Nicotianamine synthase: Gene isolation, gene transfer and application for the manipulation of metal assimilation

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List of the Abbreviations

A. tumecaciens	Agrobacterium tumefaciens
Amp^r	Ampicilin resistance
ATP	Adenosine triphosphate
b, bp	Base, base pair
BSA	Bovien serum albumin
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
D	Dalton
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylendiamine tetraacatic acid
et al.	*et alii* (and others)
g	Gram
HEPES	N-[2-Hydroxyethyl]-piperazine-N'-[2-ethansulfonic acid]
IPTG	Isopropyl-ß-D-thiogalactozide
K	Kilo
L	Litre
M	Molarity
m	Mili
MOPS	N- morpholinopropanesulfonic acid
mRNA	Messenger RNA
n	Nano
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
T-DNA	Transferred DNA
Tris	Tris-hydroxymethylaminomethane
WT	Wild type
µ	Micro

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1. Introduction

1.1. Iron as a nutrient

Iron deficiency is the most common nutritional disorder in the world. The numbers are staggering: as many as 4-5 billion people, 66-80% of the world's population, may be iron deficient; 2 billion people – over 30% of the world's population – are anemic, mainly due to iron deficiency. Subtler in its manifestations than, for example, protein-energy malnutrition, it presents the heaviest overall toll in terms of ill-health, premature death and lost earnings. Iron deficiency and anemia reduce the work capacity of individuals and entire populations, bringing serious economic consequences and obstacles to national development. Conversely, treatment can raise national productivity levels by 20%. (World Health Organization, 2001).

Iron supplements, iron-rich diets, increasing iron absorption and fortification should be the main tools to combat micronutrient malnutrition. The plants, as the major nutrient source for the people and animals, must be extensively developed and improved. Therefore, basic understanding of iron homeostasis in plants as well as the development of iron efficient crop plants is a major challenge for research and plant breeding.

1.2. Iron in Plants

Iron is a vital micronutrient involved in many cellular processes like electron transport chains in photosynthesis and respiration, enzyme activation, oxygen carrier in nitrogen fixation etc. It is essential for synthesis of the porphyrin ring as a precursor of haeme as well as chlorophyll, haeme synthesis declines under Fe deficiency conditions in the roots. This leads to physiological anoxia in the roots even though oxygen is present (Mori, 1999).

On the other side, excess supply of iron, as well as other essential and non-essential heavy metal ions, is toxic to most species. One of the general symptoms of heavy metal toxicity in higher plants is chlorosis of young leaves.

Often this is associated with low internal Fe concentrations indicating metal-induced inhibition of mobilization, uptake and/or translocation of Fe (Schmidt *et al.*, 1997). Blockage of physiological response of the plants to Fe shortage such as formation of transfer cells, medium acidification and increased Fe-reductase activity by elevated levels of divalent metal cations have been reported (Landsberg, 1982; Alcantara *et al*., 1994).

Although iron is abundant in most well aerated soils, its availability for plants is limited. In soil, iron is mainly found as stable Fe (III) compounds that are insoluble at neutral pH. Under anaerobic conditions, iron is precipitated as $Fe(OH)_3$ with very low solubility that limits the concentration of ferric irons at pH 7 to about 10^{-18} M. (Drechsel and Jung, 1998). Decreasing the pH to 4,0 increases the solubility of Fe (III) by a factor of 1000 (M a and Nomoto, 1996). However, the plants demand approximately 10^{-4} – 10^{-8} M Fe (III) for normal growth (Mori, 1999). To overcome this shortage the plants have evolved different iron acquisition mechanisms, which can be clearly divided in two groups often designated as Strategy I, and Strategy II (Römheld and Marschner, 1986).

Iron acquisition mechanism of Strategy I plants

All higher plants except *Gramineae* use the Strategy I mechanism. They facilitate iron uptake by enhanced extrusion of protons leading to acidification of the rhizosphere and thus to solubilisation of iron compounds, and release of reductors or chelators. A root-specific plasma membrane-bound reductase reduces Fe (III) to Fe (II) and the ferrous iron is taken up by root cells through Fe(II) specific membrane sites.

Enhanced Fe (III) reduction activity is the most typical feature of Strategy I. Two closely related ferric-chelate reductases genes (*FRO1* and *FRO2*) of *A. thaliana* were isolated (Robinson, 1999). They show moderate similarity to yeast ferric-chelate reductases such as *FRE1* and *FRP1*, and to human phagocytic NADPH oxidase *gp91phox*. These enzymes are involved in the transfer of electrons from cytosolic donors to FAD and then through 2 consecutive haem groups, to single electron acceptors on the opposing face of the membrane. The authors predicted that FRO2 is a trans-membrane

protein with 8 hydrophobic α-helices, 2 more than *gp91phox*. Four conservative histidine residues positioned in two trans-membrane α-helices probably coordinate the two haem groups located within the plasma membrane as it is predicted from the structure of *gp91phox* and the yeast ferric-reductases. The human gp91phox requires a second protein (*p22phox*) with 2 trans-membrane α-helices to form an active complex. In contrast, FRO2 seems to act alone. Even more, the C-terminal domain, including the two additional 2 trans-membrane α-helices shows similarity to *p22phox*.

Ferric-chelate reductases from other species seem to be active also in plants. It was reported that the ectopic expression of yeast FRE1 and FRE2 (encoding Fe (III) reductases) in tobacco leads to overall increasing of the iron reductase activity along the entire plant. The FRE2 transformants were also more tolerant to Fe deficiency in hydroponics cultures as shown by higher chlorophyll and Fe concentrations in younger leaves (Samuelsen, 1998).

Another element in the metal ion uptake system in plants is a group of transporters belonging to the ZIP family (for **Z**RT, **I**RT related **P**roteins) (Guerinot, 2000). IRT1 (**I**ron **R**egulated **T**ransporter 1), an iron deficiency inducible protein, is the first isolated *Arabidopsis ZIP* protein (Eide *et al.*, 1996). The function of IRT1 to transport Fe (II) is confirmed by functional expression in yeast. Additional studies in yeast showed that IRT1 has a broad substrate range and transports Mn^{2+} and Zn^{2+} and possibly Cd^{2+}; on the other side, cadmium, copper, cobalt and Fe (III) inhibit it (Korshunova *et al.*, 1999). Meanwhile several other metal transporters have been identified in *A. thaliana* - ZIP1-4, ZNT1, AtNramp1/3/4, COPT1 and LCT1, which makes obvious that multiple pathways of plant metal transport exist. (Clemens, 2000).

The third element of the Strategy I respond to Fe-deficiency is an enhanced acidification of the rhizosphere due to proton extrusion by a root plasma membrane H^+-ATPase. Strong increase in the plasma membrane H^+-ATPase activity occurs under Fe-deficiency (Dell'Orto et al., 2000; Rabotti and Zocchi, 1994). By pumping protons outside the cell, this enzyme contributes to enhance the solubility of Fe oxides and generates the proton motive force for ion uptake. In addition, its activity would help to maintain an adequate environment for the activity of the Fe-deficiency-induced PM Fe(III)-chelate

reductase (low apoplastic pH and membrane potential homeostasis). Unlike Fe(III)-chelate reductase, an increased H^+-*ATPase* activity was not frequently observed. The enzyme activity seems to differ considerably between plant species and genotypes (Bienfait, 1988; Buckhout *et al*., 1989; Chosack *et al*., 1991; Rabotti and Zocchi, 1994; Schmidt *et al*., 1997). It has been suggested that Fe(III) reduction and proton extrusion activities may be regulated independently (Yi and Guerinot, 1996).

Physiological disorder in the tomato mutant chloronerva

The chloronerva is a spontaneous mutant of *Lycopersicon esculentum* Mill. Cv. Bonner Beste (BB). The mutant was extensively studied during the last decades and a lot of information concerning its physiology was collected. The mutant *chloronerva* is characterized by severe disturbance of iron metabolisms. It exhibits intercostal chlorosis of young leaves, retarded growth of root and shoots, excessive root branching and permanently activated iron uptake systems such as highly active ferric-chelate reductase, and enhanced proton extrusion. It shows also other typical iron deficiency syndromes as thickening of the root tips and increased density of the root hairs. In spite of these features, the mutant accumulates iron in roots and shoots (Scholz *et al.*, 1988; Becker *et al.*, 1995). Iron-phosphate particles are formed in the chloroplasts of palisade parenchyma of the leaves, and in cytoplasm and vacuoles of rhizodermis. At the electron microscopic level, it becomes obvious that the chlorotic leaves have a reduced number of chloroplasts (about one third in comparison with normal leaves). Moreover, the amount of the thylakoid membranes in the chloroplast is also reduced and disordered (Adler and Scholz, 1986). Besides iron, several other metals are accumulated in *chloronerva* – manganese and zinc in the leaves (Stephan and Grün, 1989) and copper in the roots (Pich and Scholz, 1991; Herbik *et al.*, 1996). A similar process of metal accumulation is observed in the wild type BB under iron deficiency (Pich *et al.*, 1994). Such an increased metal concentration and especially that of iron and copper could provoke formation of free radicals (Halliwell and Getteridge, 1984; Luna *et al.*, 1994). This is in agreement with the observed 2-3 fold increasing of the activity of the catalases and

peroxidases in the roots of *chloronerva,* as well as in the wild type BB under iron deficiency (Pich and Scholz, 1993). Other stress-inducible proteins such as Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH, E.C. 1.2.1.12.) and Formiat-Dehydrogenase (FDH, E.C. 1.2.1.2.) are also up regulated (Herbik, 1997).

It was found that the *chloronerva* phenotype could be changed to wild type by grafting the mutant to BB rootstocks or by treatment with water extracts from normal plants. The chemical basis of this phenotypic normalization was shown to be a water-soluble, heat-stable, ninhydrin-positive substance, which was identified as non-proteinogenous amino acid nicotianamine (NA) (Scholz, Rudolph, 1968). NA is ubiquitous in the plant kingdom. The only known higher plant without NA remains the *chloronerva* mutant of BB.

The gene responsible for the *chloronerva* phenotype was fine-mapped onto the long arm of chromosome 1 and YAC clones surrounding the region were isolated using flanking markers (Ling et al., 1999). The *chloronerva* transcript was identified by cDNA isolation with the complementing cosmids. The gene encodes a novel protein of 35 kDa. The mutant differs from the wild type only by a single base pair change (T->C) at nucleic acid position 761 that creates a substitution of a phenylalanin by a serine at amino acid position 238.

Iron acquisition mechanisms of Strategy II

In contrast to all other higher plants, the economically most important *Gramineae* use a different iron uptake system known as Strategy II. The basic mechanism is excretion of phytosiderophores (PS), which act as chelators for ferric ions. The resulting Fe (III)-phytosiderophore complexes (Fe (III)-PS) are then taken up by root cells by a specific Fe (III)-PS transporter.

Most steps of the biosynthesis of PS have been deduced. The group of Satoshi Mori from University of Tokyo, Japan has investigated extensively the PS biochemical pathway and particularly that of mugineic acid family (MAs). The synthesis begins with methionine, which is synthesized in the roots but not transported from the leaves (Nakanishi *et al.*, 1999). The methionine is adenosylated to S-adenosyl-methionin (SAM) by ATP-dependent SAM synthetase (*SAMS*). Three molecules of SAM are used to produce

nicotianamine by Nicotianamine synthase (*NAS*), perhaps the key enzyme in MA synthesis. In Strategy II plants, NA in addition to its function in Strategy I plants, is also a precursor of MA. The Nicotianamine aminotransferase (*NAAT*) catalyzes the transfer of an amino-group and subsequent reduction at 3'-carbon of the keto acid to 2'-deoxymugineic acid (DMA) (Higuchi *et al*., 1994). This crucial step distinguishes graminaceous plants from the other members of the plant kingdom. *IDS3* and *IDS2* (iron deficiency specific clones 3 and 2) (Nakanishi *et al.*, 1997; Nakanishi *et al.*, 2000) are the putative hydroxylase encoding genes that convert DMA to MA and 3-epihydroxy-2'-deoxymugineic acid (epiHDMA), and MA to 3-hydroxymugineic acid (HMA), as well as epiHDMA to 3-epihydroxymugineic acid (epiHMA). The authors suggest that *IDS3* is an enzyme that hydroxylates the C-2' positions of DMA and epiHDMA, while *IDS2* hydroxylates the C-3 positions of MA and DMA (Kobayashi *et al.*, 2001). These phytosiderophores are produced in various ratios in the different graminaceous plants. It was found that the hydroxylated PS species possess enhanced chelate stability and affinity for iron (von Wirén *et al.*, 2000). The iron-chlorosis resistance plants as barley and rye produce mainly hydroxylated PS while non-hydroxylated DMA predominates in the susceptible species such as maize and rice. (Kawai et al., 1988).

1.3. Nicotianamine and Nicotianamine synthase

Structure and possible functions of NA

The Strategy I and Strategy II appear rather different but they share a common element. In both strategies, the NA is a key metabolite. As it is known from the *chloronerva* mutant, the lack of NA leads to catastrophic disorder of the plant physiology due to the iron starvation and iron intoxication simultaneously. In Strategy II plants, one can predict similar or even stronger consequences of NA insufficiency since NA in these plants is also a precursor in PS biosynthesis.

NA was firstly isolated from *Nicotiana tabacum* leaves (Noma *et al*. 1971). Chemically NA is (2S:3'S:3''S)N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidin-2-caboxyl acid (Kristensen and Larsen, 1974) (Fig.

1.1.). The biosynthesis of NA involves the direct condensation of three molecules of S-adenosyl-L-methionine followed by the formation of an acetidine ring (Mori and Nishizawa, 1987; Shojima et al., 1990; Kawai et al., 1988, 1990; Higuchi et al., 1994). The synthesis is catalyzed by nicotianamine synthase (NAS).

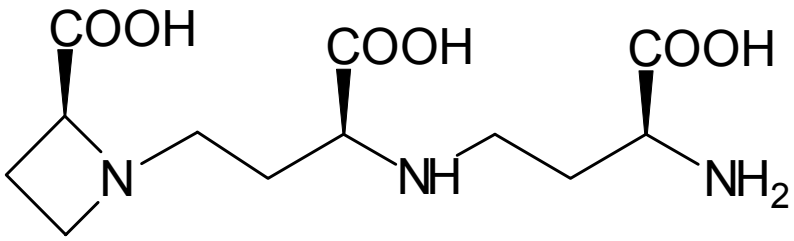

Fig. 1.1. Structural formula of Nicotianamine

NA is present in all higher plants. It is not present in the unicellular Algae and the bacteria. The only known NA-free multicellular plant is the tomato mutant *chloronerva*. Some *Streptomyces* strains as well some fungi (*Basiolobus meristoporus*) are also known to produce NA (Suzuki *et al*., 1996; Scholz *et al*., 1992)

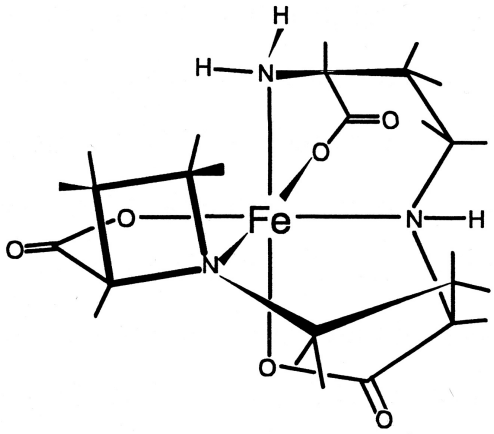

Fig. 1.2. Three-dimensional structure of NA-Fe (II) complex

The concentration of NA in the plant tissues depends on the age of the tissue and the physiological stage (Rudolph *et al.*, 1985). It varies from 10 to 400 nmol NA per gram fresh tissue, where the concentration of NA is highest

in young developing tissues as root- and shoot epical meristeme (Stephan *et al.*, 1990; Pich *et al.*, 1994).

The 3-dimensional structure and the 6 functional groups of NA are optimal to form an octahedral complex with 2-valent metals (Fig. 1.2.) (Buděšinský *et al*., 1980; Ripperger and Schreiber, 1982).

The complex possesses an unusual kinetic stability, which prevents auto-oxidation of Fe(II) to Fe(III). Therefore, it was accepted for a long time that NA binds only Fe(II) but not Fe(III). However, it was demonstrated that NA is an effective chelator of Fe(III) at physiological pH values (von Wirén *et al.*, 1999).

Many authors have speculated about the putative function of NA, but this question remains to be clarified. NA has been proposed to play a key role in the cellular distribution of Fe (Scholz *et al.*, 1992). The distribution of iron in apoplasm and symplasm of BB and *chloronerva* led to the conclusion that NA is not required for the transport of Fe(II) through the plasmalemma into the cell (Becker et al., 1992).

The function of NA is certainly related to its molecular structure since changes in the octahedral coordination structures in NA derivates destroys its capability to act as a “phenotypic normalization factor” of the *chloronerva* mutant (Scholz et al., 1988). Immunochemical detection in leaf and root cells localizes NA into cytoplasm around the vacuoles, except in the cells of the plants cultivated in a medium with high iron concentration (100 µM FeEDTA). In these plants, NA was located also within the vacuoles (Pich et al., 2001). Furthermore, an increased NA concentration has been reported due to increased Fe levels (Pich et al., 2001). The authors suggest the hypothesis that NA might play an important role in the detoxification of intracellular Fe and propose a function of NA as intracellular Fe-storage and buffering system.

Perhaps NA is a regulator of active iron keeping it available in solution and maintaining a small iron pool for all dependent cellular processes (Stephan and Scholz, 1993).

Nicotianamine synthase

The nicotianamine synthase (*NAS*, EC 2.5.1.43.) is the enzyme positioned on the metabolic crossroad of the Strategy I and Strategy II of iron acquisition.

The *NAS* synthesizes NA by direct condensation of three molecules of S-adenosyl-L-methionine followed by the formation of an acetidine ring (Kawai *et al.*, 1988). The process could be reproduced in cell-free systems (Shojima *et al*., 1989).

NASs are a new class of enzymes, their number growing rapidly since the first cloning of the *NAS* from barley (Herbik *et al.*, 1999; Higuchi *et al*., 1999) and the *chloronerva* gene of tomato (Ling et al., 1999). Today, at least 9 barley *NAS* genes are known, and at least 7 of them are unique genes.

The *NASHOR1* and *NASHOR2* genes (Herbik *et al.*, 1999) were isolated from iron-deficient barley roots. This material was a suitable source for *NAS* isolation since the iron deficiency resulted in ~5 folds increasing ot *NAS* activity in the roots of barley (Herbik, 1997). After purification, the enzyme could be enriched about 140-fold. The resulting single protein peak precisely corresponded to the peak of enzyme activity. The SDS/PAGE of the purified active protein fraction revealed the presence of three polypeptides with molecular weights of 24, 28, 38 kDa. The 28-kDa protein was the only one, which could be labelled by UV cross linking to [^{14}C]SAM, suggesting that this polypeptide binds SAM as it would be expected for *NAS*. Tryptic digestion of the 28-kDa polypeptide and micro-sequencing of the resulting peptides revealed several amino acid sequences that were used to generate oligonucleotide primers further used for 3'-RACE. A 0,6-kb DNA fragment was amplified from total RNA isolated from iron deficient barley roots. The 0,6-kb fragment was used to screen a phage library of barley cDNA clones. Several clones have been isolated. The nucleotide sequence of the full-length *NASHOR1* clone (NCBI Accession No: AF136941) predicts a polypeptide of 330 amino acids residues and molecular mass of 35 611 Da. A full-length *NASHOR2* (AF136942) clone predicts a 340 amino acids and a molecular mass of 36 312 Da. These two genes were cloned and the further analysis will be described in this work.

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

Plant species	Cultivars
Arabidopsis thaliana L.	cv. “Columbia”
Hordeum vulgare L.	cv. “Bonus“
Lycopersicon esculentum Mill.	cv. “Moneymaker”
Lycopersicon esculentum Mill.	cv. “Bonner Beste“
Lycopersicon esculentum Mill.	*chln* mutant of cv. “Bonner Beste”
Nicotiana tabaccum L.	cv. “Petit Havana”

Tab. 2.1. Used plant species

2.1.2. Bacterial strains

Microorganism	Strain	Characteristics
Escherichia coli	DH5α	RecA1, endA1, gyrA96, thi-1, hsdR17, (r_K-m_{K+}), relA1, supE44, u80ΔlacZΔM15, Tn10, $(Tet)^r$, (Sambrook et al.,1989)
Escherichia coli	HMS174 (DE3)	F^-, recA, hsdR ($rk_{12}^-mk_{12}^+$) Rif^R(DE3)
Agrobacterium tumefaciens	$C58C1Rf^r$	Deblaere *et al.* (1985)

Tab. 2.2. Used bacterial strains

2.1.3. Plasmids

Vectors	Characteristics	Source
pET12a	amp^r, T7	Novagen
pET17b	amp^r, T7	Novagen
pBinAR19	Kan^r, CaMV 35S	Höfgens and Willmitzer, 1990
pAH17	Ubi	Christensen and Quail, 1996
PCR2.1		Invitrogen
PCR2.1-TOPO		Invitrogen

Tab. 2.3. Used plasmid vectors

2.1.3.1. Vector maps

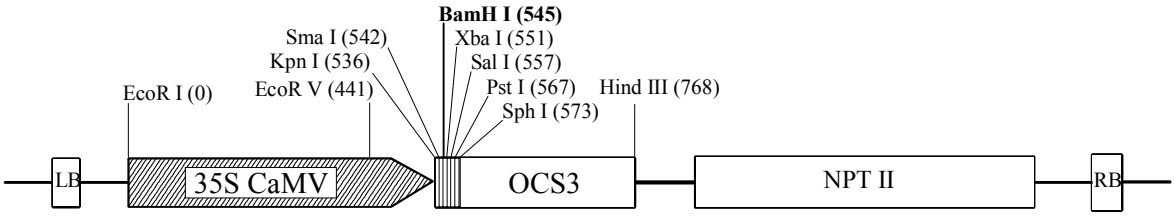

Fig. 2.1. pBinAR19 vector used for dicots transformations. *Contains expression cassette based on cauliflower mosaic virus (CaMV) 35S promoter, partial pUC19 polylinker and octopine synthase terminator (OCS3). NPT II - neomycin phosphotranspherase from Tn5 (kanamycin resistance), LB and RB - respectively left and right border.*

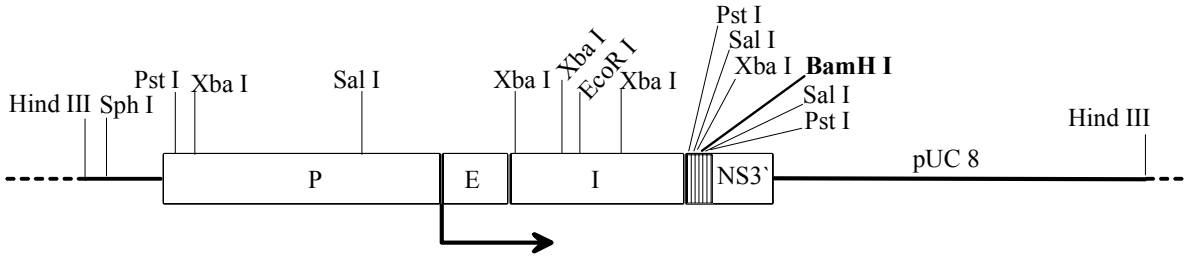

Fig. 2.2. pAHC17 vector used for monocots transformations*. The vector is based on maize Ubi-1 sequence. Ubi-1 elements: P - promoter sequence, E - exon, I - intron. NS3' - nopaline synthases 3' polyadenylated sequence. Arrow at the Ubi-1 exon indicates the transcription start site and the direction; pUC 8 - partial pUC 8 sequence.*

2.1.4. Primers and oligonucleotides

2.1.4.1. PCR primers

Name	Notes	Sequence 5'-3'
35S-P1	35S prom. primer	ATG ACG CAC AAT CCC ACT ATC CTT C
AR1	NASARA1, BamH I site	CCG CGG GAT CCA TGG GTT GCC AAG ACG AAC AAT TGG TGC AAA C
AR2	NASARA1, BamH I site	CCG CCG GAT CCC GTC CTC CTT AAG ACA ACT GTT CC
AR3	NASARA5, BamH I site	CCG CCG GAT CCA TGG CTT GCC AAA ACA ATC TCG TTG TG
AR4	NASARA5, BamH I site	CCG CCG GAT CCT ACT CGA TGG CAC TAA ACT CCT C
BC1	BCKDH, Kpn I site	AAC TTT AGC GGT ACC ACT AGG ACT TA
BC2	BCKDH, Kpn I site	ATC ACT TCG GTA CCG TTC GTG TAC AGG AGT GCT TAT
BC3	BCKDH	AAC TTT AGC TCA ACC ACT AGG ACT TA
BC4	BCKDH	CAT TTG GAA AGA GAA CAT GAA GAT T
BC5	BCKDH	GTT TCA ATG GCA ACT TGG TTT TTA AG
BC6	BCKDH	GTG ACA TTA TGC TTG AAA ATC AGT AGG
BC7	BCKDH	TTC CCA TCG ACG CGA ATA CTT C
BC8	BCKDH	TGG CCG TTT TCC AGT ATT CTA TTT C
BC9-SphI	BCKDH, Sph I site	ACA TGC ATG CGT TTC AAT GGC AAC TTG GTT TTT AAG
BC10-SalI	BCKDH, Sal I site	ACG CGT CGA CGT GAC ATT ATG CTT GAA AAT CAG TAG G
BC11-SphI	BCKDH, Sph I site	GTA TAT ATG CAT GCG TTT CAA TGG CAA CTT G
BC12-SalI	BCKDH, Sal I site	TTT CTT TTC AGT CGA CGT GAC ATT ATG CTT G
NASH1 (4-29)	NASHOR1	GAT GCC CAG AAC AAG GAG GTT GAT GC

		PCR PRIMERS CONTINUED...
NASH1 (981-956)	NASHOR1	CAC TTC CGC GTT GGT GAA CTC CTC TC
M3USal I	NASMET, Sal I site	CCT AAG GAG GTC GAC TAT GAG CTG CTA C
M3UNde I	NASMET, Nde I site	GGA GAA CCC ATA TGA GCT GCT ACA TCT AC
M3RBamH I	NASMET, BamH I site	GCG AGT GGG ATC CTT TAT CAT GGG AAG T
NA13	NASHOR1, BamH I site	CCG CCG GAT CCG ATG CCC AGA ACA AGG AGG TTG ATG C
NA14	NASHOR1, Nde I site	CCG CCA TAT GGA TGC CCA GAA CAA GGA GGT TGA TGC
NA15	NASHOR1, Nde I site	CCG CCG GAT CCC AAC GAT CAG AAG GCC ACT TG
NA16	NASHOR1, Kpn I site	CCG CCG GTA CCC GAT GCC CAG AAC AAG GAG GTT GAT GC
NA23	NASHOR2, Nde I site	CCC GGC ATA TGG GCA TGG AGG GCT GCT GCA GCA AC
NA24	NASHOR2, BamH I site	CCC GGG ATC CGG CAT GGA GGG CTG CTG CAG CAA C
NA25	NASHOR2, BamH I site	CCC GGG GAT CCC TAC GCC TCC ATC TCC TCC ATG GCG AT
NA28	NASHOR2, BamH I site	ATC CCG GAT CCT GCA TGA GGA CAA ACG GAT TAT TAC C

Tab. 2.4. Used PCR primers

2.1.4.2. Sequencing oligonucleotides

Name	Note	Sequence 5'-3'
AR9	5' CY5, NASARA1	AAA CCA GAA GAG AAG CGA GTG AGT
AR10	5' CY5, NASARA1	AGA CAA CTG TTC CTC CCT AGC TCC
AR11	5' CY5, NASARA5	TGC GTG TGA GTC GAT GTC AAA GTT
AR12	5' CY5, NASARA5	TAC TCG ATG GCA CTA AAC TCC TC
HPM1	5' CY5, HASHOR1 prom.	GGA GCA GAG GCG GAT GAT
HPM2	5' CY5, HASHOR1 prom.	CAA AAT CAA ACG CCG GTT GTA AC
HPM3	5' CY5, HASHOR1 prom.	TGT GTG ATG CCT AGC CGT TCG
HPM4	5' CY5, HASHOR1 prom.	CAC GCA TGA TCT TCC AAC GAA TG
HPM5	5' CY5, HASHOR1 prom.	GTG TGG CGC CGA ACG CTT AG
HPM6	5' CY5, HASHOR1 prom.	ATC CCA CAT TAT GCC TAC CAT CAA AC
HPM8	5' CY5, NASHOR1 prom.	TAA CCC TAA CCC CAT CAT ATC CCT AAC AC
NAH1Pm-S1	NASHOR1 prom	GG GCG AAG TGG AGT GGT GGA TG
NAH1-3'U1	NASHOR1	CGT TGC GAG GGA ATG AAA ATG AAG
NAH1-3'R1	NASHOR1	TAC TTG GCA CAC TAC CCT CGT CTG

Tab. 2.5. Oligonucleotides used for sequencing

2.1.4.3. Oligonucleotides and DNA probes used for genetical mapping and detection of the barley NAS genes

Name	Note	Sequence 5'-3'
hvNAS-Uni-R	NAS universal	GCC AGC ATG TCG GAG TAG TGC GCC TC
NASHOR1-F	NASHOR1	CTG TGC TCT AGG TCG CCA CAA CAT ACA
hvNAS2-F	hvNAS2	CTC CTG TGC CTG TCC TGA GGT ACC AAG AA
hvNAS3-F	hvNAS3	CTA CTT CAC TCA CAC TAG TGC CCA GAA AGA AG
hvNAS-Uni-F	NAS universal	ACG TCG TCT TCC TGG CCG CGC TC
hvNAS4-R	hvNAS4	TAC ATA GGT GAT AGG TGG TGG TAG GAG GAG GAG TA
hvNAS5-R	hvNAS	CCC ATC AAT GTG CAG GGT ATC ATC TG
hvNAS7-R	hvNAS7	CAC ATT TCT TTT CCT TTG CAC AGT CTC TTG
NAH1OP1	NASHOR1	TCT GGG CAT CCA TTT TAA TAC TGT ATG TTG
NAH2OP1	NASHOR2	AGC TAA GCT GAG AGG CTG TGA GAG TGA GTG

Tab. 2.6. Oligonucleotides and DNA probes used for genetical mapping and detection of the barley NAS genes.

2.1.5. Enzymes and kits

Company	*Products*
Amersham, Braunschweig	*Megaprime DNA labeling kit, Readyprime II DNA labeling kit, Restriction endonucleases*
Applied Biosystems, USA	BigDye terminator sequencing kit
Biolabs, USA	Restriction endonucleases
Biomol GmbH, Hamburg	Total RNA isolation kit
Roche (Boehringer Mannheim)	Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, Shrimp alkaline phosphatase, Klenow enzyme, Taq DNA polymerase, Expand high fidelity PCR system, Rapid DNA ligation kit, PCR nucleotide mix, Pwo DNA polymerase
Gibco-BRL, USA	Superscript II RNase H^- Reverse Transcriptase
Invitek GmbH, Berlin	Invisorb spin plant DNA extraction kit
Invitrogen, The Netherlands	TA cloning kit, TA TOPO cloning kit
Novagen, USA	His-Tag purification kit, T7-Taq purification kit.
Qiagen, Hilden	QIAquick agarose gel extraction kit, QIAqiuck PCR purification kit, Plasmid purification kits (mini, midi and maxi), Taq PCR polymerase, Taq PCR master mix, DNeasy plant DNA isolation kit, DNeasy 96 plant kit, RNeasy plant total RNA isolation kit
Stratagene, Heidelberg	Restriction endonucleases
USB, Cleveland OH, USA	Restriction endonuclease, Klenow enzyme

Tab. 2.7. Used enzymes and kits

2.1.6. Chemicals

Company	*Products*
Sigma-Aldrich	S-Adenosyl-L-methionine, Homocystein, S-Adenosylhomocystein, Methionine, Homoserin, Methylthioadenosin, Sodium carbonate, Sodium bicarbonate, Sodium chloride, $Tween_{20,}$ Potassium chloride, Sodium monophosphate, Sodium diphosphate, IPTG, X-gal, MOPS
Ambion, USA	RNAse ZAP cleaning reagent
Amersham, Braunschweig	[α^{32}P] dATP, [γ^{32}P] ATP, [α^{32}P] dCTP, Hybond-N+ nylon membrane, S-Adenosyl-L-[carboxyl-^{14}C]-methionine
Amresco, USA	Phenol
Biometra, Göttingen	Chloroform, Phenol, Phenol-chloroform, ATP, BSA, dNTPs, SDS
Difco, USA	bacto®-agar, bacto®-trypton, yeast extract
Duchefa, The Netherlands	Murashige-Skoog whole medium solid substance, Rifampicin, Kanamycin, Hygromycin, Carbenicillin
Fluka, Schweiz	DEPC
Gibco-BRL, USA	Agarose, 1Kb DNA ladder, EDTA
Invitrogen, The Netherlands	RM basis medium, induction basis medium
Kodak, USA	X-Ray films
Merck, Darmstadt	Ethanol, Ethidium bromide, Formamide, HEPES, Magnesium chloride, Sodium acetate, Sodium hydroxide, Sodium-dihydrogen phosphate, di-Sodium hydrogenphosphate, trichloroacetate, Tris base
Metabion, Planegg-Martinsried	DNA oligonucleotides
MWG-Biotech AG, Ebersberg	DNA oligonucleotides
NEN, USA	GeneScreen Plus hybridization transfer membrane
Roth, Karlsruhe	Phenol, Phenol-chloroform, Chloroform, Formaldehyde, Glycerol, Isopropanol, Lithium chloride, Sodium chloride
Schleicher&Schuell, Dassel	Blotting paper GB 002, nitrocellulose membrane BA 85
Serva, Heidelberg	X-Gal, Sodium citrate, $Tween_{20}$, tetracycline, Coomassie blue, EDTA, X-gal
Eurogentec, Belgium	Smart Ladder

Tab. 2.8. Used chemicals

2.1.7. Laboratory tools and equipment

Company	Equipment
AGS, Heidelberg	DNA gel-electrophoresis tanks
Appligene-Oncor	Vacuum blotter
Berhof GmbH, Eningen	DAP III high pressure block
BioRad, München	Gene-Pulser, Mini Electrophoretic System (Mini-Protean SDS-PAGE running cell, Mini Trans-Blot Electrophoretic transfer cell, Electro Eluter)
Biotec Fischer, Reiskirchen	Phero-stab 200 electrophoresis power supply
CBS, USA	EBS 250 power supply
DuPont, USA	Sorvall centrifuge RC 5C
Eppendorf, Hamburg	Mastercycler®5330 (DNA- thermocycler), Thermomixer 5436 and 5437, Thermomixer compact, cold centrifuge 5402, BioPhotometer
GFL, Burgwedel	Hybridization oven, water bath
Heraeus, Osterode	Centrifuges (Biofuge 13, Biofuge 15R), HERASafe laminar boxes
Hofer, San Francisco CA, USA	Transfer electrophoresis unit
Millipore, Schwalbach	Centricon protein concentrators, MilliQ water purification system
OWL	Agarose gel trays
Perkin-Elmer, USA	GenAmp PCR system 9700 (0,5 and 0,2 mL blocks)
Pharmacia, Freiburg	Photometer, Ultrospec plus
Polaroid, Offenbach	MP-4 camera
Raytest, Straubenhardt	FUJI BAS imager, imaging plates
Savant	SpeedVac SPD101B
Stratagene, Heidelberg	UV-Stratalinker® 1800, NucTrap® probe purification columns
Varian, Australia	SpectAA 10 plus AAS

Tab. 2.9. Used laboratory tools and equipment

2.1.8. Media

2.1.8.1. Bacterial media

LB medium

- 10,0 g NaCl
- 5,0 g Tryptone,
- 5,0 g yeast extract
- H_20 to 1000 mL

pH 7,4

SOC medium

- 0,580 g NaCl
- 0,186 g KCl,
- 20,0 g tryptone,
- 5,0 g yeast extract,
- 2 mL of 2 M sterile (0,2 µm filter) glucose added after autoclaving
- H_2O to 1000 mL

pH 7,4

TBY medim

- 5,0 g NaCl
- 5,0 g $MgSO_4.7H_2O$
- 10,0 g tryptone
- 5,0 g yeast extract
- H_2O to 1000 mL

pH 7,4

YEB medium

- 0,5 g $MgSO_4.7H_2O$
- 5,0 g beef extract
- 5,0 g peptone
- 5,0 g saccharose
- 1,0 g yeast extract
- H_2O to 1000 mL

pH 7,0

2.1.8.2. Plant growth media

Self made ½ MS modified medium with variable iron concentration:

Stock solutions for self-made ½ MS medium (modified) (for 1 L stock solution):

- Stock 1 (40x): 3,61 g $MgSO_4$ / 3,40 g KH_2PO_4 (or 7,38 g $MgSO_4.7H_2O$)
- Stock 2 (100x): 82,50 g NH_4NO_3
- Stock 3 (100x): 95,00 g KNO_3
- Stock 4 (100x): 16,60 g $CaCl_2$ (or 21,98 g $CaCl_2.2H_20$)
- Stock 5 (250x): 10 mM FeEDTA

For iron free medium, stock 5 was omitted.

Solutions of micro elements (1000x):

- 0,0125 g $CoCl_2.6H_2O$ (or 15,30 g $Co(NO_3)_2.6H_2O$)
- 0,0125 g $CuSO_4.5H_2O$
- 3,1000 g H_3BO_3
- 0,4150 g KI
- 8,4500 g $MnSO_4.H_2O$
- 0,1250 g $Na_2MoO_4.2H_2O$ (or 0,0927 g $(NH_4)_6Mo_7O_{24}.4H_2O$)
- 4,3000 g $ZnSO_4.7H_2O$
-

To prepare ½ MS modified medium, Stocks 1 to 5 and the Microelements were added to 1x final concentration. The Stock 5 (iron source) was added in different volumes in order to obtain the desired iron concentration in the medium. The normal iron concentration for *A. thaliana* was 40 µM FeEDTA and pH was adjusted to 5,8 by 0,1 M KOH.

Ready MS medium – Duchefa, the Netherlands

Hoagland liquid medium with variable iron concentration for tobacco, tomato and A. thaliana:

Stocks

- Stock 1 (100x) - 0,5 M $Ca(NO_3)_2$
- Stock 2 (100x) - 0,5 M $MgSO_4$
- Stock 3 (100x) - 0,5 M KNO_3 / 0,1 M $KH_2 PO_4$
- Stock 4 (1000x) - 10 mM FeEDTA

Solutions of microelements

- 5,0 mM H_3BO_3
- 4,5 mM $MnCl_2$
- 3,8 mM $ZnSO_4$
- 0,3 mM $CuSO_4$
- 0,1 mM $(NH_4)_6Mo_7O_{24}$

To prepare 1 L of Hoagland medium, 10 mL each of the Stocks 1 to 3 and 1 mL Microelements were added. The Stock 4 (iron source) was added in different volumes in order to obtain the desired iron concentration in the medium. The normal iron concentration for *A. thaliana* was 40 µM FeEDTA and pH was adjusted to 6,0 by 0,1 M KOH. For tobacco and tomato, the normal concentration was 10 mM and pH 5,5.

2.1.9. Software

DNA and protein sequence data were processed using the program package *Lasergene* version 4 and 5 of DNASTAR Inc., USA and *BLAST* (Basic Local Alignment Search Tool), (Altschul *et al.*, 1990).
The autoradiography images were analyzed by TINA 2.09 of Raytest Isotopenmeßgeräte GmbH, Germany)

2.2. Methods

2.2.1. Basic cloning methods and sequencing

The standard molecular cloning methods (e.g. restriction digestion, ligation, DNA and protein gel electrophoresis) were performed according to Sambrook *et al.*, 1989.

The transformation of *E. coli* was performed using the heat-shock procedure (Cohen *et al.*, 1972) or electroporation (Inoue *et al.,* 1990).

Plasmid DNA extraction and purification was done by using the standard methods described in Sambrook *et al.,* 1989, or by using Qiagen plasmid kit. PCR products were purified with QIAquick PCR purification kit (Qiagen).

TA based cloning was performed using the pCR2.1 or pCR2.1-TOPO vector systems (Invitrogene).

DNA fragments were isolated and purified from the agarose gel with the QIAquick kit (Qiagen).

DNA sequences were determined in the Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). DNA was detected using Fluorescence-labeled Primers by the A.L.F. Sequencer (Pharmacia LKB) and the Autoread Sequencing kit (Pharmacia). Sequences of longer DNA fragments were determined by primer walking. Alternatively, the BigDye terminator sequencing kit (Applied Biosystems) was used on ABIPrism (Applied Biosystems) sequencer.

2.2.2. Extraction of plant genomic DNA

The rapid plant DNA extraction, PCR grade, was carried out according to Edwards *et al.* (1991). The leaf tissue (~100 mg) was grinded in liquid nitrogen and then 400 µL of extraction buffer (200 mM Tris-HCl pH 8,0, 250 mM NaCl, 125 mM EDTA, 0,5% SDS) were added and the mixture was shaken for 1 min. The leaf suspension was centrifuged for 5 min at full speed and the supernatant transferred into a new tube containing 300 µL of

isopropanol. The DNA was collected by centrifugation for 10 min, washed twice with 70% ethanol, and resuspended in 100 µL of H_2O.

High purity plant DNA was extracted and purified using the following protocol:

1. Grinding of 200-300 mg young leaves by mortar and pestle in liquid nitrogen
2. Collecting of the powder into 15 mL tube
3. Adding of 3,0 mL Lysis buffer (10 mM Tris pH 8,0, 10 mM EDTA, 0,1 mM NaCl), 0,8 mL 10% SDS and 20 µL proteinase K
4. Incubation for 2 h at 56 °C and moderate shaking
5. Cooling to room temperature (RT)
6. Adding of 1 mL saturated NaCl and mixing by inversion of the tube for 15 sec
7. Centrifugation for 15 min at 4000 rpm, RT
8. Transferring the supernatant into new tube with 10 mL ethanol cooled to –20 °C
9. Collecting of the precipitated DNA with sterile glass rod and transferring into 1,5 mL tube containing 96% ethanol
10. Collecting the DNA by centrifugation and drying of the samples
11. Resuspending in 500 µL TE buffer
12. Purifying by Phenol/Chloroform extraction
13. Precipitating of the DNA by adding of 0,1 vol. 3M K-acetate, pH 5 and 1 mL 96% ethanol
14. Centrifugation to collect the DNA and removing the ethanol
15. Washing twice by 70% ethanol and drying
16. Resuspending in 100-200 µL Tris buffer, pH 8,0

Later, this method was replaced by the use of DNeasy Plant kit, DNeasy 96 plant kit (Quiagen) or by Invisorb spin plant DNA extraction kit (Invitek).

2.2.3. Extraction of genomic DNA from *Methanobacterium thermoautotrophicum*

To extract DNA from *M. thermoautotrophicum*, the method of Bintrim *et al.*, 1997 was used with minor modifications.

1. Collecting the cells from 3 mL liquid culture by centrifugation
2. Resuspending in 200 µL TE buffer
3. Sonication of the cells, 300 sec
4. Adding of lysosime (0,5 mg/mL)
5. Incubation for 30 min at 37 °C with shaking
6. Adding of proteinase K (2,0 mg/mL)
7. Incubation for 30 min at 37 °C
8. Adding of 200 µL buffer B (250 mM NaCl, 100 mM EDTA, 4% SDS) + 30 µL of 5M Guanidine isothiocyanate
9. Gently agitating by inverting the tube
10. Incubation at 68 °C for 1 h
11. Adding of 60 µL CTAB buffer (2% CTAB, 100 mM Tris-Cl pH 8,0, 20 mM EDTA, 1,4 M NaCl)
12. Incubation at 65 °C for 15 min
13. Phenol-chloroform extraction and isopropanol precipitation of the DNA
14. Dissolving the pellets in appropriate buffer

2.2.4. Southern blotting

DNA was prepared using the methods described above. For Southern hybridization DNA was digested with restriction enzymes, separated on a 0,5 to 1% agarose gel in Tris-acetate buffer (Sambrook *et al.*, 1989) and transferred onto a Hybond N^+ (Amersham) or GeneScreen Plus (NEN) membrane using alkali capillary blots according to the following protocol:

1. Pre-wetting of the membrane in distilled water for a few seconds
2. Equilibrating of the membrane in 0,4 N NaOH for 10-15 min
3. Agitating the gel in 0,25 N HCl for approximately 10 min
4. Denaturizing the DNA by soaking the gel in 0,4 N NaOH for 30 min

5. Transferring of the DNA to the membrane by using 0,4 N NaOH as a transfer solution on a capillary blot or vacuum blotter (Appligene-Oncor).
6. UV cross-linking the DNA to the membrane by using the Auto Cross link mode of UV Stratalinker 1800 (Stratagene).

2.2.5. Extraction of plant total RNA

The protocol for RNA isolation is based on the Guanidium thiocyanate (GCN) method of Chomzynski and Saccini, 1987 (modified):

Solution D:

- 250 g (GCN)
- 293 mL H_2O (DEPS treated)
- 17,6 mL 0,75 M Na Sarkosyl (sodium lauryl sarcosinate)
- Mix and store at 4 °C. Before use, add 72 µL β-mercaptoethanol to 10 mL solution D.

DEPS H_2O (RNAse free)

1 mL DEPS to 1 L H_2O, 12-24 h at 37°C with shaking. Autoclave until the smell of DEPS disappeared (2-3 times).

Procedure:

1. Homogenization of the sample in 300 µL Solution D
2. Adding of 30 µL 2M Sodium Acetate, pH 4, vortex
3. Adding of 300 µL Phenol (pH 6,6), vortex
4. Adding of 60 µL Chloroform:Isoamyl alcohol (24:1), intensive shaking for 10 sec
5. Incubation on ice for 15 min
6. Centrifugation, 10 000 g, 20 min at 4 °C
7. Transferring of the aqueous phase to clean Eppendorf tube containing 300 µL Isopropanol
8. Incubation for 1 h at -20 °C
9. Pellet the RNA, 10 000 g, 20 min, 4 °C

10. Aspirating the isopropanol
11. Washing twice in 70% ethanol and drying
12. Dissolving in H_2O (DEPS treated)
13. Determination of the concentration and A_{260}/A_{280} ratio.

Alternatively, total RNA isolation reagent (Biomol) or RNeasy kits (Qiagen) were used.
All the glass- and plastic ware used for RNA isolation were treated with RNAse ZAP cleaning reagent (Ambion) and washed with DEPS treated water.

2.2.6. Northern blotting

Formaldehyde-agarose (FA) gel electrophoresis preparation:

10x formaldehyde gel running buffer (FA gel buffer)

- 40 g MOPS (200 mM) in 800 mL 50mM Na-Acetate, pH 7,0 (with 2N NaOH)
- 20 mL 0,5 M EDTA, pH 8,0
- Adjusting to 1000 mL
- Autoclaving at 120 °C (2 bar) for 10 min.

1X FA gel running buffer (1 L)

- 100 mL 10X FA gel buffer
- 20 mL 37% formaldehyde (works without formaldehyde too)
- 880 mL RNase free water

Gel preparation - 100 mL 1% agarose

- 1,0 g agarose
- 10 mL 10x buffer MOPS buffer
- RNase-free water to 100 mL
- Melting and cooling to 65°C
- 1,8 mL Formaldehyde (37%)
- 1 µL Ethidium bromide (10 mg/mL)
- Equilibrate the gel in 1X FA gel buffer for at least 30 min.

5x RNA loading buffer (1 mL)

- 1,6 µL Saturated bromphenol blue
- 8,0 µL 0,5 M EDTA, pH 8,0
- 72,0 µL 37% Formaldehyde
- 200,0 µL 100% Glycerol
- 308,4 µL Formamide
- 400,0 µL 10X FA gel buffer
- RNase-free water to 1 mL

20xSSPE

- 3,00 M NaCl
- 0,20 M NaH_2PO_4
- 0,02 M EDTA

Adjusting the pH to 7,4

Sample preparation

- Add 1 volume of 5X RNA loading buffer per 4 vol. RNA
- 3-5 min at 65°C, keep on ice until loading into the gel.

Transfer of the RNA to the membrane

RNA was prepared using the methods described above. For Northern hybridization the RNA was separated on a 1% FA agarose gel and transferred onto a Hybond N^+ (Amersham) or GeneScreen Plus (NEN) membrane using alkali capillary blots according to the following protocol:

1. Pre-wetting of the membrane in distilled water for a few seconds
2. Equilibrating of the membrane in 10xSSPE for 15 min
3. Soaking the gel in 5 volumes distilled water for about 5 min to remove the formaldehyde from the gel, repeat 4 times
4. Transferring of the RNA to the membrane by using 10xSSPE as a transfer solution on a capillary blot or vacuum blotter (Appligene-Oncor)

5. UV cross-linking the RNA to the membrane by using the Auto Cross link mode of UV Stratalinker 1800 (Stratagene).

2.2.7. Radioactive labeling of DNA probes

Oligonucleotide labeling by terminal phosphorylation

- 100 ng oligonucleotide in less than10 µL volume
- 2,5 µL 10X polynucleotide kinase buffer
- 5,0 µL [γ-^{32}P] ATP (50 µCi)
- H_20 to 25 µL
- 1 µL T4 polynucleotide kinase (10 U/µL), vortex gently
- Incubate 1 h at 37°C
- Reaction was stopped by adding of 75 µL STE buffer (100 mM NaCl, 20 mM Tris/HCl pH 7,5, 10mM EDTA)
- Purification by NucTrap probe purification columns (Stratagene).

Labeling of long DNA probes by random priming

- 25 ng DNA probe
- 5 µL Random nonamer primers mix
- H_2O to 50 µL
- Denaturation - 5 min, 100°C
- Span down, kept on RT
- 10 µL Labeling buffer (dATP, dGTP, dTTP in Tris/HCl pH7,5, 2-mercaptoethanol and $MgCl_2$)
- 5 µL α-^{32}P dCTP (50 µCi)
- 2 µL Klenow (1 U/µL)
- Incubation 10-30 min, 37°C
- Denaturation for 5 min at 100°C and chilling on ice for use in hybridization or the reaction was stoped by addition of 5 µL 0,2 M EDTA

Alternatively, Megaprime or Readyprime II labeling systems (Amersham) were used.

2.2.8. DNA-DNA and DNA-RNA hybridization protocol

Hybridization of the Southern and Northern blot membranes was carried using the method of Church (Church and Gilbert, 1984) (modified).

Church buffer 250 mL

- 4,40 g NaH_2PO_4
- 16,55 g $Na_2HPO_4.2H_20$
- 17,50 g SDS
- 2,50 g BSA
- H_20 to 250 mL, dissolve overnight
- Filter before use to remove the remaining SDS crystals.

Phosphate buffer, 100 mL

- 74,7 mL 0,5M Na_2HPO_4
- 25,3 mL 0,5M NaH_2PO_4

Wash buffer

- 80 mL Phosphate buffer
- 50 mL 20% SDS
- 4 mL 0,5 M EDTA (pH 8,0)
- H_2O to 1 000 mL

Prehybridization

The membranes were prehybridized at 55-65 °C with Church buffer containing denaturated carrier DNA (Calf thymus DNA) with concentration of 100 µg/mL.

Hybridization and washing the membrane

New preheated Church buffer, denaturized carrier DNA and the labeled DNA probe were added to the membrane. Hybridization was carried overnight at the same temperature as the prehybridization. Then, the membrane was washed with the wash buffer (1x30 min, 4x15 min) and the signal was

detected and quantified with a Bio-Imaging analyzer BSA2000 (Fuji Photo Film Co. Ltd) or X-ray film.

Stripping of the membranes

After the hybridization, the membranes must not dry out. To strip the labeled probe, a boiled 0,5% SDS solution was poured onto the membranes and allowed to cool to room temperature.

2.2.9. Protein overexpression, isolation and purification

The protein overexpression in *E. coli* was carried according to standard methods (Sambrook *et al.*, 1989).

Purification and solubilisation of inclusion bodies

The induced cells were harvested by centrifugation and resuspended with appropriate buffer. In case of NAS overexpression, a buffer A (50 mM Tris, 1 mM EDTA, 3 mM DTT, 500 µM Methionine, pH 8,7) was used. Then the *E. coli* cells were destroyed by ultrasonication and the suspension was centrifuged at 10 000 g, 4 °C. The supernatant contained the soluble proteins while the natant contained the inclusion bodies and insoluble cell debris. Typically, the NAS protein was found in the inclusion bodies fraction.

Refolding of the inclusion bodies.

The following protocol for purification and refolding of NAS was used (for pellets obtained from 500 mL LB culture):

1. Washing the pellets with 0,1 M Tris pH 8,0
2. Resuspending of the pellets in 15 mL buffer (0,1 M Tris pH 8,0, 1mM $MgCl_2$, 0,2 mg/mL Lysozym, 1000 U/mL Benzoat)
3. Incubation for 1 h at 37 °C
4. Centrifugation at 10 000 g, 4 °C for 15 min
5. Washing of the pellets twice with buffer A and once with H_20
6. Resuspending of the pellets in 20 mL 2M Urea by shaking for 20 min at RT
7. Centrifugation at 10 000 g, RT, for 10 min

8. Dissolving of the pellets in 80-100 mL 6M Urea by shaking at RT (the protein concentration must not exceed 1 mg/mL)
9. Centrifugation at 10 000 g, RT, for 15 min
10. Dialyzing the suspension against 50 fold higher volume of Urea in buffer A with decreasing concentration of the Urea (5 M, 4 M, 3 M, 2 M, 1 M, 0M (twice)), at 4 °C for several days
11. Centrifugation at 10 000 g, 4 °C, for 20 min.

Refolded NAS protein was controlled by SDS- PAGE.

A further preconcentration of the proteins (when needed) was performed by using Centricon concentrators (Millipore).

2.2.10. Antibody preparation and purification

Rabbit was injected with 80 µg of purified NAS protein for 4 times with an interval of 1 month in between. Two weeks after the 3rd injection a serum sample was taken to check the polyclonal specificity. Two weeks after the 4th injection the animal was killed and its blood was collected. The polyclonal serum was obtained by centrifugation of the collected blood and the polyclonal IgG fraction was isolated using protein A sepharose (Amersham). Further, the IgG graction of the serum was purified according to following protocol:

1. Resuspending of about 1 mL of drained gel in 50 mL PBS buffer pH 7,0 and degassing for 10 min
2. Packaging of the gel slurry in C10/10 column (Amersham)
3. Washing of the gel with 30 mL of PBS buffer
4. Adding of 3 mg of serum protein to the surface of the column and allowing it to penetrate through the column by adding a buffer
5. Waiting for 30 min at RT to complete the binding process between IgG and protein A.
6. Washing of the column with PBS until the outlet buffer has no protein content, as measured with OD at 280 nm
7. Elution of IgG by using 0,1 M of glycine buffer pH 3,0
8. Collecting of the outlet in 3 mL collection tubes and immediately neutralization using a saturated solution of KOH

9. Precipitation of the IgG protein with 50% ammonium sulphate
10. Collecting the protein by centrifugation
11. Reconstition of the pellet in PBS pH 7,0 and
12. Dialyzing in the PBS buffer
13. Measuring of the protein content and dispensing and storing of the polyclonal IgG.

2.2.11. Western blot analysis

Soluble proteins were separated in a denaturing 12% (w/v) SDS-PAGE gel. After electrophoresis, the proteins were transferred in transfer buffer (25 mM Tris, 192 mM glycine pH 8,3, 20% v/v methanol) to the nitrocellulose membrane (Schleicher and Schuell) at 1,3 mA/cm^2 for 16 h on Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad) or on a bigger device.

Immunodetection of the blotted NAS proteins

To detect the blotted NAS protein, Alkaline-Phosphatase bound anti-rabbit antibodies were used according the following protocol:

1. Blocking of the excess protein binding sites of the membrane by incubation in 3% BSA in TBST buffer (10 mM Tris pH 8,0, 150 mM NaCl, 0,1% Tween$_{20}$) for 30 min at RT
2. Incubation of the membrane with specific NAS antibodies diluted 1:10 000 in TBST for 1 h
3. Washing of the membrane by gently shaking in TBST, 3x5 min, RT
4. Incubation with second antibodies (Anti-rabbit Ab, Alkaline-Phosphatase conjugate, Boehringen) diluted 1:10 000 in TBST for 1 h
5. Washing of the membrane by gently shaking in TBST, 3x5 min, RT
6. Developing the color as follows: adding of 90 µL NBT (75 mg/mL nitro blue tetrazolium) and 70 µL BCIP (50 mg/mL 5-bromo-4chloro-3-indolyl phosphate) (both from Novagen) per 20 mL AP buffer (100 mM Tris, pH 9,5, 100 mM $MgCl_2$) (Novagen) and incubation of the membrane at RT until color develops
7. Stopping the reaction by rinsing in 20 mM Tris, pH 8,0, 5 mM EDTA.

2.2.12. Screening of the barley BAC library

Filters containing spotted barley BAC library (cv. Morex, constructed in Clemson University, USA, provided by D. Schmidt, IPK) were hybridized with specific oligonucleotide probes (NAH1OP1) by using the following protocol:

Prehybridization solution and conditions:

- 6x SSPE
- 5x Denhardt's reagent (see below)
- 0,5% SDS
- 100 µg/mL Calf thymus DNA

Denhard's reagent (100x):

- 2% (w/v) BSA,
- 2% (w/v) Ficoll,
- 2% (w/v) PVP (polyvinylpyrrolidone)

Prehybridization was carried out at 55 °C, overnight.

Hybridization solution and conditions:

- 6x SSPE
- 5x Denhard's reagent
- 0,5% SDS
- 100 µg/mLL Calf timus DNA
- 200 ng 5'-labeled (γ-^{32}P ATP) oligonucleotide probe

Hybridization at 55°C, overnight.

Washing solution and conditions:

First wash (in the hybridization tube)

- 2xSSPE
- 0,1% SDS

Washing twice for 30 min at 55 °C

Second wash (in tray)

- 2xSSPE
- 0,1% SDS

Washing for 30 min at 55 °C

Third wash (in tray)

- 2xSSPE
- 0,1% SDS

Washing twice for 30 min at 55 °C

Fourth wash (in tray)

- 0,5 SSPE
- 0,1% SDS

Washing twice for 30 min at 55 °C

The signals were detected by exposing the membranes on X-ray films and amplification screens, for 4 days at -80 °C.

2.2.13. *Agrobacterium tumefacience* growth and treatment

Transformation of Agrobacterium tumefacience

The competent cells of *Agrobacterium tumefacience* (pGV 2260) were prepared using the $CaCl_2$ method. The *Agrobacterium* strain was grown in 50 mL of YEP medium at 28 °C until OD_{600}~ 0,5-1,0. The cells were centrifuged at 3000 rpm for 5 min and resuspended in 1 mL of 20 mM of $CaCl_2$. 100 µL aliquots of the resuspended cells were dispensed in separate Eppendorf tubes. The transformation with plant expression vectors was done using the thawing-freezing method as described by Höfgen and Willmitzer, 1988.

Total DNA preparation from Agrobacterium tumefacience

The bacteria were grown in 5 mL YEP at 28 °C for 18 hours and the pellets were collected by centrifugation in a 1,5 mL tube. After resuspension in 300 µL of suspension buffer (20 mM EDTA, 50 mM Tris pH 8,0), 100 µL of 5% sarkosyl in TE and 2,5 mg/mL proteinase K in TE were added. The mixture

was incubated at 37 °C for 2 hours, followed by two extractions with phenol, one with phenol-chloroform (1:1) and finally twice with chloroform. The supernatant was precipitated by adding 0,1 volume of 3 M K-Acetate and 2 volumes of ethanol, the DNA pellets were washed twice with 70 % ethanol and resuspended in 50µL TE buffer.

2.2.14. Plant growth and treatment

2.2.14.1. *Arabidopsis thaliana*

Growth of A. thaliana in soil

Arabidopsis plants were grown in a growth chamber at 22°C with 6000 lux of white light for 16 hours. The plants grew at these conditions until the end of maturation (~22 days after pollination).

Hydroponics cultures of A. thaliana

Using hydroponics methods in air- and light-conditioned growth chamber by using Hoagland medium with 40 µM FeEDTA performed the growth of A. thaliana under controlled conditions.

The plants were grown in 30x24 cm trays, as it is described in Fig. 2.3. The plants were grown in a chamber with 200-300 µE/cm^2 light intensity, 8 h light by 21 °C and 16 h darkness at 19 °C (short day). The seeds were germinated on rock wool bundles that were soaked into the nutrient solution. At day 3-4 the seedlings had 2 cotyledons developed and the opaque screen was removed to permit the light to the plantlets. At day 8, the nutrient solution was changed and the glass plate was shifted to allow the insertion of aeration tubes. At day 10-11 the plantlets usually were well developed (2-6 leaves), the glass plate was completely removed, and the growth tray was taken out of the germination chamber. The plants were grown further with regular changing of the nutrition medium until the day 32-36 when they were used in physiological experiments in iron limited conditions.

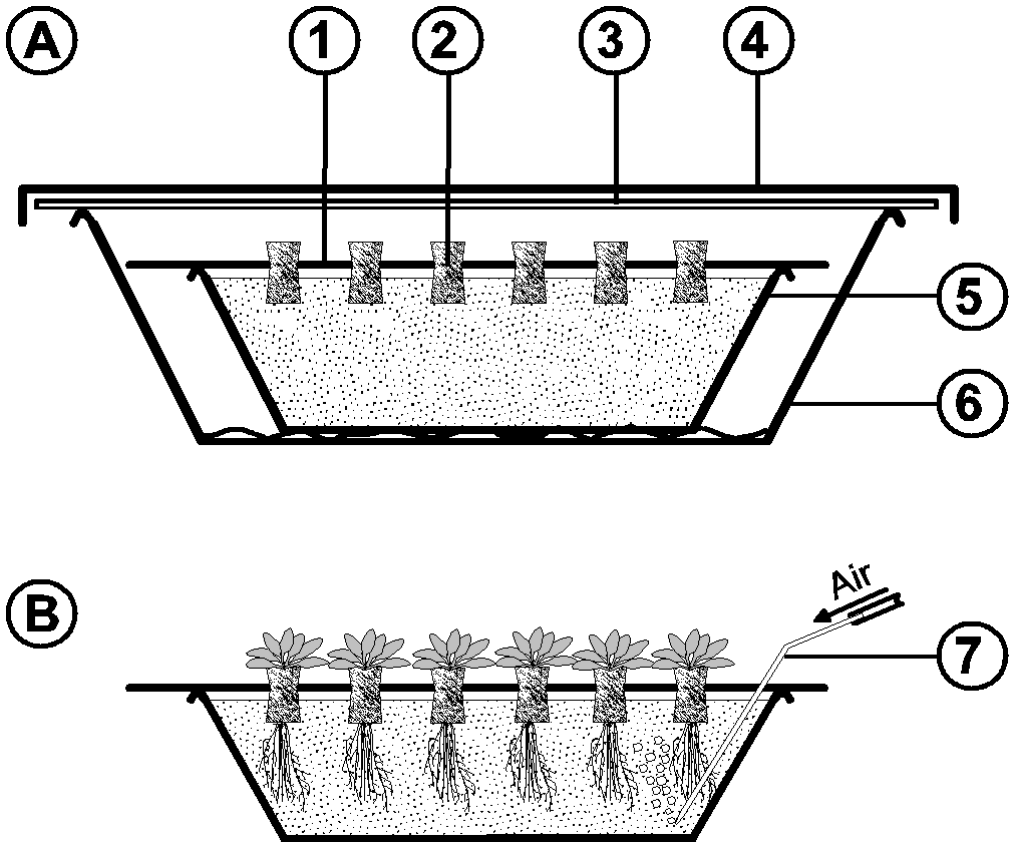

***Fig. 2.3. Hydroponics system for A. thaliana*. *Figure (A)**. The germination chamber: A small tray (5), covered with hole-plate (1) with a rock-wool bundle soaked into the nutrient solution (2) in each hole. The whole construction (1, 2, 5) was put into a bigger tray (6), covered with a glass plate (3) and opaque screen (4). The* A. thaliana *seeds were put on the top of each rock-wool bundle. Wed paper towels on the bottom of the big tray kept the humidity in the germination camera. After the germination, the opaque screen was removed to allow lighting of the seedlings. **Figure (B)**. The growth tray. After approximately 8 days, the glass plate was shifted at few centimeters and a tube for aeration (7) of the nutrition medium was inserted. After 2 weeks approximately, the seedlings were well developed. Then the small tray was taken out of the germination camera and the plants were grown until the end of the experiment by regular changing of the nutrition medium.*

Submerged A. thaliana cultures

For short-term iron limitation experiments (up to 1 week of iron limitation) and to prepare a material for screening of DNA arrays, submerged *A. thaliana* cultures were used.

1. About 10 surface-sterilized (Ethanol, NaOCl) *A. thaliana* seeds in 100 mL flasks, containing 25 mL ½ Murashige-Skoog (MS) modified medium
2. Imbibition of the seeds for 24 h at 4 °C in the dark
3. Moving the flasks in a growth chamber and growing for 10 day at constant shaking
4. Removing of the nutrition medium and washing of the plants with 0,1 mM $Ca(NO_3)_2$ for 30 sec, 0,1 mM $Ca(NO_3)_2$/1 mM EDTA for 10 min and finally again in 0,1 mM $Ca(NO_3)_2$ for 30 sec
5. Transferring of the plants in new iron-free (10% HCl washed) 100 mL flasks filled with 25 mL ½ MS modified medium with or without iron.

Plant transformation of Arabidopsis thaliana by vacuum infiltration

Transformation of *Arabidopsis* was performed based on the protocol of Bechtold *et al.* (1993). Plants of *Arabidopsis thaliana* (ecotype Columbia) were grown for three weeks under short day conditions (8 hours light, 16 hours dark) and transferred to long day (16 hours light, 8 hours dark). After three weeks, the emerging bolts were cut to induce growth of multiple secondary bolts. Vacuum infiltration of plants with *A. tumefacience* culture was done one week after clipping. Bacteria were grown until OD_{600} > 2,0, harvested by centrifugation and resuspended in three volumes of infiltration medium (OD_{600} approx. 0,8). Entire shoots of the plants were submerged into the *A. tumefacience* suspension in a beaker. Vacuum was applied by an oil pump for 5 min and then rapidly released. Plants were removed from the beaker, placed on their side and kept at high humidity under plastic warp for 24 hours, after that they were uncovered and set upright. Seeds were harvested from the siliques, sterilized by Na-hypochlorite as described before and plated onto GM selection plates containing 50 mg/L hygromycin. After two weeks, hygromycin resistant plants were transferred to soil, grown up and their seeds were collected. Stable transformation and expression of the constructs were analyzed by PCR.

2.2.14.2. *Nicotiana tabacum* growth and treatment

Transformation of tobacco and tomato

The leaf disk method was used for *Agrobacterium*-mediated transformation of tobacco and tomato as it is described in Horsch *et al*. (1985)

Hydroponics cultures of tobacco

The regenerated from leaf disks transgenic tobacco was grown in hole-plate covered trays with liquid Hoagland medium with 10 µM FeEDTA as a normal iron concentration. When seeds were used, they were surface-sterilized and germinated on MS agar plates where they grew for 2 weeks, and then the plantlets were transferred to trays with Hoagland medium.

2.2.14.3. *Lycopersicon esculentum* growth and treatment

The tomatoes were transformed and grown in a way similar to tobacco except the germination of the seeds, which was performed on wet paper towels. Than the seedling were transferred to Biolaston (PVC fibers) soaked in diluted 1:1 with water Hoagland medium as is described on Fig. 2.4..

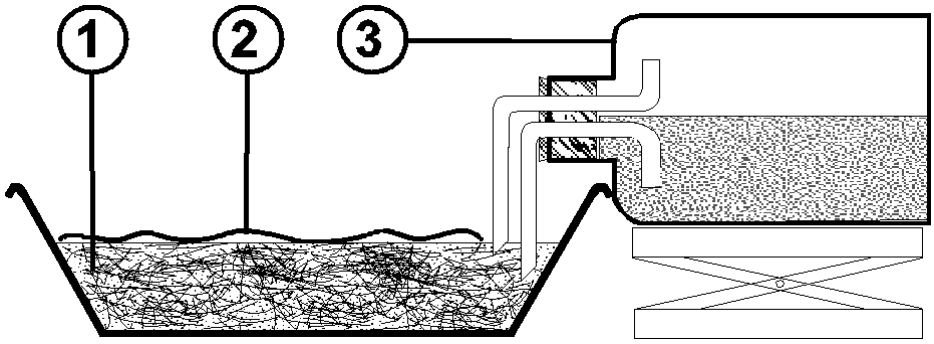

Fig. 2.4. Grow system for tomato seedlings. *A tray was filled with Biolaston (1) soaked in nutrient solution. The seeds were put on the top of a plastic mesh (2). Constant level of the nutrient solution was maintained by the system (3).*

2.2.15. Nicotianamine synthase activity assay

Preparation of the samples for determination of NAS activity in plant extracts was performed according to A. Herbik (1997).

The activity of the NAS enzyme was determined by using S-Adenosyl-L-[carboxyl-^{14}C]-methionine (^{14}C-SAM) as a substrate. 50 µL plant or bacterial extract was incubated with 20 µM ^{14}C-SAM at 30 °C, pH 8,7 for 10 min. Adding an equal amount of 99% methanol stopped the reaction. The samples were centrifuged and 0,5 to 2 µL of the supernatant was spotted on silicagel plates together with 5µL 1 mM NA solution and separated by thin layer chromatography (TLC) by using 1-propanol/water in 7:8 ratios as a mobile phase. To visualize the non-labeled NA on the TLC-plate, the latter was dried and stained by ninhydrin (300 mg Ninhydrin, 3 mL Acatic acid, 100 mL butanol) at 60 °C. The amount of the produced ^{14}C NA was quantified by using Fuji Bio-Imaging analyzer and the Software TINA 2.09.

2.2.16. Determination of the metal ions concentration

The determination of the concentration of iron, zinc and other metal ions was performed according to Stephan *et al.* (1994). Absolutely dry plant material was dissolved in 65% HNO_3 at 170°C for 3 h in High pressure block DAB III (Berhof GmbH) and the samples were measured on the Atomic absorption spectrometer SpectAA 10 Plus (Varian).

2.2.17. Determination of the Nicotianamine concentration

For determination of the NA concentration plant material was crushed in liquid nitrogen. The powder was mixed with water and the resulting pull was homogenized in a Potter-Elvehjem device after thawing. The homogenate was stirred for 1 h and deproteinized by heating to 80 °C and centrifuged at 48 000 g for 20 min. The supernatant was concentrated in a vacuum evaporator at 42 °C and lyophilized. The lyophilizate was dissolved in 0,2 M Na-citrate buffer pH 3,05. The NA concentrations were determined after postcolumn derivatization with ninhydrin at 570 nm using an amino acid analyzer (S 432, Sykam) as described in Schmidke and Stephan (1995).

2.2.18. Ferric-reductase assay

Ferric-reductase activity was used as a criterion for the response of the plant to iron insufficiency. The measurement of Fe^{3+}-chelate reduction by intact

roots was followed by observing the formation of Fe^{2+}-BPDS (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid) complex from Fe^{3+}-EDTA, according to Chaney *et al.* (1972). Plants with appropriate size were transferred into 100 mL aerated opaque glass tubes filled with Hoagland medium containing 2 µM FeEDTA. The plants were grown in this condition for 5 days. During this time, the pH of the medium was measured regularly each day. At the 5^{th} day the roots of the plants were washed in 10^{-4} M $Ca(NO_3)_2$ solution and the plants were transferred to new tubes filed with iron-free Hoagland medium with pH adjusted to the last pH value measured in the previous nutrient solution. Then, 1 mL 2 mM FeEDTA and 1 mL 8,5 mM BPDS were added to the medium. The first sample (T_0 – the base) was taken after 30 sec waiting for mixing then samples after 30 min (T_1) and 60 min (T_2) incubation time were also collected. All the samples were stored at dark until the spectrophotometric measurement of the extinction at 540 nm. At the end of the experiment, the exact volume of the reaction medium was measured in order to be used in the calculations. The iron reductase activity was estimated according the rule that 1 M $Fe(BPDS)_3$ has an extinction of 22500 units at 540 nm and the result was recalculated for the reaction volume and the time to obtain the activity in µmol reduced Fe^{3+} per hour per liter (µmol/h/L).

To control the induction of the iron-uptake mechanisms in *A. thaliana* grown to produce a material used in the screening of DNA arrays for iron-concentration dependent gene expression, a slightly modified procedure was used.

2.2.19. Chlorophyll content measurement

The samples were grinded in mortar and liquid nitrogen. Then 1 mL 100% acetone was added and the samples were extracted for 10 min with shaking. The homogenates were centrifuged for 6 min at 10000 g at 4°C. The supernatant was transferred to a new tube and the debris was extracted again with 80% acetone (10 min, vortex). The second extract was added to the first one. The A_{652} of the supernatant was measured against 80% acetone. The samples were diluted with 80% acetone where was necessary. The A_{652} value was divided with 34,5 to obtain the chlorophyll concentration in mg/mL. The quantity of pigment was normalized to the sample weight in each sample

3. Results

Iron deficiency leads to in a significant increase in NAS activity in roots of barley – a typical strategy I plant. Therefore, barley roots grown under iron deficient conditions have been chosen as a starting material for NAS isolation. A SAM-binding protein was detected in the protein extract and was used for microsequencing. Based on the amino acid sequence a specific oligonucleotide was derived and used as probe for the screening of a barley cDNA library. Two cDNA clones designated as *NASHOR1* and *NASHOR2* were isolated. Further studies were mainly focused on the *NASHOR1* gene, which was used for expression studies and generation of transgenic plants.

By homology search, two genes similar to *NASHOR1* sequences were identified in the *A. thaliana* genome on chromosome 1 and chromosome 5 and were designated as *NASARA1* and *NASARA5*, respectively. These *NAS*-like sequences were used for plant transformations.

3.1. Arabidopsis thaliana wild type experiments

Wild type *Arabidopsis thaliana* was studied in order to obtain basic physiological data concerning NA and metal metabolism in this plant.

Arabidopsis plants were grown in nutrient solution with different iron concentrations. An increase in NA was observed both in the shoots and in the roots of plants grown in iron limiting condition (0 µM and 1 µM FeEDTA) whereas in the normally iron supplied (40 µM EDTA) or iron overloaded (100 µM FeEDTA) plants the NA concentration was considerably lower (Fig. 3.1.)

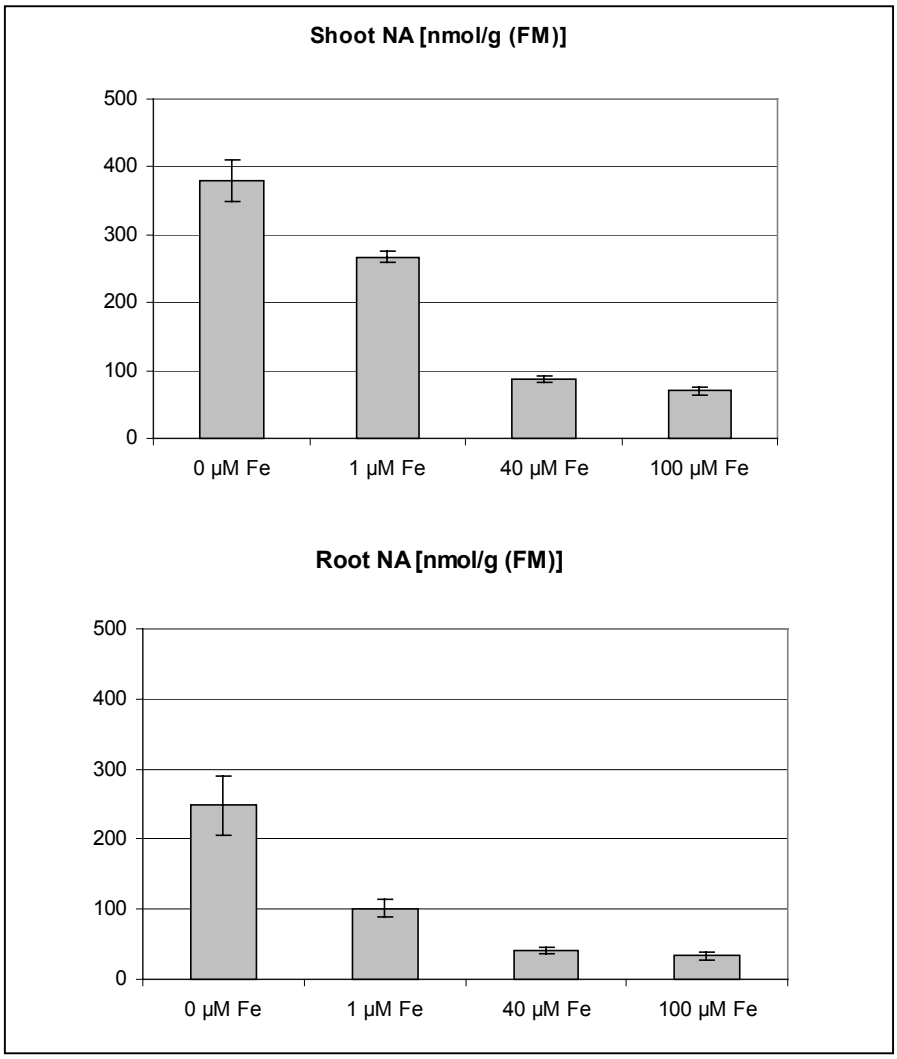

Fig. 3.1. Nicotianamine concentrations (nmol/g FW) in shoots and roots of A. thaliana plants grown in different iron concentration of the medium. *The bars represent the standard error.*

The iron concentrations were maximal in the normal iron supplied plants (Fig. 3.2.). A slight decreasing of the iron in the plant was observed in iron-overloaded plants perhaps due to an inhibitory effect of the high iron concentration in the nutrient solution. The iron in the iron-limited plants was considerably lower. This tendency was clearer in the shoots than in the roots of the plants.

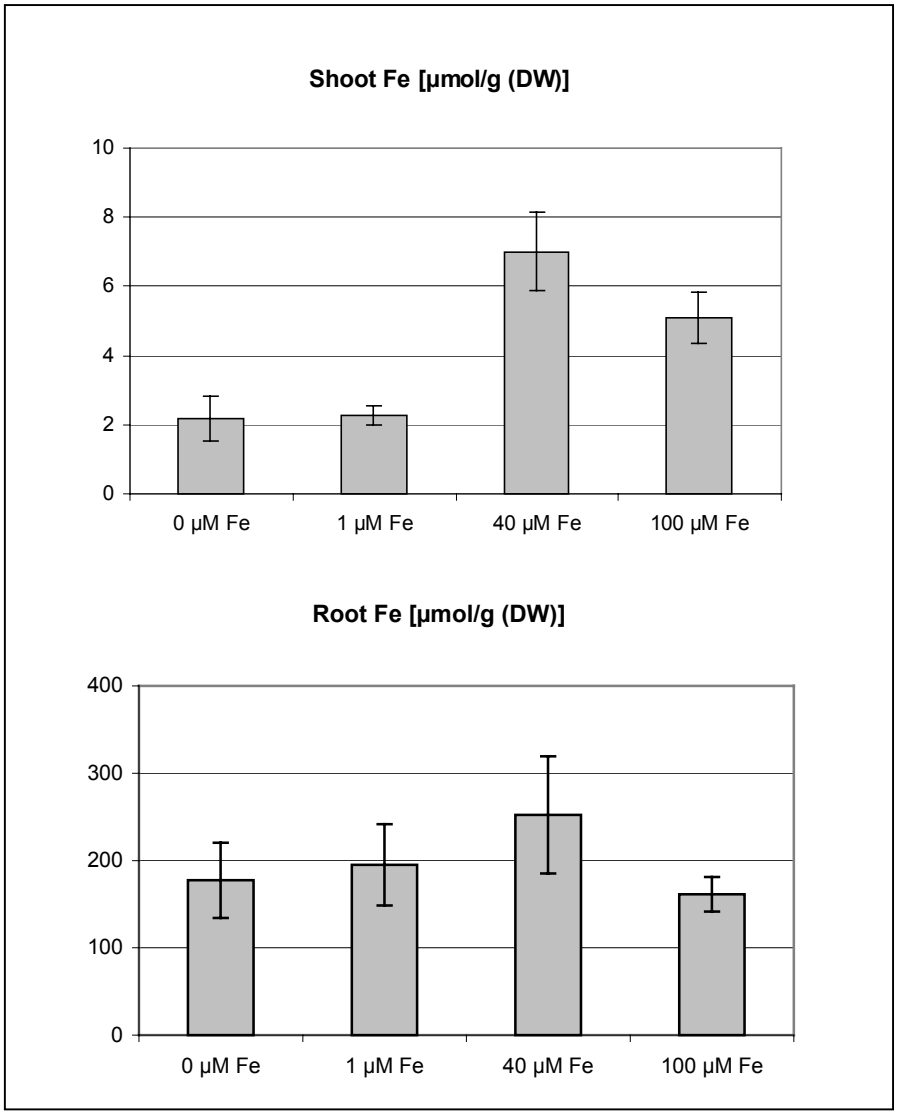

Fig. 3.2. Iron concentrations (µmol/g DW) in shoots and roots of A. thaliana plants grown in different iron concentration of the medium. *The bars represent the standard error.*

The concentrations of zinc and manganese in the plant were in reverse correlation to the iron concentration –more iron was taken up, less Zn and Mn (Fig.3.3.).

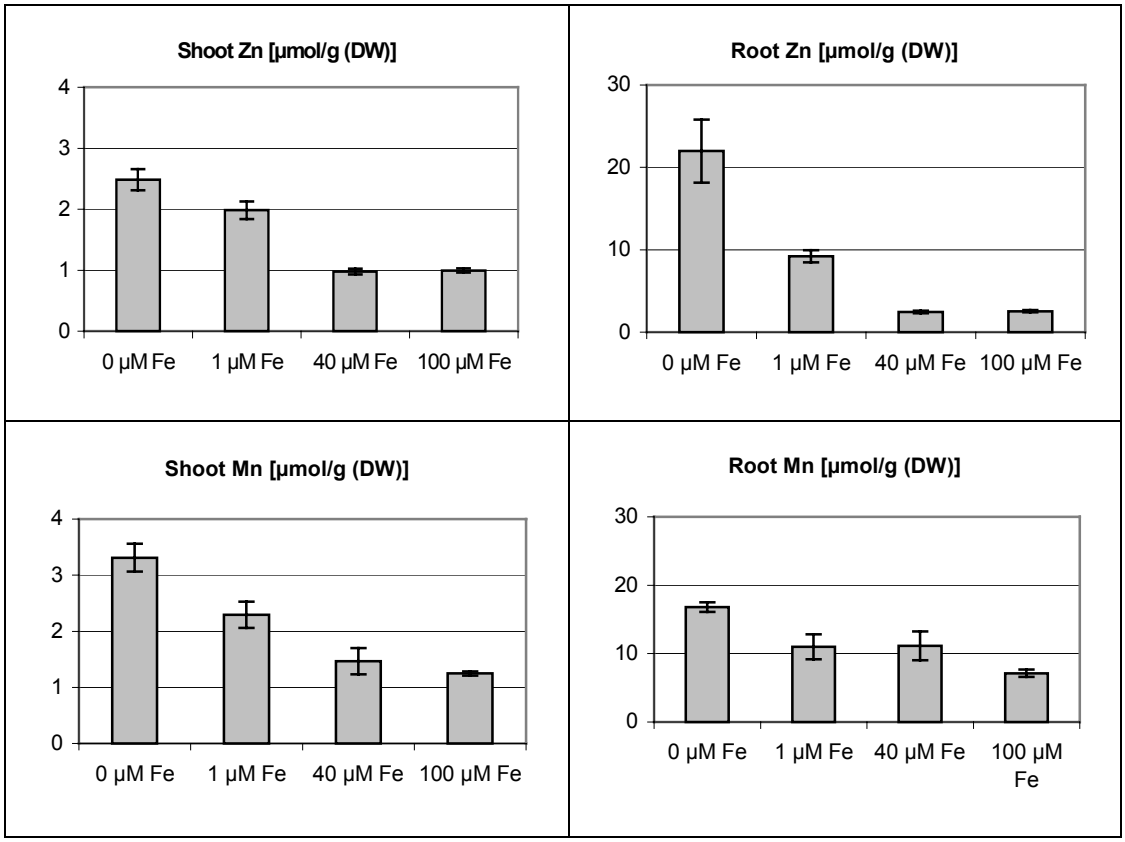

Fig. 3.3. Zinc and manganese concentrations (µmol/g DW) in shoots and roots of A. thaliana plants grown in different iron concentration of the medium. *The bars represent the standard error.*

To check whether the NAS mRNA level also changed in correlation to the iron condition, a preliminary experiment with *A. thaliana* wild type was made. The plants were grown in liquid medium with or without iron, and half of them were sprayed with 5 µM NA solution. The Northern blot results are presented at Fig. 3.4.. A full length NASARA5 cDNA was used as a DNA probe. Since the amounts of the applied RNA were not equal, the mRNA levels were normalized by the intensity of rRNA bands (Fig. 3.5.). A slight increase in the NAS mRNA can be seen in the plant grown without iron. The lowest NAS mRNA level were detected in the normally iron supplied plants, sprayed with NA. In spite of the high stringency hybridization conditions, a cross hybridization of NASARA5 DNA probe with other NAS mRNAs cannot be excluded. However, a precisely designed experiment to confirm these data is in progress.

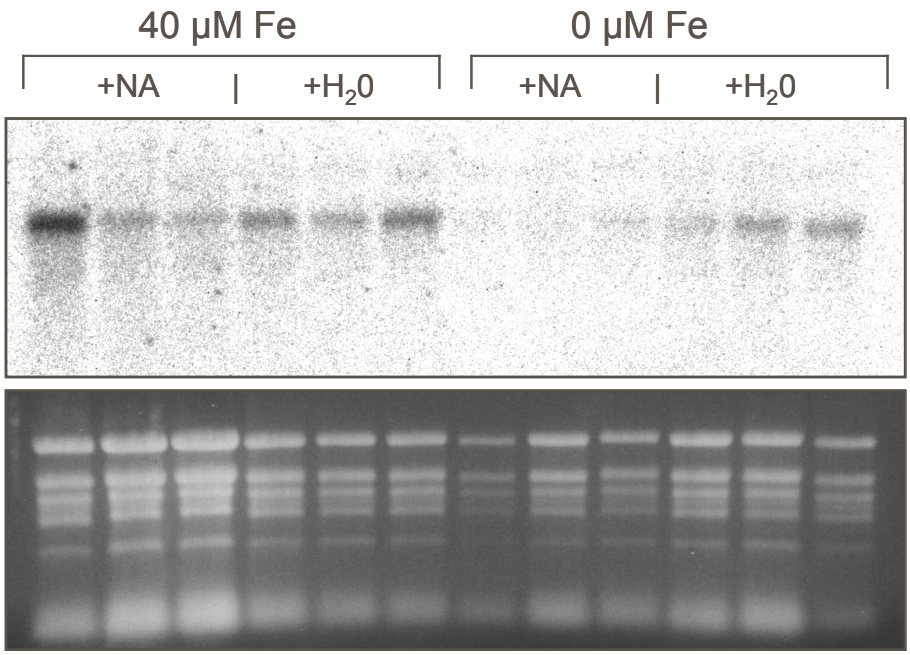

Fig. 3.4. Northern blot analysis of A. thaliana total RNA for NASARA5 transcript detection. *RNA isolated from plant grown in different condition – normal (40 µM FeEDTA) or no iron supply, and sprayed with NA or water (control), as is indicated on the top of the figure. Upper part of the figure – autoradiography of NASARA5, lower part – Eth. Br. stained agarose gel.*

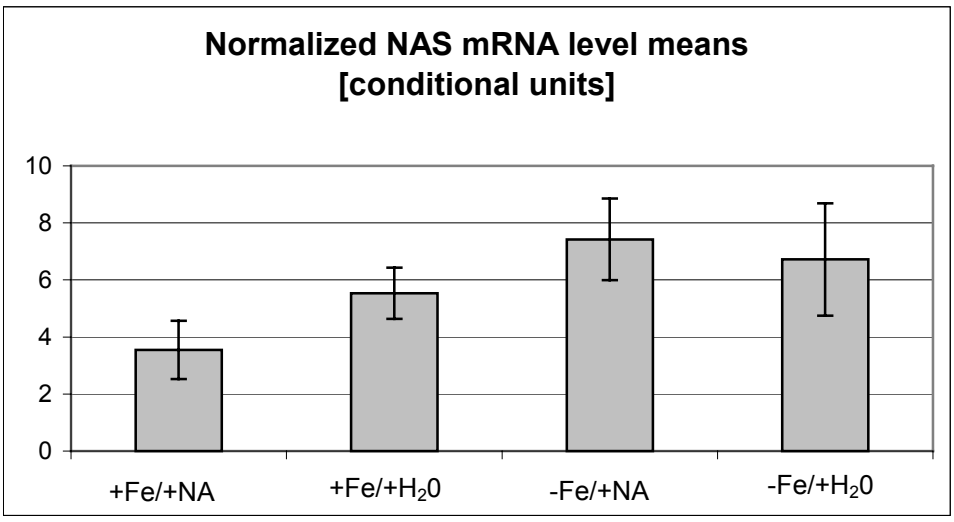

Fig. 3.5. Normalized NAS mRNA level in A. thaliana grown in different condition, mean values. *(+Fe/+NA) – normal iron supply, NA spraying; (+Fe/+H_20) – normal iron supply, H_20 spraying; (-Fe/+NA) – no iron supply, NA spraying; (-Fe/+H_2O) – no iron supply, H_2O spraying; The bars represent the standard error.*

3.1.1. Expression analysis using DNA arrays with *A. thaliana* genes

A DNA array prepared by the EU consortium REGIA (Regulatory Gene Initiative in Arabidopsis) project containing ~1 200 unique *A. thaliana* transcription factor as well as DNA array with ~8 000 unique *A. thaliana* genes (the work is in progress) were used for screening of genes with iron-dependent regulation.

The material for the DNA probe preparation was isolated from *A. thaliana* plants grown submerged in ½ MS medium. Ten day after germination the medium was changed and the plants were separated in 2 groups, one with normal iron supply and another without iron in the medium. The iron deficiency was estimated by parallel monitoring of the Fe (III)-chelate reductase (ferric reductase) activity.

The ferric reductase activity of the iron-limited plants started to increase after the first day of iron limitation. The maximum activity was reached at the 4th day and it dropped after the 5th day (Fig.3.6.). The iron-supplied plants showed constant low levels of the ferric reductase activity.

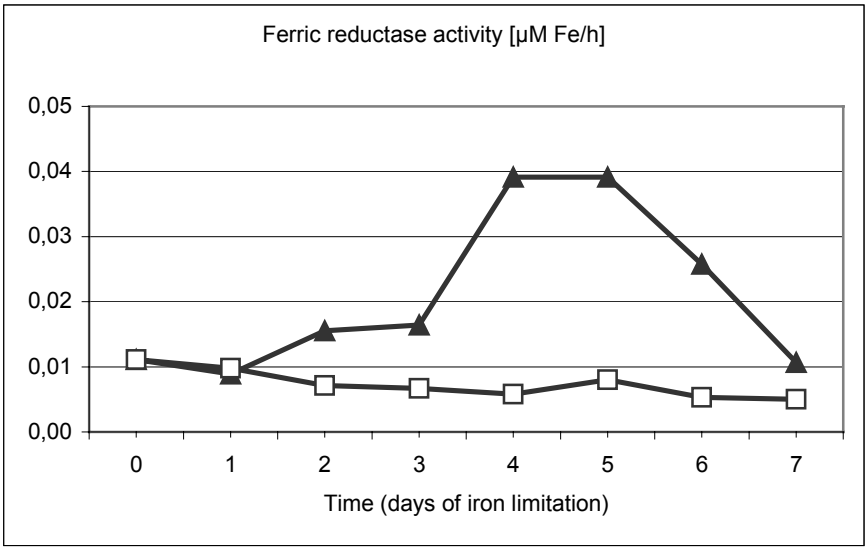

***Fig. 3.6. Ferric reductase activity of A. thaliana**. Normally iron supplied (open squares) and iron limited (black triangles) plants. On X-axis is indicated the time (days) of iron limitation.*

Samples from the day 1 to 4 were used by to prepare DNA probes and to screen the DNA arrays. That work was done in cooperation with A. Czihal (IPK Gatersleben).

Two different transcription factors belonging to the bHLH (**b**ase **H**elix-**L**oop-**H**elix) family were found to be highly induced under iron limitation (Fig. 3.7.). The complete analysis of these genes, including a transgenic plant approach is in progress.

The growth system for *Arabidopsis* using submerged cultures developed for the array experiment seems to be fast and very efficient method to prepare an iron-deficient *Arabidopsis* material for RNA and DNA isolation. It allows precise definition of the growth conditions, real iron free environment and preparation of plant material in less than 2 weeks starting from seeds. The tested plants developed iron-deficiency within 24 h and visible chlorosis of the leaves in less than 60 h of iron limitation. The use of the ferric-reductase activity to assess the iron-deficiency is also an easy and reliable approach.

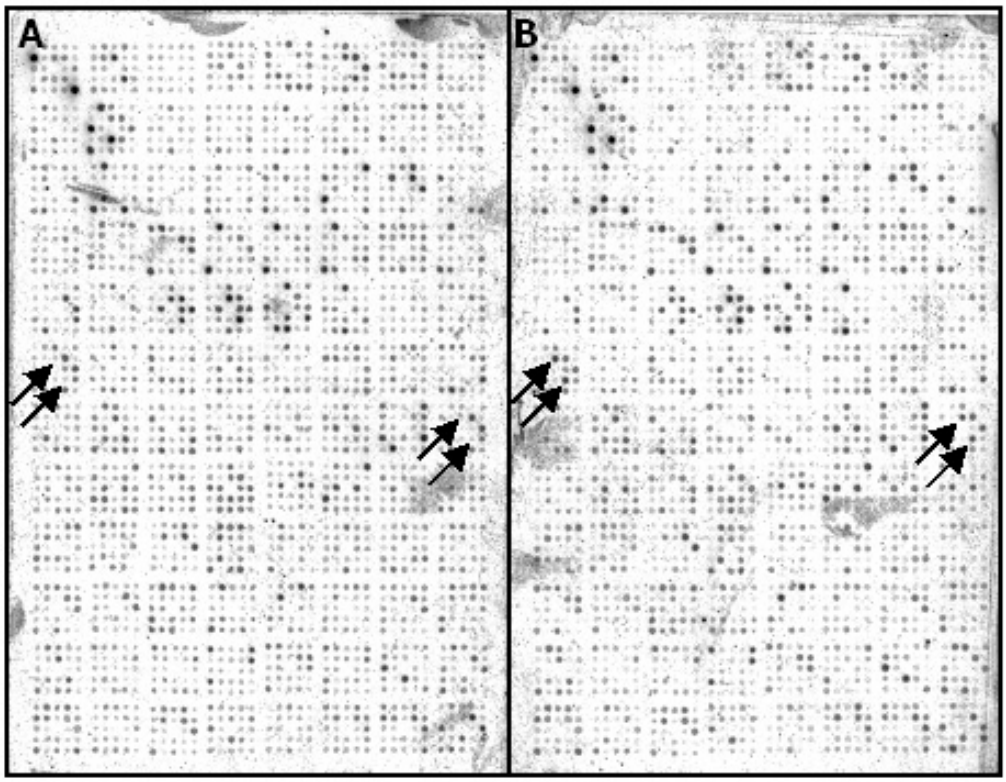

Fig.3.7. Autoradiography of the transcription factor array hybridized with DNA probes from iron sufficient (A) and iron deficient (B) A. thaliana*. Each gene is double spotted on the filter. The spots of the transcription factors that are up regulated under iron deficiency are indicated by arrow pairs.*

3.2. The NASHOR1 gene

To characterize the functions of the *NASHOR1* gene, two different approaches were used. Firstly, the gene was cloned and expressed in *E. coli*. This was done in close co-operation with Dr. G. Koch. Secondly, the gene function was analyzed in transgenic *Arabidopsis*, tobacco and tomato plants.

3.2.1. Expression of the NASHOR1 in *E. coli*

To prove that the newly found barley gene encodes a NAS enzyme, the *NASHOR1* cDNA was expressed in *E. coli*. For this purpose, the complete NAS encoding cDNA was amplified with gene specific and *Bam*H I site containing primers. After *Bam*H I digestion the fragment was cloned in both orientations into the *Bam*H I site of the expression vector pET12a. After induction by IPTG, a protein of approximately 35 kD was detected in the pellet fraction of *E. coli* extracts. This size corresponds well with to expected size of the NASHOR1 protein (Fig. 3.8.).

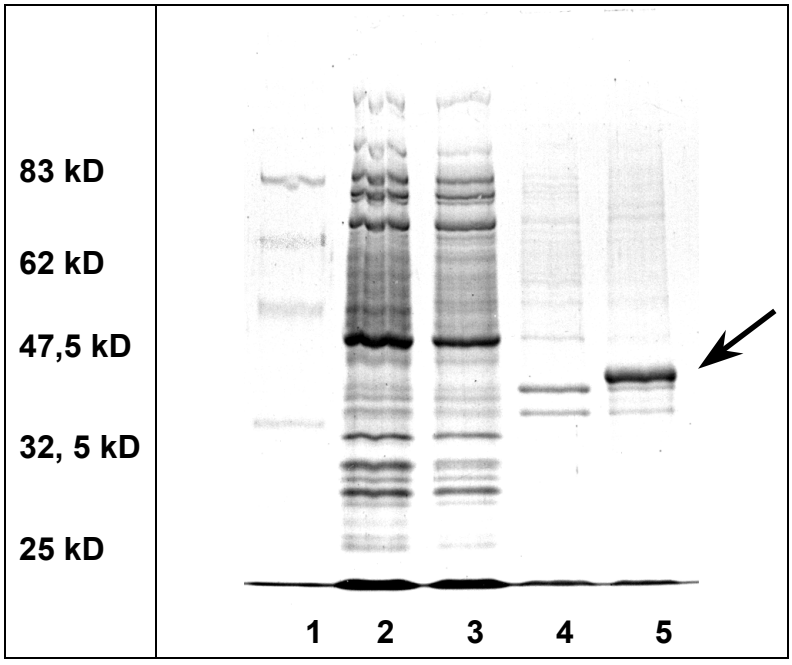

Fig. 3.8. SDS protein gel of E. coli extracts. *Lanes: 1 - protein molecular weight marker; 2 - empty vector (pET12a), supernatant; 3 - pET12a:NASHOR1, supernatant; 4 - empty vector, pellet; 5 - pET12a:NASHOR1, pellet. The arrow indicates a strong expression of a protein, corresponding in size to NASHOR1.*

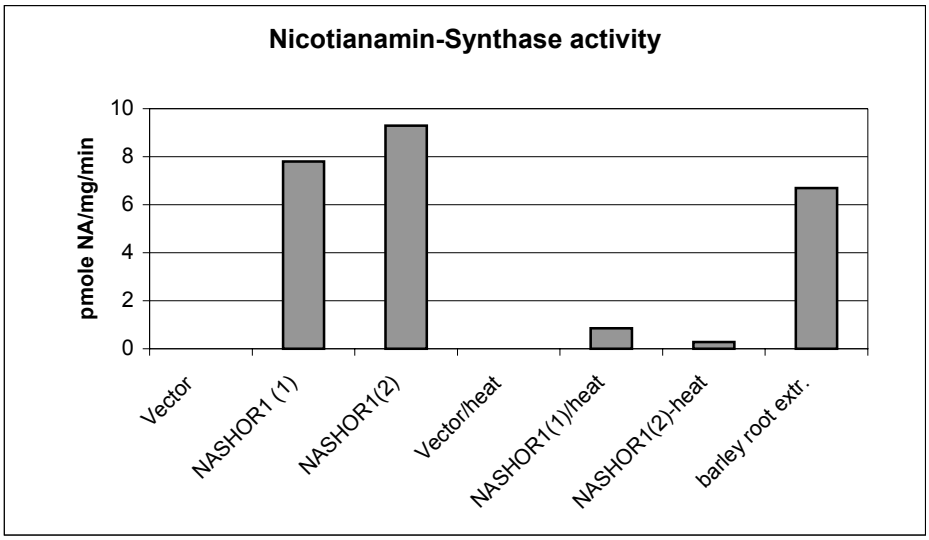

Fig. 3.9. Overexpression of NASHOR1 in E. coli*. The activity is given in pmole NA.mg^{-1}. Extracts of bacteria carrying empty expression vector (indicated as Vector) were used as negative control and iron-limited barley root extract as a positive control. NASHOR1(1) and NASHOR1(2) represents two independent clones carrying the NASHOR1 gene (tested with and without heat denaturation at 100°C for 5 min).*

As a definite proof for the correct identification of the synthesized protein, a NAS assay was performed in the *E. coli* extract. The overexpressing strains exhibited strong *NAS* activity comparable to the activity of iron-limited barley root extracts. (Fig. 3.9.).

Heat treatment resulted in a strong reduction of the enzyme activity and the empty vector control was without NAS activity.

NASHOR1 crystallization

Highly effective NASHOR1 overexpressing clones were submitted to the group of Prof. Schulz (Albert-Ludwigs-Univarsität Freiburg) who will try to solve the crystal structure of NASHOR1. In case of success, NASHOR1 will be the first NAS protein with known crystal structures.

3.2.2. *NASHOR1* expression in barley.

For a further functional analysis and to estimate its putative biotechnological value for the manipulation of iron assimilation, the *NASHOR1* gene as well as its gene promoter was studied in barley and in various transgenic plants.

3.2.2.1. The *NASHOR1* gene promoter

To study the expression of the *NASHOR1* gene and the localization of its gene product, specific DNA probes and polyclonal antibodies were generated and applied for Northern hybridization and Western blotting, respectively.

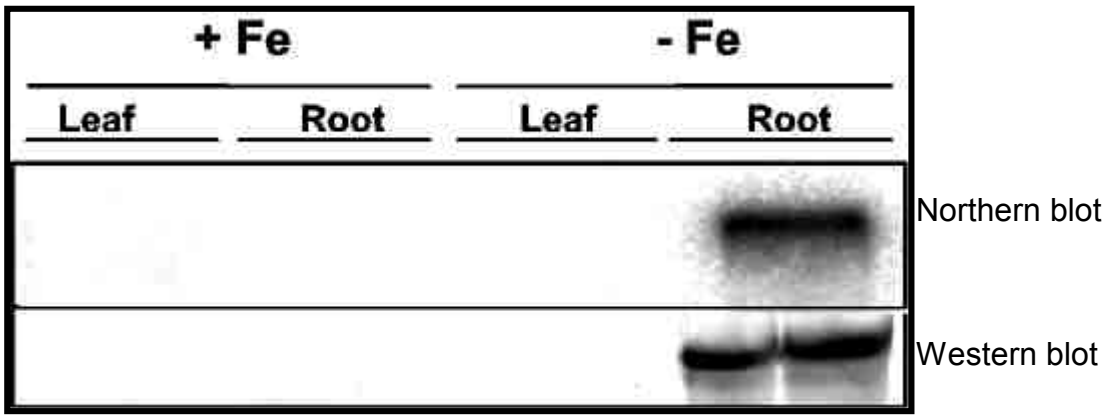

Fig. 3.10. Northern (upper part) and Western (lower part) analysis of barley roots and leaves grown under normal iron supply (+Fe) and iron limitation (-Fe).
A NASHOR1 specific transcript and corresponding gene product can be identified only in barley roots grown under iron limitation.

As shown in Fig. 3.10., both the *NASHOR1* transcript and the corresponding gene product can be detected only in barley roots grown under iron limitation. No expression could be detected in leaves as well as in roots grown under normal iron supply.

Therefore, the *NASHOR1* gene promoter obviously exhibits a rather interesting activity pattern combining root specificity with an induced activity under iron limitation. Such a promoter might be of interest for biotechnological applications. Therefore, the sequence of the gene promoter was studied in more detail.

A barley BAC library was screened with a *NASHOR1* specific DNA oligonucleotide probe detecting 17 hybridizing clones. The BAC DNA from these clones was isolated and checked by PCR for the presence of the *NASHOR1* gene. Only 2 clones (807H23 and 807I23) gave a product of the expected size (1024 bp). Restriction digestion of both clones revealed an identical fragment pattern suggesting that both clones are redundant. Thus, the BAC clone 807H23 was used for the isolation of the gene promoter.

The promoter-containing fragment of the BAC clone was identified by Southern blots using an oligonucleotide probe spanning the ATG start codon of the *NASHOR1* gene (Fig.3.11.).

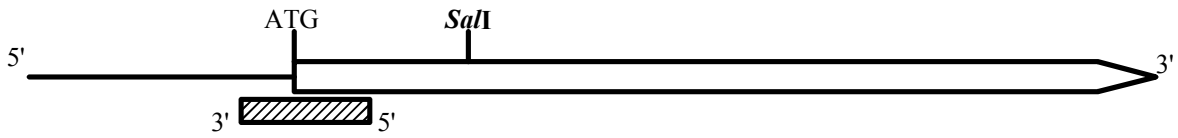

Fig. 3.11. Schematic representation of NASHOR1 cDNA including the 5' untranslated region. *The diagonal pattern box indicates the binding region of the NAH1OP1 DNA oligoprobe; the translation start codon (ATG) and the Sal I recognition site are also indicated.*

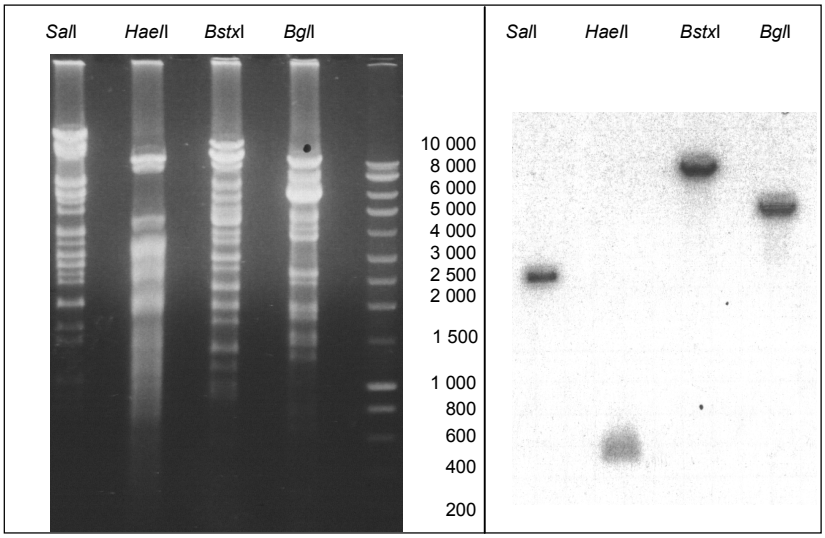

Fig. 3.12. Agarose gel separation of the digested BAC DNA (left) and autoradiography of restriction fragments that contains the 5' upstream region of the NASHOR1 gene (right). *Used endonucleases are indicated at the top of the lanes (at left) and the size of the DNA marker bands are given in bp. The DNA was transferred to a nylon membrane and hybridized with a NASHOR1 DNA oligonucleotide probe specific for the 5' region.*

The detected fragment is approximately 2 500 bp long (Fig. 3.12.). Since a *Sal* I site is found 100 bp downstream of the start codon, the *Sal* I fragment most likely includes approximately 2 kbp of the gene promoter.

The 2,5 kbp band was cloned into the *Sal* I site of the pET12a vector. The construct was used to transform *E. coli,* but the efficiency of the transformation was unusually low. To find an *E. coli* clone that contains the 2,5 kbp fragment, about 500 colonies were isolated and hybridized with the NAH1OP1 oligonucleotide probe as described in Material and methods. Only

2 positive colonies were found, which showed an atypically slow growth rate (Fig. 3.13.).

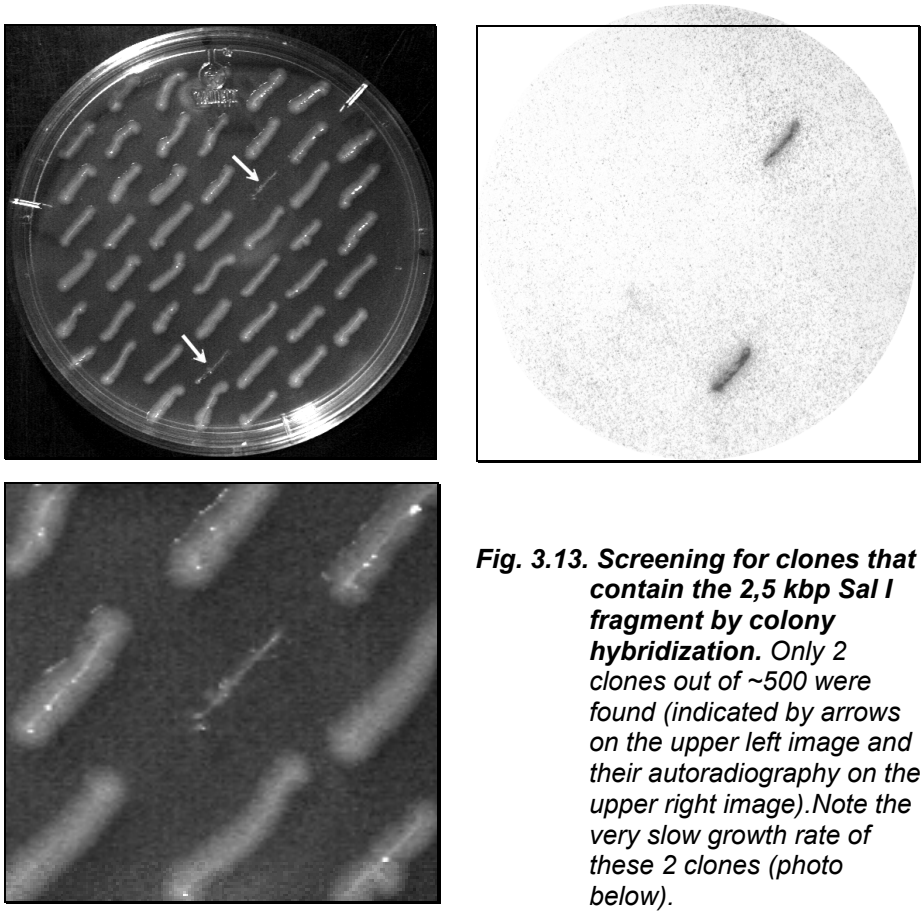

Fig. 3.13. Screening for clones that contain the 2,5 kbp Sal I fragment by colony hybridization. *Only 2 clones out of ~500 were found (indicated by arrows on the upper left image and their autoradiography on the upper right image).Note the very slow growth rate of these 2 clones (photo below).*

One of the two clones was found to contain a duplicated 2,5 kbp *Sal* I fragment and was rejected. The DNA from the other clone was purified and sequenced (Fig. 3.14).

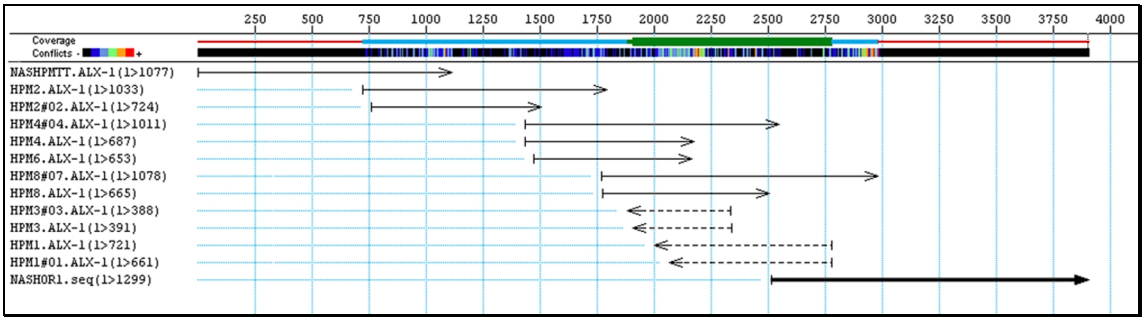

Fig 3.14. The sequencing strategy for the 2,5 kbp Sal I fragment. *A full length NASHOR1 cDNA (black arrow) is also included in the aligment.*

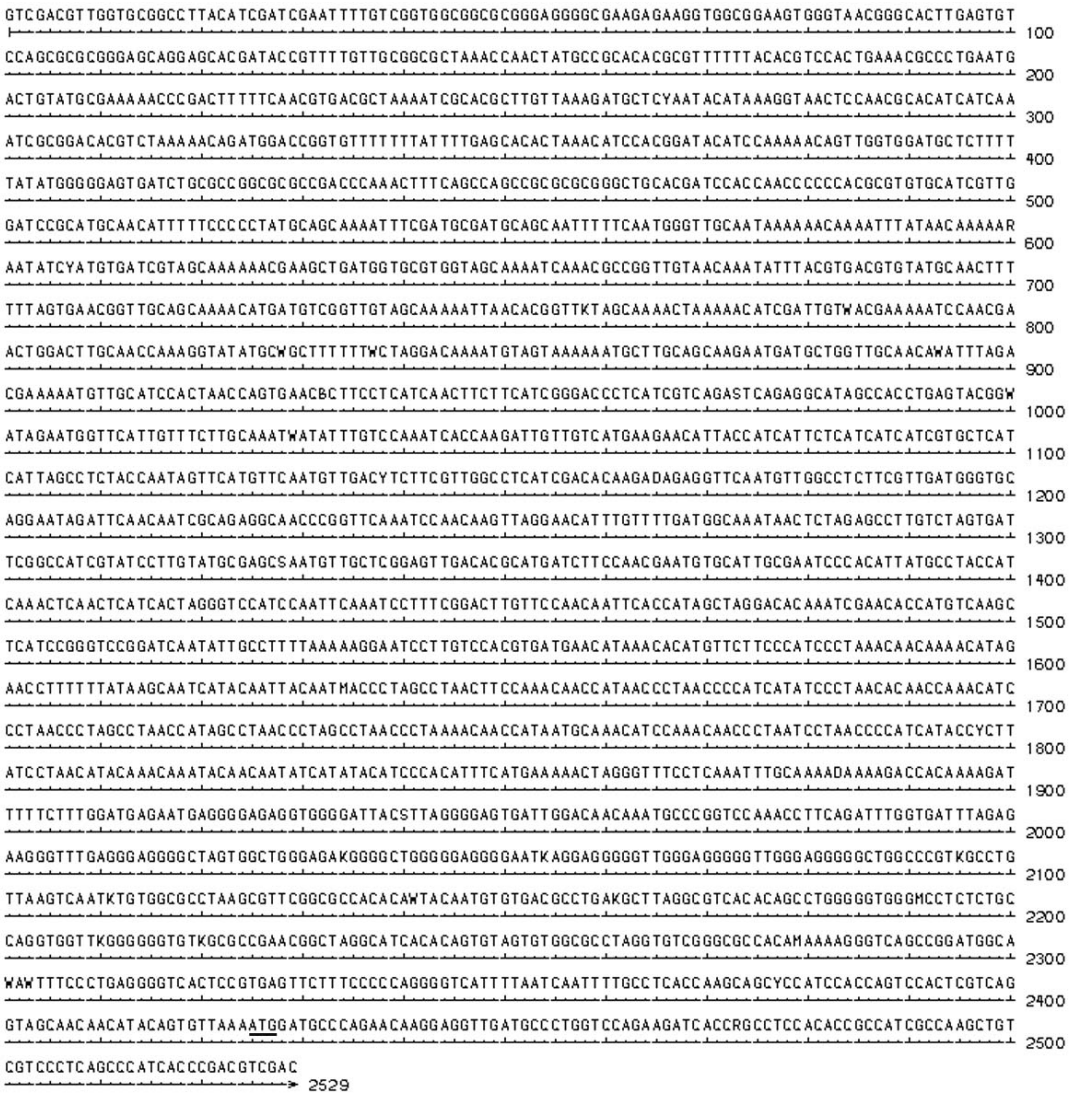

Fig. 3.15. The sequence of the 2,5 kbp Sal I fragment containing the upstream region of NASHOR1 gene. *The start codon of NASHOR1 is underlined.*

The sequencing revealed 2529 bp in total, of which 2424 bp were located in front of the start codon of *NASHOR1* gene (Fig. 3.15.). Remarkably, the GC content of the first 500 bp was relatively high (56%).

A BLAST search in the NCBI data base retrieved 2 sequence motifs in the upstream region of the *NASHOR1* gene also present in the 5`flanking regions of 3 other barley genes: the *IDS3*-, a dehydrin- and the *CF2/CF5* resistance gene (Fig. 3.16.).

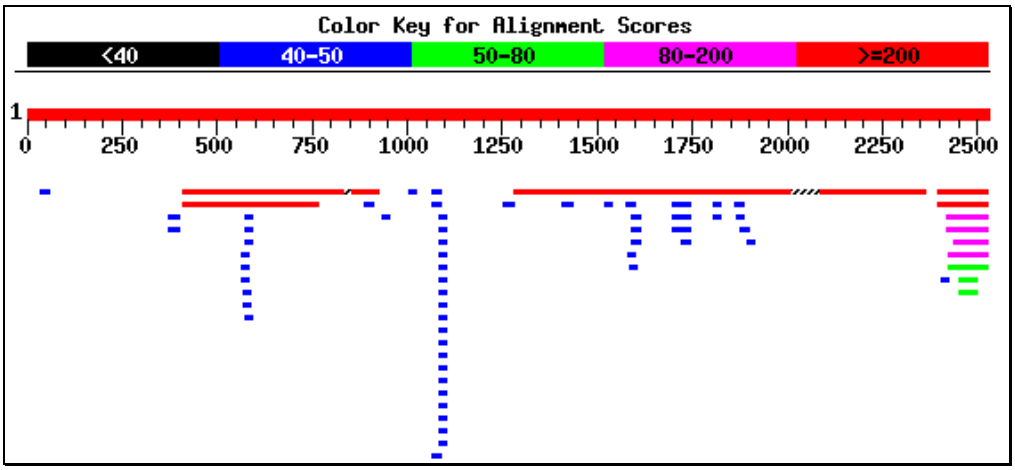

Fig. 3.16. NCBI BLAST search results with the 2,5 Kbp SalI fragment of the NASHOR1 gene upstream region*. 2 sequence motifs are also present in the 5`flanking regions of 3 other barley genes: ids3-, dehydrin- and cf2/cf5 resistance gene.*

These two motifs were called A and B (Fig. 3.17). Motif A is approx. 1 kbp long and shares 95% similarity to the 5' flanking region of the dehydrin gene *DHN12* (Accession No AF155129).

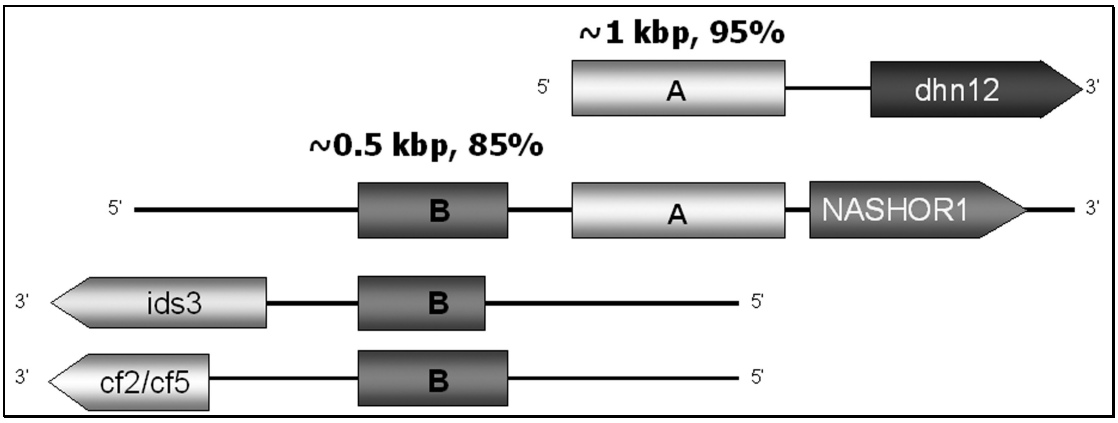

Fig. 3.17. Schematic representation of the motifs A and B found in 5' flanking regions of NASHOR1-, DHN12-, IDS3- and CF2/CF5 genes*.*

Motif B is approx. 0,5 kbp in length and shares 85% similarity with the 5' flanking regions of an iron deficiency induced *IDS3* gene (AB024007), which encodes an enzyme belonging to the same pathway of the phytosiderophore biosynthesis as *NAS*. Motif B is also found in the promoter of the *CF2/CF5* disease resistance gene (AF166121). Both motif B sequences were found in reversed direction than in the promoter of the *NASHOR1* gene.

3.2.2.2. Immunohistochemical localization of NASHOR1

The intracellular localization of NASHOR1 was performed in cooperation with the electron-microscopic group of M. Melzer (IPK). The work is still in progress but the preliminary results show that NASHOR1 is localized in the cytoplasm, not associated with any cellular structures (Fig. 3.18.).

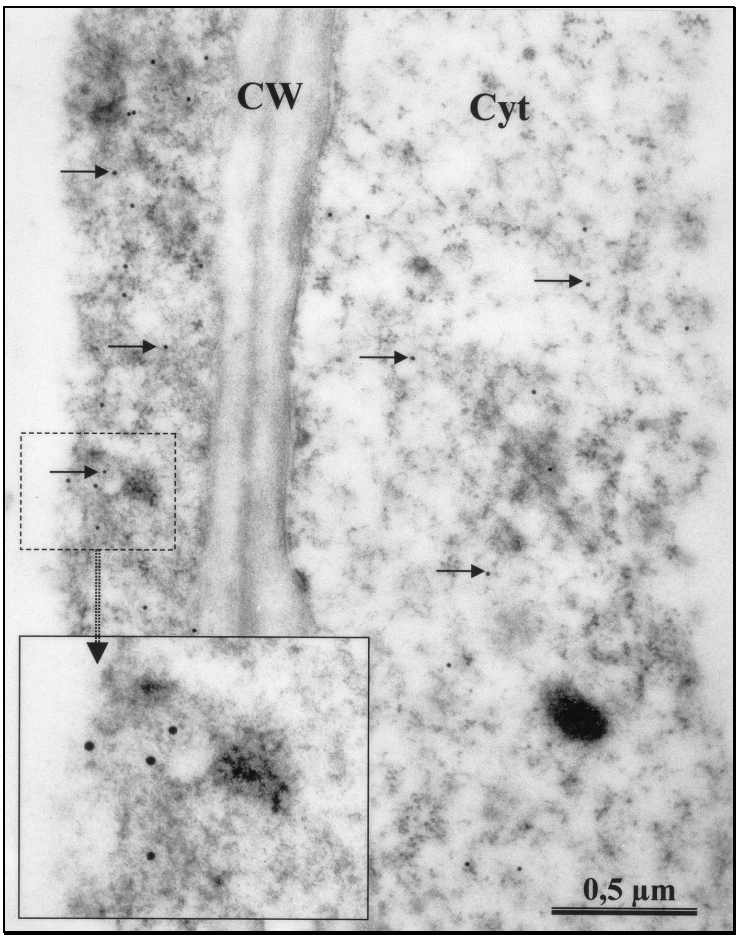

Fig. 3.18. Immmunogold localization of NASHOR1 with 10 nm protein A-gold (arrows) in root tissue of barley grown under iron deficiency. *Phloem parenchyma cells; CW –cell wall; Cyt – cytoplasm*

3.2.2.3. Chromosomal localization of barley NAS genes

To improve the iron efficiency of plants by increased NA contents, different approaches could be used. Thus, NA could be directly applied to the plants as it has been described previously (Scholz et al., 1985). Alternatively, NAS genes can be overexpressed in transgenic plants (Douchkov et al., 2002). Still another approach would be the transfer of chromosomes or chromosomal regions from iron efficient plants like barley to iron inefficient plants by

chromosomal engineering. This approach requires the chromosomal localization of *NAS*-genes.

Besides our 2 barley *NAS* genes (*NASHOR1* and *NASHOR2*) another 5 *NAS* genes were identified in the barley genome by another group (Higuchi *et al.*, 1999). These genes were designated as *hvNAS1*, *hvNAS2*, *hvNAS3*, *hvNAS4*, *hvNAS5-1, hvNAS5-2* and *hvNAS7*. An eighth gene reported from the same group and designated as hvNAS6 seems to be homologous to *NASHOR1* gene. By this way, nine different *NAS* genes have been identified in the barley genome.

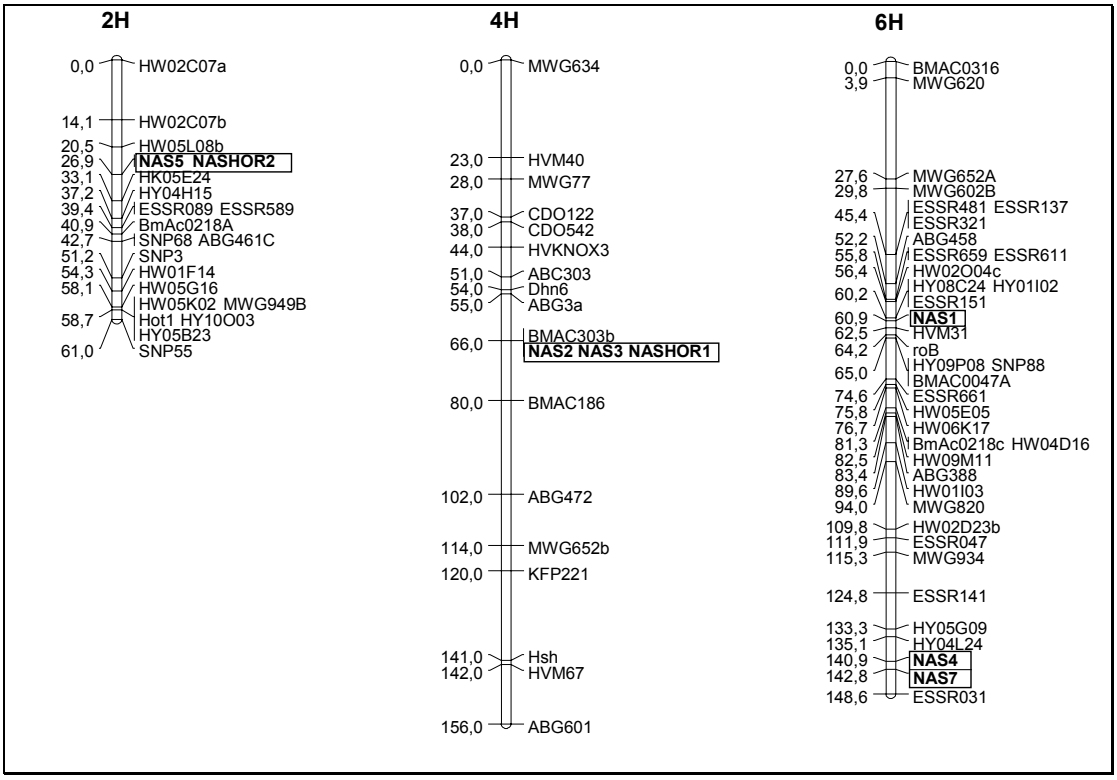

Fig. 3.19. Chromosomal localization of all known barley NAS genes.

The chromosomal localization by using specific for each barley NAS-gene oligonucleotide probes was performed in cooperation with Dr. D. Perovic, IPK, and was described in Perovic et al., 2002. *NAS* genes are located on chromosome 4HL (*NASHOR1*, *hvNAS2* and *hvNAS3*), on chromosome 6H (*NASHOR2*, *hvNAS1* and *hvNAS4*) and on chromosome 2HS (*hvNAS5*) (Fig. 3.19.).

3.2.3. *NASHOR1* gene transformation in plants

Transgenic plants

To study the putative physiological role of *NAS* genes, the barley *NASHOR1* and the *Arabidopsis NASARA1* and *NASARA5* were used to generate transgenic plants. For this purpose, the coding sequence of the *NAS* gene was cloned into the binary vector pBinAR19 and expressed under the control of the CaMV 35S promoter.

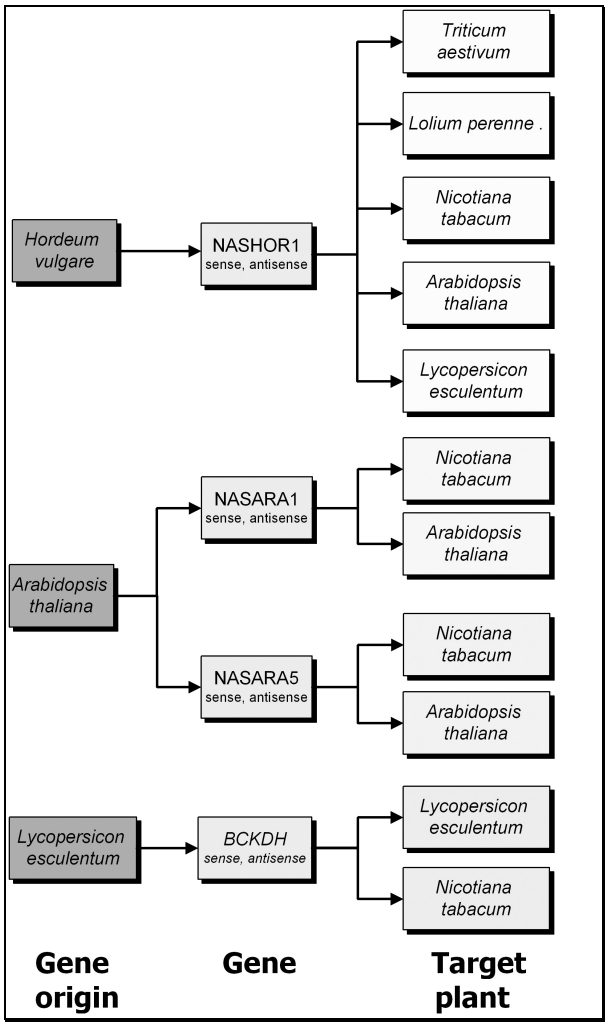

Fig. 3.20. Diagram of the plant transformations

These constructs were used for the transformation of the dicots - tobacco, tomato and *Arabidopsis*. For direct gene transfer into monocots the cDNA was

also cloned into the vector pAHC17. In this vector, the expression is controlled by the maize ubiquitin (*Ubi-1*) promoter. This construct was used for the transformation of ryegrass and wheat (in cooperation with the group of F. Altpeter, IPK).

An additional experiment was made with tomato's BCKDH gene.

In total, more than 500 independent transgenic lines based on 4 different genes in sense and antisense orientation and 5 different plant species have been generated and analyzed (Fig 3.20.).

NASHOR1 gene transformation in plants

Preliminary results indicate that at least two ryegrass lines stay green even under iron limitation when compared with wild type control plants (G. Hensel, personal communication). Furthermore, transgenic wheat lines with slightly increased iron content in seeds have been identified (G. Hensel, personal communication).

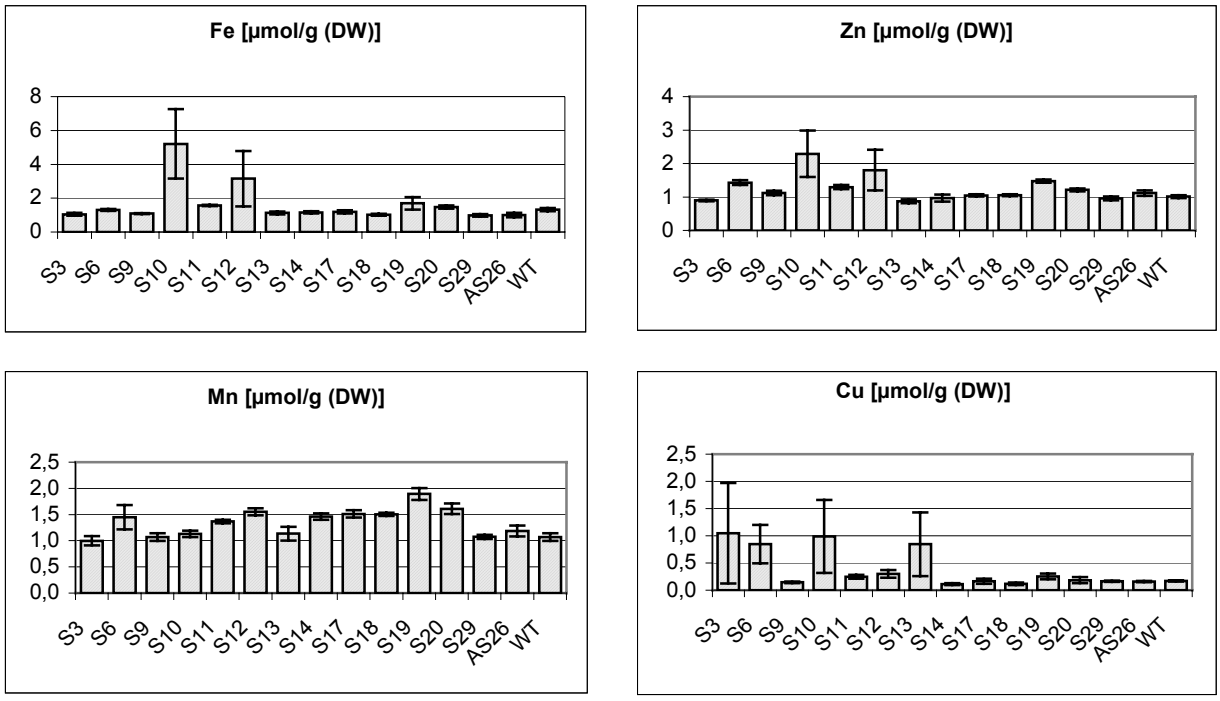

Fig. 3.21. Metal ion concentrations in leaves of 35S:NASHOR1 tobacco plants grown in medium with 0,5 µM Fe. *Each column represents the mean value of 3 plants of independent lines. S stands for sense plants, AS for antisense plants, WT for wild type control plants. The bars represent the standard error.*

The analysis of both moncot species is still in progress and the mentioned results need further confirmation.

No clear effect of the *NASHOR1* ectopic expression could be detected in the transgenic plants. Only in two tobacco lines (S10 and S12, Fig. 3.21.) an increased iron and zinc concentrations were observed.
To determine whether the described differences in metal ion concentrations ware a result of the *NASHOR1* expression, the NA concentration was measured and they were found to be unchanged (Fig. 3.22.).

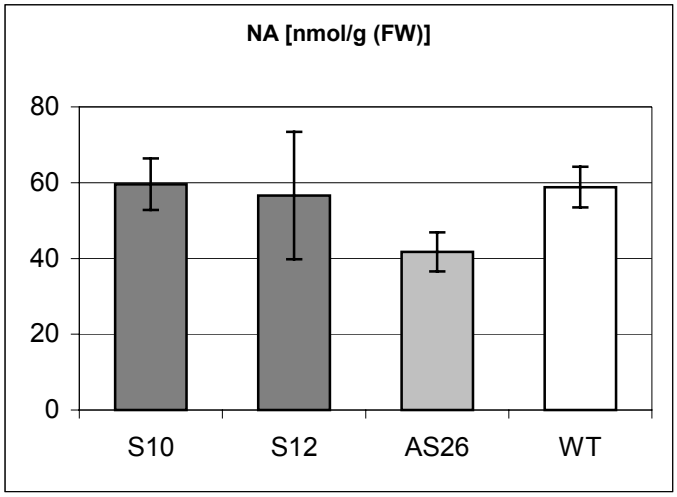

Fig. 3.22. NA concentrations of the35S:NASHOR1 sense lines S10 and S12. *The line AS26 (35S:NASHOR1, antisense) and wild type plants from the same experiment are included as controls. Each column represents the mean value of 3 to 5 plants of independent lines. The bars represent the standard error.*

Therefore, the detected differences in the iron, zinc and copper concentrations seem to be unrelated to NA concentrations. The results might reflect unknown physiological differences in individual transgenic lines.
Similar results were obtained with transgenic tomato plants transformed with the 35S:*NASHOR1* construct. No clear differences in iron concentrations between experiment and control could be observed (Fig. 3.23.).
Although the analysis is still ongoing, no phenotypic changes could be detected yet in transgenic *Arabidopsis* plants transformed with the 35S:*NASHOR1* construct.

The developing of the anti-NASHOR1 antibodies allowed checking the expression of NASHOR1 at protein level. Regardless of the high specificity and sensitivity of the antibodies, it wasn't possible to detect NASHOR1 in the PCR positive transgenic tobacco.

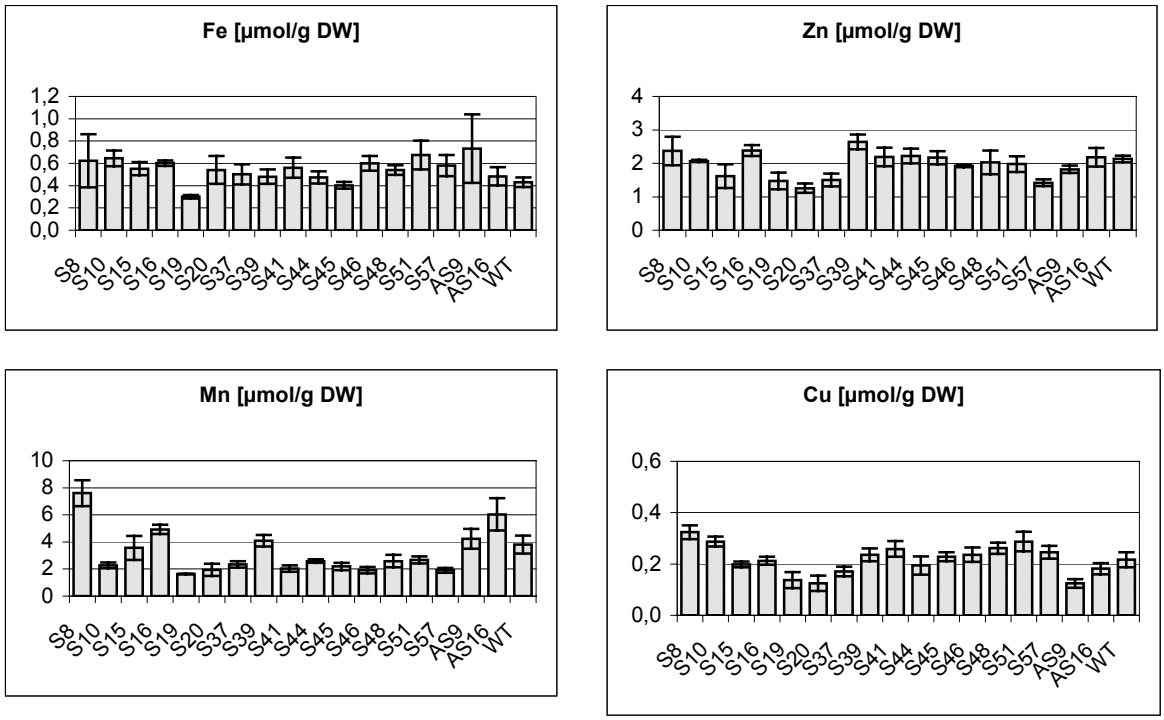

Fig. 3.23. Metal ion concentrations in leaves of 35S:NASHOR1 tomato plants. *Each column represents the mean value for 3 to 6 plants of an independent line. S stands for sense, AS for antisense and WT for wild type plants. The bars represent the standard error.*

3.3. Transgenic plants transformed with the 35S:NASARA1 construct

In addition to the described *NAS* genes from barley, a database search revealed similarity to several anonymous open reading frames (ORF) in the *Arabidopsis* genome. One ORF is found on the BAC clone T12M24 (AC003114) derived from chromosome 1 and another ORF is found on the P1 clone MDA7 (AB011476) derived from chromosome 5. These *NAS*-like sequences were designated as *NASARA1* and *NASARA5*, respectively.
To test whether the *Arabidopsis* genes *NASARA1* and *NASARA5* code for functionally active NAS, sense and antisense constructs of these genes were transferred to tobacco and *Arabidopsis.*

3.3.1. Cloning of the *NASARA1* gene

According to the genome databases, the *NASARA1* gene of Arabidopsis does not possess any intron. Therefore, it could be directly PCR amplified from genomic DNA using proofreading polymerase and primers with integrated *Bam*H I restriction sites. The PCR product was cloned into the *Bam*H I sites of the pCRII vector and resequenced. For plant transformation the *Bam*H I fragment of the pCRII:*NASARA1* construct was recloned in both orientations into the binary cloning vector pBinAR19 under the control of CaMV 35S promoter. Sense and antisense constructs were used for *Agrobacterium*-mediated transformation of tobacco and *Arabidopsis*.

3.3.2. *NASARA1* transgenic plants

In total 40 independent sense and 40 independent antisense lines of transgenic tobacco were generated with the 35S:*NASARA1* construct. These lines were transferred to liquid Hoagland medium. The presence of the *NASARA1* gene and the corresponding transcript was demonstrated by PCR and Northern analysis, respectively. The NA-concentration of the obtained T_0 transgenic plants was measured (Fig.3.24.).

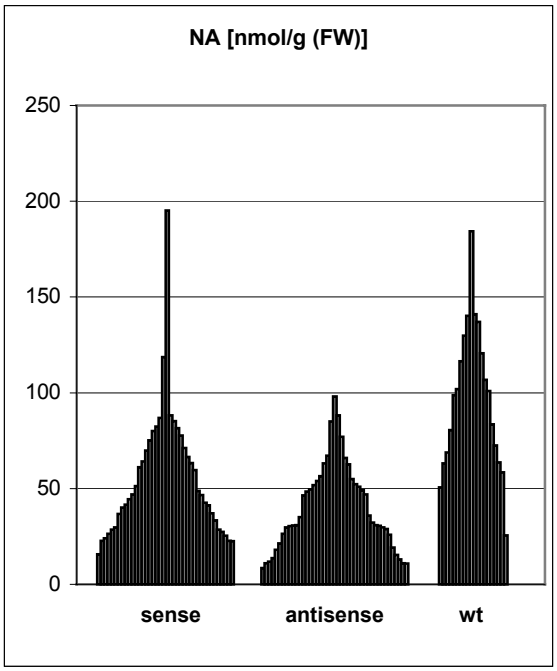

Fig. 3.24. NA concentration (nmol/g (FW)) of young leaves of T_0 35S:NASARA1 sense and antisense plants compared to wild type tobacco. *Each column represents an independent transgenic line.*

In spite of the great variation of the NA concentrations in young leaves even in wild type, the data demonstrate slightly reduced NA concentrations in antisense and in sense plants. The plants were transferred to soil and grown to obtain seeds. Seeds of six sense lines with highest and four antisense lines with lowest NA concentrations were selected for further experiments.

3.3.3. Physiological experiments with selected T_1 lines

The ectopic expression of sense and antisense *NAS* gene sequences should result in changes in the NA levels of transgenic plants. Therefore plants were grown in liquid Hoagland medium containing 0,5 µM FeEDTA (iron limitation) and samples from roots, young and old leaves were collected for the determination of NA and Fe concentrations.

NA concentration

In young leaves, NA levels are relatively constant and higher than in old leaves (Fig. 3.25.), which is in agreement with previous observations (Stephan *et al.,* 1990).

The results demonstrate no significant differences in NA concentrations between transgenic plants and wild type controls. No obvious phenotypic change could be detected.

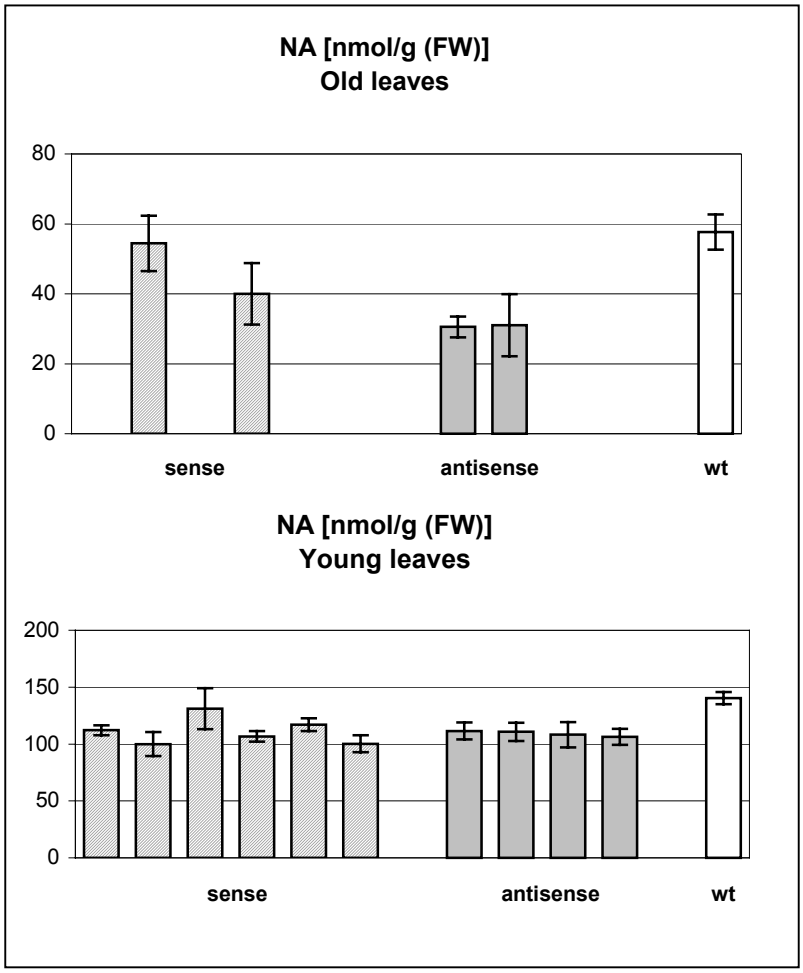

Fig. 3.25. Nicotianamine concentrations (nmol/g FW) in young and old leaves of T_1 35S:NASARA1 tobacco plants. *Hatched bars -antisense, gray bars -sense, empty bars -wild type. The NA concentration of transgenic lines does not differ significantly from wild type. Each column represents the mean value of 3 to 4 plants of an independent line. The bars represent the standard error.*

Iron concentration

As expected from the unchanged NA concentrations no obvious differences in iron concentrations could be detected in the transgenic plants in comparison to wild type (Fig. 3.26.).

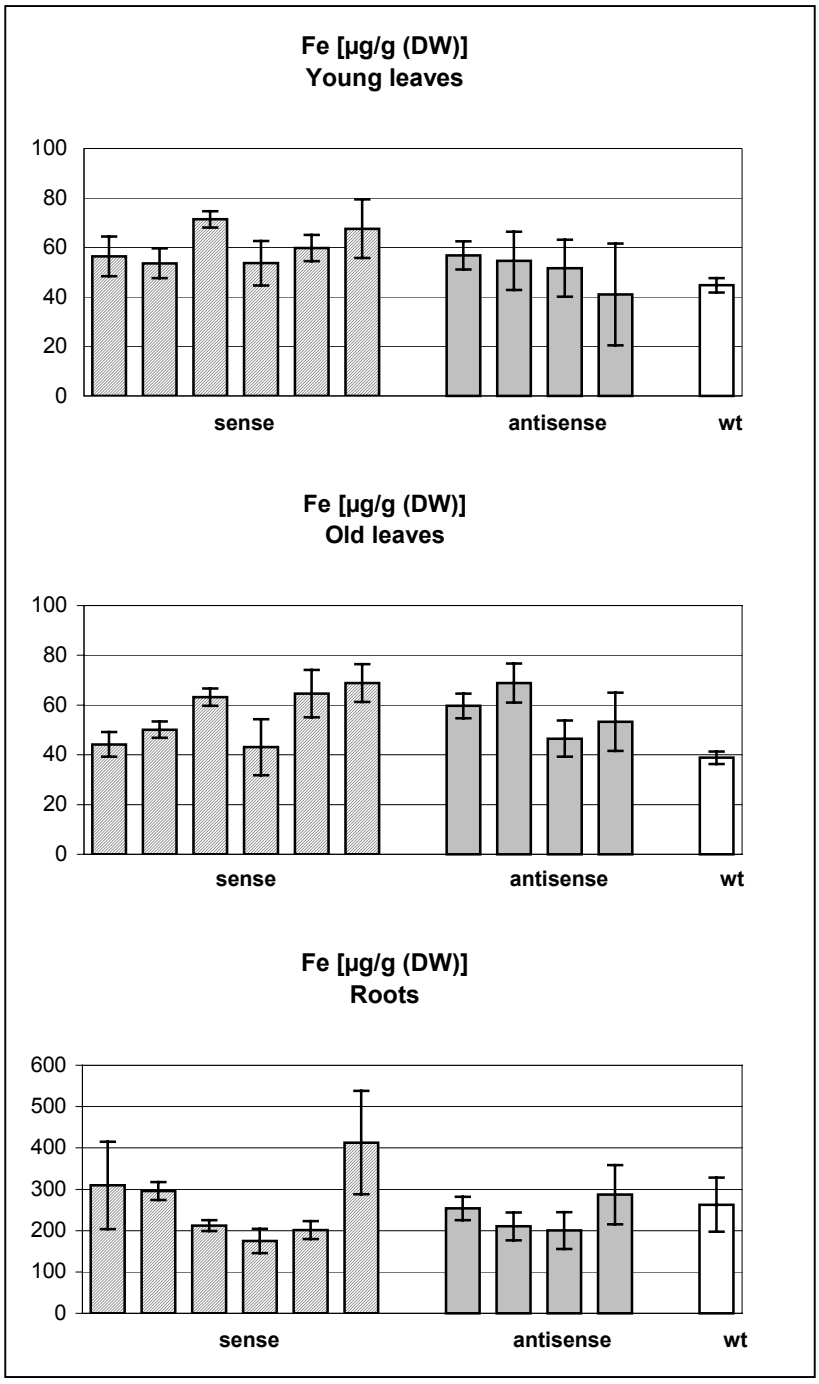

Fig. 3.26. Iron concentration in different parts of T_1 35S:NASARA1 tobacco plants. *Hatched columns -antisense, gray columns -sense, empty column -wild type. Each column represents the average value of 2 to 8 plants of an independent line. The bars represent the standard error for the corresponding data set.*

Ferric reductase activity

The ferric reductase is a major component in the iron uptake system in dicots. Increased activity of this enzyme is an indicator for iron starvation whereas plants grown under normal iron supply show low ferric reductase activity. Therefore, the ferric reductase activity was measured and found to vary in a

wide range. Some of the transgenic plants show significantly increased ferric reductase activity. However, the effect was not significant in other lines (Fig. 3.27.).

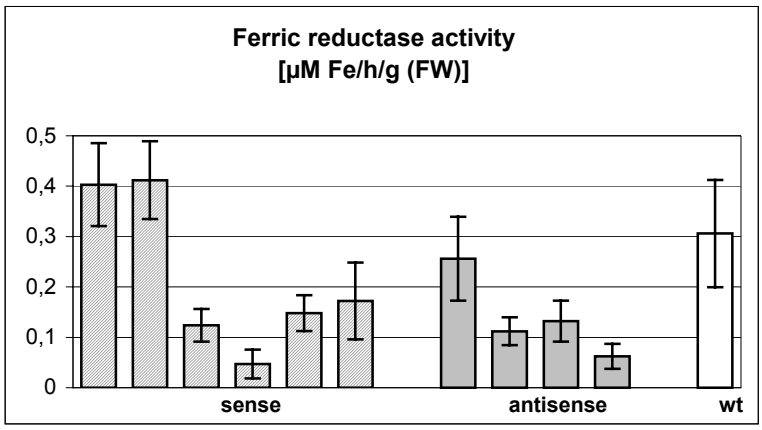

Fig. 3.27. Ferric reductase activity (µM Fe/h/g (FW)) of 35S:NASARA1 T_1 tobacco plants grown under iron limitation. *Hatched columns -antisense, gray columns -sense, empty columns -wild type. Each column represents the average value of 6 to 10 plants of an independent line. The bars represent the standard error for the corresponding data set.*

3.4. Transgenic plants transformed with the 35S:NASARA5 construct

3.4.1. Cloning of the *NASARA5* gene

The *NASARA5* gene was isolated and cloned into the binary vector pBinAR19 as described in the previous chapter for the *NASARA1* gene. The pBinAR19:*NASARA5* sense and antisense constructs were used to transform tobacco and *Arabidopsis* plants.

3.4.2. *NASARA5* transgenic plants

In total about 40 independent sense and 55 independent antisense lines of transgenic tobacco as well as 40 independent sense and 65 independent antisense lines of transgenic *Arabidopsis* were generated. The tobacco plants were transferred to liquid Hoagland medium and the NA-concentration of the obtained T_0 transgenic plants was measured (Fig.3.28.).

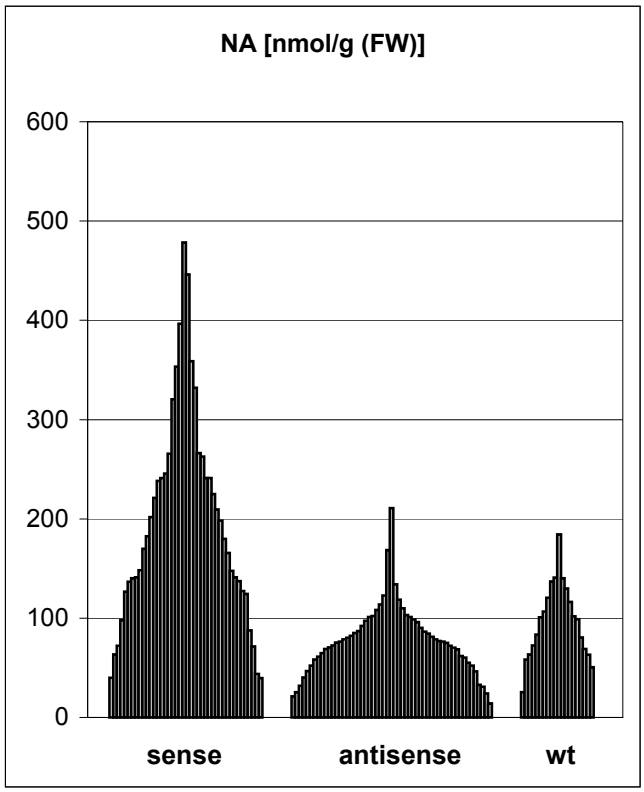

Fig. 3.28. Nicotianamine concentration (nmol/g (FW)) in leaves of T_0 35S:NASARA5 sense and antisense plants compared to wild type tobacco. *Each column represents an independent transgenic line.*

As shown for the *NASARA1* plants, the NA concentrations varied in a wide range between the lines. However, in this experiments -for the first time- NA

concentrations of more than 400 nmol/g FM could be detected. Such a high concentration of NA in the sense line was taken as a first indication for a successful overexpression of the *NASARA5* gene.

To confirm this further, three sense lines with the highest NA concentrations and three antisense lines were selected for further experiments.

3.4.3. Physiological experiments with selected 35S:*NASARA5* lines

The three selected lines (next generation) were grown in Hoagland medium supplemented with three different concentrations of iron. Samples from the first well-developed leaves were collected and analyzed for NA and iron concentrations. The results of this experiment confirm the high concentration of NA in the sense lines (Fig. 3.29.).

The differences in the iron concentration between the sense, antisense lines and wild type were not as obvious as for of NA (Fig. 3.30.). However, the correlation between NA and iron concentrations was higher in the plants grown in 1,0 µM FeEDTA in the medium and therefore this concentration was chosen for the next experiments.

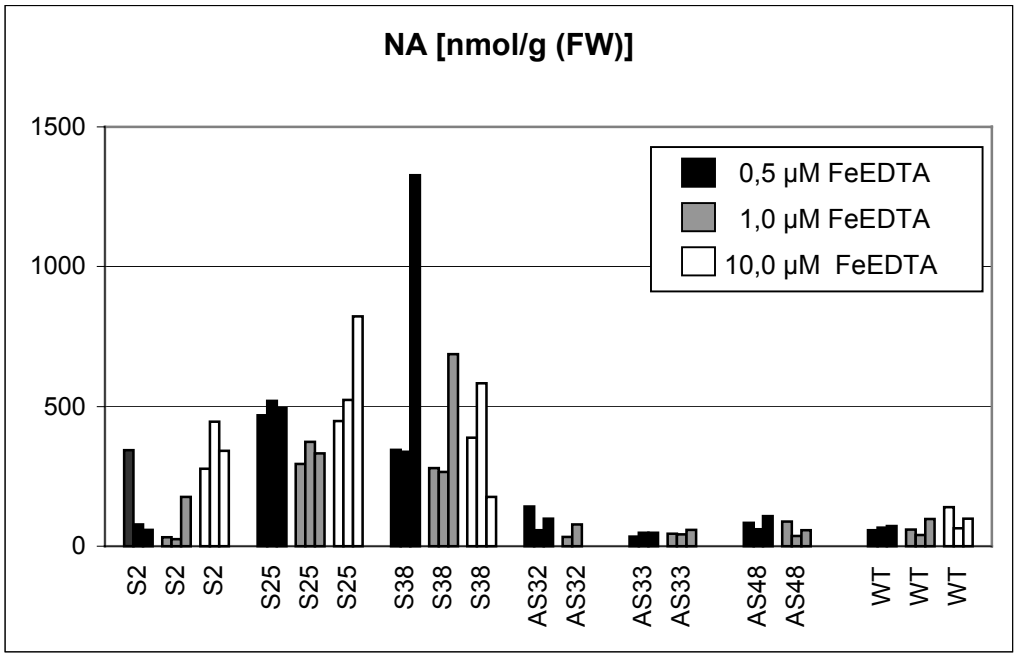

Fig. 3.29. Nicotianamine concentrations (nmol/g FW) in sense and antisense 35S:NASARA5 T_1 tobacco lines with 3 different iron concentrations in the nutrition medium. *Each column represents a single plant value grouped by transgenic line. Black columns - 0,5 µM FeEDTA, gray columns -1 µM FeEDTA, white columns - 10 µM FeEDTA. S stands for sense, AS for antisense, WT for wild type.*

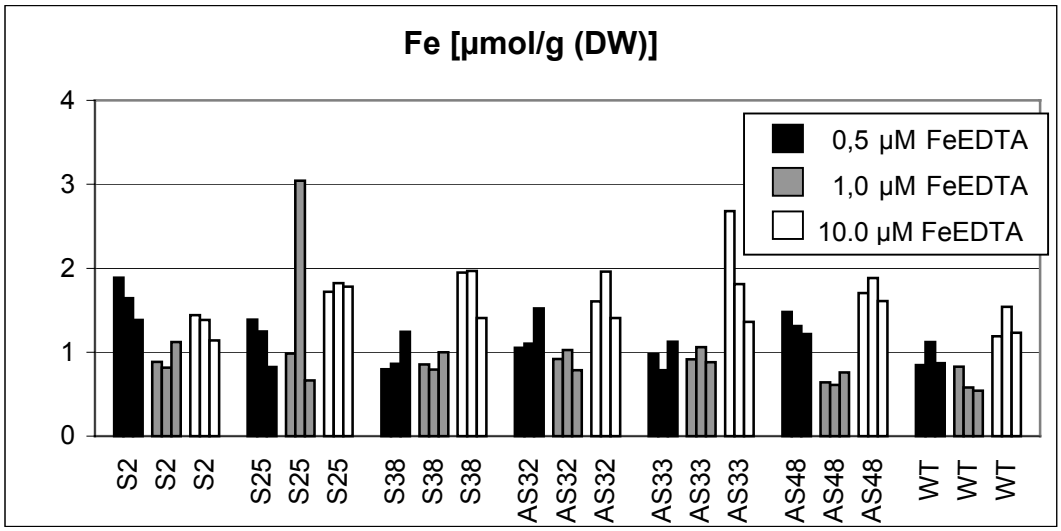

Fig. 3.30. Iron concentrations in sense and antisense 35S:NASARA5 T_1 tobacco lines in 3 different iron concentrations of the nutrition medium. *Each column represents an independent single plant value. In the line numbers S stands for sense, AS for antisense, WT for wild type. Black columns – plants grown under 0,5 µM FeEDTA, white – under 1 µM FeEDTA, gray – under 10 µM FeEDTA.*

The ferric reductase activity of these plants (Fig. 3.31.) was significantly increased under iron limitation compared to normal iron supply. However, the variation in the activity of sense and antisense plants was approximately in the same range.

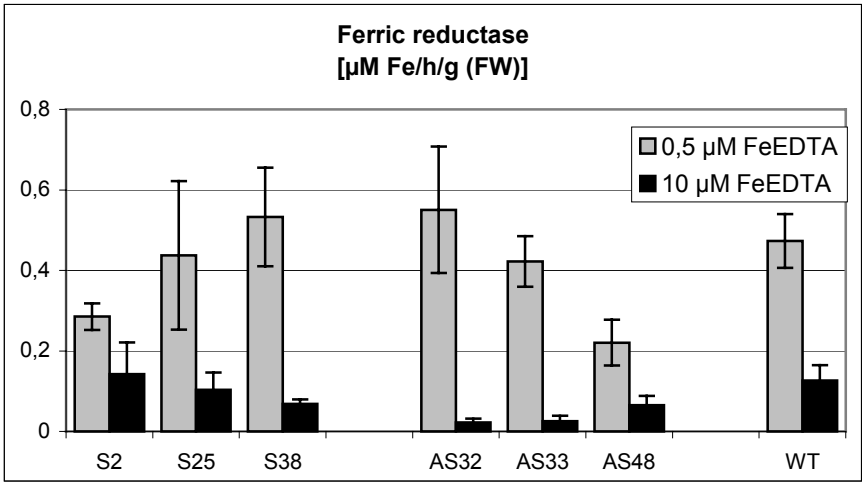

Fig. 3.31. Ferric reductase activity (µM Fe/h/g (FW)) of T_1 35S:NASARA1 tobacco plants. *Each column represents the average value of 3 plants of an independent line. The bars represent the standard error for the corresponding data set.*

A second experiment with the same generation of 35S:*NASARA5* sense plants was performed. The high NA concentrations in the transgenic plants could confirm the data obtained in the previous experiment (Fig. 3.32.).

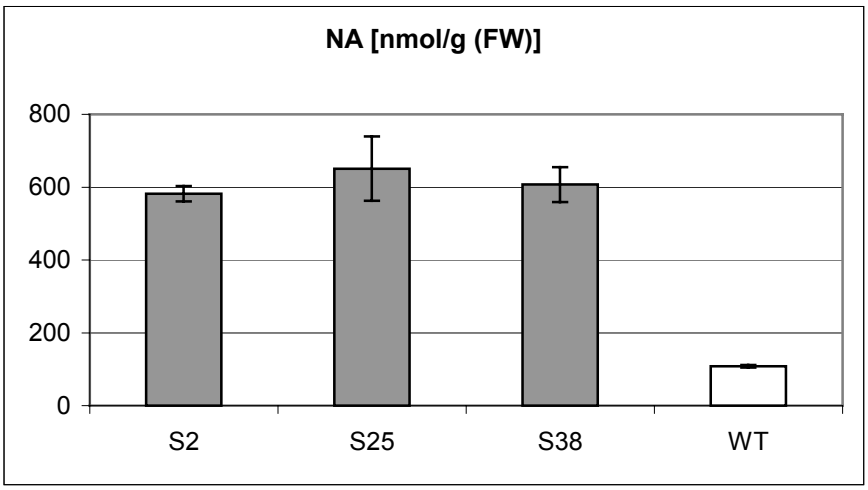

Fig. 3.32. Average NA concentrations in 35S:NASARA5 T_1 tobacco plants. *Each column represents the mean value for the line. The bars represent the standard error for the corresponding data set.*

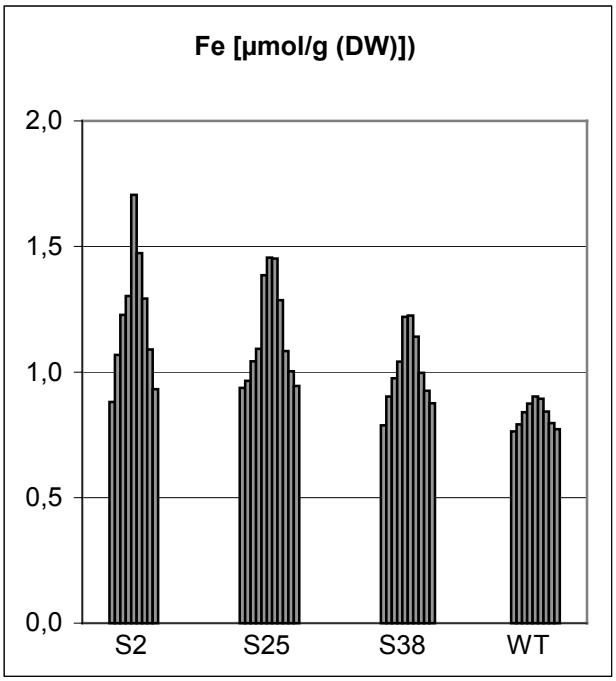

Fig. 3.33. Fe concentration (µmol/g (FW)) of young leaves of T_0 35S:NASARA5 plants compared to wild type tobacco. *Each column represents a single plant value.*

For the first time in this work, a clear increase in the iron concentration of the transgenic plants compared to the controls was observed (Fig. 3.33.). The

statistical analysis (Kruskal-Wallis one-way analysis of variance of ranks) validated the 95% level of significance of the differences between Fe concentration of lines S2 and S25 compared to wild type. Line S38 failed on this test but the mean Fe concentration of the line is still higher than wild type values as it can be seen on Fig. 3.33..

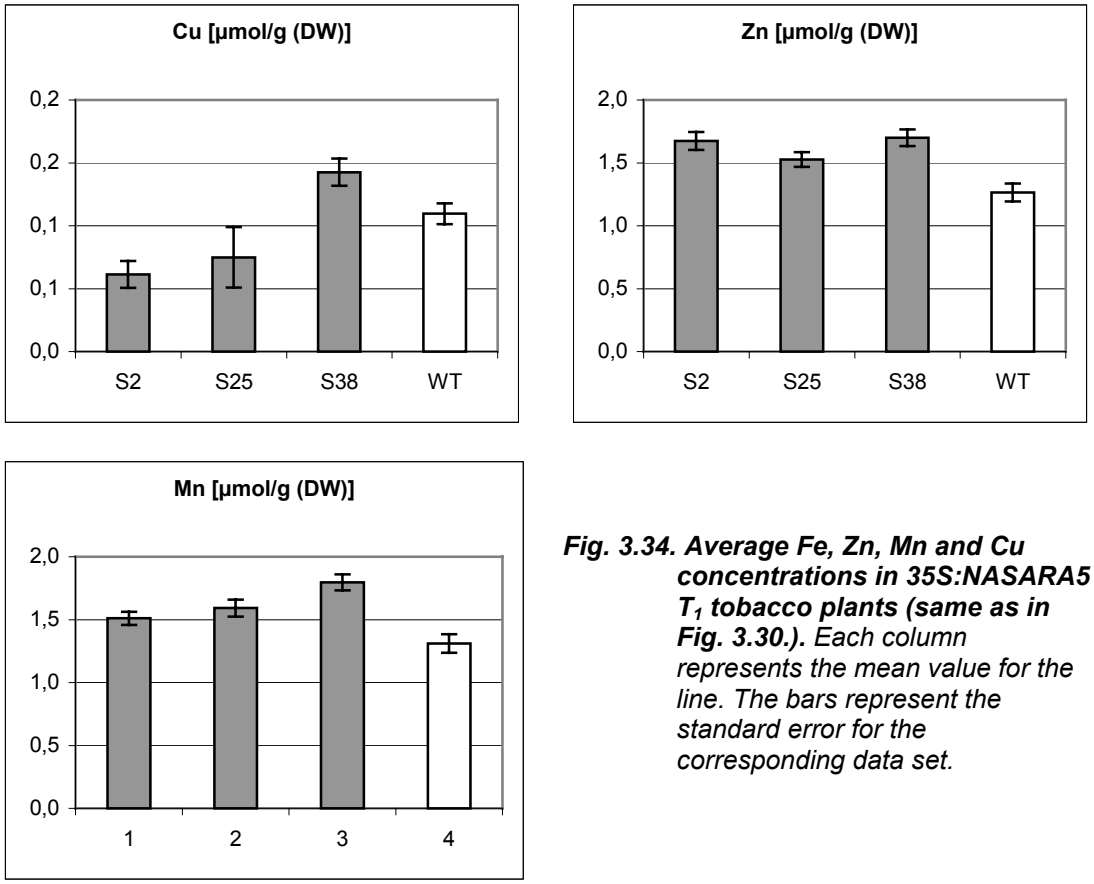

Fig. 3.34. Average Fe, Zn, Mn and Cu concentrations in 35S:NASARA5 T_1 tobacco plants (same as in Fig. 3.30.). *Each column represents the mean value for the line. The bars represent the standard error for the corresponding data set.*

The potentially improved metal uptake of the transgenic plants was confirmed also by the zinc and manganese concentrations (Fig. 3.34.).

Such an improved metal uptake should result in better growth of the plants under iron limiting conditions. This could be demonstrated by observing the phenotype. In iron deficient condition, the transgenic plants were visibly greener than the controls (Fig. 3.35.).

Fig. 3.35. Photo of the plants from the lines S2, S25 and S38 compared to the wild type. *The transgenic plants show a lower level of chlorosis than the wild type plants.*

To confirm the observed greener phenotype of the transgenic plants compared to the wild type the chlorophyll content of the youngest leaves of these plants was measured (Fig. 3.36.).

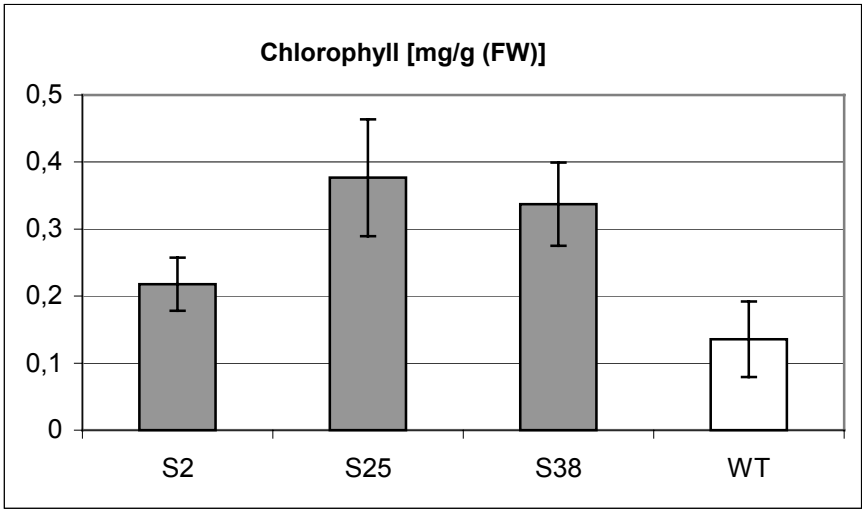

Fig. 3.36. Average chlorophyll concentrations in the youngest leaves of 35S:NASARA5 T_1 tobacco. *Each column represents the mean value for the line. The bars represent the standard deviation for the corresponding data set.*

The results showed statistically significant (95% level in Kruskal-Wallis one-way analysis of variance of ranks) increased chlorophyll content of lines S25 and S38 but not for line S2.

Similar results were observed in 35S:*NASARA5* transgenic *A. thaliana*. In one of the segregating transgenic lines, phenotype differences corresponding to the presence or the absence of the *NASARA5* insert were observed. Under iron limitation, the plants with *NASRARA5* were greener, whereas the segregated wild type plants were chlorotic (Fig. 3.37.).

***Fig. 3.37. Segregation of 35S:NASARA5* A. thaliana.** *The revertant to wild type (left) is more chlorotic than the transgenic plant (right) from the same line.*

To demonstrate even more conclusively that the high NA levels in the transgenic plants are the result of the ectopic expression of the *NASARA5* gene, segregating offspring of heterozygous T1 plants were analyzed in parallel for the presence of the transcript, the NA concentration and the iron concentration. As shown in Fig. 3.38., the presence of the transcript and the level of NA concentration correspond well, however, the correlation is not as clear for the iron concentration.

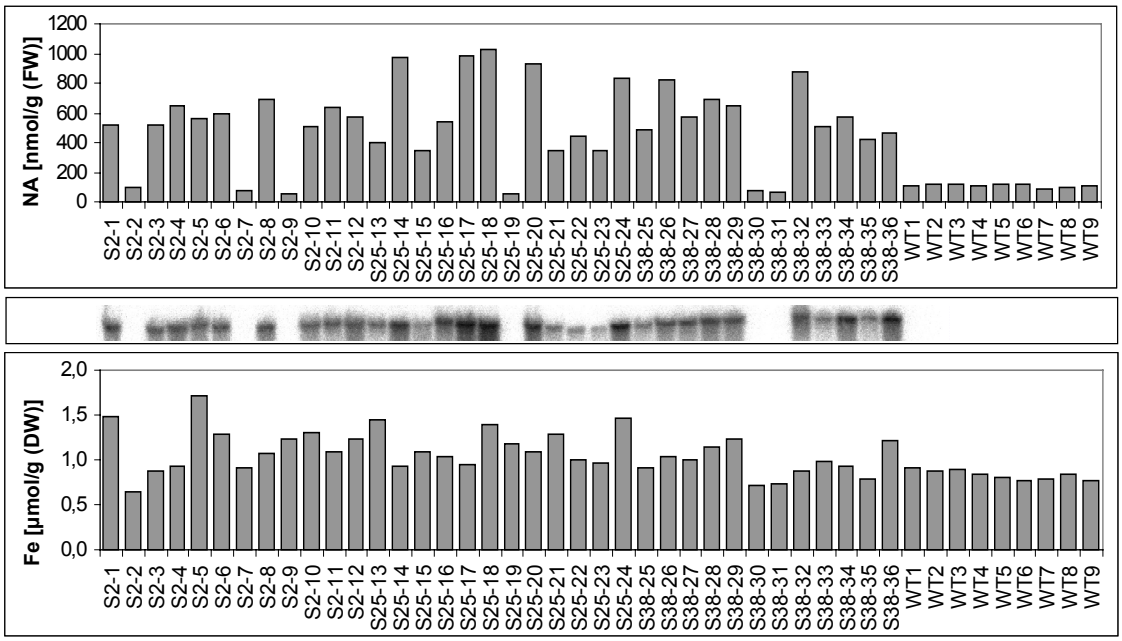

Fig. 3.38. Nicotianamine concentration (upper), Northern (middle) and iron concentration (lower) analyses of 35S:NASARA5 T1 transgenic plants.

Taken together, it is concluded that the ectopic expression of the *NASARA5* of *Arabidopsis* in tobacco results in a significant increase in the concentration of NA and - as a consequence of this - also in the concentration of iron.
These results confirmed the potential role of NAS in the manipulation of the iron homeostasis of the plants.

3.4.4. Enhanced heavy metal tolerance of *NASARA5* tobacco

At the end of the project it was decided to test the *NASARA5* tobacco and the segregated wild type plants for tolerance to high concentration of heavy metals. The transgenic lines S25-20 and S38-32 were compared to the segregated lines S25-19 and S38-31. The S2 lines failed due to technical reasons. The plants were grown in ½ MS agar plates with different concentrations of Fe (10, 100 and 500 µM as FeEDTA), Cd (0, 10, 100 µM as $CdSO_4$) and Ni (0, 50 and 500 µM as $NiCl_2$). The highest concentrations of Fe and Ni were lethal for all plants, but none of the chosen concentrations of cadmium had the same effect. However, the transgenic plants grown in

100 µM Cd developed slightly better and were greener than the wild type ones under the same conditions. Surprisingly, the most obvious differences appeared between the plants grown in 50 µM Ni. The segregating sister plants, originated from the same capsule as the transgenic ones, and the wild type controls showed an apparent intercostal chlorosis and retarded growth, whereas the transgenic plants from both lines remained green and well developed (Fig. 3.39.).

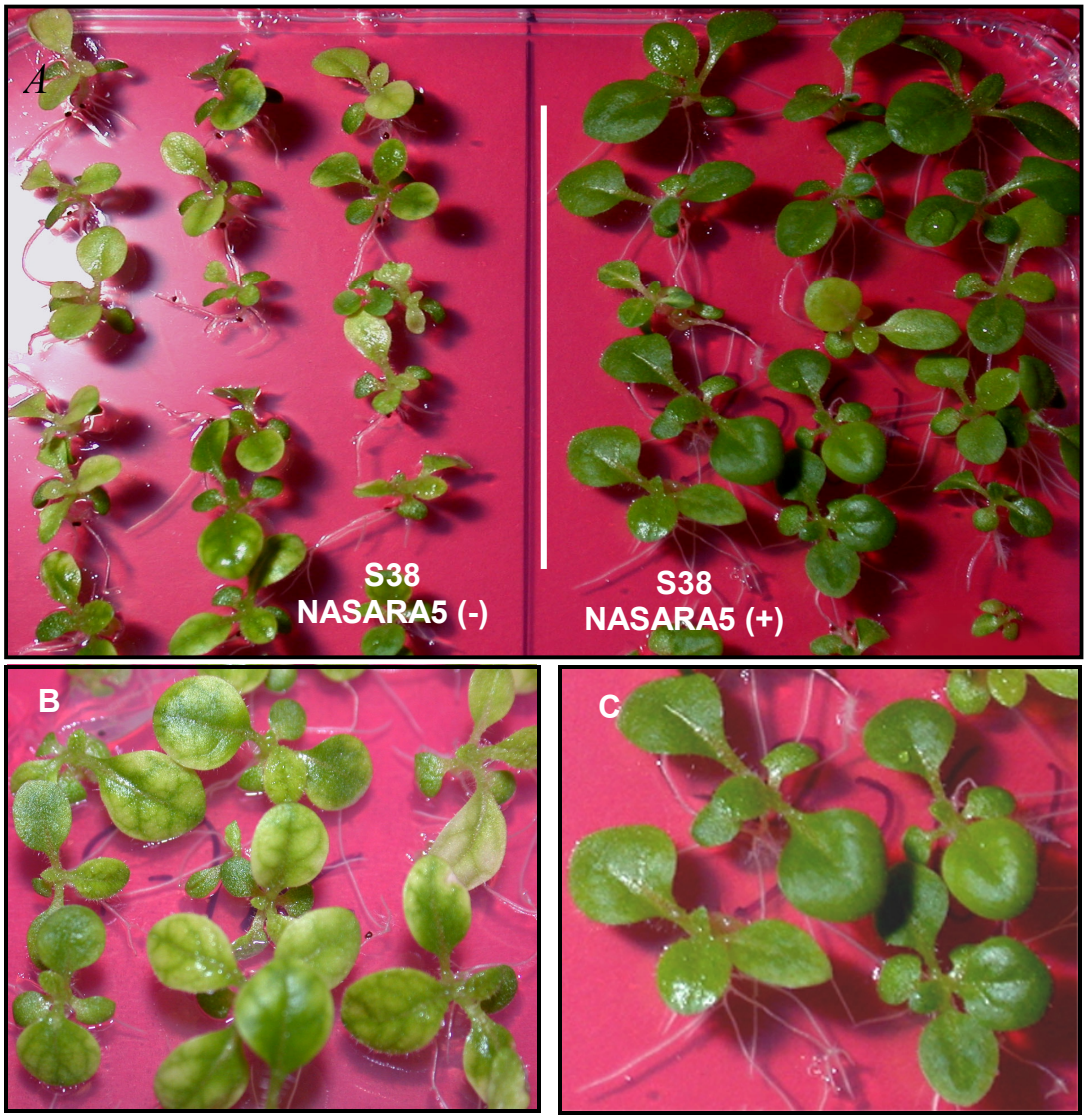

Fig. 3.39. Panel A: *NASARA5 tobacco line 38 (right) and segregating sister plants (left), grown in 50 µM Ni. The transgenic plants grow noticeably better than wild type under the same conditions.* ***Panel B and C:*** *Closer view of the plants from the same plate. The intercostal chlorosis of the NASARA5 (-) plants is obvious.*

3.5. Methanobacterium NAS-like gene

Regardless of the extensive search in the databases for NAS-like sequences in organisms outside the Plant kingdom, only one slightly similar sequence was found in the genome of the Archaebacteria *Methanobacterium thermoautotrophicum*, which was announced as putative protein MTH675 with unknown function (A. Shutov, personal communications) and was designated as *NASMET*. The similarity of *NASMET* to other *NAS* proteins was about only 20%. However, the similarity was especially obvious at the conservative regions of the *NAS* proteins.

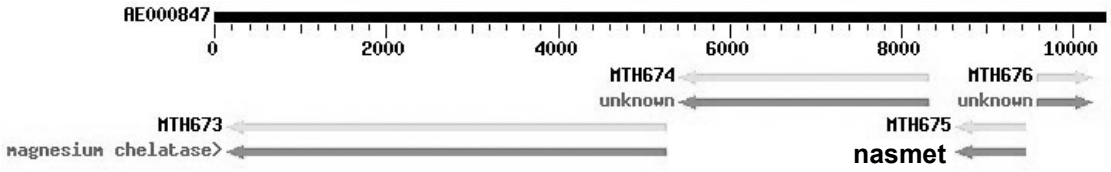

Fig. 3.40. The Methanobacterium thermoautotrophicum section of the complete genome (bases 597721 to 608106)*. The putative gene MTH675 shares a slight similarity to NAS genes.*

The *NASMET* gene was isolated and cloned from genomic DNA of *M. thermoautotrophicum.* All attempts to express this protein in *E. coli* as well as in *Bacillus* systems were unsuccessful.

3.6. NAS sequence comparison

The rapidly growing number of NAS genes allowed comparing their sequence and looking for conservative domains.

In fact, most of the NAS genes share high similarity to each other (Fig. 3.41.) that make it difficult to define the regions important for the enzyme function. The F-L-Y-P-[IV] motif is certainly one of the important domains, since, as known from *chloronerva,* the mutation of that phenylalanine leads to the complete inactivation of the enzyme.

The longest motif is [YF]-D-V(2)-F-L-A(2)-L-V-G-M but many other motifs can be defined too.

On the phylogenetic tree of the NAS proteins based on the alignment shown in Fig.3.41. one can clearly distinguish the groups of the monocot (barley and rice) and dicot (*A. thaliana* and tomato) NAS genes.

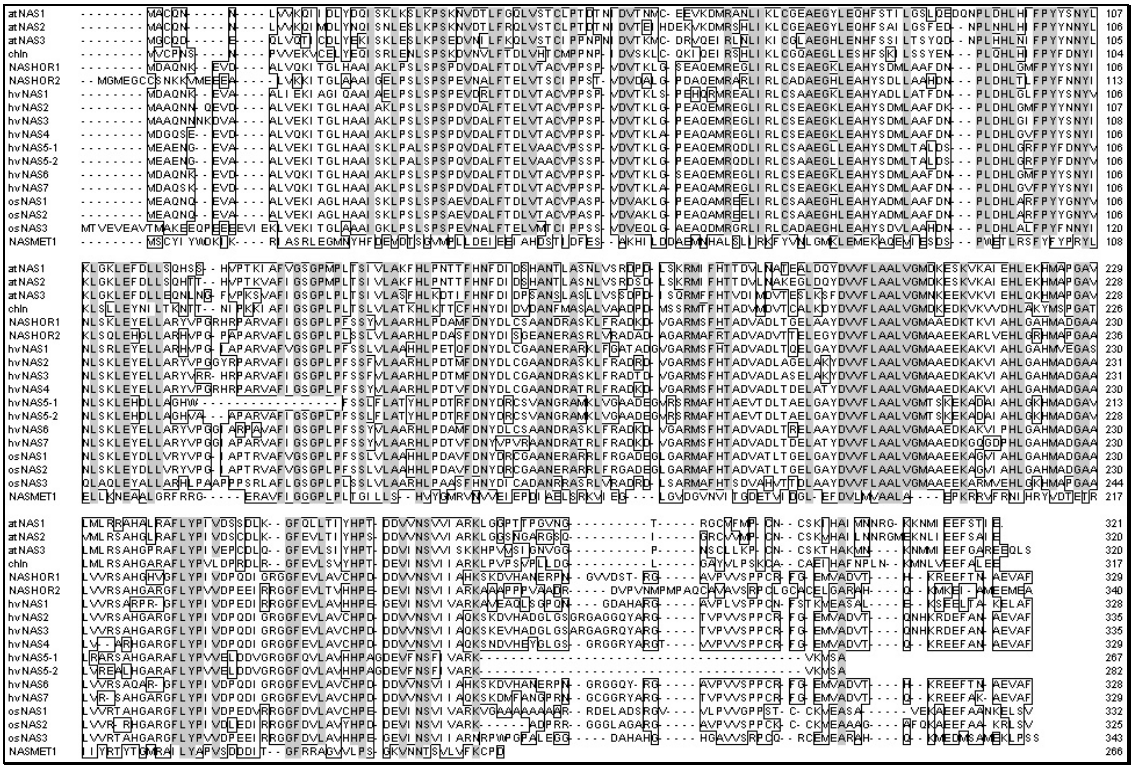

Fig. 3.41. Protein alignment of NAS sequences. *The highly conservative amino acids are shadowed; the boxed amino acids are identical to at least 3 distance units according to PAM250 table.*

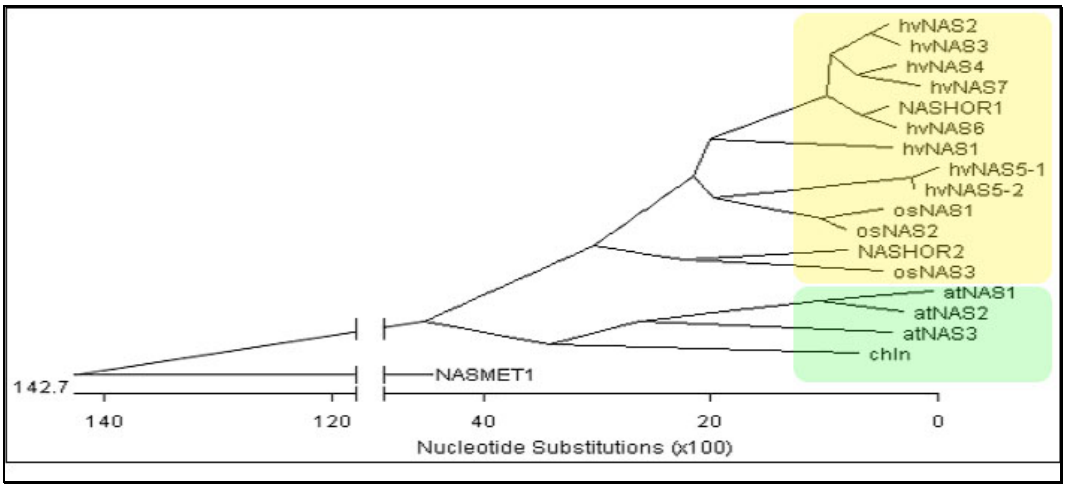

Fig. 3.42. A phylogenetic tree of the NAS proteins based on the alignment on Fig.3.41. *Notice the clear differentiation into monocot (yellow area) and dicot (green area) proteins. Interestingly, NASHOR2 is grouped far from the rest barley NAS genes.*

3.7. Branched chain α-keto acid dehydrogenase complex (BCKDH)

It is known that in so-called Strategy II plants NA is a precursor of the phytosiderophore synthesis. The metabolism and especially the degradation of NA in the Strategy I plants is less known. Therefore, some attempts were made to answer this question.

The BCKDH was suggested as an enzyme that is potentially involved in NA metabolism and in iron homeostasis in general for the reasons exposed in the Introduction.

A 953 bp 5' RACE fragment, provided by A. Giritch, was cloned in both orientations into pBinAR19 under the control of CaMV 35S promoter and the constructs were used for transformation of tomato plants.

Plants from T_1 generation were grown in soil, checked for the presence of *5'BCKDH* fragment by PCR and samples from first and second well-developed leaves were collected for NA analysis (Fig. 3.43.).

The data showed slightly increased NA concentration of the transgenic lines but both in sense and antisense lines.

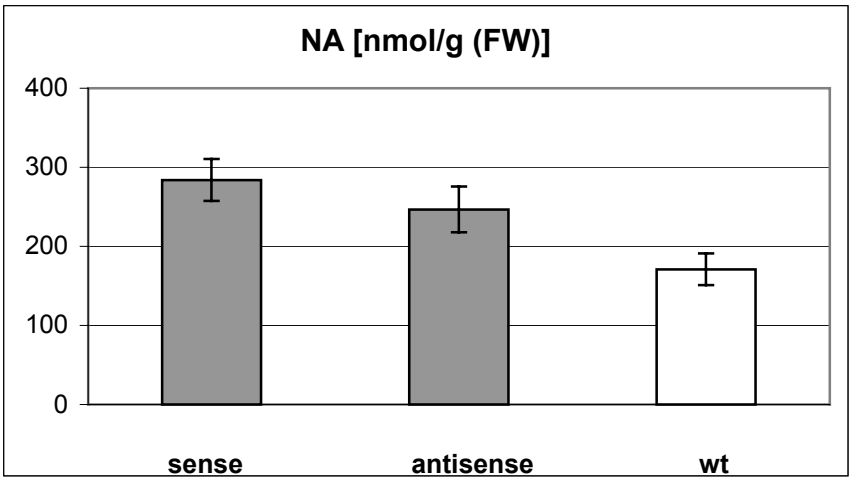

Fig. 3.43. NA concentration in sense and antisense 35S:BCKDH plants. *Each column represents the mean value for the line. The bars represent the standard error for the corresponding data set.*

Interesting results were obtained by measuring the concentrations of the amino acids in selected BSKDH antisense lines. The concentrations of Met, Ile, Leu, Val and Thr that might be influenced by *BCKDH* antisense construct, were increased in the *BCKDH* antisense plans compared to the wild type ones (Fig. 3.44.). The same was true also for Tyr, Lys, Arg and Asn. The levels of other amino acid, as Glu, Asp and Pro were not increased. The concentration of Phe, Gly, Ala, Ser and Gln varied within the transgenic lines.
However, the obtained data was poorly reproducible.

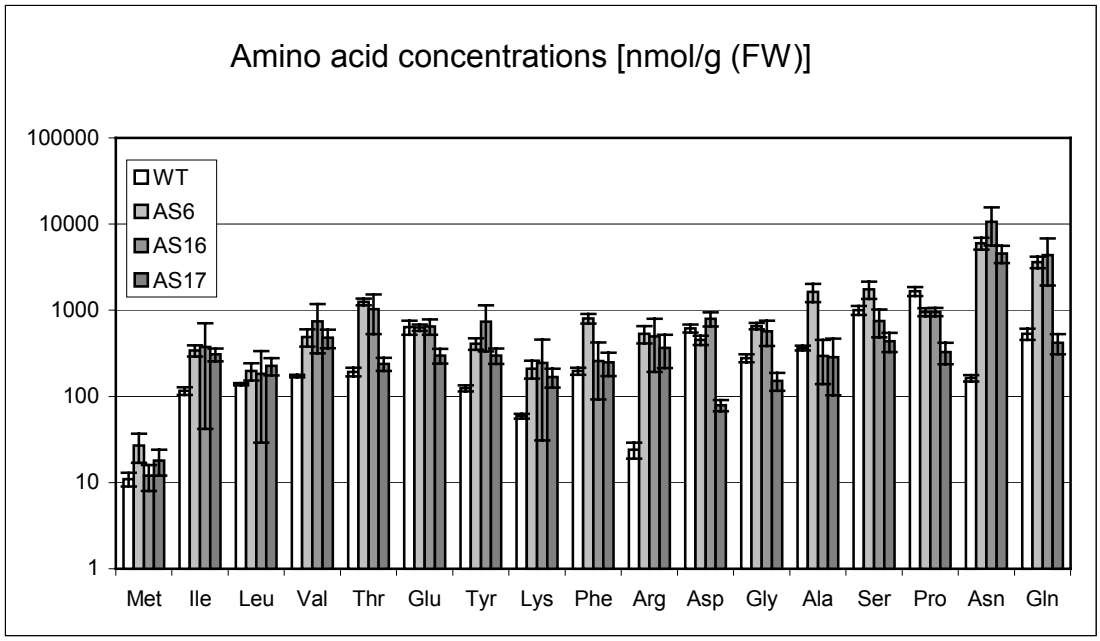

Fig. 3.44. Amino acids concentration in three antisense 35S:BCKDH lines. *Each column represents the mean value for the line. The bars represent the standard error for the corresponding data set.*

Obtaining of full length BCKDH cDNA

The full length *BCKDH* cDNA available in the databases was a result of the combination of 3'RACE and 5'RACE clones but not a sequence of a full-length clone. Therefore, we decided to acquire a full-length cDNA by RT-PCR. PCR primers were designed according to the sequence announced in the databases. The primer pair BC3/BC4 gave the full-length cDNA including 5' and 3' untranslated regions (1430 bp), the pair BC5/BC6 gave only the coding

region (1383 bp) (Fig. 3.45.). The primers BC1, BC2, BC7 and BC8 were used for proving and detection of the *BCKDH* sequence. In addition, BC9 with incorporated *Sph* I site, and BC10 with *Sal* I site were designed to be used for cloning into pET12a vector.

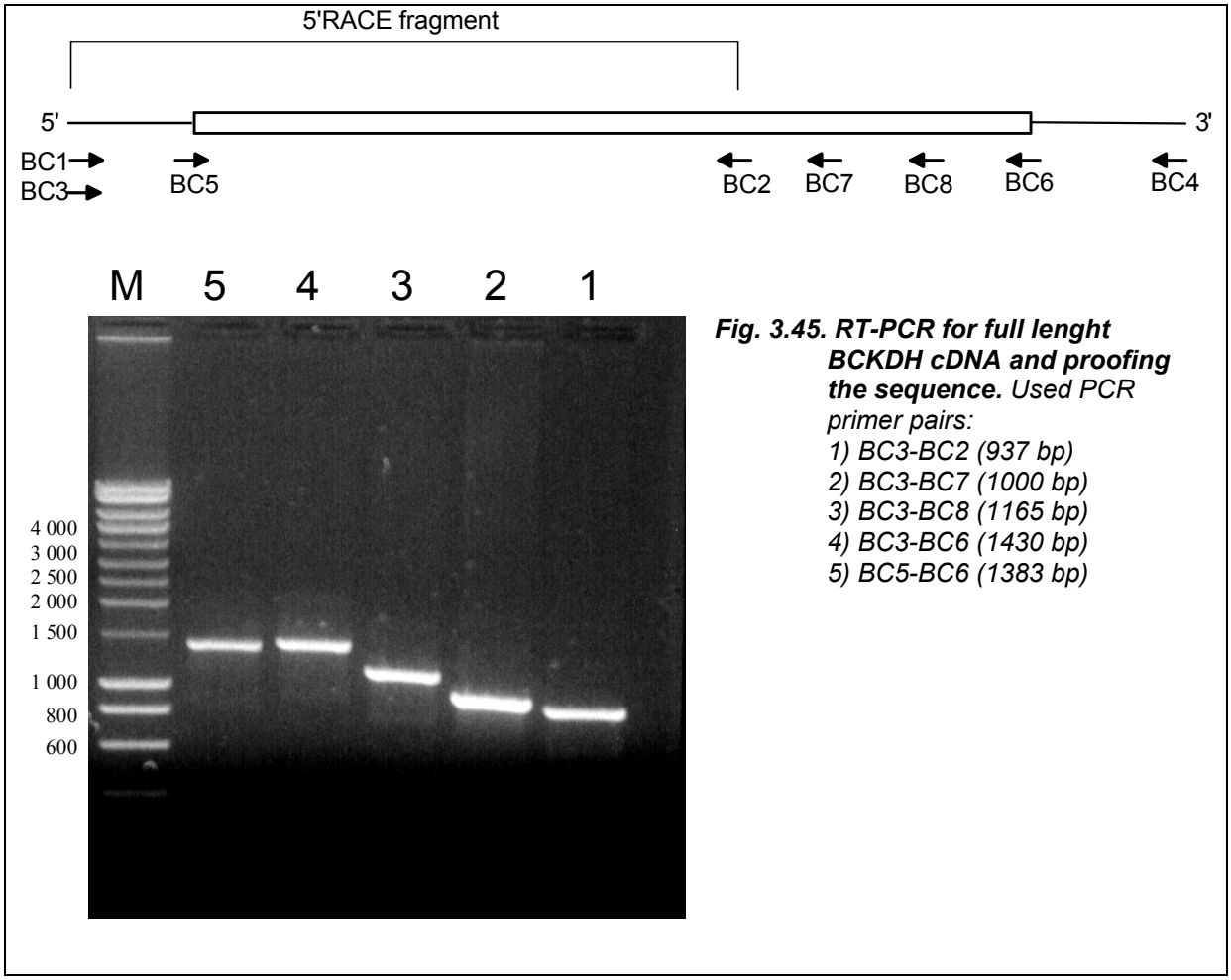

Fig. 3.45. RT-PCR for full lenght BCKDH cDNA and proofing the sequence. *Used PCR primer pairs:*
1) BC3-BC2 (937 bp)
2) BC3-BC7 (1000 bp)
3) BC3-BC8 (1165 bp)
4) BC3-BC6 (1430 bp)
5) BC5-BC6 (1383 bp)

Since the BCKDH story was turned out to be more complex developed to be a material for separate project, the work on this subject was stopped at that point.

4. Discussion

Iron is both an essential and a potentially toxic component for all living cells. In plants, a disturbance of iron acquisition causes complex harmful effects, as demonstrated by the tomato mutant *chloronerva.* This mutant lacks the nonproteinogenous amino acid nicotianamine. In Strategy I plants like tomato, failure of nicotianamine synthesis leads to the permanent activation of iron acquisition processes in roots including proton extrusion and increased ferric reductase activity. In mutant leaves, iron is accumulated to nonphysiologically high concentrations and its disturbed distribution leads to intercostal chlorosis.

Iron dependent changes of the NA concentrations in wild type A. thaliana. Function of NA

Although *A. thaliana* is a suitable model system for plant genetics and molecular biology, plant physiologists deals with iron assimilation have partially neglected it. Therefore some basic physiological experiments, concerning metal accumulation and NA concentration in wild type *A. thaliana* under different iron concentrations, have been performed in this study.

As expected, the low or zero iron concentrations in the medium correspond well with the low internal iron concentrations found. However, the highest iron concentration of the medium does not correspond to the highest internal iron concentration. Plants grown in 100 µM FeEDTA have lower iron concentration than plants supplied with normal iron concentration (40 µM). A possible reason for the decreased iron uptake could be the inhibitory effect of the high iron concentration on the cellular iron transporters (Korshunova *et al.*, 1999). The competition of iron with other metals for the metal uptake system becomes obvious by the experiments with Zn and Mn (Fig. 3.3.). These two metals were supplied at constant concentrations in the medium but the uptake of Zn and Mn shows a negative correlation to the uptake of iron.

Perhaps the most interesting result from this experiment was the dependence of the NA concentration on the iron concentration of the medium. Currently there are no clear evidences that *NAS* genes of *Arabidopsis* are regulated by

iron as it was shown for instance for the *NAS* gene of barley. For tobacco it was demonstrated that the NAS activity is certainly not induced under Fe deficiency (Higuchi *et al.*, 1995).

In contrast, in our experiment the NA concentration was strongly increased in the *Arabidopsis* plants grown with low or no iron, suggesting up regulation of the *NAS*-gene expression under condition of iron limitation. This finding is also in disagreement with the conclusions of Pich *et al.* (2001). They suggested that in tomato NA synthesis is induced by high iron availability. It is still possible that only the intracellular concentration of NA is increased by iron overload, supporting the hypothesis of a protective role of NA in the detoxification of excess iron.

The other proposed major function of NA - the long distance transport of iron in the phloem and possibly in the xylem- perhaps has to be revised too. According to von Wirén *et al.* (1999) the calculated equilibrium concentrations of different metal complexes in conditions approximating those in xylem shows that all Fe in the xylem should be complexed by citrate. Unlike the NA-Fe^{II}, the Fe^{II}-citrate complexes oxidize rapidly and Fe^{II}-citrate is converted to Fe^{III}-citrate, which should be the major form of iron in the xylem. On the contrary, NA was found to be more important than citrate in the chelation of both Zn^{II} and Cu^{II}. The calculations showed that 50% of the Zn^{II} and all Cu^{II} should be chelated by NA. In contrast to xylem, NA seems to play a more important role in phloem. The same calculation but for conditions approximating the phloem showed that all metal ions should be present as NA-complexes. Nevertheless, the work of Krüger et al. (2001, 2002) shows that all Fe in the phloem is bound to proteins. The described metal-binding protein of *Ricinus communis* shares high similarity to dehidrin proteins of the LEA (**L**ate **E**mbryogenesis **A**bundant) protein family, which are typically expressed under stress conditions as well as during seed development. These new findings require a reconsideration of the role of NA for the transport of metal nutrients in the phloem.

In spite of the results mentioned above, the increase in the NA concentration under iron deficiency as well as the knowledge collected from the *chloronerva*

mutant suggest a potential role of NA in iron uptake mechanisms and/or the remobilization of the iron. NA might be used to manipulate the plant iron deficiency response and to generate iron deficiency tolerant plants with enhanced iron acquisition. Experiments aiming at this direction are in progress (see below).

Isolation and structure of NAS

The *chloronerva* mutant has been studied for several decades and a huge amount of physiological data has been accumulated. In spite of this, the molecular basis of the mutation remained unknown. Recently the gene responsible for the *chloronerva* phenotype was fine-mapped onto the long arm of chromosome 1 using a large segregating tomato population and YAC clones surrounding the region were isolated using flanking markers (Ling et al., 1999). The *chloronerva* transcript was identified by cDNA isolation using the complementing cosmids. The gene encodes a novel protein of 35 kDa. The mutant differs from the wild type only by a single base pair change (T->C) at nucleic acid position 761 that creates a substitution of a phenylalanine by a serine at amino acid position 238 (Ling *et al.*, 1999).

Parallel experiments aimed at the isolation of the nicotianamine synthase from barley roots using an enzymatic and micro-sequencing approach (Herbik *et al.*, 1999; this work). The close cooperation and the early possibility for a comparison between these two projects led to the conclusion that a mutation in the NAS gene is the molecular basis for the *chloronerva* mutant.

The barley *NASHOR1* and *NASHOR2* genes were isolated from iron deficient barley roots by protein purification and micro-sequencing of the derived peptides. A similar approach was used by Higuchi *et al*. (1999) to isolate the genes *hvNAS1* (AB010086), *hvNAS2* (AB011265), *hvNAS3* (AB011264), *hvNAS4* (AB011266), *hvNAS5-1* (AB011267), *hvNAS5-2* (AB011268), *hvNAS6* (AB011269) and *hvNAS7* (AB019525).

In addition to the barley NAS genes, a database search revealed homology to several anonymous open reading frames (ORFs) found in the genomic DNA of *A. thaliana*. One of the ORFs was located on chromosome 1 and another

on chromosome 5 of *A. thaliana.* These two sequences, designated as *NASARA1* and NASARA*5,* were cloned and analyzed. A third *NAS*-like sequence was found on chromosome 4 of *A. thaliana.*

In parallel, Suzuki et al. (1999) reported the cloning and expression of three *A. thaliana* NAS genes designated as *atNAS1* (AB021934), *atNAS2* (AB021935) and *atNAS3* (AB021936). The *atNAS3* gene located at chromosome 1 is identical to *NASARA1* and the *atNAS1* located at chromosome 5 is identical to *NASARA5*. The third gene – *atNAS2* is located on chromosome 5. These three genes were expressed as maltose binding protein fusions in *E. coli*. All of the fusion proteins exhibited NAS activity. The expression pattern of *atNAS* genes in *Arabidopsis* was investigated by using RT-PCR since no signal was detectable by Northern hybridization. An *atNAS1* signal was detected in both roots and shoots, whereas an atNAS3 specific signal could be detected in shoots only. An *atNAS2* specific transcript was not detected in either shoots or roots.

Recently, Higuchi et al. (2001) reported the isolation of three rice *NAS*-like cDNA clones, *OSNAS1*, *OSNAS2* and *OSNAS3* from Fe-deficient rice roots. In contrast to barley, where Fe deficiency induces the expression of *NAS* genes only in roots, Fe deficiency in rice induced NAS gene expression both in roots and chlorotic leaves. The possible reason for this difference between these two Strategy II monocot species is not yet clear.

Although the databases used for BLAST search included several total bacterial genomes as well as the genome of yeast, outside of the plant kingdom a *NAS*-like sequence was found only in the genome of *Methanobacterium thermoautotrophicum* (gene MTH675 of the complete genome, accession number AE000847). The function of the gene in this organism is unknown. Remarkably, it is flanked (3,4 kb upstream) by a gene sequence (MTH673), which resembles a Mg-chelatase subunit encoding gene. Whether this fact is of functional significance remains to be determined. Interestingly, the *Methanobacteria* are known to be symbiotic species of eukaryotes and one even might speculate that the plant NAS genes originate from a horizontal gene transfer event (Herbik *et al.*, 1999).

Recently two *Zea mays NAS* genes were published only into the databases - *ZmNAS2* (AB061270) and *ZmNAS3* (AB061271). The *ZmNAS3* seems to be the result of a partial duplication event.

The amino acid sequence of the active NAS enzyme lacks any domain similarity to other enzymes, which accept SAM as a substrate as well, like DNA-methylases, other SAM depending methyltransferases and ACC synthases (Joshi and Chang, 1998). This might be explained by different substrate binding or reaction mechanisms. Whereas methylases catalyze the transfer of a methyl group and ACC-synthase catalyzes the formation of a cyclopropane ring from the methionine skeleton itself, the NAS reaction mechanism most likely involves the direct trimerization of SAM, resembling in some aspects a nonribosomal peptide synthesis.
The molecular mass of the active enzyme in purified barley roots was determined to be ~105 kDa, which is approximately triple the size of the NASHOR1 polypeptide (35 611 Da). This suggests that the native enzyme probably consists of three identical subunits. Taking into account the proposed mechanisms of NA synthesis from three molecules of SAM, one might speculate that the three subunits are necessary for the initial binding of three substrate molecules (Herbik *et al.*, 1999). Nevertheless, there are also some suggestions that the native form of NASHOR1 could be a dimer (K-H Süß, pers. comm.).

The NASHOR1 promoter

To analyze the expression pattern of different members of the barley *NAS* family, specific oligonucleotide probes were used. A *NASHOR1* specific transcript and the corresponding gene product can be identified only in barley roots grown under iron limitation. To explore the potential biotechnological value of such a root specific promoter, the upstream gene region was isolated and sequenced. The derived sequence reveals very high similarities to 5'-flanking regions of other barley genes (Fig. 4.3.). Remarkably, one of these sequences belonged to the *IDS3* gene, which encodes an enzyme involved in phytosiderophore biosynthesis, a step after *NAS* and *NAAT* (Kobayashi *et al.*,

2001). Another region of the *NASHOR1* 5'-flanking sequence was found to be very similar to the upstream region of the *DHN12* gene, encoding dehydrin12, which belongs to the LEA protein family. Interestingly, the recently isolated metal-binding protein from *Ricinus communis* is also a member of this protein family (Krüger *et al.*, 2002). The molecular basis for these similarities is not known but provokes speculations about the barley genome organization. Beforehand however, one should exclude cloning artifacts as a result of artificial rearrangements within the BAC clone.

Chromosomal mapping of the barley NAS genes

Chromosomal engineering is an interesting approach for basic research and crop-plant improvement, involving a transfer of chromosomes or chromosomal regions from iron efficient plants species to target crop-plants. Barley is a plant with an efficient iron uptake system. In contrast, wheat is rather sensitive to iron deficiency. Therefore, a transfer of barley chromosomal regions containing genes involved into the iron uptake to wheat could be a promising approach. A prerequisite for this approach is a precise chromosomal localization of the relevant genes. The chromosomal localization of all known barley NAS genes was a challenging task due to the number of NAS genes that share a high similarity. The mapping approach revealed that NAS genes in barley are located on chromosomes 2H, 4H and 6H, usually close to each other (except hvNAS1), perhaps forming gene clusters.

Transgenic plant approach

All data obtained from the tomato mutant *chloronerva*, the observed increase of the iron uptake of tomato and sunflower by NA spraying (Scholz *et al*. 1985) and the increased NA concentration in *A. thaliana* under iron deficiency together led to the suggestion that the iron physiology of the plants could be significantly influenced by an internal increase of the NA concentration. The recently isolated NAS genes and well-developed plant transformation techniques provide the prerequisites for such a transgenic approach.

No significant increase in the NA concentration could be achieved in plants transformed with the *NASHOR1* and *NASARA1* genes. At least for *NASHOR1* this could be explained by the absence of the translation product, as demonstrated by specific anti-NASHOR1 antibodies. In spite of the high sensitivity, the antibodies were not able to detect the NASHOR1 protein in the protein extracts from 35S:NASHOR1 transgenic plants. Obviously, no active NASHOR1 enzyme is synthesized. The reason for this remains unclear. A possible explanation could be that the *NASHOR1* mRNA or the protein, as a product of a monocot gene, is more prone to degradation in the dicot cells. This interpretation is favored by the successful overexpression of *NASHOR1* in monocots like *Lolium perenne* and wheat (G. Hensel, pers. comm.), however, using a different construct and promoter.

The 35S:NASARA1 construct showed a high transcription level but as in the previous case, the NA concentrations of the transgenic plants were not increased. Instead, the antisense lines showed a slight but statistically significant decrease in the NA concentration compared to the wild type. A similar decrease in NA concentration was observed in many sense lines. It remains unclear whether this was the consequence of a co-suppression effect and further experiments are needed to clarify this.

The reason for the inactivity of the *NASHOR1* and *NASARA1* genes is still waiting for an explanation. One might speculate about the processes of gene regulation. The *NASHOR1* is a member of a rather large gene family of barley NAS genes, each gene contributing only low activity. One might also consider a stringent posttranscriptional control. However, the known posttranscriptional regulatory mechanisms, as iron-dependent aconitase-mediated repression of the ferritin or transferin genes (Henze et al., 1987) should not function in the case of the *35S:NASHOR1* construct since it includes only the coding sequence of *NASHOR1* and not the 5’ or 3’ untranslated regions, that are responsible for the aconitase gene regulation. Another possible explanation could be the fact this both *NASHOR1* and *NASARA1* are tissue specific genes (Herbik *et al*., 1999; Higuchi *et al*., 1999; Suzuki *et al*., 1999). In addition to that, *NASHOR1* is highly expressed under iron limitation, suggesting the presence of mechanisms for fast degradation of the protein when it is no

longer needed. Targeted protein degradation, as for instance the ubiquitin system could be involved in the regulation of the protein concentration.

The most promising results were obtained with the *NASARA5* gene. NA concentrations of about 1300 nmol/g (FW) could be detected in some tobacco sense lines, which is among the highest values ever detected in a plant. The average NA concentration for the selected sense lines was increased approximately six times compared to wild type. The segregating plants in combination with PCR and Northern results unambiguously proved that the high NA concentrations are the result of *NASARA5* expression. The iron concentration of the transgenic sense lines was significantly higher than in wild type. Nevertheless, the correlation between NA and iron concentration within the transgenic lines was significant only for 2 of the lines. The relatively weak correlation could be explained with the assumption that NA reached its saturation level and the fluctuation beyond this level does not affect the iron concentration.

Enhanced heavy metal tolerance of NASARA5 overexpressing plants.

Higher concentrations of heavy metals have deleterious effects on plant growth and development. Therefore, plants with increased tolerance to high heavy metal concentrations might be of interest both for basic research and phyto-remediation purposes.

Various heavy metals are known to induce Fe-deficiency chlorosis in different plant species (Römheld and Marschner, 1986; Wallace *et al.*, 1992; Ma and Nomoto, 1993). Particularly Ni^{2+} is a heavy metal, which affects the induction and function of the Fe(III) reductase. The presence of Ni^{2+} influences the uptake of iron (Alcantara *et al.*, 1994). Thus, the NAS overexpressing plants with increased NA concentration might overcome the nickel-induced limitation in iron uptake and/or iron distribution. Such observation is documented in Fig. 3.39.. Challenged with 50 µM and 100 µM of Ni^{2+} ions the transgenic plants grow more rapidly and stay green. The segregating sister plants used as control remain small and show symptoms of chlorosis.

Although, the observation needs further investigation, the data suggest that a higher NA concentration might help to overcome the nickel induced limitation in iron uptake and/or iron distribution.

Nicotianamine forms very stable complexes with a number of metals. The order of affinity is as follows: $Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Fe^{2+} > Mn^{2+}$ (Scholz *et al.*, 1988; Stephan and Scholz, 1993). It is therefore tempting to suggest that heavy metals, which form NA complexes with higher stability than with Fe^{2+} could displace the iron from the NA complex causing iron deficiency. The 5-10 fold higher NA levels in the NASARA5-gene over-expressing plants could overcome this effect. Experiments using various concentrations of other heavy metals are in progress.

The branched chain α-keto acid dehydrogenase complex with putative functions for iron assimilation.

The branched chain α-keto acid dehydrogenase complex (*BCKDH*) is a mitochondrial multi-enzyme complex catalyzing the oxidative decarboxylation of branched chain α-keto acids derived from branched chain amino acids (Val, Leu, Ile) as well as from Met and Thr. The protein complex is thought to be essential for the utilization of these amino acids as a substrate for the citrate cycle (Yeaman, 1986). The *BCKDH* complex consist of three catalytic components - branched chain α-keto acid decarboxylase (E1α and E1β units); dihydrolipoyl transacylase (E2 unit) and dihydrolipoamide dehydrogenase (E3 unit). It catalyzes the oxidative decarboxylation of the α-keto acids arising from the deamination of the corresponding amino acids.

The expression of the *BCKDH* gene was reported to be light and sugar dependent in *A. thaliana.* The transcript levels of E1β and E2 subunits as well as the *BCKDH* activity was increased in leaves kept in the dark and under sugar starvation (Fujiki et al., 2000, 2001). Addition of sucrose inhibited the accumulation of the transcripts, whereas application of a photosynthesis inhibitor strongly induced the expression of these genes.

The gene encoding the E1α unit of BCKDH was found to be up-regulated in root tips of the tomato mutant *chloronerva* as well as in iron deficient wild type

plants (A. Giritch, pers. comm.). These findings suggested a potential function of BCKDH in iron metabolism. This suggestion was further supported by the following observations (A. Giritch, pers. comm.):

- An *Arabidopsis* T-DNA insertion mutant exhibits a *chloronerva*-like phenotype. The interrupted gene of unknown function shows a high homology to an ABC-transporter.
- A yeast mutant defective in this ABC-transporter shows extensive changes in iron status.
- The *Arabidopsis* gene can complement this yeast mutant. A cDNA corresponding to the yeast branched-chain amino acid transaminase (*BCAATA*) also complements the yeast mutant.
- The *BCAATA* is catalyzes the enzymatic step just before *BCKDH* in the branched-chain amino acid degradation pathway.

This information suggests that *BCKDH* could be potentially involved in the iron uptake mechanisms.

A suggested hypothesis was based on the assumption that the non-proteinogenous amino acid NA could be considered as a branched chain amino acid and therefore be accepted as a substrate of *BCKDH*. Following this hypothesis *BCKDH* was thought to be involved in the degradation of NA and the *BCKDH* activity could be a component to control the NA concentration.

To test this hypothesis, *BCKDH*-antisense constructs have been used for the transformation of tomato. However, the analysis of the NA levels in sense and antisense plants did not reveal any reproducible differences in NA concentrations.

Furthermore, the blockage of the degradation pathway of branched chain amino acids by the antisense inhibition of the *BCKDH*-gene could lead to an increased concentration of these nutritionally essential amino acids. Therefore, the concentrations of these amino acids have been measured. Again no reproducible changes could be detected.

Taken together, this side project turned out to be more complex and obviously needs a more detailed analysis.

Changed expression of transcription factor genes under iron limitation

Iron limitation leads to complex phenotypic changes partially as the result of changed gene expression programmes. The specific interaction between promoter *cis*-elements and transcription factors is a component of the underlying gene regulation.

Preliminary experiments based on an *A. thaliana* transcription factor (TF) gene *array* identified two TF genes with highly induced expression under iron limitation. They belong to the bHLH (**b**ase **H**elix **L**oop **H**elix) TF family. Interestingly, the recently isolated *FER* gene of tomato also represents a TF of the same family (Ling, 2002). The root system of the recessive *fer* mutant is unable to induce any of the characteristic responses to iron deficiency and iron uptake is completely blocked. Unfortunately, the sequence of the *FER* gene is not yet available for a comparison.

The two identified TF are the only two genes on the array up-regulated under iron deficiency, making them putative candidates for regulators of iron uptake mechanisms of *A. thaliana*. Due to their high sequence similarity both genes cross-hybridize and it is not yet decided whether they are regulated simultaneously. The structural similarity suggests functional redundancy of both gene functions. This phenomenon seems to be a wide spread safe guarding mechanism of higher organisms.

Remarkably, both TF-genes were found to be highly induced already about 10 hours after iron limitation, suggesting a rather fast sensing mechanism. Assuming that internal cellular iron pools are not depleted within 10 hours, one might speculate that a potential sensing mechanism should determine the extra-cellular iron conditions. Obviously, this speculation requires further experiments to be confirmed or rejected. Corresponding experiments on transgenic lines of tobacco and *Arabidopsis* as well as in *Agrobacterium rhizogenes* induced transgenic roots, which ectopically express both TF genes are in progress.

Summary

Iron deficiency is one of the most common nutritional disorders of the mankind. Since in most diets plants are the principal source of iron, crop plants with higher iron content could contribute to the improvement of this situation.

Iron is essential for fundamental cellular processes such as electron transfer in photosynthesis, respiration, nitrogen fixation as well as DNA synthesis. However, excessive accumulation of iron causes severe damage to cellular components. Although abundant in most well aerated soils, iron is mainly found as stable Fe (III) compounds with extremely low solubility at neutral pH. Thus, iron is nutritious, noxious and not readily available. The precise control of the iron homeostasis is a basic prerequisite for cellular function.

Plants have evolved at least two different mechanisms of iron acquisition. The non-proteinogenous amino acid nicotianamine (NA) is an essential component of both strategies. NA is ubiquitously found in the plant kingdom. The lack of NA is the principal distortion of the tomato mutant *chloronerva* and leads to severe disturbances of iron metabolisms. Recent experiments led to the conclusion that a mutation in the nicotianamine synthase (NAS) gene is the molecular defect of this mutant.

Two NAS genes of barley (*NASHOR1* and *NASHOR2*) have been isolated and characterized further by enzymatic studies after expression in *E. coli*. These enzymes catalyze the synthesis of NA from three molecules of S-adenosyl methionine and the formation of an azetidine ring. NAS genes encode a novel class of enzymes.

A *NASHOR1* gene-specific transcript and the corresponding gene product can be identified only in barley roots when grown under iron limitation. To explore the potential biotechnological value of such an iron regulated as well as root specific gene promoter, the upstream gene region has been isolated and sequenced. In addition, all barley NAS genes have been mapped in clusters on chromosomes 4 and 6.

Database searches identified two NAS-like genes in the genome of *A. thaliana*, which have been located on chromosomes 1 and 5. These two sequences, designated as *NASARA1* and *NASARA5*, have been cloned and used for gene transfer experiments.

Previous observations led to the suggestion that iron assimilation of the plants could be improved by an increase of the internal NA concentration. Therefore, gene constructs containing *NASHOR1*, *NASARA1* and *NASARA5* genes, both in *sense* and antisense orientation, under the control of the CaMV 35S promoter have been transformed into *N. tabacum*, *L. esculentum* and *A. thaliana.* Corresponding constructs controlled by the maize ubiquitin gene promoter have been used for the transformation of *L. perenne* and *T. aestivum*. In total, more than 500 independent transgenic lines have been generated and partially analyzed.

Promising results have been obtained with the *NASARA5* gene. NA concentrations of about 1300 nmol/g fresh weight could be detected in selected tobacco *sense* lines. It is among the highest concentration ever detected in plants. The average NA concentration of tobacco *sense* lines was increased 5 to 10 fold compared to wild type. Segregation analysis demonstrated that the high NA concentrations are the result of the *NASARA5* gene over-expression. The iron concentration in most of the transgenic plants was significantly higher than in wild type.

Preliminary data also suggest that the higher NA concentration due to the over-expression of the *NASARA5* gene leads to a higher nickel tolerance of transgenic tobacco plants in comparison to the segregating non-transgenic plants. When challenged with 50 µM and 100 µM of Ni^{2+} ions the transgenic plants grew more rapidly and stayed green. The segregating sister plants used as control remained small and showed chlorosis.

Initial experiments based on an array of most of the *Arabidopsis* transcription factor (TF) genes identified two TF genes with highly induced expression under iron limitation. They belong to the bHLH-TF family. Therefore, they might be candidates for regulators of iron uptake mechanisms.

Zusammenfassung

Eisenmangel ist eines der am meisten verbreiteten Ernährungsprobleme. Weltweit sind Pflanzen die wesentliche Quelle für Eisen. Kulturpflanzen mit erhöhtem Eisengehalt könnten daher zur Verbesserung der Situation beitragen.

Eisen ist eine essentielle Komponente für eine Reihe fundamentaler zellulärer Prozesse. Dazu gehören z. B. Stickstofffixierung, DNA-Synthese sowie der Elektronentransfer bei Photosynthese und Atmung. Andererseits führt eine Hyperakkumulation von Eisen zur Schädigung zellulärer Strukturen. Wenngleich Eisen ausreichend im Boden vorhanden ist, liegt es in belüfteten Böden hauptsächlich in stabilen Fe(III)-Verbindungen vor, die bei neutralen pH-Werten nahezu unlöslich sind. Eisen ist demnach gleichzeitig essentiell, toxisch und schwer verfügbar. Die präzise Kontrolle der Eisen-Homeostase ist deshalb eine notwendige Voraussetzung für zelluläre Funktionen.

Pflanzen haben zwei unterschiedliche Strategien der Eisenaufnahme entwickelt. Die nichtproteinogene Aminosäure Nicotianamin (NA) ist eine essentielle Komponente beider Strategien; sie ist im Pflanzenreich ubiquitär verbreitet. Das Fehlen von NA bei der Tomatenmutante *chloronerva* führt zu komplexen Störungen des Eisenstoffwechsels. Jüngst konnte gezeigt werden, dass der molekulare Defekt eine Mutation im Gen für Nicotianamin-Synthase (NAS) ist.

Zwei NAS-Gene aus Gerste (*NASHOR1* und *NASHOR2*) wurden isoliert und u. a. durch enzymatische Untersuchungen an dem in *E. coli* synthetisierten Enzym charakterisiert. Das Enzym katalysiert die Synthese von NA aus drei Molekülen S-Adenosyl-Methionin sowie die Bildung eines Azetidin-Rings. NAS-Gene kodieren eine neue Klasse von Enzymen.

Ein *NASHOR1*-spezifisches Transkript sowie das entsprechende Genprodukt waren ausschließlich in Wurzeln, die unter Eisenmangel wachsen, nachweisbar. Der *NASHOR1*-Genpromotor wurde isoliert. Durch Sequenzierung wurden charakteristische Promotormotive identifiziert. Alle NAS-Gene des Gerstengenoms wurden in zwei Gruppen auf den Chromosomen 4 und 6 lokalisiert.

Durch Datenbank-Analysen wurden im *Arabidopsis*-Genom zunächst zwei NAS-ähnliche Gene (*NASARA1* und *NASARA5*) gefunden. Beide Gene wurden kloniert und für Gentransfer-Experimente eingesetzt.

Frühere Beobachtungen ließen vermuten, dass erhöhte interne NA-Konzentrationen die Eisenassimilation optimieren können. Um dies näher zu untersuchen, wurden die Gene *NASHOR1*, *NASARA1* und *NASARA5* unter Kontrolle des CaMV 35S- Promotors in *sense* und *antisense*-Orientierung in *Nicotiana tabacum*, *Lycopersicon esculentum* sowie *Arabidopsis thaliana* transformiert und zur Expression gebracht. Entsprechende Genkonstrukte unter Kontrolle des Ubiquitin-Genpromotors aus Mais wurden in *Lolium perenne* und *Triticum aestivum* tranformiert. Insgesamt wurden mehr 500 transgene Pflanzenlinien erzeugt und teilweise charakterisiert.

Weiterführende Ergebnisse wurden mit dem *NASARA5*-Gen erhalten. In ausgewählten transgenen Tabaklinien wurden NA-Konzentrationen von bis zu 1300 nmol/g Frischgewicht gemessen. Dies gehört zu den höchsten bisher in Pflanzen gemessenen Werten. Im Durchschnitt wurde eine 5-10 fache Erhöhung der NA-Konzentrationen im Vergleich zum Wildtyp gefunden. Segregations-Analysen zeigten zweifelsfrei, dass die hohen NA-Konzentrationen die Folge der Überexpression des *NASARA5*-Gens sind. In den transgenen Pflanzen mit erhöhter NA-Konzentration wurde eine signifikant erhöhte Eisenkonzentration nachgewiesen.

Weitere Beobachtungen erlauben die vorläufige Schlussfolgerung, dass die erhöhte NA-Konzentration als Folge der Überexpression des *NASARA5*-Gens auch zu erhöhter Nickeltoleranz führt. Ein Vergleich des Wachstums transgener Tabakpflanzen mit segregierenden nicht-transgenen Schwesterpflanzen auf Medien mit 50 µM und 100 µM Ni^{2+}-Ionen zeigte, dass die transgenen Pflanzen besser wachsen und nicht chlorotisch werden.

Erste, auf einem *array*-Filter mit 1200 Transfaktor (TF)-Genen von *Arabidopsis* basierende Versuche ließen erkennen, dass zwei TF-Gene der bHLH-Klasse unter Eisenmangel eine erhöhte Expression zeigen. Laufende Arbeiten sollen klären, ob es sich dabei um Regulatoren der Eisenassimilation handelt.

Literature

Adler K, Scholz G (1986) Die Chloroplastenstrukturen der Mutante chloronerva von Lycopersicon esculentum Mill. im Mutantenstatus und nach Normalisierung mit Nicotianamin. *Kulturpflanze* **34:** 185-194

Alcántara E, Romera FJ, Canete M, De la Guardia MD (1994) Effects of heavy metals on both induction and function of root Fe (III) reductase in Fe-deficient cucumber (*Cucumus sativus L.*) plants. *Journal of Experimental Botany* **45:** 1893-1998

Becker R, Fritz E, Manteuffel R (1995) Subcellular localization and characterization of excessive iron in the nicotianamine-less tomato mutant *chloronerva*. *Plant Physiol* **108:** 269-275

Bienfait HF (1988) Mechanisms in Fe-efficiency reactions of higher plants. J *Plant Nutr* **11:** 605–629.

Bintrim SB, Donohue TJ, Handelsman J, Roberts GP, Goodman RM (1997) Molecular phylogeny of Archaea from soil. *Proc Natl Acad Sci USA* **94:** 277-282

Buckhout TJ, Bell PF, Luster DG, Chaney RL (1989) Iron-stress induced redox activity in tomato (*Lycopersicon esculentum* Mill.) is localized on the plasma membrane. *Plant Physiology* **90:** 151–156.

Buděšinský M, Budesinsky H, Prochazka Z, Ripperger H, Römer A, Scholz G, Schreiber K (1980) Nicotianamine, a possible phytosiderophore of general occurrence. *Phytochemistry* **19:** 2295 – 2297

Chosack E, Rubinstein B, Reinhold L (1991) Effect of iron deficiency on electron transport processes of plasmalemma-enriched vesicles isolated from cotton roots. *Plant Science* **77:** 163–172.

Church GM and Gilbert W (1984) Genomic Sequencing. *Proc Natl Acad Sci USA* **81:** 1991-1995

Clemens S (2000) Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212:** 475-486

Christensen AH and Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgen Res* **5:** 213-218

Dell'Orto M, Santi S, De Nisi P, Cesco S, Varanini Z, Zocchi G, Pinton R (2000) Development of Fe-deficiency responses in cucumber (*Cucumis sativus* L.) roots: involvement of plasma membrane H(+)-ATPase activity. *J Exp Bot* **51**(345): 695-701

Douchkov D, Herbik A, Koch G, Mock H-P, Melzer M, Stephan UW, Bäumlein H (2002) Nicotianamine synthase: Gene isolation, gene transfer and application for manipulation of plant iron assimilation. *Plant and Soil* **241:** 115-119

Drechsel H, Jung G (1998) Peptide Siderophores. *J of Peptide Science* **4:**147-181

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc Natl Acad Sci USA* **93:** 5624-5628

Fujiki Y, Ito M, Nishida I, Watanabe A (2001) Leucine and its keto acid enhance the coordinated expression of genes for branched-chain amino acid catabolism in Arabidopsis under sugar starvation. *FEBS Lett* **499**(1-2): 161-165

Fujiki Y, Sato T, Ito M, Watanabe A (2000) Isolation and characterization of cDNA clones for the e1beta and E2 subunits of the branched-chain alpha-ketoacid dehydrogenase complex in Arabidopsis. *J Biol Chem* **275**(8): 6007-6013

Guerinot ML (2000) The ZIP family of metal transporters. *Biochim Biophys Acta* **1465**(1-2): 190-198

Guerinot ML (2001) Improving rice yields-iron out the details. *Nature Biotechnology* **19:** 417-418

Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219:** 1-14

Hentze MW, Caughman SW, Rouault TA, Barriocanal JG, Dancis A, Harford JB, Klausner RD (1987) Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science* **238**(4833): 1570-3

Herbik A (1997) Proteinchemische und molekularbiologische Charakterisierung der Tomatenmutante *chloronerva*. *Dissertation, Humboldt-Universität zu Berlin*

Herbik A, Koch G, Mock H-P, Dushkov D, Czihal A, Thielmann J, Stephan UW, Bäumlein H (1999) Isolation, characterization and cDNA cloning of nicotianmine synthase from barley - a key enzyme for ion homeostasis in plants. *Eur J Biochem* **264:** 1-10

Higuchi K, Kanazawa K, Nishizawa N-K, Chino M, Mori S (1994) Purification and characterization of nicotianamine synthase from Fe-deficient barley roots. *Plant and Soil* **165:** 173-179

Higuchi K, Nishizawa N-K, Yamaguchi H, Römheld V, Marschner H, Mori S (1995) response of nicotianamine synthase activity to Fe-deficiency in tobacco plants as compared with barley. *J Exp Botany* **46**(289) 1061-1063

Higuchi K, Suzuki K, Nakanishi H, Yamaguchi H, Nishizawa N-K, Mori S (1999) Cloning of Nicotianamine Synthase Genes, Novel Genes Involved in the Biosynthesis of Phytosiderophores. *Plant Physiology* **119:** 471-479

Higuchi K, Watanabe S, Takahashi M, Kawasaki S, Nakanishi H, Nishizawa N-K, Mori S (2001) Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions. *The Plant Journal* **25**(2): 159-167

Joshi CP, Chang VL (1998) Concerved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. *Plant Mol Biol* **37:** 663-674

Kawai S, Itoh K, Takagi S (1993) Incorporation of ^{15}N and ^{14}C of methionine into the mugineic acid family of phytosiderophores in iron-deficient barley roots. *Physiologia Plantarum* **88:** 668-674

Kawai S, Takagi S, Sato Y (1988) Mugineic acid-family phytosiderophores in root-secretions of Barley, Corn and Sorghum varieties. *J Plant Nutrition* **11** (6-11): 633-642

Kobayashi T, Nakanishi H, Takahashi M, Kawasaki S, Nishizawa NK, Mori S. (2001) In vivo evidence that Ids3 from Hordeum vulgare encodes a dioxygenase that converts 2'-deoxymugineic acid to mugineic acid in transgenic rice. *Planta* **212:** 864-871

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. *Plant Mol Biol* **40**(1): 37-44

Kristensen I, Larsen PO (1974) Azetidine-2-carboxylic Acid Derivatives from Seeds of Fagus Silvatica L. and a Revised Structure for Nicotianamine. *Phytochemistry* **13:** 2791-2798

Krüger C, Hell R, Stephan UW (2001) A metal-binding LEA protein traffics micronutritiens in the phloem of Ricinus communis L. *in Plant Nutrition (Horst et al. Eds.) Kluwer Academic Publishers* 54-55

Kruger C, Berkowitz O, Stephan UW, Hell R (2002) A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of Ricinus communis L.. *J Biol Chem* **277 (28):** 25062-25069

Ling HQ, Koch G, Baumlein H, Ganal MW (1999) Map-based cloning of *chloronerva*, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. *Proc Natl Acad Sci USA* **96**(12):7098-7103

Ling HQ, Bauer P, Bereczky Z, Keller B Ganal MW (2002) The tomato fer gene encoding a bHLH protein controls iron-uptake responses in roots. *Proc Natl Acad Sci USA, published online before print **10.1073/pnas.212448699***

Luna CM, Gonzalez CA, Trippi VS (1994) Oxidative Damage Caused by an Exsess of Copper in Oat Leaves. *Plant Cell Physiol* **35**(1): 11-15

Ma JF, Nomoto K (1993) Inhibition of mugineic acid-ferric complex uptake in barley by copper, zinc and cobalt. *Physiologia Plantarium* **89**: 331-334

Ma JF, Nomoto K (1996) Effective regulation of iron acquisition in gramineous plants. The role of mugineic acids as phytosiderophores. *Physiologia Plantarum* **97:** 609-617

Mori S (1999) Iron acquisition by plants. *Curr Opin Plant Biol* **2**(3): 250-3

Mori S, Nishizawa N (1987) Methionine as a Dominant Precursor of Phytosiderophores in Gramineae Plants. *Plant Cell Physiol* **28**(6): 1081-1092

Nakanishi H, Bughio N, Matsuhashi S, Ishioka N-S, Uchida H, Tsuji A, Osa A, Sekine T, Kume T, Mori S (1999) Visualizing real time [^{11}C]methionine translocation in Fe-sufficient and Fe-deficient barley using a positron emitting tracer imaging system (PETIS). *J of Exp Botany* **50:** 637-643

Nakanishi H, Okumura N, Umehhara Y, Nishizawa N-K, Chino M, Mori S (1993) Expression of a Gene Specific for Iron Deficiency (Ids3) in the Roots of Hordeum vulgare. *Plant Cell Physiol* **34**(3): 401-410

Nakanishi H, Yamaguchi H, Sasakuma T, Nishizawa N-K, Mori S (2000) Two dioxygenase genes, Ids3 and Ids2, from Hordeum vulgare are involved in the biosynthesis of mugineic acid family phytosiderophores. *Plant Mol Biol* **44:** 199-207

Noma M, Noguchi M, Tamaki E (1971) A New Amino Acid, Nicotianamine, From Tobacco Leaves. *Tetrahedron Letters* **22:** 2017-2020

Pich A, Manteuffel R, Hillmer S, Scholz G, Schmidt W (2001) Fe homeostasis in plant cells: Does nicotianamine play multiple roles in the regulation of cytoplasmic Fe concentration? *Planta* **213:** 967-976

Pich A, Scholz G (1993) The relationship between the activity of various iron-containing and iron-free enzymes and the presence of nicotianamine in tomato seedlings. *Physiologia Plantarum* **88:** 172-178

Pich A, Scholz G, Stephan UW (1994) Iron dependant changes of heavy metals, nicotianamine and citrate in different plant organs and in xylem exudates of two tomato genotypes. Nicotianamine as possible copper translocator. *Plant Soil* **165:** 189-196

Rabotti G, Zocchi G (1994) Plasma-membrane bound H^+-ATPase and reductase activities in Fe-deficient cucumber roots. *Physiologia Plantarum* **90:** 779–785.

Ripperger H, Schreiber K (1982) Nicotianamine and analogous amino acids, endogenous iron carriers in higher plants. *Heterocycles* **17:** 447-461

Robinson NJ, Procter CM, Connoly EL, Guerniot ML (1999) A ferric-chelate reductese for iron uptake from soils. *Nature* **329:** 694-697

Römheld V and Marschner H (1986) Mobilization of iron uptake by dicotyledonous and monocotyledonous plants. II. The reduction of Fe3+ as influenced by root and inchibitors. *J. Plant Nutr.* **2**: 647-660

Rudolph A, Becker R, Scholz G, Prochazka Z (1985) The occurence of the amino acid nicotianamine in plants and microorganisms. A Reinvestigation. *Biochem Physiol Pflanzen* **180:** 557-563

Samuelsen AI, Martin RC, Mok DWS, Mok MC (1998) Expression of yeast FRE genes in transgenic tobacco. *Plant Physiol* **118:** 51-58

Schmidt W, Bartels M, Tittel J, Fuhner C (1997) Physiological effects of copper on iron acquisition processes in Plantago. *New Phytologist* **135:** 659–666.

Schmidt W, Bartels M, Tittel J, Fühner C (1997) Physiological effects of copper on iron acquisition processes in Plantago. *New Phytol* **135:** 659-666

Scholz G, Becker R, Pich A, Stephan UW (1992) Nicotianamine - A common constituent of strategies I and II of iron acquisition by plants: A Review. *J of Plant Nutrition* **15**(10): 1647-1665

Scholz G, Becker R, Stephan UW, Rudolph A, Pich A (1988) The regulation of iron uptake and possible functions of nicotianamine in higher plants. *Biochem Physiol Pflanzen* **183:** 257-269

Scholz G, Faust J, Ripperger H, Schreiber K (1988) Structure-Funktion Relationships of Nicotianamine Analogues. *Pytochemistry* **27**(9): 2749-2754

Scholz G, Rudolph A (1968) A Biochemical mutant of *Lycopersicon esculentum* Mill. Isolation and properties of the ninhydrin-positive "Normalizing Factor". *Phytochemistry* **7:** 1759-1764

Scrimshaw NS (1986) Consequences of hunger for individuals and societies. *Fed Proc* **45** (10)**:** 2421-6

Shojima S , Nishizawa N-K, Fushiya S, Nozoe S, Irifune T, Mori S (1990) Biosynthesis of Phytosiderophores. In vitro biosynthesis of 2'-deoxymugineic acid from L-methionine and nicotianamine. *Plant Physiol* **93:** 1497-1503

Shojima S, Nishizawa N-K, Mori S (1989) Establishment of a Cell-Free System for the Biosynthesis of Nicotianamine. *Plant Cell Physiol* **30**(5): 673-677

Stephan UW, Grün M (1989) Physiological disorders of the nicotianamine-auxothroph tomato mutant *chloronerva* at different levels of iron nutrition II. Iron deficiency response and heavy metal metabolism. *Biochem Physiol Pflanzen* **185:** 189-200

Stephan UW, Scholz G (1993) Nicotianamine: mediator of transport of iron and heavy metals in the phloem? *Physiologia Plantarum* **88:** 522-529

Stephan UW, Scholz G, Rudolph A (1990) Distribution of nicotianamine, a presumed symplast iron transporter, in different organs of sunflower and of a tomato wild type and its mutant *chloronerva*. *Biochem Physiol Pflanzen* **186:** 81-88

Suzuki K, Higuchi K, Nakanishi H, Nishizawa H-K, Mori S (1999) Cloning of nicotianamine synthase genes from *Arabidopsis thaliana*. *Soil Sci Plant Nutr* **45**(4): 993-1002

Suzuki K, Shimada K, Nozoe S, Tanzawa K, Ogita T (1996) Isolation of nicotianamine as a gelatinase inhibitor. *The Journal of Antibiotics* **49**(12): 1284-1285

Vijayaraghavan K (1995) Strategies for control of micronutrient malnutrition. *Indian J Med Res* **102:** 216-22

von Wirén N, Khodr H, Hider RC (2000) Hydroxylated phytosiderophore species possess and enhanced chelate stability and affinity for iron *Plant Physiology* **124:** 1149-1157

von Wirén N, Klair S, Bansal S, Briat J-F, Khodr H, Shioiri T, Leigh RA, Hider RC (1999) Nicotianamine chelates both FeIII and FeII. Implications for metal transport in plants. *Plant Physiology* **119:** 1107-1114

Wallace A, Wallace GA, Cha JW (1992) Some modifications in trace metal toxicities and deficiencies in plant resulting from interactions with other elements and chelating agents. The special case of iron. *J Plant Nutr* **15**: 1589-1598

Yeaman SY (1986) the mammalian 2-oxoacid dehydrogenases: a complex family. *Trends Biochem Sci* **11:** 283-296

Yi Y, Guerinot ML (1996) Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. *The Plant Journal* **10:** 835–84

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Declaration

Hereby I declare that all the work presented in this manuscript is my own, carried out solely with the help of the literature and aid citied.

Gatersleben, October 2002

APPENDIX

THESIS

Nicotianamine synthase: Gene isolation, gene transfer and application for the manipulation of metal assimilation

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Iron deficiency is one of the most common nutritional disorders in the world. According to data of the World Health Organization the health of more than three billion people worldwide is affected by iron deficient diet. Since in most diets plants are the principal source of iron, crop plants with higher iron content, for instance in the endosperm of cereals, could contribute to the improvement of this situation. To understand the basic molecular, biochemical and physiological processes of iron assimilation is therefore a great challenge for plant biology.

Iron is essential for fundamental cellular processes such as electron transfer in photosynthesis, respiration, nitrogen fixation as well as DNA synthesis. However, the excessive accumulation causes severe damage to cellular components due to the formation of highly reactive hydroxyl radicals by the Fenton reaction. Although abundant in most well aerated soils, iron is mainly found as stable Fe(III) compounds with extremely low solubility at neutral pH. Thus, iron is nutritious, noxious and not readily available and the precise control of iron homeostasis is a basic prerequisite for cellular function.

Plants have evolved at least two different mechanisms of iron acquisition designated strategy I and II. Strategy I plants including mainly dicots facilitate iron uptake by increased acidification of the rhizosphere due to enhanced proton extrusion and the reduction of Fe(III) to Fe(II) by an inducible plasma membrane-bound reductase. Strategy II plants which mainly include graminaceous monocots release phytosiderophores of the mugineic acid family into the rhizosphere. These

compounds act as chelators of ferric ions and are taken up by root cells as Fe(III)-phytosiderophore complexes.

The non-proteinogenous amino acid nicotianamine (NA) is an essential component of both strategies (Scholz *et al.,* 1992). NA is ubiquitously found in the plant kingdom. The lack of NA is the principal distortion of the tomato mutant *chloronerva* and leads to severe disturbances of iron metabolisms.

Map-based cloning of the *chloronerva* gene of tomato (Ling et al., 1999) as well as the isolation of NA-synthase (NAS) genes of barley using a micro-sequencing approach (Herbik et al., 1999 and this thesis) led to the conclusion that a mutation in the NAS gene is the molecular defect of the *chloronerva* mutant.

Two NAS-genes of barley (*NASHOR1* and *NASHOR2*) have been isolated and characterized further by enzymatic studies after expression in *E. coli*. NAS genes encode a novel class of enzymes catalyzing the synthesis of NA from three molecules of S-adenosyl methionine and the formation of an azetidine ring.

A *NASHOR1* gene specific transcript and the corresponding gene product can be identified only in barley roots grown under iron limitation. Thus, *NASHOR1* gene regulation comprises to different aspects: organ specific expression in roots and induction under iron limitation. To explore the potential biotechnological value of such an iron regulated and root specific gene promoter, the upstream gene region has been isolated. The sequence reveals the presence of conserved sequence regions also found in front of various other barley genes. They include for instance a dehydrin (*DHN12*) gene, a *CF2/CF5* resistance gene as well as an iron deficiency-inducible (*IDS3*) gene.

In addition to single gene transfer, chromosomal engineering including defined chromosomal regions could be an interesting alternative approach to improve the iron efficiency of crop plants. As a prerequisite for this, NAS genes have been mapped and found in clusters on barley chromosomes 2H, 4H and 6H.

Database searches identified NAS-like genes also in other plant species, including rice and *Arabidopsis thaliana*. In this model plant each one NAS-like gene has been located on chromosomes 1 and 5. These two sequences, designated as *NASARA1* and *NASARA5*, have been cloned and used for gene transfer experiments aiming to the investigation and manipulation of iron assimilation.

Previous observations led to the suggestion that iron physiology of the plants could be improved by an increase of the internal NA concentration. This suggestion could be tested by various ectopic expression approaches with different NAS genes available. Gene constructs containing *NASHOR1*, *NASARA1* and *NASARA5* genes both in sense and antisense orientation under the control of the CaMV 35S promoter have been transformed into *Nicotiana tabacum*, *Lysopersicon esculentum* and *Arabidopis thaliana.* Corresponding constructs controlled by the maize ubiquitin gene promoter have been used for the transformation of *Lolium perene* and *Triticum aestivum* (co-operation with F. Altpeter, IPK). In total, more than 500 independent transgenic lines have been generated and partially analyzed.

The most promising results have been obtained with the *NASARA5* gene. NA concentrations of about 1300 nmol/g fresh weight could be detected in selected tobacco sense lines, which is the highest concentration ever detected in a plant. The average NA concentration for tobacco sense lines was increased 5 to 10 fold compared to wild type. Segregation analysis in combination with PCR- and Northern-experiments demonstrated that the high NA concentrations are the result of the *NASARA5* gene over-expression. The iron concentration in most of the transgenic plants was significantly higher than in wild type.

Enhanced concentrations of various heavy metals have deleterious effects on plant growth and development. Plants with increased tolerance to high heavy metal concentrations might be of interest both for basic research and phyto-remediation purposes. Preliminary data suggest that the 5 to 10 fold higher NA concentration due to the over-expression of the *NASARA5* gene leads to a higher nickel tolerance of transgenic tobacco plants in comparison to the segregating non-transgenic plants. When challenged with 50 µM and 100 µM of Ni^{2+} ions the transgenic plants grew more rapidly and stayed green. The segregating sister plants used as control

remained small and showed symptoms of chlorosis. Experiments using various concentrations of other heavy metals are in progress.

Iron limitation leads to complex phenotypic changes partially as the result of changed gene expression programs. The specific interaction between promoter *cis*-elements and transcription factors is a component of the underlying gene regulation. Preliminary experiments based on an array of most of the *Arabidopsis thaliana* transcription factor (TF) genes (provided by the EU consortium REGIA) identified two TF genes with highly induced expression under iron limitation. They belong to the bHLH -TF family and are the only two genes on the array up-regulated under iron deficiency. Therefore, they might be candidates for regulators of iron uptake mechanisms. Further experiments with transgenic lines of tobacco and *Arabidopsis* as well as with *Agrobacterium rhizogenes*-induced transgenic roots, which ectopically express both TF genes, are in progress.

Publications of the results

Scientific articles

Herbik A, Koch G, Mock H-P, Douchkov D, Czihal A, Thielmann J, Stephan UW, Bäumlein H (1999) Isolation, characterization and cDNA cloning of nicotianmine synthase from barley - a key enzyme for ion homeostasis in plants. *Eur J Biochem* **264:** 1-10

Douchkov D, Herbik A, Koch G, Mock H-P, Melzer M, Stephan UW, Bäumlein H (2002) Nicotianamine synthase: Gene isolation, gene transfer and application for manipulation of plant iron assimilation. *Plant and Soil* **241:** 115-119

Douchkov D, Hell R, Stephan UW, Bäumlein H (2001) Increased iron efficiency in transgenic plants due to ectopic expression of nicotianamine synthase. *Plant Nutrition – Food security and sustainability of agro-ecosystems. Horst et al. (Eds.)* 54-55

Perovic D, Douchkov D, Bäumlein H, Graner A. (2003) Genetical mapping of barley NAS genes. *(in preparation)*

Douchkov D, Gryczka C, Hell R, Stephan UW, Bäumlein H (2003) Overexpression of NASARA5 leads to improvement in the heavy metal metabolism of the plant. (in preparation).

Posters

Douchkov D, Gryczka C, Hell R, Stephan UW, Bäumlein H (2002)**.** Increased heavy metal tolerance of transgenic plants due to ectopic expression of nicotianamine synthase. *IPK Tag, Gatersleben, Germany*

Douchkov D, Hell R, Stephan UW, Bäumlein H (2001)**.** Increased iron efficiency in transgenic plants due to ectopic expression of nicotianamine synthase. *XIV International Plant Nutrition Colloquium, Hannover, Germany.*

Douchkov D, Herbik A, Koch G, Mock H-P, Melzer M, Stephan UW, Bäumlein H (2000) Nicotianamine synthase: Gene isolation, gene transfer and application for the manipulation of plant iron assimilation. *Huston, USA*.

Stephan UW, Dochkov D, Herbik A, Koch G, Mock H-P, Bäumlein H (1999**)**. Nicotianamine synthase – key enzyme in iron homeostasis of plants. *Bioiron, Sorrento, Italy.*

Table of figures

Curriculum vitae

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EDUCATION

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