Isolation and functional analysis of \textit{MdNAS1}, with functions in improved iron stress tolerance and abnormal flower in transgenic tobacco

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**ABSTRACT**

Metal elements are essential micronutrients required by all plants for natural physiological activities. Nicotianamine is considered as the chelate substance in the transport of metal ions. In the present study, a new gene encoding NA synthase was isolated from \textit{Malus domestica} (L.) Borkh and designated as \textit{MdNAS1}. The expression levels of \textit{MdNAS1} were enriched in leaf, and phloem which were highly affected by Fe stress, indoleacetic acid (IAA) and abscisic acid (ABA) treatments in \textit{M. domestica} seedlings. Subcellular localization research revealed that \textit{MdNAS1} was localized in cytoplasmic membrane. Overexpression of \textit{MdNAS1} in transgenic tobaccos increased the tolerance to Fe stress, but also contributes to higher chlorophyll, NA, Fe, Mn, Cu and Zn contents and abnormal flowers. Moreover, the \textit{MdNAS1-OE} tobaccos had the increased expression levels of Fe uptake and transport related genes (\textit{NIFRO}, \textit{NtIRT1}, \textit{NIVIT}, \textit{NINRAMP1}, and \textit{NITYS}).

**Introduction**

Iron (Fe) is one of the necessary micronutrients for plant growth and development with many functions (López-Millán et al. 2012). Fe deficiency is a common human nutrition problem in agricultural production problems all over the world. To ensure the Fe obtainment from environment and to avert the toxicity of Fe redundancy for plants, its acquisition & dynamic equilibrium were strictly regulated (Han et al. 2013). However, for the low solubility of Fe in most soil kinds, especially for alkaline surroundings, where the content of free iron is less than $10^{-6}$ M, a suitable content for normal plant development (Schuler et al. 2012). Therefore, Fe-deficiency induces plants young leaf chlorosis which leads to massive losses in crop yield (Ling et al. 1999). Moreover, the chlorosis (a usual threat of apple) largely limits the quality and yield of \textit{Malus} plants (Yang et al. 2015).

To remedy these deficiencies, plants had formed adaptation mechanism for obtaining Fe from different environments, which had been defined as Strategy I and II (Marschner and Romheld 1994). For the responses of iron deficiency, “Strategy I” mechanism is implemented by most higher crops, except \textit{Gramineae}. The “Strategy I” plant can generate more ferric reductase-oxidase (FRO) in low iron surroundings, for accelerating the reaction from Fe$^{3+}$ to Fe$^{2+}$ and benefitting iron obtainment (Conte and Walker 2011). The Fe absorption in apple (\textit{M. domestica} included) follow the Strategy I mechanism. As an iron chelator in plants, nicotianamine may combine with iron for the transport via new leaf and phloem where the pH value is above 7. Nicotianamine can also chelate copper, manganese, zinc and other metal ions in vitro or in vivo (Takahashi et al. 2003). Many nicotianamine synthase genes had been isolated from plants, e.g. tobacco, barley, \textit{Arabidopsis}, and apple, and the expression levels were greatly affected by iron stress (Takahashi et al. 2003; Weber et al. 2004; Han et al. 2013; Yang et al. 2015). The main function of NA in dicotyledonous plant is the storage and transport of metal ions (Perovic et al. 2007). Previous studies have shown that NA was closely related to the absorption of Fe in cotton. Lacking and excess of Fe affect the expression of NA-related genes in cotton (Yang et al. 2014). Nicotianamine can maintain the balance and benifit the acquisition of metal ions, such as nickel, cobalt, zinc, copper, and iron in \textit{Staphylococcus aureus} (Ghssein et al. 2016). Furthermore, several YSL proteins, such as OsYSL2 (Koike et al. 2004), TcYSL3 (Gendre et al. 2007) and OSYSL16 (Zheng et al. 2012) were confirmed to be the plasmolocalized transporters of the metal-NA complex responsible for metal ions transposition and distribution in plant.

Moreover, some hormones, e.g. ethylene (ETH), IAA, and ABA, were known as the signalling of Fe stress in tomato and \textit{Arabidopsis} (Schmidt et al. 2000; Schikora and Schmidt 2001; Lingam et al. 2011). Moreover, studies with ethylene in different dicotyledons indicated a physiological relation between ethylene and iron-deficiency signaling. More ethylene was produced when dealt with iron deficiency (Lingam et al. 2011). Moreover, ethylene inhibitor treatments can reduce iron deficiency signaling and the expression levels of \textit{IRT1} and \textit{FRO2} genes (Lucena et al. 2006). The expression of \textit{MxNAS1} and \textit{MxNAS2} in \textit{M. xiaojinensis} was also affected by IAA and ABA treatments and the expression levels in root and mature leaf of \textit{M. xiaojinensis} all increased (Han et al. 2013, 2015). Fe-deficiency also induced a level increase of IAA in the shoot apex of \textit{M. xiaojinensis} and treatments of IAA to the shoot apex induced Fe deficiency responses (Wu et al. 2012).

The main purpose of this study was to isolate \textit{MdNAS1} (a gene encoding putative NA synthase from \textit{M. domestica}),
investigate the expression pattern and subcellular localization of MdNAS1 as well as the relationship between MdNAS1 and Fe transport, flower development in transgenic tomatoes.

Materials and methods

Plant material and treatments

According to Han et al. (2017), the tissue culture seedlings of M. domestica were grown for rapid propagation on MS medium, rooting and in Hoagland’s solution for acclimation and growth. When M. domestica seedlings grown to about 15 cm, having 8–9 mature leaves (fully expanded), they were dealt with different iron contents (40, 160, and 4 μM) in Hoagland’s solution. (4, 40, and 160 μM). For treatments of ABA or IAA, the seedlings were put into 0.1 mM ABA or 0.1 mM IAA Hoagland’s solution with normal iron level. The phloem was sampled as follows: the stem of the plant was intercepted, and then the outer bark was stripped quickly at low temperature (2–4°C). The phloem was scraped from the bark with a sterile knife. After treatments performed respectively for 0, 2, 4, 6, and 8 d, all plants including xylem, young leaf, mature leaf, and phloem were sampled, frozen with liquid nitrogen, and reserved at −80°C for RNA extraction.

Isolation and qPCR analysis of MdNAS1

The CTAB method was used for total RNA extraction of different parts, e.g. xylem, young leaf (leaf of top), mature leaf (fully unfolded), and phloem in different parts, e.g. xylem, young leaf, mature leaf, and phloem were sampled, frozen with liquid nitrogen, and reserved at −80°C for RNA extraction.

Composition of MdNAS1

The CTAB method was used for total RNA extraction of different parts, e.g. xylem, young leaf (leaf of top), mature leaf (fully unfolded), and phloem in different parts, e.g. xylem, young leaf, mature leaf, and phloem were sampled, frozen with liquid nitrogen, and reserved at −80°C for RNA extraction.

The expression analysis of MdNAS1 used real-time fluorescence quantitative PCR method (Han et al. 2013). As a control, the Actin gene was amplified from M. domestica tissues using the following primers: ActinF1, 5'-ACTCAAGT-CATGTCACAGATGCTT-3' and ActinR1, 5'-TAAAGAAGCTGCTTCTCAAGG-3') was planned to amplify the ORF sequence of MdNAS1 (XP_008370376). The MdNAS1 ORF was isolated from Malus domestica by PCR method. The acquired DNA fragment was gel purified and connected to pEASY-T1 vector (TransGen Biotech Co. Ltd, China) and sequenced (Invitrogen, Beijing).

The expression analysis of MdNAS1 used real-time fluorescence quantitative PCR method (Han et al. 2013). As a control, the Actin gene was amplified from M. domestica tissues using the following primers: ActinF1, 5'-CTGATTCATGTCACAGATGCTT-3' and ActinR1, 5'-TGGGATGA-CATGTCACAGATGCTT-3'. The primers for MdNAS1 were designed for real-time PCR from partial sequences isolated in this study, i.e. MF, 5'-ACTGAGTTCCAGGGAGATGCTT-3' and MR, 5'-ATATCTGTGGTGGAAAGCTGCTTCTCAAGG-3'. The thermal cycling program was an initial cycle of 94°C for 30 s, followed by 30 cycles of 94°C for 5 s, and 58°C for 30 s. The relative gene expression data was analyzed using the 2ΔΔCt method (Livak and Schmittgen 2001).

Subcellular localization of MdNAS1

The MdNAS1 coding region was inserted into the pSAT6-GFP-N1 (a plant transient expression vector, provided by Prof. Kedong Xu, Zhoukou Normal University) by the restriction enzyme cutting sites of HindIII and SalI. This transient expression vector contains a GFP at HindIII and SalI sites. According to Xu et al. (2014), the plasmids of MdNAS1-GFP were introduced into Allium cepa epidermal cells by injection. The fusion proteins of MdNAS1-GFP were observed and photographed by confocal microscopy.

Transformation of MdNAS1 into tobacco

To construct an expression vector for transformation of tobacco, restriction enzyme cut sites of BamHI and SacI were added into MdNAS1 cDNA at both 5' and 3' ends by PCR. To construct the pBI121-MdNAS1 vector, the products of PCR and pBI121 were digested by BamHI and SacI, and linked together through the replacing of GUS gene. The MdNAS1 gene driven under the cauliflower mosaic virus (CaMV) 35S promoter was introduced into Nicotiana tabacum cv. Xanthi ecotype tobacco by An et al. (1993). The MF and MR primers, NtUbiquitin (reference gene: U66264.1) were used for MdNAS1 PCR detection from various tobacco lines. The transformants were selected on MS medium containing 50 mg/L Kanamycin. T1 generation transformed tobaccos were used for further analysis.

Characterization of MdNAS1 in transgenic tobacco

The DNA of T1 generation transgenic tobacco and WT line was used as template, with F1 and R1 as primers, the RT–PCR analysis was carried out. Through the analysis, only the T1 lines with seedlings’ DNA containing purpose stripe were determined to be the transgenic MdNAS1–OE lines. The T1 generation transgenic tobaccos (OE-3 and OE-9, randomly chosen) and WT line were used in the subsequent experiments. Ten germinated seedlings from each line were carefully transferred to Hoagland solution supplemented with 4 μM (low level), 40 μM (normal level), and 160 μM (high level) Fe, respectively. After 15 d of growth, the appearance was observed. The leaves of each test line (WT, OE-3, and OE-9) with different treatments (high Fe level, low Fe level, and control) were collected for the following measurements of Fe, chlorophyll, NA, and citric acid (CA) contents.

Measurements of the contents of Fe, chlorophyll, NA, and citric acid (CA)

According to Kojima and Iida (1986), the Fe content in the leaves above was measured. The chlorophyll content in leaves above was measured by the method of Aono et al. (1993). According to method of Takahashi et al. (2003), the NA contents in the phloem of M. domestica seedlings dealt with different treatments (high Fe level, low Fe level, IAA and ABA) and the leaves of tobaccos above were performed by the method of high-performance liquid chromatography, all experiments used pure NA (T. Hasegawa Co. Ltd, Japan) as an external standard. Assays for CA content were performed by HPLC method with pure citric acid (Sigma, USA) as an external standard (López-Millán et al. 2012). Experiments on the contents of chlorophyll Fe, chlorophyll, NA, and CA were conducted for three times and the standard errors (± SE) were measured, respectively.

Structure investigation and measurements of Fe, Cu, Zn and Mn contents of tobacco

Forty strains (T1) of each transgenic and WT tobacco seedlings were sown on the culture matrix (nutrient soil: vermiculite ratio was 3:1) with normal management in a growth
chamber at 25 ± 1°C under a 16 h light (50 μmol/m²/s) /8 h dark regime. According to Han et al. (2015), the flower structure of every test lines were investigated and recorded. During the whole growth process, all tobacco lines' development status and flower were observed and recorded with photograph.

The flower samples (including pistil, stamen, calyx, and petal) of each test line (WT, OE-3, and OE-9) were collected for determination the contents of metal elements. According to Kojima and Iida (1986), the Fe, Cu, Zn and Mn contents in new leaf and flower samples were measured.

**The qPCR analysis of genes related to Fe uptake and transport in tobaccos**

The genes related to Fe uptake and transport (FRO, IRT, VIT, NRAMP, YSL) in the leaves of T1 transgenic tobacco and WT were also analyzed by qRT-PCR method. The qRT-PCR was performed as described above. Primers specific to genes related to Fe uptake and transport were as following: NtFRO: 5′-CAATTATAATGGCCTACTGGTATAC-3′, and NtFOR: 5′-GTTCACACAGGATGTCTCCCTTTG-3′ for NtFRO gene (XM_016631470.1); NtIRT1F: 5′-GACAACTCTATGTCACAATAC-3′, and NtIRT1R: 5′-CCGGCAGAAGCCTTTGA-3′ for NtIRT1 gene (XM_016611068.1); NtVITF: 5′-GGAGAGGAGGAGTGAAGAGGCAAT-3′, and NtVITR: 5′-AGTTCTGCTGGACAATAATCCCAA-3′ for NtVIT gene (XM_016594956.1); NtNRAMP1F: 5′-TTACTGGGCCGGGTTTTTTAATGGAG-3′, and NtNRAMP1R: 5′-TAGAGGTGGCCACTCCCAAAC-3′ for NtNRAMP1 gene (NM_001325280.1); NtYSLF: 5′-GAAGAGCAT-3′, and NtYSLR: 5′-CATTAAGAGAGGGATAATCCAG-3′ for NtYSL gene (XM_016652756.1).

**Statistical analysis**

Duncan multiple range tests were performed by using SPSS 13.0 program. Statistical differences between the treatments and control were referred to as significant *p ≤ 0.05, **p ≤ 0.01.

**Results**

**Isolation of MdNAS1 gene from M. domestica**

Sequence analysis showed that the MdNAS1 cDNA has a complete open reading frame of 978 bp, the predicted MdNAS1 comprises 325 amino acids (Figure S1) with a theoretical isoelectric point of 5.36 and a predicted molecular weight of 36.3 kDa.

**Phylogenetic relationship of MdNAS1 with other NAS proteins**

To investigate the evolutionary relationship among plant NAS proteins, seven proteins of NAS genes from different species were analyzed by DNAMAN (v6.0) analyse software. As shown in Figure 1(a), the deduced amino acid sequence of MdNAS1 includes a LIRL-box in the C-terminus and one conserved NAS domain in the N-terminal region. The LIRL-box contains the plant-specific LIRLCGEAEG sequence which serves as a DNA-binding motif of NAS (Ling et al. 1999).

Comparing the amino acid sequences of MdNAS1 with other NAS proteins, we found that MdNAS1 has a high identity to the NAS protein family. Additionally, a phylogenetic tree (neighbour-joining) was constructed with the full-length amino acid residues (Figure 1(b)) by DNAMAN (v6.0). The results showed that MdNAS1, MxNAS1 (ABD64879, a NAS protein from Malus xiaojinensis, Han et al. 2013), FvNAS1 (XP_004302239, from Fragaria vesca) and CsNAS1 (XP_006472196, Citrus sinensis) clustered together. LjNAS1 (BAH22562, Lotus japonicus, Hakoyama et al. 2009), VvNAS1 (XP_002282175, Vitis vinifera) and RcNAS1 (XP_002512510, Ricinus communis) clustered into another group.

**Analyses of MdNAS1 expression level and NA content in M. domestica**

The expression profile of MdNAS1 in various M. domestica tissues under normal iron stress (40 µM) was investigated using real-time PCR assay. Expression of MdNAS1 was enriched in leaf, phloem of stem and root, but very low in the xylem (Figure 2(a)). The results showed that MdNAS1 increased in new leaf and phloem under low Fe stress, IAA and ABA treatments (4 µM) (Figure 2(b,d)) at the beginning and reached the maximums at 6 d, but slightly decreased at 8 d, whereas the expression levels of MdNAS1 in these parts decreased under high Fe stress. The expression level of MdNAS1 in mature leaf was just opposite to the above parts under Fe stress (Figure 2(c)) and had the same trend when dealt with IAA and ABA. The NA content in phloem increased and reached the maximums at 8 d when dealt with low Fe stress, IAA and ABA treatments, but decreased with high Fe stress (Figure 2(e)).

**Subcellular localization of MdNAS1**

As shown in Figure 3, the MdNAS1-GFP fusion protein was targeted into the cytoplasmic membrane, whereas the control GFP alone was distributed throughout the cytoplasm. These results showed that the MdNAS1 is a cytoplasmic membrane localization protein.

**Overexpression of MdNAS1 confers increased Fe stress tolerance, higher Fe, chlorophyll and NA contents, but lower CA content**

In order to investigate the role of MdNAS1 in response to iron stress in plants, we generated transgenic tobacco with overexpression of MdNAS1 under the control of the CaMV 35S promoter. Among 15 transformed lines, six of them (OE-2, OE-3, OE-5, OE-7, OE-8 and OE-9) were confirmed by using RT–PCR analysis with WT line as control (Figure 4(a)). As shown in Figure 4(b), no significant difference existed in appearance between WT and MdNAS1-OE (OE-3 and OE-9) lines after 15 d of growth in Hoagland solution with normal Fe concentration (40 µM). However, when dealt with low Fe stress (4 µM), the WT line had obvious chlorotic appearance while transgenic tobacco has no obvious chlorotic appearance. Transgenic tobaccos had better appearance than WT in high iron concentration (160 µM).
As shown in Table 1, the transgenic tobaccos (OE-3 and OE-9) also had higher Fe, chlorophyll, and NA contents, but lower CA content than WT tobacco, especially when dealt with high and low Fe treatments.

**Overexpression of MdNAS1 resulted in deformed flowers in transgenic tobacco**

In addition to the changes of the contents of chlorophyll and NA, the transgenic MdNAS1 tobacco (OE-3 and OE-9) flowers developed markedly morphological abnormalities (Figure 5). The flowers of WT tobacco have 5 petals, 5 sepals, 5 stamens, and one pistil (Figure 5(a,i,m,p)). In contrast, MdNAS1-OE tobacco produced five types of abnormally shaped flowers: (1) projected petals (Figure 5(b–e,g,h)); (2) Chimeric flower organ. Petaloid filaments (Figure 5(e–g,n,p)) were observed; (3) Dehiscent flower. Dehiscent flowers were observed (Figure 5(h)), with the corolla split open; (4) Abnormal number of flower organs. This type of flower showed supernumerary stamens and petals (Figure 5(c,d,h,j,r)) or a decreased number of petals and stamens (Figure 5(b,g,l,q)); (5) Twisted flower organ. Curved filaments were observed (Figure 5(k)).

### Table 1. Influences of MdNAS1 overexpression with different Fe contents (40, 160, and 4 µM) on contents of Fe, Zn, chlorophyll, NA, and citric acid (CA) of tobaccos.

| Parameter          | 4 µM   | 40 µM  | 160 µM |
|--------------------|--------|--------|--------|
|                    | WT     | OE-3   | OE-9   | WT     | OE-3   | OE-9   | WT     | OE-3   | OE-9   |
| Fe content (µg·g⁻¹ DW) | 45.1D  | 74.2C  | 76.2C  | 72.9C  | 97.1C  | 96.9C  | 132.2B | 177.1A | 182.4A |
| Chlorophyll content (mg·g⁻¹ FW) | 0.59D  | 1.18C  | 1.16C  | 1.97A  | 1.99A  | 2.03A  | 1.15C  | 1.66B  | 1.71B  |
| NA content (µg·g⁻¹ FW) | 81.7D  | 189.4A | 196.8A | 65.3C  | 108.5C | 112.6C | 77.5D  | 153.7B | 156.8B |
| CA content (µg·g⁻¹ FW) | 81.9A  | 74.7B  | 76.6B  | 65.3B  | 55.8C  | 57.3C  | 56.4B  | 46.7D  | 48.3D  |

Note: Each data represents the mean of 3 results with 10 replicates. Means within a column followed with various alphabets were significantly different at P < 0.01 (DW, FW for dry weight and fresh weight, respectively).
Overexpression of MdNAS1 increased Fe, Cu, Zn, and Mn contents

Metal concentrations (Fe, Cu, Zn, and Mn) in flowers (including pistil, stamen, petal and calyx) of MdNAS1-OE tobaccos (OE-3 and OE-9) were also analyzed (Figure 6), in the whole flower (Figure 6(a)), new leaf (Figure 6(b)), calyx (Figure 6(c)) and petal (Figure 6(d)) of the MdNAS1 tobacco samples, the concentrations of Zn and Fe significantly increased as compared to WT tobacco. The concentrations of Cu and Mn also increased but insignificantly. These results indicate that NA promoted the transport of metal ions, particularly Fe and Zn to young leaves and flowers.

**Over-expression of MdNAS1 contributed to higher expression levels of Fe uptake and transport related genes**

In order to research what caused the higher contents of metal elements and abnormal flowers in transgenic tobaccos (OE-3 and OE-9), the expression levels of 5 Fe uptake and transport related genes (NtFRO, NtIRT1, NtVIT, NtNRAMP1, and NtYSL) in all lines were also analyzed by qRT-PCR method. The expression levels of 5 genes all increased obviously in both trial transgenic tobacco lines (OE-3 and OE-9) than wild-type, especially for NtFRO, NtIRT1 and NtYSL, which had very significant difference with WT (Figure 7).

**Discussion**

Sequence homologous analysis showed that MdNAS1 is a new member of NAS family. There are 94.1%, 88.3%, 87.2%, 80.3%, 78.2%, 77.9% amino acid homologies between MdNAS1 and MxNAS1, FvNAS1, CsNAS1, RcNAS1, VvNAS1, LjNAS1, respectively (Figure 1(b)). All the NAS proteins include a conserved NAS domain in the N-terminal and one LIRL-box in the C-terminus region (Marschner and Romheld 1994; Herbik et al. 1999; Weber et al. 2004). This result showed that the NAS family is highly conserved during evolutionary process. Previous studies had reported that NAS genes were widely distributed in apple, castor bean, grape, orange, and strawberry, which were known to be involved in metal transport (Ling et al. 1999).

The expression levels of MdNAS1 in the active parts such as young leaf and phloem (Figure 2(b,d)) were just opposite to the mature leaf under Fe stress treatments, which increased when dealt with high Fe stress and decreased with low Fe stress (Figure 2(c)). In low iron environment, Fe was preferentially provided to active organs and transferred from old leaf to active organs such as young leaf. Consequently, the expression levels of MdNAS1 in new leaf increases, while decreases in mature leaf. When dealt with high Fe treatment, the MdNAS1 expression level increases in mature leaf for dislodging iron toxicity to maintain the regular physiological activity of active organs. For this reason, MdNAS1 has different expression patterns under low and high Fe stresses in mature leaf and new leaf. The expression level of MdNAS1 increased in M. domestica, stimulating the synthesis of NAS protein and NA (Figure 2(e)) when dealt with low Fe, IAA and ABA treatments. Therefore, higher NA content in transgenic plant facilitates the uptake of Fe from low iron condition (Koike et al. 2004; Deinlein et al. 2012; Yang et al. 2015).

Certain plant hormones, such as ABA, IAA and ETH were regarded as the signals of iron stress in advanced plants (Schmidt et al. 2000; Schikora and Schmidt 2001; Lingam et al. 2011). Fe-deficiency induced an obvious increase of IAA level in the branch apex and treatments of IAA to the branch apex induced Fe deficiency responses in M. xiaojinensis (Wu et al. 2012). In the present study, the expression levels of MdNAS1 were affected by IAA.
and ABA treatments in the leaf, and phloem of *M. domestica* seedlings. Hence, we reckon that iron transposition is probably influenced by *MdNAS1* gene. ABA and IAA treatments were also discovered to influence the expression level of *MxNAS2* in *M. xiaojinensis* (Yang et al. 2015).

Subcellular localization study showed that *MdNAS1* protein was localized in cytoplasm membrane (Figure 3). Some other NAS were also found to localize in cytoplasm membrane (Ling et al. 1999; Han et al. 2013). Moreover, the NAS2 from rice was localized in vesicles (Nozoye et al. 2014). Presumably, the *MdNAS1* may have an uncertain or unknown region which affects the result of subcellular localization experiment, leading to the localization in cytoplasm membrane.

Overexpression of *MdNAS1* gene improved the tolerance to low or high Fe stress in transgenic plants (Figure 4(b)). We speculate that the *MdNAS1* gene plays a crucial role in helping tobacco to survive from Fe stress through regulating the syntheses of NAS and NA. Higher content of NA in *MdNAS1-OE* tobacco helps to acquire iron from Fe-deficiency environment. Moreover, high concentration of NA can also help chelate superfluous iron for detoxification when the plant was exposed to high-Fe condition (Deimlein et al. 2012). Hence, the transgenic plant could have higher endurance to Fe stress. The high NA concentration in *MdNAS1-OE* tobaccos induced higher iron content, which leads to the higher chlorophyll content (Table 1). The overexpression of *MdNAS1* gene in transgenic tobaccos also contributed to the lower content of citric acid (Table 1). It is possible that most of the metal ions were combined to the higher NA levels, and these was less need for NA to metal ions.

More importantly, we firstly found that overexpression of *MdNAS1* resulted in deformed flowers in transgenic tobaccos (Figure 5), including abnormal shape and the number of floral organs. The proportion of flowers with normal number of petals in *MdNAS1-OE* tobaccos was less than 50%, while the ratio in WT line was more than 98%. Previous study showed that increased expression level of *MxNS2* in transgenic tobaccos resulted in floral morphological abnormalities (Han et al. 2015). Metal micronutrients (particularly Cu and Zn) participate in normal flower development (Conte and Walker 2011). Metal ions are critical for reproductive development of plants, because they are components of many very important proteins during this period (Kim and Guerinot 2007).

NA is an essential substance for the metal ions transport in veins (Haydon et al. 2012). It has been testified that NA can chelate metal elements through new leaf and phloem in plants for their transport (Schuler et al. 2012). Metal ions are very important for plant growth and development, because they are crucial components of many important enzymes. High concentration of metal ions could influence the enzyme activity (Takahashi et al. 2003). The contents of metal ions (especially Fe & Zn) in flower and new leaf of transgenic tobaccos changed obviously (Figure 6), which probably affect the activity of very important enzymes in flower development. The elevated concentration of NA leads to the increased contents of metal micronutrients in transgenic tobacco.

Several genes had been found to affect the Fe uptake and transport, such as *FRO, IRT1, VIT*, *NRAMP1*, and *YSL*. In this study, the expression levels of 5 Fe uptake and transport related genes increased obviously in transgenic tobacco lines (Figure 7), especially these genes involved in metal ions

![Figure 3. Subcellular localization of *MdNAS1*. Transient expressions in onion epidermal cells of 35S-GFP and 35S-*MdNAS1*-GFP translational product were visualized by fluorescence microscopy. The transient vector harboring 35S-GFP and 35S-*MdNAS1*-GFP cassettes were transformed into onion epidermal cells by particle bombardment. The photos were taken in the bright light (left), in the dark for GFP images (right) after incubation for 24 h.](image-url)
reduction and transposition, such as *NtFRO*, *NtIRT1* and *NTYSL*.

Overexpression of *MdNAS1* enhanced the iron stress tolerance in transgenic tobacco, but also contributed to higher chlorophyll and NA contents. Moreover, the transgenic plants had increased contents of Fe, Cu, Zn and Mn in flower and new leaf than WT line. Clarifying the role of different domains of *MdNAS1* in metal stress response will be helpful in breeding stress-resistant *Malus* by gene transformation.

![Figure 5](image)

**Figure 5.** Flowers and floral organs of WT and *MdNAS1*-OE transformed tobaccos (OE-3 and OE-9). (a) Wild-type flower; (b–h) *MdNAS1*-OE tobacco flowers; (b–e) Flower with projected petal; (f–g) Petaloid stamen flowers; (h) Dehiscent flower; (i) Column and stamen filaments of WT tobacco flowers; (j–l) Column and stamen filaments of *MdNAS1*-OE tobacco; (m) Stamen of WT tobacco flowers; Petaloid stamen; (n–o) Stamen of *MdNAS1*-OE tobacco flowers; (p) Calyx of WT flower (a); (q–r) Calyces of *MdNAS1*-OE tobacco flowers (b, c and f). Scale bars: 1 cm.

![Figure 6](image)

**Figure 6.** The contents of metal elements (Fe, Cu, Zn and Mn) in flower. Metal concentrations in pistil (a), stamen (b), calyx (c), petal (d) of *MdNAS1*-OE tobacco (OE-3 and OE-9) and WT tobacco. All treatments were repeated at least three times. For each part (pistil, stamen, calyx and petal) of flower, the content of each metal element in WT tobacco was reckoned as control to the transgenic lines.
Disclosure statement
No potential conflict of interest was reported by the authors.

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References
An G, Watson BD, Chiang CC. 1986. Transformation of tobacco, tomato, potato, and Arabidopsis thaliana using a binary Ti vector system. Plant Physiol. 81:301–305.

Aono M, Kubo A, Saji H, Tanaka K, Kondo N. 1993. Enhanced tolerance to photo-oxidative stress of transgenic Nicotiana tabacum with high chloroplastic glutathione reductase activity. Plant Cell Physiol. 34:129–136.

Conte SS, Walker EL. 2011. Transporters contributing to iron trafficking in plants. Mol Plant. 4:464–476.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.

Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen C, Lobinski R, Lemaire D, Richaud P, et al. 2016. Biosynthesis of a tetracyclic terpene from Senecio vulgaris. Sci Rep 6:30529.