SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
PLANT MOLECULAR BIOLOGY
MOLECULAR ENGINEERING IN PLANTS

Received
26 June 2014

Accepted
29 September 2014

Published
24 October 2014

Correspondence and requests for materials should be addressed to J.J. (jiangjiafu@njau.edu.cn) or F.C. (chenfd@njau.edu.cn)

A bHLH transcription factor regulates iron intake under Fe deficiency in chrysanthemum

Min Zhao, Aiping Song, Peiling Li, Sumei Chen, Jiafu Jiang & Fadi Chen

College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China.

Iron (Fe) deficiency can represent a serious constraint on crop growth and productivity. A number of members of the bHLH transcription factor family are known to be involved in the plant Fe deficiency response. Plants have evolved two distinct uptake strategies when challenged by Fe deficiency: dicotyledonous and non-graminaceous species rely mostly on a reduction strategy regulated by bHLH transcription factors, whereas rice relies on a chelation strategy, also regulated by bHLH transcription factors. *CmbHLH1*, a bHLH transcription factor which is localized within the nucleus, was isolated from chrysanthemum. Its transcription was up-regulated both by Fe deficiency and by the exogenous application of abscisic acid. The roots of transgenic chrysanthemum plants in which *CmbHLH1* was up-regulated were better able than those of the wild type chrysanthemum cultivar to acidify their immediate external environment by enhancing the transcription of the H^+-ATPase encoding gene *CmHA*. However, there was no effect of the transgene on the efficiency of uptake of either manganese or zinc. Here, Chrysanthemum *CmbHLH1* contributed to Fe uptake via H^+-ATPase mediated acidification of the rhizosphere. ABA may be positively involved in the process.

Iron (Fe), as an essential element for plant growth and development, is involved in both the respiratory electron transport chain and chlorophyll synthesis. Fe deficiency, especially where the soil pH is non-acidic, is well recognized to represent a constraint on crop growth and productivity. The plant response to Fe deficiency and the regulation of cellular Fe homeostasis have been intensively studied.

Plants have evolved two distinct strategies to cope with Fe deficiency. In the reduction strategy, root plasma membrane H^+-ATPases act to acidify the rhizosphere, thereby increasing the local solubility of Fe^1. Fe^{3+} is then reduced to Fe^{2+} by a membrane-bound ferric-chelate reductase enzyme such as AtFRO2, and this appears to be a major rate-limiting step in Fe uptake[2]. Subsequently, Fe^{2+} is transported into the root by plasma-membrane divalent cation transporters such as IRT1, a plasma-membrane divalent cation transporter regulated by the bHLH (basic helix-loop-helix) transcription factor FIT[3–5]. The alternative strategy involves chelation, here, the mechanism operates through the release of phytosiderophores, which avidly bind Fe^{3+} [6,7]. The chelated complex is transported into the roots via YSL transporters such as the product of *OsYSL15*, a rice gene which is strongly induced by Fe deficiency throughout the root[8].

Certain bHLH-related transcription factors, for example *OsIRO2*, are Fe deficiency-inducible[9]. The promoter sequence of *OsIRO2* harbors IDE Fe deficiency-responsive pathway *cis*-acting elements[10]. OsbHLH133 regulates the transport of Fe between the root and the shoot[11]. A number of bHLH transcription factors are also thought to be involved in the regulation of Fe uptake in *A. thaliana* roots exposed to Fe deficient growing conditions[5,12]. These include PYE, an Fe deficiency inducible transcription factor which is especially strongly expressed in the root pericycle. The targets of PYE are genes implicated in metal homeostasis[12]. PYE is not only a regulator of the chelation strategy, but may also play a role in the reduction strategy[12].

Dimerization of bHLH plays a key role in its function. PYE dimerizes with certain bHLH homologs, notably ILR3 and bHLH115; the former is a component of metal ion-mediated auxin sensing in the *A. thaliana* root[13]. ILR3 may control metal homeostasis through the regulation of genes involved in Fe transport[12]. Peiffer et al. have demonstrated that the FIT/bHLH038 heterodimer induces Fe acquisition genes in soybean[14]. Interactions between the bHLH transcription factor FIT and EIN3/EIL1 also promote Fe acquisition, revealing a molecular connection between the regulation of Fe acquisition and ethylene signaling in *A. thaliana*.[15].

Chrysanthemum is one of the most popular of ornamental species[16]. As for many plants, Fe is essential for its normal growth and development. A visible symptom of Fe deficiency in chrysanthemum is interveinal chlorosis

on the leaf. As yet there is little information available regarding the regulation of Fe uptake in chrysanthemum. Here, we have identified *CmbHLH1*, a member of the bHLH family of transcription factors. Its sequence proved to be highly similar to that of *AtILR3*, and under Fe deficiency, its transgenic up-regulation improved the plant's ability to absorb Fe. The present paper represents the first report to show how a bHLH1 transcription factor regulates the Fe deficiency response of chrysanthemum.

Results

The *CmbHLH1* gene in chrysanthemum cultivar 'Jinba'. The *CmbHLH1* full-length cDNA was isolated from chrysanthemum 'Jinba' using primers derived from the chrysanthemum ESTs described by Chen et al.[17], consisting of a 630 bp open reading frame, an 86 bp 5′-UTR and a 271 bp 3′-UTR. The predicted product of *CmbHLH1* was a 210 residue polypeptide. The sequence contained a specific DNA binding/dimerization domain in its central region (positions 50–111), and an oligopeptide AAYPAAVAAA region (159–168) of low compositional complexity. A phylogenetic analysis based on established bHLH sequences showed that CmbHLH1 was highly similar to the *A. thaliana* protein ILR3 (NP_200279.1) (Fig. S1).

Subcellular localization of CmbHLH1. Based on the online software ProtComp v. 9.0 (www.softberry.com), CmbHLH1 was predicted to localize to the nucleus. A transient assay, involving the biolistic introduction of a *CmbHLH1-GFP* fusion into onion epidermal cells, showed that GFP was expressed largely in the nucleus (Fig. 1a–c). In contrast, in control cells transformed with *GFP* alone, the GFP signal was dispersed throughout the cell (Fig. 1d–f).

The effect of Fe deficiency and ABA (abscisic acid) treatment on the transcription of CmbHLH1. The qPCR analysis suggested that there was little evidence for any Fe deficiency induced up- or down-regulation of *CmbHLH1* over the first 12 h, but that transcript abundance peaked by around 48 h (Fig. 2a). This was taken to indicate that *CmbHLH1* may be involved in the regulation of genes active in Fe uptake. *CmbHLH1* was also induced by ABA treatment (Fig. 2b), which suggested that ABA signaling may be involved in Fe uptake in chrysanthemum.

The responsiveness of *CmbHLH1* promoter to Fe deficiency and ABA. The CmbHLH1 promoter sequence harbored two IDE2 and three ABRE motifs (Fig. 3a). To experimentally confirm the responsiveness of the *CmbHLH1* promoter to Fe deficiency and/or ABA, a *CmbHLH1pro::GUS* fusion was transiently transformed into tobacco leaves. GUS activity was significantly enhanced when the leaves were challenged by either Fe deficiency or ABA treatment (Fig. 3b).

The ionic content of the root and its influence over the local rhizosphere pH. Two independent sense transgenic lines showing a high level of *CmbHLH1* transcription, and similarly two independent antisense low level *CmbHLH1* transcription transgenic lines, were selected (Fig. 4a). However no additional morphological alterations have been observed between sense, antisense transgenic plants and wild type (Fig. S2). After a three day exposure to Fe deficiency, a bromocresol purple based test showed that the sense transgenics were more capable of acidifying the external medium than were either the control or the antisense transgenics (Fig. 4b). Inspection of the transcript abundance of *CmHA*, a gene encoding a proton pump ATPase gene, showed that, compared to the control non-transgenic plants, this gene was up-regulated in the sense transgenics, and down-regulated in the antisense ones (Fig. 4c). The former, when exposed to Fe deficiency, accumulated roughly twice as much Fe as the non-transgenic control plants, while the antisense lines accumulated less Fe than the control (Fig. 5). There was no significant difference between the performance of the transgenics and the control plants with respect to either their Zn or their Mn content (Fig. 5). This observation suggested the involvement of C*mbHLH1* in the acidification response, which may be the first step employed by chrysanthemum plants to reduce the rhizosphere pH and hence improve the plant's uptake of Fe.

Discussion

Fe deficiency has been shown to up-regulate a number of *A. thaliana bHLH* transcription factors, including *FIT*, *bHLH38/39* and *PYE*, all of which are involved in Fe import[5,12,18]. *A. thaliana* also possesses an FIT-independent pathway, in which Fe homeostasis is controlled by bHLH100 and bHLH101[19]. Here, we have shown that Fe deficiency induced the transcription of a *bHLH* gene in chrysanthemum. Alignment of its product with homologs from other species revealed a conserved (as yet unnamed) region at the C terminus, which in the

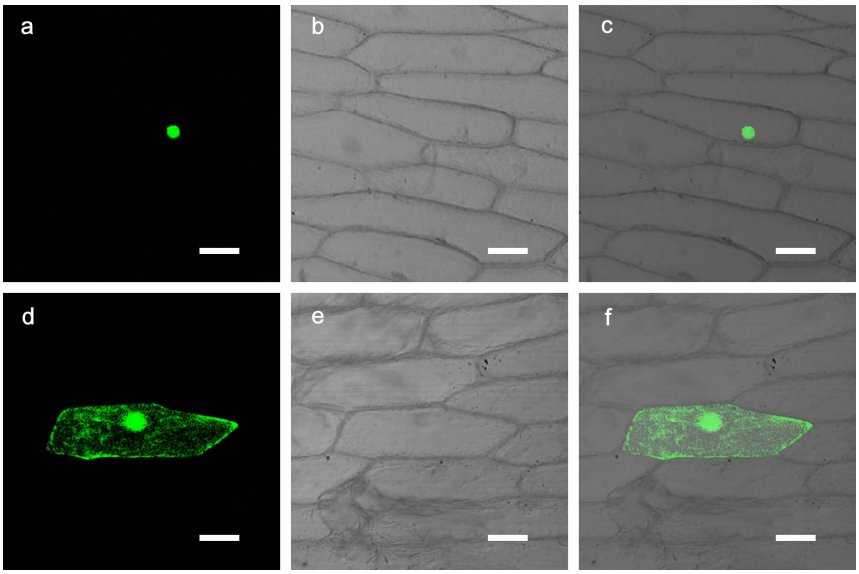

Figure 1 | Subcellular localization of CmbHLH1 in onion epidermis cells. (a–c) Cells transformed with *35S::CmbHLH1-GFP*. (d–f) Control cells transformed with *35S::GFP*. Images captured (a, d) under bright light to show cell morphology, and (b, e) against a dark field to display GFP fluorescence. The images in (c) and (f) are merged from, respectively, (a) and (b), and (d) and (e). Bars, 50 μm.

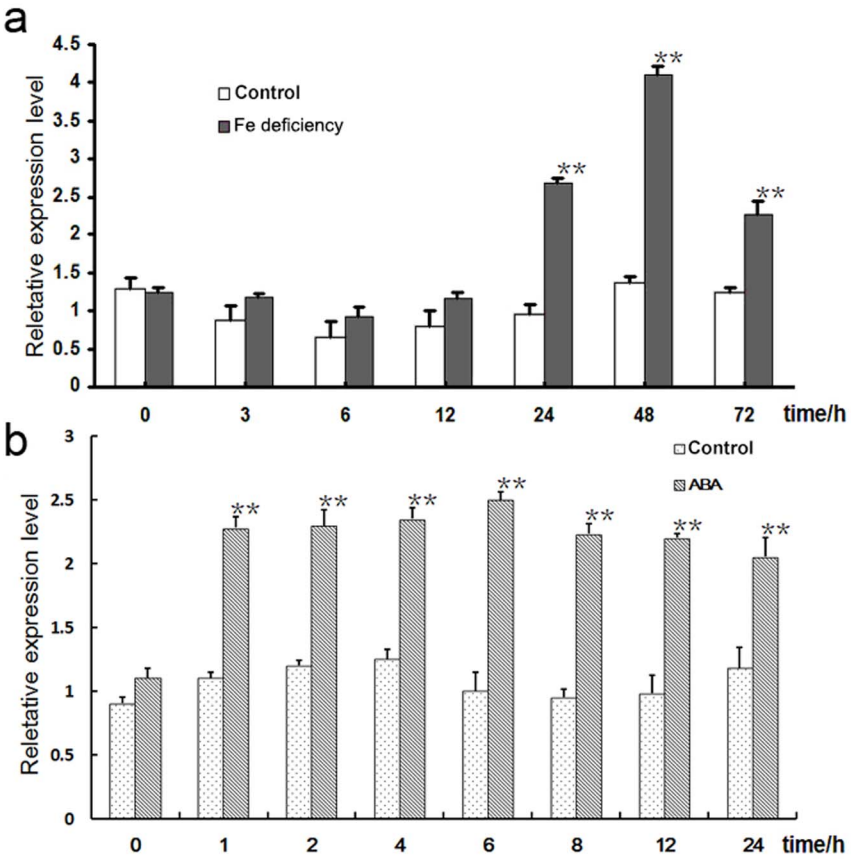

Figure 2 | *CmbHLH1* transcription in response to (a) Fe deficiency and (b) the application of exogenous ABA. **, difference significant at $P < 0.01$.

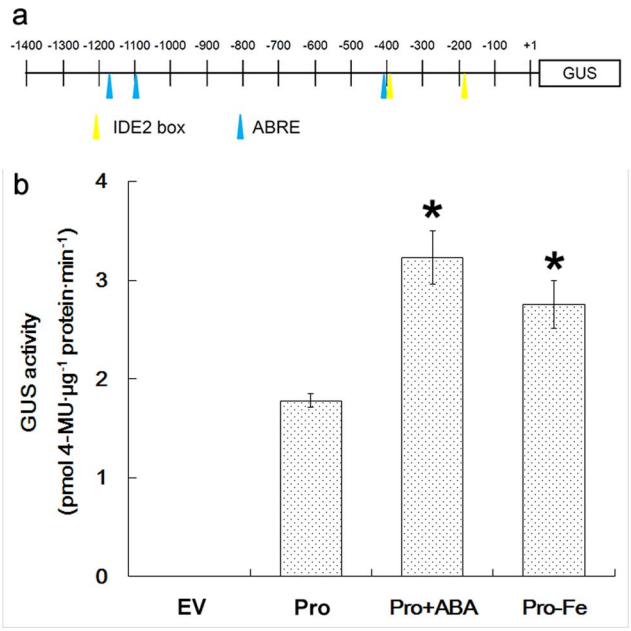

Figure 3 | The responsiveness of the *CmbHLH1* promoter to Fe deficiency and ABA treatment. (a) The pORE-*CmbHLHpro::GUS* construct. The IDE2 box and ABRE elements are highlighted. (b) GUS activity analysis of transgenic tobacco leaves in response to ABA treatment. EV: empty vector control, pro: pORE-*CmbHLH1pro::GUS*, +ABA: ABA treated plants, -Fe: plants subjected to Fe deficiency. *, difference significant at $P < 0.05$.

A. thaliana protein ILR3 has been implicated as being essential for normal function[13]. CmbHLH1 activity clearly enhanced the ability of the roots to acidify their external environment (Fig. 4b), perhaps by promoting the release of plasma membrane H^+-ATPases[1], as implied by the up-regulation of *CmHA* in the *CmbHLH1* sense transgenic chrysanthemum plants (Fig. 4c). A one unit reduction in pH can increase the solubility of Fe ions by 1,000 fold[20]. Under Fe deficient conditions, the *A. thaliana* reductase AtFRO2 (and similarly the pea enzyme PsFRO1) both reduce Fe^{3+} to Fe^{2+}[2], and these ions are then transported by plasma-membrane divalent cation transporters such as IRT1[3,4]. The present data have shown that the transcription of *CmFRO3* was not disturbed in any of the transgenic lines (Fig. S3a), even though the up-regulated *CmbHLH1* transgenic plants were able to accumulate more Fe than the down-regulated ones (Fig. 5). The suggestion is that CmbHLH1, unlike PYE, acts as a positive regulator of Fe intake.

The *CmbHLH1* promoter sequence harboured two IDE2 motifs (Fig. 3a). In barley, rice, tobacco and *A. thaliana*, IDE is associated with the induction of transcription of *bHLH* under Fe deficient conditions[21]. IDEF2, a rice transcription factor which binds to IDE2, is thought to regulate a number of genes responsive to Fe deficiency (for example, the two bHLH transcription factors *OsIRO2* and *OsIRO3*), and particularly those implicated in Fe uptake and utilization during the initial response to stress[10,21,22]. In chrysanthemum, the presence of two IDE2 binding sites in the *CmbHLH1* promoter may make a contribution towards the Fe-deficiency induced expression of *CmbHLH1* (Fig. 3).

ABA is involved in a wide range of physiological processes in plants. An increase in ABA level was observed in cucumber roots under salt stress conditions, where increased ABA induced the expression of PM-H^+-ATPase gene expression[23]. *CmbHLH1* was inducible by exogenously applied ABA (Fig. 2), but the transgenic

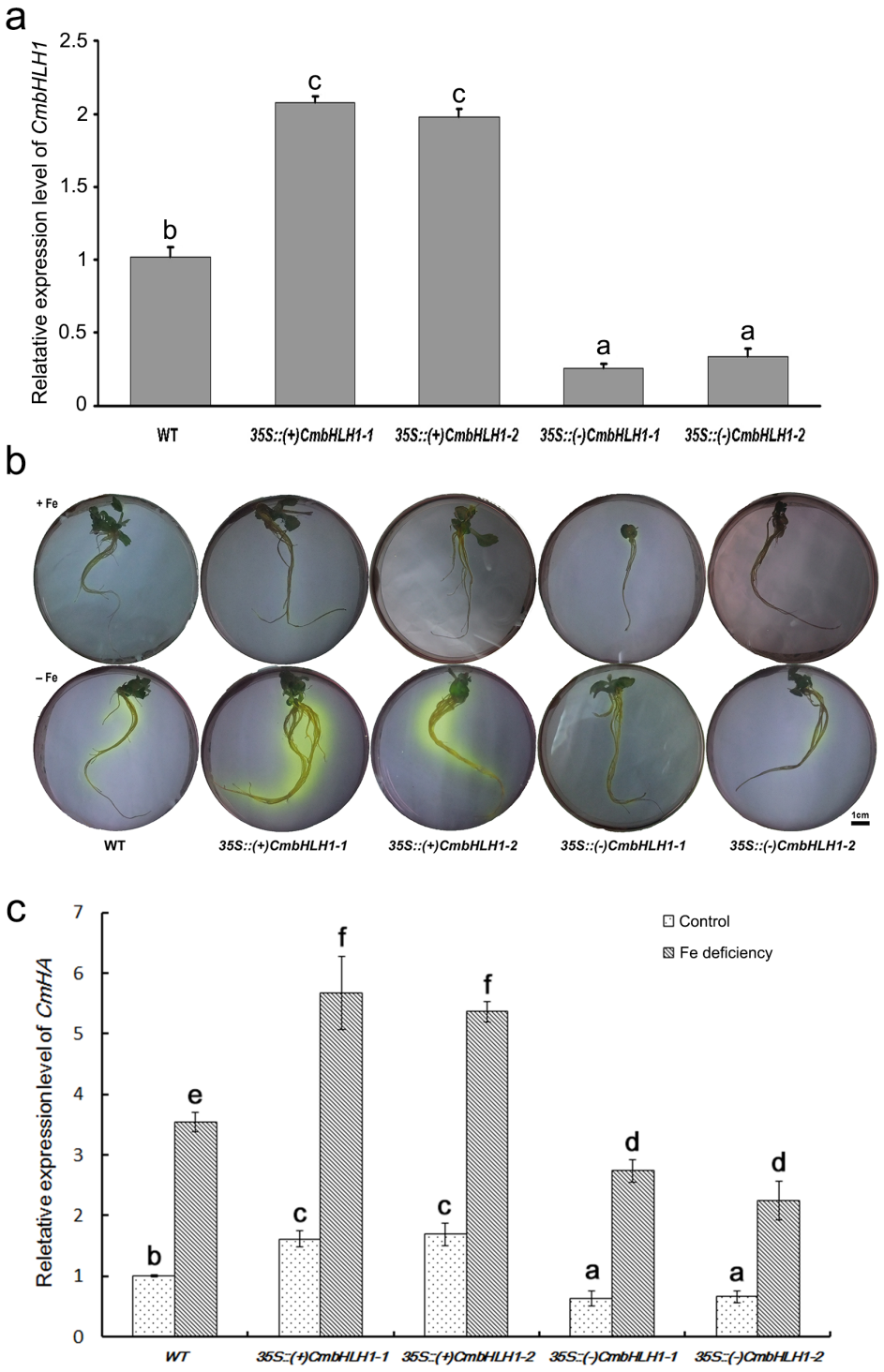

Figure 4 | The acidification capacity of *CmbHLH1* transgenic plants and expression profiles of *CmHA* in *CmbHLH1* transgenic plants subjected to Fe deficiency. (a) CmbHLH1 transcription level in sense and antisense transgenic plants was evaluated in whole seedlings at three week old stage. (b) The acidification capacity of *CmbHLH1* transgenic plants in Fe deficient growing conditions, as indicated by the bromocresol purple dye. The roots of two independent sense transformants were both able to reduce the local pH to <5.2. (c) The transcription of *CmHA* in *CmbHLH1* transgenic plants subjected to Fe deficiency for 48 h. Values labeled with a different superscript differ from one another at $P < 0.05$.

up-regulation of *CmbHLH1* had no effect on the transcription of the ABA marker gene *PP2C* (Fig. S3b). We found *PP2C* was also induced by Fe deficiency (Fig. S4), the induction of *PP2C* was more quickly than *CmbHLH1* (Fig. 2a). All these result suggest that *CmbHLH1* may not regulate ABA signalling as a feedback, but instead that ABA increases H^+-ATPase activity via the induction of *CmbHLH1*.

bHLH transcription factors regulate Fe uptake through their dimerization with certain other proteins. In *A. thaliana*, ILR3 interacts with BTS under Fe deficient conditions[12], while in soybean, the formation of a FIT/bHLH038 heterodimer induces a set of Fe acquisition genes[14]. An interaction between FIT and EIN3/EIL1 is thought to promote Fe acquisition[15]. The possibility that CmbHLH1

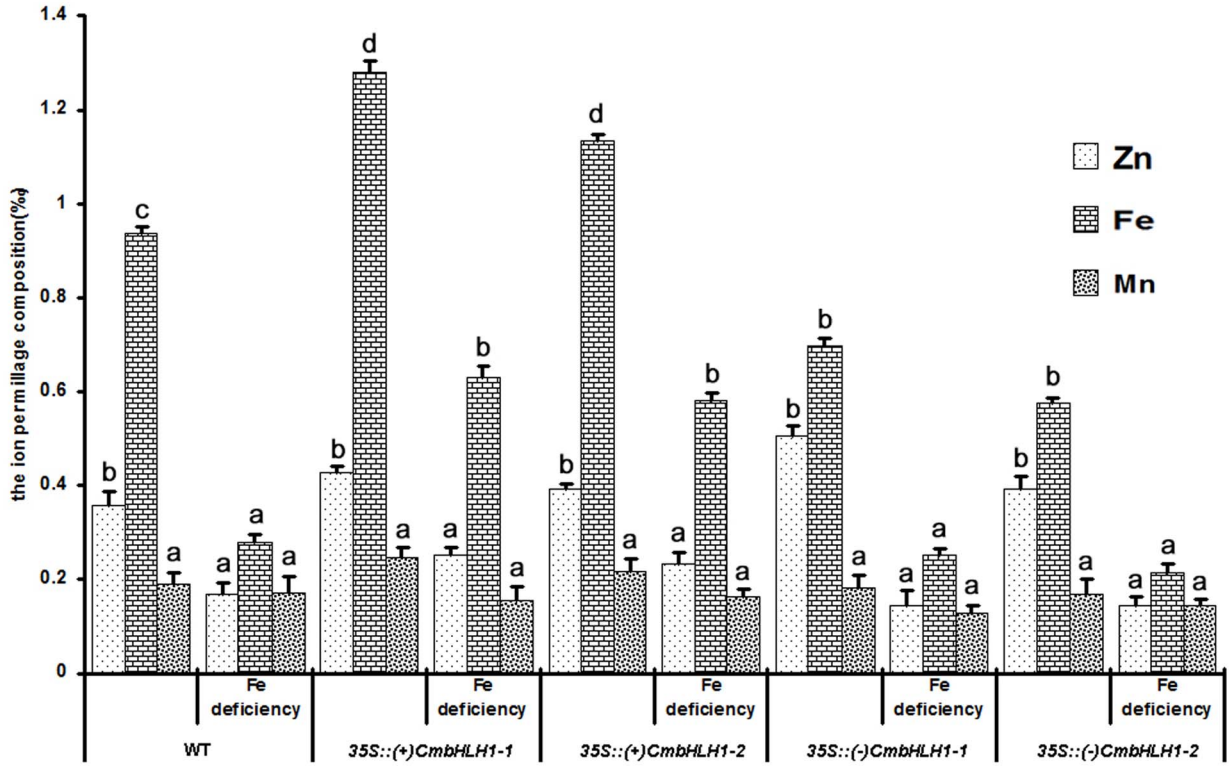

Figure 5 | Ion content in the roots of *CmbHLH1* sense and antisense transgenic plants. The sense transgenic lines contained more Fe than either the non-transformed control or the antisense transformant, while the content of Mn and Zn was unaffected by the presence of either transgene. Values labeled with a different superscript differ from one another at $P < 0.05$.

needs to interact with other transcription factors or to self-dimerize in order to promote Fe intake is a topic of our ongoing research.

Conclusions

We have described a chrysanthemum *bHLH* type transcription factor gene which was up-regulated in the root both under conditions of Fe deficiency and by the provision of exogenous ABA. Transgenic chrysanthemum plants in which *CmbHLH1* was up-regulated were better able to acidify their immediate root environment, and were more effective in taking up Fe when the medium was Fe deficient. The data suggested that ABA may be involved in the *CmbHLH1*-regulated absorption of Fe.

Methods

Plant materials and growing conditions. Seedlings of the chrysanthemum cultivar 'Jinba' were obtained from the Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Plants were grown in a greenhouse where the day/night temperature was maintained at approximately 25°C/18°C and the relative humidity held at ~70%. The same cultivar was also used for the purpose of transformation by cultivating it on MS medium[24].

Isolation of CmbHLH1 and its promoter. 'Jinba' roots were frozen in liquid nitrogen and stored at −70°C. Total RNA was extracted from roots following the protocol provided with the TRIzol reagent (Invitrogen, USA). First strand cDNA was synthesized from DNaseI treated RNA, and served as template for a PCR using *CmbHLH1* specific primers (sequences given in Table S1) designed from the *bHLH* sequence retrieved from the chrysanthemum EST library described by Chen et al.[17]. Both a 3′ and a 5′ RACE were carried out to obtain the full length cDNA sequence, using primers detailed in Table S1. The full open reading frame was amplified by the primer pair CmbHLH1-Full-F/-R (Table S1) and inserted into the pMD19-T vector (Takara, Japan) for sequencing. The *CmbHLH1* promoter sequence was isolated using the thermal asymmetric interlaced PCR (TAIL-PCR) method[25], employing the primers detailed in Table S1.

Phylogenetic and promoter sequence analysis. The structural domain of the predicted polypeptide was annotated using SMART 5 software (http://smart.embl.de/)[26]. An unrooted tree was constructed using the Neighbour Joining Method implemented in the software package MEGA v5[27]. The robustness of each node of the tree was assessed by bootstrap analysis, each comprising 1,000 replications. The promoter sequence was scanned by PLACE software (http://www.dna.affrc.go.jp/PLACE/) to identify plant cis-acting elements[28].

Subcellular localization of CmbHLH1. The subcellular localization of CmbHLH1 was identified using a transient assay based on onion epidermal cells. The *CmbHLH1* open reading frame, lacking its stop codon, was amplified using a Phusion® High-Fidelity PCR kit (New England Biolabs, Ipswich, MA, USA) with the primer pair CmbHLH-Dra-F/-Not-R (Table S1), and the resulting amplicon was cloned into pMD19-T for sequencing. The same fragment was inserted both into the *Dra*I and *Not*I cloning sites of the pENTR™1A dual selection vector (Invitrogen) using T4 DNA ligase (Takara), and into pEarleyGate 103[29] using LR Clonase™ II enzyme mix (Invitrogen). The latter plasmid includes *GFP* as a marker for transgene expression. The construct was bombarded into the onion cells and GFP expression was monitored by confocal laser microscopy[30].

Transcription analysis. The transcription analysis of *CmbHLH1* in 'Jinba' seedlings exposed to Fe deficiency was carried out using three week old seedlings grown in a greenhouse under natural light (14 h light/10 h dark, with the light intensity ranging from 50 to 100 μmol m^{-2} s^{-1}) in a 2 : 1 mixture of garden soil and vermiculite. The day/night temperature was maintained at 18°C/15°C and the relative humidity at 70–75%. At the end of this period, the plants were transferred to a liquid MS medium, held for three days, and then transferred to a liquid MS medium in which $Fe_2(SO_4)_3$ was replaced by 300 μM of the iron chelator ferrozine. Control plants were left to grow in liquid MS medium. The plant hormone ABA was added to the hydroponic solution at a concentration of 0.38 mM (the parallel control experiment was run without the addition of ABA). The roots were exposed to this medium for either 0, 3, 6, 12, 24, 48 or 72 h. RNA was extracted from the roots, and the transcript abundance of *CmbHLH1*, *CmHA*, *CmFRO3* and *CmPP2C* assayed using quantitative PCR (qPCR), based on primers listed in Table S1. The qPCR assay was conducted using iTaq SYBR Green Supermix (Bio-Rad) and *GAPDH* (DK941612) was used as a reference gene[31]. The comparative Ct method (Applied Biosystems bulletin) was used to estimate transcript abundance. Each sample was tested at least in triplicate, and the relative expression abundance was normalized using *GAPDH* as the internal control. The assay included three biological replication.

Transient expression of *CmbHLH1pro::GUS* in tobacco. A *CmbHLH1pro::GUS* plasmid was constructed as follows: first, the *CmbHLH1* promoter sequence was amplified with the primer pair Pro-SacII-F/-NheI-R (Table S1), then this amplicon was inserted into the *Sac*II and *Nhe*I cloning sites of the pORE-R1 vector[32]. Transient transformation of tobacco was achieved following Sparkes et al.[33]. After infiltration, the tobacco plants were held in the dark for two days, then moved to a growth cabinet

providing a 14 h photoperiod at a constant temperature of 21°C. The Fe deficiency and ABA treatments followed the methods described above, and the infiltrated leaves were harvested 48 h after the treatment had been imposed. GUS activity was expressed in the form nmol 4-methylumbelliferone generated per min per μg protein[34,35]. Three independent assays were performed per treatment.

Transgene construction and plant transformation. Both a sense and an antisense version of the *CmbHLH1* open reading frame were amplified from 'Jinba' cDNA using the primer pairs CmbHLH1-Sense-F/-R and CmbHLH1-Antisense-F/-R, respectively (sequences given in Table S1). Each amplicon was inserted separately into the *Bam*HI/*Sac*I cloning site of the pCAMBIA1301 vector, and placed under the control of the CaMV 35S promoter. 'Jinba' leaf discs were agro-infected as described by Das et al.[36]. Putative transformants were selected on the basis of hygromycin resistance, and confirmed by both a PCR and an RT-PCR assay.

Ion content of the root and the rhizosphere acidification. A 100 mg sample (three replicates) of the roots exposed to Fe deficiency was rinsed and digested in 5 mL 10 M HNO_3, then neutralized with NaOH. The aqueous phase was analyzed for the presence of Fe, zinc (Zn) and manganese (Mn) ions using an inductively coupled plasma-mass spectrometer, following Morrissey et al.[37]. The pH in the medium immediately surrounding the roots was measured[38]; for each transgenic line, three plants were transferred to an Fe deficient medium for three days, then plated on 1% agar containing 0.006% w/v bromocresol purple and 0.2 mM $CaSO_4$ (pH 6.5) for a further 24 h. The indicator dye color is pH responsive.

1. Santi, S., Cesco, S., Varanini, Z. & Pinton, R. Two plasma membrane H^+-ATPase genes are differentially expressed in iron-deficient cucumber plants. *Plant Physiol. Biochem* **43**, 287–292 (2005).
2. Robinson, N. J., Procter, C. M., Connolly, E. L. & Guerinot, M. L. A ferric-chelate reductase for iron uptake from soils. *Nature* **397**, 694–697 (1999).
3. Eide, D., Broderius, M., Fett, J. & Guerinot, M. L. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Nat. Acad. Sci. USA* **93**, 5624–5628 (1996).
4. Guerinot, M. L. The ZIP family of metal transporters. *BBA-Biomembranes* **1465**, 190–198 (2000).
5. Colangelo, E. P. & Guerinot, M. L. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant Cell* **16**, 3400–3412 (2004).
6. Aciksoz, S. B., Ozturk, L., Gokmen, O. O., Römheld, V. & Cakmak, I. Effect of nitrogen on root release of phytosiderophores and root uptake of Fe (III)-phytosiderophore in Fe-deficient wheat plants. *Physiol. Plant.* **142**, 287–296 (2011).
7. Schaaf, G. *et al.* A putative function for the Arabidopsis Fe–phytosiderophore transporter homolog AtYSL2 in Fe and Zn homeostasis. *Plant Cell Physiol.* **46**, 762–774 (2005).
8. Lee, S. *et al.* Disruption of OsYSL15 leads to iron inefficiency in rice plants. *Plant Physiol.* **150**, 786–800 (2009).
9. Ogo, Y. *et al.* The rice bHLH protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. *Plant J.* **51**, 366–377 (2007).
10. Ogo, Y. *et al.* A novel NAC transcription factor, IDEF2, that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. *J Biol. Chem.* **283**, 13407–13417 (2008).
11. Wang, L. *et al.* Identification of OsbHLH133 as a regulator of iron distribution between roots and shoots in Oryza sativa. *Plant Cell Environ.* **36**, 224–236 (2013).
12. Long, T. A. *et al.* The bHLH transcription factor POPEYE regulates response to iron deficiency in Arabidopsis roots. *Plant Cell* **22**, 2219–2236 (2010).
13. Rampey, R. A. *et al.* An Arabidopsis basic helix-loop-helix leucine zipper protein modulates metal homeostasis and auxin conjugate responsiveness. *Genetics* **174**, 1841–1857 (2006).
14. Peiffer, G. A. *et al.* Identification of candidate genes underlying an iron efficiency quantitative trait locus in soybean. *Plant Physiol.* **158**, 1745–1754 (2012).
15. Lingam, S. *et al.* Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in Arabidopsis. *Plant Cell* **23**, 1815–1829 (2011).
16. Singh, H. Current status of floriculture–national and international scenario. *Commercial floriculture. FAO and DAC, Ministry of Agriculture, Govt. of India, New Delhi*, 1–26 (2000).
17. Chen, S. *et al.* Analysis of expressed sequence tags (ESTs) collected from the inflorescence of chrysanthemum. *Plant Mol. Biol. Rep.* **27**, 503–510 (2009).
18. Sivitz, A., Grinvalds, C., Barberon, M., Curie, C. & Vert, G. Proteasome-mediated turnover of the transcriptional activator FIT is required for plant iron-deficiency responses. *Plant J.* **66**, 1044–1052 (2011).
19. Sivitz, A. B., Hermand, V., Curie, C. & Vert, G. Arabidopsis bHLH100 and bHLH101 control iron homeostasis via a FIT-independent pathway. *PLoS One* **7**, e44843 (2012).
20. Guerinot, M. L. & Yi, Y. Iron: nutritious, noxious, and not readily available. *Plant Physiol.* **104**, 815 (1994).
21. Kobayashi, T. *et al.* The spatial expression and regulation of transcription factors IDEF1 and IDEF2. *Ann. Bot.* **105**, 1109–1117 (2010).
22. Kobayashi, T. *et al.* The rice transcription factor IDEF1 is essential for the early response to iron deficiency, and induces vegetative expression of late embryogenesis abundant genes. *Plant J.* **60**, 948–961 (2009).
23. Janicka-Russak, M. & Kłobus, G. Modification of plasma membrane and vacuolar H^+-ATPases in response to NaCL and ABA. *J. Plant Physiol.* **164**, 295–302 (2007).
24. Murashige, T. & Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497 (1962).
25. Zhao, M. *et al.* PCR primers targeting cis-acting promoter elements in plants. *Russ. J. Plant Physiol.* **59**, 413–418 (2012).
26. Letunic, I. *et al.* SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res.* **34**, D257–D260 (2006).
27. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739, doi:10.1093/molbev/msr121 (2011).
28. Higo, K., Ugawa, Y., Iwamoto, M. & Korenaga, T. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* **27**, 297–300 (1999).
29. Earley, K. W. *et al.* Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629 (2006).
30. Song, A. *et al.* Isolation and characterisation of *Chrysanthemum crassum SOS1*, encoding a putative plasma membrane Na^+/H^+ antiporter. *Plant Biol. (Stuttg)* **14**, 706–713 (2012).
31. Gu, C. *et al.* Reference gene selection for quantitative real-time PCR in chrysanthemum subjected to biotic and abiotic stress. *Mol. Biotechnol.* **49**, 192–197 (2011).
32. Coutu, C. *et al.* pORE: a modular binary vector series suited for both monocot and dicot plant transformation. *Transgenic Res.* **16**, 771–781 (2007).
33. Sparkes, I. A., Runions, J., Kearns, A. & Hawes, C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature Protoc.* **1**, 2019–2025 (2006).
34. Jefferson, R. A. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405 (1987).
35. Gallagher, S. R. *GUS protocols: using the GUS gene as a reporter of gene expression.* (Access Online via Elsevier, 1992).
36. Das, D., Reddy, M., Upadhyaya, K. & Sopory, S. An efficient leaf-disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). *Plant Cell Rep.* **20**, 999–1005 (2002).
37. Morrissey, J. *et al.* The ferroportin metal efflux proteins function in iron and cobalt homeostasis in Arabidopsis. *Plant Cell* **21**, 3326–3338 (2009).
38. Marschner, H., Römheld, V. & Ossenberg-Neuhaus, H. Rapid method for measuring changes in pH and reducing processes along roots of intact plants. *Zeitschrift für Pflanzenphysiologie* **105**, 407–416 (1982).

Acknowledgments

This study is supported by the National Natural Science Foundation of China (Grant No. 31071820, 31071825,31272202), Natural Science Foundation of Jiangsu Province (Grant No. BK2011641, BK2012773), the Fundamental Research Funds for the Central Universities (KYZ201112, KYZ201147), the Program for New Century Excellent Talents in University of Chinese Ministry of Education (Grant No. NCET-10-0492), Youth Science and Technology Innovation Fund (KJ2011009), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Author contributions

A.S., M.Z. and P.L. performed the experiments; M.Z. wrote the manuscript; S.C. and J.J. edited the manuscript; A.S., S.C. and J.J. revised the manuscript; J.J. and F.C. designed the experiments.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhao, M. *et al.* A bHLH transcription factor regulates iron intake under Fe deficiency in chrysanthemum. *Sci. Rep.* **4**, 6694; DOI:10.1038/srep06694 (2014).