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Cloning and characterization of the nicotianamine synthase gene in *Eruca vesicaria* subsp *sativa*

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ABSTRACT. Nicotianamine (NA) is a ubiquitous metabolite in plants that bind heavy metals, is crucial for metal homeostasis, and is also an important metal chelator that facilitates long-distance metal transport and sequestration. NA synthesis is catalyzed by the enzyme nicotianamine synthase (NAS). *Eruca vesicaria* subsp *sativa* is highly tolerant to Ni, Pb, and Zn. In this study, a gene encoding *EvNAS* was cloned and characterized in *E. vesicaria* subsp *sativa*. The full-length *EvNAS* cDNA sequence contained a 111-bp 5'-untranslated region (UTR), a 155-bp 3'-UTR, and a 966-bp open reading frame encoding 322-amino acid residues. The *EvNAS* genomic sequence contained no introns, which is similar to previously reported *NAS* genes. The deduced translation of *EvNAS* contained a well-conserved NAS domain (1-279 amino acids) and an LIKI-CGEAEG box identical to some *Brassica* NAS and to the LIRL-box in most plant NAS, which is essential for DNA binding. Phylogenetic analysis indicated that EvNAS was most closely related to *Brassica rapa*

NAS3 within the Cruciferae, followed by *Thlaspi* NAS1, *Camelina* NAS3, and *Arabidopsis* NAS3. A reverse transcription-polymerase chain reaction indicated that *EvNAS* expression was greatest in the leaves, followed by the flower buds and hypocotyls. *EvNAS* was moderately expressed in the roots.

Key words: *Eruca vesicaria* subsp *sativa*; Nicotianamine synthase; Gene cloning

INTRODUCTION

Nicotianamine (NA) is a key ligand that is involved in metal transport and homeostasis in plants (Inoue et al., 2003; Douchkov et al., 2005). NA is a ubiquitous metabolite in plants that is able to bind heavy metals both *in vitro* and *in vivo*, and that is crucial to the homeostasis of essential metals such as iron, zinc, manganese, nickel, and copper (Takahashi et al., 2003; Pianelli et al., 2005). NA is also an important metal chelator that facilitates long-distance metal transport and sequestration (Ling et al., 1996, 1999; Mari et al., 2006; Dreyfus et al., 2009). The synthesis of NA from three molecules of *S*-adenosyl-L-methionine is catalyzed by the enzyme nicotianamine synthase (NAS; Kawai et al., 1988; Shojima et al., 1989). Vacchina et al. (2003) demonstrated that in yeast cells expressing *TcNAS1* cDNA from *Thlaspi caerulescens* (now *Noccaea caerulescens*), NA bound nickel *in vivo*, resulting in a reduction in nickel cytotoxicity. Pianelli et al. (2005) reported enhanced nickel resistance both at the cellular and whole-plant level in *Arabidopsis thaliana* transgenic lines that had over-accumulated NA. They also observed a direct correlation between the amounts of NA accumulated *in planta* and the nickel resistance phenotype. Moreover, these plants can accumulate up to 0.25% dry weight of their aerial parts of nickel on nickel-contaminated soil without any symptom of toxicity. Pianelli et al. (2005) also demonstrated that improved nickel tolerance can be achieved by increasing resistance at the cellular level by NA accumulation, and that such a strategy can be envisaged for phytoremediation purposes.

The results of our previous study indicated that *Eruca* is highly tolerant to Ni, Zn, and Pb (Zhi et al., 2015). Here, we report the cloning and characterization of full-length cDNA and genomic DNA sequences of the *EvNAS* gene from *Eruca vesicaria* subsp *sativa*. The expression profiles of *EvNAS* in different tissues were also examined.

MATERIAL AND METHODS

Plants and tissue collection

E. vesicaria subsp *sativa* 'hubu-14' (*E. sativa* 'hubu-14'), which is highly tolerant to Ni, Zn, and Pb (Zhi et al., 2015), was used for cloning the *NAS* gene. Seeds were germinated on filter paper immersed in liquid Murashige and Skoog medium without sugar or organic components. Seven days after seed inoculation, the roots and hypocotyls were harvested separately and frozen immediately in liquid nitrogen and stored at -80°C. Young leaves and flower buds were collected from the field and also frozen immediately in liquid nitrogen and stored at -80°C.

Cloning of full-length cDNA and *EvNAS* genomic DNA

Total RNA was isolated using TRIzol® Total RNA Extraction Reagent (Takara) according to

the manufacturer protocol. First-strand cDNA was synthesized with 1 µg total RNA and 1 µL ReverTra Ace® (100 U, Toyobo) following the manufacturer instructions. *NAS* sequences from different higher plants (*Arabidopsis halleri*, JQ619642.1; *Arabidopsis thaliana*, AB181237.1; *Brassica rapa*, XM_009144131; *Camelina sativa*, XM_010425211.1; *Citrus sinensis*, XM_006472133.1; *Cucumis melo*, XP_008453616.1; *Lotus japonicas*, BAH22562.1; *Malus baccata*, ABD64879.1; *Medicago truncatula*, XP_003591220.1; *Nicotiana tomentosiformis*, XP_009619408.1; *Nicotiana sylvestris*, XP_009804361.1; *Noccaea caerulescens*, JF714218.1; *Solanum lycopersicum*, XP_004230307.1; and *Tarenaya hassleriana*, XM_010545902) were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and some highly conserved regions were retrieved. A pair of degenerated primers (NAS core-FP and NAS core-RP; Table 1) was designed according to these highly conserved regions to amplify a 284-bp fragment of the *NAS* coding region using leaf and root cDNAs as templates. Two gene-specific primers (5'-RACE-NAS and 3'-RACE-NAS; Table 1) were designed for 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE to clone full-length *EvNAS* cDNA using a SMARTer™ RACE Amplification Kit (Clontech). Primers (NAS-FP and NAS-RP; Table 1) based on sequences of the 5'-RACE and 3'-RACE fragments were used to amplify the full-length *EvNAS* from cDNA and genomic DNA. All of the polymerase chain reaction (PCR) products were cloned into a pMD-18T vector (Takara) and sequenced. At least five independent clones were sequenced for each PCR product to ensure sequencing accuracy.

Table 1. Primers and sequences used in the study.

PCR system	Primer name	Primer sequence (5'-3')	Amplicon size/bp
Core fragment PCR	NAS core-FP	TCACGTCCATCGTTCTTGCTTCA	284
	NAS core-RP	AGCACCAGGAGCCATGTGTTTCTG	
5'-RACE	5'-RACE-NAS	GGGCAAGCAGTGGTATCAACGCAGAGT	674
3'-RACE	3'-RACE-NAS	GTACTCTGCGTTGATACCACTGCTTGC	741
cDNA full-length PCR	NAS-FP	GGGGACCCGAGAAACACAATAT	1143
	NAS-RP	ACATTACACCAACATACAACACAAAGAC	
Genomic DNA PCR	NAS-FP	GGGGACCCGAGAAACACAATAT	1143
	NAS-RP	ACATTACACCAACATACAACACAAAGAC	
qRT-PCR	NAS qRT-FP	CGTCGTGTTTCTAGCAGCTCTCGTTGG	277
	NAS qRT-RP	AGCACGATGGCCCACCTAAAATACTCTC	
	actin qRT-FP	CGCCGCTTAACCCTAAGGCTAACAG	322
	actin qRT-RP	TTCTCTTTAATGTCACGGACGATTT	

PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Sequence analysis

ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to perform open reading frame (ORF) analysis and deduce the amino acid sequence. The genomic organization of the *EvNAS* genomic DNA was determined using Splign (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). The protein theoretical isoelectric point (pI) and molecular weight (Mw) were computed using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Nucleotides, as well as the derived amino acid sequences of *EvNAS*, were BLASTP-searched with reported NAS gene sequences of different species using the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST). A motif scan of the deduced *EvNAS* protein was conducted using the Pfam database (http://pfam.sanger.ac.uk/), the secondary structure was predicted by SOPMA (http://npsa-pbil.ibcp.fr/), and a phylogenetic tree was constructed using DNAMAN (Lynnon Biosoft).

Expression analysis by real-time quantitative reverse transcription-PCR (qRT-PCR)

To investigate the *EvNAS* mRNA expression profile, total RNA from leaves, roots, hypocotyls, and flower buds were extracted for real-time qRT-PCR. The constitutively expressed actin gene was used as an internal control to normalize the transcript levels of the target gene. The primers described in Table 1 (NAS qRT-FP and NAS qRT-RP) were used to detect *EvNAS* expression levels. The qRT-PCRs were performed in triplicate according to the manufacturer (Bioer) instructions using the comparative Ct method, and the results are presented as means and standard deviations.

Statistical analysis

Statistical analysis was conducted using the SPSS software (version 18.0, SPSS Inc., Chicago, IL, USA). Spearman's rank correlation was used to assess relationships between *EvNAS* expression levels in different tissues of *E. vesicaria* subsp *sativa*, and analysis of variance was conducted to identify gene transcripts that exhibited significant ($P < 0.05$) changes in relative abundance in the different tissues.

RESULTS

Cloning and sequence analysis of *EvNAS*

By using degenerated primers based on highly conserved sequences of *NAS* from different species, a 284-bp conserved fragment was amplified from *E. vesicaria* subsp *sativa* root, hypocotyl, leaf, and flower bud mRNA. By aligning and splicing the sequences of the conserved region, a 1235-bp full-length *EvNAS* cDNA was predicted to contain a 111-bp 5'-untranslated region (UTR), a 966-bp coding region, and a 155-bp 3'-UTR (Figure 1). A 1143-bp sequence was amplified from cDNA and genomic DNA using primers based on 3'- and 5'-RACE sequences. ORF Finder indicated that the *EvNAS* cDNA sequence contained a 966-bp ORF.

Analysis of deduced amino acid sequence

The pI of the EvNAS protein was 5.70 and the Mw was 35954.75 Da. A comparison of amino acid sequence homologies and biochemical properties of NAS from different plant species is provided in Table 2.

The BLASTP analysis revealed that the deduced amino acid sequence of EvNAS shared the highest identity (94.1%) with *B. rapa* NAS3 (GenBank accession No. XM_009149927.1), followed by *T. caerulescens* NAS1 (AJ300446.1, 90.4% identity), *C. sativa* NAS3 (XM_010459903.1, 89.4% identity), *A. halleri* NAS2 (JQ619642.1, 87.3% identity), and *A. halleri* NAS3 (AJ580399.1, 87.0% identity). The putative secondary structure of the deduced amino acid sequence is presented in Figure 2.

Multiple alignments of the EvNAS protein with NAS from other species are presented in Figure 3. The motif scan in Pfam confirmed that the EvNAS protein contains an intact NAS domain (1-279 amino acids) and a LIKI-CGEAEG box identical to some *Brassica* NAS and similar to the LIRL-box of most plant NAS.

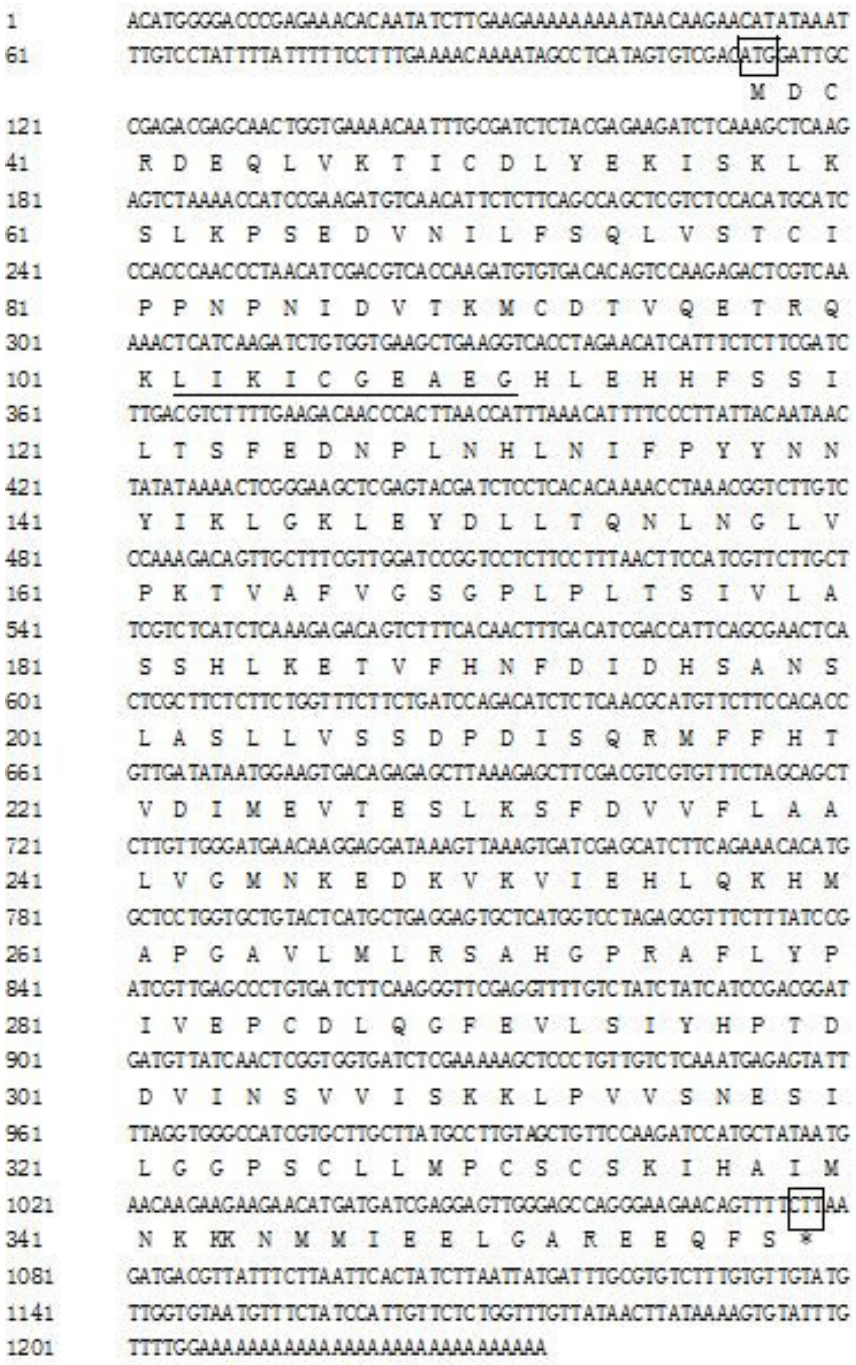

Figure 1. Full-length NAS cDNA sequence of *Eruca vesicaria* subsp *sativa* and the deduced amino acid sequence. The initiator codon and stop codon are boxed, and sequences before the initiator codon and after the stop codon are the 5'-untranslated region (UTR) and the 3'-UTR, respectively. The LIKI-CGEAEG box is underlined.

Table 2. Homologies of amino acid sequences and biochemical properties of nicotianamine synthase (NAS) in different plant species.

NAS from plants	Accession No.	No. of amino acid	Similarity (%)	Identity (%)	Molecular weight	Isoelectric point
Eruca vesicaria subsp *sativa* NAS		322	-	-	35,954.75	5.7
Brassica rapa NAS3	XM_009149927.1	323	96.0	94.1	36,076.79	5.51
Thlaspi caerulescens NAS1	AJ300446.1	321	93.8	90.4	35,896.92	5.7
Camelina sativa NAS3	XM_010459903.1	322	92.5	89.4	35,857.66	5.52
Arabidopsis halleri NAS2	JQ619642.1	320	90.7	87.3	35,683.44	5.85
Arabidopsis halleri NAS3	AJ580399.1	320	90.1	87.0	35,749.47	5.69
Arabidopsis thaliana NAS4	AB181237.1	324	84.3	74.4	36,349.59	5.08
Brassica rapa NAS1	XM_009144131.1	323	83.3	74.4	36,207.38	5.23
Noccaea caerulescens NAS4	JF714218.1	322	82.7	74.4	36,201.37	5.12
Solanum lycopersicum NAS	NP_001296307	317	74.4	59.6	35,300.68	5.72
Arabidopsis thaliana NAS1	AED90808.1	320	73.2	64.0	35,544.93	6.15
Camelina sativa NAS1	XM_010425211.1	321	72.4	63.2	35,670.86	5.95
Arabidopsis thaliana NAS2	NP_200419.1	320	72.3	63.1	35,676.79	6.05
Arabidopsis thaliana NAS3	NM_100794	320	72.0	64.0	35,574.78	6.04
Malus baccata NAS2	ABD64879.1	325	72.0	58.2	36,090.44	5.33
Brassica rapa NAS2	XM_009128720.1	320	71.7	63.4	35,745.13	5.62
Camelina sativa NAS2	XP_010450614.1	320	71.7	63.4	35,555.58	5.85
Zea mays NAS2	NP_001104862.1	327	62.8	47.7	35,621.68	6.11
Brassica rapa NAS4	XP_009122978.1	278	61.1	50.3	31,456.48	6.63
Hordeum vulgare NAS6	BAA74586.1	328	61.0	46.7	35,349.55	5.18
Oryza sativa japonica NAS3	BAF22621.1	343	60.7	44.7	36,981.03	5.53
Hordeum vulgare NAS4	BAA74583.1	329	60.4	46.2	35,395.36	5.04
Hordeum vulgare NAS7	BAA74587.1	329	59.9	45.7	35,243.39	5.1
Hordeum vulgare NAS2	AAD32651.1	340	59.8	42.9	36,310.46	5.18
Hordeum vulgare NAS3	BAA74581.1	335	59.8	45.6	3,612.35	5.53
Hordeum vulgare NAS1	BAA74580.1	328	59.7	44.2	35,143.8	5.3
Oryza sativa japonica NAS2	BAF11808.1	326	59.5	45.0	34,483.94	5.56
Oryza sativa japonica NAS1	BAF11809.1	332	58.4	43.4	34,930.33	5.08
Zea mays NAS1	NP_001105504.1	364	53.4	39.6	39,030.33	5.42
Zea mays NAS3	NP_001151345.1	601	33.8	25.5	65,822.96	6.08

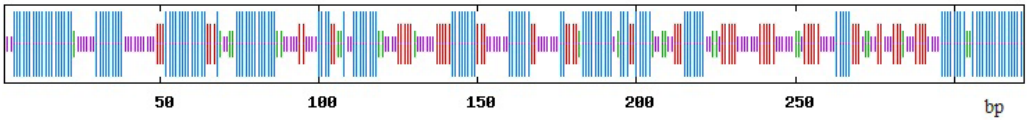

Figure 2. Secondary structure of EvNAS predicted by SOPMA. The longest, second longest, third longest, and shortest lines represent alpha helixes, extended strands, beta turns, and random coils, respectively.

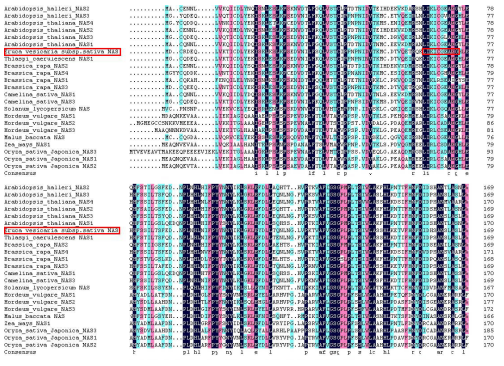

Figure 3. Multiple alignments of the N-terminus of nicotianamine synthase (NAS) proteins from various plant species. The Eruca NAS and LIKI-CGEAEG are presented in the boxes.

Sequence alignments and phylogenetic analysis

As shown in the phylogenetic tree (Figure 4), the NAS proteins were clustered into two main groups: NAS from monocots (Group M) and NAS from dicots (Group D). The monocot group included NAS from *Elaeis*, *Hordeum*, *Oryza*, and *Zea mays*. In the dicot group, EvNAS and the other Cruciferae NAS were clustered with two *Tarenaya* NAS and formed a separate subgroup (subgroup C). Within the Cruciferae, EvNAS was most closely related to *B. rapa* NAS3, followed by *Thlaspi* NAS1, *Camelina* NAS3, and *Arabidopsis* NAS3.

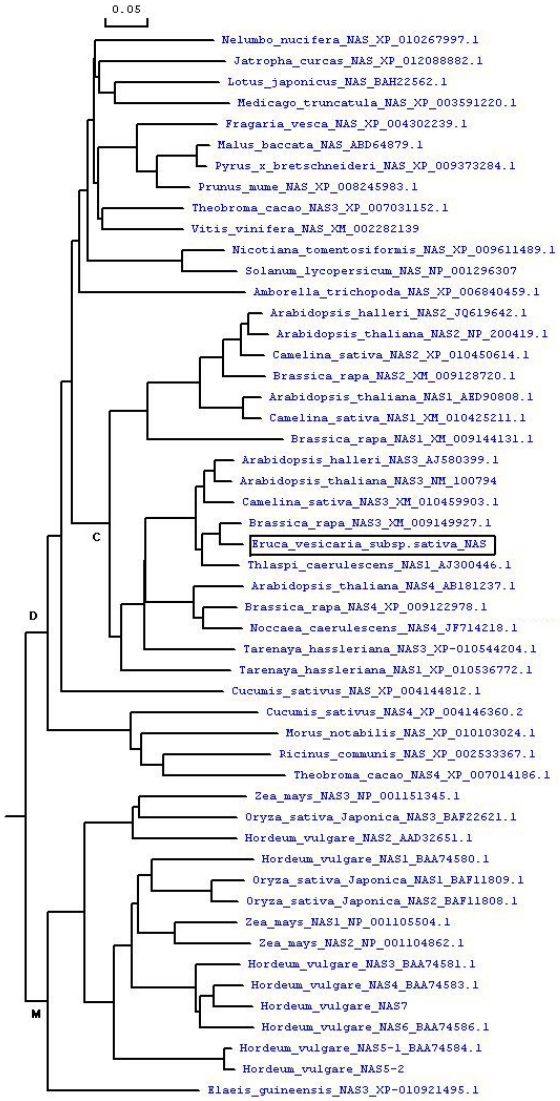

Figure 4. Phylogenetic relationships between EvNAS and nicotianamine synthase (NAS) proteins from different plant species. The Eruca NAS is boxed.

Tissue-specific expression of *EvNAS*

In this study we found that *EvNAS* was expressed at different levels in different tissues of *E. vesicaria* subsp *sativa* and was highest in the leaf, followed by the flower bud and hypocotyl. The *EvNAS* expression level in the roots was moderate; indeed, the *EvNAS* expression level in the leaves was almost three times higher than that in the roots (Figure 5).

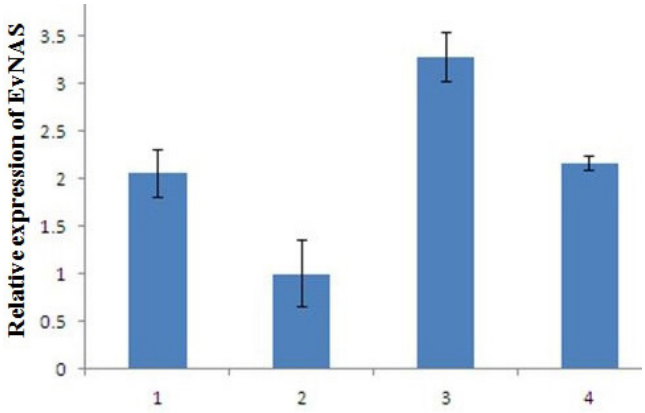

Figure 5. Relative mRNA expression levels (means ± standard deviations) of *Eruca vesicaria* subsp *sativa* nicotianamine synthase. The expression of EvNAS in hypocotyl is normalized as 1.0 and that in root, leaf and flower bud is presented as relative to that in hypocotyl. Data are reported as means ± SD. 1, root; 2, hypocotyl; 3, leaf; 4, flower bud.

DISCUSSION

In this study, we cloned full-length *NAS* cDNA and genomic DNA sequences from *E. vesicaria* subsp *sativa.* The *EvNAS* genomic sequence contained no introns, which is similar to that previously reported in other species (Ling et al., 1999; Higuchi et al., 2001; Mizuno et al., 2003; Zhou et al., 2013). The deduced translation of *EvNAS* contained a well-conserved NAS domain (1-279 amino acids) and a LIKI-CGEAEG box identical to some *Brassica* NAS and similar to the LIRL-box of most plant NAS, which is essential for DNA binding (Yang et al., 2015). In the phylogenetic tree (Figure 4) EvNAS and the other Cruciferae NAS were clustered with two *Tarenaya* NAS and formed a separate subgroup (subgroup C). This was to be expected, because *Tarenaya* and the Cruciferae species belong to the Capparales. Within the Cruciferae, EvNAS was most closely related to *B. rapa* NAS3, followed by *Thlaspi* NAS1, *Camelina* NAS3, and *Arabidopsis* NAS3. Higuchi et al. (1999) reported that the expression of *NAS* in barley is induced by Fe deficiency and is root specific, while *OsNAS1* expression in rice is induced by Fe deficiency in both the roots and shoots (Ito et al., 2009). In maize, a RT-PCR revealed that all 10 *ZmNAS* genes were only observed in root tissue, except for *ZmNAS6* (Zhou et al., 2013). In tomato, *NAS* is expressed in the leaves and roots regardless of Fe status (Ling et al., 1999). In *A. thaliana*, *AtNAS1* and *AtNAS2* have been detected in both shoots and roots, while *AtNAS3* was present only in the shoots (Suzuki et al., 1999). In *A. halleri*, *NAS1* and *NAS4* are expressed in both the roots and the leaves, while *NAS2* is predominantly expressed in the roots and *NAS3* almost exclusively in the leaves (Deinlein et al., 2012). In *Malus xiaojinensis*, *MxNas1* expression is enriched in the roots and leaves (Han

et al., 2013), while *MxNAS2* expression is more enriched in the leaf, root, and phloem than in the xylem (Yang et al., 2015). Here in this study we found that the expression of *EvNAS* highest in the leaf, followed by the flower bud and hypocotyl. The *EvNAS* expression level in the roots was moderate.

Conflicts of interest

The authors declare no conflict of interest.

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