner, 1995).
Kallar gras is a C_4 plant with high tolerance to salinity, alcalinity, as well as waterlogged conditions. In association with *Azoarcus* Sp BH72, it is able to grow without fertilizer, indicating that bacterial N_2-fixation may contribute to the plants' N-demand. Furthermore, the transcription of *Azoarcus nifH*, encoding for the structural gene of nitrogenase, could be detected in inoculated roots by *in situ*-hybridization (Hurek *et al.*, 1997). *Azoarcus* Sp BH72 is also able to successfully penetrate rice- and millet plants, without causing a pathogenic response (Hurek *et al.*, 1994a). Upon inoculation, plant growth of rice is strongly promoted, N_2-fixation however, is apparently not involved. On the contrary, millet plants were shown to be supplied with significant amounts of bacterial-derived nitrogen (Stein *et al.*, 1997). There is no current data concerning the interaction between *Azoarcus* Sp BH72 and tomato plants. In laboratory cultures, *Azoarcus* has been capable of efficiently fixing dinitrogen. A specific feature is the development of a hyperinduced state, characterized by high respiration rates coinciding with efficient N_2-fixation below 35nM O_2 (Hurek *et al.*, 1994b).

1.4 Scope of the thesis

The mechanisms by which associative N_2-fixing bacteria contribute to the benefit of their host plants, are still a matter of debate. This is due at least partly to the lack of appropriate tools, providing sufficient sensitivity to detect metabolites found excreted in small amounts by both plant and bacteria and significant in providing key signals in the plant-microbe-interaction. One of the most interesting metabolites on the side of the bacteria is undoubtedly NH_4^+, the final product of N_2-fixation. As many genes are known to respond to small traces of substrates by increasing the amount of corresponding enzymes, an approach has been made to test the suitability of a plant gene and to investigate, whether plants are supplied with bacterial-derived NH_4^+.

- In the first part of the thesis, the fundamentals of NH_4^+-transport in tomato plants should be investigated, mainly by means of northern blot analyses. The *LeAMT1;2* gene should be used as a probe to scrutinize the factors, influencing its expression. Among these factors are different compounds involved in N- and C-metabolism, potassium transport, and plant growth regulation, as well as environmental factors like pH, day- and nighttime, and mechanical perturbations of the root system. Furthermore, the minimum NH_4^+-concentration for induction, and the kinetics of the response to NH_4^+-exposure and N-starvation should be determined.

- In physiological studies, the uptake rate of NH_4^+, the NH_4^+-content of the root and the exudate pattern should be analyzed under conditions, comparable to those of the northern blot experiments.

- In the second part of the thesis, an insight in the nature of the plant-microbe-interaction should be gained by following the expression of *LeAMT1;2* in the presence of diazotrophic wild types and several mutants of *Azospirillum,* affected in different aspects of N_2-fixation, colonizing abilities, and IAA-production. Furthermore, the involvement of *LeAMT1;2* in pathogen response should be analyzed, using different strains of the genus *Herbaspirillum* for inoculation. In addition, the expression pattern should be determined in dependence of bacterial density and time of inoculation. Finally, the supernatants of semisolid grown cultures should be tested to evaluate, whether bacterial excretion products are capable of inducing *LeAMT1;2*.

- The colonization pattern of *Azospirillum brasilense* and *Azoarcus* on tomato roots should be determined, and their influence on root morphology should be statistically evaluated in comparison to control plants treated with different plant growth regulators.

- N_2-fixation in association with plants should be followed by means of the acetylene reduction assay, the ^{15}N-enrichment-technique, and a *nifH-gusA*-fusion, harboured in *Azospirillum brasilense*. Furthermore, the NH_4^+-content of inoculated roots should be determined after long-term incubation.

2 Materials and methods

2.1 Materials

2.1.1 Plant

Lycopersicon esculentum (L.) cv. Carmello. Seeds were purchased from 'Novartis Seeds B.V.', The Netherlands. This French hybrid usually matures in 70 days and has an indeterminate growth habit, whereby it continues to grow and bear fruit throughout the growing season. Abundantly yielding heavy, thick, full flavored tomatoes, Carmello is considered to be disease and crack resistant.

2.1.2 Bacteria

Strain	Relevant characteristics
Azospirillum brasilense	
Sp7 (DSM 1690)	Wild type strain (WT), isolated from the rhizosphere of *Digitaria decumbens* (Tarrand *et al.*, 1978)
7317	*nifD*::Tn*5*-mutant of Sp7 (Nif$^-$), fixes no nitrogen (C. Elmerich, Institute Pasteur, Paris)
SpM7918	*ipdc*::Tn*5*-mutant of wild type strain Sp6 (Ipdc$^-$), attenuated in IAA-synthesis (Costacurta *et al.*, 1994)
A7030 NM313	Km^r, Tn*5*-*lacZ*-induced mutant of A7030[1] (Mob$^-$), no chemotactic response (van Rhijn *et al*., 1990)
C3	EDA-resistant mutant of Wa5[2](GlnA$^-$, Nif$^+$), fixes nitrogen constitutively, impaired in GS-activity, excretes ammonium (Christiansen-Weniger and Van Veen, 1991)
7029	*glnA*-mutant of Sp7 (GlnA$^-$, Nif$^-$), fixes no nitrogen, impaired in GS-activity, excretes ammonium (Gauthier and Elmerich, 1977)
7628	Km^r, *glnB*::*kan*-mutant of Sp7 (GlnB$^-$, Nif$^-$), fixes no nitrogen, P_{II}-defect contributes to ammonium excretion (de Zamaroczy *et al.*, 1993)
7611	Spe^r, Sm^r, *glnZ*::Ω-mutant of Sp7 (GlnZ$^-$, Nif$^+$), fixes nitrogen, P_Z-defect does not contribute to ammonium excretion (de Zamaroczy, 1998)
7628 (pAB5320) (referred to as 7628b)	Km^r, Tc^r, *glnB*::*kan*-mutant of Sp7 (GlnB$^-$, Nif$^+$), fixes nitrogen constitutively, P_{II}-defect contributes to ammonium excretion (This study)
FT 326	FT-resistant mutant of Sp7, excretes high amounts of IAA (Hartmann *et al.*, 1983)
Sp7 (pFAJ31.13)	Tc^r, wild type strain Sp7, carrying the *gus*A reporter plasmid pFAJ31.13 (Vande Broek, 1994)
Sp245 (pFAJ21)	Tc^r, wild type strain Sp245, carrying the *nifH-gusA* fusion plasmid pFAJ21 (Vande Broek, 1994)
Azotobacter vinelandii	
Lipman 1903 AL (DSM 2289)	Wild type strain, free living diazotroph, isolated from field soil, U.S.A. (Skerman *et al.*, 1980)

Acetobacter diazotrophicus	
Pal5 (ATCC 49037)	Wild type strain, isolated from roots and stems of sugarcane, endophytic strain, Brazil (Gillis *et al.*, 1989)
Azoarcus	
BH72	Wild type strain, isolated from the rhizosphere of *Leptochloa fusca* cv. Kunth, endophytic strain (Reinhold-Hurek *et al.*, 1993a)
Escherichia coli	
S 17-1 (pAB5320)	*pro thi hsdR recA,* chromosomal integration of RP4-2-Tc::Mu-Km::Tn7, Sm^r, Tp^r, Tra^+, Tc^r, donor for mobilization of pAB5320 by conjugation (Arséne *et al.*, 1996)
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 DlacU169 (ϕ80lacZDM15)* (Gibco BRL)
Herbaspirillum seropedicae	
Z67 (ATCC 35897)	Wild type strain, isolated from rice roots, Brazil (Baldani *et al.*, 1986)
Z78 (ATCC 35893)	Wild type strain, isolated from sorghum roots, Brazil (Baldani *et al.*, 1986)
Herbaspirillum rubrisubalbicans	
M4 (BCCM / LMG 2286)	phytopathogen, causing mottled stripe desease on sugar cane (Hayward, 1962)

[1] A7030: Nitrosoguanidine-induced Sm^r mutant of Sp7 (Franche and Elmerich, 1981)
[2] Wa5: Wild type strain of *Azospirillum brasilense*, isolated from a greenhouse-grown summer wheat (Christiansen-Weniger, 1988)

2.1.3 Plasmids

Plasmid	Relevant characteristics
pAB5320	pVK100 derivative, carrying a N-terminal truncated *Azospirillum brasilense nifA*-gene (Arséne *et al.*, 1996)
pFAJ31.13	Tc^r, pLAFR3 derivative, carrying a constitutively expressed *Azospirillum brasilense* promoter::*gusA* fusion (Vande Broek, 1994)
pFAJ21	Tc^r, pRK290 derivative, carrying the 3.8 kb *Eco*RI fragment of pFAJ20 with the translational *nifH-gusA* fusion (Vande Broek, 1994)
pFL61	Amp^r, shuttle vector, carrying the *LeAMT1;2*-gene from *Lycopersicon esculentum* within *Not*I-restriction sites (Minet *et al.*, 1992)
pBS II KS ±	Amp^r, cloning vector, carrying the *18S-rRNA*-gene from *Arabidopsis thaliana* within *Eco*RI-restriction sites (Stratagene)

2.1.4 Molecular biology products and kits

Agarose *	peqlab Biotechnologie GmbH
Albumin, bovine, Fraction V Powder *	Roth
Ammonia, test-combination	Boehringer Mannheim
Bromophenol blue *	Fluka
[α-^{32}P]-deoxycytidine phosphate (10 MB/25 µl)	Hartmann Analytik
Diethyl pyrocarbonate *	Sigma
EDTA *	Sigma

Ethanol *	Merck
Ethidium bromide *	Sigma
Formaldehyde (37.5 %) *	Merck
Formamide (deionized) *	Merck
Glycerol *	Sigma
Jetsorb-DNA-extraction-kit	Genomed
β-Mercaptoethanol *	Sigma
MOPS *	Fluka
Promega-Midi-Caps-DNA-Purification-Kit	Promega
Ready To Go DNA Labelling Kit (-dCTP)	Amersham Pharmacia Biotech
Restriction endonuclease *Eco*RI (10 U/µl)	Boehringer Mannheim
Restriction endonuclease *Not*I (10 U/µl)	Boehringer Mannheim
RNeasy Plant Mini Kit	Qiagen
Sephadex G50 (DNA grade fine) *	Sigma
SmartLadder	Eurogentec
Sodium dodecyl sulfate *	Biomol
Tris-HCl *	Biomol
Wizard Minipreps DNA Purification System	Promega
Xylene cyanole FF *	Sigma

All other molecular biology products were purchased from Merck.

* Products were purchased free of nucleases.

2.1.5 Chemicals

Apart from cane sugar all chemicals were of analytical grade.

Abscisic acid	Sigma
Acetylene	Widmann Gase GmbH
Agar	Difco
Alanine	Merck
Ampicillin	Sigma
Aspartic acid	Sigma
Bacto peptone	Sigma
Cane sugar	supermarket
Cronex, TF-Fixer (for X-ray film)	Du Pont
Developer (for X-ray film)	Adefo
Dimethylarsinic acid	Sigma
Ethylene	Linde AG
Fructose	Merck
Gibberellic acid	Sigma
Glucose	Merck
Glutamic acid	Sigma

Glutamine	Sigma
Glutaraldehyde	Sigma
Hydrogen peroxide	Merck
Indole-3-acetic acid	Sigma
Kanamycin	Sigma
Kinetin	Sigma
DL-Methionine DL-sulfoximine	Sigma
$^{15}N_2$	Chemotrade
α-Naphtaleneacetic acid	Sigma
β-Naphtaleneacetic acid	Sigma
Nitrilotriacetic acid	Sigma
Nitrogen, liquid	Linde AG
Salicylic acid	Sigma
Selenium	Merck
Sodium hypochlorite	Riedel de Haën
Spectinomycin	Sigma
Streptomycin	Serva
Sucrose	Merck
Sulfuric acid	Merck
Tetracycline	Sigma
X-Gluc	Duchefa
Yeast extract	Merck

All other chemicals were purchased from Merck.

2.1.6 Buffers and solutions

Unless otherwise stated, bi-distilled water was used as the solvent as well as for the necessary dilutions.

DEPC-H_2O *	0.1% (v/v) diethyl pyrocarbonate
Digestive solution (Kjeldahl)	500 ml sulfuric acid, 50 g K_2SO_4, 500 mg selenium
DMA-buffer	52.8 ml 2 M dimethylarsinic acid (pH 7.2), 7.2 ml 25% glutaraldehyde
DNA-loading buffer	0.25% (w/v) bromophenol blue, 100 mM EDTA, 0.25% (w/v) xylene cyanole FF, 50% (v/v) glycerol
GUS-staining-solution **	100 ml KP_i-buffer, 50 mg X-Gluc, 33 mg $K_3Fe[CN]_6$, 42 mg $K_4Fe[CN]_6$

10x MEN-buffer **	200 mM MOPS, 47 mM sodium acetate, 6.4 mM EDTA (disodium salt), dissolved in DEPC-H_2O, pH 7.0
KP_i-buffer, pH 7	13.6 g/l KH_2PO_4, 17.4 g/l K_2HPO_4
NaP_i-buffer, pH 7.2	68.4 ml 1 M Na_2HPO_4, 31.6 ml 1 M NaH_2PO_4
P_i-buffer ***	250 mM NaP_i-buffer, 7% SDS (w/v), 1 mM EDTA, 1% BSA, pH 7.2
PBS *	0.88% (w/v) NaCl, 2.9 mM KH_2PO_4, 7.1 mM K_2HPO_4, pH 7.2
RNA-loading buffer	100 µl MEN-buffer, 175 µl formaldehyde (37.5%), 500 µl formamide (deionized), 50 µl DNA-loading buffer, 10 µl ethidiumbromide (5 mg/ml), DEPC-H_2O ad 1000 µl
Saline *	0.88% (w/v) NaCl
SSC-buffer (20x) *	3 M NaCl, 0.3 M sodium citrate (trisodium salt), pH 7.0
TBE agarose gel (1%)	1% agarose (w/v) in 1x TBE-buffer, 0.1 µg/ml ethidiumbromide
TBE-buffer (10x) *	0.9 M Tris, 0.9 M boric acid, 2 mM EDTA (disodium salt), pH 8.3
TE-buffer *	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Tfb I-buffer **	30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2 \cdot 4\ H_2O$, 15% glycerol, pH 5.8
Tfb II-buffer **	10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH 6.5

* Solution was autoclaved (DEPC-H_2O was autoclaved 2 times)

** Solution was filtersterilized (0.22 µ-filter unit)

*** Solution was filtered (0.45 µ-filter unit)

2.1.7 Media

All media were prepared with bi-distilled water and autoclaved before use. NH_4Cl was added by filtersterilization.

Burk-medium 5 g glucose, 0.64 g K_2HPO_4, 0.16 g KH_2PO_4, 0.2 g NaCl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 2.5 mg $FeSO_4 \cdot 7\ H_2O$, 2.9 mg H_3BO_3, 1.2 mg $CoSO_4 \cdot 7\ H_2O$, 0.1 mg $CuSO_4 \cdot 5\ H_2O$, 0.09 mg $MnCl \cdot 4\ H_2O$, 2.5 mg $Na_2MoO_4 \cdot 2\ H_2O$, 2.5 mg $ZnSO_4 \cdot 7\ H_2O$, 100 mg nitrilotriacetic acid, 1000 ml H_2O, pH 7 (Dalton 1980, modified)

Burk-agar Burk-medium, 15 g agar

LB-medium 10 g Bacto-peptone, 5 g yeast extract, 10 g NaCl, 1000 ml H_2O (Miller, 1972)

LB-agar LB-medium, 15 g agar

LGI-P-medium 100 g cane sugar, 100 mg NaCl, 200 mg $MgSO_4 \cdot 7\ H_2O$, 20 mg $CaCl_2 \cdot 2\ H_2O$, 2.0 mg $Na_2MoO_4 \cdot 2\ H_2O$, 10 mg $FeCl_3 \cdot 6\ H_2O$, 2.5 ml bromothymol blue (0.5% in 0.2 N KOH), 200 mg K_2HPO_4, 600 mg KH_2PO_4, 1000 ml H_2O, pH 6 (Döbereiner *et al.*, 1995)

LGI-P-agar LGI-P-medium, 20 g agar, 16 mg yeast extract

MPCL-basic-medium 1.02 g $CaCl_2 \cdot 2\ H_2O$, 0.348 g K_2SO_4, 0.48 g $MgSO_4 \cdot 7\ H_2O$, 0.445 g KCl, 0.408 g KH_2PO_4, 8.07 mg NaFe(III)EDTA, 3.44 mg H_3BO_3, 0.65 mg $MnSO_4 \cdot H_2O$, 0.85 mg $ZnSO_4 \cdot 7\ H_2O$, 0.36 mg $CuSO_4 \cdot 5\ H_2O$, 0.18 mg $Na_2MoO_4 \cdot 2\ H_2O$, 1000 ml H_2O, pH 6 (Lavigne, 1987, modified)

MPCL-low-K^+-medium 1.02 g $CaCl_2 \cdot 2\ H_2O$, 0.1128 g K_2SO_4, 0.48 g $MgSO_4 \cdot 7\ H_2O$, 0.0877 g KH_2PO_4, 1.086 g MES, 8.07 mg NaFe(III)EDTA, 3.44 mg H_3BO_3, 0.65 mg $MnSO_4 \cdot H_2O$, 0.85 mg $ZnSO_4 \cdot 7\ H_2O$, 0.36 mg $CuSO_4 \cdot 5\ H_2O$, 0.18 mg $Na_2MoO_4 \cdot 2\ H_2O$, 1000 ml H_2O, pH 6 (Lavigne, 1987, modified)

MPCL-low-NO_3^--medium	MPCL-basic-medium supplemented with 5µM KNO_3
MPCL-low-NH_4^+-medium	MPCL-basic-medium supplemented with 5µM NH_4Cl
MPCL-high-NO_3^--medium	1.64 g $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.202 g KNO_3, 0.348 g K_2SO_4, 0.48 g $MgSO_4 \cdot 7\ H_2O$, 0.296 g KCl, 0.408 g KH_2PO_4, 8.07 mg NaFe(III)EDTA, 3.44 mg H_3BO_3, 0.65 mg $MnSO_4 \cdot H_2O$, 0.85 mg $ZnSO_4 \cdot 7\ H_2O$, 0.36 mg $CuSO_4 \cdot 5\ H_2O$, 0.18 mg $Na_2MoO_4 \cdot 2\ H_2O$, 1000 ml H_2O, pH 6 (Lavigne, 1987, modified)
NFb-medium	5 g DL-malic acid, 4.5 g KOH, 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.02 g $CaCl_2 \cdot 2\ H_2O$, 0.7 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g NH_4Cl, 66 mg NaFe(III)EDTA, 2.8 mg H_3BO_3, 2.35 mg $MnSO_4 \cdot H_2O$, 2 mg $Na_2MoO_4 \cdot 2\ H_2O$, 0.24 mg $ZnSO_4 \cdot 7\ H_2O$, 0.08 mg $CuSO_4 \cdot 5\ H_2O$, 1000 ml H_2O, pH 6.8 (Döbereiner, 1980, modified)
NFb-VK-medium	NFb-medium, 0.5 g NH_4Cl
NFb-VK-agar	NFb-VK-medium, 15 g agar, 20 mg yeast extract
NFb-semisolid-medium	NFb-medium, 1.8 g agar
YEP-medium	10 g Bacto-peptone, 10 g yeast extract, 5 g NaCl, 1000 ml H_2O
YEP-agar	YEP-medium, 15 g agar

2.1.8 Stock solutions of antibiotics

Antibiotic	stock solution	working solution
Ampicillin	100 mg/ml H_2O	100 mg/l
Kanamycin	25 mg/ml H_2O	25 mg/l
Tetracycline	10 mg/ml ethanol	10 mg/l
Streptomycin	5 mg/ml H_2O	5 mg/l
Spectinomycin	75 mg/ml H_2O	75 mg/l

Where appropriate, antibiotics were added by filtersterilization (0.22 µ-filter). Media containing antibiotics are provided with the corresponding index (e.g., LB_{Amp}-agar).

2.1.9 Techware and further material

Cellulose acetate filter units (0.45 µ)	Sartorius
Cuvettes, disposable	Plastibrand
Exposure cassette	Appligene
Falcon tubes (15 ml, 50 ml)	Becton & Dickinson
Hybond-N	Amersham Pharmacia Biotech
LTK-008 set (for removal of RNAse-contamination)	racksys Laborequipment GmbH
Minisart FP 030/3, filter units (0.22 µ)	Sartorius
Polaroid-film 667 (ISO 3000/36°)	Polaroid
Parafilm M	Roth
Silicone closures	neoLab
Silicone sponge closures	Sigma
Thoma chamber	Marienfeld, laboratory glassware
Ultra-micro cuvet 105.202-QS (100 µl)	Hellma
Whatman 3MM filter paper	Schleicher & Schuell
Hyperfilm MP	Amersham Life Sciences

2.1.10 Lab equipment

Amino acid analyzer	LC 5000/5001, Biotronik GmbH & Co., Berlin
Ammonia analyzer	Gamma Analysen Technik GmbH-Wescan, Bremerhaven
Autoclave	Varioklav, model 500E, H+P Labortechnik GmbH, München
Balances	Sartorius research R300S, Sartorius, Göttingen Sartorius research R200D, Sartorius, Göttingen Sartorius laboratory L2200P, Sartorius, Göttingen
Centrifuges	model GS 15-R, Beckman, München microcentrifuge Biofuge pico, Heraeus Inst., Osterode model J2-MC5415C, Beckman, München
Clean Bench	NSF 49 BS 5726, 'biohazard' , Clean Air, Woerden, The Netherlands
Climatic chamber	Vötsch Industrietechnik GmbH, Balingen-Frommern
Cameras	Canon EOS 1000FN Polaroid MP4, land camera, USA
Critical Point Dryer	Kritisch-Punkt Apparatur CPD 030, Balzers Union
Deepfreezer	–85° C: UF85-460T, Colora Messtechnik GmbH, Lorch –20° C: GTS 2606/02, Bosch, Berlin
Gas chromatograph	Fractovap, model 4200-01, Carlo-Erba Strumentazione, fitted with stainless steel columns (90 cm, 0.3 cm in diameter) containing Porapak N80/100 mesh with printer BD41, Kipp & Zonen, Solingen

Geiger counter	Contamat FHT 111G, Melit GmbH, Hinwil
Gel electrophoresis units	Easy cast Horizontal minigel system, models B1 & B3, peqlab Biotechnologie GmbH, Erlangen
Hot plate	Ceran 500, Harry Gestigkeit GmbH, Düsseldorf
Hot plate stirrer	IKA Combimag RCT, Ika Werk, Staufen i. Br.
HPLC	model 4500i, Dionex Corporation, Sunnyvale
Ice machine	UBE 125-100, Ziegra-Eismaschinen, Isernhagen
Incubator	BvW40, Memmert, Schwabach
	model 5420, Eppendorf-Netheler-Hinz GmbH, Hamburg
Light meter	LI-250, Li-Cor Inc., Lincoln, Nebraska
Microscopes	phase contrast microscope (63x – 400x), Zeiss, Oberkochen
	binocular Stemi SV11 (6x – 660x), Zeiss, Oberkochen
	digital scanning electrone microscope DSM940, Zeiss, Oberkochen
	Axiophot with camera, Zeiss, Oberkochen
Microwave	model 5001B, TEC
Water distillation unit	Fistreem water still, Fisons, Loughborough, England
Water purification systems	Mili-Q plus, reagent grade water purification system, Eschborn
	Mili-RO 10 plus, reverse osmosis water purification system, Eschborn
^{15}N-analyzer	NOI 5, VEB Straton, Fürstenwalde
	with printer BD8, Kipp & Zonen, Solingen
Oven	KM 170, Heraeus, Hanau
	TV 500L, Memmert, Schwabach
pH-electrode	WTW, Weilheim
pH meter	model 350, Knick, Berlin
Power supply	55-E815, peqlab Biotechnologie GmbH, Erlangen
Rotary shaker	MTS4, IKA Works, Inc., Wilmington, USA
spectrophotometer	Spectronic 301, Milton Roy, USA
	Uvikon 932, Bio-Tek, Kontron Instruments, Neufahrn
Sputter coater	model SCD 040, Balzers Union
Thermomixer	model 5436, Eppendorf-Netheler-Hinz GmbH, Hamburg
UV-Transilluminator	Fluo Link, Biometra, Göttingen
Vacuum pump	1004 A, Alcatel, France
	with pressure gauge VAP 5, Vakuubrand, Wertheim
Vortex mixer	MS1 Minishaker, IKA Works, Inc., Wilmington, USA
Waterbath	model 1083, GFL, Burgwedel

2.1.11 Data processing

Software: Microsoft® Word 97 SR-1 (Microsoft)
CorelDRAW 7(TM), Version 7.373 (Corel Corporation)
Microcal™ Origin™ 5.0 (Microcal Software, Inc.)
Scion Image (Scion Corporation)

2.2 Methods

2.2.1 Growth conditions and handling of organisms

2.2.1.1 Bacterial cultures

Bacterial strains, used in this study, are listed in Section 2.1.2. The various culture media used were NFb-medium for strains of the genera *Azospirillum* (YEP-medium for *glnA*-mutants), *Herbaspirillum* and *Azoarcus*, LGI-P-medium for *Acetobacter diazotrophicus*, a modified Burk´s medium for *Azotobacter vinelandii*, and LB-medium for *Escherichia coli* (Section 2.1.7). Antibiotics were added as appropriate (Sections 2.1.2 and 2.1.8).
The growth of overnight cultures was carried out in 100 ml-Erlenmeyer flasks containing 50 ml of corresponding medium. All strains were grown aerobically at 30° C or, in the case of *Escherichia coli* at 37° C. During incubation, the flasks were vigorously shaken at 140 rpm.

2.2.1.1.1 Adjustment of bacterial cell density

In order to adjust bacterial cell densities rapidly to a desired value, a standard curve was prepared to relate the cell number of a culture, obtained from microscopic count, to its turbidity (OD_{578}-units), measured with a spectrophotometer.
For this purpose, overnight cultures of *A. brasilense* Sp7 and *Azoarcus* BH72 were adjusted to different OD_{578}-units, where the cell numbers were simultaneously determined using a Thoma counting chamber (total area of a large square: 0.0025 mm^2, space between coverslip and slide: 0.1 mm).
Recorded data from both measurements was finally plotted on a double logarithmic scale in order to derive an equation formula. As strains of the genus *Azotobacter, Acetobacter, and Herbaspirillum* were referred to only once, cell densities were adjusted solely through the use of a Thoma counting chamber.

2.2.1.1.2 Preservation of bacterial cultures

Working strains of the genera *Azospirillum, Herbaspirillum,* and *Azoarcus* were preserved in 5 ml quantities on NFb-semisolid-medium (Section 2.1.7), contained in 10 ml-test tubes. Mutant strains, incapable of fixing nitrogen, were streaked out on

NFb-VK-agar. Working strains of *Acetobacter diazotrophicus, Azotobacter vinelandii*, and *Escherichia coli* were incubated on LGI-P-, Burk-, and LB-agar, respectively, and as in the liquid media, antibiotics were supplemented, where appropriate. Strains were grown aerobically at 30° C or, in case of *E. coli* at 37° C, and transferred weekly to new media.
For long-term preservations, well-grown overnight cultures of all strains were centrifuged for 15 min at 10000 rpm and resuspended in 5 ml PBS. Sterile glycerol was added giving final concentration of 15%, and the culture was subdivided into aliquots of 1 ml. Finally, strains were frozen at –85° C and stored for further usage.

2.2.1.1.3 Acetylene reduction assay

Activity of the nitrogen fixing strains was measured by the acetylene reduction assay (ARA), and thereby using the fact that nitrogenase not only reduces N_2 but also several other triply bonded compounds, such as acetylene.
The ability of the genera *Azospirillum*, *Herbaspirillum*, and *Azoarcus* to fix nitrogen was tested by using a nitrogen-free NFb-semisolid-medium (Section 2.1.7). After three days of growth, the culture vials were hermetically sealed with rubber stoppers, and 10% of the atmosphere was replaced by acetylene using a syringe. Following a further incubation period lasting at least one hour, acetylene production was measured using a gas chromatograph fitted with a stainless steel column (90 cm, 0.3 cm in diameter, containing Porapak N80/100 mesh) and equipped with a hydrogen flame ionization detector.

2.2.1.2 Plant cultures

Tomato seeds were surface sterilized by a 2 min immersion in 70% (v/v) ethanol followed by an incubation period in 7% sodium hypochlorite lasting 15 min. Using sterile distilled water the seeds were subsequently washed four times (10 min each) and stored in the last change of water for 16 hrs at 4° C in the dark. Throughout sterilization, the flask containing the seeds was shaken at a rate of 120 rpm at room temperature.
Treated seeds, placed on moistened filter paper in a sealed petri plate, were incubated in the dark at room temperature. In order to verify sterility, an aliquot of sterilized seeds was put on LB-agar and incubated at 35° C. After 96 hrs, uniform seedlings selected from the filter paper were placed on perforated discs, which were positioned in tubes containing 40 ml of a sterile nutrient solution based on the MPCL-basic-medium (Section 2.1.7). To maintain sterility as well as the air supply, tubes

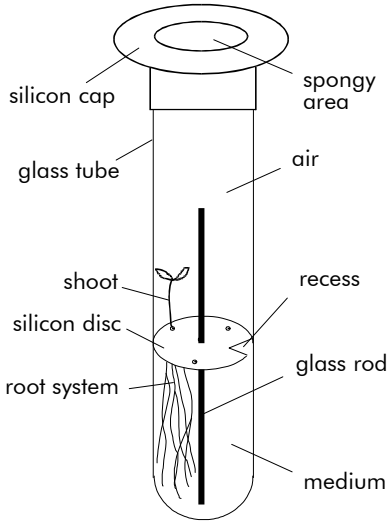


Figure 2.1 Hydroponic unit, used for growth of tomato plants

were closed by silicone sponge closures (Figure 2.1), whereby each tube contained up to three plants. These hydroponic units were grown at 24° C with the shoots illuminated for 16 hrs per day by a light flux density of 200 µmol · m^{-2} · s^{-1} and thereby exposed to a relative humidity of 65%. To protect the roots from light, the medium area was wrapped in black foil. After a growth period of 14 days, the hydroponic units were used for experiments. In order to overcome large changes in nutrient concentration, solutions were replaced regularly, usually after 7, 10, and 13 days of growth. For this purpose, the discs were equipped with a recess, making a quick exchange of medium possible without harming the root system.

2.2.1.2.1 Strategies of harvest

During these first 14 days of growth in the hydroponic units all plants were exposed to the same medium. At the day of harvest (usually day 14), this medium was renewed once more for 6 hrs. This period, referred to as pre-induction-period, started at the beginning of illumination and ensured that all plants were in the same physiological and, with regard to the *LeAMT1;2*-expression, transcriptional state.
Subsequently, plants were shifted to a medium, of which the specific influence on the expression of *LeAMT1;2* should be investigated. During this induction-period of 6 hrs, the medium was renewed once (usually after 4 hrs). Conclusively, plant roots were harvested, immediately frozen in liquid nitrogen, and stored in 50 ml-Falcon tubes at –85° C for further processing. The scheme in Figure 2.2 illustrates the principle, which the analysis of *LeAMT1;2*-expression followed.

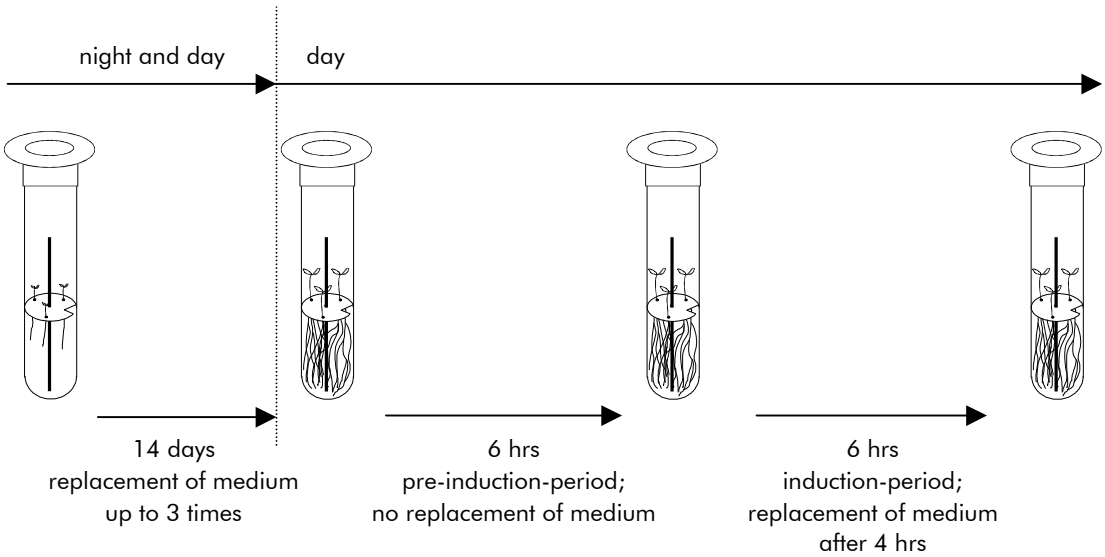


Figure 2.2 Experimental design for expression analysis of *LeAMT1;2*. For each factor to be investigated, 15 plants, contained in 5 tubes, were harvested. At first, plants were grown for 14 days. At the day of harvest, plants passed through a pre-induction-period starting with the day-period. In the following induction-period, plants were exposed to the condition that should be analyzed with respect to expression of *LeAMT1;2*. Root tissue was finally harvested after a further lapse of 6 hrs, during which the medium was renewed once.

2.2.1.2.2 Continuous cultivation

In order to ascertain that the pouring out and refilling of tubes does not harm plant roots, an experiment was designed specifically to keep plant cultures in a constant environment.
For this purpose, similar tubes were used as described in Section 2.2.1.2. These tubes however, were equipped with an inlet and an outlet enabling continuous renewal and removal of medium at a desired rate, controlled by a flow-rate regulator between medium reservoir and tubes.

2.2.1.2.3 Kinetic experiments

Kinetic experiments were carried out in order to follow the gene expression over a course of time. Plants subject to kinetic experiments, passed through the pre-induction-period as previously described and were then exposed to the condition of interest (induction-period). Thereupon, plants were harvested at regular intervals within a defined time period, and after 4 hrs, media of the remaining plants were usually renewed.

2.2.1.2.4 Inoculation experiments

In the bacterial experiments, where the expression of *LeAMT1;2* was analyzed in the presence of an inoculant, host plants were initially grown for 14 days in MPCL-low-NO_3^--medium. In order to ensure that all nitrogen traces were used up before the adding of bacteria, nitrogen-free MPCL-basic-medium was used in the pre-induction-period.
Bacterial overnight cultures were harvested by centrifugation (15 min at 10000 rpm), then double-washed and resuspended in 5-10 ml of nitrogen-free MPCL-basic-medium. The cell densities were then determined spectrophotometrically (Section 2.2.1.1.1), and a corresponding aliquot was diluted in MPCL-basic-medium to adjust the inoculum level (usually 10^7 cfu/ml). Unless a time course study with bacteria was carried out, the induction-period, starting with the addition of bacteria, was prolonged to 10 hrs. During this time period, where upon completion it was still daytime, the medium was not changed.

2.2.1.2.5 Use of bacterial supernatants

Bacterial supernatants, containing products excreted during growth, were tested for their capacity to regulate *LeAMT1;2*-transcription. For this purpose, five tubes containing nitrogen-free semisolid NFb-medium were prepared for each strain used in this study. As some strains were incapable to grow in a nitrogen-free medium, overnight cultures of all strains were firstly prepared in appropriate media. Subsequently, 50 µl of each culture was spinned down, then washed twice and resuspended in PBS. These washed cultures were finally used to inoculate semisolid NFb-media. Bacteria were incubated for 7 days at 37° C, after which tubes were

vigorously vortexed and spinned down for 5 min at 10000 rpm in a table top centrifuge. The liquid phase was then filtersterilized giving a volume of nitrogen-free MPCL-basic-medium suitable for the supply of all plants (dilution 1:20). As the medium was not renewed, the induction period was shortened to 3 hrs.

2.2.2 Microbial molecular biology

2.2.2.1 DNA transfer

The introduction of plasmid-DNA into bacterial cells took place either through transformation or conjugation. Strains of *Escherichia coli* received DNA through heat-shock transformation, whereas strains of *Azospirillum brasilense* were conjugated to donor cells of *Escherichia coli* S17-1. Prior to transformation, the cells were made receptive to plasmids (competent) through calcium treatment, which resulted in permeable cell membranes.

2.2.2.1.1 Making of competent cells

1 ml of an overnight culture of *Escherichia coli* DH5α-cells, grown at 37° C in 10 ml LB-medium, was transferred to 50 ml of fresh LB-medium and incubated at 37° C on a rotary shaker (140 rpm). When the culture reached an OD_{578} of 0.45 to 0.55, the cells were put on ice for 10 min and spinned down at 4° C (10 min at 3600 rpm). The pellet was resuspended in 20 ml of ice-cold Tfb I-buffer (Section 2.1.6), and the cells were put on ice for a further 5 min. Following a consequent centrifugation step at 4° C (5 min at 3600 rpm), bacteria were resuspended in 2 ml of ice-cold Tfb II-buffer (Section 2.1.6) and then put back on ice for 10 min. Finally, the cells were subdivided into aliquots of 100-200 µl and frozen at –85° C.

2.2.2.1.2 Heat-shock-transformation

For the purposes of amplification and storage, the vectors carrying cDNA of *LeAMT1;2* and *18S-rRNA* (Section 2.1.3) were introduced into competent cells of DH5α. Deep-frozen cells with artificially induced competence were thawn on ice for 15 min and 1 to 5 ng of the vector (pFL61 and pBS II KS ±, respectively) was pipetted to the cell-suspension, which was then incubated for another 20 min on ice. Subsequently, the cells were shifted to 42° C for 2 min (heat-shock) before being put back on ice for 15 min. After the addition of 1 ml LB-medium, bacteria were incubated for 1 hr at 37° C (recovery period) and, finally, aliquots of 10 to 100 µl were spreaded evenly over the surface of selective LB_{Amp}-agar using a sterile glass spreader. These plates were incubated at 37° C overnight after which, 5 to 10 colonies were picked and transferred to 50 ml of liquid LB_{Amp}-medium. Cultures were incubated overnight at 37° C on a rotary shaker (140 rpm) and tested for the presence of the transferred plasmid using plasmid isolation and restriction analysis

(Sections 2.2.2.2, 2.2.4.1, and 2.2.4.2). A positive culture was taken for long-term-preservation (Section 2.2.1.1.2).

2.2.2.1.3 Conjugation

As heat-shock-transformation proved ineffective with strains of *Azospirillum brasilense* (Holguin *et al.*, 1999), biparental bacterial mating was used to transfer plasmid pAB5320, carrying a N-terminal truncated *A. brasilense nifA*-gene, from *Escherichia coli* S17-1 to *Azospirillum brasilense* 7628. The nitrogen fixation ability of the latter strain was therefore restored.
Taking the antibiotic resistances into consideration (Section 2.1.2), cultures of both strains were grown overnight; 100 µl of each strain then being transferred to 5 ml of fresh medium. After 2 hrs of growth, where both strains entered the exponential phase, cells were centrifuged for 5 min at 6000 rpm. The pellets were resuspended in saline by pipetting, washed twice by further centrifugation steps, then mixed with three times more acceptor than donor, and spotted on YEP-agar. The agar plate was placed in a box covered with wet towels to ensure a humid atmosphere and after overnight incubation at 37° C, the mating mixture was completely scraped from the agar surface before being resuspended in saline. Serial dilutions of this suspension were plated on $NFb_{Km\text{-}Tc}$-VK-agar (Section 2.1.7) and incubated at 30° C.
After two days of growth, putative recipients of pAB5320 were transferred on NFb-agar as well as NFb-semisolid-medium, supplemented with both tetracycline and kanamycin. Strains were identified by light microscopy as well as by the acetylene reduction assay, which proved a successful conjugative transfer of pAB5320 to *Azospirillum brasilense* 7628 to have taken place.

2.2.2.2 Isolation of plasmid-DNA

Prior to isolation, plasmid-DNA was amplified within an overnight culture of transformed *Escherichia coli* DH5α-cells, using LB_{Amp}-medium. Isolation followed the protocol of either the ‚Wizard Minipreps DNA Purification System' or the ‚Promega-Midi-Caps-DNA-Purification-Kit', in case higher yields were needed. The concentration of plasmids, finally eluted in TE-buffer was determined spectrophotometrically as described in Section 2.2.3.3.

2.2.3 Plant molecular biology

2.2.3.1 General handling of RNA

As RNA in sample material or solution is subject to degradation by ubiquitous RNases, numerous precautions were taken while dealing with RNA. While handling reagents and RNA samples, a lab smock and nitril gloves were worn, thus preventing possible RNase contamination due to contact with the skin.

Pestle and mortar, glassware and metal spatulas were oven-baked at 220° C for at least 12 hours before use. Disposable plasticware (e.g. pipet tips or polypropylene tubes) was autoclaved twice, whereas non-disposable plasticware and electrophoresis tanks were treated with LTK-008™ (racksys Laborequipment GmbH) before being rinsed thoroughly with DEPC-H_2O. Solutions were treated with 0.1% DEPC, a strong inhibitor of RNAse.

2.2.3.2 Isolation of total RNA

Root tissue from 15 plants, originating from one sample of a specific experiment, was ground in liquid nitrogen using pestle and mortar. Total RNA-isolation was then carried out, following the protocol of the 'RNAeasy™ plant mini kit' (Qiagen) and RNA was finally eluted in 30 µl RNAse-free water before being stored at –85° C for further processing.

2.2.3.3 Quantitation of nucleic acids

The absorbance values of DNA and RNA at 260 nm (A_{260}) were used to estimate the concentration of nucleic acids in solution. At this wavelength, an absorbance unit of 1 corresponds to a RNA concentration of 40 µg/ml (standard, Stratagene). Prior to measurement, RNA-samples were diluted in DEPC-H_2O to get A_{260}-values between 0.15 and 0.5. The RNA, having recovered from isolation, was therefore determined by the formula:

$$\text{Total RNA [µg/ml]} = A_{260} \cdot 40\ \text{µg/ml} \cdot \text{dilution factor}$$

Based on this formula, the RNA-concentration of each sample was determined at least twice, and the mean value was calculated for future applications.
In addition, the absorbance values at 280 nm (A_{280}) and 320 nm (A_{320}) were measured. While the absorbance at 320 nm provided a background level, the absorbance ratio A_{260}/A_{280} of a sample could be considered an estimate of RNA purity. The ratio for pure aqueous RNA is 2.0.
The concentration of DNA was determined in a similar manner. As an A_{260}-value of 1 corresponds to a concentration of 50 µg/ml for a solution of double-stranded DNA the above formula was changed as follows:

$$\text{Total DNA [µg/ml]} = A_{260} \cdot 50\ \text{µg/ml} \cdot \text{dilution factor}$$

2.2.3.4 RNA gel electrophoresis

Prior to northern transfer to a nylon membrane, RNA gel electrophoresis was performed to check integrity and concentration of purified RNA or in order to fractionate RNA. To check RNA integrity, a denaturing agarose gel electrophoresis was carried out as follows: 0.3 g agarose was added to 21.5 ml DEPC-H_2O in a

baked Erlenmeyer flask. Using a microwave, the solution was brought to the boil twice, between which it was mixed by swirling the flask. After cooling to 60° C, 3 ml 10x MEN-buffer and 5.5 ml formaldehyde (37.5%) were added in a fume hood. Finally containing 1% of agarose, the solution was then poured into a gel mould and allowed to solidify for 30 min. A 10-well comb was used during this time to create slots for the samples to be separated. After the comb was removed carefully, the gel was placed in the electrophoresis apparatus and submerged in 1x MEN as running buffer.
An equal amount of each RNA-sample (usually 1-2 µl) was pipetted into a volume of RNA loading buffer three times greater. The suspension was heated to 65° C for 10 min before being stored on ice for 5 min. Samples were then loaded into gel slots and a potential of 80 V was applied for 1 hr. Then the gel was documented under UV-transillumination. The intensity of ethidiumbromide stained ribosomal bands was compared with the results of the absorbance measurements (Section 2.2.3.3), while integrity of RNA was assessed according to the sharpness of visible bands.
If the gel was to be subject to northern transfer after electrophoresis, gel and RNA-samples were prepared similarly. Depending on the electrophoresis apparatus used, however, the gel size was scaled up to a volume of 80 to 168 ml and generally contained 1% (w/v) agarose, 1x MEN-buffer and 2.42 M formaldehyde. Slots were created by 8- or 12-well combs, providing a capacity for samples holding at least 60 µl. To prepare samples, equal amounts of total RNA (10 μg) were suspended in RNA loading buffer lacking ethidiumbromide. The incubation time of samples was extended to 15 min at 65° C, and gel running conditions were changed to give a reduced voltage of 40 V with an extended running time of 4 hrs, where the running buffer was changed once.

2.2.3.5 Northern blot

After electrophoresis, the gel was incubated twice for 10 min in 10x SSC-buffer with gentle agitation (40 rpm). Subsequently, an upward capillary transfer was assembled, as shown in Figure 2.3. A baked glass plate was placed over the edges of a large glass tray, containing 10x SSC as transfer buffer. A single piece of Whatman 3MM filter paper was pre-moistened in 10x SSC and placed on the plate, such that each end hung to the bottom of the tray submerged in transfer buffer. The next layers, all of which were cut to the size of the gel, were made up with a further piece of pre-moistened Whatman 3MM paper, followed by the gel itself positioned upside-down and the nylon membrane also soaked in 10x SSC. Any air bubbles trapped between the layers were removed during the assembly of the blot. On the top of the membrane, 3 more pieces of dry Whatman 3MM filter paper as well as a layer of paper towels, approximately 10 cm in height, were piled. A glass plate was placed on top, with a weight of 4 g/cm^2 gel. Surrounding the bottom of the gel, four slices of Parafilm were placed such that contact was avoided with the paper above and below the gel.
After 20 hrs of transfer, all paper on top of the nylon membrane was removed and the well-positions on the blot were marked with a pen. The filter was briefly rinsed in

10x SSC and allowed to dry at 60° C. RNA was crosslinked to the nylon membrane using UV-illumination (0.8 J/cm^2), and finally, the membrane was stored at 4° C for further processing.

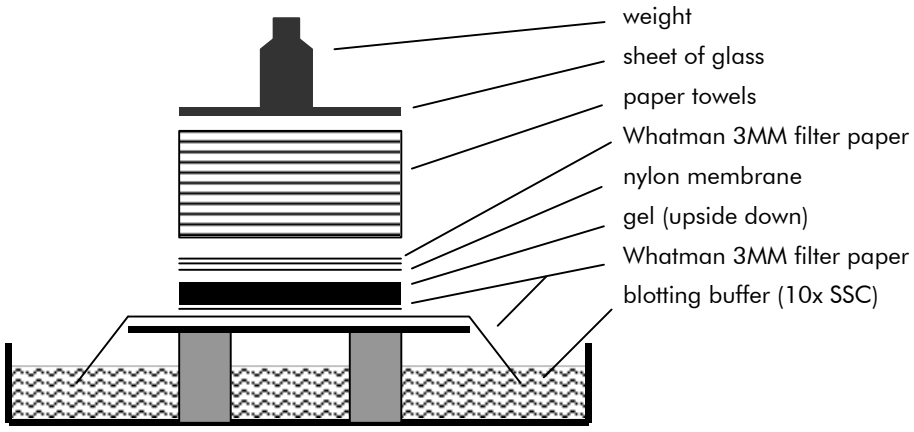


Figure 2.3 Transfer of electrophoretically fractionated RNA on a nylon membrane by means of a capillary blot. Whatman 3MM filter layers below the gel and nylon membrane were pre-moistened in 10x SSC-buffer.

2.2.4 Preparation of probes

In order to follow the expression of *LeAMT1;2* under given conditions, the amount of *LeAMT1;2*-transcripts within the entire RNA-population was determined. For this purpose, the nylon membrane containing the immobilized RNA was hybridized with a radioactively labelled *LeAMT1;2*-cDNA probe. To verify sample loading during gel electrophoresis, a *18S-rRNA*-cDNA probe of *Arabidopsis thaliana* was used as a control. This gene was assumed to have been equally expressed under all given conditions. The various preparation of probes included cleavage of plasmid DNA, fragment separation by DNA electrophoresis, elution of DNA fragments from agarose, as well as random prime labelling of probes.

2.2.4.1 Cleavage of plasmid-DNA with restriction endonucleases

Cleavage of both pFL61 and pBS II KS ± occurred via restriction enzymes purchased from Boehringer Mannheim. As the *LeAMT1;2*- and the *18S-rRNA*-gene were flanked by *Not*I- and *Eco*RI-restriction sites (Figure 2.4), respectively, the corresponding endonucleases were used according to the manufacturer´s instructions. For each microgram of DNA to be cut, 4 units of enzyme were added. For both of the endonucleases, 1x incubation buffer supplied with the enzyme was used. Restriction was carried out for at least 1 hr at 37° C in a final volume of 10 µl.

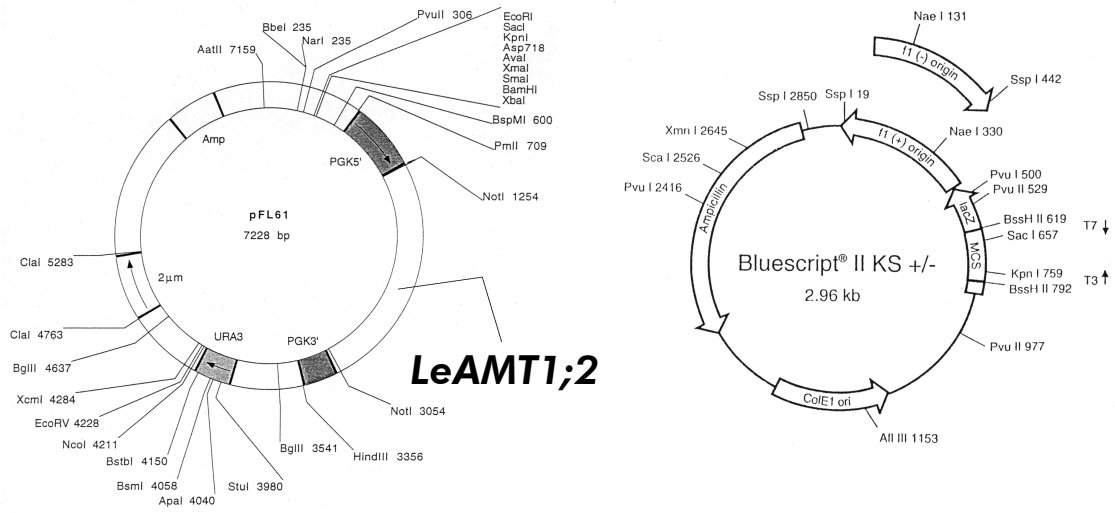


Figure 2.4 Restriction maps. (a) Restriction map of the pFL61 shuttle vector (Minet *et al.*, 1992). *LeAMT1;2* is flanked by *Not*I-restriction sites. (b) Restriction map of pBS II KS ± (Stratagene). The *18S-rRNA*-gene was cloned in *Eco*RI restriction sites (not shown), which is part of the multiple cloning site (MCS). Both plasmids encode for ampicillin resistance.

2.2.4.2 DNA gel electrophoresis

DNA gel electrophoresis was employed either to size fractionate DNA molecules, resulting from restriction enzyme digestion or to quickly determine purity and concentration of eluted fragments. Prior to gel casting, a microwave was used to dissolve agarose in 1x TBE-buffer giving a final concentration of 1% (w/v). A mould was fitted with a 10-well comb before the warm gel solution was poured in and allowed to solidify for 30 min. The gel was then submerged in 1x TBE buffer, contained in a horizontal electrophoresis apparatus. DNA samples were mixed with DNA loading buffer (5:1) and loaded into the sample wells. Electrophoresis was carried out at 80 to 100 V until the required separation has been achieved. In order to assign bands to specific sizes and concentrations, a DNA size marker (‚SmartLadder', Eurogentec) was co-electrophoresed (Figure 2.5). After electrophoresis, the gel was placed on a UV-transilluminator in order to view the separation pattern of the fluorescent, ethidium bromide-stained DNA. When necessary, the result was photographed with a Polaroid camera.

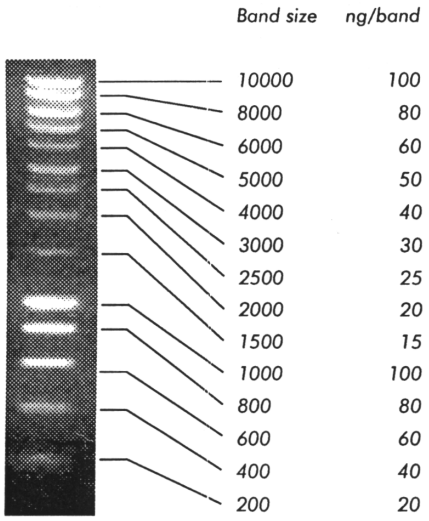


Figure 2.5 SmartLadder (Eurogentec). The DNA size marker allows the assignment of band sizes at the same time as their concentration.

2.2.4.3 Elution of DNA fragments from agarose gels

The band of interest was excised with a blade, and DNA-fragments were eluted from this gel slice following the protocol of the ‚Jetsorb-DNA-extraction-Kit' (Genomed).
In order to determine the purity and concentration of the fragment in a final eluate of 20 µl, an aliquot of 2 µl was subject to DNA gel electrophoresis (Section 2.2.4.2). A size marker (‚SmartLadder') allowed the identification of the band as well as the determination of its concentration.

2.2.4.4 Random prime labelling

To use DNA-probes in hybridization experiments, the fragments were radioactively labelled by random priming. The labelling of both *LeAMT1;2*- and *18S-rRNA*-cDNA followed the protocol of the ‚Ready To Go DNA Labelling Kit (-dCTP)' (Amersham). An amount of 150 ng of template-DNA was used for each probe that was labelled. The tube containing the complete reconstituted reaction mix was finally incubated for 45 min at 37° C, and the reaction was stopped with the addition of 150 µl of 0.5 M TE-buffer.

2.2.4.5 Purification of probes

In order to remove unincorporated nucleotides and short-sized DNA-fragments from the reaction mix, probes were purified with Pasteur pipettes as columns. The columns were basally stuffed with cotton, filled with Sephadex G50 (DNA grade fine) and equilibrated with 0.5 M TE-buffer before use. The reaction mix from Section 2.2.4.4. was pipetted into the column and subsequently rinsed with small portions of 0.5 M TE-buffer. The first 600 µl of radioactive outflow was collected and taken for hybridization.

2.2.4.6 Hybridization

Membranes were recognizably marked and successively submerged in a plastic box containing 125 µl P_i-buffer/cm^2 membrane. They were then prehybridized in a waterbath at 68° C for at least 2 hrs with constant agitation. Subsequently, the probe previously denatured by boiling, was added to fresh P_i-buffer, then the membranes were submerged again and incubated as described above. After overnight incubation, membranes were washed twice for 30 min in P_i-buffer at 68° C and residual radiation was checked with a Geiger counter.

2.2.4.7 Autoradiography

Membranes, sealed in plastic bags, were exposed overnight to X-ray film at –85° C using an exposure cassette. The film was developed, washed with tap water and fixed before being washed again and dried at room temperature. The localization of radioactivity on the membrane was revealed by photographic emulsion as a pattern

with silver grains. Mean densities of signals were quantified using the ‚Scion image'-software and adjusted to signal intensities obtained from hybridization with the *18S-rRNA*-probe.

2.2.4.8 Stripping of membranes

To re-hybridize membranes with the *18S-rRNA*-probe, the *LeAMT1;2*-hybrids were removed by boiling the blots in distilled water for 30 min. This step was repeated reducing incidence of residual radiation, then membranes were subject to further hybridizations as described in Section 2.2.4.6.

2.2.5 Analysis of root tissues and supernatants

Samples of root tissues and supernatants of plant and bacterial cultures were tested for organic acids, amino acids, free ammonium, and soluble carbohydrate concentrations. The amino acids were separated by ion exchange chromatography (Biotronik LC 5001 analyzer, ninhydrin post-column derivatization), then identified and quantified using a mixture of amino acids (Benson standard PANB). The organic acids (malate, oxalate) and carbohydrates were separated by a model 4500i Dionex Ion Chromatograph with a Carbopac column and detected by conductivity, identified and quantified using a mixture of organic acids and carbohydrates. Free ammonium was determined using an ammonium analyzer (GAT-Wescan). With the exception of the ammonium analyses within the scope of the kinetic experiment (Section 2.2.5.3), all analyses were carried out in the Research group of Prof. Dr. W. Kaiser (Julius-von-Sachs-Institut, Würzburg).

2.2.5.1 Analysis of ammonium content in plant roots

The analysis of root ammonium content was carried out in order to determine a correlation between the *LeAMT1;2*-expression under particular circumstances, and the level of ammonium in the root. Plants used in this study were grown for 14 days in MPCL-low-NH_4^+-medium as described in Section 2.2.1.2 including a pre-induction-period (Section 2.2.1.2.1). They were then shifted to MPCL-basic-medium, supplemented with 50 µM NH_4^+ (pH 6 and pH 4.7), or 5 mM NH_4^+, or 50 µM NH_4^+ + 1 mM MSX, or 50 µM NH_4^+ + 15 mM glutamine, or 50 µM NH_4^+ + 15 mM glutamate.
Three to five replicates were prepared for each sample. Plants were harvested after 6 hrs of incubation, during which the medium was changed after 4 hrs. After harvest, the roots were gently washed twice in an ice-cold, isotonic $CaCl_2$-solution (53 mM) and dabbed using a paper towel. The fresh weight was determined with scales. Roots were then gound in liquid nitrogen using a pestle and mortar, and 10 ml of distilled water were added per gram of the fresh weight. The ice crystals which formed immediately in liquid nitrogen, were likewise ground. The fine powder was transferred to a 2 ml-Eppendorf tube, and the sample was boiled for two minutes in

order to destroy residual enzyme activity irreversibly. Finally, cell debris was spinned down at 13000 rpm, and the supernatant was immediately frozen at –20° C for analysis.

2.2.5.2 Analysis of ammonium content in bacterial supernatants

Wild types and mutant strains of the genera *Azospirillum* and *Azoarcus* were grown for seven days in nitrogen-free semisolid NFb-medium, enabling bacteria to fix dinitrogen from the atmosphere. Cultures were then vigorously vortexed, destroying the gel state and centrifuged for 5 min at 10000 rpm. The liquid supernatant was transferred to a new Falcon tube using filtersterilization and boiled for 2 min as described in Section 2.2.5.1. Samples were frozen at –20° C for analysis.

2.2.5.3 Analysis of ammonium content in the plant medium

In order to determine the ammonium uptake kinetics of roots, plants were initially grown for 14 days, as described in Section 2.2.1.2. To distinguish the different states of nitrogen nutrition, growth was performed under a low-NO_3^--, a high-NO_3^-- or a low-NH_4^+-regime. Medium of the latter sample was changed for the last time 1 hr before the experiment started in order to obtain an induced state of ammonium transport.
During the experiment, plants were exposed to MPCL-basic-medium supplemented with 50 µM NH_4^+. Plant medium samples of 1 ml were collected at regular intervals over the next 27 hrs, and the discs carrying the plants were adjusted to medium level, as there was no replacement of removed medium. Samples were frozen at -20° C for further processing.
After the experiment, roots were harvested, gently dabbed on a paper towel, and the fresh weight was determined with scales.
As previously mentioned in Section 2.2.5, ammonium content of medium samples was determined with the ‚Ammonia test-combination' from Boehringer Mannheim, according to the manufacturer´s instructions.

2.2.5.4 Analysis of plant medium for root exudates

The analysis of root exudates, regarding to organic acids, soluble carbohydrates, and amino acids, was carried out as described in Section 2.2.5. In order to meet the strong dilution effect, to which compounds were subject to after excretion, media were not changed throughout the overall incubation period of 25 days. As growth started, medium was either rich in nitrogen (MPCL-high-NO_3^--medium) or poor (MPCL-low-NO_3^--medium), thus resulting in plants with a nitrogen-repleted or nitrogen-depleted state. Plant medium was frozen at –20° C for analysis.

2.2.6 Root colonization

Colonization of plant roots with *Azospirillum brasilense* was followed using a *gusA*-expressing wild type strain, while the colonization pattern of inoculated roots with *Azoarcus* BH72 was documented through the use of electron microscopy.

2.2.6.1 Analysis of root colonization by *Azospirillum brasilense*

A strain of *Azospirillum brasilense* Sp7, carrying the *gusA* reporter plasmid pFAJ31.13 (Vande Broek, 1994) was used to follow the colonization pattern on the tomato roots. In pFAJ31.13, *gusA* was fused with an *Azospirillum brasilense* promoter and showed constitutive expression in cells harbouring the plasmid. The activity of *b-glucuronidase*, the enzyme encoded by *gusA*, was monitored using X-Gluc as the substrate. Hence, bacteria were detected as blue zones on the root surface.
Plants subject to root colonization experiments, were grown for 14 days in MPCL-low-NO_3^--medium as described in Section 2.2.1.2. *Azospirillum brasilense*, carrying pFAJ31.13, was then added giving a final density of 10^7 cfu/ml as described in Section 2.2.1.2.4. After 48 hrs of incubation roots were harvested, washed twice in PBS and stained in GUS-staining solution at 37° C (Section 2.1.6). When first signs of blue staining were observed, roots were removed from the staining solution and washed three times in KP_i-buffer. The colonization pattern was then observed using bright- and dark-field microscopy and documented photographically.

2.2.6.2 Analysis of root colonization by *Azoarcus* Sp BH72

The colonization pattern of *Azoarcus* on tomato roots was followed using a digital scanning microscope. Growth and inoculation of plants was carried out as for the *gusA*-strain of *Azospirillum brasilense* described in the previous section. After 48 hrs of incubation, root segments about 1 cm in length were fixed in DMA-buffer for 1 hr and then in aqueous solutions of 30, 50, 70, 90 and 99.8% ethanol sequentially dehydrated in each for 20 min. The specimen was then dried in a CO_2-atmosphere in a critical point drying apparatus and coated with gold using a sputtering system. Root segments were observed and photographically documented using a digital scanning electron microscope. This part of the work was carried out in the Research group of Prof. Dr. H. Diekmann (Institut für Mikrobiologie, Hannover).

2.2.7 Nitrogen fixation assays

Three methods were applied to show evidence of nitrogen (N_2) fixation in the association of tomato plants with strains of *Azospirillum* or *Azoarcus*:

1. Wild type strain *Azospirillum brasilense* Sp245 carrying the *nifH-gusA* fusion plasmid pFAJ21 (Vande Broek, 1994) was used to monitor *b-glucuronidase* activity during incubation with tomato plants.

2. Acetylene reduction was used for semiquantitative studies to determine the potential nitrogen-fixation activity in the plant-bacteria-system.
3. The ^{15}N-isotope technique was used to follow the incorporation of $^{15}N_2$ in plants via diazotrophic bacteria.

2.2.7.1 Monitoring N_2 fixation using the GUS reporter gene

The expression of bacterial *nifH* encoding for dinitrogenase reductase was followed using the *nifH-gusA* fusion plasmid pFAJ21. Cells harbouring the plasmid express *b-glucuronidase*, when fixing nitrogen.
Plants were grown for 14 days, in MPCL-low-NO_3^--medium as described in Section 2.2.1.2. An overnight culture of *Azospirillum brasilense* WT carrying pFAJ21 was then prepared for inoculation as described in Section 2.2.1.2.4 (10^7 cfu/ml), resuspended in MPCL-basic-medium, and added to the hydroponic unit. After 96 hrs of incubation, the medium was replaced by MPCL-basic-medium supplemented with 0.5 mg/ml X-Gluc. Plant roots were then incubated until the first signs of blue-staining were detected. Roots were observed by bright-field microscopy and photographically documented.

2.2.7.2 Monitoring N_2 fixation using ARA

Acetylene reduction assays were conducted in plant tubes, containing hydroponically grown tomato plants, inoculated with strains of *Azospirillum brasilense* and *Azoarcus*, respectively. In addition to the wild types, the *nifD*-mutant of *Azospirillum brasilense* (strain 7317) was used as inoculant. Prior to inoculation, plants were grown for 14 days in MPCL-low-NO_3^--medium. Diazotrophic bacteria were then added to give a final density of 10^7 cfu/ml or 10^8 cfu/ml, as described in Section 2.2.1.2.4. Media were used as liquid as well as in a semisolid state, and both were partly supplemented with 0.1% fructose. After 1, 3, or 6 days, tubes were sealed with airtight rubber stoppers, and acetylene was added to give a final concentration of about 10% (v/v) in headspace. The headspace was analyzed within regular intervals for ethylene content by flame ionization gas chromatography.

2.2.7.3 Monitoring N_2 fixation using ^{15}N-enriched gas technique

Strains, used in this study, were the wild types of *Azoarcus* and *Azospirillum brasilense*. Plants were grown for 10 days before inoculants were added, using three different approaches. Firstly, inoculation density was increased to 10^8 cfu/ml in liquid medium. Secondly, inoculation density was adjusted to 10^7 cfu/ml using semisolid medium. Thirdly, inoculation occurred as described in the second approach and fructose was added to give a final concentration of 0.1%. After the addition of bacteria, plants were incubated for 6 days enabling bacteria to colonize the roots. Plant tubes were then sealed with airtight rubber stoppers, and the headspace was enriched with 20 atom% $^{15}N_2$ by replacing a corresponding amount of the gaseous phase. Plants were then incubated for 6 days and harvested by separating the roots

and shoots. Plant material was dried at 60° C for 4 days, and dry weight of each sample was determined with scales. Subsequently, tissue was mechanically ground to a fine powder and subject to Kjeldahl analysis.

2.2.7.3.1 Semimakro-Kjeldahl analysis

The Kjeldahl method is an analytical method serving to determine nitrogen in the trinegative state in certain organic compounds. The wet digestion of dry plant material was carried out following a procedure given by Bergersen (1980).
5-10 mg of each tissue sample to be analyzed was incubated for 15 min in a mixture of 500 µl ‚digestive solution' (Section 2.1.6) and 50µl H_2O_2 (35%) at 330° C. Digestion was conducted in 10 ml Erlenmeyer flasks, which were closed in order to avoid evaporation. After digestion of the sample was complete, the digestion mixture was adjusted to pH 4-6, using 3.6 N NaOH. Subsequently, ammomium content was enzymatically analyzed using the ‚Ammonia test-combination' from Boehringer Mannheim, according to the manufacturer´s instructions. The nitrogen concentration of harvested tissue was then calculated by simply dividing the determined ammonium content by the amount of sample, originally used for Kjeldahl digestion.

2.2.7.3.2 Determination of ^{15}N-enrichment

In order to determine, whether the $^{15}N/^{14}N$ ratio of inoculated tissues was measurably different from the constant $^{15}N/^{14}N$ ratio of 0.3663% present in the atmosphere, the ^{15}N-enrichment in sample tissues was determined by emission spectroscopy. Based on the nitrogen concentration of harvested tissues calculated from Kjeldahl analysis, 7.5 µg of nitrogen were loaded in a discharge tube, mixed with CaO and copper filings, evacuated to give about 0.4 kPa pressure and finally sealed using a welding torch. CaO was preheated at 1000° C for 12 hrs before use. Bound nitrogen, contained in sealed tubes was then converted to N_2 gas following the method first described by Dumas (Fiedler and Proksch, 1975). Percentual abundance of ^{15}N was then estimated using an emission spectrometer.

2.2.8 Plant morphology

Plant morphology was examined after inoculation of plants with plant growth regulators indole-3-acetic acid, abscisic acid, kinetin, and gibberellic acid. They were used at final concentrations of 0.5 µM and 5 µM, respectively. Among the parameters measured were shoot length, root weight, number of lateral roots, and overall length of root. For each sample, data of 10 replicates was collected and compared with plant morphology in the absence of plant growth regulators. A two-population t-test was employed to test if the means of parameters were equal.
Plants received growth regulators immediately after transfer to the hydroponic units when seedlings showed root lengths of approximately 1-2 cm. Media were based on MPCL-basic-medium supplemented with 50 µM KNO_3. Growth regulators were

added by filtersterilization and medium was replaced on a weekly basis. Plants were harvested after 21 days of incubation.
After harvest, roots were analyzed by spreading out each root system on black background. Individual root segments were isolated from each other in order to count the number of lateral roots. Stretching of segments enabled the measurement of root length using a ruler. Roots were subsequently dried at 60° C until weight remained constant. Shoot length was also determined using a ruler.

2.2.8.1 Bacterial induced plant morphology

Plant morphology was also observed in the presence of bacteria. Apart from the wild type strains of *Azoarcus* and *Azospirillum brasilense*, strains FT326 (IAA-hyperproducing strain) and SpM7918 (IAA-hypoproducing strain) were used for inoculation.
Bacteria were added to plant medium as soon as seedlings were transferred to hydroponic units. Inoculation density was adjusted to 10^7 cfu/ml and MPCL-basic-medium supplemented with 50µM KNO_3 was taken as a basis. After 7 days of incubation, upon which bacteria were supposed to have colonized, medium was replaced by MPCL-basic-medium supplemented with 50µM KNO_3. Plants were harvested at day 21 of incubation, and parameters mentioned in Section 2.2.8 were analyzed.

3 Results

3.1 Basics

3.1.1 Relating optical density to total cell count

Strains of *Azospirillum brasilense* and *Azoarcus* were adjusted to four different OD_{578} units and related to the total cell number, simultaneously determined by direct microscopic count. The graphs reflecting these relationships are shown in Figure 3.1.

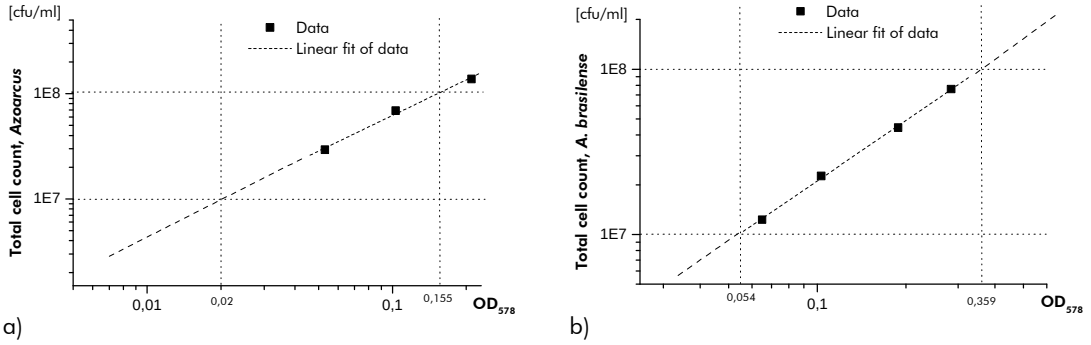


Figure 3.1 Total cell number as a function of turbidity presented on a double logarithmic scale. Total cell number was determined by direct microscopic count, whereas turbidity was measured in a spectrophotometer at 578 nm. Data is presented as a scatter graph and fitted lineary. Auxiliary lines, intersecting with the graphs, show the cell densities most frequently used, namely 10^7 and 10^8 cfu/ml. Plotted are *Azoarcus* Sp BH72 (a) and *Azospirillum brasilense* (b).

The mathematical formula, reflecting the relationship between turbidity units (OD_{578}) and total cell count (TCC), is as given.

$$OD_{578} = \left(\frac{TCC}{10^{intercept}} \right)^{\frac{1}{slope}}$$

Slope- and intercept values for strains were calculated to give the below results.

Strain	Intercept	Slope
Azospirillum brasilense	8.5419	1.2175
Azoarcus Sp BH72	8.9132	1.1211

3.2 *Root colonization*

3.2.1 Root colonization by *Azospirillum brasilense*

Root colonization by *Azospirillum brasilense* was monitored by means of reporter plasmid pFAJ31.13, showing constitutive expression of *gusA* (Section 2.2.6.1).

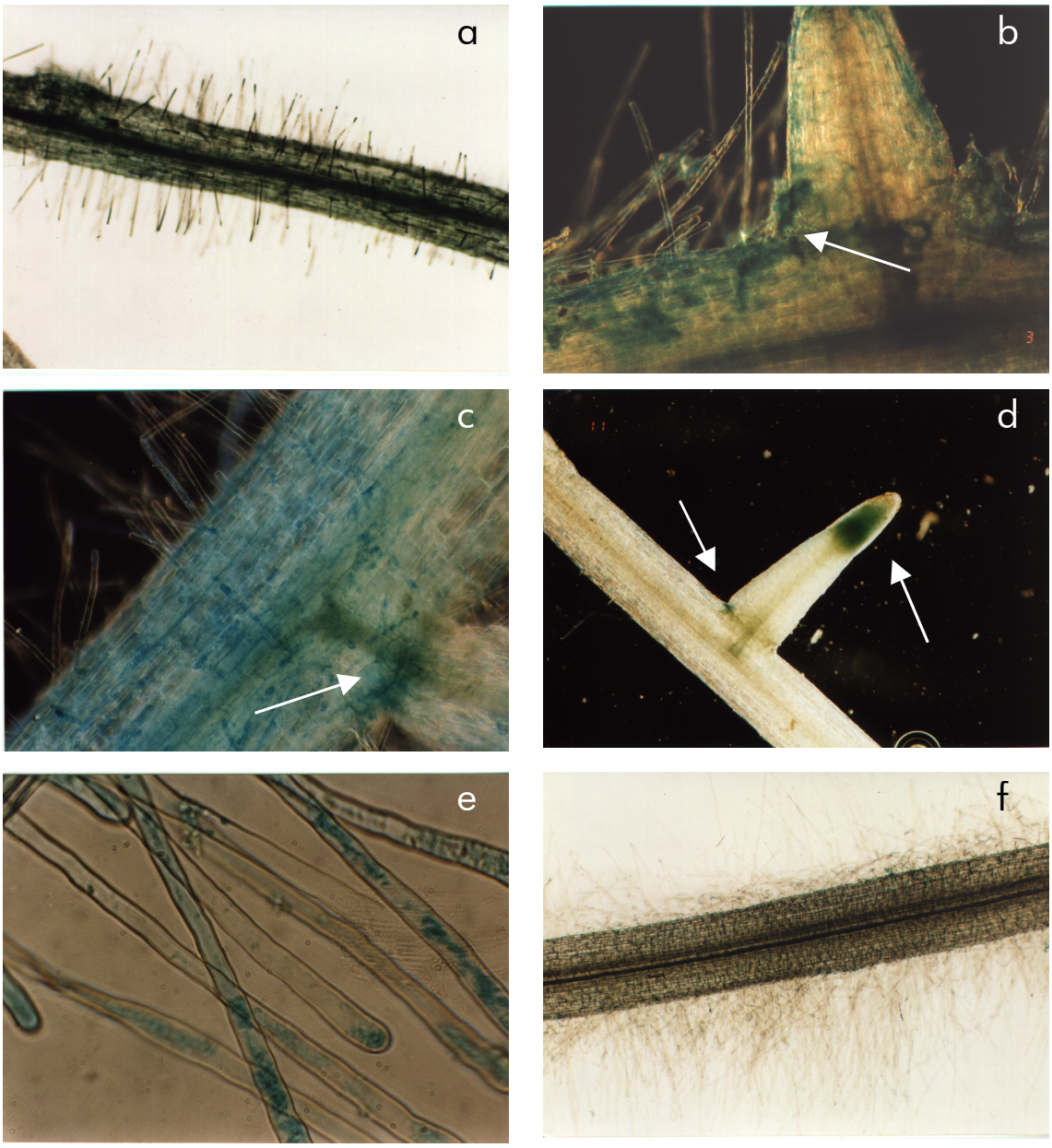


Figure 3.2 Colonization of tomato roots by *Azospirillum brasilense* expressing *gusA*. Magnitude is noted in parentheses. (a) Photograph of a root segment from the root hair zone, showing a widespread colonization, pronounced especially on root hair tips (50 x). (b) and (c) The sites of lateral root emergence were also preferentially colonized (200 x). (d) Emergence of a young lateral root. First signs of staining appeared frequently on root tips or at the basis of laterals (50 x). (e) Root hairs of tomato, showing intensive staining (400 x). (f) Control plants grown without inoculum (50 x). In all treatments staining with X-Gluc was performed for at least 48 hrs at 35° C.

The colonization pattern of *Azospirillum brasilense* on roots of tomatoes was observed on at least 20 different plants. Figure 3.2 illustrates the main sites of colonization, such as root tips, sites of lateral root emergence, and root hairs. In control plants incubated without inoculum, no background staining was observed even after one week of incubation in the presence of X-Gluc, proving *b-glucuronidase* activities were solely restricted to bacteria.

3.2.2 Root colonization by *Azoarcus* Sp BH72

Colonization of tomato roots by *Azoarcus* Sp BH72 was carried out using a digital scanning microscope. Photographs are presented in Figure 3.3.

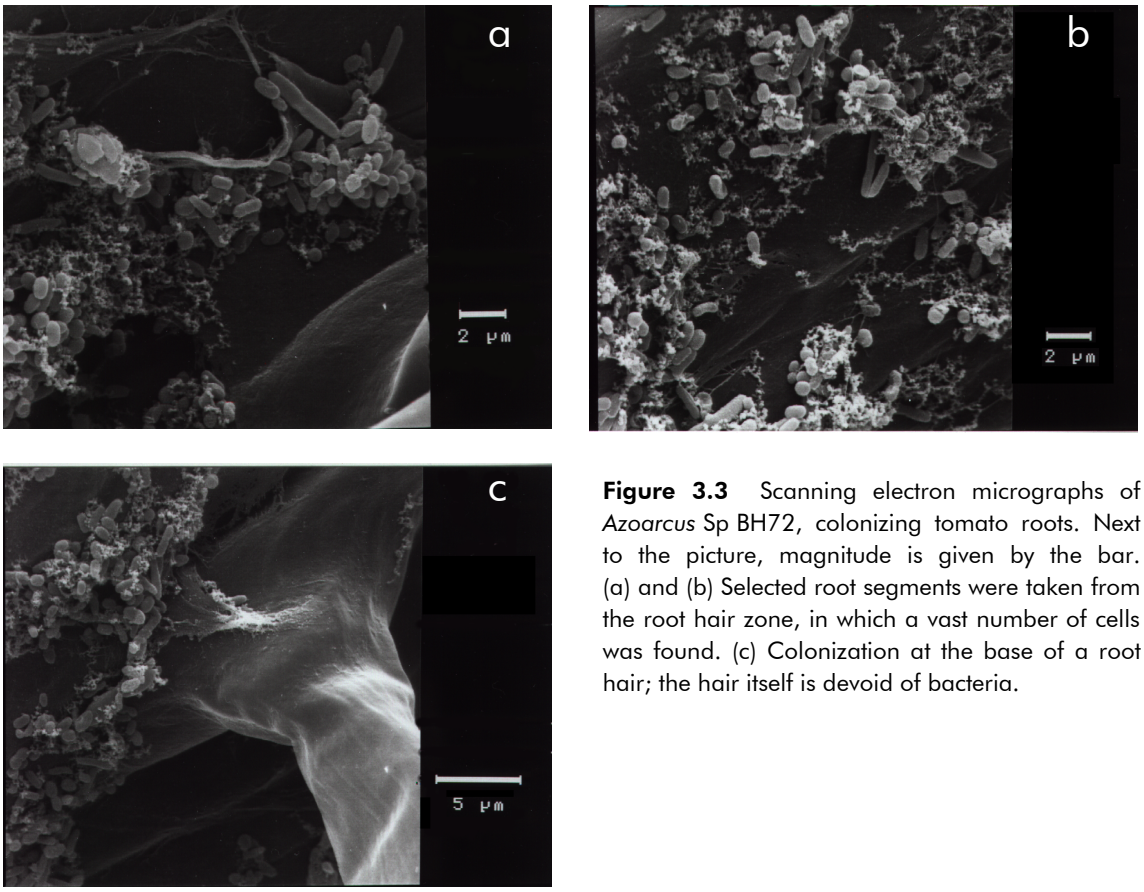


Figure 3.3 Scanning electron micrographs of *Azoarcus* Sp BH72, colonizing tomato roots. Next to the picture, magnitude is given by the bar. (a) and (b) Selected root segments were taken from the root hair zone, in which a vast number of cells was found. (c) Colonization at the base of a root hair; the hair itself is devoid of bacteria.

As seen in Figure 3.3, the root segments prepared for SEM-observation, showed a huge number of bacterial cells on the entire surface.

3.3 *Expression of NH_4^+-transporter, LeAMT1;2*

3.3.1 *LeAMT1;2*-expression without inoculum

In order to obtain a more detailed insight in the expression of *LeAMT1;2*, numerous experiments were carried out, in which the expression of the gene was followed without the participation of bacteria. Among the substrates tested were key metabolites of nitrogen metabolism, as well as phytohormones and substances affecting processes in the cell membrane. Furthermore, kinetic experiments were conducted in order to evaluate the time of response after ammonium had been added. In all cases, root-RNA was subject to northern analysis. Leaf-tissue was not examined.

3.3.1.1 *LeAMT1;2*-expression in response to ammonium

This experiment was originally designed to evaluate any presence of stress with respect to the expression of *LeAMT1;2* when replacing the plant medium. For this purpose, three different approaches were tested, namely, the medium was changed once, hourly or continuously after the start of the induction period.
Plants were previously grown in MPCL-low-NO_3^--medium. During the induction period, in which plants received 5 µM NH_4^+ instead of nitrate, plants were harvested before shift as well as after 1, 2, and 4 hrs of incubation. Results are presented in Figure 3.4.

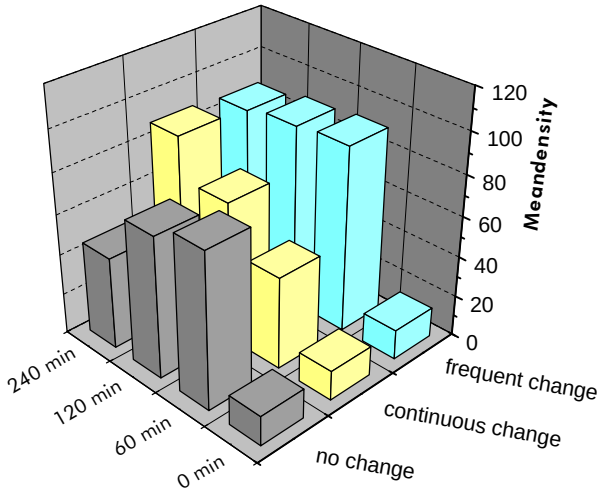


Figure 3.4 Expression of *LeAMT1;2* over a course of time after first exposure to NH_4^+. In all three approaches of medium replacement, induction could be observed after only 1 hr of incubation. Leaving out further changes of medium during induction period, however, resulted in a continuous decline of the signal. In contrast, the expression was maintained at a high level, when frequent changes of medium occurred. A continuous supply of fresh medium caused an increasing signal in time, where peak expression was gained after 4 hrs.

In order to scrutinize threshold concentration, below which no induction can be observed , a similar experiment was carried out. In this case, nitrogen depleted plants previously grown in MPCL-low-NO_3^--medium, were shifted to MPCL-basic-medium supplemented with ammonium, with a concentration ranging from 1 – 5 µM. The expression pattern revealed peak expression of *LeAMT1;2* at an external ammonium concentration of only 2 µM (Figure 3.5). Even in the presence of only 1 µM ammonium the signal intensity exceeded the background level by 1.5-fold.

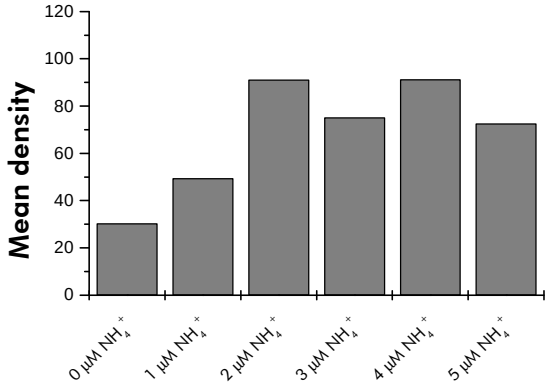


Figure 3.5 Expression of *LeAMT1;2* in the low micromolar range. Nitrogen-depleted plants were shifted from MPCL-low-NO_3^--medium to different concentrations of ammonium covering 1 – 5 µM. Peak expression was observed in the presence of only 2 µM ammonium.

3.3.1.2 *LeAMT1;2*-expression in response to nitrogen starvation

In order to evaluate, whether a continuous lack of nitrogen causes an increase of *LeAMT1;2*-transcripts, plants were grown for 14 days in MPCL-high-NO_3^--medium, which resulted in a nitrogen-repleted state. Plant roots were then shifted to nitrogen-free MPCL-basic-medium and harvested before shift as well as after 6, 24, 96, and 168 hrs of incubation. The results obtained from this experiment are shown in Figure 3.6. As it turned out, the expression level remained very low throughout incubation period.

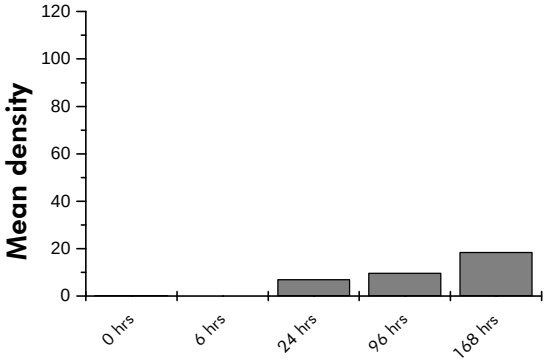


Figure 3.6 *LeAMT1;2*-expression in response to nitrogen starvation. No transcripts were detected in nitrogen-repleted plants. First signs of expression were not observed before 24 hrs of incubation in nitrogen-free medium. The level of expression remained very low throughout the experiment, although signal intensity measured at 96 hrs had doubled after 168 hrs of incubation.

3.3.1.3 *LeAMT1;2*-expression in response to daytime

This experiment should clarify, if the expression of ammonium transporter *LeAMT1;2* is subject to continuous variations, with peak values at daytime and low values during the night. The revelation of such a diurnal pattern would relate *LeAMT1;2*-expression to light interception.

Throughout the experiment, plants were grown in MPCL-low-NH_4^+-medium. At the outset of day 14, plants were harvested every three hours over a period of 21 hrs.

With the exception of the very first sample, media were always replaced 2 hrs before the next harvest. The diurnal expression pattern is shown in Figure 3.7.
During day period, signals revealed an increased intensity with time of light exposure, although the expression temporarily decreased after 9 hrs of daytime, suggesting a type of oscillation.

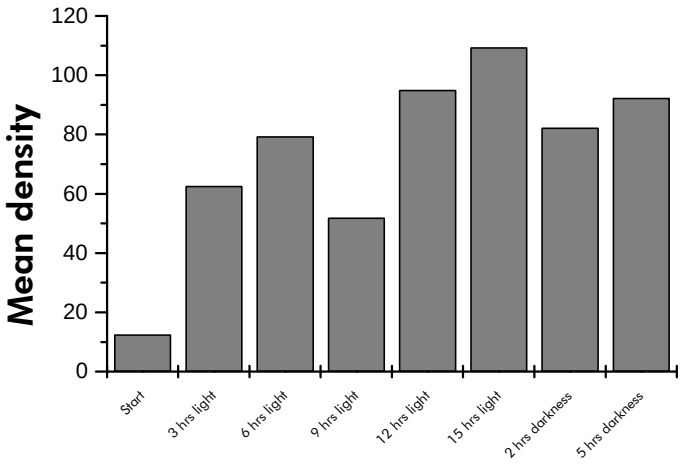
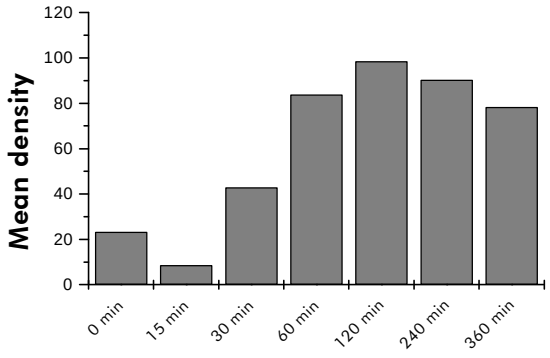


Figure 3.7 *LeAMT1;2*-expression in response to daytime. Plants were harvested every 3 hrs throughout the day accompanied by a regular renewal of the medium. Signal intensities generally were high, with a tendency towards peak expression at the end of daytime, although a temporary decrease of expression after 9 hrs of light exposure occurred. As the night period started, only a slight decrease in the transcript-level could be observed.

3.3.1.4 *LeAMT1;2*-expression in response to nitrate

The following experiments deal with aspects of nitrogen assimilation and metabolism in plants. The first experiment in this context was designed to test the *LeAMT1;2*-expression for a large nitrate supply over a course of time. In order to obtain plants both with nitrogen depletion and having grown up without nitrate supply, MPCL-low-NH_4^+-medium was used during the first 14 days of incubation. To avoid expression being conditional on the presence of ammonium, medium was changed for the last time 24 hrs before the induction period started. The kinetics of expression was finally followed in MPCL-NO_3^--medium, thus plants were provided with 15 mM nitrate.
As shown in Figure 3.8, initial expression observed before shift was weak. The transcript-level, however, increased quickly by severalfold when nitrate was supplied, whereby a peak was reached after 120 min of exposure. Afterwards, signal intensities decreased slightly but remained high until 360 min after the first exposure.

Figure 3.8 *LeAMT1;2*-expression in response to a high concentration of nitrate. The expression pattern is reflected by a bell-shaped histogram.



3.3.1.5 *LeAMT1;2*-expression in response to nitrite

One of the key metabolites in nitrogen metabolism, the toxic intermediate nitrite, was tested for its influence on *LeAMT1;2*-expression. Plants were initially grown for 14 days in MPCL-low-NH_4^+-medium. They were then shifted to a MPCL-low-NH_4^+- or a nitrogen-free medium, each sample being either in the presence of 5 mM KNO_2 or in the absence. The effect of nitrite is shown in Figure 3.9.
The strongest signal was observed in the presence of ammonium alone, whereby intensity was more than three times higher than that in nitrogen-free medium. Nitrite turned out to be a strong repressor of *LeAMT1;2*-transcription, independent of the presence of ammonium.

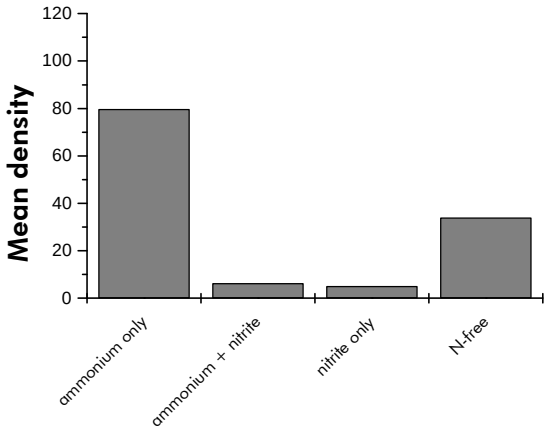


Figure 3.9 *LeAMT1;2*-expression in response to externally supplied nitrite. In roots, nitrite caused a strong decrease of signal intensities, even in the presence of ammonium. Peripheral columns confirmed the strong response to ammonium, as previously observed.

3.3.1.6 *LeAMT1;2*-expression in response to amino acids and sugar

Diverse amino acids and sugar compounds were tested for their capacity to affect the expression of *LeAMT1;2*. Considering the nitrogen status, the carbohydrates sucrose, glucose, and fructose were used in combination with ammonium. In addition, amino acids glutamine, alanine, and γ-aminobutyric acid (GABA) were supplied to nitrogen-depleted plants to test a possible feedback inhibition of *LeAMT1;2*-transcription in the presence of ammonium. Finally, methionine sulfoximine (MSX), a specific inhibitor of glutamine synthetase, was added to ammonium-supplemented media, in order to examine the consequence in blocking the most important assimilation pathway for ammonium.
Plants exposed to the above mentioned carbohydrates were previously grown in MPCL-high-NO_3^-- and MPCL-low-NO_3^--medium, resulting in either nitrogen-repleted or -depleted plants. They were then shifted to MPCL-basic-medium, supplemented with 50 µM ammonium and 10 mM of the corresponding C-sources.
Plants exposed to amino acids were grown for 14 days in MPCL-low-NH_4^+-medium. During the induction period this medium was additionally supplemented with nitrate,

glutamine, and alanine (15 mM each). Control plants received a nitrogen-free medium. As GABA was used in the frame of a different experiment, these plants were previously treated like the carbohydrate-supplemented samples. Final concentration of GABA however, was also 15 mM.
Plants that received MSX (1 mM) were grown previously in MPCL-low-NH_4^+-medium. They were then shifted to 50 µM and 5 mM ammonium, respectively in the presence of MSX or in the absence. Results are presented in Figure 3.10.

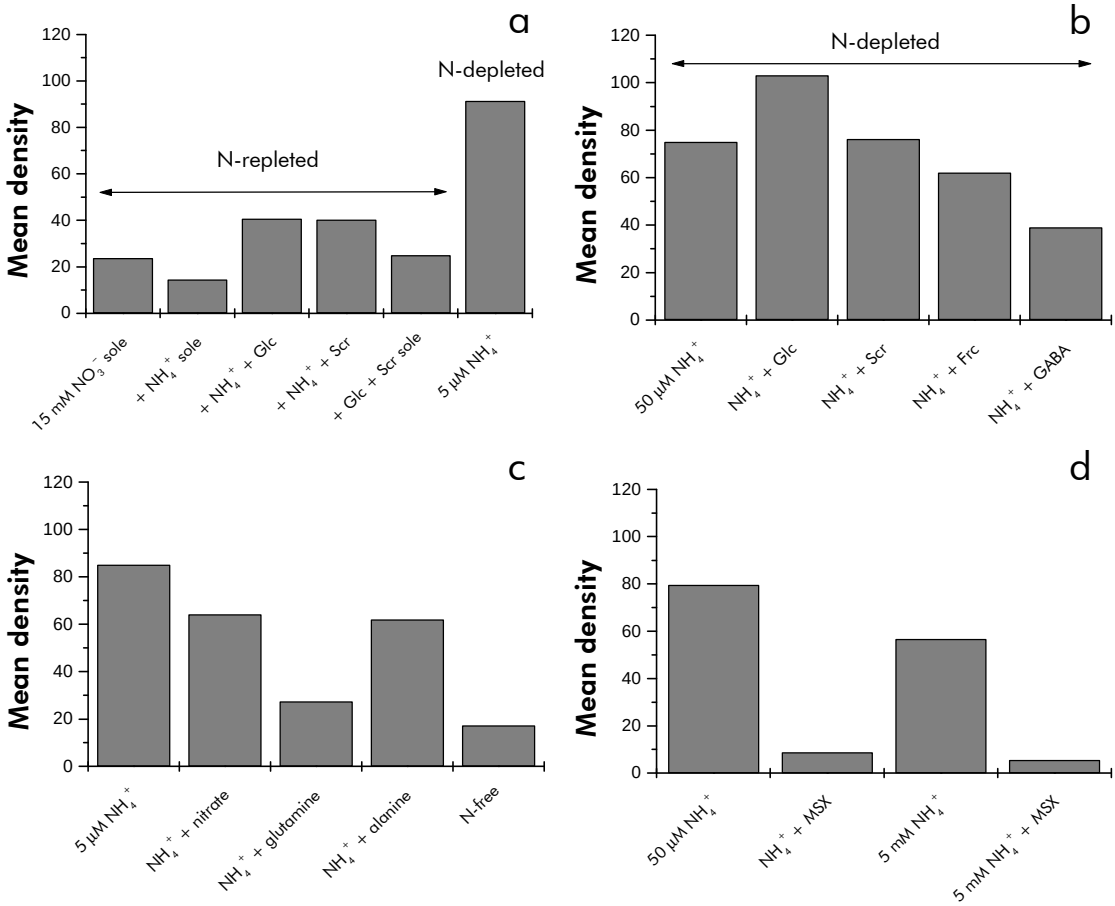


Figure 3.10 *LeAMT1;2*-expression in response to amino acids and sugar. (a) Nitrogen-repleted plants revealed generally a low transcript-level irrespective of ammonium supply. The additional presence of glucose or sucrose increased *LeAMT1;2*-expression slightly, whereas the signal intensity caused by both sugars alone (Glc + Scr sole: 5 mM each) did not exceed the level observed in control plants (15 mM NO_3^- sole). To illustrate weak expression, a signal caused by 5 µM NH_4^+ in nitrogen-depleted plants was added. (b) Nitrogen-depleted plants showed high transcript-levels in the presence of NH_4^+ and were affected differently by the presence of sugar. While glucose enhanced the expression level in addition, sucrose showed no effect and fructose caused the level to lower slightly. GABA was revealed to be a repressing agent. (c) The strong signal intensity caused by ammonium alone (column 1) decreased in the presence of all the three alternative nitrogen-sources. Gluatmine however, was by far the most efficient agent concerning *LeAMT1;2*-repression. In contrast, glutamate and aspartate, which were tested once each at a concentration of 1.5 mM, could not reduce an ammonium-induced signal intensity (data not shown). (d) MSX strongly repressed *LeAMT1;2*-expression in the presence of both 50 µM and 5 mM ammonium. In plants not receiving MSX, the signal intensity was inversely proportioned to the ammonium concentration supplied.

3.3.1.7 *LeAMT1;2*-expression in response to external pH

Three different pH values were chosen in order to examine the influence of the external proton concentration on the expression of *LeAMT1;2*. In addition, plants were grown either in MPCL-high-NO_3^- medium or MPCL-low-NO_3^--medium, resulting in a corresponding nitrogen status. During the induction period, plants received nitrogen-free MPCL-basic medium adjusted to pH 4.7, 6.0, or 6.8. Results are shown in Figure 3.11.

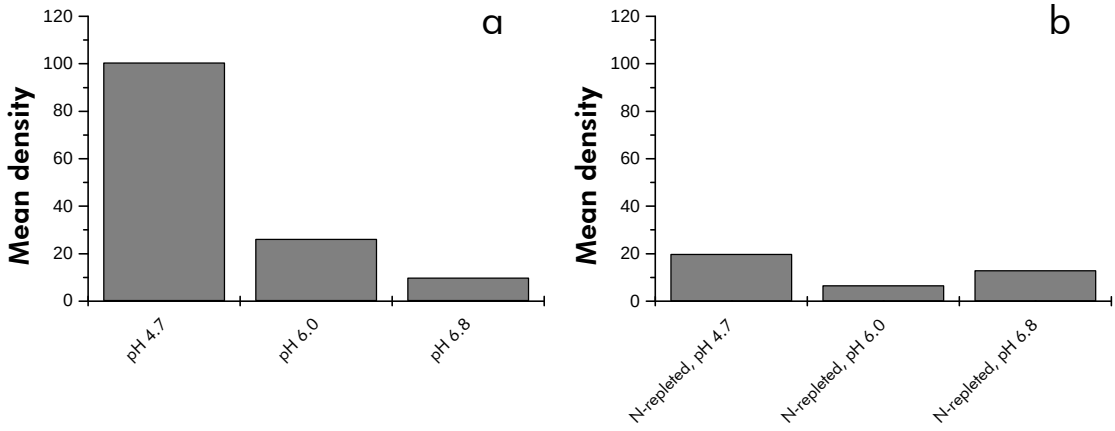


Figure 3.11 *LeAMT1;2*-expression in response to pH. (a) Nitrogen-depleted plants show a strong dependence on external pH, regardless of the absence of ammonium. High transcript-levels could be observed in acid media while the expression was much lower at pH 6 or 6.8. (b) The pH-effect was neutralized when plants had received high amounts of nitrogen before. In this case, the expression independent of the pH was generally low.

3.3.1.8 *LeAMT1;2*-expression in response to cesium and potassium

This experiment was designed to investigate the importance of potassium channels for the *LeAMT1;2*-expression as a consequence of a possible initial mediation of NH_4^+-transport. For this purpose, cesium (Cs^{2+}), a well-known inhibitor of potassium channels, was supplied together with ammonium at a low potassium concentration. As potassium was a part of many compounds in the MPCL-basic-medium, a specific medium was designed, containing only 1.3 mM K^+ (MPCL-low-K^+-medium). As a result, concentrations of phosphate, sulphate, and chloride were also reduced (Section 2.1.7). To test the role of K^+ as putative antagonist for the uptake of ammonium, an elevated K^+-concentration (130 mM) was supplied in combination with ammonium. Plants were grown for 14 days in MPCL-low-K^+-medium supplemented with 5 µM NO_3^- as the sole source of nitrogen. During the induction period, plants were shifted to MPCL-low-K^+-medium supplemented with 5 µM ammonium as well as 13 mM $CsCl_2$, 13 mM KCl or 130 mM KCl. Control plants received only ammonium or nitrate (5 µM each). Results are shown in Figure 3.12.

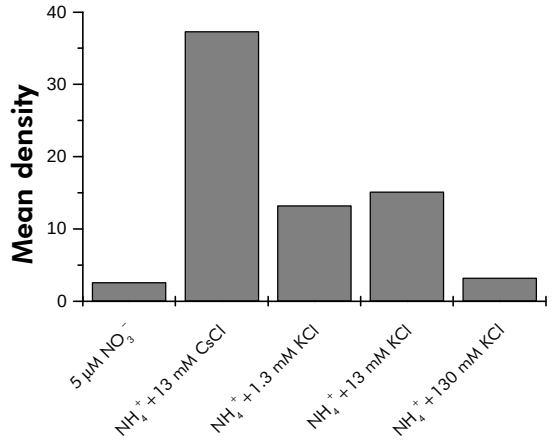


Figure 3.12 *LeAMT1;2*-expression in response to cesium and potassium. The strongest signal could be observed in the presence of cesium. There was no difference in signal intensities with 13 mM K^+ in comparison to 1.3 mM K^+. In the presence of 130 mM K^+, however, the signal was strongly reduced. The level caused by control plants (5 µM NO_3^-) was likewise low.

3.3.1.9 *LeAMT1;2*-expression in response to plant growth regulators

As plant growth regulators, formerly referred to as plant hormones, have been shown to influence many processes during plant growth and differentiation, their effect on the expression of ammonium transporter *LeAMT1;2* was investigated. Among tested substances were abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellic acid (GA_3), the synthetic cytokinin kinetin (Kin), and naphtalene acetic acid (both as 1-NAA and 2-NAA). Two different concentrations were used for the experiments namely 0.5 µM and 5 µM.

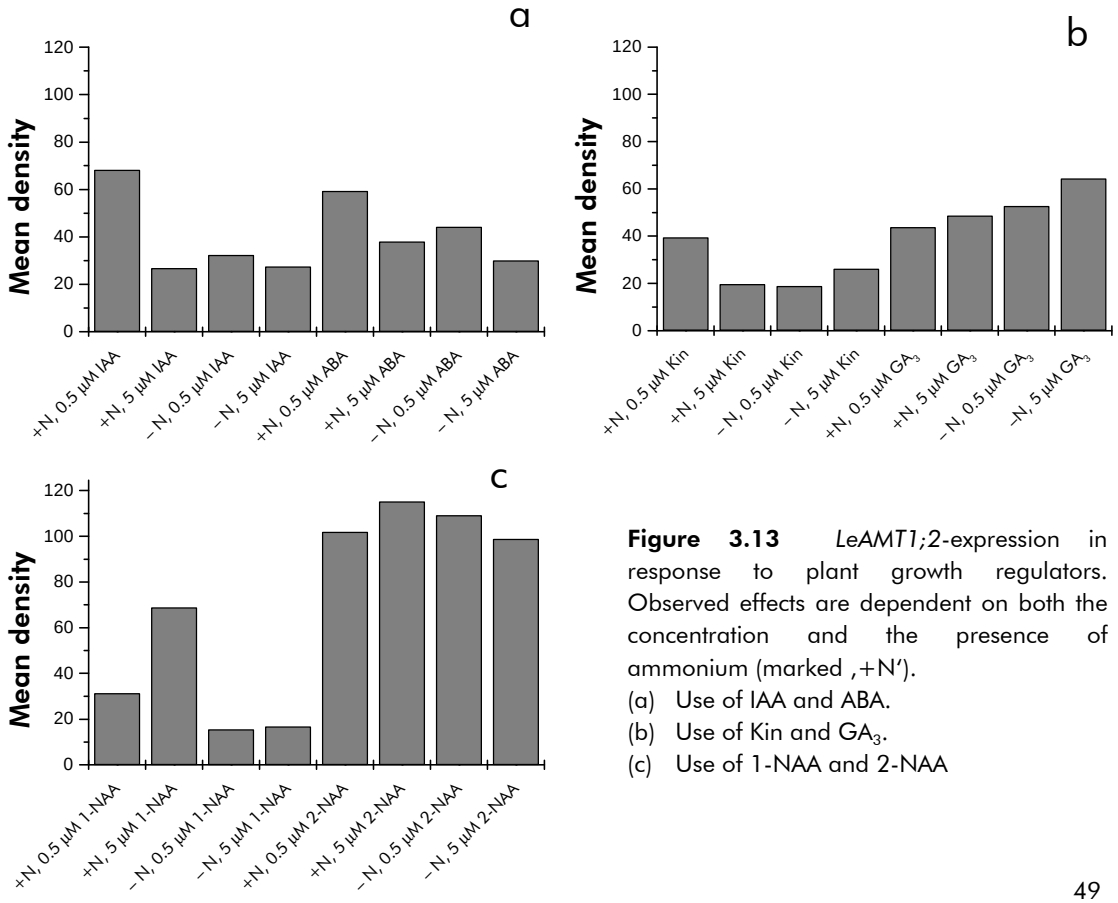


Figure 3.13 *LeAMT1;2*-expression in response to plant growth regulators. Observed effects are dependent on both the concentration and the presence of ammonium (marked ‚+N').
(a) Use of IAA and ABA.
(b) Use of Kin and GA_3.
(c) Use of 1-NAA and 2-NAA

In addition, plants were exposed to either ammonium-supplemented or nitrogen-free medium, enabling a better distinction between inducing and repressing effects.
All plants were previously grown in MPCL-low-NO_3^--medium. Plants were then shifted to MPCL-basic medium supplemented with the corresponding plant growth regulators, and in addition, half of the plants also received ammonium (5 µM). Results are presented in Figure 3.13.
A summary of the effect of individual substances and used concentrations follows.

IAA: IAA shows a clear concentration-dependent effect. *LeAMT1;2*-expression is repressed in the presence of higher IAA-concentrations, irrespective of the presence of ammonium. Nevertheless, it should be noted that the effect is much more distinct, if ammonium is added.

ABA: ABA shows exactly the same effect as IAA. However, in plants, exposed to 5 µM ABA the ´ammonium-effect´ of induction is clearer. In addition, the difference between the two concentrations is more pronounced in the absence of ammonium in comparison with the IAA-samples.

Kin: Kinetin shows a clear concentration-dependent effect in the presence of ammonium, that is, repression at higher concentrations. In the absence of ammonium, the signal of the 5 µM-sample is increased slightly, although a low intensity of both signals remains.

GA_3: Irrespective of the presence of ammonium, GA_3 induces *LeAMT1;2* with all conditions tested. Signal intensities, although weakly pronounced, increase both in the absence of ammonium and at higher GA_3-concentrations. These observations contrast with the effects caused by the above growth regulators.

1-NAA: In the presence of ammonium, 1-NAA was the only substance to show a very clear up-regulation of *LeAMT1;2* at higher concentrations. In the absence of ammonium however, there is no such effect; the transcript-level remains low.

2-NAA: Among all tested substances, the strongest signals are observed in the presence of 2-NAA. It happens to be irrelevant, whether ammonium is added or not.

Although signal intensities coming from different northern blots are not able to be compared, Figure 3.14 may contribute a better overview with respect to the effect of plant growth regulators. In the absence of ammonium, it can be seen that with the exception of the GA_3- and 2-NAA-samples, signal intensities are markedly reduced or remain low, if the concentration of the growth regulator rises. In the presence of ammonium, the clear down-regulation at higher concentrations is only valid for IAA, ABA and Kin. With the other substances present, signals remain high or even increase as by 1-NAA.
On closer examination of the ´ammonium effect´ of *LeAMT1;2*-induction, it can be seen that in samples containing 0.5 µM growth regulator the signal is increased in the presence of ammonium, with the exception of plants having received GA_3 or 2-NAA. Comparing the samples exposed to 5 µM growth regulator, the difference caused by NH_4^+-supplement is much less distinct, with the only exception being plants having received 1-NAA.

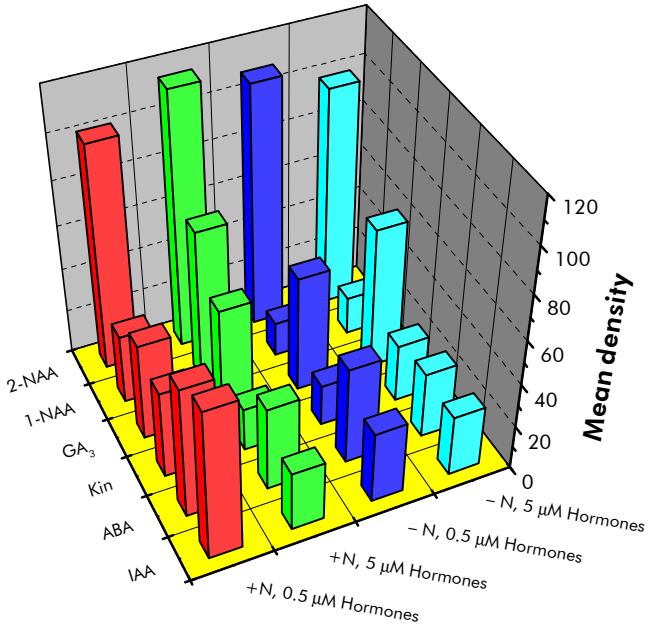


Figure 3.14 Overall view of the effect of plant growth regulators on the expression of *LeAMT1;2*. Note that a direct comparison of signal intensities between single hormones is not possible, as they originated from different northern blots. The diagram, however, quickly enables single effects caused by both the concentration of growth regulators and the presence of ammonium to be determined.

To determine, if the nitrogen status of plants has to be considered, when the effect of growth regulators is examined, a further experiment has been conducted.
In this case, plants were previously grown in MPCL-high-NO_3^--medium resulting in nitrogen-repleted plants. They were then shifted to nitrogen-free MPCL-basic-medium supplemented with either 0.5 µM IAA, ABA, GA_3 or Kin. Results are shown in Figure 3.15.

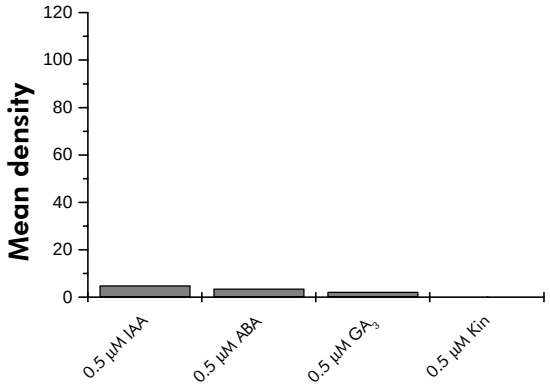


Figure 3.15 *LeAMT1;2*-expression in nitrogen-repleted plants in response to growth regulators. Mean densities remained close to zero, indicating a strong repressing effect due to the plant´s high nitrogen status. The effects of growth regulators, previously observed in nitrogen depleted plants, were thus neutralized.

Indole-3-acetic acid was subject to closer inspection. Plants were exposed to different concentrations of IAA, ranging from 1 nM to 5.7 µM. Two northern blots were prepared, covering lower and higher concentrations each.
Plants were previously grown in MPCL-low-NO_3^--medium. They were then shifted to nitrogen-free MPCL-basic medium supplemented with different concentrations of IAA. Results are shown in Figure 3.16.

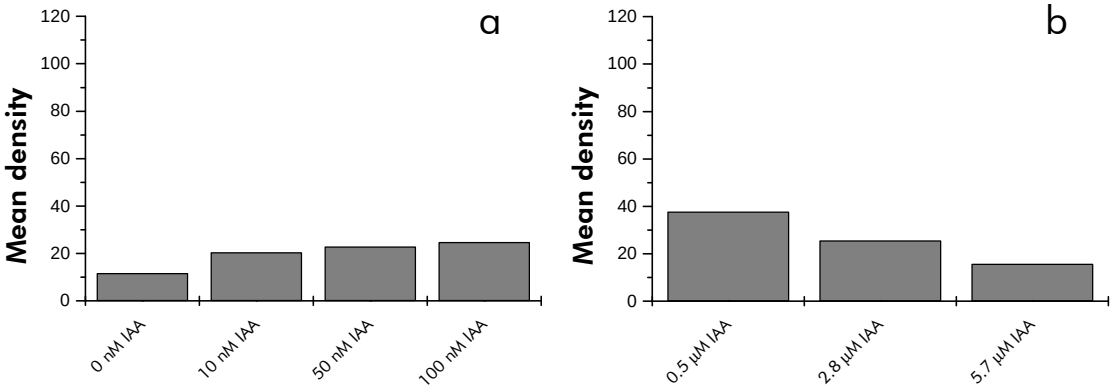


Figure 3.16 *LeAMT1;2*-expression in response to IAA. (a) In the very low concentration range, there is a slightly increasing signal intensity from 10 nM to 100 nM IAA. Control plants (0 nM IAA) showed the lowest level of induction. (b) A decline of signal intensity was observed from 0.5 µM upwards.

In the presence of IAA, a concentration-dependent effect was revealed, although not very well pronounced. According to that, peak expression is given in the range from 100 nM to 500 nM IAA.

3.3.1.10 *LeAMT1;2*-expression in young seedlings germinated on filter

To elucidate, whether signals developed in nitrogen-free medium represented background activity of *LeAMT1;2*, thus visible in any kind of medium, RNA was isolated from young seedlings, before transfer to the hydroponic units occurred. Figure 3.17 shows the transcript-level of such seedlings in comparison with plants, which were exposed to nitrogen-free medium.

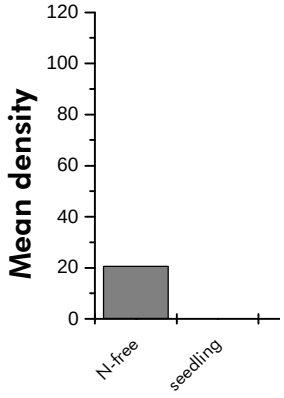


Figure 3.17 *LeAMT1;2*-expression in seedlings germinated on filter paper. No transcripts could be detected, before plants were finally transferred to the hydroponic units, suggesting that the signal obtained in nitrogen-free medium is probably caused by real background activity of *LeAMT1;2* and not by unspecific cross-hybridization.

3.3.2 *LeAMT1;2*-expression with inoculum

This section deals with the results obtained from inoculation experiments. In order to evaluate whether the induction of ammonium transporter *LeAMT1;2* is induced by ammonium, which was previously fixed and excreted by diazotrophic bacteria, experiments were designed such that plants were responsive to externally supplied ammonium. Therefore, based on former results, plants were previously grown in a nitrogen-poor environment. This led to nitrogen-depleted plants, which used to reveal a sensitive response to traces of ammonium in the plant medium.

3.3.2.1 *LeAMT1;2*-expression in response to diazotrophic wild types

First experiments were conducted using bacterial wild types of the strains *Azospirillum brasilense* Sp 7, *Azoarcus* Sp BH72, *Azotobacter vinelandii*, and *Acetobacter diazotrophicus* Sp Pal5. As a control, plants received nitrogen-free MPCL-basic-medium. Inoculation experiments were carried out as described in Section 2.2.1.2.4. Results are presented in Figure 3.18.

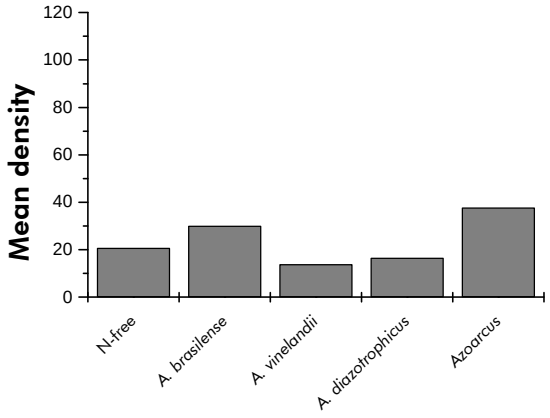


Figure 3.18 *LeAMT1;2*-expression in response to bacterial wild types. An increase of signal intensity could be observed in the presence of *Azospirillum brasilense* Sp 7 and *Azoarcus* Sp BH72. By contrast, transcript-levels caused by *Azotobacter vinelandii* and *Acetobacter diazotrophicus* did not exceed background level in nitrogen-free medium.

As first experiments revealed induction of *LeAMT1;2* by *Azospirillum brasilense* Sp 7 and *Azoarcus* Sp BH72, both bacteria were further investigated.

3.3.2.2 *LeAMT1;2*-expression in response to mutant strains

Numerous mutant strains of *Azospirillum brasilense* were used to further investigate the nature of the effect, diazotrophic bacteria exert on the expression of *LeAMT1;2*. Among mutants tested were strains impaired in or completely devoid of

- nitrogen fixation ability (strains 7317, 7029, 7628),
- IAA-production (strain SpM7918),
- chemotaxis (strain A7030),
- ammonium assimilation (strains C3, 7029), and
- regulation of nitrogen metabolism (strains 7628, 7628b, 7611).

In addition, wild type strains of *Azospirillum brasilense* and *Azoarcus* were used. Relevant characteristics of all strains are listed in Section 2.1.2. Simultaneously, nitrogen fixation ability of pure bacterial cultures was tested, using nitrogen-free semisolid NFb-medium. Cultures, subject to the acetylene reduction assay, were grown for one week and exposed to acetylene for 40 hrs. Ethylene produced was converted to 1 hr, and rate of fixation was compared to the results of northern blot analysis (Figure 3.19).

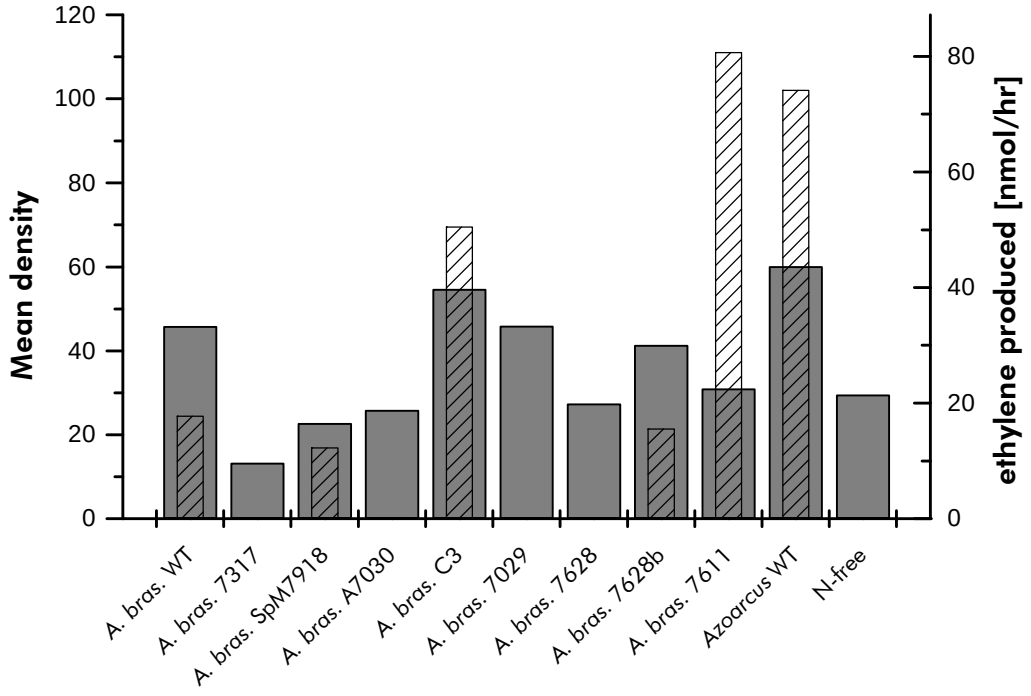


Figure 3.19 *LeAMT1;2*-expression in response to diverse mutant and wild type strains. Signal intensities are presented in grey columns. Additionally, nitrogen fixation rates of pure cultures are outlined by striped columns. Even if not in all cases, nitrogen fixation was to some extent correlated with the expression of ammonium transporter, *LeAMT1;2*. High transcript-levels were also detected in strain 7029, which is, however, well known for excretion of ammonium. A further exception is given by strain 7611 showing the highest rate of nitrogen fixation but a relatively low transcript-level.

In addition to the wild types, several mutant strains were able to up-regulate *LeAMT1;2*-expression in tomato roots. The effect was most pronounced in *glnA*-mutants C3 and 7029. Both strains are impaired in glutamine synthetase-activity and, as a result, excrete ammonium to the medium. Strain 7628b, affected in the nitrogen regulatory protein P_{II}, did also rise transcript-level of *LeAMT1;2*. Since this strain was transformed with pAB5320, carrying an *Azospirillum brasilense* 5´-end deleted *nifA*-gene, the strain fixes nitrogen constitutively. In contrast, strain 7628 lacking pAB5320 exhibits a Nif^--phenotype. All other strains did not clearly exceed the transcript-level observed in the nitrogen-free control, although some of them were able to fix nitrogen (strains 7611, Spm7918, and A7030).

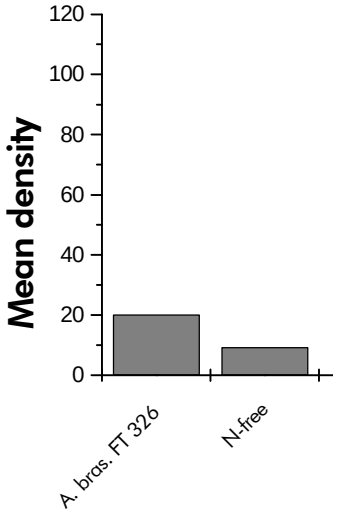


Figure 3.20 *LeAMT1;2*-expression in response to IAA-hyperproducing strain FT326.

Besides mutants tested above, *Azospirillum brasilense* FT326, an IAA-hyperproducing strain, which was provided later during the thesis, was exposed to tomato roots, and *LeAMT1;2*-expression was compared with a control exposed to nitrogen-free medium.

The transcript-level, caused by FT326, exceeded the level observed in the control twice (Figure 3.20). However, no statement can be made about the expression in comparison with the wild type or the *ipdc* $^-$-mutant (SpM7918), which produces only 10% of the constitutive IAA-level.

3.3.2.3 *LeAMT1;2*-expression in response to bacterial supernatants

This experiment was designed to evaluate, whether substances excreted by diazotrophic bacteria affect *LeAMT1;2*-expression. The preparation of bacterial supernatants is described in Section 2.2.1.2.5. Simultaneously, the supernatants of bacterial cultures were analyzed for ammonium content as described in Section 2.2.5.2. Results of expression and analysis are presented in Figure 3.21.

The amount of transcripts in all plants exceeded the level, which could be detected in control plants (last column). The strongest inductions were caused by the supernatants of *Azospirillum brasilense* strains wild type, 7029, and 7628. However, apart from the plants supplied with the C3-supernatant, all transcript-levels were substantially increased.

Regarding ammonium, most concentrations, finally supplied, ranged between 0.39 and 1.42 µM NH_4^+, thereby in the order of the threshold concentration described before (Section 3.3.1.1). As the high ammonium concentration found in the supernatant of strain C3 did not coincide with the low level of *LeAMT1;2*-transcription, it is unlikely that the signals are attributable to ammonium content only.

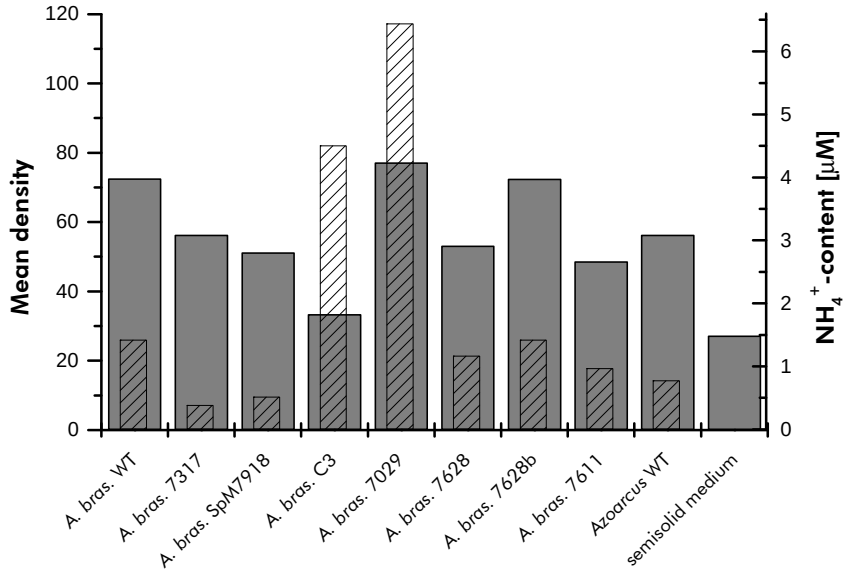


Figure 3.21 *LeAMT1;2*-expression in response to bacterial supernatants (grey columns). In addition, independently grown pure cultures of bacteria were analyzed for ammonium content in the supernatant (striped columns). The actual ammonium content found was divided by 20, taking into consideration that supernatants were diluted before addition to plants. Transcript-levels did not always coincide with ammonium content.

3.3.2.4 Role of *LeAMT1;2*-expression in pathogen response

To elucidate the role of *LeAMT1;2* in pathogen response, three members of the genus *Herbaspirillum* were used as inoculants for plants. Among these were the beneficial strains Z67 and Z78 of *Herbaspirillum seropedicae* as well as the phytopathogen *Herbaspirillum rubrisubalbicans* M4. Furthermore, one of the key metabolites in pathogen defense, salicylic acid, was tested for its influence on *LeAMT1;2*-expression. While bacteria were added for 10 hrs as usual, the plants, which received salicylic acid (50 µM) were already harvested after 6 hrs. Results are presented in Figure 3.22.

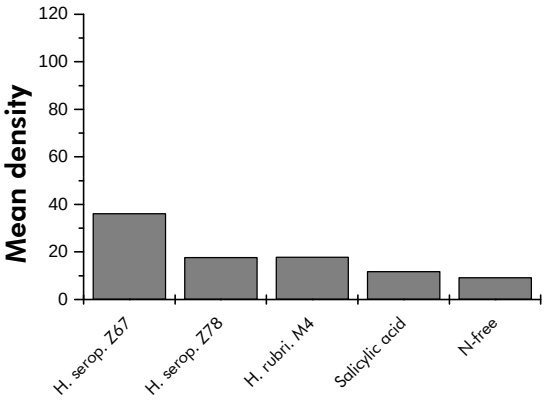


Figure 3.22 *LeAMT1;2*-expression in response to a potential pathogen. The strongest signal was observed in the presence of beneficial wild type strain Z67. However, the likewise beneficial strain Z78 as well as the phytopathogen M4 caused only weak expression of *LeAMT1;2*. The expression in the presence of salicylic acid was similarly low as in the control plants.

3.3.2.5 *LeAMT1;2*-expression in response to inoculation density

Apart from the normally used inoculation density of 10^7 cfu/ml, the wild types of *Azospirillum brasilense* Sp 7 and *Azoarcus* Sp BH72 were additionally employed at densities of 10^6 and 10^8 cfu/ml, respectively. Densities were adjusted spectophotometrically, using the equation formula described in Section 3.1.1. Results are shown in Figure 3.23.

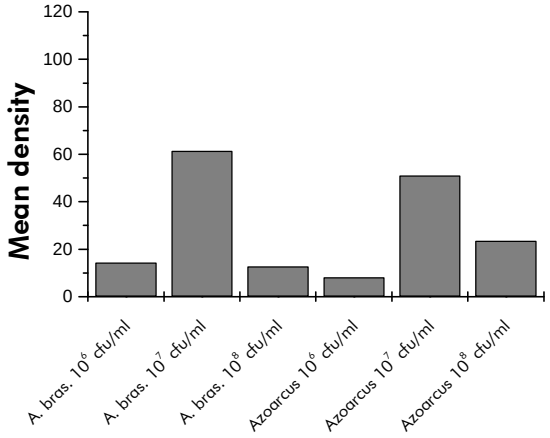
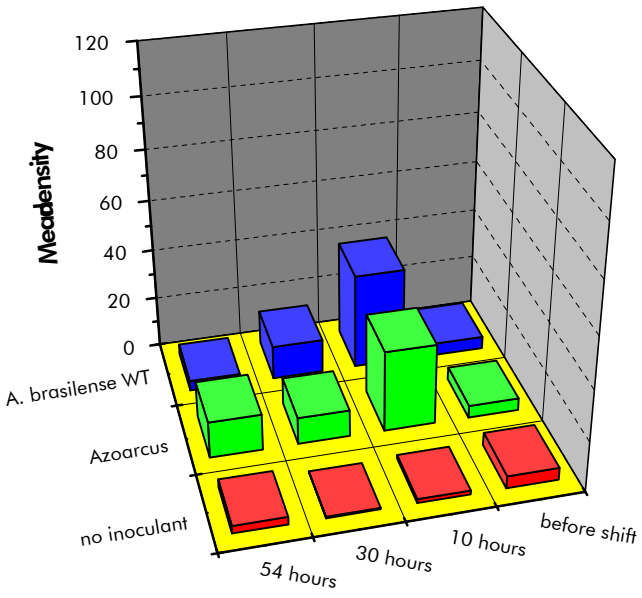


Figure 3.23 *LeAMT1;2*-expression in response to inoculation density. It turned out that a cell density of 10^7 cfu/ml resulted in the strongest signal both in *Azospirillum* and *Azoarcus*. Densities adjusted beyond that value led to transcript-levels which were much lower.

3.3.2.6 *LeAMT1;2*-expression in response to inoculation time

In order to determine the long-term effect of an inoculation, tomato plants were incubated over a period of three days in the presence of *Azospirillum* and *Azoarcus* wild types. During incubation, the medium was not changed any more. Control plants received nitrogen-free medium. Plants were harvested after 10, 30 and 54 hrs of incubation. Results are presented in Figure 3.24.

Figure 3.24 *LeAMT1;2*-expression in response to long-term inoculation. The plants, which were harvested at the day of bacterial addition (10 hrs), revealed a strong expression in comparison to the control. However, signals decreased strongly at day 2 in the presence of both bacteria, although they still exceeded the control level. After 54 hrs, the transcript-level in the presence of *Azospirillum brasilense* was equal to that in the control. Plants incubated with *Azoarcus* still revealed a slightly increased signal.
Note that signal intensities between single bacterial samples are not able to be compared, as they originate from different northern blots.



The high expression in the presence of bacterial wild types observed after 10 hours of incubation was not maintained in the long run, indicating the short-term nature of the strong effect observed at day 1.

3.4 Analysis of Root tissues and supernatants

The analysis of root tissues concerned the determination of root ammonium content after different treatments, comparable to those, applied for the purpose of *LeAMT1;2*-expression analysis. Furthermore, supernatants of plants were analyzed for carbohydrates, organic acids, amino acids, and also for ammonium, of which the uptake was followed.

3.4.1 Analysis of root ammonium content

The experiment was designed to determine the accumulation of ammonium in plant roots after treatment with different key substances of nitrogen metabolism. Among compounds tested were glutamine, glutamate, MSX, and ammonium. The latter was used both at different concentrations and at different pH values. Root ammonium content was analyzed as described in Section 2.2.5.1. Results are presented in Figure 3.25.

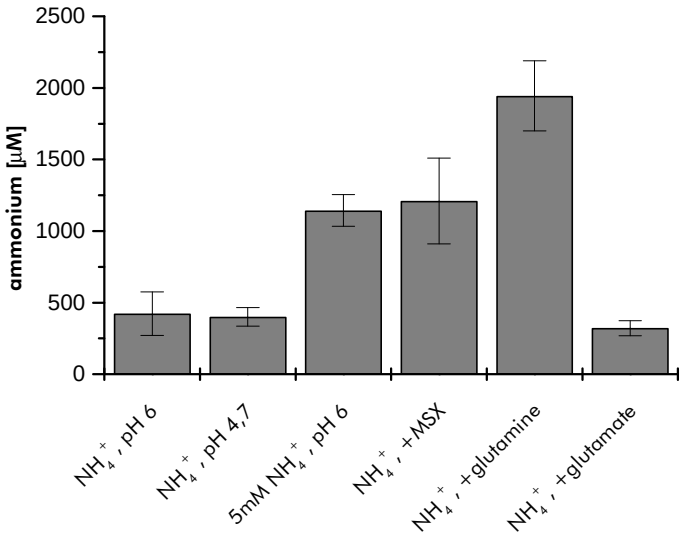


Figure 3.25 Analysis of root ammonium content after different treatments as indicated below columns. Unless otherwise stated, NH_4^+ was supplied at a concentration of 50 µM. Highest ammonium contents were found in the presence of glutamine, whereas high concentrations of glutamate did not rise internal ammonium. Levels were also increased in the presence of high external ammonium concentrations as well as after MSX-supply. The pH value of the medium had no significant effect on root ammonium content.

3.4.1.1 Analysis of root ammonium content after inoculation with diazotrophic bacteria

Nitrogen-depleted plants, previously grown for 14 days in MPCL-low-NO_3^--medium, were incubated for 10 days in the presence of diazotrophic bacteria (10^7 cfu/ml). Subsequently, roots were analyzed for ammonium content to evaluate, whether bacteria elevate the ammonium concentration in root tissues. Wild types of *Azoarcus* and *Azospirillum* as well as the Nif$^-$-mutant of the latter strain were used as inoculants. Control plants received nitrogen-free medium. Six plants were prepared for each sample.
Results, obtained from this experiment (Figure 3.26), revealed that plant tissues inoculated with bacteria contain very low amounts of ammonium, which do not substantially exceed the control level. Highest concentrations were measured in roots inoculated with *Azoarcus* Sp BH72. However, a Student´s *t*-test revealed no significance, taking p□0.05 as significant.

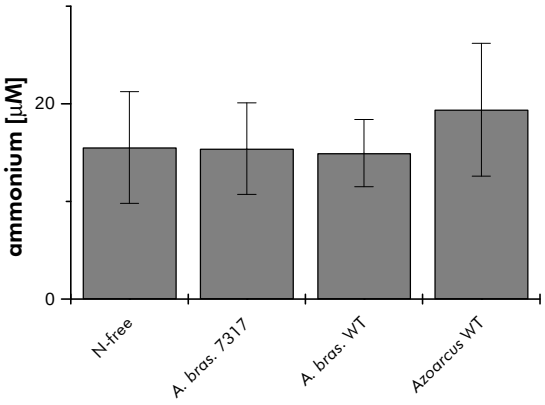


Figure 3.26 Analysis of root ammonium content in the presence of diverse inoculants. Ammonium concentrations, found in roots, remained very low in all samples analyzed.

3.4.2 Analysis of plant supernatant for root exudates

The experiment was carried out to identify potential compounds in the plant medium, which might serve as energy source or chemotactic attractant for bacteria. Growth of plants and analysis of supernatants were carried out as described in Sections 2.2.5 and 2.2.5.4. The analysis revealed the presence of glucose, fructose, and sucrose in the medium surrounding nitrogen-depleted plants. Concentrations found were:

Glucose: 10.19 µM
Fructose: 11.46 µM
Sucrose: 1.84 µM

No amino acids or organic acids were found in the supernatants of neither nitrogen-depleted nor nitrogen-repleted plants. Furthermore, the medium of nitrogen-repleted plants was free of carbohydrates, as well.

3.4.2.1 Analysis of ammonium content in plant medium

The uptake of ammonium from plant medium was followed using nitrogen-repleted and nitrogen-depleted plants. The latter were used both in an ammonium-induced and non-induced state. The experiment was carried out as described in Section 2.2.5.3. Two replicates were prepared for each sample. Data obtained was polynomially fitted for regression. Results are presented in Figure 3.27.

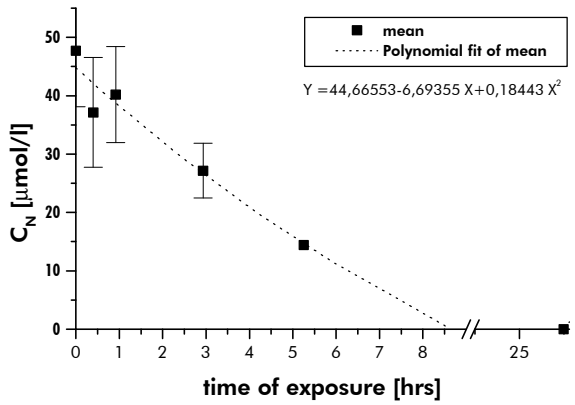
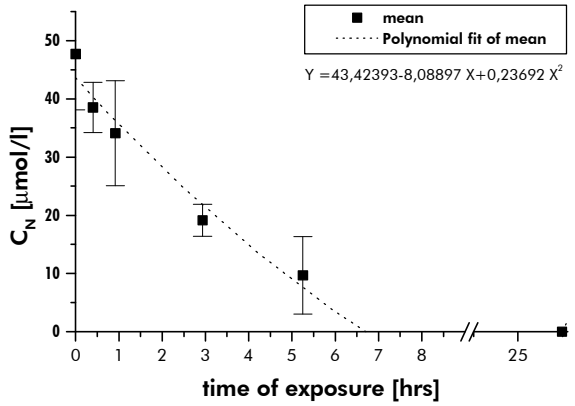
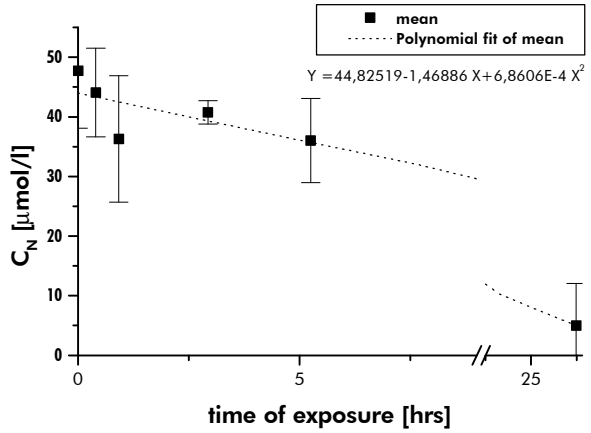


Figure 3.27 Analysis of NH_4^+-content in plant media, supplemented with 50 µM NH_4^+, in a course of time. Formulas for polynomial fits are shown in each graph.

(a) Uptake kinetics in nitrogen-depleted, pre-induced plants. According to the polynomial fit ammonium was completely taken up after 8.8 hrs of exposure.



(b) Uptake kinetics in nitrogen-depleted, non-induced plants. According to the polynomial fit all ammonium was taken up after 6.7 hrs of exposure.



(c) Uptake kinetics in nitrogen-repleted, non-induced plants. According to the polynomial fit ammonium was exhausted not before 31 hrs of exposure.

The mean fresh weight (FW) of roots per sample (three each) was determined to be:

0.4268 g for nitrogen-depleted, pre-induced plants,
0.4558 g for nitrogen-depleted, non-induced plants and
0.3838 g for nitrogen-repleted, non-induced plants.

Uptake rates are generally described by the formula

$$\text{uptake rate} = \frac{\text{abs. amount taken up [nmol]}}{\text{root fresh weight [g]} \times \text{time [hr]}}$$

Considering the absolute amount of ammonium initially supplied (50 µM = 2 µMol in 40 ml), the time needed to completely take it up (8.8 hrs, 6.67 hrs and 31 hrs), and the root fresh weight, uptake rates were as follows:

533 nmol/g FW·hr for nitrogen-depleted, pre-induced plants,
658 nmol/g FW·hr for nitrogen-depleted, non-induced plants and
168 nmol/g FW·hr for nitrogen-repleted, non-induced plants.

3.5 Nitrogen fixation assays

In order to follow nitrogen fixation in the association between tomato plants and diazotrophic bacteria, three approaches were used to provide scientific proof: the acetylene reduction assay (ARA), the ^{15}N enrichment gas-technique, and the *b-glucuronidase* (GUS) reporter system.

3.5.1 Monitoring nitrogen fixation using ARA

As described in Section 2.2.7.2, the acetylene reduction was determined in the association between tomato plants and diazotrophic bacteria. Ethylene (C_2H_2) produced during incubation was measured in a cumulative way. The closed atmosphere in the tubes led to considerable stress for the plants, which resulted in yellowing and loss of leaves after 96 hrs of incubation at latest. This phenomenon was independent of the presence of acetylene in the atmosphere. That is why plants were partly incubated for either 3 or 6 days before acetylene was added. This should ensure that bacteria were able to efficiently colonize the roots of healthy plants.
In plant tubes, containing the *nifD*$^-$-mutant of *Azospirillum brasilense* as well as in the control tubes containing no inoculum at all, ethylene production was never measured, indicating that plants caused no ethylene background due to hormone production. All measurements were carried out twice, and the mean value was taken from two replicates prepared for each sample. Results are presented in Figure 3.28.

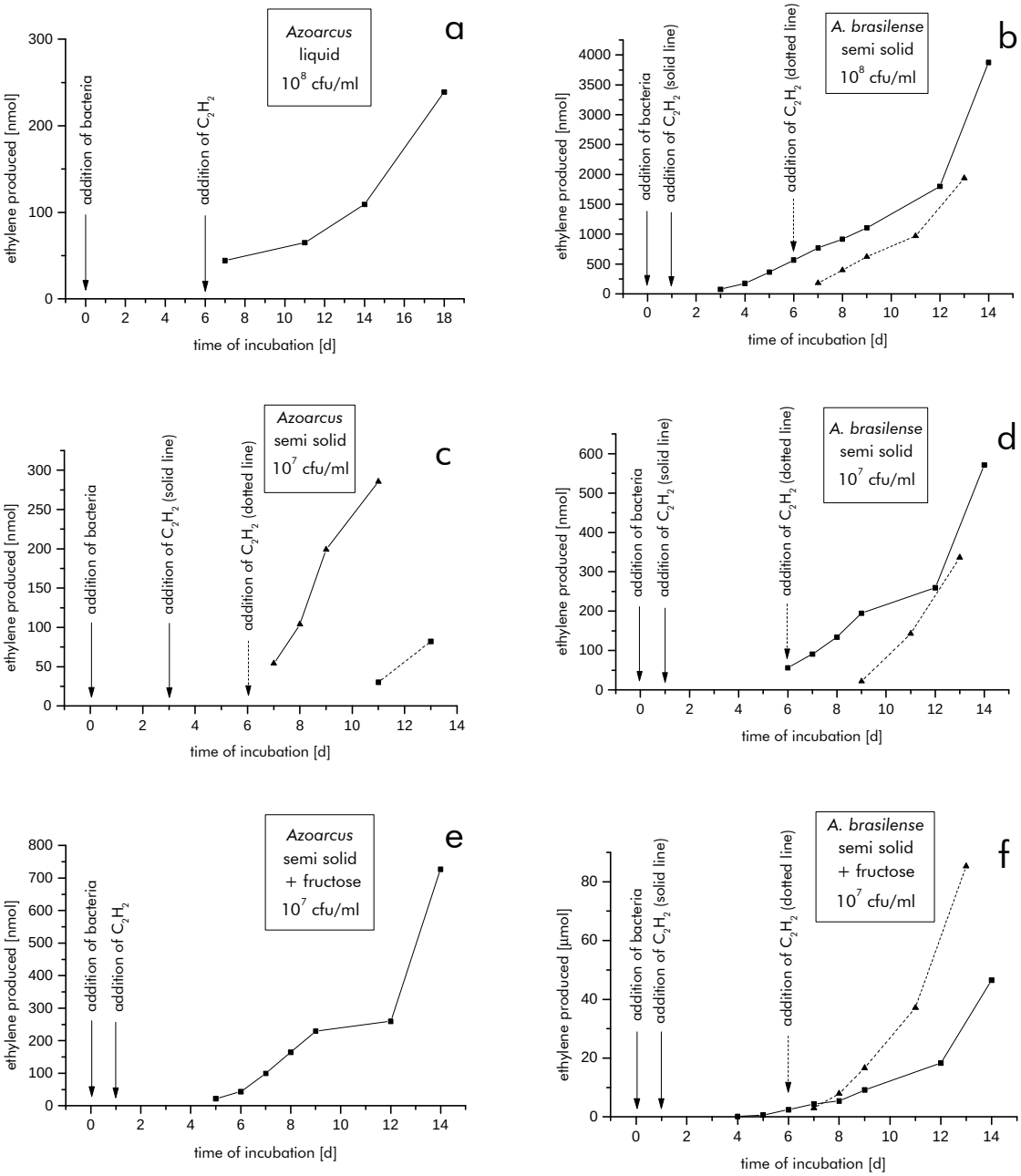


Figure 3.28 Acetylene reduction in the association between plant and bacteria. Bacteria were added at the time of day zero. Acetylene was added either after 1, 3 or 6 days of incubation as indicated by arrows. Ethylene production (y-axis) was then recorded at regular intervals for a maximum of 13 days (x-axis). Curves are either drawn dotted or solid, indicating to which arrow they belong. (a) C_2H_4-production of *Azoarcus* Sp BH72 (10^8 cfu/ml) in liquid medium. (b) C_2H_4-production of *Azospirillum brasilense* (10^8 cfu/ml) in semisolid medium. (c) C_2H_4-production of *Azoarcus* Sp BH72 (10^7 cfu/ml) in semisolid medium. (d) C_2H_4-production of *Azospirillum brasilense* (10^7 cfu/ml) in semisolid medium. (e) C_2H_4-production of *Azoarcus* Sp BH72 (10^7 cfu/ml) in semisolid medium supplemented with 0.1% fructose. (f) C_2H_4-production of *Azospirillum brasilense* (10^7 cfu/ml) in semisolid medium supplemented with 0.1% fructose.

The rate of acetylene reduction turned out to be dependent on four factors: inoculation density, medium-consistency, colonization time, and, above all, an additional carbon source in the medium. Apart from one sample (*Azoarcus*, Figure 3.28a), no ethylene was ever measured, when bacteria were suspended in liquid media. Even in the case of *Azoarcus*, which was inoculated at a density of 10^8 cfu/ml after all, ethylene production remained initially low and began to rise not before day 14 of incubation. The addition of fructose to liquid medium had no effect on ethylene production, irrespective of the inoculum used.
In semisolid media, bacteria formed a pellicle, characteristic for diazotrophic bacteria fixing nitrogen. However, unless cell densities were increased (Figure 3.28b), acetylene reduction remained low and was revealed with delay (Figure 3.28c+d). When *Azospirillum brasilense* was enabled to colonize before acetylene was added, the delay between C_2H_2-injection and ethylene production was shorter, and the rate was stronger (steeper slope). This was also observed in the presence of fructose (Figure 3.28f). *Azoarcus* could not efficiently benefit from fructose-supplement (Figure 3.28e) in comparison with *Azospirillum*. Semisolid media, however, turned out to promote nitrogen fixation in *Azoarcus*, too. In general, it should be noted that fixation rates did not increase stronger before plants visibly suffered from the closed atmosphere within the tube.

3.5.2 Monitoring nitrogen fixation using ^{15}N-enrichment gas technique

This experiment was carried out following the results of the acetylene reduction assay. Tomato plants were subject to ^{15}N-analysis as described in Section 2.2.7.3 using wild types of *Azospirillum brasilense* and *Azoarcus* as inoculants. The ^{15}N-contents of harvested tissues, separated in shoots and roots, are presented in Figure 3.29.

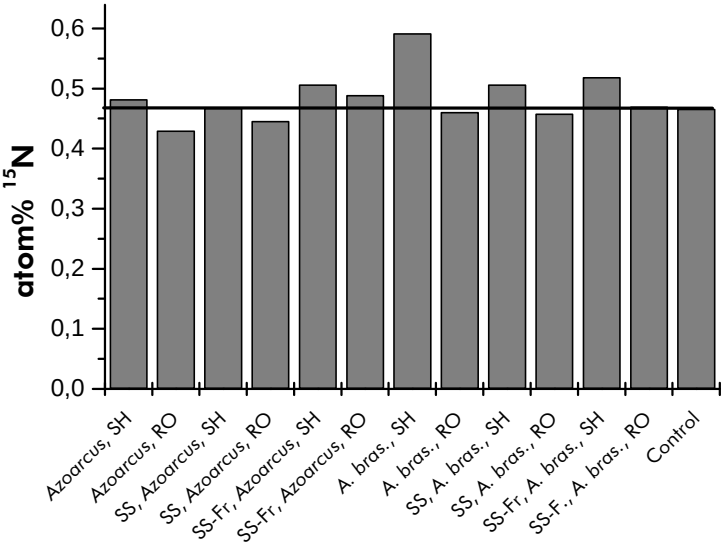


Figure 3.29 ^{15}N-content of plants inoculated with *Azospirillum brasilense* and *Azoarcus*, respectively. Plants were either inoculated in semisolid medium (marked ‚SS'), partly supplemented with fructose (marked ‚Fr.'), or at a density of 10^8 cfu/ml in liquid medium (no further indication). Control plants were grown both in the absence of inoculum and ^{15}N-gas. Roots (RO) and shoots (SH) were separated before analysis. Bar indicates ^{15}N-level of control plants.

In all cases, shoot-samples from plants inoculated with *Azospirillum brasilense* showed an elevated ^{15}N-content, which was highest, when inoculation density was adjusted to 10^8 cfu/ml. A slightly elevated level could also be observed in tissue samples inoculated with *Azoarcus* in semisolid medium and supplemented with fructose. All root-samples revealed ^{15}N-contents, which did not exceed the control level. As the experiment was carried out only once, no statement can be made about the statistical significance of the results.

3.5.3 Monitoring potential nitrogen fixation using GUS

Nitrogen fixation was also followed using an *Azospirillum brasilense* strain harbouring *nifH-gusA* fusion plasmid pFAJ21. Plants were grown and inoculated as described in Section 2.2.7.1. First signs of weak blue staining were observed not before day 11 after addition of *Azospirillum brasilense* and day 7 after addition of substrate X-Gluc to plant medium, respectively. Affected areas were mainly localized in the root hair zone. Microscopic observations are presented in Figure 3.30.

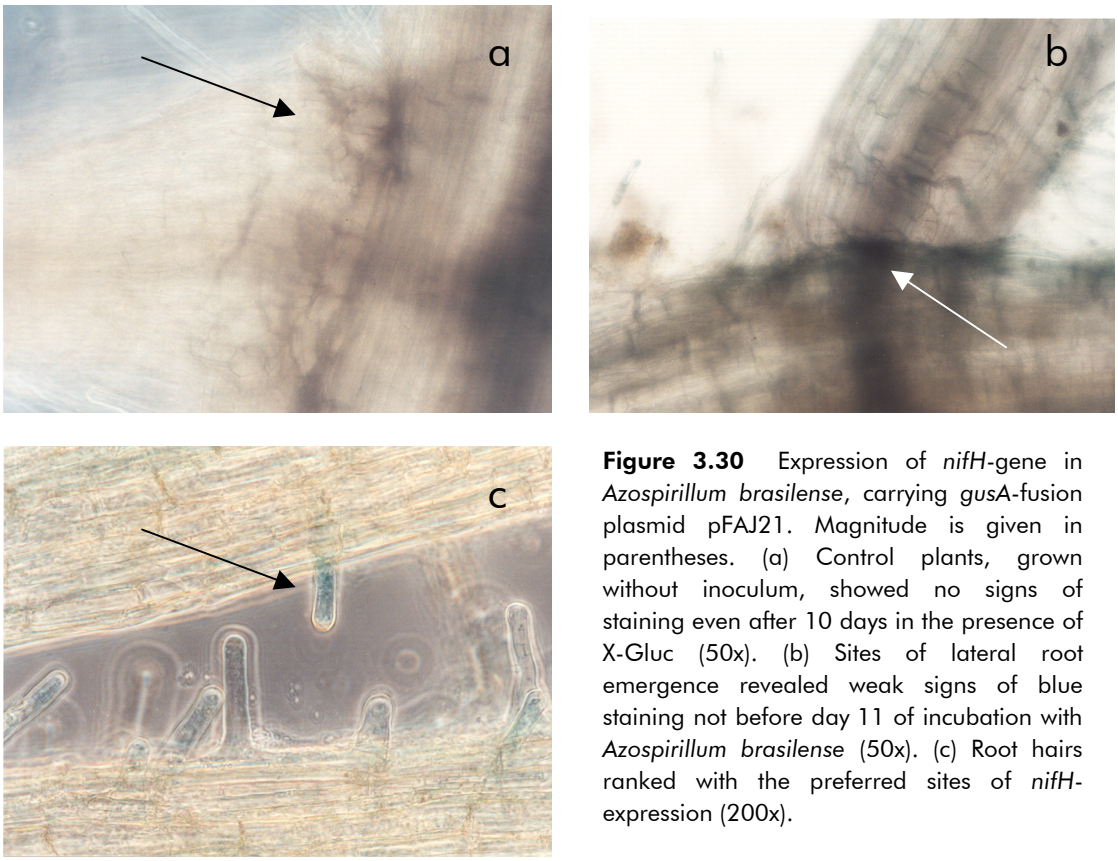


Figure 3.30 Expression of *nifH*-gene in *Azospirillum brasilense*, carrying *gusA*-fusion plasmid pFAJ21. Magnitude is given in parentheses. (a) Control plants, grown without inoculum, showed no signs of staining even after 10 days in the presence of X-Gluc (50x). (b) Sites of lateral root emergence revealed weak signs of blue staining not before day 11 of incubation with *Azospirillum brasilense* (50x). (c) Root hairs ranked with the preferred sites of *nifH*-expression (200x).

In contrast to the blue staining caused by the constitutively *gusA*-expressing strain (Section 3.2.1), the *b-glucuronidase*-activity in this study was strongly restricted to root hairs and some sites of lateral root emergence.

3.6 *Plant root morphology*

Plant root morphology was investigated after application of plant growth regulators IAA, ABA, Kin. and GA_3. Furthermore, wild types of *Azoarcus* and *Azospirillum brasilense* as well as two mutants of the latter strain, affected in IAA-production, were tested for their effects on root morphology. Plant growth and parameters analyzed are described in Section 2.2.8. The standard error of differences in measured parameters referred to control was calculated by analysis of variance, and Student´s *t*-test was performed, taking $p \leq 0.05$ as significant.

3.6.1 Root dry weight

Root dry weight was significantly enhanced by both mutants of *Azospirillum brasilense*, affected in IAA-production (Figure 3.31). Among growth regulators, gibberellic acid caused a significant decrease of root weight, when supplied at a concentration of 5 µM. In general, root dry weight was decreased, when the concentration of a growth regulator was increased.

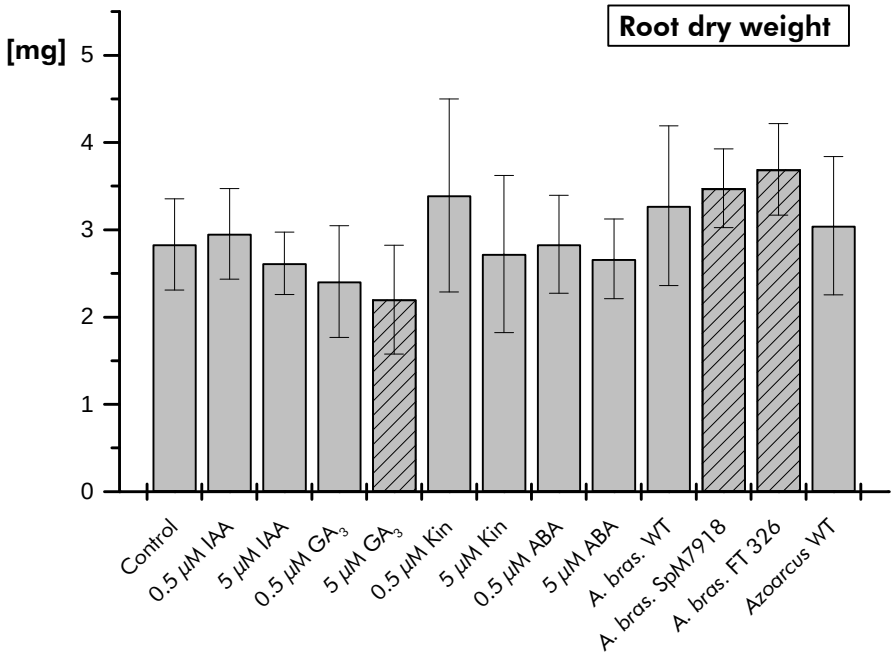


Figure 3.31 Root dry weight after treatment of plants with different plant growth regulators and diazotrophic bacteria, respectively, for three weeks. Control plants received nitrate only (50 µM). All measurements which differed significantly from control are presented as striped columns.

3.6.2 Number of lateral roots

The number of lateral roots was significantly increased in the presence of IAA (Figure 3.32). Emerged roots, however, remained short. Abscisic acid had the same effect. Both IAA and ABA showed a positive correlation with the supplied

concentration of plant growth regulator. Kinetin markedly decreased the number of lateral roots, if supplied at a concentration of 5 µM. Concerning bacteria, yet again the mutants affected in IAA-production caused significant changes. However, while there was a decreased number of lateral roots in the presence of strain SpM7918, the IAA-hyperproducing strain FT326 gave rise to the opposite.

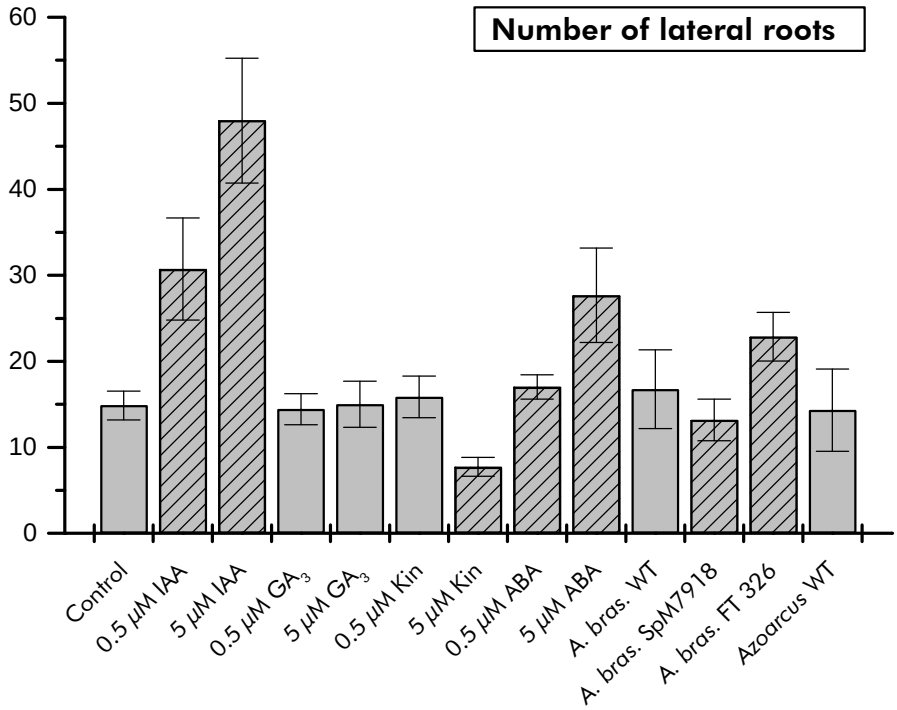


Figure 3.32 Number of lateral roots after treatment of plants with different plant growth regulators and diazotrophic bacteria, respectively, for three weeks. Control plants received nitrate only (50 µM). All measurements which differed significantly from control are presented as striped columns.

3.6.3 Root length

Apart from gibberellic acid, all treatments with plant growth regulators significantly decreased root length (Figure 3.33). This effect was especially pronounced in the presence of IAA, Kin and 5 µM ABA, indicating a concentration-dependent effect for the latter substance. The same phenomenon was observed in the presence of *Azospirillum brasilense* WT and the IAA-hyperoducing strain FT326. No treatment increased root length significantly.

3.6.4 Shoot length

The most pronounced effect on shoot growth was observed in the presence of gibberellic acid, which strongly promoted shoot growth (Figure 3.34). The effect was positively correlated with the concentration supplied. Shoot growth was also

enhanced in the presence of 5 µM IAA as well as in the presence of all bacteria, apart from strain SpM7918.

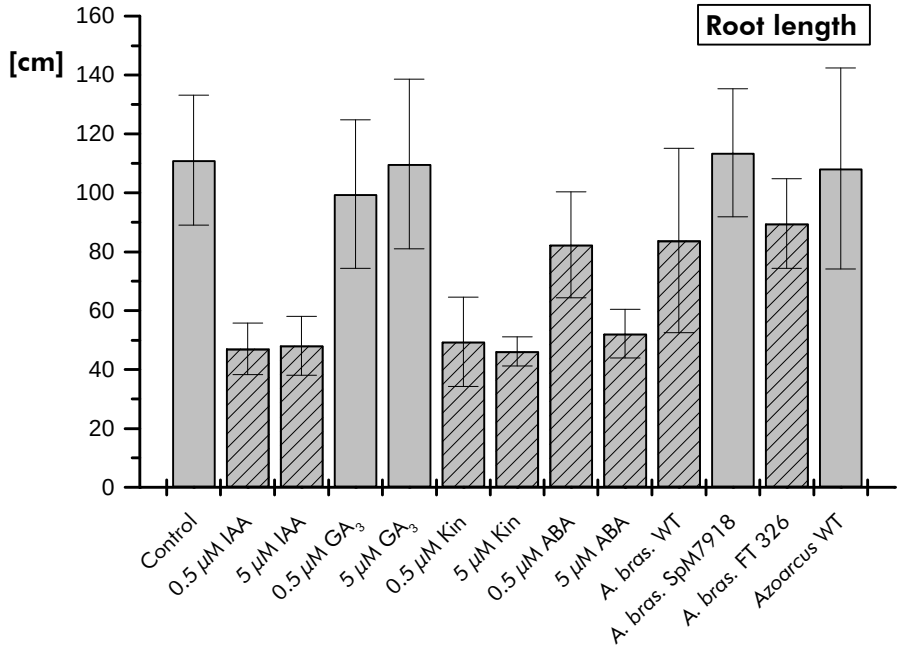


Figure 3.33 Root length after treatment of plants with different plant growth regulators and diazotrophic bacteria, respectively, for three weeks. Control plants received nitrate only (50 µM). All measurements which differed significantly from control are presented as striped columns.

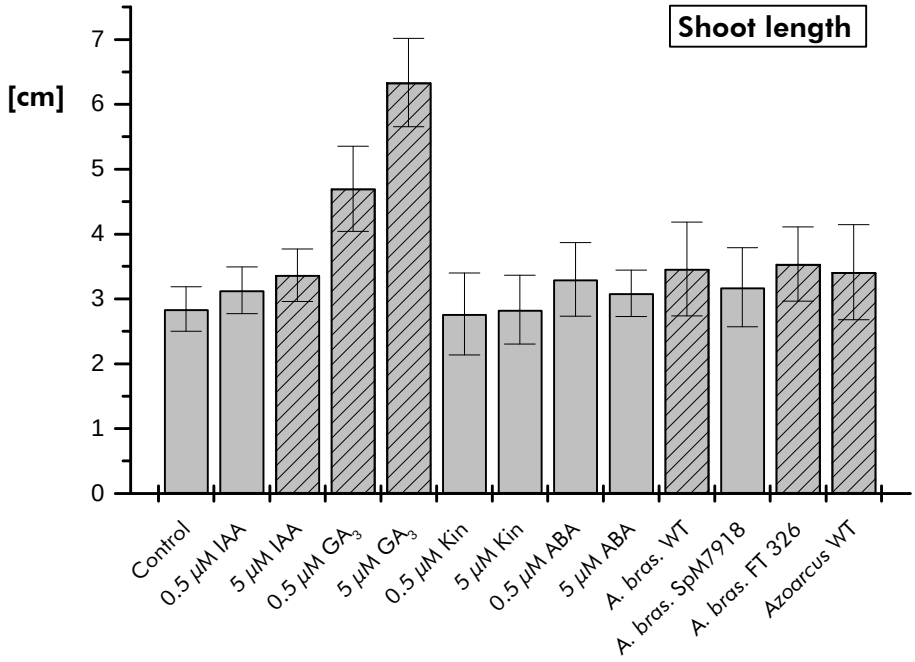


Figure 3.34 Shoot length after treatment of plants with different plant growth regulators and diazotrophic bacteria, respectively, for three weeks. Control plants received nitrate only (50 µM). All measurements which differed significantly from control are presented as striped columns.

3.6.5 Further effects of plant growth regulators on plant morphology

Besides statistical analyses, the following observations were made during plant growth and after harvest.

Control: Plants, grown in the presence of 50 µM NO_3^- as sole nitrogen-source developed a luxuriant root system, consisting of long main and lateral roots. Root hairs were hardly developed (Figure 3.35). There were also no roots of higher order.

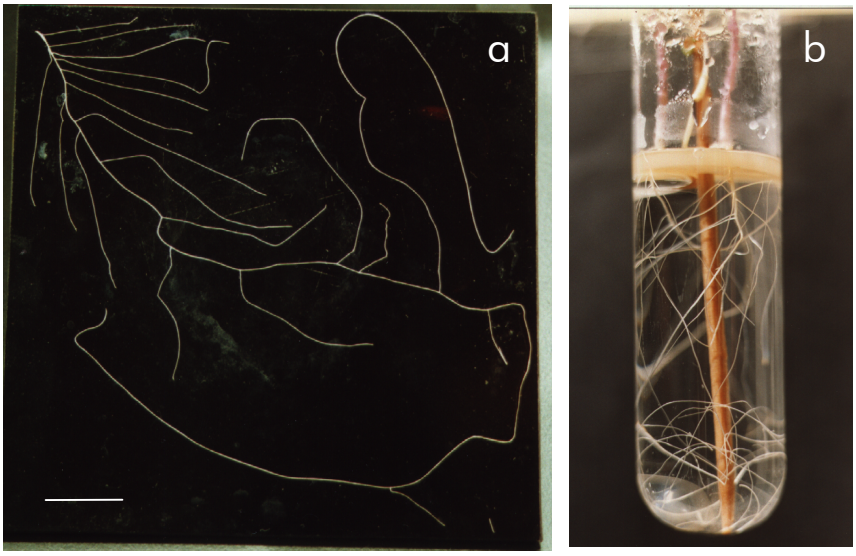


Figure 3.35 Root morphology in the presence of 50 µM NO_3^-. (a) The overall root system of control plants was strongly developed. Bar represents 2 cm. (b) *in vivo*-view of roots in plant tubes.

IAA: The most pronounced effect of IAA on roots concerned the laterals, which initially emerged in large numbers but remained short and very bushy during residual growth (Figure 3.36). The tips of lateral roots were frequently splitted, and the entire main root stayed shorter. In contrast to plants which received only 0.5 µM IAA, the tenfold concentration of IAA caused a strong development of lateral roots of the 2nd order. Furthermore, root hairs could be detected in the presence of both concentrations.

GA_3: While the root system remained rather inconspicuously, the shoot was strongly affected with regard to lengthening. Side roots were long but seemed to be thinner than the main root. No roots of higher order were observed. In the presence of 5 µM GA_3, the main root did also stay thin (Figure 3.37).

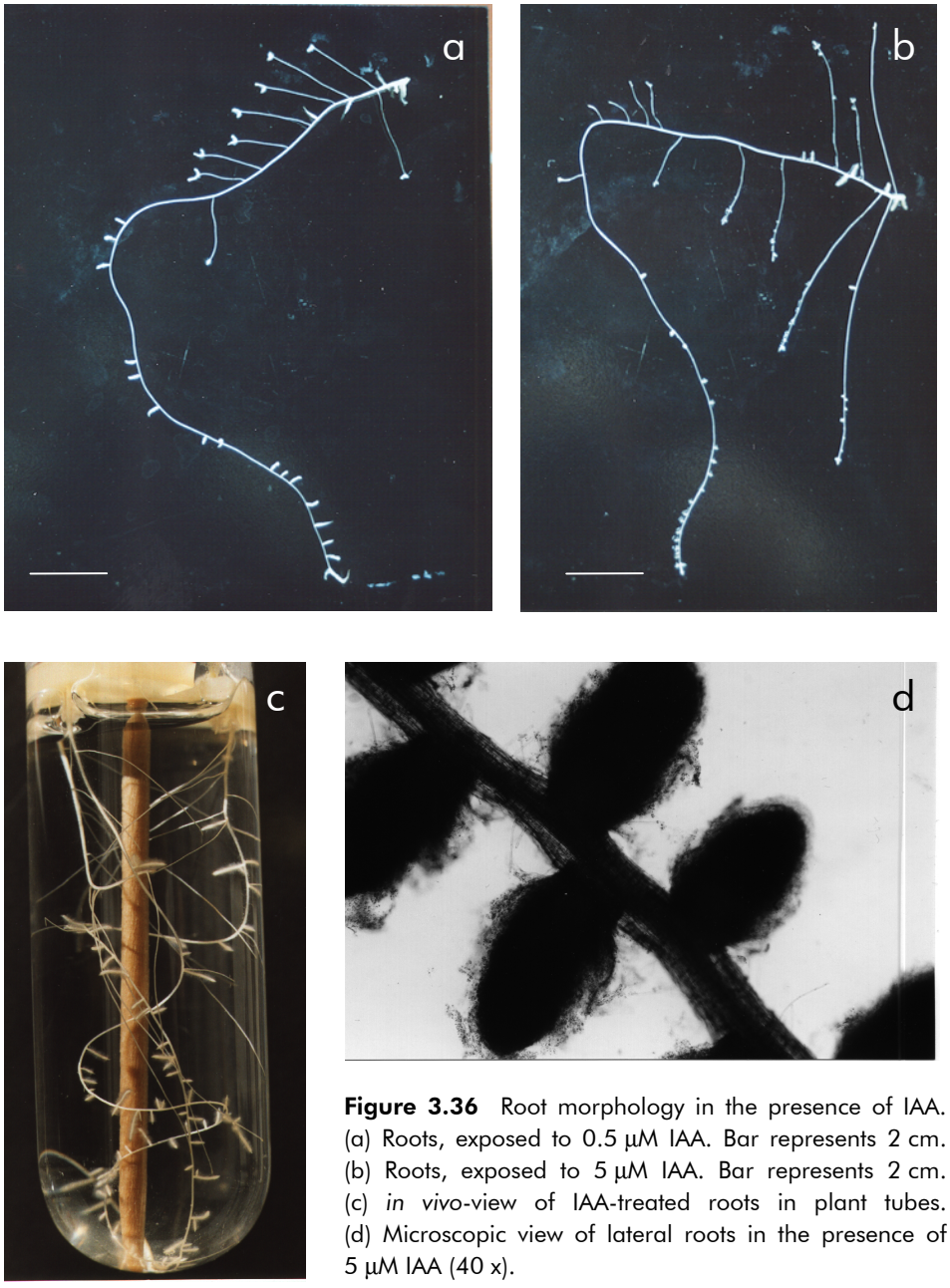


Figure 3.36 Root morphology in the presence of IAA. (a) Roots, exposed to 0.5 µM IAA. Bar represents 2 cm. (b) Roots, exposed to 5 µM IAA. Bar represents 2 cm. (c) *in vivo*-view of IAA-treated roots in plant tubes. (d) Microscopic view of lateral roots in the presence of 5 µM IAA (40 x).

Kin: At the sites of lateral root emergence, nodule-like thickenings were observed (Figure 3.38). Root tips appeared as thick, curled, and bushy structures. In the presence of the tenfold concentration, the number of lateral roots remained low and was restricted to the upper parts of the root system. The overall root growth was strongly inhibited, and no roots of higher order were observed. The entire root, however, seemed to be thicker than in the other samples described above (Figure 3.38).

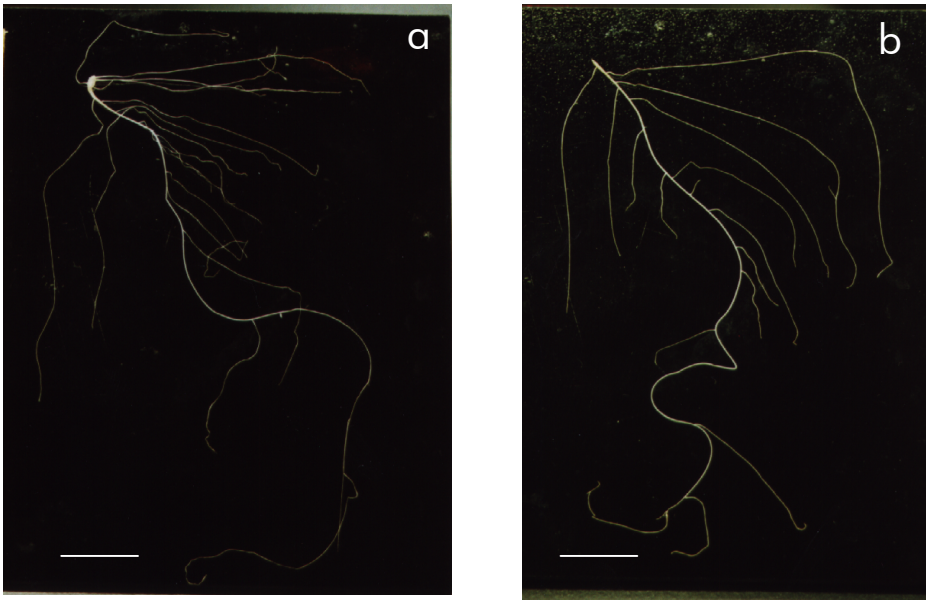


Figure 3.37 Root morphology in the presence of GA_3. The root system did not differ visibly from the control roots. Root segments, however, seemed to be thinner, especially in the presence of 5 µM GA_3 (b). In the 0.5 µM-sample (a), only lateral roots were affected. Bars represent 2 cm each.

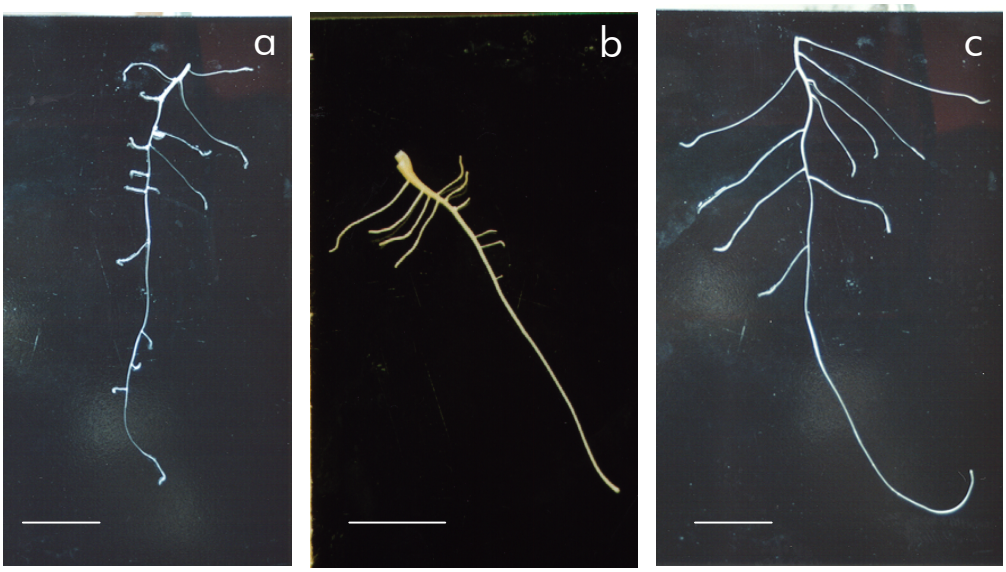


Figure 3.38 Root morphology in the presence of Kin. Root development was strongly inhibited. (a) Roots, exposed to 0.5 µM Kin. (b) and (c) Roots, exposed to 5 µM Kin. Lateral roots were restricted to the upper parts of the main root. Bars represent 2 cm each.

ABA: Abscisic acid caused small, bushy lateral roots, which were frequently splitted at the tips. Splitting at the tip of the main root was also observed. The addition of the tenfold concentration (5 µM) gave rise to many small laterals along the main root. Sites of lateral root emergence appeared bushy and thickened. At both concentrations, roots of higher order developed (Figure 3.39).

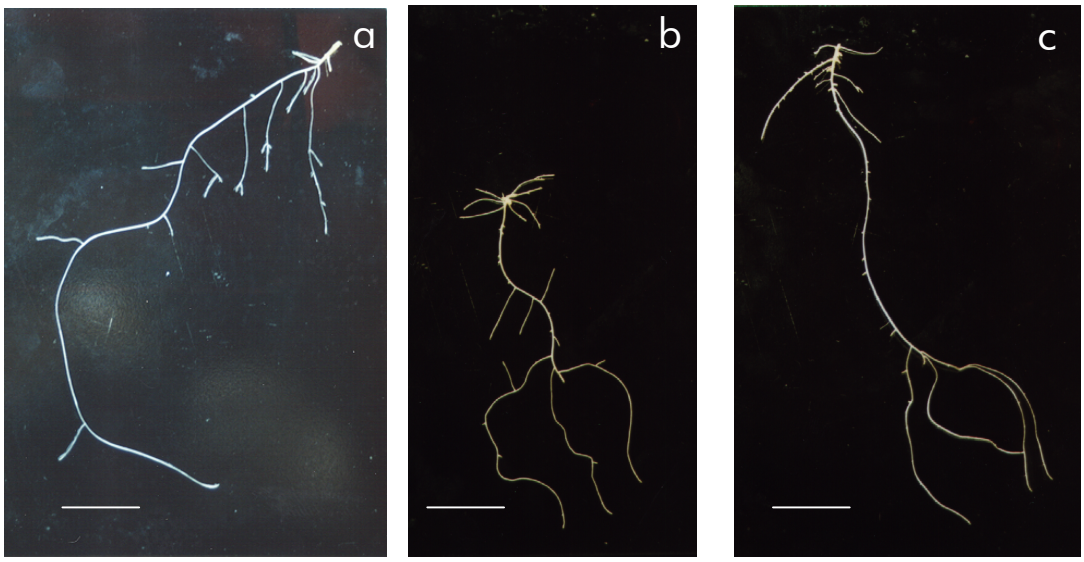


Figure 3.39 Root morphology in the presence of ABA. (a) – (c) Roots, exposed to 5 µM ABA. Most pronounced effect was splitting, curling, and thickening of root tips, as well as promotion of lateral root development. Bars represent 2 cm.

3.6.6 Further effects of diazotrophic bacteria on plant morphology

Azospirillum brasilense:

About 7 days after the inoculation with *Azospirillum brasilense* and the IAA-hyperproducing strain FT326 started, an auxin-like phenotype began to develop. Along the entire main root, lateral roots emerged, which in contrast to the auxin treatment, did not remain short but grew substantially stronger. The sites of lateral root emergence appeared thickened and very bushy. In addition, FT326 caused splitting, curling, and thickening at the tips of lateral roots. Roots of a higher order were found in the presence of both strains. Roots incubated with strain SpM7918 developed long lateral roots, mainly emerging in the upper parts of the main root. Roots of higher order were hardly observed (Figure 3.40).

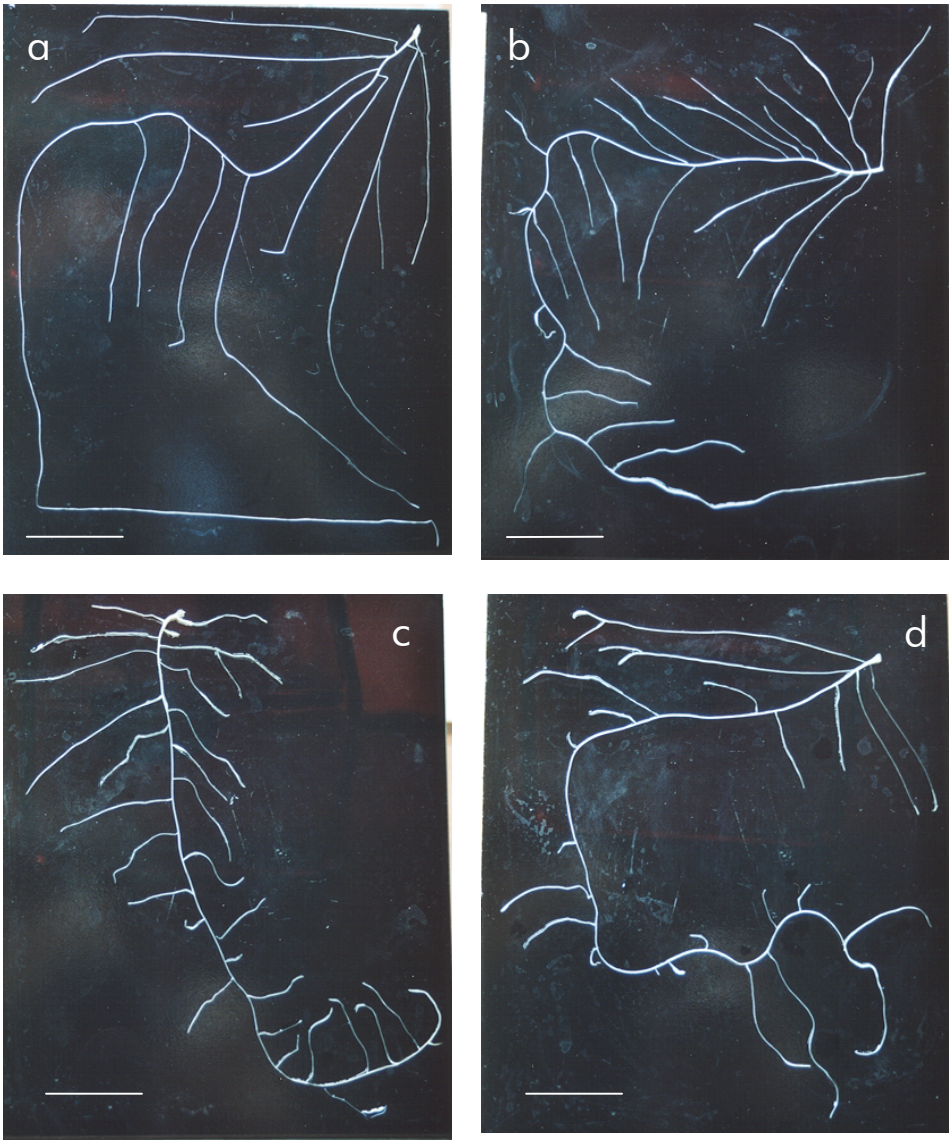


Figure 3.40 Root morphology in the presence of *Azospirillum brasilense*. Bars represent 2 cm each. (a) Roots, exposed to strain SpM7918. Lateral roots appeared as long as in the control plants and were restricted to the upper part of the main root. (b) Roots, exposed to the wild type of *Azospirillum brasilense*. Lateral roots emerged along the entire main root. (c) and (d) Roots, exposed to the IAA-hyperproducing strain FT326. Lateral roots frequently appeaered but remained short in comparison to the wild type. Root-splitting, thickening, and curling was observed at the tips of laterals. (e) *in vivo*-view of roots, inoculated with the wild type of *Azospirillum brsilense*. Sites of lateral root emergence frequently appeared hairy in comparison to control plants.

Azoarcus Sp BH72:

In the presence of *Azoarcus* Sp BH72, the longest roots among all examined samples were found. This was valid for both main and lateral roots. Roots of higher order were also observed (Figure 3.41).

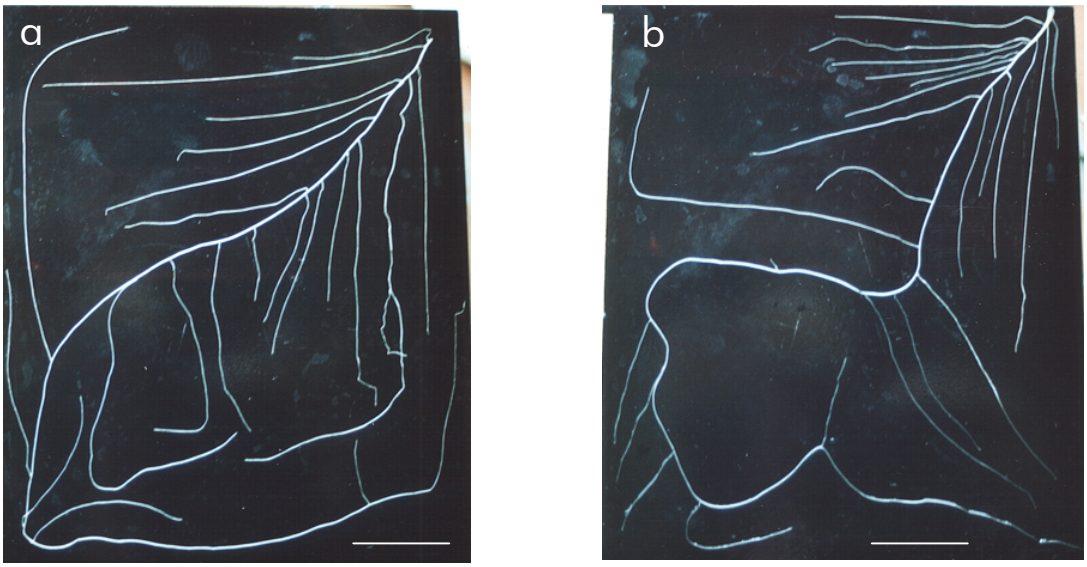


Figure 3.41 Root morphology in the presence of *Azoarcus* Sp BH72. Bars represent 2 cm each. The overall root system appeared as one of the longest, among all roots investigated.

4 Discussion

The purpose of this thesis was to shed some light on the interaction between diazotrophic bacteria and their host plants with regard to the supply of NH_4^+ derived from N_2-fixation. The line of approach to evaluate this aspect was to use a plant gene that responds in a highly sensitive way to traces of externally supplied NH_4^+. A very promising gene that lent itself to this approach is *LeAMT1;2*, encoding for a high-affinity-NH_4^+-transporter. Preliminary work, carried out within the scope of a diplomathesis (Stanke, 1996), characterized this gene as an inducible component of the entire NH_4^+-uptake system in tomato roots. First results revealed the NH_4^+-concentration and pH of the nutrient solution as well as the nitrogen status of the plant seedlings as crucial factors, influencing the expression of *LeAMT1;2*. Under certain circumstances (pH 6.0, N-depleted plants), the expression turned out to be a reciprocal function of the externally supplied NH_4^+-concentration, a property that made this gene extremely useful for the above mentioned purpose.
Considering the large number of complex reactions that are assumed to occur within the plant upon inoculation, various experiments preceded in pure plant cultures to obtain data on the transcriptional regulation of this gene. That is why the first part of the discussion deals with experiments, carried out under sterile, well-defined conditions. Based on these results, the inoculation experiments were then designed such that the expression of the *LeAMT1;2*-gene would have finally pointed to a bacterial effect. This aspect of the thesis is dealt with in the second part of the discussion. To properly draw conclusions from the transcriptional analyses, additional experiments were carried out concerning the uptake rate and the tissue content of ammonium at different treatments. Referring to the inoculation experiments, the exudate pattern of tomato plants and the morphological response to diverse plant growth regulators were analyzed, whereas bacteria were tested for their capability to colonize tomato roots, fix dinitrogen in the association, and promote root growth.

4.1 Regulation of LeAMT1;2

The hydroponic technique used in this work for growing plants ensured to experimentally control the culture conditions by simply exchanging the medium. Furthermore, media were maintained sterile, as growth of fungi or nitrifying organisms could rapidly alter the nitrogen content and -source of a medium (Padgett and Leonard, 1993).
As most experiments required N-depleted plants at the start of the induction period, the frequency of medium exchange during the initial 14 days was kept low, and within the first week of growth, the solution was not changed at all in order to exhaust seed reserve supply. It was considered important, however, that the composition of the nutrient solution and the pH remained constant throughout the induction period, and as the rate of N-uptake, either as NH_4^+ or as NO_3^-, is known to be much greater than that of any other ion (Cox and Reisenauer, 1973), the medium was changed for the last time not later than 2 hrs before harvest. The duration of the induction period

was fixed at 6 hrs, as physiological studies on several plants revealed an increased NH_4^+-uptake rate over a period of 5 to 6 hrs upon first exposure to this ion (Clarkson and Lüttge, 1991; Goyal and Huffaker, 1986; Morgan and Jackson, 1989). The induction period was always started 6 hrs after the onset of the day period, thereby ensuring on the one hand that light-stimulated metabolic activities already started and, on the other hand, harvest could occur before the night period began.

As roots are very sensitive to perturbation, the physical transfer of plants from one solution to another has frequently been shown to produce transplant shock (Bloom and Sukrapanna, 1990; Larsson *et al.*, 1991; Macduff *et al.*, 1997; Cárdenas-Navarro *et al.*, 1998), which might influence the induction of NH_4^+- or NO_3^--absorption. To preclude that the technique to exchange medium affects *LeAMT1;2*-expression, the kinetics of expression upon first exposure to NH_4^+ was followed over a period of 4 hrs using three different approaches (see Section 3.3.1.1).

Irrespective of the technique, used for the replacement of the solution, *LeAMT1;2*-transcript-levels increased already after 1 hr of exposure to NH_4^+, thereby ruling out transplant shock as a possible cause of the observed gene expression. The prompt response reflects the highly inducible character of *LeAMT1;2* and reveals kinetics, comparable to that in *Escherichia coli* (Jayakumar *et al.*, 1987). In barley, a similarly fast response (80 min) was derived from uptake measurements, when N-free grown seedlings were exposed to external NH_4^+ (Mäck and Tischner, 1994). The expression patterns in the three approaches, however, differed from each other and gave rise to new questions concerning the kinetics of *LeAMT1;2*-expression.

Frequently changing the medium resulted in a steady highly-induced state of expression. In contrast, the omission of further changes ended in the halving of the initially strong signal after 4 hrs of exposure, indicating a high turnover-rate of *LeAMT1;2*-mRNA in this approach. To elucidate, whether this decrease was caused by the exhaustion of the initially supplied NH_4^+-concentration, the removal of NH_4^+ from a medium was measured in a course of time, starting with a concentration of 50 µM (see Section 3.4.2.1). The application of this method is controversial, as NH_4^+-efflux is not considered, which would underestimate the actual uptake (Fried *et al.*, 1965). For tomato plants, however, the efflux of NH_4^+ was shown to be negligible at an external concentration of 50 µM (Evans *et al.*, 1996).

Both pre-induced and non-induced plants efficiently scavenged all traces of NH_4^+ from the medium within 8.8 and 6.7 hrs, respectively. The corresponding uptake rates were determined to be 0.533 and 0.658 µmol/g FW·hr, thus rather low compared to rates obtained for higher plants (including tomato) by other authors, ranging from 1.25 – 4 µmol/g FW·hr (Mäck and Tischner, 1994; Kronzucker *et al.*, 1996; Goyal and Huffaker, 1986; Jackson *et al.*, 1993; Kosola and Bloom, 1994). However, considering that media were neither stirred (like in the northern experiments) nor maintained at a constant NH_4^+-level, this discrepancy is not surprising. Interestingly, NH_4^+-uptake was not delayed in non-induced plants and even higher than in the pre-induced plants. This is probably due to the rapid filling of the ‚Apparent Free Space' in the root cortex, as frequently observed in plants upon first exposure to NH_4^+ (Breteler and Siegerist, 1984; Henriksen *et al.*, 1992; Nicolaud and Bloom, 1998) but it might also reflect a feedback signal as a consequence of

prolonged NH_4^+-exposure in pre-induced plants. However, as tomato plants were N-depleted and the applied NH_4^+-concentration was well below the critical concentration for NH_4^+-toxicity (Cox and Reisenauer, 1973; Bennett and Adams, 1970), the latter hypothesis is unlikely. The immediate uptake in non-induced plants might also be mediated by the second HATS for NH_4^+, LeAMT1;1, which was indeed shown to be strongly expressed in N-depleted plants (Lauter *et al.*, 1996; von Wirén *et al.*, 2000).
According to the calculated uptake rates, the amount of NH_4^+, contained in 40 ml of MPCL-low-NH_4^+-medium, is completely taken up within 20 min by nitrogen-depleted, non-induced plants, supporting the idea that the above mentioned halving of the signal intensity after 4 hrs is caused by the exhaustion of NH_4^+ in the medium. These findings suggest that the external NH_4^+-supply is a prerequisite for the transcription of *LeAMT1;2* and emphasize the necessity to replace the medium during the induction-period.
The continuous replacement of medium resulted in a slow increase of the *LeAMT1;2*-transcript-level over time, showing peak expression after 4 hrs of exposure. Bearing in mind that the slow flow-rate, by which NH_4^+ was released into the hydroponic unit, led to a strong initial dilution in the root medium, one might conclude that there is a certain threshold-level of NH_4^+, below which *LeAMT1;2* is not induced. Therefore, the slow induction is probably due to the delayed exceeding of this threshold-concentration. When *LeAMT1;2*-expression was followed in response to different NH_4^+-concentrations in the low micromolar range, it turned out that this gene already shows peak-expression in the presence of 2 µM NH_4^+ (Section 3.3.1.1), and even at 1 µM NH_4^+ the transcript-level is slightly increased. In tomato, the apparent K_m, defined as the nutrient concentration at which the plant's NH_4^+-uptake is 50% of the V_{max}-rate, was reported to range between 5 and 20 µM (Smart and Bloom, 1993). The *AtAMT1;1*-gene, another member of the *AMT1* gene family in *Arabidopsis thaliana*, was even shown to possess an affinity in the nanomolar range (Gazzarrini *et al.*, 1999). These values are consistent with the low NH_4^+-concentrations that are already sufficient to rise the *LeAMT1;2*-transcript-level.

Withholding nitrogen from a growing plant for an appropriate period usually results in an enhanced capacity to absorb nitrogen when the supply is restored (Peuke and Kaiser, 1996; Ullrich *et al.*, 1984; Rawat *et al.*, 1999). As NH_4^+ and NO_3^--transporter genes provide an important molecular target to regulate the nitrogen stress response in plants, the involvement of LeAMT1;2 in this process was analyzed by shifting N-repleted plants to N-free conditions for 1 week, during which plants were harvested 5 times (Section 3.3.1.2). The expression pattern revealed a strong repression of *LeAMT1;2* in N-repleted plants, and a one-week exposure to N-free conditions did only result in a slight increase of transcript-levels. This kind of response is highly unusual for a HATS, as, firstly, N-starvation does obviously not induce *LeAMT1;2*, and, secondly, the induction requires the presence of NH_4^+ suggesting that sensing of NH_4^+ is not a primary function of LeAMT1;2. The lack of stress response and the substrate-inducibility are well-known properties of HATS for NO_3^- (Siddiqi *et al.*, 1992; Ono *et al.*, 2000), whereas similar regulated NH_4^+-uptake

systems were only found in fungi so far (Breiman and Barash, 1980; Roon *et al.* 1975). Recently, a high-affinity phosphate transporter from tomato roots, *LePT1*, was shown to be regulated similarly, that is repressed under conditions of P_i-starvation (Daram *et al.*, 1998).
A possible function of LeAMT1;2 might be the retrieval of leaking NH_4^+ that is generated endogenously and subject to efflux (von Wirén *et al.*, 2000). This could explain the slightly increasing *LeAMT1;2*-expression during the week of N-starvation. Endogenous generation of NH_4^+ is characteristic of plants and assumed to occur as a consequence of protein- and amino acid catabolism (Feng *et al.*, 1998; Morgan and Jackson, 1989; Lam *et al.*, 1996). During N-starvation, the catabolism of organic nitrogen is even enhanced and the vacuolar NO_3^--pool is rapidly declined (Morgan and Jackson, 1988a; see also Section 1.2). Both processes might temporarily increase the cell-NH_4^+-content and thus, the efflux of this ion, which requires an efficient retrieval system, especially under conditions of N-stress.
Efflux of NH_4^+, however, is also typical of N-repleted plants, even when NO_3^- is the sole N-source (Wang *et al.*, 1993a; Morgan and Jackson, 1988b) but *LeAMT1;2*-transcripts could never be detected in N-repleted tomato plants. This result matches the physiological data obtained from the uptake study that was carried out with N-repleted, non-induced plants after exposure to 50 µM NH_4^+ (Section 3.4.2.1). The removal of NH_4^+ in this experiment turned out to occur at a very low rate (168 nmol/g FW·hr) and the polynomial fit of the data predicts the complete removal of NH_4^+ not before 31 hrs of exposure. In this context, the uptake of NH_4^+ by the HATS was often reported to be severely repressed in N-repleted plants (see Section 1.2.1.1), which may be mandatory in minimizing toxic accumulation of NH_4^+ in the cytoplasm above the assimilatory capacity of the roots (Lee, 1993). As the C-content of roots is negatively correlated with the N-status of the whole plant (Jackson *et al.*, 1976), the suppression of NH_4^+-uptake in N-repleted plants is possibly due to the lack of photosynthates serving both as energy source and receptor-molecules for incoming nitrogen.

Such a linkage between NH_4^+-uptake and C-availability in roots is assumed to be responsible for the frequently observed daily variations in the uptake-rates for NH_4^+ showing increase during the day- and decrease during the night-period, in the more recent papers referred to as diurnal regulation (Macduff *et al.*, 1997; Ourry *et al.*, 1996; Cárdenas-Navarro *et al.*, 1998). Observed over the whole day, the expression pattern of *LeAMT1;2* in the presence of 5 µM NH_4^+ revealed that this gene is also subject to a diurnal regulation (Section 3.3.1.3). The transcript-level was initially low, increased during the day-period, peaked at the end of the day and slightly decreased during the night. Of note is that the level showed a transitory decrease 9 hrs after the onset of light, giving the impression of an oscillating signal.
The low transcript-level at the start of the day coincides with the low uptake rate that was frequently observed here (Ourry *et al.*, 1996). Bearing in mind, however, that there was no change of medium prior to the first harvest, the low level might also reflect the response to N-stress (see above). The increase in signal intensity during day and the slight decrease at night is likewise consistent with the physiological data:

considering that the translocation of C-sources usually continues throughout the night, albeit with a decreasing rate, the assimilation of NH_4^+ is only partly carbon-limited. Evidence that the decrease in signal intensity after 9 hrs of exposure was not caused by a technical problem, is given by Macduff *et al.* (1997), who measured highest NH_4^+-uptake rates during the first half of the day. Very recent results showed that the transcript-levels of the two HATS for NO_3^- in tomato roots, are diurnally oscillating showing peak expression 4 hrs after the onset of light and at the end of the day-period (Ono *et al.*, 2000). As other genes of the *AMT1* family were also proven to be subject to a diurnal regulation (Gazzarrini *et al.*, 1999; von Wirén *et al.*, 2000), and *LeAMT1;2* already revealed a diurnal expression pattern in tomato leaves, it is likely that *LeAMT1;2*-expression in tomato roots is regulated likewise, showing 1 to 2 peaks within a day. An oscillating signal is assumed to be caused by multiple factors, such as carbon translocation to the roots, feedback regulation from N-assimilates, and phytochrome-mediated regulation.

Further evidence that NH_4^+-uptake in N-repleted plants may be inhibited by the lack of C-sources is provided by the effect of sugars, externally supplied to the plants (Figure 3.10a in Section 3.3.1.6). The addition of glucose and sucrose slightly increased the signal in N-repleted plants exposed to NH_4^+. Sugars only had no effect, once again proving substrate-inducibility of *LeAMT1;2*. Of note is, however, that in the absence of sugars, NH_4^+ is not able to induce *LeAMT1;2* in N-repleted plants, characterizing the N-status or the C-deficiency as factors with overriding importance regarding *LeAMT1;2*-transcription. Furthermore, a comparison of the NH_4^+-induced transcript-level in N-depleted and N-repleted but carbon-supplemented plants reveals a considerable difference, indicating that the lack of C-sources is not solely responsible for the down-regulation of *LeAMT1;2*. In maize, the addition of glucose was shown to stimulate the uptake of NH_4^+ by N-repleted plants (Morgan and Jackson, 1988a), an observation that would match the transcriptional response of *LeAMT1;2*.
Adding carbohydrates to N-depleted, NH_4^+-exposed plants (Figure 3.10b in Section 3.3.1.6) resulted in different signal intensities according to the source. While glucose enhanced the anyhow high transcript-level, sucrose showed no effect, and fructose even slightly decreased the level. It is unlikely that the activity of *LeAMT1;2* and its transcription is limited by the root's capacity to assimilate the supplied amount of NH_4^+ (50 µM), as roots of N-depleted plants are rich in soluble carbohydrates (Jackson *et al.*, 1976). It might be speculated, however, that the observed differences are a consequence of the strong accumulation of carbohydrates in the root or alternatively reflect antagonistic effects on the uptake processes of NH_4^+ and sugars, both of which are assumed to be energized by the plasma membrane-bound H^+-ATPase (Riesmeyer *et al.*, 1992).

Several metabolites, interfering with the uptake and assimilation of NH_4^+, could markedly affect *LeAMT1;2*-expression. The exposure of N-depleted plants to a high NO_3^--regime resulted in an accumulation of *LeAMT1;2*-transcripts (Section 3.3.1.4) within 60 min, characterizing NO_3^- as a further inducer for *LeAMT1;2* as also shown

by von Wirén *et al.* (2000). In N-depleted plants, the uptake-capacity for NO_3^- is assumed to be already high or rapidly enhanced upon first exposure to this ion (Bloom *et al.*, 1992), and the enzymes, responsible for the reduction of NO_3^- are known to be synthesized within minutes (Crawford, 1995; Tsay *et al.*, 1993; Ono *et al.*, 2000). Therefore, the internal NH_4^+-level probably increased rapidly in these plants, and the simultaneous rise in the *LeAMT1;2*-transcript-level suggests that the signal for its induction may also arise internally. Hence, *LeAMT1;2* might be regarded as a high-affinity NH_4^+-uptake system that is regulated by the internal NH_4^+-content. Bearing in mind that internal NH_4^+ is subject to efflux and has to be retrieved under conditions of N-starvation, the expression of a retrieval-system like LeAMT1;2 appears logical under these circumstances. *LeAMT1;2*-expression is obviously subject to a negative feedback-signal, as expression in this experiment has peaked after 120 min of exposure to NO_3^- and slightly decreased thereafter. This down-regulation is certainly not caused by the exhaustion of NO_3^- in the medium because the initial concentration was rather high (15 mM) and the medium was renewed during the induction-period. It appears that either the accumulation of NH_4^+ or an NH_4^+-assimilation product, i.e. the plant's N-status, is responsible for this feedback. It might be speculated that *LeAMT1;2* is completely down-regulated as soon as the plant releases the signal for a repleted N-status on the whole-plant-level, as a retrieval-system in such plants is redundant (see also below). Alternatively, the down-regulation of *LeAMT1;2* might be due to a decrease in NO_3^--reduction and internal NH_4^+-supply as a consequence of increasing C-deficiency in the root (Aslam *et al.*, 1979; Ashley *et al.*, 1975; Oaks and Hirel, 1985).
In several plants and fungi, the initial NH_4^+-uptake rate was shown to be enhanced upon pretreatment with NO_3^- (Kronzucker *et al.*, 1996; Goldsmith *et al.*, 1973; Kleiner, 1981). These findings are consistent with the induction of an NH_4^+-uptake system by NO_3^-.

The addition of nitrite (NO_2^-) to N-depleted plants severely repressed *LeAMT1;2*-expression even in the presence of NH_4^+ (Section 3.3.1.5), thereby sharply contrasts with the response to NO_3^-. Due to its toxicity, NO_2^- has to be reduced to NH_4^+ immediately after uptake (Aslam *et al.*, 1979). Considering the high initial NO_2^--supply, it might be assumed that the internal NH_4^+-content in this experiment is strongly increased throughout the induction period, again indicating that the intracellular accumulation of NH_4^+ or an assimilation-product of NH_4^+ represses *LeAMT1;2*. In barley, nitrite inhibited NH_4^+-uptake on the physiological level (Aslam *et al.*, 1996), coinciding with the transcriptional response in tomato.

In N-depleted plants, glutamine and γ-aminobutyrate (GABA) repressed the expression of *LeAMT1;2* in the presence of NH_4^+ (Figure 3.10b+c in Section 3.3.1.6). NO_3^- and alanine had only a moderate repressive effect, whereas aspartate and glutamate even increased the level slightly (data not shown). When MSX, an inhibitor of glutamine synthetase (GS), was added, no transcripts could be detected, irrespective of whether the NH_4^+-concentration was 0.05 or 5 mM (Figure 3.10d). In the absence of MSX, however, the expression level was inversely correlated with the

supplied NH_4^+-concentration. Such a pattern is not unusual, as, for example, the NH_4^+-transporters *MEP1* and *MEP2* in *Saccharomyces cerevisiae* and the phosphate transporters *LePT1* and *LePT2* in tomato were shown to be regulated just the same way (Marini *et al.*, 1994a; Marini *et al.*, 1994b; Liu *et al.*, 1998). The analysis of NH_4^+-content in similar treated roots revealed that the accumulation of NH_4^+ in plant roots is positively correlated with the externally supplied concentration (Section 3.4.1) and hence, negatively correlated with the *LeAMT1;2*-transcript-level. Some authors suggest that at a high NH_4^+-supply, the low-affinity uptake-system is responsible for the toxic accumulation of NH_4^+ in the plant (Dubois and Grenson, 1979; Rawat *et al.*, 1999), thereby postulating that this system is not subject to any feedback-regulation upon NH_4^+-accumulation. In contrast, N-starved plant roots were determined to contain approximately 1/30 of the amount in roots, exposed to 50 µM NH_4^+ (Section 3.4.1.1). A highly-elevated NH_4^+-content was also measured in the MSX-treated roots and, interestingly, the strongest rise of internal NH_4^+ was caused by the addition of glutamine.

The latter finding is rather puzzling, as the question raises for the origin of this high level. Conceivable is either an increased NH_4^+-uptake, a negative feedback on NH_4^+-assimilation, or the catabolism of glutamine via glutamate, catalyzed by GDH (see Section 1.2.2). The latter case is not probable, as the addition of glutamate did not markedly rise the internal NH_4^+-level. An increased NH_4^+-uptake is also unlikely, as at least the down-regulated transcript-level of *LeAMT1;2* and numerous reports about inhibition of NH_4^+-uptake in the presence of glutamine would not match this hypothesis (Raper *et al.*, 1991; Syrett and Peplinska, 1988; Lee *et al.*, 1992). In many plants, the glutamine level is assumed to reflect the plant's N-status, and high glutamine levels prevent the assimilation of NH_4^+ due to a feedback inhibition of the GS/GOGAT-pathway (Sitte *et al.*, 1991). Therefore, the observed accumulation of NH_4^+ is probably due to the inhibition of the GS by glutamine. Whether glutamine itself may serve as a repressor of *LeAMT1;2*, is not apparent from the available data, as it might be concluded from the MSX-experiment that the *LeAMT1;2*-expression in roots of glutamine-treated plants is just regulated by the intracellular NH_4^+-pool. According to the single measurements (not shown) repression is revealed both at very high (> 890 µM) and very low (< 20 µM) NH_4^+-levels, whereas *LeAMT1;2* is strongly induced at internal NH_4^+-concentrations between 210 and 570 µM NH_4^+. Within this critical range, a retrieval system for NH_4^+ might perfectly fulfill its function, as efflux of NH_4^+ would lead to indispensable losses. At higher contents NH_4^+-efflux may be crucial to avoid the toxic accumulation of NH_4^+ in the plant, which is why a retrieval system is rather disadvantageous under these circumstances. Consistent with this hypothesis, a treatment of wheat roots with MSX was shown to increase the NH_4^+-efflux rate (Morgan and Jackson, 1989). At very low contents the efflux of NH_4^+ might be negligible.

In *Arabidopsis thaliana*, the *AtAMT1*-gene, which is highly expressed under conditions of N-starvation, was shown to be down-regulated by glutamine, whereas an elevated NH_4^+-level, caused by MSX-treatment, did not repress *AtAMT1*, indicating that, unlike *LeAMT1;2*, the intracellular NH_4^+-level does not regulate *AtAMT1* (Rawat *et al.*, 1999). This is no contradiction, as the studies of N-transport often revealed the

existence of several permeases with different affinities, specifities, capacities, and regulation properties (Marini *et al.*, 1994b), enabling plants to adapt to many different growth conditions (Rawat *et al.*, 1999).
In tomato roots, the inhibition of the GS/GOGAT-pathway by MSX was shown to result in NH_4^+-assimilation via GDH, catalyzing the synthesis of glutamate (Magalhaes, 1991). Glutamate, in turn, can then be used both for transamination with pyruvate to yield alanine and decarboxylation to yield GABA. Under conditions of NH_4^+-excess, these three amino acids may accumulate in the plant, however, GABA revealed to be the only substrate, which could down-regulate *LeAMT1;2*, indicating that GABA might be a key metabolite in the nitrogen-metabolism of tomato. Considering the GDH-pathway of NH_4^+-assimilation, the accumulation of GABA upon MSX-treatment and its repressive effect on *LeAMT1;2*-expression cannot be ruled out. Bearing in mind that the NH_4^+-contents of roots treated with either MSX or 5 mM NH_4^+ were approximately the same but the repression in the presence of MSX was much stronger, a cumulative effect on *LeAMT1;2*-repression by GABA and a high NH_4^+-level is possible.
The slight decrease of *LeAMT1;2*-expression in the presence of NO_3^- may reflect a moderating effect of NO_3^- on NH_4^+-uptake that was already observed in plants by some authors (Deignan and Lewis, 1988). However, as low concentrations of NH_4^+ (< 200 µM) were shown to stimulate NO_3^--absorption (Lee and Drew, 1989; Smart and Bloom, 1998), it is rather likely that NO_3^- is taken up and immediately reduced as a consequence of N-deficiency. Therefore, the slight down-regulation of *LeAMT1;2* is probably of the same nature as in the presence of NO_3^- only (Section 3.3.1.4, see also above).

The solution's pH turned out to be a further crucial factor for the evaluation of the *LeAMT1;2*-response. In earlier studies, within the scope of a diplomathesis (Stanke, 1996), the induction of *LeAMT1;2* by external NH_4^+ was severalfold enhanced, when the pH was dropped to 4.7, and the peak expression was shifted from 5 to 50 µM NH_4^+. In this study, the pH-effect was tested in the absence of external NH_4^+, which resulted in a similar response (Section 3.3.1.7). Hence, the external proton-concentration is positively correlated with the *LeAMT1;2*-transcript-level in roots. In N-repleted plants, however, this effect was cancelled, which is why the plant´s N-status is confirmed to be a factor of overriding importance for *LeAMT1;2*-expression.
The NH_4^+-content of plant roots exposed to 50 µM NH_4^+ was only slightly higher at pH 6.0 than at pH 4.7 (Section 3.4.1). At the same time, however, the *LeAMT1;2*-level in the latter roots was much higher (Stanke, 1996). Dyhr-Jensen and Brix (1996) have shown that the affinity of the transporter for NH_4^+ is decreased with pH. Therefore, the increased level in acid media may rather reflect the lower affinity of *LeAMT1;2* than the response to the intracellular NH_4^+-concentration. It appears as if the plant needs to compensate for the decreased affinity by synthesizing more transporter-proteins.
The fact that the higher expression of *LeAMT1;2* in acid media is independent of the presence of external NH_4^+ is at first puzzling. However, this observation supports earlier findings that the signal for *LeAMT1;2*-expression is likely to arise internally. As

already noted, endogenous NH_4^+-generation is possible by different processes, namely NO_3^--reduction or catabolism of organic N. The first-mentioned process was assumed to be responsible for the observed *LeAMT1;2*-expression in N-depleted plants exposed to NO_3^- (see above). The latter process, however, was reported to occur upon N-starvation and is therefore probably responsible for the moderate increase of *LeAMT1;2*-transcript-level after 1 week of N-starvation (see above).
pH-activity profiles have frequently proven that NH_4^+-uptake has its optimum between pH 6 and 7 and is inhibited in acid media (Marcus-Wyner, 1983; Findenegg, 1987; Chaillou *et al.*, 1991; Ninnemann *et al.*, 1994). The latter is assumed to be a consequence of unfavourable conditions for the H^+-ATPase-activity, as the uptake of NH_4^+ is accompanied by the extrusion of protons (Venegoni *et al.*, 1997; Smart and Bloom, 1998). Furthermore, the assimilation of NH_4^+ releases protons internally, which have to be removed from the cytoplasm (Ganmore-Neumann and Kafkafi, 1980; Henriksen *et al.*, 1992; Salsac *et al.*, 1987). As a result of the reduced H^+-ATPase-activity, however, the extrusion of protons cannot keep pace with the continuous assimilation-process, which is why the internal pH is also assumed to decrease at a prolonged time of NH_4^+-nutrition (Kirkby and Mengel, 1967; Jungk, 1970; Cao *et al.*, 1993). The reduced NH_4^+-uptake in acid media causes N-stress in plants (Tolley-Henry and Raper, 1989; Tolley-Henry and Raper, 1986), thereby leading to an endogenous generation of NH_4^+ (Jackson *et al.*, 1993). The acid conditions, however, seriously interfere with the reassimilation of this NH_4^+, which consequently accumulates within the root (Barker *et al.*, 1966). Therefore, the pH-response may rather confirm the function of *LeAMT1;2* in NH_4^+-retention, and the comparatively higher signal in acid media is probably due to the decreased affinity to NH_4^+. On the other hand, recent results indicate that the cytosolic pH may serve as a second messenger, being involved in cellular metabolism, ion-channel-activities, and transcriptional regulation of different genes (Lapous *et al.*, 1998; Zimmermann *et al.*, 1999). Hence, a direct role of the pH in regulating *LeAMT1;2*-transcription cannot be excluded.
The stronger down-regulation of *LeAMT1;2* in response to a higher pH may reflect significant N-supply by the uncharged species NH_3, thereby the presence of an energy-demanding uptake system is hardly necessary. However, the N-nutrition by NH_3 is highly controversial, and at pH 6.0, used for most experiments, no NH_3-diffusion was shown to occur in tomato (Evans *et al.*, 1996).

In many plants, potassium was shown to counteract the detrimental effect of NH_4^+-nutrition (see Section 1.2.1.2). Comparing the *LeAMT1;2*-expression in N-depleted plants, exposed to NH_4^+ and different K^+-concentrations (Section 3.3.1.8), it turned out that a high potassium level (130 mM) markedly repressed *LeAMT1;2*-expression, whereas a low K^+-concentration (1.3 mM) did not affect *LeAMT1;2*-transcript-levels compared to the control (13 mM K^+). However, the addition of cesium (Cs^+), an inhibitor of voltage-gated inward and outward rectifying K^+-channels (Maathuis and Sanders, 1995; Bouteau *et al.*, 1999), led to a strong induction of *LeAMT1;2* in spite of the low K^+-level (1.3 mM) in the medium.

Potassium was frequently observed to inhibit NH_4^+-accumulation in higher plants (Barker *et al.*, 1967; Ajayi *et al.*, 1970; Maynard *et al.*, 1966). However, the uptake of NH_4^+ itself is not inhibited by K^+ (Rosen and Carlson, 1984; Cao *et al.*, 1993), unless the transport is mediated by the LATS for K^+. The possible uptake of NH_4^+ via K^+-channels was proven several times (Cao *et al.*, 1995; Ninnemann *et al.*, 1994) but due to their lower conductance for NH_4^+, the K^+-channel-mediated uptake is assumed to be significant only at external NH_4^+-concentrations from 100 µM upwards (von Wirén *et al.*, 1997; Gazzarrini *et al.*, 1999). The conductivity might be increased, however, through high activities of the plasma membrane-bound H^+-ATPase, which hyperpolarizes the membrane potential and enables NH_4^+ to enter the cell via K^+-channels at even lower concentrations (Venegoni *et al.*, 1997).
Probably, K^+ inhibits NH_4^+-accumulation in plant cells rather by the promotion of NH_4^+-assimilation (Wilcox, 1964). Furthermore, the cellular pH and the ionic balance is assumed to be controlled by potassium, as protons in the cytoplasm might be exchanged for external K^+-ions (Cao *et al.*, 1993). The uptake of NH_4^+ in several plants was shown to be accompanied by K^+-efflux instead of proton-extrusion, thereby preventing a drop in the external pH (Marcus-Wyner, 1983; Pan *et al.*, 1985; Morgan and Jackson, 1988a).
Therefore again, the increased *LeAMT1;2*-level in the presence of Cs^+ might result from the internal accumulation of NH_4^+ due to the inhibition of K^+-uptake. The very recent isolation of a Cs^+-sensitive K^+-uptake channel from root hairs of tomato (Hartje *et al.*, 2000), and the characteristic K^+-sensitivity of the *AMT1*-gene family (see Section 1.2.1.3) suggest that K^+ and NH_4^+ might tightly interact both on the level of uptake and assimilation. Of note is that a lack of potassium was shown to increase the cellular content of the plant growth regulators IAA and cytokinine, both of which inhibit root growth (Cao *et al.*, 1993). As plant growth regulators are known to control numerous metabolic pathways and uptake activities in plants (Zimmermann *et al.*, 1999), it cannot be precluded that *LeAMT1;2*-expression is affected by these regulators (see also below).
Furthermore, it should be noted that the strong down-regulation of *LeAMT1;2* in the presence of 130 mM K^+ could also be caused by an osmotic shock, as many authors have shown that the supply of a high-salt-regime severely inhibits the uptake of nitrogen (Clarkson and Lüttge, 1991; Klobus *et al.*, 1988; Jayakumar *et al.*, 1985).

The effect of plant growth regulators on the expression of *LeAMT1;2* was tested, as some of them are known to be excreted by *Azospirillum brasilense* (Bashan and Levanony, 1990). As RNA of control plants, exposed to NH_4^+ and N-free conditions only, was not loaded on the gels, no statements are possible regarding the absolute effect of each growth regulator. Therefore, effects of individual growth regulators can only be deduced on the basis of earlier results showing that plants, exposed to NH_4^+, usually develop a strong signal, whereas plants, exposed to N-free conditions, generate no detectable transcripts (see above).
Upon addition of growth regulators, various responses could be observed and the effects were dependent on both the supplied concentration and the presence of NH_4^+ (Section 3.3.1.9). Comparing the signals of NH_4^+-exposed and N-free-grown plants,

it is noticeable that gibberellic acid (GA_3) and 2-naphtaleneacetic acid (2-NAA) could neutralize the NH_4^+-inducible feature of *LeAMT1;2*-expression. On examination of the other samples that were simultaneously loaded on the gel (kinetin and 1-NAA, respectively), it appears that this is rather achieved by induction than by repression.
In several studies, GA_3 was shown to have multiple effects on plant development. It promotes seed germination, stem elongation, and flowering (Gray and Estelle, 1998). By breaking dormancy, proteins and amino acids in the seed are degraded (de Klerk, 1998), and, as shown in mustard seedlings, enzymes of the N-assimilation pathway, namely nitrate reductase and glutamine synthetase, are activated (Chanda *et al.*, 1998). In maize coleoptiles GA_3 was reported to be responsible for cytoplasmic acidification (Gehring *et al.*, 1994). All three aspects were previously assumed to potentially rise the cytoplasmic NH_4^+-level. Therefore, it might be speculated that GA_3 increases the *LeAMT1;2*-transcript-level indirectly by means of endogenous NH_4^+-generation.
In this context, the expression of *LeAMT1;2* was analyzed in young seedlings, which were germinated on a filter and not yet exposed to hydroponic conditions (Section 3.3.1.10). Although NH_4^+ is assumed to be contained in seeds and released upon break of seed dormancy and germination (Oliveira *et al.*, 1997; Mäck and Tischner, 1994), no transcripts could be detected under these circumstances. It is possible, however, that efflux of endogenously generated NH_4^+ is dramatically enhanced in the hydroponic units (Deignan and Lewis, 1988), which might contribute to the increase of *LeAMT1;2*-expression.
The very strong response to 2-NAA, however, is puzzling, as 2-NAA displays only weak auxin activities (Imhoff *et al.*, 2000) and is commonly employed to inhibit auxin influx. In addition, it also perturbs auxin-efflux but shows a high affinity towards auxin-binding proteins (ABPs), some of which are integrated membrane proteins (Zettl *et al.*, 1994). As the molecular mechansims that mediate reception and signal transduction upon binding of plant growth regulators to ABPs are largely unknown to date (Zimmermann *et al.*, 1999; Guilfoyle *et al.*, 1998), the cause of the strong induction of *LeAMT1;2* in response to 2-NAA remains unclear for the time being and needs to be investigated in more detail.
In the presence of indole-3-acetic acid (IAA), abscisic acid (ABA), kinetin and 1-NAA, the inducible effect of NH_4^+ is visible. However, apart from 1-NAA, this is only valid for the lower concentration (0.5 µM), as it turned out that the tenfold concentration decreased *LeAMT1;2*-expression to the level obtained in the absence of N. This is consistent with the frequent observation that the effect of plant growth regulators is always a matter of the supplied concentration (Pilet, 1998), and in most cases, higher levels reveal inhibiting effects (Thiel and Weise, 1999). Amazingly, 1-NAA shows a reverse effect, that is the signal increases with the concentration of 1-NAA. Like IAA, 1-NAA is an auxin but it is more active and more stable (Chen *et al.*, 1995; Christiansen-Weniger, 1998). In contrast to IAA, it is not dependent on the presence of an active uptake system, as it is able to passively cross the membrane (Estelle, 1998). On the other hand, 1-NAA is much more subject to an actively mediated efflux than IAA (Imhoff *et al.*, 2000). Whether these differences account for the observed effect, is not apparent from the available data.

The down-regulation of *LeAMT1;2* by an increased level of plant growth regulators is most pronounced in the presence of IAA. In many studies, the effects of IAA were well recorded: cell-elongation, cell-division, cell-differentiation, adventitious root formation and inhibition of root growth are only some of the observed effects (Guilfoyle *et al.*, 1998; de Klerk, 1998). The mechanism, by which auxins affect plant growth are partly understood. It was shown, that upon addition of IAA, (or active derivatives) the membrane-bound H^+-ATPase is activated, thereby extruding protons and hyperpolarizing the membrane potential. This leads to an increased influx of K^+ and an acidification of the cell wall, finally resulting in cell-elongation (Catala *et al.*, 2000; Thiel and Weise, 1999). At the same time, protons are released from the vacuole, and, hence, the intracellular pH drops as well (Zimmermann *et al.*, 1999). As the acidification of the cytoplasm is assumed to be a possible factor influencing *LeAMT1;2*-expression (see above), the IAA-induced change in pH may be responsible for the observed response. When different IAA-concentrations were tested in the absence of NH_4^+, it turned out that *LeAMT1;2*-expression showed a concentration-dependent pattern, peaking in the range between 100 and 500 nm IAA. At higher concentrations (> 5 µM) *LeAMT1;2* is strongly repressed confirming that the concentration of a growth regulator needs to be tightly regulated in order to achieve a desired effect.

In contrast to IAA, ABA inhibits both ATPase-activity and K^+-influx. The cellular pH shifts to more alkaline conditions (Zimmermann *et al.*, 1999) and specific enzymes of the N-assimilation-pathway were shown to be affected, that is activation of nitrate reductase and inhibition of glutamine synthetase (Chanda *et al.*, 1998). In spite of the reverse action, the addition of ABA revealed the same pattern as IAA regarding *LeAMT1;2*-expression. It might be speculated that in the more alkaline range, the formation of the uncharged species NH_3 is promoted, especially under conditions of GS-inhibition. This might probably lead to a higher efflux out of the root by means of diffusion and an increased demand for an NH_4^+-retrieval system.

Kinetin is a cytokinin and its main effects on plant growth are the promotion of cell division and inhibition of root and shoot formation (de Klerk, 1998). Like ABA, kinetin activates nitrate reductase and inhibits glutamine synthetase (Chanda *et al.*, 1998). The pattern of *LeAMT1;2*-expression in the presence of kinetin is similar to IAA and ABA, although less pronounced and even inverse in the absence of nitrogen.

When IAA, ABA, GA_3, and kinetin were tested for their influence on *LeAMT1;2*-expression in N-repleted plants, no transcripts could be detected indicating that plant growth regulators are not able to overcome the strong repressive effect exerted by the plant's high N-status.

As a consequence of multiple, very complex, and not yet fully understood processes that occur upon the addition of plant growth regulators, the observed patterns are difficult to explain, and most attempts to draw conclusions from the known mechanisms on the expression of *LeAMT1;2* are highly speculative. No data are available to date about possible effects of plant growth regulators on the uptake process of NH_4^+.

In conclusion, the response of *LeAMT1;2*-expression to N-supply suggests that both plant N-nutritional status and substrate availability have to be considered. The kinetics of *LeAMT1;2*-expression reveals that this gene may rapidly adapt to environmental signals, thereby displaying an economic pattern, that is driven by immediate demand. NH_4^+-concentrations of 2 µM turned out to be already sufficient to cause induction of *LeAMT1;2* emphasizing its high affinity. Of note is further that the presence of NH_4^+ is required to affect *LeAMT1;2*-transcription as N-starvation did not induce *LeAMT1;2*. The revelation of the diurnal rhythm suggests that C-sources are required for *LeAMT1;2*-mediated uptake of NH_4^+. Support for this hypothesis is given by the fact that in N-depleted, C-starved plant roots, no transcripts could ever be detected unless a C-source was externally added.
The strong *LeAMT1;2*-response of N-depleted plants to NO_3^- reveals that the signal for the induction may arise internally, thus characterizing LeAMT1;2 as a retention system for NH_4^+ in times of N-stress. On the other hand, the strong repression of *LeAMT1;2* in response to NO_2^-, MSX and high external NH_4^+-concentrations leads to the assumption that the intracellular NH_4^+-level is a major determinant for the observed pattern. As noted, the plant's N-status is of overriding importance for the *LeAMT1;2*-transcription. Whether glutamine serves as the primary signal of cellular N-status, is not certain, as the analysis of the NH_4^+-content revealed high values upon the addition of glutamine.
The pH of the nutrient solution as well as the K^+-status of the plant seedlings are further factors to be considered. Acidification of the medium and inhibition of K^+-uptake increased the *LeAMT1;2*-transcript-level, probably as a consequence of intracellular NH_4^+-accumulation.
Finally, plant growth regulators IAA, ABA, GA_3, kinetin, 1-NAA, and 2-NAA could affect *LeAMT1;2*-expression. GA_3 and 2-NAA turned out to be the only compounds, causing *LeAMT1;2*-induction in the absence of NH_4^+, whereas the other growth regulators, apart from 1-NAA, revealed a repressive effect at higher concentrations.

4.2 LeAMT1;2, a target for fixed dinitrogen?

The capability of beneficial bacteria to colonize plants is generally considered crucial for plant growth promotion. Therefore, prior to northern blot analyses the colonization pattern of both *Azospirillum brasilense* and *Azoarcus* Sp BH72 was investigated. *Azospirillum brasilense* was detected by means of a plasmid encoded and constitutively expressed *gusA*-gene, whereupon it turned out that after 48 hrs of incubation the entire surface of tomato roots was already colonized (Section 3.2.1). Intensity of blue zones identified the basis of lateral root emergence, root tips, and root hairs as preferred sites of colonization. This pattern was also found by other authors, according to which *Azospirillum* bacteria are even capable of colonizing all zones of a root depending on the plant species, the time of inoculation and the composition of the medium (Bashan and Levanony, 1990; Kennedy and Tchan, 1992; Hadas and Okon, 1987).

Lateral root emergence is assumed to cause lesions (Michiels *et al.*, 1989; Kennedy and Tchan, 1992), which are major sources of leaking root exudates (Rovira, 1969) serving as attractants for the chemotactic response of *Azospirillum brasilense* (Bashan, 1999). The colonization of root hairs was also reported for wheat but required N-free conditions in the medium (Vande Broek, 1993), which were also applied in this study. GUS-staining of tomato root tips forms a contrast to wheat roots. Uninoculated roots remained white confirming that tomato plants do not exhibit β-glucuronidase activity. On the other hand, GUS-staining was reported to produce artefacts (Hurek *et al.*, 1994a) as a consequence of diffusing indoxyls, which did not precipitate yet. As the assay was stopped as soon as first signs of staining were visible, the risk of unspecific GUS-staining was minimized.
A vast number of *Azoarcus* cells was found in the root hair zone (Section 3.2.2). In contrast to *Azospirillum*, colonization of root hairs by *Azoarcus* was not observed (Figure 3.3c). However, due to the technical efforts, only a small segment of the root was analyzed, and the available data is not sufficient for an accurate assessment of root hair colonization. *Azoarcus* cells were embedded in a non-identifiable substance, which might probably represent some kind of fibrilar material that was often observed in root surface colonies of *Azospirillum brasilense* (Bashan and Holguin, 1997). In rice and Kallar grass, *Azoarcus* was shown to preferentially colonize the apical region of the root behind the meristem but colonization of the root interior was also observed (Hurek *et al.*, 1994a).
Whenever the expression of *LeAMT1;2* was evaluated in response to an inoculum, the induction-period was prolonged to 10 hrs, which was considered sufficient to obtain an appropriate colonization density on roots. Studies on the colonization process of *Azospirillum* revealed 2 distinct phases referred to as absorption and anchoring (Michiels *et al.*, 1991). While the absorption phase leads to a rapid colonization by a vast number of bacteria within 2 hrs of incubation, the anchoring phase ensures the irreversibility of this process, as bacteria bind very tightly to the surface by means of their polar flagellum. The latter phase, however, is assumed to start not before 8 hrs of incubation and is favoured by the addition of fructose (Zaady *et al.*, 1993). As the preparation of the inoculum required a twofold washing-step by centrifugation, it is possible that the polar flagellum was partly broken off when bacteria were added to the plants, as reported by Arsène *et al.* (1994). Furthermore, as fructose would have affected *LeAMT1;2*-expression (see above), it was omitted from the medium. Therefore at the time of harvest, bacteria were probably absorbed but not firmly attached to the root surface.

To ensure that plants were highly responsive to externally supplied NH_4^+ when bacteria were added, N-depleted plants were used for inoculation. Moreover, the risk of introducing unspecific sources of nitrogen into the medium was minimized by using N-free MPCL-basic-medium during pre-induction-period and bacterial overnight cultures that were thoroughly washed before.
When diazotrophic wild types were used for inoculation, the transcript-levels of *LeAMT1;2* increased in the presence of *Azospirillum brasilense* and *Azoarcus* Sp BH72 (Sections 3.3.2.1 and 3.3.2.2), whereas *Azotobacter vinelandii* and *Acetobacter*

diazotrophicus had no effect compared to the control. Likewise, strain 7317 of *Azospirillum brasilense*, displaying a Nif^--phenotype due to a defect in the structural genes of nitrogenase, did not rise the *LeAMT1;2*-level (Section 3.3.2.2). As the effect of *Azospirillum* and *Azoarcus*-wild type strains compared with the Nif^--mutant was obtained in 4 experiments independently from each other, this might indicate that nitrogen fixation is involved in this response. However, it should be kept in mind that strain 7317 does not proliferate well. Based on this assumption, the negative effect of *Azotobacter* and *Acetobacter* might tie up with the fact that conditions for N_2-fixation were unfavourable for these strains. Nitrogenase-activity of *Acetobacter diazotrophicus* was determined to have its optimum in the presence of 10% sucrose in an acid environment (Reis and Döbereiner, 1998), thus the inoculation in an N- and C-free MPCL-basic-medium is likely to inhibit N_2-fixation. Although *Azotobacter vinelandii* was shown to promote growth of tomato (Jackson *et al.*, 1964), its colonization abilities are rather poor with this plant (Brown, 1974), which was considered important before.

Numerous mutants of *Azospirillum brasilense* were used for inoculation to investigate the nature of the observed effects in more detail (see Section 2.1.2 for relevant characteristics). Besides wild type strains of *Azospirillum* and *Azoarcus*, strains C3, 7029, and 7628b showed transcript-levels that markedly exceeded that of the N-free control without inoculum (Section 3.3.2.2). An acetylene reduction assay (ARA) carried out in pure, semisolid-grown cultures of each strain revealed that N_2-fixation abilities may not be solely responsible for the observed effects, albeit it should be considered that N_2-fixation in pure cultures is assumed to be different from N_2-fixation in the association (Han and New, 1998). In particular, this is reflected by the extreme cases of strain 7029, showing no nitrogenase-activity but a strong effect on *LeAMT1;2*-expression, and strain 7611, showing high nitrogenase-activity but little influence on the *LeAMT1;2*-transcript-level. However, on closer examination of the strain characteristics, the data may become understandable. Strains C3 and 7029 are impaired in *glnA*-activity, that is the assimilation of NH_4^+ via glutamine synthetase is restricted. Hence, in case of intracellular NH_4^+-excess, these strains rather excrete than assimilate NH_4^+. Like plants, bacteria may generate NH_4^+ endogenously (Kleiner, 1984), which is why NH_4^+ might be excreted by the Nif^--strain 7029. By contrast, strain C3 shows a Nif^+-phenotype, even under unfavourable conditions, and parts of the excreted NH_4^+ may originate from N_2-fixation, which would explain why *LeAMT1;2*-expression is even stronger in the presence of this strain. The other extreme case, strain 7611, is defective in the *glnZ*-gene, encoding for a P_{II}-like protein of yet unknown function. A recent study confirmed that strain 7611 is an efficiently N_2-fixing strain but it also reveals an NH_4^+-uptake rate that is twice as high as in the wild type strain (de Zamaroczy, 1998). Therefore, little amounts of the fixed nitrogen might be available for the plant, probably leading to the absence of the *LeAMT1;2*-response.
Strain 7628 is a regulatory mutant of *Azospirillum brasilense* defective in the *glnB*-gene. *glnB* encodes for the signal transduction protein P_{II}, which is, among other things, responsible for the activation of NifA and GS. *glnB*-mutants do not fix N_2 and

excrete NH_4^+ when grown with NO_3^-. It appears contradictory that while both strains 7628 and 7029 reveal a Nif^--phenotype and might excrete NH_4^+, only the latter is able to increase the *LeAMT1;2*-transcript-level. However, it might be speculated that this difference is due to a pleiotropic effect in *glnB*-mutants, which were reported to be impaired in swarming properties and in the control of general energy metabolism (de Zamaroczy *et al.*, 1996). This, in turn, could have led to poor colonization abilities of strain 7628, thereby negatively affecting *LeAMT1;2*-transcription (see above). *LeAMT1;2*-expression was markedly higher in the presence of strain 7628b, which had received a plasmid-borne 5´ end-deleted *nifA* gene from *Azospirillum brasilense* by means of conjugation. Hence, nitrogen-fixing ability in strain 7628b was restored indicating that N_2-fixation is involved in the *LeAMT1;2*-response.
Direct evidence that colonization abilities are crucial for the expression of *LeAMT1;2*, comes from strain A7030, which is a generally non-chemotactic mutant. Although strain A7030 is capable of fixing dinitrogen, it reduced only low amounts of acetylene in semisolid medium, probably due to a lack of aerotaxis. Inoculation of tomato roots with strain A7030 could not rise the *LeAMT1;2*-level above the control, which is most probably rooted in the poor colonizing abilities.
To evaluate the importance of bacterial auxin-production, a low-IAA-producing strain (SpM7918) and an IAA-hyperproducing strain (FT326) were tested for their influence on *LeAMT1;2*-expression. Although strain SpM7918 revealed a nitrogenase-activity similar to that of strain 7628b, the transcript-level of *LeAMT1;2* was markedly lower, indicating that auxins might play a role in the observed response. The signal obtained in the presence of strain FT326 was twice as strong as in the control. However, no statement is possible in comparison to the wild type, as these strains were not co-electrophoresed.
Evidence that *LeAMT1;2*-expression in these experiments required viable cells was provided by inoculation of tomato with autoclaved wild type strains of *Azospirillum brasilense* and *Azoarcus* Sp BH72. *LeAMT1;2*-level in these plants did not exceed the control level (data not shown). Killed bacteria usually show weak attachment properties to plant cells (Eyers *et al.*, 1988).

The response of *LeAMT1;2*-expression to the filtersterilized semisolid culture medium of the above used strains revealed the existence of compounds in the bacterial supernatants that have a high capacity to influence *LeAMT1;2*-expression (Section 3.3.2.3). According to El-Khawas and Adachi (1999), the optimum concentration of bacterial supernatants that is beneficial to hydroponically grown plant cultures is 6-8%. In this experiment, supernatants had to be diluted 1:20 (= 5%) in order to supply all plant tubes. Nevertheless, in all samples the *LeAMT1;2*-transcript-level exceeded that of the control. However, there are marked differences in the individual signal intensities, which partly challenge the hypothesis that N_2-fixation is solely responsible for the observed effects. To get an idea about the actual NH_4^+-content in the plant medium at the time of addition, the supernatants of identically grown bacterial strains (henceforth referred to as ‚*strain*-sample', e.g. ‚7029-sample') were analyzed for NH_4^+. Based on this analysis, the NH_4^+-content in almost all plant media was in the range of the determined threshold concentration for *LeAMT1;2*-

induction (see above) and thus, below the level that would cause peak-expression. Under these circumstances, the *LeAMT1;2*-transcript-level would run parallel with the NH_4^+-concentration and not reciprocal.

The highest amounts of NH_4^+ were measured in the ‚*glnA*-mutant-samples', emphasizing their capability to excrete NH_4^+. However, while the *LeAMT1;2*-transcript-level in the presence of the ‚7029-sample' was indeed the highest of all, the ‚C3-sample' caused the lowest signal, apart from the control. This observation is rather puzzling if only NH_4^+ is assumed to be responsible indicating that other bacterial compounds than NH_4^+ affect *LeAMT1;2*-expression. This hypothesis is supported by the expression in the other samples, as it is doubtful that the high signal intensities could be solely explained by NH_4^+-levels, which are in part below the determined threshold for *LeAMT1;2*-induction.

The measured NH_4^+-concentrations match quite well the characteristics of the strains, and apart from the ‚C3-sample' they are roughly reflected by the *LeAMT1;2*-transcript-level, too. The NH_4^+-content was lowest with the ‚7317-sample', certainly as a result of the Nif^--phenotype of strain 7317, yet the transcript-level of *LeAMT1;2* exceeded that of the control approximately twofold. Strain 7628 is also a Nif^--phenotype but its supernatant shows an NH_4^+-level that is comparable to the wild type. This is consistent with the finding that *glnB*-mutants might excrete NH_4^+ (de Zamaroczy *et al.*, 1996), probably derived from endogenous sources, as already speculated above. Interestingly, the third Nif^--strain, 7029, revealed the highest level of NH_4^+ in the supernatant, as already noted. This difference between strains 7628 and 7029 would explain the discrepancy observed before with these bacteria (see above): both strains are Nif^- and excrete NH_4^+ but obviously, as revealed here, there is more excretion by strain 7029. It appears as if the above mentioned pleiotropic effect in *glnB*-mutants also affects endogenous NH_4^+-generation or -excretion. In accordance with the NH_4^+-contents, the *LeAMT1;2*-transcript-level caused by the ‚7029-sample' is markedly higher than that caused by the ‚7628-sample'.

The NH_4^+-content was slightly increased in the ‚7628b-sample', indicating that strain 7628b excretes fixed nitrogen, as also shown by de Zamaroczy *et al.* (1996). The *LeAMT1;2*-transcript-level in tomato roots was likewise elevated in the presence of the ‚7628b'-sample. The low NH_4^+-content in the ‚7611-sample' supports the idea that strain 7611, despite its high N_2-fixation-activity, hardly loses any NH_4^+ to the medium (see above) and confirms that the low signal obtained in the presence of strain 7611 (Section 3.3.2.2) is probably due to a strong retention of NH_4^+.

The ‚*Azospirillum* WT-sample' was slightly higher in NH_4^+-content than the ‚7611-sample', thereby revealing the less efficient retrieval of NH_4^+ in the wild type. The *LeAMT1;2*-transcript-level caused by the ‚*Azospirillum* WT-sample', however, was one of the highest obtained. Despite the high N_2-fixation rates (Section 3.3.2.2), the ‚*Azoarcus* wild type-sample' contained relatively low amounts of NH_4^+, which is either due to a low excretion rate or a high retention rate. However, as *Azoarcus* turned out to be the most efficient inoculant for rising the *LeAMT1;2*-transcript-level, it is evident that other factors than NH_4^+ in the supernatant might influence the *LeAMT1;2*-expression. It is also conceivable that *Azoarcus* releases more NH_4^+ in the association than in pure culture.

The NH_4^+-content in the ‚SpM7918-sample' was comparable to that in the ‚7317-sample', which might query the involvement of auxins in regulating *LeAMT1;2*, as the low signal in the presence of the strain itself (Section 3.3.2.2) might have been caused by low amounts of excreted NH_4^+ as well. According to today's state of knowledge there is no direct linkage between auxin-metabolism, N_2-fixation, and NH_4^+-excretion. Therefore, no satisfying explanation can be given for the low amounts of NH_4^+ in the supernatant of strain SpM7918.
Assuming that the actual threshold-concentration for the *LeAMT1;2*-induction was lower than originally determined and peak-expression would be reached in the range from 1 to 6.5 µM, the transcript-levels caused by bacterial supernatants would perfectly match an NH_4^+-stimulated induction pattern with the exception of the ‚C3-sample'. Bearing in mind that, firstly, the NH_4^+-content analysis was carried out with strains other than that used for inoculation, and, secondly, measurements in the micromolar range are easily subject to little deviations, it is probable that NH_4^+-contents in the plant media were actually slightly higher than indicated. Furthermore, it should be noted that the the possibility of NH_4^+-enrichment in the supernatant by ammonification of dead cells cannot be precluded.

The involvement of *LeAMT1;2* in pathogen response was mainly tested for two reasons: the putative promoter sequence of *AtAMT1*, another gene of the *AMT1*-multigene-family from *Arabidopsis thaliana*, revealed the presence of the ASF1-motif suggesting transcriptional regulation by salicylic acid and auxins (Becker [1], personal communication). Salicylic acid is a signal transducer, responsible for the induction of systemically acquired pathogen resistance in plants (Gaffney *et al.*, 1993). Secondly, the attachment of pathogens to plant roots causes accumulation of NH_4^+ in plants (Barker, 1999), a process that most probably leads to the induction of *LeAMT1;2*. Furthermore, it was shown that the pathogen response of plants is accompanied by the extrusion of protons or potassium ions (Schaller *et al.*, 2000), which occurs upon NH_4^+-supply as well (Smart and Bloom, 1998). The extrusion of protons was also observed 5 hrs after the addition of *Azospirillum* bacteria to plant roots (Bashan and Holguin, 1997) but in tomato, no symptoms of disease develope upon inoculation with *Azospirillum* (Bashan, 1998).
When different strains of the genus *Herbaspirillum* were tested (Section 3.3.2.4), it turned out that the wild type strain Z67 induces *LeAMT1;2* above the control-level, whereas strain Z78 had little effect, indicating that strain Z67 might contribute fixed nitrogen to the plant. Both bacteria are considered to be beneficial to their host plants rice and sorghum, respectively. The different signal-intensities indicate that the ability to affect *LeAMT1;2*-expression might even vary within a given genus. *Herbaspirillum rubrisubalbicans* Sp M4, is a phytopathogenic strain that causes mottled stripe disease in some sugar cane varieties, in Napier grass, and sorghum (Texeira [2], personal communication). This strain, however, did not markedly increase the *LeAMT1;2*-level in tomato. In the presence of salicylic acid, the signal was even lower and did hardly

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exceed the control level. These results suggest that *LeAMT1;2* is probably not involved in the pathogen response.

In many studies the effect of diazotrophic bacteria turned out to be dependent on the inoculation density, as the addition of a supraoptimum inoculum level was frequently shown to be rather detrimental than beneficial to the plant (Okon and Kapulnik, 1986; El-Khawas and Adachi, 1999). By contrast, too low levels might be inefficient (Dubrovsky *et al.*, 1994). The optimum level has to be determined for a given plant-microbe-interaction. For tomato, this *Azospirillum* level was reported to be 10^6 cfu/ml (Bashan *et al.*, 1989). However, Hadas and Okon (1987) found best growth promotion in the presence of 10^8 cfu/ml.
When different inoculum levels of both *Azospirillum brasilense* and *Azoarcus* Sp BH72 were tested for their influence on *LeAMT1;2*-expression, a cell density of 10^7 cfu/ml for both bacteria turned out to increase the *LeAMT1;2*-level strongest (Section 3.3.2.5). Cell densities below (10^6 cfu/ml) and above (10^8 cfu/ml) this level decreased the signal, although *LeAMT1;2*-transcription was slightly more tolerant to a higher level of *Azoarcus* cells. Too high cell densities may cause severe stress to tomato plants possibly due to to high levels of plant growth regulators, which are excreted by bacteria.
In all inoculation experiments, the colonization densities for both bacteria were adjusted spectrophotometrically, thereby providing a rapid method to reach the desired inoculum level. However, the calculated densities do not fit very well to the measurements of other authors. According to Nelson and Knowles (1978), who worked on *Azospirillum*, an OD_{578} of 0.079 corresponds to a cell density of $1.9 \cdot 10^8$ cfu/ml. A similar relationship was obtained by Bashan *et al.* (1989). Based on the equation formula used in this study (Section 3.1.1), however, a level of $1.9 \cdot 10^8$ cfu/ml would cause an OD_{578} of 0.608, that is almost tenfold higher. This suggests that the spectrophotometrically determined cell densities in this study actually underestimate the real densities.

When *LeAMT1;2*-expression was followed over a period of three days, it turned out that the strong *LeAMT1;2*-expression in the presence of both *Azospirillum brasilense* and *Azoarcus* Sp BH72 is a short-term effect (Section 3.3.2.6). The high transcript-levels that could be observed after 10 hrs reduced to a much lower level after 30 hrs of inoculation. At day 3 the *LeAMT1;2*-expression in the presence of *Azospirillum brasilense* was just as low as in the control, whereas plants inoculated with *Azoarcus* still revealed a slightly increased transcript-level.
On the assumption that *LeAMT1;2* is expressed under conditions of bacterial NH_4^+-supply, the result of the long-term experiment indicates, that N_2-fixation stops or is at least strongly impaired already at the second day of inoculation. There are several possible explanations for this observation. For a long-term growth stimulation, inoculants are required, which are able to survive in the rhizosphere and continuously release phytoeffective metabolites (Höflich *et al.*, 1994). The plant, on the other hand, has to provide assimilates, serving as substrates for bacteria. As the plant-bacteria culture in this experiment was initially suspended in N- and C-free MPCL-

basic medium, one might assume that the availability of carbon and nitrogen would soon become the major limiting factor for growth and metabolism of bacteria. In particular, N_2-fixation is an energy-demanding process, strongly dependent on the presence of energy sources (Burris, 1991). However, the only source of energy that is available in the hydroponic unit is released by the plant root, usually as a mixture of sugars and organic acids, referred to as root exudates (Rovira, 1969). The analysis of the plant medium after 25 days of growth revealed the presence of micromolar amounts of sucrose, glucose, and fructose (Section 3.4.2) but no traces of organic acids or amino acids were found. In addition, carbohydrates were only found in the supernatant of N-depleted plants, whereas N-repleted plants were completely devoid of organic compounds. Certainly, the plants were N-depleted upon addition of bacteria, but sucrose and glucose cannot be utilized by *Azospirillum brasilense* (Michiels *et al.*, 1989). Fructose, however, may not only serve as energy source but promotes also the irreversible attachment of *Azospirillum* bacteria on the root surface (see above). Furthermore, fructose was shown to attract *Azospirillum* chemotactically (Reinhold *et al.*, 1985). Considering the time of incubation that preceded the analysis of the plant medium, one might conclude that the exudation rate for fructose is rather slow, and as the medium was renewed upon the start of the induction period, it was probably free of carbon sources during the first days. It should be noted, however, that *Azospirillum* is assumed to stimulate the exudation of plant roots (Bashan and Levanony, 1990), hence, the C-content of the medium could differ in quality and quantity upon inoculation. Like *Azospirillum brasilense*, *Azoarcus* Sp BH72 can hardly utilize sugars for growth (Reinhold-Hurek *et al.*, 1993b), and even fructose cannot serve as energy source.

A second aspect to be considered is the low amount of nitrogen in the mixed culture. The inoculation experiments aimed at scientific evidence for the contribution of N_2-fixation to the plant´s N-status. Therefore, nitrogen had to be omitted from the medium, as nitrogenase-activity requires N-concentrations in the low micromolar range (Nelson and Knowles, 1978; Liang *et al.*, 1992). Dubrovsky *et al.* (1994) have shown that *Azospirillum* bacteria do not multiply but still survive in the association for at least three days, when external nitrogen is omitted. To evaluate the fundamental question, whether *Azospirillum brasilense* and *Azoarcus* Sp BH72 fix dinitrogen during the inoculation with tomato plants, three approaches were tested.

The acetylene reduction assay (ARA) revealed that nitrogenase activity of both bacteria is strongly inhibited in liquid media during the first two weeks of incubation. Nitrogenase activity was only observed, if *Azoarcus* was applied at a cell density of 10^8 cfu/ml and allowed to colonize for 6 days before acetylene was added. But even in this case, the activity was rather low, and not detectable before day 11 of inoculation. In no other sample, acetylene reduction was observed irrespective of the bacterium, the cell density, the addition of fructose, and the time of acetylene injection, most probably due to unfavourable high concentrations of O_2 in liquid media (Zhang *et al.*, 1993). Likewise, in hydroponic maize cultures, *Azospirillum brasilense* showed no acetylene reduction (Zuberer and Alexander, 1986). In principle, O_2-gradients in liquid media may be generated by a high respiration of

roots (Michiels *et al.*, 1989) or by the formation of bacterial cell aggregates on the root surface (Barak *et al.*, 1982; Hurek *et al.*, 1994b).
The situation was different when semisolid media were used. Due to the O_2-gradient in this medium, *Azospirillum* bacteria formed a pellicle at the site of the optimum oxygen concentration. The amount of produced ethylene was even stronger when fructose was added to the medium or when the inoculum level was rised to 10^8 cfu/ml. Irrespective of the time period that precedes acetylene injection, ethylene production developed with delay, probably due to the slow diffusion of both acetylene into and ethylene out of the medium (Lee and Watanabe, 1977). Such a delay in response was also observed with acetylene-reduction of *Azospirillum brasilense* in wheat (Han and New, 1998). When *Azospirillum* was allowed to colonize before acetylene was added, ethylene production was subject to the same delay but then developed stronger, as indicated by the steeper slope (Figure 3.28d + f). This might reflect that the ‚pre-injection-period' is used to better establish the plant-microbe interaction. In contrast to *Azospirillum*, *Azoarcus* revealed generally low ethylene production, and the strain could not benefit from the semisolid state of the medium or the addition of fructose.
The acetylene reduction assay is generally considered unsuitable for assessment of N_2-fixation, as only short-term effects may be observed (Danso, 1995). 48 hrs after the injection of acetylene, plants began to suffer from the sealed atmosphere probably due to CO_2-stress. Conditional on diffusion rates, however, acetylene reduction just started at that time point.

In the ^{15}N-experiment the situation was similar (Section 3.5.2). Exposure of plants to a $^{15}N_2$-enriched atmosphere required sealing of the plant tube and caused severe symptoms of CO_2-stress in the plant (Boddey, 1987), leading to the abscission of leaves after 4 days of incubation. Nevertheless, when plants were analyzed for ^{15}N-content, all samples which exceeded the control were shoot-derived samples and the enrichment was strongest, when *Azospirillum brasilense* was added to liquid medium at a density of 10^8 cfu/ml. This observation contradicts the results from the ARA, which never revealed acetylene reduction of *Azospirillum brasilense* in liquid medium. As this experiment was carried out only once, however, no statistical significance of the enrichment can be given, and the presentation of the result should only have a qualitative character. According to Boddey (1987), the incorporation of biologically fixed nitrogen can only be regarded as significant if the enrichment of the plant exposed to labelled gas was greater than two times the standard deviation of the enrichment of plants from replicate unexposed cores.

From a technical point of view, the third approach to monitor N_2-fixation was the least problematical one, as sealing of plant tubes was not necessary (Section 3.5.3). The expression of a bacterial *nifH*, encoding for dinitrogenase reductase was followed, using the *nifH-gusA* fusion plasmid pFAJ21. *Azospirillum* bacteria, harbouring the plasmid, expressed *b-glucuronidase*, when fixing nitrogen. First weak signs of blue staining were observed not before day 11 of incubation. This coincides with the ARA-measurements, which revealed ethylene production of *Azoarcus* in liquid

media after exactly the same time period. It might be speculated that the establishment of the associative interaction requires several days and that N_2-fixation may contribute to the beneficial effect at later stages. Support for this hypothesis is also given by the ^{15}N-experiment. At the day of harvest plants were inoculated for 12 days, hence, a time, at which N_2-fixation probably just started. Furthermore, the analysis of root-NH_4^+-content (Section 3.4.1.1) revealed very low concentrations after 10 days of inoculation with diazotrophic bacteria. Thus, at the day of harvest, N_2-fixation was probably not involved yet in the interaction.

In this context, the *nifH-gusA*-strain revealed first signs of blue-staining on wheat roots likewise after 11 days of inoculation (Vande Broek *et al.*, 1993). In that study, oxygen-partial pressure and carbon-availability in hydroponic cultures were determined to limit nitrogenase-activity in N-free liquid media, and blue staining by the GUS-fusion was restricted to the lower parts of the root, where the oxygen-concentration was considered adequate for nitrogenase activity. Further evidence that the establishment of an association requires a prolonged incubation time is given by Zaady *et al.* (1993), who reported that the strongest effects of *Azospirillum* inoculation are visible after 2 - 3 weeks of inoculation.

The blue-staining in this study was restricted to some sites of lateral root emergence and root hairs, which are also known to be the sites of preferential *LeAMT1;2*-expression. The fact that N_2-fixing bacteria colonize a site on the root that is quite close to a high density of LeAMT1;2-transporter proteins, might ensure that the plant could benefit from N_2-fixation. As NH_4^+, the end product of N_2-fixation, is subject to futile retention cycles across the bacterial membrane (Kleiner, 1984), one might assume that parts of this NH_4^+ may escape from retrieval and are available to the tomato plant. The N-depleted tomato plant, on the other hand, shows a high receptivity for externally supplied NH_4^+ and responds with a rise of the *LeAMT1;2*-transcript-level. As *LeAMT1;2* needs to be induced first, it might be speculated whether first traces of NH_4^+ enter the root via K^+-channels that were already found in root hairs of tomato (see above). The uptake by K^+-channels might be promoted by the hyperpolarization of the membrane that was observed upon *Azospirillum* inoculation, as already noted.

Finally, the assumption that N_2-fixation starts not before day 11 would also explain the decrease of the *LeAMT1;2*-level already at day 2 of inoculation but the question arises for the cause of the initially strong transcription. Bearing in mind that inoculants were derived from overnight cultures, which were exposed to N-rich and complex media, respectively, it might be speculated that bacteria may have stored some reserve supply of nitrogen and carbon, and as the retrieval system for leaking NH_4^+ is known to be repressed under conditions of N-excess (Kleiner, 1985), there may also be some NH_4^+-excretion at the first day of the experiment.

This study provides considerable evidence that *LeAMT1;2* is indeed induced by bacterial derived NH_4^+, although other factors than NH_4^+ cannot be precluded as inducing agents. Evidence that *Azospirillum* may interfere with the plant's nitrogen metabolism comes from maize roots, in which *Azospirillum* was found to increase GDH-activity upon inoculation, a gene that is involved in the assimilation of nitrogen (Ribaudo *et al.*, 1997). The nature of this interaction, however, was not resolved.

Certainly, from an economical point of view the most important aspect of the plant-*Azospirillum*-interaction is the contribution of bacterial fixed nitrogen to the plant's N-status. However, there are also other modes of action, by which *Azospirillum* bacteria promote plant growth. For instance, the secretion of phytohormones is assumed to markedly affect root- and shoot habitus (Rudnick *et al.*, 1997). That is why the root morphology was statistically evaluated in response to different plant growth regulators and inoculants (Section 3.6).

Azospirillum brasilense was reported to produce auxins (mainly IAA), gibberellins, cytokinins and abscisic acid (Bashan and Levanony, 1990; Costacurta and Vanderleyden, 1995; El-Khawas and Adachi, 1999). Upon inoculation, roots reveal a markedly changed morphology (Okon, 1985), that is an increased dry weight, an enlarged surface (Fulchieri and Frioni, 1994), an increase in lateral roots and root hair density (Eyers *et al.*, 1988; Bashan, 1998), and an increased root hair length (Dubrovsky *et al.*, 1994).

In this study, the significance of an effect was evaluated towards a control exposed to 50 µM NO_3^-. As the addition of NO_3^- is also known to promote the growth of lateral roots and the whole root system (Drew and Saker, 1975; Ganmore-Neumann and Kafkafi, 1980; Granato and Raper Jr., 1989), the control plants developed a luxuriant root system. Compared to this control, the wild type of *Azospirillum* significantly enhanced shoot length but decreased root length. At day 7 of inoculation with the wild type, the root developed numerous small lateral roots and mimicked the auxin-induced morphology, as IAA was shown to stimulate lateral root generation (Reed *et al.*, 1998).

The IAA-hyperproducing strain, FT326, revealed the same effects as the wild type. In addition, the number of lateral roots and the root dry weight were significantly increased, indicating that IAA is involved in these changes. The IAA-low-producing strain SpM7918 did also increase dry weight but the number of lateral roots was significantly decreased.

Comparing the photographs of roots colonized by these three strains, it is conspicuous that strain SpM7918 causes less but long lateral roots, strain FT326 causes many but very short lateral roots, whereas the wild type exhibits a habitus that is in between these two mutants. It was reported that a prolonged exposure to IAA inhibits root growth but the short application of smaller doses rather promotes it (Brown, 1974). This might be reflected by the three strains, as SpM7918 produces only 10% of the constitutive level, whereas IAA-production by FT326 is severalfold enhanced. Furthermore, in this study it was frequently observed that the sites of lateral root emergence developed an increased root hair density (Figure 3.40e). As these are the preferred sites of colonization, bacteria are most probably responsible for this effect.

In the presence of *Azoarcus* Sp BH72 none of the measured parameters was significantly changed, albeit the longest roots were among these. To date, there are no reports about phytohormon-production in *Azoarcus* Sp BH72 but the results suggest that the amount of excreted growth regulators is below the efficient level.

Among the tested growth regulators, only the higher concentrated GA_3-sample significantly decreased root dry weight. This is probably due to a higher dry weight accumulation in the shoot as both GA_3-concentrations significantly enhanced hypocotyl elongation (positively correlated). The other parameters did not change significantly and root morphology was not visibly different from the control. These observations on GA_3 are confirmed by other authors (Gray and Estelle, 1998; Chanda *et al.*, 1998). Abscisic acid increased the number of lateral roots (positively correlated) but decreased the length of the entire root (negatively correlated), thereby ABA-treatment caused a stocky habitus accompanied by splitted root tips. Several reports on the effect of ABA confirm the inhibition of root growth (Chanda *et al.*, 1998; Pilet, 1998). Kinetin had a pronounced effect on root development. It decreased the length of the root (independent of the supplied concentration), and at higher concentrations, the number of lateral roots (positively correlated) is also decreased resulting in a thickset habitus, as was also observed by Chanda *et al.* (1998). Auxin-treatment led to a strong increase in the number of lateral roots (positively correlated), whereas the root length was significantly decreased, independent of the supplied concentration. The strong inhibition of root growth was also observed by many other authors (de Klerk, 1998).

‚Ammonium does not induce ammonium absorption in tomatoes' (Nicoulaud and Bloom, 1998) and ‚Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation' (Bashan *et al.*, 1989). These two papers strongly challenge the main statements of this study. The investigation of the transcriptional regulation of *LeAMT1;2*, however, clearly proves that this gene is an inducible component of the entire NH_4^+-uptake system in tomato roots and likely fulfills its function in the retention of NH_4^+ inside the cell. Nicoulaud and Bloom (1998) used either N-repleted plants or plants exposed to NO_3^- and found that NH_4^+-absorption declined or remained constant after first exposure to NH_4^+. Therefore, they concluded that NH_4^+-absorption is not induced. However, the findings in this study reveal that NH_4^+-inducibility is neutralized in N-repleted plants, and furthermore, NO_3^--pretreatment may already induce the NH_4^+-transporter. It might be concluded from this study that the expression of NH_4^+-transporters, at least that of *LeAMT1;2*, is a matter of numerous factors, which have to be considered. In this context, N-depleted plants at pH 6.0 provide an inducible system upon first exposure to the substrate NH_4^+.

Northern blot analyses with *LeAMT1;2* have proven to be a valuable tool to evaluate, whether or not traces of bacterial-derived NH_4^+ are delivered to the plant. There is strong evidence that *LeAMT1;2* is expressed upon inoculation with NH_4^+-excreting strains. Bacterial NH_4^+-excretion is a prerequisite for the N-supply of a host plant, and the development of sustainable agriculture is partly dependent on strains that are capable of both efficient N_2-fixation and significant NH_4^+-excretion. The *LeAMT1;2*-gene may finally help to uncover this aspect of the interaction.

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6 Appendix

6.1 Veröffentlichungen

Die vorliegende Arbeit wurde auszugsweise veröffentlicht:

Stanke, R., Becker, D., Frommer, W.B., Fendrik, I., Vanderleyden J., Hedrich, R. (1998). Genetic Regulation of Ammonium Transport in Tomato Roots. In: Biological Nitrogen Fixation for the 21st Century. Elmerich, Kondorosi, Newton (eds.), Kluwer Academic Publishers, Dordrecht, 348.

Stanke, R., Becker, D., Fendrik, I., Vanderleyden J., Hedrich, R. (2000). Regulation of ammonium transport in tomato roots colonized by *Azospirillum brasilense*. In: Nitrogen Fixation: From Molecules to Crop Productivity. Pedrosa, Hungria, Yates, Newton (eds.), Kluwer Academic Publishers, Dordrecht, 437.

6.2 Eidesstattliche Versicherung

Ich versichere, daß ich die vorliegende Arbeit selbständig verfaßt und nur die angegebenen Hilfsmittel verwendet habe. Die Arbeit wurde in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegt.

Rüdiger Stanke
Hannover, im Juli 2000

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6.4 Lebenslauf

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Hochschulausbildung

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5/96 bis 7/96 der katholischen Universität Leuven, Belgien

10/96 Kurzvortrag auf dem „7. International Symposium on Nitrogen Fixation with Non-Legumes" in Faisalabad, Pakistan

7/97 Posterpräsentation auf dem „11. International Congress on Nitrogen Fixation" in Paris, Frankreich

9/99 Posterpräsentation auf dem „12. International Congress on Nitrogen Fixation" in Foz do Iguaçu, Brasilien

Wehrdienst

1989 bis 1990 Wehrdienst bei der Flugabwehr in Hannover-Langenhagen

Schulausbildung

1976 bis 1982 Grundschule und Orientierungsstufe in Hannover
1982 bis 1989 Gymnasium Lutherschule in Hannover. Abschluß: Abitur