

# Overexpression of a *Malus xiaojinensis* *Nas1* Gene Influences Flower Development and Tolerance to Iron Stress in Transgenic Tobacco

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**Abstract** Iron (Fe) is one of the essential micronutrients required by all living organisms. Expression of *MxNas1* of *Malus xiaojinensis* is enriched in active tissues, including leaves, roots, and phloem, and these seedlings are highly affected by Fe stress and indoleacetic acid treatment, but weakly affected by abscisic acid treatment. Subcellular localization has revealed that *MxNas1* is preferentially localized in cell membranes. When this gene is introduced into tobacco, it promotes synthesis of nicotianamine (NA) synthase and increases NA content. Overexpression of *MxNas1* improves tolerance to Fe stress in transgenic tobacco, but also leads to misshapen flowers. Higher levels of *MxNas1* expression in transgenic tobacco plants also contributes to delayed flowering and increased levels of Fe and Zn in flowers. In addition to its role in metal transport in plants, NA may be involved in the regulation of metal transfer within cells. These results suggest that NA excess influences functions of metal-requiring proteins, including some of the transcription factors.

**Keywords** Iron · *MxNas1* · Strategy I · Transgenic tobacco · Real-time PCR

## Abbreviations

NA Nicotianamine  
ABA Abscisic acid

|               |  |
|---------------|--|
| IAA           | Indoleacetic acid                                      |
| IBA           | Indole-3-butytric acid                                 |
| 6-BA          | 6-Benzylaminopurine                                    |
| MS            | Murashige and Skoog medium                             |
| <i>MxNas1</i> | <i>Malus xiaojinensis</i> nicotianamine synthase gene1 |
| Real-time PCR | Real-time polymerase chain reaction                    |
| GFP           | Green fluorescent protein                              |
| CaMV          | Cauliflower mosaic virus                               |
| WT            | Wild type  |
| OE            | Overexpression   |

## Introduction

Metal ions, such as Fe, Mn, Zn, and Cu are essential elements for plant growth and development (López-Millán et al. 2009). However, these ions (particularly Fe) have poor solubility in most types of soil (Guerinot and Yi 1994; Han et al. 2012), particularly in calcareous soil solution where the concentration of free Fe is far below  $10^{-6}$ M, a required concentration for optimal plant growth (Han et al. 1998). Therefore, Fe deficiency is a worldwide problem for crop production. Iron deficiency chlorosis is a very common disease in apples, especially in North China (Gao et al. 2011), and largely limits the growth, yield, and quality of apples (Zhang et al. 2012).

To avoid such deficiencies, plants have developed sophisticated regulating mechanisms to acquire Fe from the soil, which have been classified into two strategies (strategies I and II) by Marschner and Romheld (1994). ‘Strategy I’ plants produce more ferric reductase-oxidase under Fe-deficiency stress, to reduce Fe(III) to Fe(II) and benefit Fe uptake (Zhang et al. 2009). The absorption and utilization of Fe in apple (*Malus xiaojinensis* included) follows the reduction mechanism of ‘Strategy I’ (Li et al. 2006a). Several

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changes have also been observed at the metabolic level in order to sustain the increased Fe uptake capacity of Fe-deficient plants.

In response to Fe deficiency, all non-graminaceous higher plants appear to adopt ‘Strategy I’, in which the role of the nicotianamine (NA) synthesis gene has not yet been determined. Additionally, NA can chelate Fe(II) for Fe transport through phloem (Stephan et al. 1994) and citric acid can chelate Fe(III) for its transport through xylem (Rellán-Álvarez et al. 2008) in plants. Recent study indicated that a transporter, FRD3, is necessary for efficient Fe translocation to the plant sap (Durrett et al. 2007). The *Arabidopsis* mutant, *frd3*, which performs Strategy I responses, showed constitutive expression regardless of the external Fe supply, therefore it has provided molecular evidence of the role of NA in iron transport (Rogers and Gueriot 2002; Green and Rogers 2004).

To identify Fe-efficient genotypes, we have collected more than 40 genus *Malus* samples of various species and ecotypes. Previous studies indicated that *M. xiaojinensis* is a Fe-efficient apple genotype (Han et al. 1994a, b). However, the role of NA synthesis gene in *M. xiaojinensis* and *MxNas1* (a gene encoding putative NA synthase from *M. xiaojinensis*, Genbank accession no. DGB0458) is not very clear. The *Nas1* gene plays a key role in synthesizing NA synthase, but the relationship between the *Nas1* gene and iron transport or plant development remains unclear. In this study, we detected the expression of *MxNas1* in different organs and investigated the relationship between the expression of *MxNas1* and indoleacetic acid (IAA), abscisic acid (ABA) as well as Fe stress treatments. Importantly, over-expression of *MxNas1* improved plant tolerance to Fe stress in transgenic tobacco, and resulted in late-flowering and abnormally shaped flowers.

## Materials and Methods

### Plant Material and Growth Conditions

*M. xiaojinensis* Cheng and Jiang test-tube seedlings are rapidly propagated on Murashige and Skoog medium (MS) + 0.5 mgL<sup>-1</sup> 6-BA + 0.5 mgL<sup>-1</sup> indole-3-butyric acid (IBA) for 1 month, and then returned to MS + 1.0 mgL<sup>-1</sup> IBA for one and a half months for rooting. Finally, the seedlings are transferred to Hoagland solution for 1 month for growth. When the plants have eight to nine mature leaves (fully expanded), plants were exposed to different iron concentrations Hoagland nutrient solutions (4, 40, and 160 μM). For IAA and ABA treatments, seedlings were respectively put in 0.1 mM IAA and 0.1 mM ABA Hoagland solution with normal Fe concentration (40 μM). All samples of control and treated plants were taken after

treatments of respectively 0, 6, 12, 24, and 48 h, and were frozen immediately in liquid nitrogen, and stored at -80 °C for RNA extraction.

### Real-Time PCR Analysis of *MxNas1* Expression

Total RNA was extracted separately from root, phloem, xylem, young leaf (partly expanded), and mature leaf (fully expanded) using the CTAB method (Zhang et al. 2005). First-strand cDNA was synthesized with 1 μg total RNA and 1 μL superscript II enzyme (Invitrogen, USA) according to the manufacturer protocol. As a control, the *Actin rRNA* gene was amplified from *M. xiaojinensis* tissues using the following primers: *Mx18SF*, 5'-ACACGGGGAGGTAGTGACAA-3' and *Mx18SR*, 5'-CCTCCAATGGATCCTCGTTA-3'.

The cDNA was diluted fivefold, and 2 μL of the dilution was used for quantitative RT-PCR. The iQ SYBR Green Supermix (Applied Biosystems, Foster City, CA, USA) was used for amplification on an Applied Biosystems 7500 RT-PCR system (Yang et al. 2011). The expression levels of each sample were normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels. The methods for *MxNas1* gene expression were designed for real-time polymerase chain reaction (real-time PCR) as follows. The primers for *MxNas1* were designed for real-time PCR from partial sequences isolated in this work. The primers for *MxNas1* (DGB0458) genes were designed from partial sequences published in the GenBank databases. The primer sets used are MF, 5'-GAGTCGACATGTGTTGC CAGGGA-3' and MR, 5'-CAGGATGTTTTAAG\*AAAGCTGCT-3'. The thermal cycling program was one initial cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s (Jia et al. 2012). The relative gene expression data were analyzed using the 2<sup>-ΔΔCT</sup> method.

### Subcellular Localization of the MxNas1 Protein

The *MxNas1* ORF was cloned into the *XbaI* and *SamI* sites of the pSAT6-GFP-N1 vector. This vector contains a modified redshifted green fluorescent protein (GFP) at *XbaI-SamI* sites. The *MxNas1*-GFP construct was transformed into onion epidermal cells by particle bombardment as described earlier (Xu et al. 2011). The transient expression of the *MxNas1*-GFP fusion protein was observed under confocal microscopy.

### Tobacco Transformation

To construct an expression vector for tobacco, the full-length *MxNas1* cDNA was ligated into the vector pBI121 (Clontech) by replacing the *gus* gene. The *MxNas1* gene driven under the cauliflower mosaic virus (CaMV) 35S promoter was introduced into tobacco plants by *Agrobacterium*-mediated

GV3101 transformation (An et al. 1988). *Nicotiana tabacum* cv. Xanthi ecotype tobacco plants were transformed using the vacuum infiltration method. Transformants were selected on MS medium containing  $50 \mu\text{g mL}^{-1}$  kanamycin. The primers for PCR detection are MF and MR, *18S rRNA* gene was amplified from various tobacco tissues for Actin.  $T_1$  generation plants were used for further analysis.

#### Analysis of Fe Stress Tolerance of Transgenic Tobacco

The  $T_1$  generation plants of lines *MxNas1*-OE and wild type (WT) were used in the subsequent experiments. Twenty germinated seedlings from each line were carefully transferred to Hoagland solution supplemented with  $4 \mu\text{M}$  (low Fe concentration),  $40 \mu\text{M}$  (normal level), and  $160 \mu\text{M}$  (high concentration) Fe. After 10 days of growth, the appearance was observed.

#### Detection of the Contents of Chlorophyll and NA

According to Aono et al. (1993), we measured the chlorophyll content of the  $T_1$  generation plants of lines *MxNas1*-OE (OE-2 and OE-9) and wild type.

Assays for the content of NA were performed by the method of high-performance liquid chromatography according to Higuchi et al. (2001). All experiments used pure NA (T. Hasegawa Co. Ltd, Japan) as an external standard. Experiments on the contents of chlorophyll and NA were conducted three times and the standard errors ( $\pm\text{SE}$ ) were measured, respectively.

#### Determination of Metal Concentrations

According to Kojima and Iida (1986), new leaf and flower samples (including whole flower, petal, pistil, and stamen) weighing 100–200 mg were respectively placed with 2 mL of nitric acid in a sealed polytetrafluoroethylene vessel with a stainless steel jacket. The vessel was heated from room temperature to  $150^\circ\text{C}$  in an oven for 90 min and then kept at  $150^\circ\text{C}$  for 6 h. After cooling to room temperature, samples were filled to a constant volume and metal concentrations were determined using inductively coupled plasma emission spectrometry (SPS1200 VR; Seiko, Tokyo, Japan).

## Results

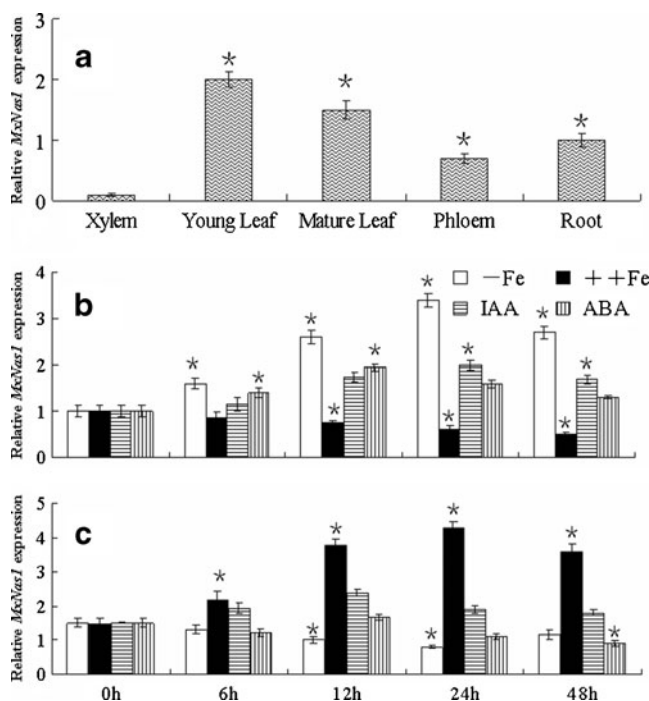
#### Expression Analysis of *MxNas1* in *M. xiaojinensis*

The expression level of the *MxNas1* in these *M. xiaojinensis* tissues under normal Fe treatment was investigated using the real-time PCR assay. These *M. xiaojinensis* tissues were collected as described in “Materials and Methods”. Expression of *MxNas1* was enriched in root and leaf, which was

also detected in the phloem of stem, but was very low in the xylem (Fig. 1a). This expression pattern indicates that *MxNas1* may play its role in active organs. To further analyze *MxNas1* expression in *M. xiaojinensis*, we prepared total RNA from root (Fig. 1b) and mature leaf (Fig. 1c) of *M. xiaojinensis* in different treatments. The results show that the expression of *MxNas1* increases in roots under a low Fe concentration ( $4 \mu\text{M}$ ), IAA, and ABA treatments. Conversely, it decreases in roots under a high Fe concentration ( $160 \mu\text{M}$ ; Fig. 1b). The expression of *MxNas1* in mature leaf (Fig. 1c) was just the opposite to root under Fe stress. Treatments of IAA, ABA, and different Fe stresses affect the expression of *MxNas1* in roots and leaves. IAA and ABA are considered as signals of Fe and other stresses in plants.

#### Localization of *MxNas1* in the Cell Membrane

The presence of a NA synthase, which commonly serves as a DNA-binding domain, suggests that *MxNas1* is a



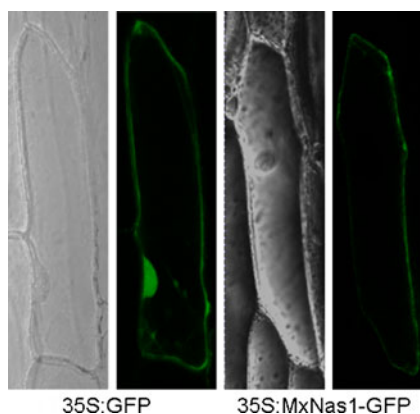
**Fig. 1** Time-course expression patterns of *MxNas1* in *M. xiaojinensis* using real-time PCR. **a** Expression patterns of *MxNas1* in young leaf (partly expanded of top) and mature leaf (fully expanded), root, xylem, and phloem in normal iron concentration ( $40 \mu\text{M}$ ). **b** Expression patterns of *MxNas1* in a low concentration of Fe ( $4 \mu\text{M}$ , -Fe), high concentration of Fe ( $160 \mu\text{M}$ , ++Fe), treated with  $0.1 \text{ mM}$  IAA (IAA) and  $0.1 \text{ mM}$  ABA (ABA) in roots at the following time points: 0, 6, 12, 24, and 48 h. The expression amounts were normalized to that of *Mx18S*. **c** Expression patterns of *MxNas1* in mature leaf of *M. xiaojinensis* at the same conditions as above (root). Each data (mean  $\pm$  SD,  $n=3$ ) represents the average of three independent plants; error bars indicate the standard deviation. Asterisks above the error bars indicate a significant difference between the treatment and control (0 h) using Student's *t* test ( $p \leq 0.05$ )

functional gene. To examine subcellular localization of MxNas1 protein, the MxNas1–GFP fusion protein was introduced into onion epidermal cells by particle bombardment. As shown in Fig. 2, the MxNas1–GFP fusion protein was targeted into the cell membrane, whereas the control GFP alone was distributed throughout the cytoplasm. These results showed that the MxNas1 protein is a cell membrane localization protein.

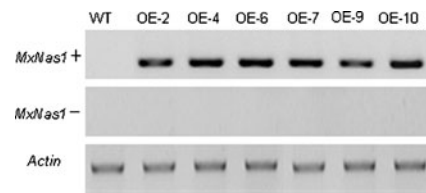
#### Overexpression of *MxNas1* Confers Tolerance to Fe Stress in Transgenic Tobacco

In order to investigate the role of *MxNas1* in response to Fe stresses in plant, we generated transgenic tobacco plants with overexpression of *MxNas1* under control of the CaMV 35S promoter. Among 13 lines of transformants, six independent transgenic lines (OE-2, OE-4, OE-6, OE-7, OE-9, and OE-10) were confirmed by using RT-PCR analysis (Fig. 3).

The T<sub>1</sub> transgenic *MxNas1*-OE lines (OE-2 and OE-9) and wild-type tobacco seedlings were grown in soil for germination, then the seedlings were grown in Hoagland solution supplemented with 4  $\mu$ M (low Fe stress), 40  $\mu$ M (normal Fe level), and 160  $\mu$ M (high Fe stress) Fe, respectively. As shown in Fig. 4, after 10 days of growth, the appearance was observed. In normal Fe concentration (40  $\mu$ M) solution, tobaccos of both types grow well. The wild type has obvious chlorotic appearance, but transgenic tobacco has no obvious chlorotic appearance in Fe deficiency (4  $\mu$ M) Hoagland solution. Transgenic plants of both lines have better appearance than wild type in high Fe concentration (160  $\mu$ M).



**Fig. 2** Subcellular localization of MxNas1. Transient expression in onion epidermal cells of 35S–GFP and 35S–DgZFP–GFP translational product was visualized by fluorescence microscopy. The transient vector harboring 35S–GFP and 35S–MxNas1–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 20 h

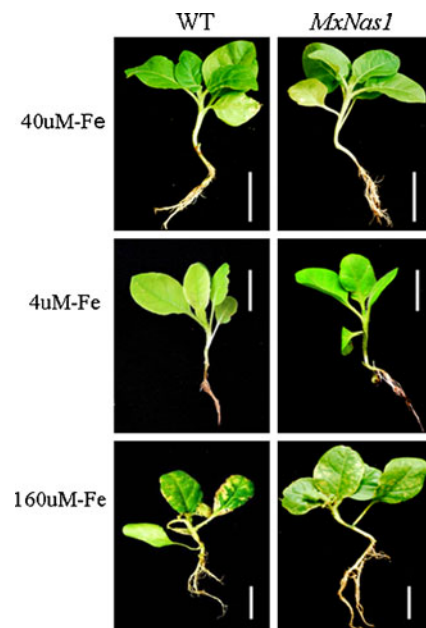


**Fig. 3** Expression of *MxNas1* in transgenic tobacco. The expression level of *MxNas1* in wild-type (*WT*) and *MxNas1*-OE transgenic T<sub>0</sub> lines. The results of semi-quantitative RT-PCR. Ethidium bromide staining of PCR products using *MxNas1*-specific primers with (*top*) and without (*middle*) prior reverse transcription, and the PT-PCR products with *18S rRNA* gene primers (*bottom*) as *Actin*

As shown in Fig. 5, the transgenic tobaccos (lines OE-2 and OE-9; Fig. 5b and d) exhibited phenotypes of decreased growth and reduced leaf size (Fig. 5e right), compact plant shape and delayed flowering for about 15 days, compared to those of wild-type tobacco (Fig. 5a, c, and e; left). The transgenic tobaccos also have higher contents of chlorophyll (Fig. 5f) and NA (Fig. 5g) than wild type.

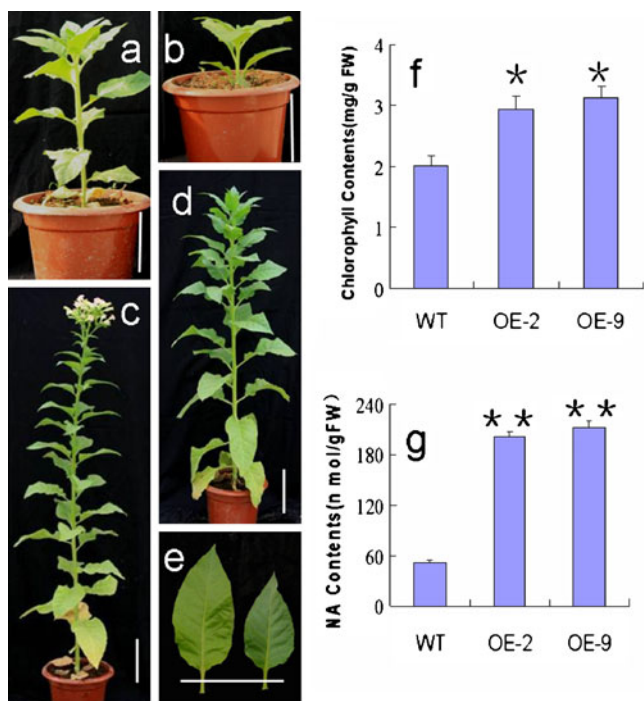
#### Overexpression of *MxNas1* Confers Abnormally Shaped Flowers

In addition to changes of plant form, and contents of chlorophyll and NA, the *MxNas1* tobacco (lines of OE-2 and OE-9) inflorescence developed marked morphological abnormalities (Fig. 6). The flowers of wild type had five



**Fig. 4** Overexpression of *MxNas1* in tobacco improved Fe stress tolerance in *MxNas1* transgenic lines (OE-2 and OE-9). WT and *MxNas1* were respectively shown the wild-type and transgenic tobacco seedlings phenotype grown in Hoagland solution supplied with 4  $\mu$ M (low Fe concentration), 40  $\mu$ M (normal level), and 160  $\mu$ M (high concentration) Fe for 10 days



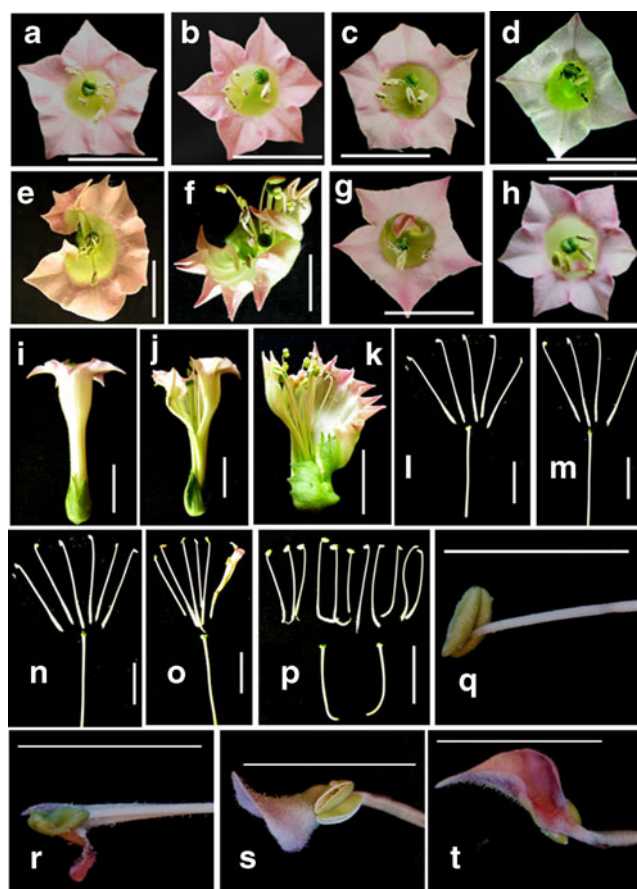


**Fig. 5** Overexpression of *MxNas1* in tobacco. The phenotype of the *MxNas1*-OE T<sub>1</sub> lines (**b** and **d**) and wild-type (**a** and **c**) tobacco at 25 and 90 days after transplanting in the soil. **e** Comparison of adaxial side of leaves in *MxNas1*-OE (right) and wild-type (left) tobacco. **f–g** Comparison of the contents of chlorophyll (**f**) and NA (**g**) in leaf of wild-type (WT) and *MxNas1* transgenic lines (OE-2 and OE-9). All treatments are repeated at least three times. Scale bars 15 cm in (**a–e**). Significant differences between *MxNas1*-OE lines and wild-type (WT) were shown by the *t* test, \* $p \leq 0.05$ ; \*\* $p \leq 0.01$

petals, five stamens, and one pistil (Fig. 6a, i, and l). In contrast, *MxNas1* tobacco produced four types of abnormally shaped flowers: (1) projected petals (Fig. 6b–f and h). (2) Chimeric flower organ. Petaloid filaments (Fig. 6f–h, o, r, s, and t) were observed. (3) Dehiscent flower. Dehiscent flowers were observed (Fig. 6e, f, j, and k), with the corolla split open. (4) Abnormal number of flower organs. This type of flower showed supernumerary stamens and petals (Fig. 6b, c, e, f, h, j, k, n, o, and p) or a decreased number of petals and stamens (Fig. 6d and m).

#### Overexpression of *MxNas1* Increased Fe, Cu, Zn, and Mn Concentrations in Young Leaves and Flowers

Metal concentrations in the young leaves and flowers (including whole flower, petal, pistil, and stamen) of *MxNas1*-OE tobaccos (OE-2 and OE-9) were also analyzed (Fig. 7). In young leaves (Fig. 7a), whole flower (Fig. 7b), petal (Fig. 7c), pistil (Fig. 7d), and stamen (Fig. 7e) of the *MxNas1* tobacco samples, the concentrations of Fe and Zn significantly increased as compared WT tobacco. The concentrations of Cu and Mn also increased but insignificantly. These results indicate that NA promoted the transport of



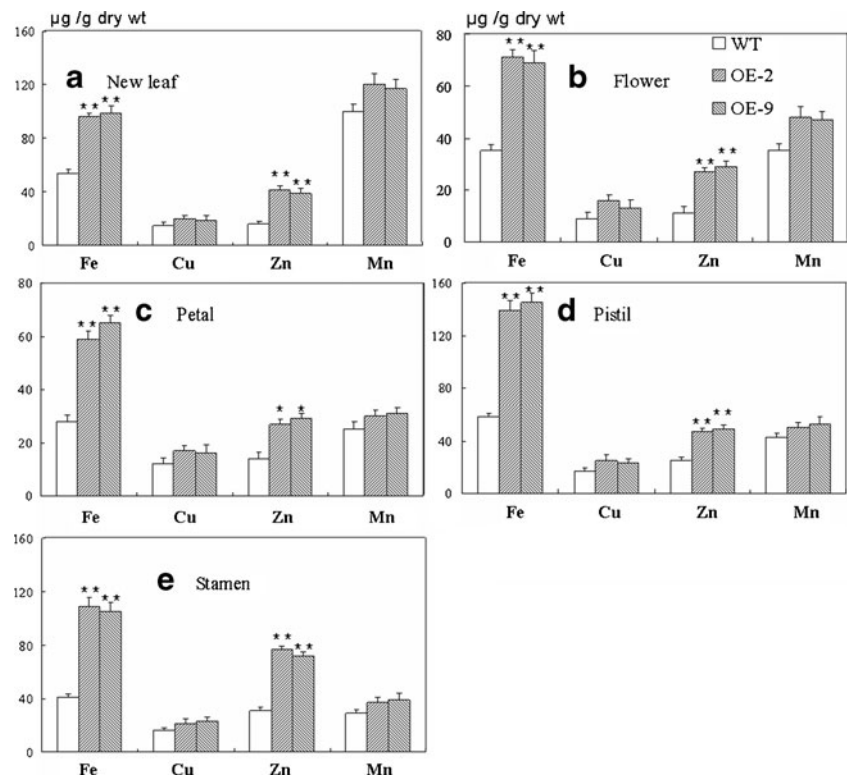
**Fig. 6** Inflorescences of wild-type tobacco and *MxNas1*-OE tobacco (OE-2 and OE-9). **a** Wild-type flower, **b–h** *MxNas1*-OE tobacco flowers, **b–d** flower with projected petal, **e–f** staminoid petal, **g–h** petaloid stamen, **i** side view of the flower shown in (**a**), **j** side view of the flower shown in (**e**), **k** side view of the flower shown in (**f**), **l** pistil and stamen filaments of wild-type flower (**a** and **i**), **m** pistil and stamen filaments of (**d**), **n** pistil and stamen filaments of (**b**), **o** pistil and stamen filaments of (**h**), **p** pistils and stamen filaments of (**k**), **q** stamen of wild-type flower in (**a** and **i**), **r–t** petaloid stamen of the stamen filament shown in (**g** and **h**) that seems to be a petal. Scale bars 1 cm in **a–t**

metal ions, particularly Fe and Zn to young leaves and flowers.

#### Discussion

mRNA expression of *MxNas1* was more enriched in root and leaf than that in phloem, while expression was nearly undetectable in xylem. This expression pattern indicates that *MxNas1* may play its role in active organs. Treatments of IAA, ABA, and different Fe stresses affect the expression of *MxNas1* in roots. Because IAA and ABA are considered as signals of Fe stress in plants (Schmidt et al. 2000; Schikora and Schmidt 2001), IAA and ABA treatments affect the expression of *MxNas1*, we believe that *MxNas1* has

**Fig. 7** Metal concentrations in young leaves and flowers. Metal concentrations in young leaves (a), whole flower (b), petal (c), pistil (d), and stamen (e) of *MxNas1*-OE tobacco (*OE-2* and *OE-9*) and wild-type (*WT*) tobacco. All treatments are repeated at least three times. Significant differences between *MxNas1*-OE lines and wild type were shown by the *t* test, \* $p \leq 0.05$ ; \*\* $p \leq 0.01$



probably participated in Fe transport. The results show that the expression of *MxNas1* in *M. xiaojinensis* is upregulated in root under low Fe condition and down-regulated under high Fe stress. It is possible that *MxNas1* plays a key role in regulating the responses of *M. xiaojinensis* to different treatments of Fe stress. When exposed to low Fe treatment, *M. xiaojinensis* increases the expression of *MxNas1* to accelerate the synthesis of Nas and NA. Consequently, higher concentration of NA in plants will promote uptake of Fe from a poor Fe environment (Deinlein et al. 2012). In contrast, the expression of *MxNas1* in roots was down-regulated in a rich Fe environment to reduce the synthesis of Nas and NA, so the uptake of Fe from the environment is decreased.

Overexpression of *MxNas1* enhanced the tolerance to Fe stresses of high and low concentrations in transgenic tobacco and increased contents of chlorophyll and NA. It is possible that *MxNas1* plays a crucial role in helping plants to survive Fe stress by regulating the synthesis of NA. Higher content of NA in *MxNas1*-OE tobacco helped to extract Fe from a poor Fe environment. Meanwhile, high concentration of NA is also helpful in chelating redundant Fe for detoxification when plants were exposed to a high Fe environment (Palmer and Guerinot 2009).

NA is essential for the transport of Fe, Mn, Cu, and Zn in veins (Haydon and Cobbett 2007). The concentrations of Zn and Fe were significantly higher in *MxNas1*-OE tobacco young leaves and flowers than in wild-type tobacco, which means that there must be some relationships between NA

and Fe translocation in plants. It has been demonstrated that NA can chelate these four metal ions (Fe, Zn, Mn, and Cu) for their transport through phloem in plant (Schuler et al. 2012). Several researchers have reported an increase of NA content under Fe deficiency in phloem (Abadia et al. 2002). Metal ions are very important for plant growth, because they are important components of many critical proteins or enzymes. High content of metal ions can affect enzyme activity, which could depress the growth rate. Additionally, high concentration of NA, as a result of *MxNas1* overexpression in transgenic tobacco, is also helpful in chelating redundant metal ions for detoxification when plants were exposed to heavy metal stress (Haydon et al. 2012).

NA is essential for reproductive growth. Normal flower development requires a specific NA concentration more strictly than that for normal leaf development (Takahashi et al. 2003). In this study, transgenic tobacco plants have higher contents of chlorophyll, NA, and metal ions and show improved tolerance to Fe stress. Leaves of transgenic tobacco grow well with increased content of chlorophyll caused by higher Fe concentration, but the shape of flower has changed and there are chimeric flower organs. The combination of NA with Fe(II) is essential for normal flower development. Other metal ions (particularly Zn and Cu) also participate in normal flower development (Conte and Walker 2011). NA, acting as a metal carrier, can help to transfer metal ions to organs such as leaves, developing pistils, and anthers. It also plays a role in the maturation of pollen and seeds. NA also could be involved in regulating functions of metal-

requiring proteins (such as Zn finger proteins), so it may affect the number of flower organs, determine the shape of flower organs, and probably serve as a regulator of transcription factors.

Metal ions such as Fe, Cu, Zn, and Mn are very important for reproductive development of plants, because they are important components of many critical proteins during this stage (Kim and Guerinot 2007). It has been reported that Cu stress can cause male sterility (Dell 1981) and affect seed yield and quality (Bhakuni et al. 2009); Mn deficiency affects pollen productivity and viability (Dordas 2009). Zn stress would lead to decreased pollen fertility (Sharma et al. 1990) and seed quality (Chatterjee and Khurana 2007); Zn is also related to female fertility, because Zn finger Polycomb group proteins are necessary for proper female gametophyte and seed development (Grossniklaus et al. 1998; Brive et al. 2001). Zn plays an essential role in some key structural motifs of transcriptional regulatory proteins, including Zn finger, Zn cluster, and RING finger domains. Furthermore, transcriptional factors, including many Zn finger proteins, participate in flower development (Kapoor et al. 2002; Li et al. 2006b). In this study, the contents of metal ions (especially Zn) changed markedly, which probably affect the activity of critical proteins in reproductive development, and the function of transcriptional regulatory proteins, such as Zn finger, Zn cluster, and RING finger domains, and the abnormally shaped flowers of transgenic tobacco were produced as a result.

To our knowledge, this work is the first report regarding expression analysis by real-time PCR and function characterization of *MxNas1* gene through transgenic tobacco. Clarifying the role of different domains of *MxNas1* under metal stress response and that in abnormally shaped flowers will be helpful in breeding stress-resistance *Malus* by gene transformation. Further gene transfer experiments are required to identify the function of *MxNas1* using knockout and RNAi technique, or through gene transformation into other *Malus*.

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