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Scopoletin 8-hydroxylase: a novel enzyme involved in coumarin biosynthesis and iron-deficiency responses in Arabidopsis

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Abstract
Iron deficiency is a serious agricultural problem, particularly in alkaline soils. Secretion of coumarins by Arabidopsis thaliana roots is induced under iron deficiency. An essential enzyme for the biosynthesis of the major Arabidopsis coumarins, scopoletin and its derivatives, is Feruloyl-CoA 6'-Hydroxylase1 (F6'H1), which belongs to a large enzyme family of the 2-oxoglutarate and Fe2+-dependent dioxygenases. We have functionally characterized another enzyme of this family, which is a close homologue of F6'H1 and is encoded by a strongly iron-responsive gene, At3g12900. We purified At3g12900 protein heterologously expressed in Escherichia coli and demonstrated that it is involved in the conversion of scopoletin into fraxetin, via hydroxylation at the C8 position, and that it thus functions as a scopoletin 8-hydroxylase (S8H). Its function in plant cells was confirmed by the transient expression of S8H protein in Nicotiana benthamiana leaves, followed by metabolite profiling and biochemical and ionomic characterization of Arabidopsis s8h knockout lines grown under various iron regimes. Our results indicate that S8H is involved in coumarin biosynthesis, as part of mechanisms used by plants to assimilate iron.

Keywords: Abiotic stress, Arabidopsis, enzyme activity, fraxetin, Fe- and 2OG-dependent dioxygenase, mineral nutrition, plant–environment interactions.

Introduction
Iron (Fe) is an essential micronutrient for all living organisms. In plants, chloroplasts and mitochondria have high Fe requirements (Nouet et al., 2011). Fe is abundant in soils, but its availability is often limited due to soil conditions, which can be highly heterogeneous, such as the pH and redox presence of co-elements (Moosavi and Ronaghi, 2011; Marschner, 2012). Fe deficiency is a serious agricultural problem, particularly in alkaline and calcareous soils (Mengel, 1994). These types of soils, which represent approximately 30% of the world’s cropland, are characterized by a higher pH, which, in combination with the presence of oxygen, leads to precipitation of Fe in the form of insoluble ferric oxides (Fe2O3) (Morrissey and Guerinot, 2009).

Higher plants have developed two different types of strategies in response to Fe limitation. The first (Strategy I), which occurs in all plants except graminaceous species, is a
reduction-based strategy, where the release of protons into the rhizosphere is enhanced by Fe-deficiency-induced proton-translocating adenosine triphosphatases (Marschner and Roemheld, 1994; Kim and Guerinot, 2007), such as AHA2 in Arabidopsis (Santi and Schmidt, 2009). As a result, at lower pH the ferric oxide precipitates are dissolved, and plasma-membrane-bound Ferric Chelate Reductase 2 (FRO2) (Robinson et al., 1999) catalyses the reduction of ferric ions (Fe³⁺) into more soluble, and bioavailable to plants, ferrous ions (Fe²⁺). Fe²⁺ ions are then transported across the plasma membrane into the root epidermal cells by the divalent metal transporter Iron Regulated Transporter 1 (IRT1) (Varotto et al., 2002; Vert et al., 2002). A second strategy (Strategy II), used by graminaceous species, is based on the release of soluble mugineic acid family phytosiderophores (PS) from the root epidermis, which form complexes with Fe²⁺ (Takagi et al., 1984; Marschner and Roemheld, 1994; Kim and Guerinot, 2007). The resulting Fe³⁺–PS complexes are then transported into the root epidermis via a high-affinity uptake system without requiring a reduction step (Curie et al., 2001; Kim and Guerinot, 2007).

The precise mechanisms underlying the responses of Strategy I plants to the low Fe availability in calcareous soils are not clear. However, it is well documented that Fe deficiency enhances the release of reductants/chelators (mainly phenolics) in many dicots (Marschner and Roemheld, 1994; Jin et al., 2007). Recently, it was shown that Fe deficiency induces the secretion of secondary metabolites, such as scopoletin and its derivatives, by Arabidopsis roots (Rodriguez-Celma et al., 2013a; Fourcroy et al., 2014), and that a Feruloyl-CoA 6'-Hydroxylase1 (F6’H1) is required for the biosynthesis of the Fe³⁺-chelating coumarin esculetin, which is released into the rhizosphere as part of Strategy I-type Fe acquisition (Schmid et al., 2014).

Scopoletin and its corresponding glycoside, scopolin, are the predominant coumarins in Arabidopsis roots. However, many other coumarin compounds, such as skimmin, esculetin, fraxetin, and the recently discovered coumarinolignans, are also present in the roots of this model plant (Rohde et al., 2004; Bednarek et al., 2005; Kai et al., 2006; Kai et al., 2008; Schmid et al., 2014; Schmidt et al., 2014; Ziegler et al., 2016; Sisó-Terraza et al., 2016; Ziegler et al., 2017). In a previous study, we reported a significant natural variation in scopoletin and scopolin accumulation between various Arabidopsis accessions (Siwinska et al., 2014). It is interesting that Strategy I plants differ considerably between plant species and genotypes in their tolerance of Fe deficiency. In Arabidopsis, an accumulation of various coumarins, in particular scopoletin and fraxetin, together with their corresponding glycosides, was shown to be highly induced in response to Fe-limited conditions (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). To date, no enzymes involved in the last step of fraxetin biosynthesis have been identified.

The biosynthesis of coumarins and their accumulation occur in response to Fe deficiency, but the exact mechanisms of action underlying these processes have remained largely unknown. To better understand these mechanisms, we have selected and functionally characterized an enzyme with a previously unknown biological role, encoded by the At3g12900 gene, which shares significant homologies with the F6’H1 and F6’H2 enzymes described by Kai et al. (2008) as being involved in the synthesis of scopoletin, and which is highlighted in the literature as being strongly iron-responsive (Lan et al., 2011; Rodriguez-Celma et al., 2013b; Mai et al., 2015; Mai and Bauer, 2016). This led us to identify the At3g12900 oxidoreductase as a scopoletin 8-hydroxylase (S8H) involved in the biosynthesis of fraxetin, which is associated with the regulation of Fe homeostasis in Arabidopsis.

Materials and methods

Plant material

The Arabidopsis thaliana Col-0 accession was used as the wild type (WT). The s8h-1 (SM_3.27151) and s8h-2 (SM_3.23443) T-DNA insertional mutant lines (Supplementary Fig. S1 at JXB online) were identified in the SM (Suppressor-mutator) collection (http://signal.salk.edu; Tissier et al., 1999) of single-copy d5pm insertions (in the Col-0 background). Seeds of both s8h lines are available at the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). Genotyping of s8h-1 and s8h-2 was done using protocols and primers described in Supplementary Table S1. Selected s8h-1 homozygous mutants were tested for S8H gene expression by quantitative real-time reverse transcription–PCR (qRT–PCR) (Supplementary Fig. S2, Supplementary Table S2). A lack of S8H transcript in the s8h-2 mutant background was confirmed by RT–PCR using primers shown in Supplementary Table S2. Nicotiana benthamiana seeds were kindly gifted by Dr Etienne Herbach (INRA, Colmar, France).

Growth conditions

Hydroponic cultures

Plants were grown in two types of modified Heeg solution (Heeg et al., 2008), described in detail in Supplementary Table S3. The first solution (10× Heeg) was prepared as previously described (Ihnatowicz et al., 2014); a second solution (1× Heeg) contained 10-fold lower concentrations of microelements (except for Fe²⁺). Arabidopsis seeds were surface sterilized by soaking in 70% ethanol for 2 min and subsequently kept in 5% calcium hypochlorite solution for 8 min. Afterwards, seeds were rinsed three times in autoclaved deionized water. The surface-sterilized seeds were sown on tip-cut 0.65% agar-filled tubes or on microcentrifuge tube lids filled with solidified Heeg medium, which were placed into tip boxes with control hydroponic solution (40 µM Fe²⁺). After a few days’ stratification at 4 °C, boxes with plants were kept in a controlled environment (16 h light at 22 °C/10 µmol m⁻² s⁻¹ and 8 h dark at 20 °C). Approximately 3 weeks later, plants were transferred either to a freshly made control solution or to Fe-deficient (10 µM Fe²⁺) or Fe-depleted (0 µM Fe²⁺) solutions. In the first set of experiments hydroponic solutions were fully changed once per week, while in the second set of experiments boxes containing old nutrient solution were replenished by the addition of fresh medium.

In vitro cultures on plates

Surface-sterilized seeds were sown on Petri dishes containing different homemade Murashige and Skoog (MS) media: (i) half-strength MS medium (0.5 MS) with macroelements at half strength and microelements/vitamins at full strength, and (ii) one-quarter-strength MS medium (0.25 MS) with macroelements at one-quarter strength and microelements/vitamins at half strength. Both types of media contained 1% sucrose, 0.8% agar supplemented with 4 mg l⁻¹ glycine, 200 mg l⁻¹ myo-inositol, 1 mg l⁻¹ thiamine hydrochloride, 0.5 mg l⁻¹...
pyridoxine hydrochloride, and 0.5 mg l⁻¹ nicotinic acid. For stratification, plates were kept in the dark at 4 °C for 72 h and then placed under defined growth conditions [16 h light (~70 µmol m⁻² s⁻¹) at 22 °C and 8 h dark at 20 °C].

In vitro liquid cultures

Ten-day-old seedlings from agar plates were transferred into a 250 ml wide-mouth Erlenmeyer flask containing 20 ml sterile 0.25 MS liquid medium. Plants grown in liquid cultures were incubated on rotary platform shakers at 120 rpm. Ten days after transfer, 20 ml of fresh medium was added to each flask. Plants were harvested 18 days after transfer (on the 29th day of culture); leaves and roots were frozen separately in liquid nitrogen and stored at −80 °C.

Two weeks after germination, N. benthamiana seeds were transplanted and cultured independently for 3 additional weeks in plant growth chambers under a photoperiod of 16 h light (120 µmol m⁻² s⁻¹) at 24 °C and 8 h dark at 22 °C with 70% humidity.

Chemicals

The following acids were used: ferulic (Alrich), coumaric (Sigma), cinnamic (Sigma), and caffeic (Fluka). Coumarins used were: coumarin (Sigma), daphnetin (Sigma), esculetin (Sigma), esculin (Sigma), fraxetin (Extrasyntèse), fraxin (Extrasyntèse), isocoumarin (Polypeptides), limetin (Herboreal), scoparon (Herboreal), 6-methoxycoumarin (Apin Chemicals), 7-methoxycoumarin (Herboreal), scopoletin (Herboreal), scopolin (Akin Chemicals Inc.), umbelliferone (Extrasyntèse), skimmia (Akin Chemicals Inc.), and 4-methylumbelliferon (Sigma). The CoA thiol esters of the coumarinates (cinnamoyl-CoA, p-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA) were enzymatically synthesized as described by Vialart et al. (2012). P-coumarate and coenzyme A (CoA) were purchased from Sigma-Aldrich. Kanamycin, chloramphenicol, and isopropyl-β-d-thio-galactopyranoside (IPTG) were purchased from Duchefa.

HPLC

Chromatographic separation was performed using an HPLC Shimadzu system with diode array detector/UV detector. The injected volume was 50 µL. Samples were separated on an Interchim µBondapack C18, 5 µm. Samples were separated on an Interchim µBondapack C18, 5 µm. Enzymatic activities were assessed immediately after the purification of S8H. The protocol was adapted from Kai et al. (2008). The reaction was done in a 200 µl volume at a saturating concentration of FeSO₄ (0.5 mM), α-ketoglutarate (5 mM), sodium ascorbate (5 mM) in 0.1 M potassium phosphate buffer at optimal pH (7.0), with 200 µM substrate and 2.6 µg of purified enzyme. Reaction mixtures were incubated for 10 min at an optimal temperature of 31.5 °C. Reaction mixtures with CoA esters were additionally incubated at the end of the reaction with 20 µl of 5 M NaOH for 20 min at 37 °C to hydrolyse the ester bond, and subsequently with 20 µl of acetic acid in order to close the lactone ring. In samples with acids and coumarins as substrates, the reaction was stopped by the addition of 2 µl of trifluoroacetic acid and incubation at 20 °C for 20 min. Reaction mixtures were then centrifuged for 30 min at 10000 g; the supernatant was recovered, filtered (0.22 µm), and analysed by high-performance liquid chromatography (HPLC).

Measurement of enzymatic activities

Enzymatic activities were assessed immediately after the purification of S8H. The protocol was adapted from Kai et al. (2008). The reaction was done in a 200 µl volume at a saturating concentration of FeSO₄ (0.5 mM), α-ketoglutarate (5 mM), sodium ascorbate (5 mM) in 0.1 M potassium phosphate buffer at optimal pH (7.0), with 200 µM substrate and 2.6 µg of purified enzyme. Reaction mixtures were incubated for 10 min at an optimal temperature of 31.5 °C. Reaction mixtures with CoA esters were additionally incubated at the end of the reaction with 20 µl of 5 M NaOH for 20 min at 37 °C to hydrolyse the ester bond, and subsequently with 20 µl of acetic acid in order to close the lactone ring. In samples with acids and coumarins as substrates, the reaction was stopped by the addition of 2 µl of trifluoroacetic acid and incubation at 20 °C for 20 min. Reaction mixtures were then centrifuged for 30 min at 10000 g; the supernatant was recovered, filtered (0.22 µm), and analysed by high-performance liquid chromatography (HPLC).

Construction of binary vector and Agrobacterium tumefaciens strains

The amplified S8H ORF (details of the PCR are provided in Supplementary Table S4) was first cloned into the pCR®8/GW/TOPO® TA cloning kit (Invitrogen) (Vialart et al., 2003), provided by D. Baulcombe (Department of Plant Science, University of Cambridge, UK) for transient expression in N. benthamiana leaves.

Construction of pET28a+ expression vector and E. coli Rosetta 2 strain

The ORF of S8H was amplified by PCR (5′ primer: 5′-GGATCCGG GTATCAATTCGAGACACAAA-3′ and 3′ primer: 5′-CTCGA GCTCGCCACGTGCGAAGTCGAG-3′) and cloned between the BamHI and XhoI sites of pET28a+. The recombinant plasmid was introduced into the competent E. coli Rosetta 2 (Novagen) strain by heat shock.

Heterologous expression and purification of S8H

The E. coli Rosetta 2 strain transformed with pET28a+-S8H was cultured at 37 °C overnight in 10 ml lysogeny broth (LB) (0.5% yeast extract, 1% tryptone, and 1% NaCl; Sambrook and Russell, 2001) supplemented with 100 mg l⁻¹ kanamycin and 33 mg l⁻¹ chloramphenicol. A 2 ml pre-culture was transferred to 1 litre of fresh LB containing 100 mg l⁻¹ kanamycin and 33 mg l⁻¹ chloramphenicol. The induction of S8H expression was adapted from the protocol developed by Oganesyan et al. (2007). Transformed cells were cultured at 37 °C until OD₆₀₀nm reached 0.6. Salt stress with 0.5 M NaCl and heat stress at 47 °C were applied for 1 h in the presence of 2 mM betaine. The temperature was then set at 20 °C for 1 h, and finally the expression of S8H was initiated by adding 1 mM IPTG. Cells were harvested after 14 h by centrifugation for 20 min at 4000 g at 4 °C, and the pellet was resuspended in 4 ml potassium phosphate buffer, pH 8.0, with 10 mM imidazol. The cell suspension was sonicated (Bandelin SONOPLUS apparatus) for five periods of 20 s with an interval of 30 s at 50–60% power, and subsequently centrifuged for 20 min at 10000 g at 4 °C. The purification of soluble recombinant His-tagged proteins was done using TALON Metal Affinity Resin (TAKARA) as described by the supplier. The purified proteins were eluted with 0.1 M potassium phosphate buffer and 200 mM imidazol solution (pH 8.0).

Measurement of enzymatic activities

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HPLC

Chromatographic separation was performed using an HPLC Shimadzu system with diode array detector/UV detector. The injected volume was 50 µl. Samples were separated on an Interchim C18 Lichrospher OD2 (250 × 4.0 mm, 5 µm) column with the flow speed set to 0.8 ml min⁻¹. Separation was performed by elution with the following programme (A) H₂O and 0.1% acetic acid (B) methanol and 0.1% acetic acid: 0–35 min gradient 10–70% B, 35–36 min isocratic elution and column regeneration. The wavelength was set at 338 nm to monitor the formation of fraxetin.

Sample analysis was performed on a Nexera UHPLC system (Shimadzu Corp., Kyoto, Japan) coupled with an LCMS 2020 mass spectrometer (Shimadzu). The chromatographic column was a C18 reverse phase (Zorbax Eclipse Plus, Agilent Technologies, Santa Clara, CA, USA) 150 × 2.1 mm 1.8 µm. Elution of the compounds was performed as described by Dugrand et al. (2013). To confirm the in vitro metabolism of scopoletin into fraxetin, separation was conducted on an Ultimate 3000 chromatographic chain coupled with an LTQ- XL mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA), as described by Karamat et al. (2012).
for 10 min at 4000 g and the pellet was resuspended in 20 ml sterile deionized water. This step was repeated three times in order to remove all traces of antibiotics. *N. benthamiana* leaves were infiltrated with a suspension of recombinant strains at an OD♥<sub>600</sub> of 0.4 for pBIN61-P19 bacteria and 0.2 for pBIN-S8H or pBIN-F6'H2 bacteria. The infiltrated plants were stored in a growth chamber for 96 h. In parallel, for each experiment, *N. benthamiana* were infiltrated with LBA4404 (pBIN-GFP) as a positive control. Expression of green fluorescent protein was assessed by examination under a binocular microscope.

**Extraction of polyphenols from *N. benthamiana* leaves**

Freshly harvested infiltrated *N. benthamiana* leaves were crushed in liquid nitrogen and a 100 mg sample was mixed with 800 µl 80% methanol. The solution was vigorously mixed for 30 s and then centrifuged for 30 min at 10000 g. The supernatant was transferred to a fresh tube and the pellet was submitted to a second extraction with 800 µl 80% methanol. Both supernatants were pooled and vacuum dried. The pellet was resuspended in 100 µl MeOH/H2O 80%/20% v/v and analysed by UHPLC.

**Preparation of methanol extracts from Arabidopsis roots**

Arabidopsis roots were frozen in liquid nitrogen and ground with a pestle and mortar. A 50 mg sample of plant tissue was mixed in 500 µl of 80% methanol supplemented with 2.5 µM 4-methylumbelliferylone as an internal standard. Samples were sonicated for 10 min and centrifuged for 10 min at 10000 g. Supernatants were transferred to fresh tubes, vacuum dried, resuspended in 100 µl MeOH/H2O 80%/20% v/v, and analysed by UHPLC.

**Extraction of root exudates from nutrient solutions**

Nutrient solutions from Arabidopsis in vitro cultures were collected 18 days after the onset of Fe treatments. Phenolic compounds were retained in a BAKERBOND™ C18 column (J. T. Baker Chemical Co., Phillipsburg, NJ, USA), eluted from the cartridge with 3 ml of 100% methanol, and dried in a centrifugal evaporator. Dry extracts were stored at –20 °C until further analysis.

**Trace element analysis**

Arabidopsis roots were first lyophilized and subsequently milled before microwave (MARS)-assisted digestion in concentrated nitric acid (Aristar). Metal concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo iCAP Q, Bremen, Germany).

**Measurement of chlorophyll**

Chlorophyll extraction with acetone was done according to the method of Porra *et al.* (1989). The absorbances of the diluted supernatants were measured at 750.0, 663.6, and 646.6 nm. After measurement, a formula was used to convert absorbance measurements to mg of chlorophyll (Porra *et al.*, 1989).

**Statistical analysis**

All treatments included at least three biological replicates. Data processing and statistical analyses (pairwise comparisons using t-tests, except for trace element analysis) were done using Excel 2010 (Microsoft). Statistical significance of differences observed in trace element analysis was analyzed by R version 3.3.2 (2016-10-31) (R Core Team 2014, https://www.R-project.org/) with the use of the agricolae package.
be a scopoletin 8-hydroxylase (S8H) (Fig. 3) that catalyses hydroxylation at the C8 position of scopoletin. The optimal reaction conditions were determined to be pH 8.0 ± 0.1 at 31.5 ± 0.4 °C (Supplementary Fig. S5A, B) and the optimal incubation time was set at 10 min (Supplementary Fig. S6A). Under these experimental conditions, we determined the kinetic characteristics of the enzyme as being $K_{m}$ 11 ± 2 µM, $K_{cat}$ 1.73 ± 0.09 s$^{-1}$ (Supplementary Fig. S7). We also demonstrated that the enzyme efficiency was best in the presence of 50 µM Fe$^{2+}$ (Supplementary Fig. S6B) and that higher concentrations of Fe$^{2+}$ significantly reduced the enzyme activity, leading to decreased fraxetin synthesis.

In vivo activity of scopoletin 8-hydroxylase in N. benthamiana

To confirm its function in plant cells, we transiently expressed the S8H enzyme in N. benthamiana leaves. Our analysis showed that the presence of this enzyme did not increase the production of fraxetin in comparison to a control infiltration performed with A. tumefaciens transformed with an empty vector (Fig. 4). However, when we conducted a double infiltration leading to the overexpression of S8H and F6$’$H2 in tobacco leaves, we detected a significantly higher level of fraxetin in comparison to leaves transiently expressing S8H alone ($P$=0.01) (Fig. 4). This result is consistent with an increase of the scopoletin pool that occurs due to F6$’$H activity, which can be further transformed into fraxetin. It also confirms that At3g12900 functions as a S8H.

Characterization of independent s8h mutant alleles grown in Fe-depleted hydroponic conditions

To gain insight into the physiological role of S8H, we identified two independent mutant lines with non-functional S8H dioxygenase ($s8h$-1 and $s8h$-2) and investigated their behaviour in various types of culture. Because the literature has reported that fraxetin accumulation is induced under conditions of Fe deficiency, we investigated the behaviour of the mutants in Fe-depleted hydroponic solutions. Plants were grown in a control hydroponic solution (40 µM Fe$^{2+}$) for 3 weeks and subsequently transferred to Fe-depleted solution (0 µM Fe$^{2+}$) and cultured for an additional 3 weeks. In Fe-depleted solutions both mutant lines were clearly paler in colour than Col-0 control plants (Fig. 5A). The mutant phenotype was linked with a lower chlorophyll a+b content and chlorophyll a/b ratio (Fig. 5B). Targeted metabolite profiling of methanolic extracts from the roots of plants grown in hydroponic systems showed a highly significant increase in scopolin ($P$<0.01) and scopoletin ($P$<0.01) concentration in roots grown in Fe-depleted conditions for both mutant lines, and a significant increase in

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**Fig. 1.** Nucleic acid sequence-based tree. The phylogenetic analysis was performed on available nucleic acid sequences of genes encoding 2-oxoglutarate (2OG)- and ferrous iron Fe$^{2+}$-dependent dioxygenase (2OGD) from the Arabidopsis genome. The phylogenetic tree was made using MEGA 6 software. Black circles indicate genes (At3g13610 and At1g55290) encoding enzymes involved in scopoletin biosynthesis (F6$’$H1 and F6$’$H2 respectively), and their closest homologue based on the nucleic acid sequences, a gene (At3g12900) encoding a dioxygenase of unknown biological function (characterized in this study as S8H). The black triangle indicates a gene (At4g22880) encoding the anthocyanin synthase (ANS) enzyme. Nucleic sequences downloaded from TAIR were selected based on the presence of sequences coding for Fe-binding (His-X-Asp-X-His) and 2OG-binding (Arg-X-Ser) motifs (Wilmouth et al., 2002).
umbelliferone accumulation in \( s8h-1 \) compared with Col-0 plants (Fig. 5C).

Biochemical and ionomic characterization of plants cultivated in liquid cultures with different Fe concentrations

Since coumarins are mostly stored in roots, all tested genotypes were cultivated \textit{in vitro} in liquid cultures, in order to obtain enough root biomass for analysis of trace elements and media for extraction of root exudates and metabolic profiling. After 3 weeks of growth, no visible phenotypic differences could be observed among WT and mutant plants: all genotypes grown in Fe-deficient medium (10 \( \mu \)M Fe\(^{2+} \)) were paler and plants grown in Fe-depleted medium (0 \( \mu \)M Fe\(^{2+} \)) were chlorotic (Supplementary Fig S8). Chlorophyll content was slightly higher in both mutant lines in all media tested, while the chlorophyll a/b ratio was significantly higher in Col-0 plants in control media and slightly higher in Fe-deficient and Fe-depleted media (Supplementary Fig. S9). However, the targeted metabolite profiling of root extracts and root exudates showed that under Fe-depleted conditions both mutant lines accumulated lower levels of various coumarins (scopoletin, scopolin, umbelliferone, and skimmin) (Fig. 6A) but secreted significantly higher amounts of scopoletin in comparison to WT plants (Fig. 6B).

The trace element analysis of plants grown in liquid culture media with various Fe levels indicated that, when grown in Fe-depleted medium (0 \( \mu \)M Fe\(^{2+} \)), the Fe content of both mutant lines (\( s8h-1 \) and \( s8h-2 \)) was significantly lower (\( P<0.05 \)) in comparison to the corresponding control (Col-0) (Fig. 7). The concentration of a range of other microelement heavy metals (Mn, Zn, Cu, Co, and Cd) was also significantly lower in the \( s8h \) mutants compared with control plants (\( P<0.05 \)) grown in Fe-depleted medium (Fig. 7).

As phosphate (P) and Fe deficiency interact with respect to Fe-induced coumarin secretion (Ziegler \textit{et al.}, 2016), and our soil experiments found that Col-0 and \( s8h-1 \) phenotypes were dependent on the P to Fe ratio (Supplementary Fig. S10, Supplementary Table S6), another observation from the trace element analysis is the lower levels of P content in both mutants (significantly lower in the \( s8h-1 \) line, \( P<0.05 \)) in comparison to Col-0 plants under Fe-depleted (0 \( \mu \)M Fe\(^{2+} \)) conditions (Supplementary Figure S11A). Plants grown in Fe-deficient (10 \( \mu \)M Fe\(^{2+} \)) and Fe-optimal (50 \( \mu \)M Fe\(^{2+} \)) media did not show changes in trace element contents (Supplementary Fig. S11A–C), with the exception that there was a significantly higher Mo content in \( s8h \) mutants grown in Fe-deficient medium (Supplementary Fig. S11C).
Phenotyping of s8h mutants grown on MS plates and in hydroponic solutions with various concentrations of Fe and other microelements

The impact of trace elements on Arabidopsis growth was further investigated by cultivating the s8h mutants and control plants on plates and hydroponic cultures characterized by different concentrations of Fe and other microelements. To observe the growth of both rosettes and roots, the MS plates were placed in a vertical and a horizontal position. The growth of all genotypes was more extensive on 0.25 MS medium than on the corresponding plates with 0.5 MS medium, irrespective of the concentration of Fe (Supplementary Fig. S12). These differences were particularly striking after 3 weeks of cultivation. When grown on 0.25 MS Fe-deficient (10 µM Fe²⁺) medium, the s8h mutants showed a strong reduction in fresh weight (Supplementary Fig. S13A), were much paler, and had shorter and fewer lateral roots compared with Col-0 plants (Fig. 8A). On 0.5 MS Fe-deficient medium, both genotypes displayed a significant reduction in shoot and root growth, but s8h mutants showed a smaller rosette size than Col-0 plants (Fig. 8B). Under both Fe-depleted conditions (0 µM Fe²⁺), mutant lines and Col-0 plants showed significantly smaller and chlorotic shoots, while roots were markedly shorter (Fig. 8A, B). However, the WT plants grew slightly more. The shoots of s8h mutants grown on 0.25 MS Fe-optimal (50 µM Fe²⁺) medium were clearly larger than the WT plants; this was not the case on 0.5 MS medium with optimal Fe content (Fig. 8A, B).

Examination of plants grown on Fe-deficient plates (10 µM Fe²⁺) under UV light showed that both s8h mutant lines secreted a higher level of fluorescent compounds into the medium compared with WT plants (Supplementary Fig. S14A); this observation might be related to the significantly higher amounts of scopoletin detected in Fe-deficient liquid culture solution of s8h plants (Fig. 6B). This difference in fluorescence was not observed in Fe-optimal conditions (50 µM Fe²⁺), in which the mutant plants seemed to accumulate slightly more fluorescent compounds in their roots compared with WT plants (Supplementary Figure S14B).

Similar to plants grown in vitro, plants cultured in hydroponic conditions also showed phenotypic variation in plant responses, which was dependent not only on Fe but also on the concentrations of other micronutrients. Both s8h mutants were paler than Col-0 plants when grown in 1× Heeg Fe-depleted (0 µM Fe²⁺) solution, which contains 10-fold lower concentrations of microelements than the other hydroponic solution used (Supplementary Table S3). This phenotype was associated with lower amounts of chlorophylls a and b. Under Fe-deficient (10 µM Fe²⁺) conditions, both mutant lines were much larger than WT plants, with greatly increased fresh weight (Supplementary Figure S15B). Surprisingly, when grown with optimal Fe (40 µM Fe²⁺) but lower amounts of other micronutrients (Supplementary Table S3), all genotypes were significantly smaller but did not show any chlorosis or changes in chlorophyll content (Supplementary Fig. S15A). In contrast, when plants were cultivated in 10× Heeg solution, characterized by

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**Fig. 3.** Last step of fraxetin biosynthesis in Arabidopsis thaliana catalysed by scopoletin 8-hydroxylase (S8H). The Fe²⁺- and 2-oxoglutarate-dependent dioxygenase (F6ʹH) catalyses the ortho-hydroxylation of feruloyl-CoA before the lactone ring formation to produce scopoletin. Subsequently, S8H catalyses hydroxylation at the C8 position of scopoletin, leading to fraxetin production.

**Fig. 4.** Fraxetin content in Nicotiana benthamiana leaves. Transient expression was carried out using Agrobacterium tumefaciens transformed with the pBIN empty vector (control), pBIN:S8H, pBIN:F6ʹH2, and simultaneously with vectors encoding F6ʹH2 and S8H. Fraxetin content was quantified using UHPLC. Data are presented as mean ±SD. *P<0.05.
higher micronutrient concentrations (Supplementary Table S3), there were no visible differences among s8h mutants and WT plants when grown in the presence of different Fe levels (Supplementary Fig. S16). Under optimal and Fe-deficient conditions, all plants displayed a WT-like phenotype with normal pigmentation, but in solutions without Fe all plants were smaller and chlorotic. In these hydroponic experiments, both solutions (1× Heeg and 10× Heeg) were fully exchanged weekly.

There appears to be a crucial role for root-secreted coumarins in the acquisition of Fe (Tsai and Schmidt, 2017). Therefore, the hydroponic culture experiment was repeated without changing the nutrient solution weekly. Instead, the root chambers were replenished by the addition of fresh medium to keep the volume of solution in each culture constant, and to retain potentially secreted coumarins. When grown in 1× Heeg solution, under Fe-depleted conditions (0 µM Fe²⁺) both s8h mutants became chlorotic (Supplementary Fig. S17C), as previously observed (Supplementary Fig. S15C). In contrast, the growth of all genotypes was not affected in Fe-optimal solution and under Fe deficiency (40 and 10 µM Fe²⁺, respectively) (Supplementary Fig. S17A, B); this could be due to a higher accumulation of coumarin in hydroponic solutions that were not fully changed. Similar to previously conducted hydroponic experiments, all plants grown in 10× Heeg solution under Fe-deficient and Fe-optimal conditions did not show any sign of chlorosis or growth retardation (Supplementary Figure S18A, B). Under Fe-depleted conditions, all genotypes were only slightly smaller compared with their growth optimal conditions, and they had normally pigmented leaves with no changes in chlorophyll content (Supplementary Figure S18C); only the shoots of the mutant lines seemed to be slightly brighter in colour.

Discussion

In the Arabidopsis genome there are more than 100 genes encoding enzymes sharing homologies with dioxygenases. The 2OGD family is the second largest enzyme family in plants; its members are involved in various oxygenation/hydroxylation reactions (Kawai et al., 2014), including the biosynthesis of coumarins, which are important compounds contributing to the adaptation of plants to biotic and abiotic stresses (Kai et al., 2008; Vialart et al., 2012; Matsumoto et al., 2012; Rodriguez-Celma et al., 2013a). Among the most common stress factors leading to plant growth disorders and chlorosis are a deficiency or excess of micronutrients, which can result in various physiological disorders (Marschner, 2012). Maintaining nutrient homeostasis in cells is crucial for the proper functioning of plants, and the mechanisms governing mineral uptake and transport must be strictly controlled.
Plants use different strategies to compensate for Fe limitation. Recently, it was shown that one of the Arabidopsis Fe$^{2+}$- and 2OG-dependent dioxygenases, the scopoletin synthase F6'H1 (Kai et al., 2008), is required for the biosynthesis of the Fe$^{3+}$-chelating coumarin esculetin, which is released into the rhizosphere as part of Fe uptake by Strategy I plants (Schmid et al., 2014).

Here, we have analysed a strongly Fe-responsive gene of previously unknown function, At3g12900, which shares high homology with the Fe$^{2+}$- and 2OG-dependent dioxygenase family, and revealed its possible contribution to Fe homeostasis in plants. The At3g12900 gene was selected on the basis of its high homology to the previously described F6'H1 dioxygenase, which has been shown to play a crucial role in Fe acquisition under alkaline soil conditions (Schmid et al., 2014), and the literature data on plant responses to Fe deficiency at the transcriptome and proteome level (Lan et al., 2011; Rodriguez-Celma et al., 2013b; Fourcroy et al., 2014; Schmidt et al., 2014). Similar to F6'H1, the protein encoded by At3g12900 accumulates by several-fold in Fe-deficient roots in comparison to Fe-sufficient roots (Lan et al., 2011). Analysis of large microarray datasets found that both genes (At3g12900 and At3g13610, encoding F6'H1) were positively correlated with genes actively involved in the response to Fe deficiency (Vigani et al., 2013) such as IRT1, FRO2, CYP82C4 (Murgia et al., 2011), and Ferroportin/Iron-Regulated (JREG2) (Morrissey et al., 2009), and a gene encoding metal tolerance protein (MTP3; Arrivault et al., 2006). Moreover, according to the expression data available at TAIR, At3g12900 is expressed specifically in the roots. The root tissue is the site of coumarin accumulation induced in response to various environmental stresses, including Fe limitation (Schmid et al., 2014).

We determined the substrate specificity of the enzyme encoded by At3g12900 to test its possible involvement in coumarin biosynthesis as an important part of the Fe uptake.
strategy in Arabidopsis. An in vitro enzymatic activity assay revealed that this enzyme is involved in the conversion of scopoletin into fraxetin via hydroxylation at the C8 position; consequently, the enzyme was named S8H. The Michaelis constant (\( K_m = 11 \mu M \)) determined in our in vitro experiments was similar to those reported for the biosynthetic enzymes of specialized metabolism (Kai et al., 2008; Vialart et al., 2012). These results, taken together, suggest that the hydroxylation of scopoletin (and, as an effect, the synthesis of fraxetin) is the main activity of the S8H enzyme. The experiments performed...
with \textit{in vitro}-produced enzymes also provided evidence that S8H activity was dependent on the concentration of Fe\textsuperscript{2+}.

Transient expression of the S8H protein in \textit{N. benthamiana} and a subsequent metabolic analysis done on the infiltrated leaves further confirmed the results of the \textit{in vitro} assay. A simultaneous transformation with the S8H and F6’H2 heterologous genes in tobacco leaves resulted in a significantly higher accumulation of fraxetin; the concentration of fraxetin was intermediate when F6’H2 alone was expressed, and much lower when empty vector or S8H alone were expressed. The latter finding could be explained by the fact that tobacco plants do not synthesize scopoletin in a constitutive manner, but do synthesize fraxetin. The overproduction of F6’H2 therefore induces the synthesis of scopoletin from feruloyl-CoA, which is naturally present in tobacco leaves (Kai et al., 2008), thus providing the substrate for the reaction catalysed by S8H, resulting in a significantly higher fraxetin content.

To better understand the link between Fe homeostasis in plants and the biosynthesis of fraxetin, and consequently to show that the \textit{in vitro} enzyme activity of S8H is relevant \textit{in vivo}, we performed a detailed phenotypic characterization of Col-0 plants and two independent s8h mutant lines grown under different Fe regimes using various types of culture. Plants were grown in hydroponic solution, soil mixes, \textit{in vitro} liquid cultures, and on MS plates with various concentrations of Fe and other micronutrients. Our results clearly showed that the s8h plants, which carry mutated S8H alleles, are strongly affected by Fe-deficient conditions. Targeted metabolite profiling of s8h mutants demonstrated that coumarin profiles are significantly modified in mutant roots grown in Fe-depleted conditions. We detected higher concentrations of scopoletin in exudates from s8h mutant roots grown in liquid cultures. This finding was associated with lower levels of various coumarins and lower Fe content in mutant roots as compared with WT plants. Furthermore, the rosettes of s8h plants grown in 1× Heeg Fe-depleted hydroponic solution were clearly paler than those of Col-0 plants, and these changes were associated with striking changes in the metabolite profiles of coumarins in mutant roots. In comparison to Col-0 plants, under the 1× Heeg Fe-depleted condition we detected a significantly higher accumulation of scopoletin and scopolin in s8h roots that would suggest the inhibition of scopoletin-hydroxylation-dependent synthesis of fraxetin in mutant tissues. It is most likely that the \textit{in vitro} culture conditions in a small volume of liquid solution favour the increased secretion of exudates, and therefore we observed lower levels of various coumarins in s8h roots grown in liquid cultures linked to a significantly higher scopoletin content in exudates of mutant roots. The phenotypic differences between WT and mutant genotypes were also apparent on MS plates containing Fe-deficient medium, on which the s8h mutants showed chlorosis, significant growth retardation, and secreted an increased level of fluorescent compounds compared with WT plants.

Taking into account the results of soil experiments in which plants were cultured in soil mixtures with different chemical compositions, and the fact that s8h-1 mutants were larger than Col-0 plants grown in a soil mixture characterized by a relatively low level of P and high level of Fe, it will be also interesting to test the growth and metabolic profiles of roots and root exudes of s8h mutants grown in hydroponic solutions with various availability of P. This would be particularly interesting in the light of recent reports on the common and antagonistic regulatory pathways between phosphate (P) and Fe deficiency-induced coumarin secretion (Ziegler et al., 2016). Another area for further investigation is linked to the ICP-MS results indicating that, a range of other metals (Mn, Zn, Cu, Co, and Cd) were also significantly decreased in the s8h mutants. This is also linked to the phenotypic variation in plant growth that we observed on MS plates and in hydroponic cultures, which was dependent not only on Fe but also on the concentration of other micronutrients. This finding could be explained by the well-known phenomenon of interdependence of individual micronutrients in relation to each other (Ihnatowicz et al., 2014), and the fact that various metals interfere with Fe-deficiency responses (Lešková et al., 2017). Nevertheless, our work indicates that to get a broad overview of plant responses to nutrient deficiencies and to better understand the physiological role of the genes/enzymes involved, one needs to consider using various types of cultures, solution, and media in experimental procedures.

Among the numerous targeted metabolite profiling experiments conducted in this study, we repeatedly obtained results clearly showing significantly changed coumarin profiles in both s8h mutant lines studied. We observed a significantly higher content of scopoletin in the root tissue in hydroponic experiments and in the root exudates in liquid cultures. Scopoletin is a substrate for the reaction catalysed by S8H. The lack of S8H in the s8h mutant background could lead to the accumulation of higher levels of scopoletin and its corresponding glycoside, scopolin.

At the moment, we have no explanation for the fact that only small amounts of fraxetin were detected in some of the Col-0 samples under Fe-deficiency conditions (data not shown). It should also be noted that unexpectedly, in one experimental replicate, we detected relatively high amounts of fraxin and a low level of fraxetin in the s8h-1 mutant background, as well as low amounts of fraxin in some other Col-0 and s8h replicates. It cannot be excluded that these observations could be explained by the presence of another enzyme involved in fraxetin biosynthesis or an alternative metabolic pathway being induced in the s8h mutant background. The fraxetin synthesized in Col-0 plants could also be further demethylated to 6,7,8-trihydroxycoumarin, with a beneficial effect for plants under Fe-deficient conditions; alternatively, fraxetin could be directly involved in increasing Fe availability. These possibilities cannot be excluded, taking into account the catechol-type structure of fraxetin, and so they need to be further investigated. As reported by Schmid et al. (2014), the Fe-deficient chlorotic phenotype of f6’h1 seedlings grown under low Fe availability could be reversed by exogenous application of esculetin, esculin, and scopoletin. In parallel, the result of \textit{in vitro} assay showed that only esculetin was able to chelate and mobilize Fe\textsuperscript{2+} (Schmid et al., 2014). This suggests that compounds bearing an
orthocatechol moiety, such as esculetin and fraxetin, may be involved in the secretion of coumarin for Fe acquisition. The beneficial effect of exogenous application of scopoletin on reversing the chlorotic phenotype of f6′h1 demonstrated by Schmid et al. (2014), could be due to the activity of the S8H enzyme catalysing the hydroxylation of scopoletin, leading to fraxetin formation. Whatever mechanisms underlie such plant responses, given the results of in vitro enzyme activity assays and the observation of significant changes in the metabolite profiles of coumarin metabolism in s8h mutants grown in Fe-depleted conditions, it seems evident that the At3g12900 oxidoreductase is an S8H involved in fraxetin biosynthesis.

The present results indicate that At3g12900 is an important candidate for having a key role in Fe acquisition by plants. Fraxetin and other root-released phenolic compounds, mainly esculetin deriving from scopoletin and scopolin, have been suggested to be Fe chelators or Fe uptake facilitators in Arabidopsis (Schmid et al., 2014; Fourcroy et al., 2014; Schmidt et al., 2014; Brumbarova et al., 2015), and esculetin and fraxetin or fraxetin-derived compounds are possibly involved in the transport of Fe ions into the plant cell under Fe-deficient conditions. The precise physiological function of the phenolic compounds synthesized by plants under Fe-deficiency stress and the possible mechanism underlying plant responses to Fe limitation under calcareous conditions remain unknown (Schmid et al., 2014). Elucidating the biological role of the S8H enzyme involved in coumarin biosynthesis is a prerequisite to understanding the function of fraxetin in Fe acquisition.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Schematic representation of independent T-DNA mutant lines for the At3g12900 gene.

Fig. S2. Lack of S8H transcript in the s8h-l T-DNA mutant line confirmed by qRT-PCR.

Fig. S3. Multiple-sequence alignment of amino acid sequences of F6′H1, F6′H2, S8H and ANS enzymes.

Fig. S4. Results of recombinant His-tagged S8H protein purification.

Fig. S5. The optimal pH and temperature for in vitro activity of S8H.

Fig. S6. Characterization of Arabidopsis thaliana S8H enzyme activity.

Fig. S7. Kinetic parameters $V_{\text{max}}$ and $K_m$ of S8H.

Fig. S8. Phenotypic appearance of Col-0 and s8h plants grown in vitro in liquid cultures with various levels of Fe.

Fig. S9. Chlorophyll content and chlorophyll a/b ratio of Col-0 and s8h plants grown in vitro in liquid cultures with various levels of Fe.

Fig. S10. Phenotyping and biochemical characterization of Col-0 and s8h-l plants grown in soil mixes with different Fe availability.

Fig. S11. Trace element content of Col-0 and s8h plant roots grown in vitro in Fe-depleted liquid culture.

Fig. S12. Phenotypic appearance of 2-week-old Col-0 and s8h-l plants grown on 0.25 MS and 0.5 MS media.

Fig. S13. Fresh weight of 3-week-old Col-0 and s8h plants grown in vitro on 0.25 MS and 0.5 MS media.

Fig. S14. Phenotypic appearance of Col-0 and s8h plants observed under UV light.

Fig. S15. Phenotypic characterization of Col-0 and s8h plants grown in 1× Heeg hydroponic solution (fully changed once per week) with 0–40 μM Fe$^{2+}$ content.

Fig. S16. Phenotypic characterization of Col-0 and s8h plants grown in 10× Heeg hydroponic solution (fully changed once per week) with 0–40 μM Fe$^{2+}$ content.

Fig. S17. Phenotypic characterization of Col-0 and s8h plants grown in 1× Heeg hydroponic solution (refilled with a fresh medium) with 0–40 μM Fe$^{2+}$ content.

Fig. S18. Phenotypic characterization of Col-0 and s8h plants grown in 10× Heeg hydroponic solution (refilled with a fresh medium) with 0–40 μM Fe$^{2+}$ content.

Table S1. Genotyping of s8h mutant lines.

Table S2. Lack of S8H transcript in the s8h mutant backgrounds.

Table S3. Modified Heeg solutions used in hydroponic cultures.

Table S4. PCR amplification of the S8H ORF.

Table S5. Compounds used in the analysis of in vitro substrate specificity of S8H enzyme.

Table S6. Chemical analysis of soil mixes originating from different batches used in two experimental replicates.

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