Isolation and functional analysis of MxNAS3 involved in enhanced iron stress tolerance and abnormal flower in transgenic Arabidopsis

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ABSTRACT

Metal trace elements, such as Fe, Zn, and Mn, are necessary micronutrients required by all plants. In this study, the MxNAS3 gene was cloned from Malus xiaojinesis and MxNAS3 was localized in the cytoplasmic membrane. The expression level of MxNAS3 in root and new leaf was higher than in mature leaf and phloem, which was greatly influenced by high and low Fe stresses, IAA and ABA treatments in M. xiaojinesis. Over-expression of MxNAS3 in transgenic Arabidopsis thaliana contributed to enhanced Fe stress tolerance, as well as higher levels of root length, fresh weight, concentrations of chlorophyll, nicotianamine, Fe, Zn, and Mn, especially under high and low Fe stresses. More importantly, it was the first time for us to find that higher expression of MxNAS3 in transgenic A. thaliana contributed to misshapen flowers. Moreover, the MxNAS3-OE A. thaliana had increased expression levels of flowering-related genes (AtYSL1, AtYSL3, AtAFDL, AtAP1, ATMYB21, and AtSAP).

Introduction

Iron (Fe) is one of the necessary micronutrients for plant growth and development with many functions (Marschner et al. 1986). Figuring out the way through which metal elements accumulate in crops would have great impacts on health for human and plant (Lahner et al. 2003). To ensure Fe is obtained from the environment and to avert the toxicity of Fe redundancy for plants, its acquisition and dynamic equilibrium are strictly regulated (Robinson et al. 1999). However, for most of the soil types have low solubility of Fe, especially for alkaline surroundings where the content of free Fe is very low, less than $10^{-6}$ M (a suitable content for normal plant development) (Wu et al. 2005). Hence, plant chlorosis (Fe-deficiency induced) causes great yield losses (Herbik et al. 1999). Additionally, chlorosis (a usual threat of apple) largely limits the quality and yield of Malus plants (Briat et al. 1995).

To remedy these deficiencies, crops have adaptation mechanism for obtaining Fe from different environments, which had been defined as Strategies I and II (Marschner et al. 1986). For the responses of Fe deficiency, the ‘Strategy I’ mechanism is implemented by higher crops, except Gramineae. The ‘Strategy I’ plant can generate more FRO in low Fe surroundings, for accelerating the reaction from Fe$^{3+}$ to Fe$^{2+}$ and benefiting Fe obtainment (Kochian and Lucas 1991). The Fe absorption strategy in Malus plant (M. xiaojinesis included) follows the ‘Strategy I’ mechanism (Han et al. 1994). As a Fe chelator in plants, nicotianamine (NA) may combine with Fe for transport via new leaf and phloem where the pH value is above 7. NA can also chelate Cu, Mn, Zn, and other metal ions in vitro or in vivo (Schmidke et al. 1999). Many nicotianamine synthase (NAS) genes had been isolated from plants, e.g. tobacco, barley, Arabidopsis, and apple, and the expression levels were greatly affected by Fe stress (Higuchi et al. 1999; Ling et al. 1999; Weber et al. 2004; Yang et al. 2015). Moreover, several YSL proteins (metal-NA transporter) had also been confirmed to convey the complexes of Fe-NA in plant cells, which strongly indicate that NA serves as the chelating substrate for transposition of Fe and perhaps other ions (Curie et al. 2001).

Previous researches showed that M. xiaojinesis was a Fe-efficient genotype Malus plant (Han et al. 1994). Many genes of M. xiaojinesis related to the higher tolerance to Fe deficiency have been cloned and researched during the past two decades. The expression levels of those members in M. xiaojinesis were obviously increased under low Fe treatment and the transgenic plants possessed enhanced tolerances to high and low Fe stresses than WT line. Those genes were mostly involved in absorption and transposition of Fe in M. xiaojinesis. Higher expression levels of MxNAS1 and MxNAS2 enhanced the tolerances to high and low Fe stresses, but also contributed to delay flowering and abnormal flowers in transgenic tobaccos (Han et al. 2013; Yang et al. 2015).

Certain hormones, e.g. indoleacetic acid (IAA), ABA, and ethylene (ETH) were considered as signaling factors of Fe deficiency in Lycopersicon esculentum and A. thaliana (Marschner et al. 1986; Bates and Lynch 1996; Romero et al. 1999). The levels of MxNAS1 and MxNAS2 in M. xiaojinesis increased obviously with treatments of IAA and ABA (Han et al. 2013; Yang et al. 2015). Moreover, studies with ETH in different dicotyledons indicated a physiological relation between ETH and Fe-deficiency signaling (Romera et al. 1999). More ethylene was produced during Fe deficiency (Romera and Alcántara 2004). Moreover, ethylene inhibitor
treatments can reduce Fe deficiency signaling and the expression levels of IRT1 and FRO2 genes (Molassiotis et al. 2005; Lucena et al. 2006).

The functions of MxNAS1 and MxNAS2 had also been researched, which played a key role in synthesizing NA synthase, and increased expression levels of MxNAS1 and MxNAS2 contributed to higher Fe stress tolerance in transgenic tobaccos. Over-expressions of MxNAS1 and MxNAS2 genes in transgenic tobaccos contributed to changed flowering, misshapen floral organs, and higher metal contents (Han et al. 2013; Yang et al. 2015). However, which is the key member of MxNAS family and whether another gene of this family has the similar effect are still unclear.

In this study, a new NAS gene was cloned from M. xiaojinensis and was named as MxNAS3. The expression levels of MxNAS3 gene in various tissues, with different treatments, e.g. high and low Fe stresses, IAA, and ABA were researched in this work. Moreover, over-expression of the MxNAS3 gene contributed to higher tolerances to high and low Fe stresses. Under high and low Fe treatments, the MxNAS3-OE plants had higher levels of weight, root total length, NAS activity, and the concentrations of NA, Fe, Zn, Mn, and chlorophyll. Additionally, it was the first time for us to find that over-expression of the MxNAS3 gene contributed to abnormal floral organs and higher expression levels of flowering-related genes in transgenic A. thaliana.

Materials and methods

Material and treatments

According to Han et al. (2017), the tissue culture seedlings of M. xiaojinensis were grown on MS medium for rapid propagation, rooting and in Hoagland’s solution for acclimation and growth. When M. xiaojinensis seedlings were grown to about 15 cm, they were dealt with Hoagland’s solution with normal Fe level, 400 μM (high level) and 4 μM (low level) Fe, respectively. After 14 d cultivation in different treatments, the growth situations were recorded with a camera.

MxNAS1 isolation and qPCR analysis of MxNAS1, MxNAS2, and MxNAS3

The CTAB method was used for total RNA extraction of different parts, e.g. mature leaf (fully unfolded), root, phloem, and young leaf (leaf of top) in M. xiaojinensis. The roots and leaves could be cut directly, for phloem the brown skin of the stem needs to be scraped with a knife, then the green part is cut off carefully, so that the xylem is not affected. All the samples (including the seedlings treated for 0, 2, 4, 8, 12, 24, and 36 h) were collected for RNA preparation (Han et al. 2015). The cDNA (first strand) was generated according to the program of script II (Invitrogen, USA). PCR was carried out to acquire the MxNAS3 sequence with M. xiaojinensis cDNA as the template. The primers of F1 and R1 (Table S1) were designed according to Malus domestica NAS gene 3 (MdnNAS3, accession number in Malus gene pool: MDP0000835599) to identify the whole open reading frame (ORF) sequence. Then the primers of F1 and R1 were used to clone the MxNAS3 ORF sequence from M. xiaojinensis by the PCR method. The acquired DNA fragment was gel purified and connected to the pEASY-T1 vector (TransGen Biotech Co. Ltd, China) and sequenced (Invitrogen, Beijing).

MxNAS3 gene expression analysis was carried out using the real-time fluorescence quantitative PCR (qPCR) method (Han et al. 2017). The Malus Actin was used as the reference gene with primers of ApActF1 and ApActR1 (Table S1). The primers for qPCR analyses of MxNAS1, MxNAS2, and MxNAS3 are provided in Table S1. The qPCR was incubated under the following procedure: 1 cycle of 94°C for 35 s, then 30 cycles at 94°C for 10 s, 57°C for 35 s. Each sample was analyzed in triplicate. According to Livak and Schmittgen (2001), the expression levels were analyzed with the 2ΔΔC value.

Subcellular localization of MxNAS3

The MxNAS3 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 SacI and Smal. This transient expression vector contains a green fluorescent protein (GFP) at SacI-Smal sites. According to Xu et al. (2014), the plasmids of MxNAS3-GFP were introduced into Allium cepa epidermal cells by injection. The fusion proteins of MxNAS3-GFP were observed and photographed by confocal microscopy.

Vector construction and agrobacterium-mediated A. thaliana transformation

The MxNAS3 gene was added to the sites of XbaI and Kpn1 with the PCR method. The pBI121-MxNAS3 plasmid was constructed by connection with the PCR products and pBI121, which were both digested by restriction enzymes of XbaI and Kpn1. The pBI121-MxNAS3 plasmids were transformed into A. thaliana by the Agrobacterium-mediated method (An et al. 1986). The transgenic lines were selected on MS medium with 40 mg dm⁻³ kanamycin. The primers of MF and MR were used for RT-PCR detection of leaves of A. thaliana, with the Actin2 gene (At3g18780) as the reference gene (AtActF and AtActR, Table S1). The PCR systems were executed for 30 and 25 circulations for MxNAS3 and AtActin, respectively.

Fe stress tolerance analysis

The transgenic A. thaliana of T3 generation and WT line were used in consecutive researches. Thirty seedlings of every line were transferred to MS mediums containing 100 μM (normal level), 400 μM (high level) and 4 μM (low Fe level) Fe, respectively. After 14 d cultivation in different treatment mediums, the growth situations were recorded with a camera. The root total length and fresh weight of each line were also surveyed. Twenty transgenic A. thaliana from each test line (OE-3, OE-8, randomly chosen, and WT) were gathered in this experiment.

Measurements of the concentration of chlorophyll, Fe, Mn, Zn, and NA

The chlorophyll contents of transgenic A. thaliana (OE-3 and OE-8) and WT were analyzed by Aono et al. (1993). By the method of Takahashi et al. (2003), we measured the metal element contents in leaf. According to Higuchi et al. (1999),
the NA contents were measured by the high-performance liquid chromatography (HPLC) method with pure NA as an external standard. Ten seedlings of each treatment were collected, and then all parts of A. thaliana were sampled for experiments, mainly containing leaves and stem segments. The measurements of each index above were repeated for 5 times, 3 values (closer to average) were calculated and the standard deviation (±SD) were also analyzed.

**Microscopic investigation of A. thaliana flowers**

The seeds of each transgenic A. thaliana (OE-3 and OE-8) and the WT line were grown on MS medium for germination and growth for about 10 d. Then, 40 strains of every test line were moved to the nutrition matrix (ratio of vermiculite and culture soil is 1:3) with regular supervision in a greenhouse. According to Han et al. (2017), the microscopic structure of A. thaliana floral organs of every test lines was investigated and recorded.

**Expression analysis of flowering-related genes in A. thaliana**

The flowering-related genes in the leaves of T3 transgenic A. thaliana and WT line were also analyzed by the qRT-PCR method. The qRT-PCR was performed as described above. Primers specific to the flowering-related genes A. thaliana yellow stripe like 1 gene (AtYSL1, At4g24120), A. thaliana yellow stripe like 3 gene (AtYSL3, At1g53550), A. thaliana abnormal flower development inbred line gene (AtAFDL, At1g52910), A. thaliana APETALA1 gene (AtAP1, At1g69120), A. thaliana myeloblastosis 21 gene (AtNAS1, AB021935), A. thaliana sterile apetala gene (AtSAP, At3g18780), and the reference gene (AtActin2, At3g18780) were also measured by the qRT-PCR method. The qRT-PCR was performed as described above. Primers specific to the flowering-related genes A. thaliana yellow stripe like 1 gene (AtYSL1, At4g24120), A. thaliana yellow stripe like 3 gene (AtYSL3, At1g53550), A. thaliana abnormal flower development inbred line gene (AtAFDL, At1g52910), A. thaliana APETALA1 gene (AtAP1, At1g69120), A. thaliana myeloblastosis 21 gene (AtNAS1, AB021935), A. thaliana sterile apetala gene (AtSAP, At3g18780), and the reference gene (AtActin2, At3g18780) were also measured by the qRT-PCR method.

**Expression analysis of NAS genes in A. thaliana**

The expression levels of A. thaliana NAS genes (including AtNAS1, AtNAS2, and AtNAS3) in the roots of WT and MxNAS3-OE lines A. thaliana were also measured by the qPCR method. Primers specific to A. thaliana nicotianamine synthase 1 gene (AtNAS1, AB021934), A. thaliana NAS2 gene (AtNAS2, AB021935), A. thaliana NAS3 gene (AtNAS3, NM_100794) and the reference gene (AtActin2, At3g18780) were also measured by the qRT-PCR method.

**Phylogenetic relationship of MxNAS3 with other NAS proteins**

In order to research the phylogenetic relationship of the MxNAS3 among NAS proteins, 31 NAS from different sources were clustered analyzed with software of DNAMan 6.0. As shown in Figure 3, the three A. thaliana NAS proteins, AtNAS1 (BAA74589), AtNAS2 (BAA74590), and AtNAS3 (NP_172395) cluster together. CoNAS1 (OMP10287, from Corchorus olitorius), FvNAS3 (XP_004309951, from Fragaria vesca), PmNAS3 (XP_008223712, from Prunus mume), MdNAS3 (MDP0000835599, from M. domestica), MxNAS3 (from M. xiaojinensis), GmNAS1 (XP_006598174, from Glycine max), PeNAS3 (XP_011006104, from Populus euphratica) RcNAS1 (XP_002533367, from Ricinus communis), and ZjNAS3 (XP_024934556, from Ziziphus jujub) are grouped into the second cluster. The seven Hordeum vulgare NAS proteins, HvNAS1 (AB010086), HvNAS2 (AB011265), HvNAS3 (AB011264), HvNAS4 (AB011266), HvNAS5 (AB011268), HvNAS6 (AB011269), and three Oryza sativa NAS proteins, OsNAS1 (AB021746), OsNAS2 (AB023818), OsNAS3 (AB023819) are grouped into the third cluster, then followed by SINAS1 (NP_001296307.1, from Solanum lycopersicum) on its own cluster. LjNAS1 (BAH22562, from Lotus japonicus) and MxNAS3 (XP_003591220, from Medicago truncatula) were grouped into the fourth cluster. The two M. xiaojinensis NAS proteins, MxNAS3 among NAS proteins, 31 NAS from different sources were clustered analyzed with software of DNAMan 6.0. As shown in Figure 3, the three A. thaliana NAS proteins, AtNAS1 (BAA74589), AtNAS2 (BAA74590), and AtNAS3 (NP_172395) cluster together. CoNAS1 (OMP10287, from Corchorus olitorius), FvNAS3 (XP_004309951, from Fragaria vesca), PmNAS3 (XP_008223712, from Prunus mume), MdNAS3 (MDP0000835599, from M. domestica), MxNAS3 (from M. xiaojinensis), GmNAS1 (XP_006598174, from Glycine max), PeNAS3 (XP_011006104, from Populus euphratica) RcNAS1 (XP_002533367, from Ricinus communis), and ZjNAS3 (XP_024934556, from Ziziphus jujub) are grouped into the second cluster. The seven Hordeum vulgare NAS proteins, HvNAS1 (AB010086), HvNAS2 (AB011265), HvNAS3 (AB011264), HvNAS4 (AB011266), HvNAS5 (AB011268), HvNAS6 (AB011269), and three Oryza sativa NAS proteins, OsNAS1 (AB021746), OsNAS2 (AB023818), OsNAS3 (AB023819) are grouped into the third cluster, then followed by SINAS1 (NP_001296307.1, from Solanum lycopersicum) on its own cluster. LjNAS1 (BAH22562, from Lotus japonicus) and MxNAS3 (XP_003591220, from Medicago truncatula) were grouped into the fourth cluster. The two M. xiaojinensis NAS proteins,
MxNAS1 (ABD64879), MxNAS2 (XP_008370376), and CsNAS (XP_006472196, from Citrus sinensis), FvNAS1 (XP_004302239, from Fragaria vesca) were grouped into the fifth cluster, and then followed by RcNAS (XP_002512510, from Ricinus communis) and VvNAS (XP_002282175, from Vitis vinifera) on the sixth cluster.

Expression analysis of MxNAS3 in M. xiaojinensis

Under normal Fe concentration, the expressions of MxNAS3 in M. xiaojinensis were higher in young leaf and root, but lower in the mature leaf and stem (Figure 4(a)). In treatments with low Fe, IAA and ABA, the MxNAS3 levels in young leaf, root, and phloem increased quickly and reached the highest value at 8 h, and then declined at 12 h (Figure 4(b, d, and e)). However, the expression levels of MxNAS3 in the above places declined when dealt with high Fe stress (160 µM). The expressions trend of old leaf was contrary to those organs under high and low Fe stresses, whereas had the similar pattern in treatments with the two plant hormones (Figure 4(c)).

Expression levels and pattern comparison of MxNAS1, MxNAS2, and MxNAS3 in M. xiaojinensis

As shown in Figure S1, under normal Fe conditions (40 µM), the expression levels of MxNAS3 in active parts (new leaf, root, and phloem) were higher than MxNAS1 and MxNAS2. When dealt with Fe-deficiency treatments, the expression levels of MxNAS3 in root and new leaf of M. xiaojinensis seedlings increased more quickly with a higher amplitude than MxNAS1 and MxNAS2, which reached a maximum at 8 h with 6.1–6.3 times increase, but for MxNAS1 and MxNAS2, which reached a maximum at 24 and 12 h with 3.7–3.9 and 4.4–4.7 times increase, respectively (Figure S1).

Subcellular localization of MxNAS3

The fusion protein of MxNAS3-GFP was targeted only onto the cell membrane. However, the GFP of the pSAT6-GFP-N1 vector was distributed throughout the A. cepa epidermal cell (Figure 5). The results above suggested that MxNAS3 is localized in the cytoplasmic membrane.
Over-expression of the MxNAS3 gene contributed to improved Fe stress tolerance

Among the 13 transgenic *A. thaliana* lines, 7 lines (OE-1, OE-3, OE-4, OE-5, OE-6, OE-8, and OE-9) were affirmative to be the transgenic *A. thaliana* (Figure 6(a)). There was no obvious different phenotypes between the MxNAS3-OE and wild-type *A. thaliana* with normal Fe content (100 μM). The transgenic *A. thaliana* can grow well, whereas the wild-type plant had noticeable chlorotic appearance when exposed to lower Fe treatment (4 μM). The MxNAS3-OE *A. thaliana* grew better than wild-type line in high Fe content (400 μM), the WT line (left) were smaller and weaker (Figure 6(b)).

The MxNAS3-over-expression *A. thaliana* (OE-3, OE-8) had a longer root length and higher fresh weight than the wild-type, especially when exposed to high and low Fe treatments (Table 1). The values of root total length and fresh weight of transgenic plants were 1.4–1.9 and 1.9–2.1 folds, respectively, bigger than those of the wild-type. The over-expression of MxNAS3 gene in transgenic *A. thaliana* also contributed to the higher chlorophyll and NA contents.

![Figure 4.](image-url)
than in wild-type plants, when dealt with high and low Fe stresses. The wild-type plants were weaker and wilted than MxNAS3-OE lines, with the lower contents of chlorophyll under Fe stress. The NA contents in transgenic plants under high and low contents were 3.8 and 4.6 folds, respectively, higher than in WT (Table 1). Higher MxNAS3 expression level in transgenic A. thaliana also contributed to higher Fe, Zn, and Mn contents.

**Over-expression of MxNAS3 contributed to misshapen floral organs in transgenic A. thaliana**

The floral organs of MxNAS3-OE A. thaliana developed obviously morphological and numerical abnormalities (Figure 7). The normal flowers of A. thaliana have four petals, six stamens, four sepals, and one pistil (Figure 7(a-g)). However, the transgenic plants developed two kinds of misshapen flowers: (1) floral organs deformity: curling petals (Figure 7(j,k)), curling sepals (Figure 7(h–k)), and short stamens (Figure 7(i,j)); (2) abnormal floral organs number: increased or decreased number of sepals, petals, stamens, or pistil were shown in (Figure 7(b–l)), respectively.

The sepal number of MxNAS3-OE A. thaliana changed obviously (Figure S2). Among the different changes in transgenic A. thaliana flowers, the largest ratio of flower with 5 sepals was 36.7%, followed with 6, 4, and 3 sepals, the proportions were 30.4%, 20.2%, and 11.1%, and those flowers having 9 petals accounted the smallest ratio, 1.7%. However, the ratio of misshapen flowers in wild-type plant was only 1.6%.

**Over-expression of MxNAS3 contributed to higher expression levels of flowering-related genes**

In order to research what caused the abnormal flowers in transgenic tobaccos (OE-3 and OE-8), the expression levels of 6 flowering-related genes (AtYSL1, AtYSL3, AtAP1, AtMYB21, AtMYB21, AtSAP) in all lines were also analyzed by qRT-PCR method. The expression levels of six genes all increased obviously in both trial transgenic A. thaliana lines (OE-3 and OE-8) than wild-type, especially for AtYSL1, AtYSL3, and ATMYB21, which had a very significant difference with WT (Figure 8).

**Over-expression of MxNAS3 did not change the expression levels of AtNAS1 genes**

As shown in Figure S3, for the expression levels of AtNAS1, AtNAS2, and AtNAS3 genes, there were no difference between WT A. thaliana and MxNAS3-OE lines (OE-3 and OE-8) under normal Fe condition (100 µM). The expression levels of AtNAS2 and AtNAS3 in MxNAS3-OE lines were slightly lower than WT.

### Table 1. Influences of MxNAS3 transformation on MS medium with various iron contents (100, 400, and 4 µM) on root total length, fresh weight, contents of Fe, Zn, Mn, chlorophyll, and NA of A. thaliana.

| Parameter                  | 4 µM          | 100 µM        | 400 µM        |
|----------------------------|---------------|---------------|---------------|
| Root length (cm)           | 13.5A         | 24.3A         | 26.9A         |
| Fresh weight (mg FW)       | 211B          | 434A          | 442A          |
| Fe content (µg g⁻¹ DW)     | 43D           | 77C           | 75C           |
| Zn content (µg g⁻¹ DW)     | 29C           | 64A           | 65A           |
| Mn content (µg g⁻¹ DW)     | 51C           | 84A           | 86A           |
| Chlorophyll content (µg g⁻¹ FW) | 0.49D         | 1.08C         | 1.11C         |
| NA content (µg g⁻¹ FW)     | 61B           | 289A          | 276A          |

Notes: Each data represents the mean of 3 results with 10 replicates. Means within a column followed with various alphabets were significantly difference at p < .01 by SAS (DW, FW for dry weight and fresh weight, respectively).
When dealt with Fe-deficiency treatments (4 µM), the expression levels of *AtNAS1*, *AtNAS2*, and *AtNAS3* all increased in *MxNAS3*-OE *A. thaliana* and WT lines, the increased amplitudes in WT *A. thaliana* were bigger than in *MxNAS3*-OE line.

**Discussion**

Homology analysis suggested that *MxNAS3* belonged to the NAS family (Figure 2). The NAS family contains one THFD-box at the C-terminal and a NAS region at the N-terminus. The THFD-box is a special binding site of the upstream promoter, and exists in all of the NAS proteins (Herbik et al. 1999; Weber et al. 2004). The phylogenetic relationship showed that *MxNAS3* was more closely related to *MdNAS3* (MDP0000835599), *PmNAS3* (XP_008223712), *FvNAS3* (XP_004309951), *GmNAS1* (XP_006598174), *PeNAS3* (XP_011006104), *RcNAS1* (XP_002533367), *CoNAS1* (OMP10287), and *ZjNAS3* (XP_024934556) (Figure 3).

Former studies had shown that NAS genes have diffusely existed in plants, which participated in many physiological activities, such as metal ions transposition. Many NAS genes had been isolated from barley, *Arabidopsis*, maize, rice, and apple, their proteins displayed the NAS activity, and overexpressations of these genes in transgenic plants increased tolerances of Fe or Zn stress (Higuchi et al. 1999; Suzuki et al. 1999; Mizuno et al. 2003; Lee et al. 2011; Han et al. 2013).

*MxNAS3* expression levels were higher in the root and young leaf than those in mature leaf and phloem in normal Fe content (Figure 4(a)). Under low Fe stress, *MxNAS3* enhanced the expression level to stimulate the NAS and NA synthesis. Therefore, a higher content of NA in *M. xiaojinensis* facilitates the Fe obtainment from poor Fe environment (Koike et al. 2004; Han et al. 2013). For Fe-deficiency stress, Fe is preferentially supplied to more active organs, such as root and young leaf. Therefore, the expressions of *MxNAS3* in young leaf and root increased, while they declined in the inactive part (Figure 4). Under high Fe treatment, the levels increased in mature leaf in order to remove Fe toxicity to keep the usual physiologic activity of active organs, e.g. young leaf and root. Hence, *MxNAS3* responded oppositely to high and low Fe treatments in mature leaf and root.

Several hormones, e.g. IAA, ETH, and ABA, are believed to be signaling Fe deficiency of vegetation (Schmidt et al. 2000; Schikora and Schmidt 2001; Lingam et al. 2011). Moreover, Fe-deficiency treatment contributed to an increase in the IAA content in the shoot apex of the *M. xiaojinensis* and IAA treatment on the shoot apex altered the Fe-deficiency responses (Wu et al. 2012). In the present research, treatments of IAA and ABA increased the expression levels of *MxNAS3* in *M. xiaojinensis* seedlings. Therefore, we speculated that *MxNAS3* had possibly affected Fe transport.

Results of intracellular localization showed that the *MxNAS3* protein was localized in the cytoplasmic membrane (Figure 5). Other NAS were also proved to be localized in the cell membrane (Ling et al. 1999; Han et al. 2013). OsNAS2 protein was localized onto vesicles (Nozoye et al. 2014). ZmNAS3 of maize was distributed throughout the cytoplasm of *Allium cepa* epidermis cells (Mizuno et al. 2003). Moreover, *MxNAS2* of *M. xiaojinensis* was localized onto the cell membrane and vesicles (Yang et al. 2015). The sequence of *MxNAS3* (only 103 amino acids) is shorter than other NAS proteins, which probably affected the localization result of *MxNAS3*, leading to only onto cytoplasmic membrane.

Higher expression levels of the *MxNAS3* gene increased the tolerances to high and low Fe stress in transgenic *A. thaliana* (Figure 6(b)). It was probable that the *MxNAS3* gene played an important role in assisting the transgenic *A.
thaliana to survive from high and low Fe treatments through adjusting the NA synthesis. Over-expression of MxNAS3 in transgenic A. thaliana promoted the productions of NAS and nicotianamine. Correspondingly, increased NA content in transgenic A. thaliana is beneficial to the Fe obtainment in Fe-deficient surroundings (Koike et al. 2004). When transgenic A. thaliana was exposed to high Fe stress, the higher NA content is beneficial for detoxification through chelating plethoric Fe (Palmer and Guerinot 2009; Deinlein et al. 2012). Hence, it was the origin why the MxNAS3-OE A. thaliana had improved tolerances to both high and low Fe stresses. Additionally, higher levels of Fe, Zn, and Mn induced by higher NA content led to higher chlorophyll concentration in MxNAS3-OE A. thaliana. Over-expressions of MxNAS1 and MxNAS2 also improved the tolerances to high and low Fe stresses in transgenic tobaccos (Han et al. 2013; Yang et al. 2015).

Higher expression level of MxNAS3 gene in transgenic A. thaliana also contributed to misshapen flowers (Figure 7). NA aminotransferase gene (HvNAAT) was introduced into transgenic tobaccos, which led to abnormal flowers and sterile (Takahashi et al. 2003). We had discovered the abnormal floral organs in MxNAS1-OE or MxNAS2-OE tobaccos, but had not found this phenomenon in transgenic A. thaliana. A probable cause was that the effects of MxNAS1 and MxNAS2 were not powerful enough. Metal ions take part in the normal development of flower in the plant (Conte and Walker 2011). Metal elements (particularly Fe and Zn) are crucial for reproductive organ development in the plant, because these metal ions are components of many important enzymes in this process (Kim and Guerinot 2007). The concentrations of metal elements (Fe, Zn, and Mn) in transgenic A. thaliana changed markedly (Table 1), which could affect the activities of crucial enzymes in reproductive organ development, and the functions of transcription factors. Several flower-related genes in A. thaliana had been found to affect floral organ developments, such as AtSAP (Byzova et al. 1999), AtYSL1 and AtYSL3 (Chu et al. 2010), AtAFDL and AtAPI (Qi et al. 2011), and AtMYB21 (Qi et al. 2015). In this study, the expression levels of 6 flower-related genes all changed obviously in transgenic A. thaliana lines (Figure 8), especially these genes involved in metal ions transposition, such as AtYSL1, AtYSL3, and AtMYB21. The elevated metal ion contents in transgenic A. thaliana were due to increased NA content. NA, a metal chelate, can assist in transporting metal elements to the reproductive organs, e.g. petal, developing stamen, and pistil. NA may participate in the regulation of metal-requiring enzymes, so it could change the structure and quantity of floral organs. Therefore, over-expression of the MxNAS3 gene in transgenic A. thaliana contributed to abnormally shaped flowers.

Under normal Fe conditions, the expression levels of MxNAS3 in new leaf, root, and phloem were higher than MxNAS1 and MxNAS2. Compared with MxNAS1 and MxNAS2, the expression levels of MxNAS3 in new leaf and root of M. xiaojinensis seedlings increased more rapidly to a higher extent under low Fe treatments (Fig. S1). The concentrations of metal ions and NA in MxNAS3-OE A. thaliana were higher than MxNAS1-OE and MxNAS2-OE plants. Therefore, MxNAS3 has more possibility to be a key gene of the MxNAS family than MxNAS1 and MxNAS2.

Higher expression levels of MxNAS3 increased the tolerances to high and low Fe stress and contributed to misshapen flowers in transgenic A. thaliana. But over-expression of MxNAS3 did not change the expression levels of A. thaliana NAS genes (Figure S3). From these results above, we concluded that improved Fe stress tolerance and misshapen flowers in transgenic A. thaliana were induced by higher contents of NA and metal elements, which were not caused by the changes of A. thaliana NAS (AtNAS1, AtNAS2, AtNAS3) expression levels.

Over-expression of the MxNAS3 gene may increase the tolerances to high and low Fe stresses, but also contribute to misshapen flowers in A. thaliana. Expounding the effects of various regions of the MxNAS3 gene in Fe stress reaction and abnormal floral organs would be beneficial to breed Fe stress-resistance Malus plant by transgene. The effect of MxNAS3 containing abnormal flower organs shows its higher potential use value in ornamental flower breeding.

Disclosure statement
No potential conflict of interest was reported by the authors.

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