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Silicon enhances leaf remobilization of iron in cucumber under limited iron conditions

Jelena Pavlovic[1], Jelena Samardzic[2], Ljiljana Kostic[1], Kristian H. Laursen[3], Maja Natic[4], Gordana Timotijevic[2], Jan K. Schjoerring[3] and Miroslav Nikolic[1,*]

[1]Plant Nutrition Research Group, Institute for Multidisciplinary Research, University of Belgrade, Kneza Viseslava 1, 11030 Belgrade, Serbia, [2]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444-A, 11010 Belgrade, Serbia, [3]Plant and Soil Science Section, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark and [4]Faculty of Chemistry, University of Belgrade, Studentski trg 12–16, 11000 Belgrade, Serbia
**For correspondence. E-mail mnikolic@imsi.bg.ac.rs*

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• **Background and Aims** Retranslocation of iron (Fe) from source tissues enhances plant tolerance to Fe deficiency. Previous work has shown that silicon (Si) can alleviate Fe deficiency by enhancing acquisition and root to shoot translocation of Fe. Here the role of Si in Fe mobilization in older leaves and the subsequent retranslocation of Fe to young leaves of cucumber (*Cucumis sativus*) plants growing under Fe-limiting conditions was investigated.
• **Methods** Iron (^{57}Fe or naturally occurring isotopes) was measured in leaves at different positions on plants hydroponically growing with or without Si supply. In parallel, the concentration of the Fe chelator nicotianamine (NA) along with the expression of nicotianamine synthase (NAS) involved in its biosynthesis and the expression of yellow stripe-like (YSL) transcripts mediating Fe–NA transport were also determined.
• **Key Results** In plants not receiving Si, approximately half of the total Fe content remained in the oldest leaf. In contrast, Si-treated plants showed an almost even Fe distribution among leaves with four different developmental stages, thus providing evidence of enhanced Fe remobilization from source leaves. This Si-stimulated Fe export was paralleled by an increased NA accumulation and expression of the YSL1 transporter for phloem loading/unloading of the Fe–NA complex.
• **Conclusions** The results suggest that Si enhances remobilization of Fe from older to younger leaves by a more efficient NA-mediated Fe transport via the phloem. In addition, from this and previous work, a model is proposed of how Si acts to improve Fe homeostasis under Fe deficiency in cucumber.

Key words: Cucumber (*Cucumis sativus*), iron retranslocation, leaves, nicotianamine, nicotianamine synthase (NAS), phloem transport, silicon, yellow stripe-like (YSL) transporters.

INTRODUCTION

Silicon (Si) and iron (Fe) are the second and the fourth most abundant minerals, respectively, in the earth's crust. While the essentiality of Fe for plants was discovered in the middle of the 19th century (see Römheld and Nikolic, 2006), Si is still not accepted as an essential element. However, its beneficial effect on plant growth and development, especially under stress conditions, is well documented in the literature (for a review, see Liang *et al*., 2015). In aerated soils, only a small proportion of Fe is available for higher plants, and in calcareous soils, which account for one-third of the world's agricultural soils, the availability of Fe does not even satisfy plant requirements (Vose, 1982; Guerinot and Yi, 1994; Lindsay, 1995). Iron deficiency is a major nutritional disorder responsible for reduction in both yield and quality of a wide range of crops (Grusak and Della Penna, 1999; Alloway, 2008). Iron deficiency in crops thus has a strong negative impact on human health worldwide (Welch, 2004; White and Broadley, 2009).

In recent years, considerable progress has been made in understanding the physiological and molecular mechanisms by which plants increase uptake and bioavailability of Fe (for reviews, see Walker and Connolly, 2008; Jeong and Guerinot, 2009; Thomine and Vert, 2013). However, information regarding long-distance transport of Fe and its distribution within shoots is still lacking. Several studies indicate that during senescence and/or Fe deprivation, substantial mobilization of Fe and its subsequent retranslocation via the phloem may occur from vegetative tissues such as leaves and stems (Zhang *et al*., 1995, 1996; Shi *et al*., 2011; Waters and Sankaran, 2011). Retranslocation of Fe is a complex process that involves Fe solubilization and phloem loading in the source tissue, phloem transport and phloem unloading in the sink tissue (Shi *et al*., 2012). Phloem transport of Fe is suggested to be of particular importance for the growth and quality of crops under Fe-limiting conditions because young leaves, fruits and seeds acquire Fe almost exclusively through the phloem. However, the means by which the molecular machinery functions to remobilize Fe to the shoot and redistribute it between source and sink tissues is much less understood.

Nicotianamine (NA) is a plant-specific non-proteinogenic amino acid synthesized by the condensation of three molecules

of *S*-adenosylmethionine in a reaction catalysed by nicotianamine synthase (NAS). As a hexadentate metal chelator, NA complexes both Fe^{2+} and Fe^{3+}, with a higher affinity for Fe^{3+}, but forming a more stable complex with Fe^{2+} (von Wirén *et al*., 1999). In graminaceous (Strategy 2) roots, NA is the direct precursor of phytosiderophores of the mugineic acid family (Mori, 1999) that strongly chelate Fe^{3+} in the rhizosphere thereby enabling Fe acquisition (Römheld and Marschner, 1986). It has also been proposed that NA plays a key role in Fe complex formation for phloem loading and translocation of Fe in plants, as well as in intracellular Fe chelation and short-distance transport (Scholz *et al*., 1992; Hell and Stephan, 2003). In tomato (*Solanum lycopersicum*), NA increases within both root and leaf cells in response to increasing Fe levels (Pich *et al*., 2001). Three arabidopsis (*Arabidopsis thaliana*) *NAS* genes expressed in leaves showed varied patterns of Fe regulation; *AtNAS1* was not Fe regulated, whereas *AtNAS3* was down-regulated and *AtNAS4* upregulated by Fe deficiency (Klatte *et al*., 2009).

The phloem-based long-distance Fe transport to sink tissues (e.g. young leaves) involves loading of Fe into the companion cell/sieve element complex and its unloading into corresponding sink tissues (Zhai *et al*., 2014). However, the contribution of phloem-specific transporters to loading/unloading of Fe has long been a subject of debate and remains insufficiently clear. The main contributors to this process are several yellow stripe-like (YSL) proteins, which are members of the oligopeptide transporter (OPT) family. It has been demonstrated that arabidopsis AtYSL1 andAtYSL3, and rice (*Oryza sativa*) OsYSL2 mediate Fe–NA transport and facilitate Fe loading into seeds (Waters *et al*., 2006; Chu *et al*., 2010; Ishimaru *et al*., 2010). Zhai *et al*. (2014) recently demonstrated that another member of the OPT family in arabidopsis, AtOPT3, also mediates Fe loading into the phloem in leaves as well as regulating Fe transport to developing tissues. The relevant contributors to Fe remobilization from source leaves in cucumber (*Cucumis sativus*), however, are as yet unknown.

It has recently been reported that Si nutrition can alleviate Fe deficiency stress in Strategy 1 species such as cucumber and soybean (*Glycine max*) (Gonzalo *et al*., 2013; Pavlovic *et al*., 2013). Pavlovic *et al*. (2013) demonstrated that application of Si increased the root apoplastic Fe pool and enhanced the expression of proteins involved in reduction-based Fe uptake. Moreover, Si influenced the expression of genes involved in biosynthesis of Fe-mobilizing compounds, thus resulting in enhanced accumulation of organic acids and phenolics which mediate increased Fe availability in the rhizosphere and mobilization of root apoplastic Fe (Pavlovic *et al*., 2013). It has also been shown that application of Si can facilitate mobility and xylem translocation of Fe towards the shoot, along with the accumulation of Fe-chelating compounds such as citrate in xylem sap and leaf tissues (Pavlovic *et al*., 2013; Bityutskii *et al*., 2014).

The objective of the present work was to gain further insight into the mechanisms involved in Si-mediated remobilization of Fe from older to younger leaves of cucumber plants under Fe-limiting conditions. We conducted stable isotope experiments with ^{57}Fe in order to trace Fe distribution in the shoot. In addition, we focused on the distribution of NA as a potential Fe chelator relevant to Fe mobilization in source leaves and its subsequent retranslocation to the young leaves. Total NA content and the expression of NAS, a key enzyme involved in NA biosynthesis, as well as the expression of YSL transporters for the Fe–NA complex were therefore also determined at different developmental stages of the leaves.

MATERIALS AND METHODS

Plant materials and growth conditions

After soaking in 1 mM $CaSO_4$ overnight, seeds of cucumber (*Cucumis sativus* L. cv. Chinese long) were germinated for 4 d between two sheets of filter paper moistened with saturated $CaSO_4$. The seedlings were then transferred to an Fe-free nutrient solution (four plants per 2·5 L plastic pot) containing (in mM): 0·7 K_2SO_4, 0·1 KCl, 2·0 $Ca(NO_3)_2$, 0·5 $MgSO_4$, 0·1 KH_2PO_4, and (in μM): 0·5 $MnSO_4$, 0·5 $ZnSO_4$, 0·2 $CuSO_4$, 0·01 $(NH_4)_6Mo_7O_{24}$ and 10 H_3BO_3. After 7 d of pre-culture, the cotyledons were removed and plants were transferred to a complete nutrient solution supplied with 10 μM Fe^{III}-EDTA, either ^{57}Fe enriched or with natural abundance of Fe isotopes (here named Fe). After 3 d, Fe was again withheld from the nutrient solution, and plants were grown for another 11 d in Fe-free nutrient solution, either without (–Si) or with (+Si) supply of 1·5 mM monosilicic acid [$Si(OH)_4$] freshly prepared and monitored according to Pavlovic *et al*. (2013). The first green leaf (L0), fully expanded during the pre-culture, was removed at the time of withdrawal of Fe from the nutrient solution (for a schematic presentation of the experimental set-up, see Supplementary Data Fig. S1). In addition, to minimize further mobilization of Fe from the root apoplast and subsequent enzymatic Fe^{III} reduction preceding Fe^{2+} uptake, the pH of the nutrient solution was kept above 7·0 by buffering with $CaCO_3$ (0·2 g L^{-1}) and checked daily. The nutrient solutions were renewed completely every 2 d and continuously aerated.

Plants were grown under controlled environmental conditions in a growth chamber with a light/dark regime of 16:8 h, temperature regime of 24:20 °C, photon flux density of 450 μmol m^{-2} s^{-1} at plant height, and relative air humidity of about 70 %.

At harvest, root apoplastic Fe was removed by a reductive extraction as described below. Subsequently, plants were divided into the following parts: root, stem (together with leaf petioles and main midrib) and leaves (blades devoid of main midrib) collected from four different positions (from the base to the youngest leaf): L1 (developed during 3 d Fe treatment), L2, L3 and L4 (including apex), which all appeared after the Fe treatment. Samples were oven dried at 70 °C for 48 h, weighed and pulverized in a ceramic grinder.

Spectral plant analysis diagnostic (SPAD) measurement

The chlorophyll content in leaves was estimated non-destructively as SPAD units, using a portable Chlorophyll Meter SPAD-502 device (Minolta Camera Co., Osaka, Japan).

Determination of Si

Dry plant material (0·2 g) was digested with 3 mL of concentrated HNO_3 + 2 mL of H_2O_2 in a microwave oven

(Speedwave MWS-3$^+$; Berghof Products + Instruments GmbH, Eningen, Germany) for 1 h. Samples were diluted with about 15 mL of H_2O, transferred into 25 mL plastic flasks, 1 mL of hydrofluoric acid was added and samples were then left overnight. After addition of 2·5 mL of 2 % (w/v) H_3BO_3, the flask volume was adjusted to 25 mL with deionized H_2O, and Si was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; SpectroGenesis EOP II, Spectro Analytical Instruments GmbH, Kleve, Germany) after a final dilution of the samples of 1:100 (v/v) with deionized H_2O.

Determination of Fe in root apoplast and plant tissues

After washing in a solution containing 0·5 mM $CaSO_4$ and 5 mM MES (pH 5·5) for 10 min, intact roots of each plant were transferred into a 40 mL incubation solution containing 5 mM MES (pH 5·5), 0·5 mM $CaSO_4$ and 1·5 mM 2,2′-bipyridyl, followed by incubation for 10 min under reductive conditions by adding 0·5 g of solid sodium dithionite under continuous N_2 bubbling through the solution. Apoplastic Fe was removed as the red Fe^{II}[bipyridyl]$_3$ complex and determined by measuring the absorbance at 520 nm using a extinction coefficient of 8·65 mM^{-1} (Bienfait *et al*., 1985).

For Fe determination in plant tissue, dry plant material (0·2 g) was microwave digested in 3 mL of concentrated HNO_3 + 2 mL of H_2O_2 for 1 h. Samples were then transferred into 25 mL plastic flasks and the volume was adjusted to 25 mL with deionized H_2O. Fe was determined by ICP-OES. The analytical accuracy of total concentrations was evaluated using certified reference material (GBW 10015 Spinach, Institute of Geophysical and Geochemical Exploration, Langfang, China).

Preparation of $^{57}Fe^{III}$EDTA and ^{57}Fe determination in leaf tissue

$^{57}Fe^{III}$EDTA was freshly prepared from $^{57}Fe_2O_3$ (96·64 % isotopic enrichment; Isoflex, San Francisco, CA, USA) as previously described by Pavlovic *et al*. (2013).

Dry leaf material (0·5 g) was microwave digested in 8 mL of concentrated HNO_3 for 1 h. ^{57}Fe was determined by quadrupole inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7500ce, Agilent Technologies, Manchester, UK). The instrument was equipped with a PFA microflow nebulizer and was used in hydrogen mode to eliminate spectral interference(s). Instrument settings were as described previously (Laursen *et al*., 2009); however, prior to analysis, the hydrogen flow of the octopole ion guide was optimized to give maximum interference-free ^{56}Fe and ^{57}Fe signals, lowest possible backgrounds and accurate $^{56}Fe/^{57}Fe$ values according to the natural abundance ratio of 43·3 (IUPAC values). This was evaluated by hydrogen ramping on non-enriched samples and standards, which yielded accuracies of 100 ± 5 % of the true $^{56}Fe/^{57}Fe$ ratio at the optimal hydrogen flow rate (7 mL min^{-1}). All samples were diluted to 3·5 % (v/v) HNO_3 prior to analysis, and external calibration was conducted to obtain total ^{56}Fe and ^{57}Fe concentrations using a commercially available standard solution (P/N 4400-132565, CPI International, Amsterdam, The Netherlands). The analytical accuracy of total concentrations was evaluated using certified reference material (Spinach NCS ZC73013, China National Analysis Center for Iron and Steel, Beijing, China). Concentration data were accepted if the accuracy exceeded 90 % of the certified reference value. The accuracy of $^{56}Fe/^{57}Fe$ isotope ratios was evaluated by analysis of four non-enriched samples, resulting in an average isotope ratio of 44·3 ± 2·3. Data were processed by the Masshunter Workstation Software, version B.02.01 (Agilent Technologies, Manchester, UK).

NA extraction and analysis

Deep-frozen leaf samples were homogenized using a mortar and pestle in liquid N_2 and extracted in deionized water at 80 °C for 30 min. After centrifugation at 5000 *g* for 30 min at 4 °C, the supernatant was collected and subjected to a liquid chromatography–mass spectrometric (LC-MS) analysis of NA, according to a modification of the method of Yamaguchi and Uchida (2012).

The samples were separated using a Syncronis C18 column (100 × 2·1 mm, 1·7 µm particle size) (Thermo Fisher Scientific Inc., Bremen, Germany). The mobile phase consisted of (A) water + 0·01 % acetic acid and (B) acetonitrile. A linear gradient program at a flow rate of 0·25 mL min^{-1} was used: 0·0–1·0 min 5 % B, 1·0–5·0 min from 5 to 95 % (B), 5·0–7·0 min 95 % B, 7·0–7·1 min from 95 to 5 % (B), then 5 % (B) for 8 min. The injection volume was 5 µL. The Thermo Scientific Orbitrap LC-MS system consisted of a quaternary pump (Accela 600), an autosampler and a linear hybrid ion trap-orbitrap MS (LTQ Orbitrap XL) with heated electrospray ionization (HESI). A standard 100 mg L^{-1} stock solution of NA (Toronto Research Chemicals, North York, Canada) was prepared by dissolution in ultrapure water (0·055 µS cm^{-1}) and was further diluted at concentrations of 0·050, 0·075, 0·100, 0·250, 0·500, 0·750 and 1·000 mg L^{-1} for the calibration curve.

The mass spectrometer was operated in positive mode; HESI-source parameters were as follows: source voltage 5 kV, capillary voltage 30 V, tube lens voltage 90 V, capillary temperature 300 °C, sheath and auxiliary N_2 flow 232 and 8 (arbitrary units). The mass spectra were acquired by full range acquisition covering 230–1000 *m/z*. The normalized collision energy of the collision-induced dissociation cell was set at 35 eV.

Thermo Xcalibur software (version 2.1) was used for instrument control, data acquisition and data analysis. Nicotianamine was quantified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation and characteristic retention time. Quantification was carried out according to the exact mass search method by comparing the retention times and exact mass of the available standard.

RNA extraction and real-time quantitative PCR

Leaf tissue samples (0·5–1 g f. wt) were frozen in liquid nitrogen and ground thoroughly using a mortar and pestle. RNA was isolated using the RNeasy® Mini Kit (Qiagen) as described in the RNeasy® Mini Handbook.

Prior to cDNA synthesis, DNA was removed from RNA samples using Ambion DNA-free DNase Treatment and Removal Reagents. First-strand cDNA was synthesized from 5 μg of RNA with M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) and random hexamer primers (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. The cDNAs were diluted 1:5 with nuclease-free water and aliquots were used for real-time PCR with primers designed for cucumber. Real-time PCRs were performed in a 25 μL volume containing 500 nM of each primer and 1× SYBER Green PCR master mix (Applied Biosystems). Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystem) using parameters recommended by the manufacturer (i.e. 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 1 min). Accumulation of PCR products was detected in real time and the results were analysed with 7500 System Software (Applied Biosystems).

The primers used in this study are: for *NAS1*, 5'-GGA GTTCGAGGTGGTGTTTC-3' and 5'-CCACCACCGGATAA ACAAAC-3'; for *NAS4*, 5'-TCCCAAAAACCGAGTTTCAC-3' and 5'-GAAACACCACCTCGAACTCC-3'; for *YSL1*, 5'-T GCTTTTGCTTTCTTGACCTC-3' and 5'-TAGGAGCCATC TTATCTTATGGC-3'; for *YSL3*, 5'-tccatgcttttaagccaagc-3' and 5'-cgggaccttgtactcaccat-3'; and for *ACTIN* (*ACT*), 5'-GC TGGCATATGTTGCTCTTG-3' and 5'-CGATGGTGATGAC TTGTCCA-3'. Levels of transcription were calculated with the $2^{-\Delta Ct}$ method using *ACT* as an internal control. Each PCR was done in triplicate and included no template controls. To determine the amplification efficiency of real-time PCRs, cDNAs were diluted 5-, 10-, 20- and 40-fold. The calculated PCR efficiency [E(%) = (10 − 1/slope − 1) × 100] was between 90 and 100 % (–3·6 > slope > –3·1).

Sequence data from this article can be found in the GenBank/EMBL data libraries and EST database (http://www.icugi.org/) under the following accession numbers (in parentheses): *CsNAS1* (XM004158701), *CsNAS4* (XP004144812), *CsYSL1* (XM004163525), *CsYSL3* (XP004150025) and *CsACT* (AB010922).

Statistical analysis

Each independent experiment with four replications (individual plants) per treatment was repeated 2–3 times. Data from a representative experiment were subjected to analysis of variance using the statistical software Statistica 6 (StatSoft, Inc., Tulsa, OK, USA), and means were compared by Tukey's test at the 5 % significance level ($P \leq 0{\cdot}05$).

RESULTS

Effect of experimental conditions on plant growth and Fe movement during experimentation

To study the effect of Si nutrition on Fe mobility in leaves, we carried out an experiment with Fe-deprived cucumber plants which were supplied with Fe (^{57}Fe-enriched or naturally occurring isotopes with ^{56}Fe prevailing) for a short period (3 d) and then transferred to Fe-free nutrient solution with or without Si supply for 11 d (see Fig. S1). The Si concentration in the leaves of Si-treated plants was 6-fold higher than that of those grown in the absence of Si (Table 1). Addition of Si did not affect root or stem dry biomass, but strongly stimulated leaf growth, resulting in > 30 % higher total plant dry biomass in Si-fed plants (Table 2). Buffering the pH of the nutrient solution above 7·0 markedly decreased mobilization of Fe from the root apoplast (Table 3); hence, the total Fe content in the root apoplast remained at the same level in both Si-treated and untreated plants at the end of the experiment (Table 4). Furthermore, the concentration of Fe in the xylem sap was very low during the first 2 d when Fe was excluded from the nutrient solution, while afterwards throughout the experiment no Fe was detected (Supplementary Data Table S1). Addition of $CaCO_3$ did not *per se* show any negative effect on plant growth, which was inhibited exclusively by Fe deficiency and amended by supply of Si (Table S2).

TABLE 1. *Silicon concentrations in different cucumber tissues*

Treatment	Si concentration (mg g^{-1} d. wt)		
	Root	Stem	Leaves
–Si	4·0 ± 0·5[a]	7·5 ± 1·5[a]	3·4 ± 0·2[a]
+Si	5·9 ± 0·8[b]	12·0 ± 1·4[b]	21·5 ± 1·8[b]

Seven-day-old Fe-deprived plants were supplied with 10 μM Fe for 3 d and then transferred to Fe-free nutrient solution with or without supply of 1·5 mM $Si(OH)_4$ for 11 d.

Data shown are means ± s.d. ($n = 4$).

Significant differences ($P < 0{\cdot}05$) between treatments are indicated by different letters.

TABLE 2. *Effect of Si nutrition on dry biomass of cucumber*

Treatment	Dry biomass (g per plant)			
	Root	Stem	Leaves	Total
–Si	0·52 ± 0·04[a]	0·9 ± 0·1[a]	2·2 ± 0·2[a]	3·6 ± 0·1[a]
+Si	0·58 ± 0·07[a]	1·0 ± 0·2[a]	3·2 ± 0·1[b]	4·8 ± 0·1[b]

Seven-day-old Fe-deprived plants were supplied with 10 μM Fe for 3 d and then transferred to Fe-free nutrient solution with or without supply of 1·5 mM $Si(OH)_4$ for 11 d.

Data shown are means ± s.d. ($n = 4$).

Significant differences ($P < 0{\cdot}05$) between treatments are indicated by different letters.

Si increases Fe remobilization from old to young leaves

Although there were no significant differences between Si treatments in total Fe content measured in the root symplast, stem and leaves (Table 4), Si supply successfully prevented symptoms of Fe chlorosis in young leaves, following 11 d of Fe deprivation (Fig. 1A). In contrast, plants grown in the absence of Si developed chlorotic leaves following withdrawal of Fe from the nutrient solution. The SPAD readings in younger leaves at positions L3 and L4 (for leaf positions, see Fig. 2A and Fig. S1) of Si-fed plants were significantly higher than in the older leaves at positions L1 and L2 (Fig. 2A). In these leaves, chlorophyll content was markedly lower as compared with the unfed Si plants.

TABLE 3. *Effect of the nutrient solution pH (–/+$CaCO_3$) on the amount of Fe removed from the root apoplast of cucumber throughout the entire experiment*

Si treatment	Fe amount (μg per plant)		Relative decrease (%)
	–$CaCO_3$ (pH 5·0)	+$CaCO_3$ (pH 7·0)	
–Si	66·2 ± 6·7^b	11·7 ± 2·1^a	92
+Si	87·8 ± 6·7^c	17·0 ± 2·3^a	81

Seven-day-old Fe-deprived plants were supplied with 10 μM Fe for 3 d and then transferred to Fe-free nutrient solution, unbuffered (pH < 5·0) or buffered with $CaCO_3$, (0·2 g L^{-1}; pH > 7·0), with or without supply of 1·5 mM $Si(OH)_4$ for 11 d. Data shown are means ± s.d. ($n = 4$).

Significant differences ($P < 0·05$) between treatments are indicated by different letters.

TABLE 4. *Effect of Si supply on the Fe content in different cucumber tissues*

Treatment	Fe content (μg per plant)			
	Root		Stem	Leaves
	Apoplast	Symplast		
–Si	103 ± 11^a	14 ± 2^a	28 ± 3^a	141 ± 16^a
+Si	92 ± 16^a	17 ± 3^a	31 ± 5^a	150 ± 13^a

Seven-day-old Fe-deprived plants were supplied with 10 μM Fe for 3 d and then transferred to Fe-free nutrient solution with or without supply of 1·5 mM $Si(OH)_4$ for 11 d.

Data shown are means ± s.d. ($n = 4$).

Significant differences ($P < 0·05$) between treatments are indicated by different letters.

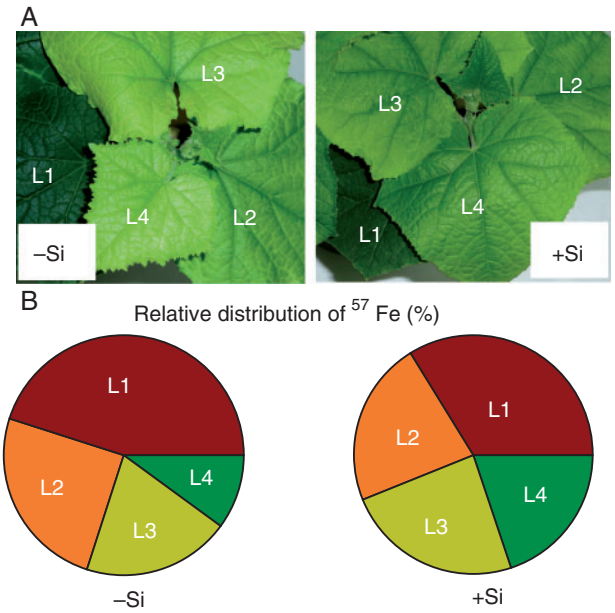

FIG. 1. Effect of Si supply on redistribution of ^{57}Fe in the leaves of cucumber plants 11 d after withdrawal of ^{57}Fe from the nutrient solution. (A) Cucumber plants at the end of the experiment; (B) relative distribution of ^{57}Fe. Leaf positions (from the base to the youngest leaf): L1, L2, L3 and L4. Plants were precultured in Fe-free nutrient solution for 7 d, subsequently subjected to 10 μM $^{57}Fe^{III}EDTA$ (96·64 % enriched) for 3 d and then transferred to an Fe-free nutrient solution with or without supply of 1·5 mM $Si(OH)_4$ for 11 d. Data shown are means ± s.d. ($n = 4$). Significant differences ($P < 0·05$) between treatments are indicated by different letters.

The Fe concentration in the youngest leaves (L4) of the Si-treated plants was about 2-fold higher than that in the untreated plants, whereas it was significantly lower in the oldest leaves (L1) (Fig. 2B). Silicon nutrition thus increased remobilization of Fe from old leaves to the youngest expanding leaves; this was further confirmed in the ^{57}Fe experiment. Although total shoot Fe content remained the same in both –Si and +Si plants (Table 4), Si supply affected the relative leaf ^{57}Fe distribution (Fig. 1B). For unfed Si plants, approx. 50 % of the total ^{57}Fe content remained in the oldest leaf (L1), whereas those receiving Si showed an almost even ^{57}Fe distribution between leaves at different positions, resulting in alleviation of Fe deficiency symptoms (Fig. 1A).

Si increases leaf NA concentration and affects the expression of NAS *genes differently*

The concentration of NA increased gradually from the older to younger leaves (Fig. 3). Silicon nutrition significantly increased the NA concentration in L1, L2 and especially in L3 leaves, while affecting NA concentrations less in the youngest (L4) leaves (Fig. 3). In the unfed Si plants, the expression levels of *NAS1* showed a distinct profile depending on leaf position, with the lowest values observed in the oldest leaf (L1) (Fig. 4A). Supply of Si upregulated *NAS1* expression in both the older (L1, L2) and younger (L3) expanded leaves. However, there was no significant difference between –Si and +Si treatments in *NAS1* expression levels of the youngest (L4) expanding leaves (Fig. 4A). The expression of *NAS4* was not influenced either by leaf developmental stages (unlike the clear leaf age-dependent expression pattern for *NAS1*; see Fig. 4A) or by Si supply (Fig. 4B).

Si upregulates YSL1, but not YSL3 leaf transporter for the Fe–NA complex

The expression of *YSL1* was strongly upregulated in the leaves of Si-supplied plants, with the highest expression levels observed in the oldest (L1) leaf (Fig. 5A). The transcript abundance of *YSL3* was highest in the oldest leaves and gradually decreased towards the shoot apex in both –Si and +Si plants. Silicon supplementation resulted in distinctly higher expression levels of *YSL3* in younger fully expanded leaves (L2 and L3), while in the oldest expanded leaf (L1) it was lower, and in the youngest expanding (L4) leaf was at the level recorded in the –Si plants (Fig. 5B).

DISCUSSION

In the present work we tested and confirmed the hypothesis that Si supply enhances remobilization of Fe from older to younger cucumber leaves. We recently demonstrated that Si mitigates Fe deficiency in cucumber plants by increasing the apoplastic Fe pool in the roots and by accumulation of Fe-mobilizing compounds in roots (citrate and catechine) and leaves (citrate), thus

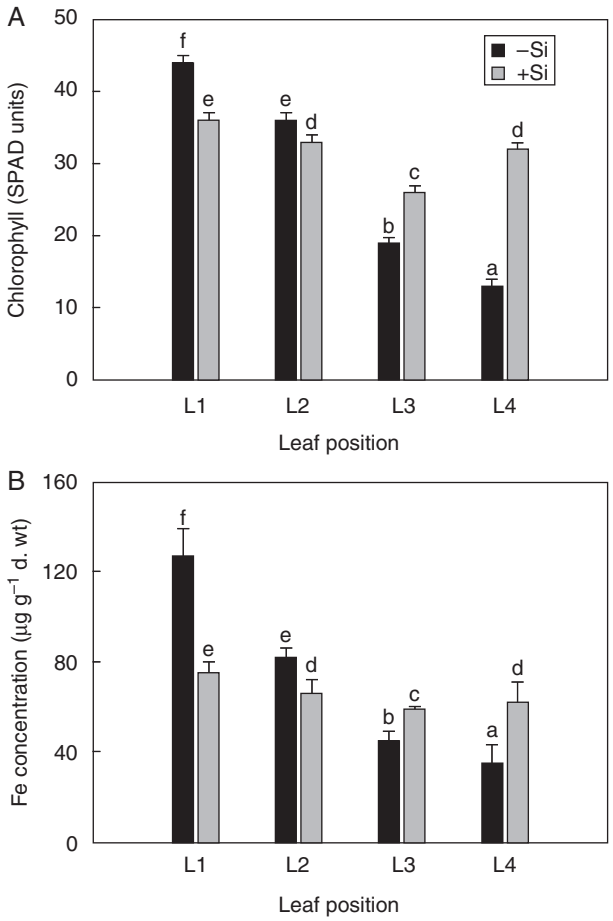

FIG. 2. Effect of Si supply on spectral plant analysis diagnostic (SPAD) readings (A) and Fe concentration (B) in cucumber leaves at different positions. Leaf positions (from the base to the youngest leaf): L1, L2, L3 and L4. Iron-deprived 7-day-old plants were treated with 10 μM Fe for 3 d and then transferred to an Fe-free nutrient solution with or without supply of 1·5 mM $Si(OH)_4$ for 11 d. Data shown are means ± s.d. ($n = 4$). Significant differences ($P < 0·05$) between treatments are indicated by different letters.

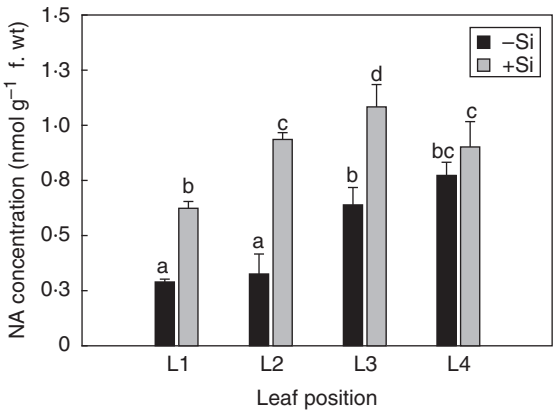

FIG. 3. Effect of Si supply on the total nicotianamine (NA) concentration in cucumber leaves at different positions. Leaf positions (from the base to the youngest leaf): L1, L2, L3 and L4. The plants were treated as described in the legend of Fig. 2. Data shown are means ± s.d. ($n = 4$). Significant differences ($P < 0·05$) between treatments are indicated by different letters.

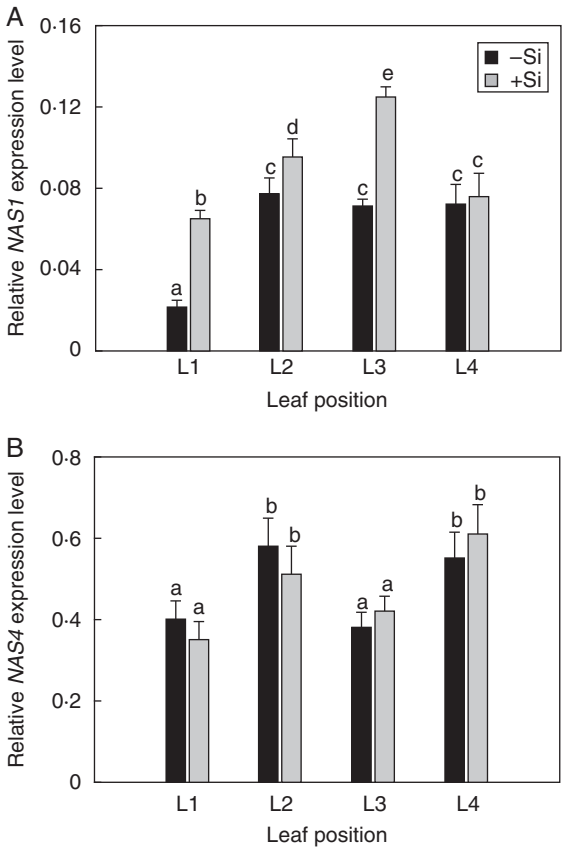

FIG. 4. Effect of Si supplyon the relative expression level of *NAS1* (A) and *NAS 4* (B) in cucumber leaves at different positions. Leaf positions (from the base to the youngest leaf): L1, L2, L3 and L4. The plants were treated as described in the legend of Fig. 2. Data shown are means ± s.d. ($n = 4$). Significant differences ($P < 0·05$) between treatments are indicated by different letters.

enhancing Fe acquisition and translocation towards apical shoot parts (Pavlovic *et al*., 2013; Bityutskii *et al*., 2014). A major limitation in many retranslocation studies conducted so far is that the contribution of the apoplastic Fe pool in roots has not been adequately considered, as pointed out by Shi *et al*. (2012). As apoplastic Fe pools in cucumber roots are usually large (Cesco *et al*., 2002), only a small change of this pool during the long-term experiment may significantly contribute to Fe translocation to sink leaves. In the present study, mobilization of Fe from the root apoplastic pool and subsequent root to shoot translocation of Fe was controlled by maintaining a high pH in the Fe-free nutrient solution (Table 3; Table S1).

The experiment with enriched ^{57}Fe clearly demonstrated that Si nutrition can induce mobilization of ^{57}Fe from older leaves and can also enhance its retranslocation into the younger leaves developed after withdrawal of ^{57}Fe from the nutrient solution (Fig. 1B). As a consequence of undeveloped xylem structures and a low transpiration rate, young expanding leaves are almost exclusively dependent on Fe import from the phloem. It is well documented that Fe can be mobilized from source organs and retranslocated to sink tissues, including young leaves via the phloem (Hell and Stephan, 2003; Römheld and Schaaf, 2004). For instance, Zhang *et al*. (1995) reported that under Fe deficiency up to 20 % of the total amount of both apoplastic and

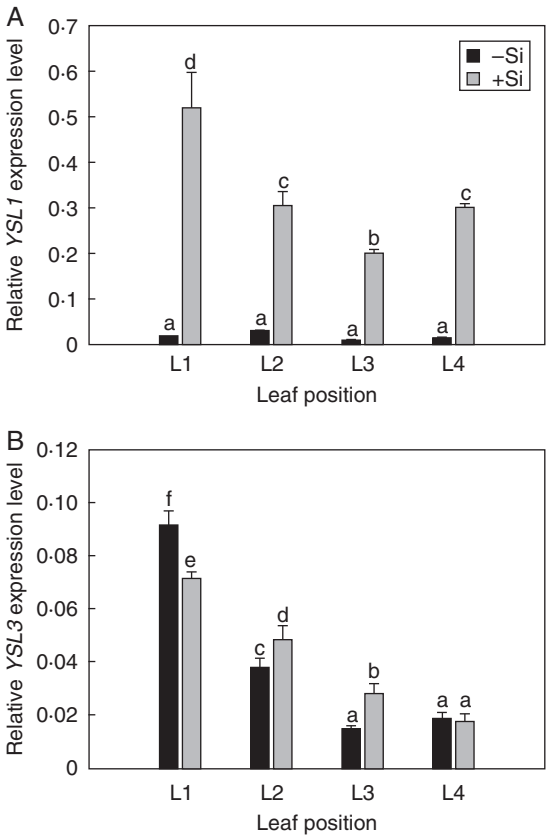

FIG. 5. Effect of Si supply on the relative expression of *YSL1* (A) and *YSL3* (B) genes coding for Fe–NA transporters. Leaf positions (from the base to the youngest leaf): L1, L2, L3 and L4. The plants were treated as described in the legend of Fig. 2. Data shown are means ± s.d. ($n = 4$). Significant differences ($P < 0{\cdot}05$) between treatments are indicated by different letters.

symplastic Fe can be mobilized from primary leaves of beans (*Phaseolus vulgaris*) and retranslocated towards young leaves and the shoot apex. Retranslocation of Fe can be even higher under conditions of induced senescence (Shi *et al*., 2012).

Shoot remobilization of Fe depends on the presence of NA, since the lack of Fe redistribution between older and younger leaves in the NA-free tomato mutant *chloronerva* can be overcome by application of NA (Scholz, 1989; Stephan and Scholz, 1993). In arabidopsis, using the mild and severe *nas4x-1* and *nas4x-2* mutants, Schuler *et al*. (2012) demonstrated that NA is involved in both long-distance transport of Fe to young leaves via the phloem and lateral transport of Fe from the vasculature to the mesophyll. Two members of the NAS protein family have so far been identified in cucumber (i.e. CsNAS1 and CsNAS4). However, no data exist in the literature on their expression pattern and specific functions in different tissues and organs of cucumber. A phylogenic tree of NAS amino acid sequences of cucumber and arabidopsis (Supplementary Data Fig. S2) suggests that *NAS* genes are most probably taxon specific, therefore leaving it as an open question whether or not their physiological function in cucumber is similar to that in arabidopsis. In the present work, we investigated the NA accumulation along with the expression of *NAS1* and *NAS4* genes in order to elucidate the mechanisms of Si-mediated distribution of Fe in cucumber leaves under limiting Fe conditions. Addition of Si upregulated NAS1 transcription and subsequently increased the NA concentration in fully expanded cucumber leaves (Fig. 4A), which resulted in enhanced remobilization of Fe from older leaves (L1, L2) and increased Fe concentration in younger leaves (L3, L4) without symptoms of Fe deficiency chlorosis (Fig. 2B; also see Fig. 1A). In contrast to NAS1, the NAS4 expression profile was unaffected by Si supply (Fig. 4B). The unaffected transcripts of NAS4 (upregulated by Fe deficiency; Klatte *et al*., 2009) appear to be attributable to the similar Fe status of the –Si and +Si plants (183 and 198 μg Fe per plant, respectively; calculated from Table 4). Therefore, Si upregulation of Fe-independent NAS1 transcripts seems to be crucial for increased leaf NA concentration in Si-fed plants, although the exact mechanisms for this remain for future investigation.

The expression pattern of arabidopsis AtYSL1 and AtYSL3 that mediate Fe–NA transport depends on the Fe status and leaf age, being more intensively expressed in the presence of Fe and in older leaves (Le Jean *et al*., 2005; Waters *et al*., 2006; Curie *et al*., 2009). The arabidopsis *ysl1ysl3* double mutant displays strong interveinal chlorosis, and has a reduced leaf Fe concentration (Waters *et al*., 2006), suggesting that YSL1 and YSL3 act as key mediators in foliar loading/unloading of Fe. From the phylogenic tree of YSL sequences in cucumber and arabidopsis, cucumber CsYSL1 was most closely related to arabidopsis AtYSL3 as well as CsYSL3 to AtYSL3, indicating the similarity of their physiological functions (Supplementary Data Fig. S3). Our experiment in cucumber showed that Si supply did not influence expression of *YSL1* and *YSL3* genes in the same manner (Fig. 5). Waters *et al*. (2006) proposed that during vegetative growth, decreased YSL expression during Fe deficiency restricts delivery of Fe to source tissues, making it more available for younger actively growing tissues. Addition of Si increases YSL1 expression, especially in the oldest leaves, indicating that this transporter plays an important role in Fe translocation to the younger leaves (Fig. 5A). The expression pattern of YSL3 depended on the leaf developmental stage (decreasing from older to younger leaves) but was slightly upregulated by Si in younger expanded leaves (L2 and L3; see Fig. 5B), thus implying that increased expression levels of YSL3 transcripts is a consequence of increased Fe content in the leaves of Si-treated plants rather than a direct influence of Si. Interestingly, YSL1 transcripts were almost absent in all examined leaves of non Si-treated plants, and addition of Si increased the relative YSL1 expression dramatically (Fig. 5A). This suggests that the upregulation of YSL1 is not caused by Si-induced change in plant Fe status (Table 4), but by Si accumulation in the leaf tissues (Table 1). However, the question of how Si might affect transcriptional activation of *YSL1* genes is still unresolved.

Whether and how Si acts in priming plants in response to biotic and perhaps abiotic stresses still remains unclear. A microarray study of Fauteux *et al*. (2006) in arabidopsis showed that Si supply did not impact expression of defence-related genes unless plants were powdery mildew stressed. These results suggest that Si treatment had no effect on the metabolism of unstressed plants but that it had beneficial properties attributable to modulation of a more efficient response to pathogen stress. However, until similar studies with high Si-accumulating plants

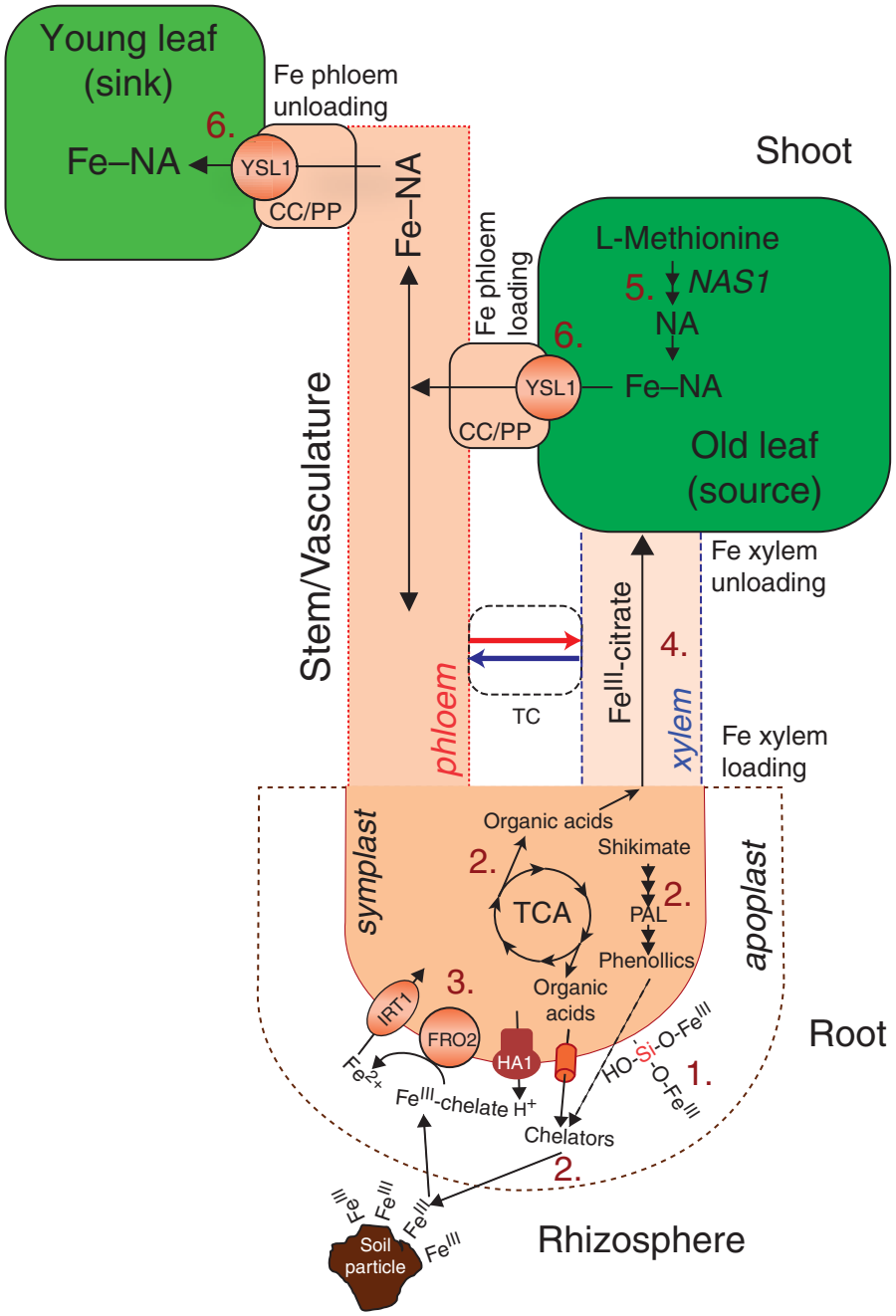

FIG. 6. The proposed model of Si-mediated alleviation of Fe deficiency in cucumber. In roots, Si increases Fe pools in the root apoplast (1) and enhances the accumulation of Fe-chelating compounds (organic acids and phenolics) in roots by stimulating expression of genes related to their biosynthesis (TCA cycle; shikimate and phenylpropanoid pathway; see Pavlovic *et al.*, 2013) for improved mobilization of Fe from the rhizosphere and reutilization of root apoplastic Fe (2), followed by the upregulated expression levels of the proteins involved in Strategy 1 reduction-based Fe uptake, i.e. HA1, FRO2 (in cucumber also known as CsFRO1; Waters *et al.*, 2007) and IRT1 (3). Silicon also increases root to shoot movement of Fe complexed by citrate (and malate) via xylem (4). In leaves, Si enhances expression of *NAS1* genes responsible for NA biosynthesis (see Fig. 4A) in sink leaves (5) and expression of *YSL1* genes responsible for Fe phloem loading and phloem transport of NA-chelated Fe from sink to source leaves (see Fig. 5), as well as for unloading of the Fe–NA complex arriving via the phloem in young leaves (6). CC/SE, companion cell/sieve element; TC, transfer cell.

can be carried out, the findings of previous work on arabidopsis have to be interpreted with caution because of a lack of the Lsi1 transporter in this species (Ma and Yamaji, 2015) and thus its inability to take up a large amount of Si. Also, Si showed an alleviating effect on plants stressed by abiotic factors such as drought and toxic metals, but had no effect on unstressed plants (e.g. Hattori *et al.*, 2003; Liang *et al.*, 2007). Relatively little is known concerning the possibility of an active role for Si in plant metabolism under abiotic stress conditions, particularly on the molecular aspects of Si-mediated nutrient efficiency (Liang *et al.*, 2015). Our previous study showed the ability of Si to modulate root activity of Fe acquisition in cucumber at an

early stage of Fe deficiency stress through regulation of gene expression levels of the proteins involved in this process (Pavlovic *et al*., 2013), which confirmed that Si played a limited role in the transcriptome of this plant species in the absence of stress. In the present study, Si nutrition modulated the expression of NAS1 involved in the biosynthesis of NA, resulting in more efficient remobilization of Fe from source to sink leaf tissues via Si-upregulated YSL1 transporter for the Fe–NA complex. Taken together, the benefits of Si are aligned with a response to transcriptomic changes induced by Fe deficiency at both root and leaf levels.

In conclusion, our results show for the first time that Si induces Fe mobilization in older leaves and increases its retranslocation to younger expanding leaves. Supply of Si enhances expression of the *NAS1* gene responsible for NA biosynthesis and hence increased NA accumulation, which in turn enhances chelation of Fe and phloem loading of Fe–NA in the source (expanded) leaves to facilitate phloem transport of Fe and enhanced phloem unloading of Fe in the sink (expanding) leaves. Finally, summarizing the results presented in this and our previous studies (Pavlovic *et al*., 2013; Bityutskii *et al*., 2014), we propose a model of how Si acts to improve Fe homeostasis under Fe deficiency in cucumber (see Fig. 6).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: concentration of Fe in the xylem sap of cucumber plants after withdrawal of Fe from the nutrient solution. Table S2: dry biomass of cucumber plants grown in the nutrient solution supplied with $CaCO_3$. Figure S1: schematic presentation of the experimental set-up and movement of Fe within cucumber plants. Figure S2: the NJ phylogenetic tree of the NAS family in cucumber and arabidopsis. Figure S3: the NJ phylogenetic tree of cucumber and arabidopsis YSL proteins.

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