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The transition metal chelator nicotianamine is synthesized by filamentous fungi

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Abstract **Nicotianamine is an important metal ligand in plants. Surprisingly, recent genome sequencing revealed that ascomycetes encode proteins with similarity to plant nicotianamine synthases (NAS). By expression in a Zn^{2+}-hypersensitive fission yeast mutant we show for a protein from *Neurospora crassa* that it indeed possesses NAS activity. Using electrospray-ionization-quadrupole-time-of-flight mass spectrometry we prove the formation of nicotianamine in *N. crassa*. Transcript level is strongly upregulated under Zn deficiency as shown by real-time PCR. These findings demonstrate that nicotianamine is more widespread in nature than anticipated and provide further evidence for a function of nicotianamine as a cytosolic chelator of Zn^{2+} ions.**

Keywords: Metal homeostasis; Metal tolerance; Metal chelation; Zinc

1. Introduction

Transition metals such as Fe, Cu, Zn are Janus-headed components of living systems. They are on the one hand essential as co-factors of a vast number of proteins, but can be deleterious when present in excess or incorrectly distributed. A highly regulated and complex homeostatic network maintains transition metal content within the narrow physiological range between deficiency and toxicity. Also, it ensures correct targeting and distribution of transition metals both intra- and intercellularly [1,2]. Degree of control and the challenge for metal homeostasis are illustrated by the fact that according to data for yeast and bacteria there are virtually no free hydrated metal ions present inside a cell [3–5].

Low molecular weight transition metal ligands play key roles in metal homeostasis. Generally, they are important for the distribution of metal ions by keeping metal ions mobile intracellularly. Furthermore, in multicellular organisms there is often transfer of metal ions from cell to cell, sometimes over wide distances. For instance in plants, metal ions taken up by root cells need to be translocated to the shoot via the xylem. Also, there is re-distribution from older plant parts to growing tissues through the phloem [6]. Precipitation of metal ions or their interaction with non-target sites along the way has to be suppressed. In addition, low molecular weight ligands can assist in the acquisition of essential metal ions. Best known in plants is the role of phytosiderophore secretion for Fe nutrition in grasses. Compounds such as mugeneic acids are able to chelate insoluble Fe(III) [2]. The resulting complexes are taken up by specialized transporters such as yellow stripe1 (YS1) from maize [7]. A fourth function is the buffering of intracellular metal ion availability under conditions of metal excess. An example is the metal-activated synthesis of phytochelatins, peptides of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$–11) [8,9].

In plants the non-proteinogenic amino acid nicotianamine is known to act as a metal ligand, presumably for a range of micronutrients. It was found to normalize the intercostal chlorosis and apparent Fe deficiency symptoms of the tomato mutant *chloronerva* [6]. Because of these mutant phenotypes nicotianamine (NA) has mostly been implicated in Fe homeostasis. It is assumed to represent the principal cytosolic Fe chelator in plants [2], required for radial transport of Fe in roots and distribution of Fe in the leaves. A likely second function is a contribution to transport between cells and translocation of Fe via the phloem. NA forms stable complexes in vitro also with Cu(II), Zn(II), Ni(II) and Mn(II) ions [10,11]. There is evidence accumulating for a role of NA in the homeostasis of these micronutrients as well. In the *chloronerva* mutant, root Cu content is higher than in wildtype plants while shoot content is lower, suggesting NA-dependent Cu translocation via the xylem [12]. More recent data obtained for a transgenic tobacco line rendered NA deficient through the ectopic expression of a barley nicotianamine aminotransferase indicated that NA is important for the transport of Cu and Zn into young leaves. Content of these metals was significantly reduced in NAAT tobacco compared to wildtype [13].

Nicotianamine is formed from three molecules of *S*-adenosylmethionine by the enzyme nicotianamine synthase (NAS). Genes encoding nicotianamine synthases were isolated in two ways. NAS proteins were purified from barley [14,15]. The *chloronerva* gene from tomato was isolated by map-based cloning [16]. At the time of cloning, nicotianamine synthases appeared to be a plant-specific gene family with just a one distantly related gene in the archeon *Methanobacterium thermoautotrophicum* [15]. This was expected from the data on the ubiquitous occurrence of nicotianamine in the plant

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Abbreviations: Cap-LC-ESI-QTOF-MS, capillary liquid chromatography coupled to electrospray-ionization-quadrupole-time-of-flight mass spectrometry; CID, collision-induced dissociation; FMOC, 9-fluorenylmethylchloroformate; NA, nicotianamine; NAS, nicotianamine synthase

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kingdom [17]. We report here on the detection of nicotianamine and of functional nicotianamine synthases in filamentous fungi, namely *Neurospora crassa*.

2. Materials and methods

2.1. Fungal strains and cultivation

N. crassa strain CBS 195.57 was obtained from the Centraalbureau voor Schimmelcultures (Utrecht, Netherlands) and cultivated on malt peptone agar. For DNA/RNA extraction and NA analysis, *N. crassa* was grown in liquid culture (Vogel's medium N) [18] at 25 °C under gentle shaking. *Schizosaccharomyces pombe zhf* cells were grown as described [19].

2.2. Cloning, expression analysis, functional characterization in S. pombe

The gene encoding *N. crassa* hypothetical protein XP_958379 (=NcNAS) was PCR amplified from *N. crassa* genomic DNA, isolated using an established protocol [20]. The primers used added *Not*I sites for cloning into the *S. pombe* expression vector pSGP72 (5′: CGCGGCGGCCGCATGCCTGCCCTTCTCTCAGTC, 3′: CGCGGCGGCCGCAACCCCTAACCCTAGCAACAAC). Expression in *S. pombe*, monitoring of expression by Western analysis, and growth assays were carried out as described [21].

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's protocol. First strand cDNA was synthesized with first strand cDNA synthesis kit (Fermentas). One microgram of DNaseI-treated total RNA was used. Quantitative real-time PCR was performed in an ABI Prism 7000 (Applied Biosystems, Foster City, USA). Each sample was analyzed in triplicate. Primers used were: fw AGGAGAAGAAATCAACCGCTTACTG, rev GATCTGCTGGATTACGCTTGGAG. *N. crassa* elongation factor 2 (Accession No. AF258620) served as constitutive control. Primers used were: fw TCGCTACTGACGACAGAGAGAAGG and rev GGATCATCATCTCCAACAAGCAGTC. For the calculation of the threshold cycle (C_T) values the mean value of each triplicate was used. To normalize target gene expression, the difference between the C_T of NcNAS and the C_T of EF-2 was calculated (=ΔC_T value); relative transcript level = $1000 \times 2^{-\Delta C_T}$.

2.3. Nicotianamine analysis

For NA detection in *S. pombe*, cell pellets were suspended in 50 μL water. Samples were vigorously vortexed with 30 mg glass beads (425–600 μm, Sigma) for 2 min, heated at 80 °C for 2 min, vortexed again for 2 min. Twenty microliters extract were diluted with 60 μL 0.5 M boric acid (pH 7.7) and derivatized by addition of 20 μL 9-fluorenylmethylchloroformate (FMOC) (10 mM, Sigma) in acetone. After 1 min the reaction was quenched by addition of 20 μL adamantane-1-amine hydrochloride (20 mM, Sigma) in acetone–water 3:1 (v/v). Two microliters of the derivatized extract were injected and separated using a capillary LC system (Ultimate, Dionex) equipped with a C_8-phase (Luna 3μ C8(2) 100 Å, 150 × 0.3 mm, Phenomenex) using the following gradient at a flow rate of 5 μL min^{-1}: 0–5 min, isocratic 95% A (H_2O/0.1% HCO_2H), 5% B (CH_3CN/0.1% HCO_2H); 5–25 min linear from 5% B to 95% B. Eluted compounds were detected by an API QSTAR Pulsar Hybrid QTOF-MS (Applied Biosystems) equipped with an ion spray source in positive ion mode. Ions were detected within *m/z* 500–800 applying an accumulation time of 2 s. NA-FMOC (t_r = 23.7 min) was quantified using reconstructed ion chromatograms (*m/z* 526.1–526.3) corresponding to protonated NA-FMOC (calc. for $C_{27}H_{32}N_3O_8^+$: 526.2184) and an external calibration curve obtained by derivatization of a dilution series prepared from an authentic standard.

For NA detection in *N. crassa*, 300 mg of freshly ground mycelium were extracted twice with 1000 μL water at 80 °C for 30 min. The combined extracts were evaporated at 20 °C using a SpeedVac. The resulting residue was dissolved in 60 μL 0.5 M boric acid (pH 7.7) and derivatized as described. Two microliters extract were separated using a modified gradient: 0–40 min linear from 20% B to 70% B. For detection of NA-FMOC the mass spectrometer was operated in product ion mode. Collision-induced dissociation (CID) was accomplished by applying a collision energy of 20 or 35 eV.

2.4. Elemental analysis

Harvested mycelium was washed four times with Millipore water at 4 °C for 10 min under shaking. Samples were lyophilized for 48 h and DW was recorded. Samples were than digested in 3 ml 65% HNO_3/1 ml 30% H_2O_2. The volume was adjusted to 8 ml with Millipore water. Zn and Fe content were determined by atomic absorption spectroscopy using an AAnalyst 800 (Perkin–Elmer, Überlingen, Germany).

3. Results

In recent years the genomes of a few filamentous fungi have been sequenced. A search of GenBank with the AtNAS2 protein sequence using the BLASTp and tBLASTn algorithms revealed that the genomes of the ascomycetes *N. crassa* and *Magnaporthe grisea* contain genes that encode hypothetical proteins with similarity to plant nicotianamine synthases: *N. crassa* hypothetical protein XP_958379 [22], identity with AtNAS2 22.5%; *M. grisea* hypothetical protein XP_365204 [23], identity with AtNAS2 21.9%. In addition, a partial sequence in *Podospora anserina* was annotated as a putative nicotianamine synthase (Accession No. AAO25955). Fig. 1 shows an alignment with both monocot and dicot NAS proteins highlighting conserved stretches of amino acids. We retrieved all non-plant protein sequences similar to AtNAS2 with an *E* value <0.01 from GenBank and constructed a phylogenetic tree including several NAS sequences from monocots (barley, rice, maize) and dicots (tomato, *A. thaliana*) (Fig. 2). The sequences fall into two main clusters: proteins from Archaea species that are mostly annotated as putative proteins or as methyl transferases (including the initially found bacterial sequence) and nicotianamine synthases. Within the nicotianamine synthase cluster the monocot and dicot sequences fall into distinct groups. A third, and more distantly related group, contains the two fungal sequences. Thus, the question arose whether filamentous fungi express functional NA synthases. The alignment revealed blocks of conserved sequence. However, the phenylalanine in position 238 in LeNAS, which is mutated in *chloronerva* to a serine and apparently essential for activity [16], is not conserved in the two fungal sequences (Fig. 1).

We had previously shown that expression in the Zn^{2+} hypersensitive *S. pombe* mutant *zhf* [19] is a suitable way of demonstrating NAS function of proteins. NA formation in these mutant cells partially rescues the Zn^{2+} hypersensitivity phenotype, most likely due to the formation of stable intracellular NA–Zn complexes [21]. Thus, we cloned the putative NAS gene (=NcNAS) – including a predicted intron of 50 bp – from *N. crassa* genomic DNA and inserted it into an *S. pombe* expression vector. *S. pombe zhf* cells transformed with NcNAS were checked for expression of the protein. Immunoblot analysis using an HA monoclonal antibody recognizing the triple HA tag added to the C-terminus demonstrated strong expression, controllable by suppression of the *nmt1* promoter with thiamine (Fig. 3A). Two bands were detected in cells carrying the NcNAS gene. Following transfer to medium without thiamine a strong increase in expression could be observed, peaking at about 6 h. The detected bands corresponded to sizes of about 41 and 32 kDa. The expected size of NcNAS-HA is 40.5 kDa (37 + 3.5 kDa for the HA tag). A sequence comparison of fragments generated by PCR amplification of NcNAS from pSGP72-NcNAS vector DNA and from cDNA of NcNAS-HA-expressing *zhf* cells demonstrated that the predicted 50 bp intron was correctly spliced out (not shown).

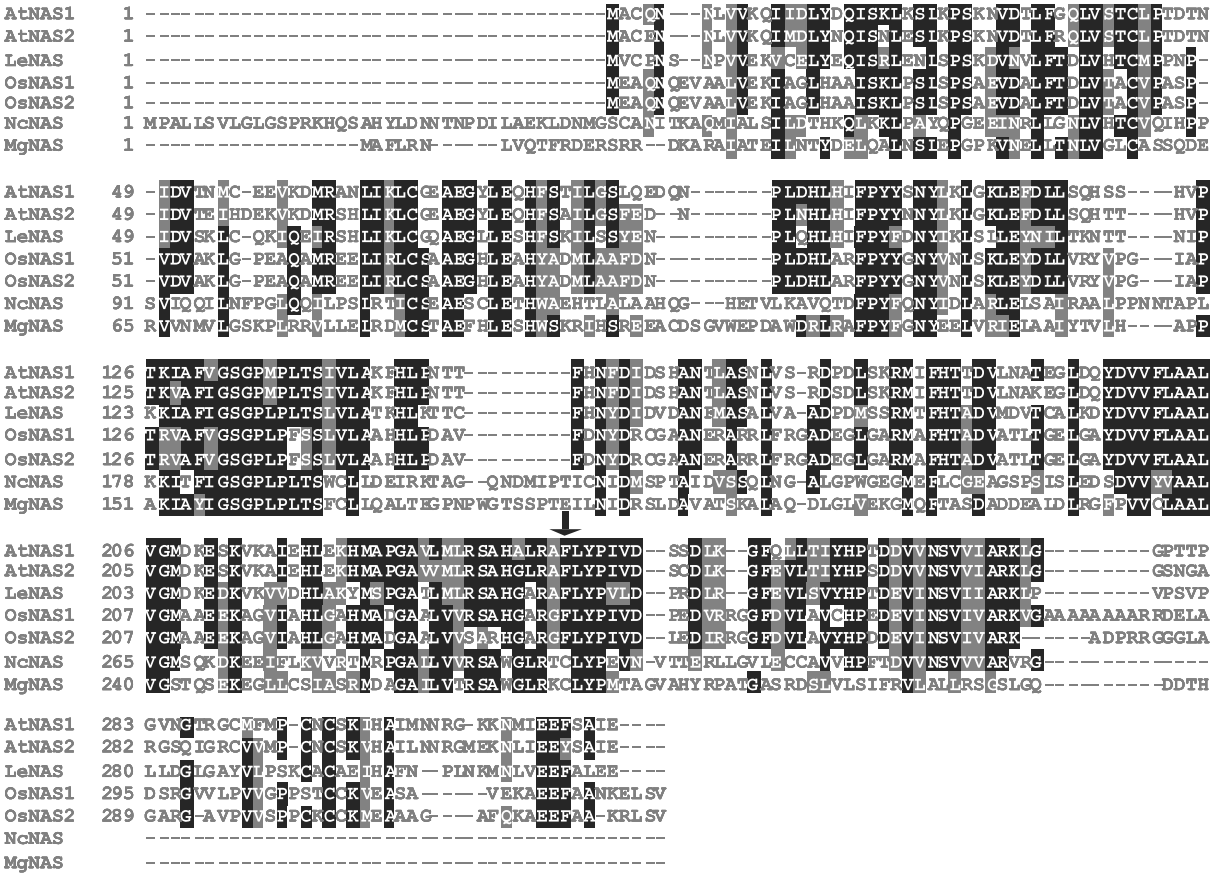

Fig. 1. Putative NAS sequences from *Neurospora crassa* and *Magnaporthe grisea* (NcNAS, MgNAS, respectively) were aligned with representative NAS sequences from monocots (OsNAS1 and 2 from rice) and dicots (AtNAS1 and 2 from *A. thaliana*, LeNAS from tomato) using the ClustalW algorithm. The output was generated with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Black shading indicates amino acid residues that are conserved in the majority of sequences. Substitutions by similar amino acids are shaded in grey. The amino acid altered in the *chloronerva* null mutant NAS of tomato is marked with an arrow (phenylalanine 238 is in the mutant changed to a serine).

NcNAS-HA expressing cells were then grown in the presence of elevated Zn^{2+} levels. In comparison to cells transformed with empty vector a strong increase in growth under excess Zn^{2+} was observed both on agar plates and in liquid culture (Fig. 3B and C). In control medium both strains grew equally well. At 150 μM Zn^{2+}, however, *zhf* cells carrying the empty vector showed a reduction in growth rate of about 82.9 (±7.4)% while growth of *zhf* cells expressing NcNAS-HA was reduced by only 27.1 (±20.4)% ($n = 8$). Strong expression of NcNAS was required as there was no phenotype in cells growing in the presence of thiamine (data not shown). To confirm NA accumulation in NcNAS-expressing *zhf* cells we analyzed FMOC-derivatized extracts by CapLC-ESI-QTOF-MS [24]. NA-FMOC was unequivocally detected as proven by co-elution with a reference, the exact mass, and CID-MS analysis. Nine hours after de-repression, about 67 ng/mg d.w. NA were detected in *zhf* cells carrying pSGP72-NcNAS, 24 h after de-repression about 234 ng/mg d.w. No NA-FMOC was detectable in *zhf* cells carrying the empty vector. We concluded that the *N. crassa* genome encodes a protein with NAS activity.

Next we searched for nicotianamine synthesis in *N. crassa*. Mycelium was extracted using a protocol established for plant roots. Following concentration and FMOC derivatization a signal with a mass of 526.221 and a retention time corresponding to NA-FMOC standard was detected. The theoretical mass of NA-FMOC ($[M+H]^+$) is 526.218. Co-injection showed that the signal from *N. crassa* mycelium co-migrated with NA-FMOC standard (Fig. 4A). Product ion spectra of the *m/z* 526 from *N. crassa* and NA-FMOC standard were almost identical (Fig. 4B and C). Thus, we unequivocally demonstrated synthesis of nicotianamine in *N. crassa*. The NA content in *N. crassa* under standard conditions was determined as about 110 ng/g f.w.

Plant NAS gene expression is responsive to changes in external micronutrient supply [25,26]. Therefore, we checked whether NcNAS transcript level changes upon exposure to Zn or Fe deficiency. *N. crassa* was grown for 7 d in medium devoid of either Zn^{2+} or Fe^{2+}. This led to a reduction of total Zn and Fe content by about 60% and 50%, respectively, as shown by atomic absorption spectroscopy (Fig. 5A). Growth was significantly slowed as compared to control cultures. Under Zn deficiency dry weight was reduced by >90% and in Fe-free medium by about 50% (data not shown). When we analyzed NcNAS transcript levels by quantitative real-time PCR we found a dramatic upregulation (about 30-fold) in Zn-deficient mycelium but not in Fe-deficient mycelium (Fig. 5B).

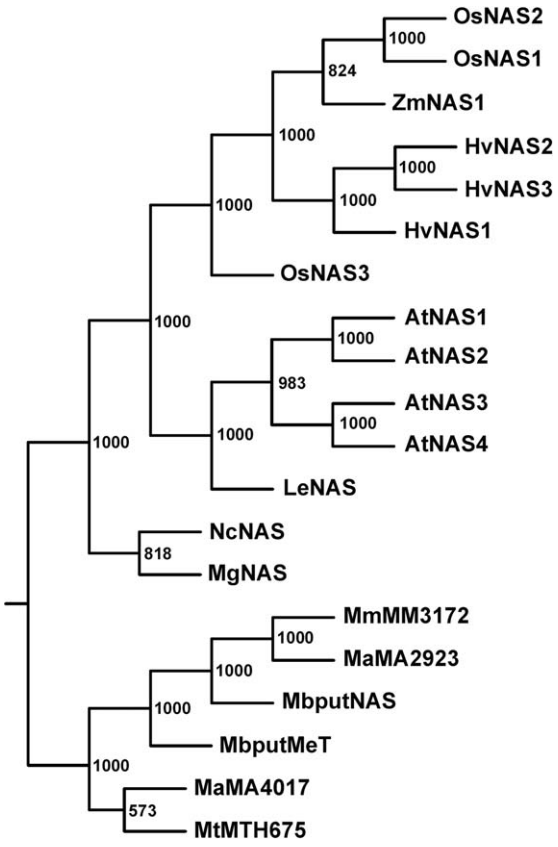

Fig. 2. Protein sequences with similarity to plant NAS proteins (highest e-score 0.007) were identified through BLASTp and tBLASTn searches and aligned with selected plant NAS sequences using ClustalW. All subsequent analyses were carried out with the PHYLIP 3.6 program (Joseph Felsenstein, University of Washington, Seattle, USA). Aligned sequences were bootstrapped with 1000 replicates and distances measured using the Jones-Taylor-Thornton model. Following UPGMA clustering a consensus tree was constructed. Numbers at the branch points indicate bootstrap fraction. Sequences included in the analysis were (Accession No. in brackets): HvNAS1 (AF136941_1), HvNAS2 (Q9ZQV7), HvNAS3 (BAA74581), ZmNAS1 (BAB87846), OsNAS1 (Q9SXQ7), OsNAS2 (Q9FEG8), OsNAS3 (BAC21363), LeNAS (=chloronerva) (CAB42052), AtNAS1 (At5g04950), AtNAS2 (At5g56080), AtNAS3 (At1g09240), AtNAS4 (At1g56430), *Methanosarcina macei* hypothetical protein MM3172 (NP_635196), *Methanosarcina acetivorans* C2A hypothetical protein MA2923 (NP_617818), *Methanosarcina barkeri* putative NAS MbputNAS (ZP_00543445), *Methanosarcina barkeri* putative methyltransferase MbputMeT (YP_304122), *Methanosarcina acetivorans* C2A hypothetical protein MA4017 (NP_618886), *Methanothermobacter thermautotrophicus* str. Delta H hypothetical protein MTH675 (NP_275817).

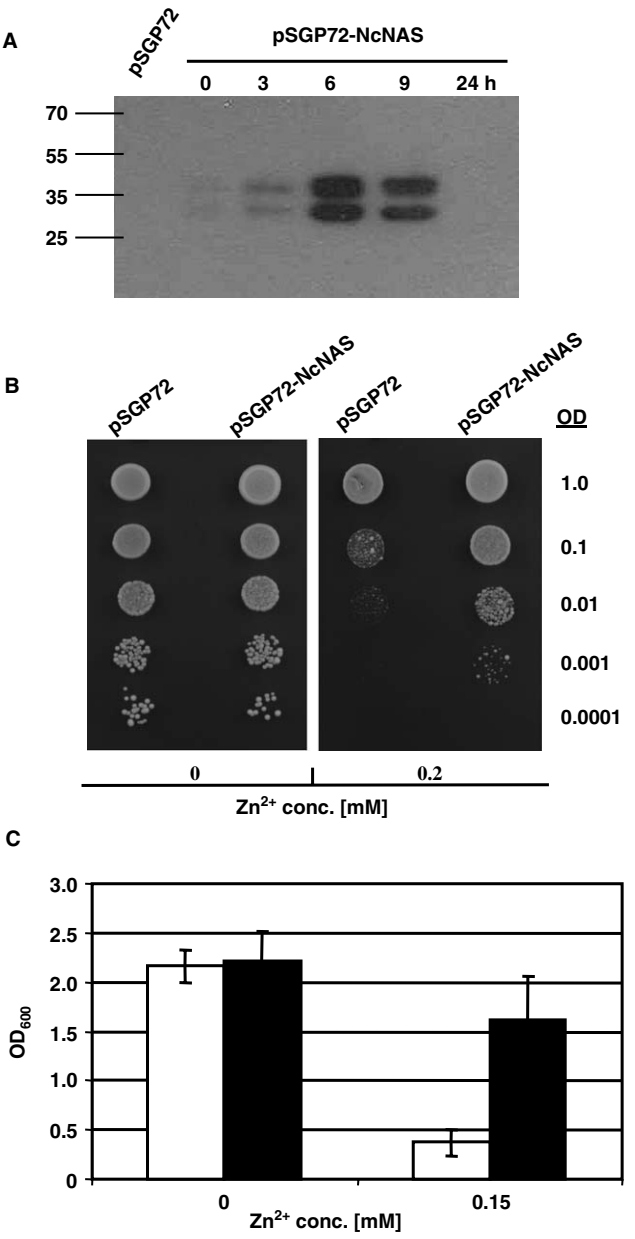

Fig. 3. Expression of *Neurospora crassa* NAS (NcNAS) partially rescues the Zn^{2+} hypersensitivity of *S. pombe zhf* mutant cells. (A) A triple HA tag was added to the C-terminus of NcNAS. Regulatable expression under control of the thiamine-repressed nmt1 promoter was monitored by SDS–PAGE, Western analysis and immunostaining using a monoclonal anti-HA antibody. Shown are the results for cells carrying either the empty vector pSGP72 or cells carrying pSGP72-NcNAS and grown in the absence of thiamine for 0–24 h. (B) Growth of *zhf S. pombe* cells carrying either the empty vector or expressing NcNAS-HA was assayed by spotting serial dilutions of cells (OD_{600} is shown on the right) on EMM plates with (right) or without (left) added Zn^{2+}. (C) Growth of *zhf S. pombe* cells carrying either the empty vector (white bars) or expressing NcNAS-HA (black bars) in EMM medium with or without added Zn^{2+}. OD_{600} was measured after 18–20 h. Shown are the means of eight independent experiments. Error bars indicate SD.

4. Discussion

The proven transition metal ligand nicotianamine has so far been widely considered a metabolite typical for plants. There is only one mention of possible NA occurrence in a basidiomycete of the genus Polyporus in a study that assessed distribution of NA within the plant kingdom [17]. Also, NA has been discussed mostly in the context of long-distance transport of Fe (and possibly other micronutrients) in the phloem and of Cu in the xylem [2,12]. The proof of a functional NAS and NA synthesis in filamentous fungi reported here (i) shows that nicotianamine might be far more widespread than previously anticipated, and (ii) provides strong additional evidence for a physiological role of NA as an intracellular – most likely cytosolic – ligand for transition metal cations, in particular Zn^{2+}.

While phylogenetic analysis placed the recently annotated NAS-like sequences from fungi in a group with proven plant

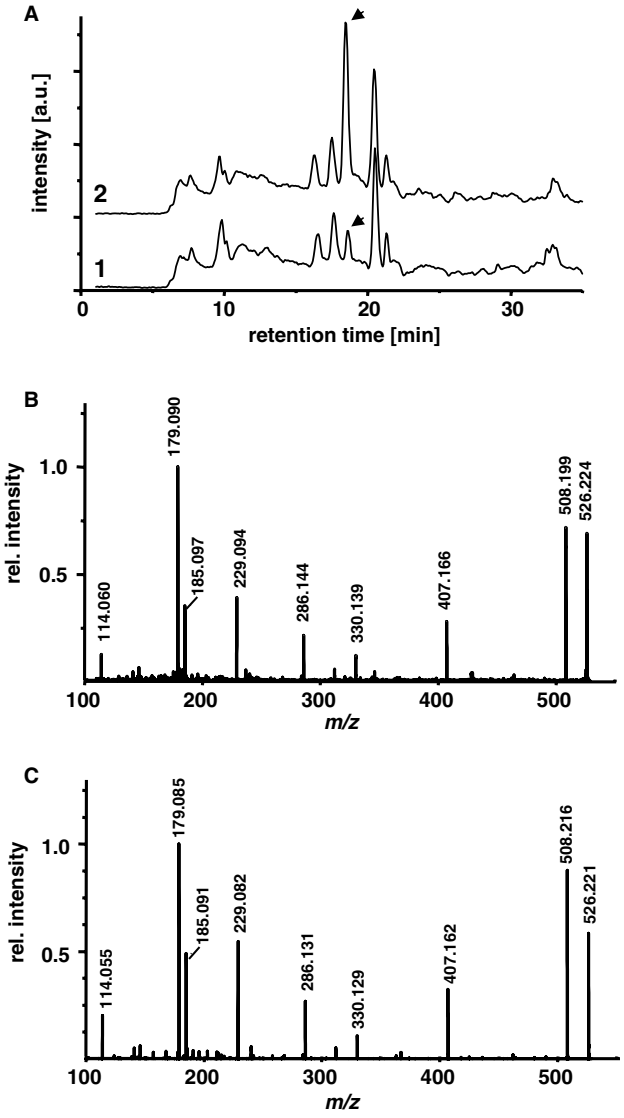

Fig. 4. Unequivocal detection of nicotianamine in *N. crassa*. (A) The ion traces for *m/z* 526–526.5 of derivatized fungal extract (1), and after spiking with NA standard (2). The arrow indicates the NA peak. (B and C) Product ion spectra for *m/z* 526 from FMOC-derivatized fungal extract and for the FMOC-derivatized standard.

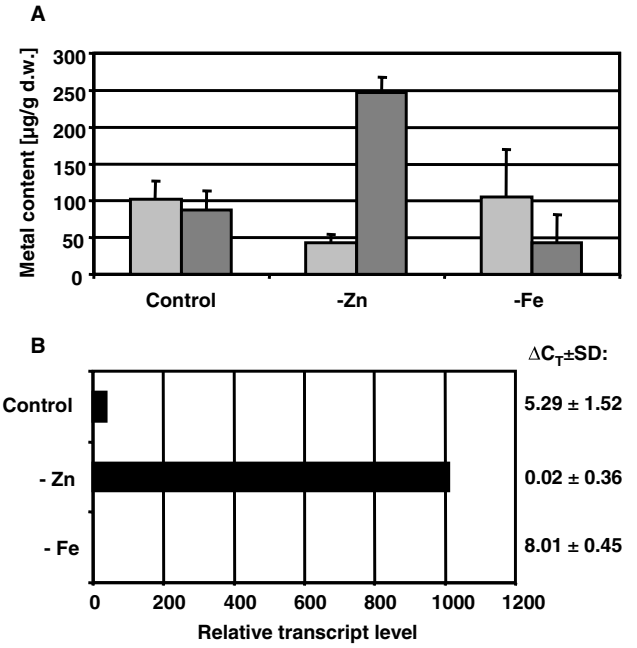

Fig. 5. *NcNAS* expression is strongly upregulated under Zn-deplete conditions. (A) Total Zn (light grey) and Fe (dark grey) content of mycelium cultivated under control conditions, in medium without Zn^{2+} or without Fe^{2+}. Shown are the results for one experiment, analyzed in triplicates. Very similar results were obtained for two additional independent experiments. (B) Quantitative real-time PCR analysis of *NcNAS* expression in control medium and under conditions of either Zn or Fe deficiency. The summary of three independent experiments, each measured in triplicates, is shown. Expression of elongation factor 2 served as a constitutive control.

NAS proteins, inspection of sequence alignments suggested that the fungal proteins would not show NAS activity. The phenotype of *S. pombe zhf* cells expressing NcNAS, however, clearly demonstrated NAS function. NA accumulation was unequivocally detected by capillary liquid chromatography coupled to electrospray-ionization-quadrupole-time-of-flight mass spectrometry (Cap-LC-ESI-QTOF-MS) analysis. Furthermore, NAS expression resulted in a strong increase in Zn^{2+} tolerance, showing again that NA forms stable complexes with Zn(II) in the cytosol of *zhf* cells. Given the high degree of conservedness of metal homeostasis in eukaryotes we hypothesize that NA forms stable complexes with Zn(II) also in plant cells. The main chelation, distribution and sequestration pathways for transition metal ions, and thus the suite of potential binding partners for Zn(II), are likely to be very similar in yeast and plant cells.

A hallmark of filamentous fungi is cytoplasmic continuity. The cellular compartments of hyphae are delineated by incomplete septa that allow passage of cytoplasmic components [27]. Synthesis of NA in *N. crassa* – which was unequivocally demonstrated by Cap-LC-ESI-QTOF-MS – demonstrates occurrence of NA in organisms other than vascular plants where long-distance transport of micronutrients is required. This finding is a strong hint for a role of NA as an intracellular, most likely cytosolic, ligand for transition metal cations. It is hypothesized to be involved in distribution of Zn and other micronutrients across the fungal mycelium. The NA content we determined for *N. crassa* mycelium cultivated in a standard growth medium is much lower than found in most plant samples analyzed so far. However, we do not know how NA synthesis is modulated in response to varying micronutrient supply. Our expression analysis indicated that NcNAS expression is indeed responsive to Zn deficiency, a culture condition that we clearly established as indicated by the effects on total Zn content and growth rate. This observation provides additional support for a role of NA in intracellular Zn chelation. NA might protect against potentially high rates of Zn^{2+} uptake under conditions of Zn starvation. A similar function is assumed for the Fe-siderophore ferricrocin which accumulates in *N. crassa* grown in Fe-deplete medium [28].

The proof of NA synthesis in filamentous fungi offers interesting perspectives for future studies. First, there is no multigene family as in most plants. Thus, *N. crassa* could serve as an additional system to study the physiological function of NA. The generation of knock-out strains is now a routine procedure [29] (“Neurospora Genome Project”, http://www.

dartmouth.edu/~neurosporagenome/index.html). Second, the fungal proteins might represent valuable material for the elucidation of NAS structure and catalytic mechanism as their amino acid sequences deviate substantially from those of plant NAS proteins, yet the proteins show NAS activity.

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