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REGULAR ARTICLE

Secretion time of phytosiderophore differs in two perennial grasses and is controlled by temperature

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Abstract Some perennial grasses secrete phytosiderophores from the roots in response to Fe-deficiency. Here, we characterized the pattern of phytosiderophore secretion by *Lolium perenne* (cv. 'Tove') and *Poa pratensis* (cv. 'Baron'), which are used to correct iron-deficiency induced chlorosis in fruit trees grown on calcareous soils. Both species showed a distinct diurnal rhythm in phytosiderophore secretion, but the secretion time differed between species; the secretion peak time was about 2 h earlier in *L. perenne* than in *P. pratensis* under the same growth conditions. The secretion time was shifted by changing temperature during the collection of phytosiderophores in both *L. perenne* and *P. pratensis*. Increasing root-zone temperature resulted in earlier secretion, while lowering the temperature resulted in delayed secretion. Furthermore, this shift of secretion time was achieved by changing the temperature around the root-zone. Shading treatment during the secretion period did not affect the secretion time in either species. These results indicate that the secretion of phytosiderophore is triggered by the temperature around the roots, but not light, in these two perennial grasses.

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Introduction

Graminaceous plants secrete phytosiderophores (Fe-chelating substances) from their roots to acquire insoluble Fe(III) from calcareous soils. Since the first phytosiderophore, named mugineic acid, was identified from Fe-deficient barley (Takemoto et al. 1978), a total of nine mugineic acids have been identified in different graminaceous plants (Ueno et al. 2007). Most research on phytosiderophores has been done on barley and rice. Great progress has been made in cloning genes related to mugineic acid biosynthesis and uptake of the Fe(III)-mugineic acid complex. More than ten genes involved in different steps of mugineic acid synthesis have been isolated and functionally characterized, mainly in barley (e.g. Higuchi et al. 1999; Higuchi et al. 2001; Kobayashi et al. 2001; Takahashi et al. 1999; Nakanishi et al. 2000; Kobayashi et al. 2005). Three genes encoding transporters (YS, Yellow Stripe) of the Fe(III)-mugineic acid complex have been identified in maize (ZmYS1, Curie et al. 2001), barley (HvYS1, Murata et al. 2006) and rice (OsYSL15, Inoue et al. 2009). However, the genes responsible for the secretion of mugineic acids have not been cloned yet.

In addition to graminaceous crops, secretion of phytosiderophores has also been reported in wild grasses. Gries and Runge (1992) investigated 30 calcicolous and calcifugous grass species and found that calcicolous species secreted more Fe-chelating substances than calcifugous species, although they did not identify the compounds secreted. Ma et al. (2003) found that *Festuca rubra* (cvs. 'Rubina' and 'Barnica') secreted 2'-deoxymugineic acid (DMA) in response to Fe-deficiency. Recently, Ueno et al. (2007) identified two novel phytosiderophores, Hydroxy-2'-deoxymugineic acid (HDMA) and hydroxy avenic acid A (HAVA), secreted from the roots of Fe-deficient *Lolium perenne* and *Poa pratensis*, respectively. *Festuca rubra*, *L. perenne* and *P. pratensis* are commonly grown in association with fruit trees, and have the effect of preventing or ameliorating Fe-deficiency in orchard trees (Tagliavini et al. 2000). The effect of these perennial grasses in improving Fe-chlorosis has been attributed to the release of phytosiderophore from their roots (Ma et al. 2003; Cesco et al. 2006; Ueno et al. 2007). The secretion of phytosiderophore in *F. rubra* has been characterized in detail, but has not been examined yet in *L. perenne* and *P. pratensis*. Therefore, the objective of this study is to characterize the phytosiderophore secretion patterns of *L. perenne* and *P. pratensis*. We found that the secretion time of phytosiderophore differs between these species and is controlled by the temperature around the root zone.

Materials and methods

Plant materials and growth conditions

Seeds (0.5 g) of *Lolium perenne* (cv. 'Tove') and *Poa pratensis* (cv. 'Baron') were germinated in the dark at 20°C on a mesh in contact with deionized water in a 3.5 L pot. After germination, seedlings were pre-cultured in 1/5 Hoagland nutrient solution (pH5.3) as described previously (Ueno et al. 2007). Nutrient solution was renewed every 3 days. After 7 days of culture, the seedlings were transferred to 1/5 Hoagland nutrient solution without Fe and used for the following experiments. Plants were grown in a temperature-controlled greenhouse at 20°C with natural sunlight. All experiments were performed with three replicates.

Diurnal rhythm of phytosiderophore secretion

Lolium perenne and *P. pratensis* were grown in a nutrient solution without Fe for 13 (*L. perenne*) or 16 days (*P. pratensis*) at 20°C, after which diurnal change in the secretion of phytosiderophores was investigated in the two species. Before collection, the roots were washed with deionized water twice, and then placed in 1.2 L of deionized water at 20°C. Root exudates were collected every 2 h from 6:00 to 20:00 h at local time from the same pot. Water was replaced after each collection. Air and water temperatures were also recorded at each sampling time. The amount of phytosiderophore secreted was determined as described below.

Effect of temperature and light on the phytosiderophore secretion

The effect of temperature on phytosiderophore secretion was investigated by moving the Fe-deficient plants (3-week-old) to temperature-controlled greenhouse at 25, 15, or 10°C 2 days before phytosiderophore collection under the same natural sunlight. At the time of exudate collection, temperature of the water was the same as the air temperature.

To investigate the effect of root-zone temperature on the secretion of phytosiderophores, plants cultured without Fe for 13 days (*L. perenne*) or 30 days (*P. pratensis*) at 15°C were transferred to pots containing deionized water at 10, 15, or 25°C before collection at 6:00 h. The collection was performed in a temperature-controlled greenhouse at 15°C under natural sunlight. The water used for the collection was pre-warmed (to 25°C) or pre-cooled (to 10°C) before the collection, and water temperatures were maintained by placing the pots in a 20 L containers filled with water maintained at different temperatures by heating or cooling during collection. Since the shoot was not in contact with water directly at all and there was a great airflow in the temperature-controlled greenhouse and a high intensity of natural sunlight, the temperature of the shoots was hardly affected by the root-zone temperature. The collection started from 06:00 h (sunrise around 5:00 to 5:30). From 06:00 to 18:00 h, root exudates were collected every 3 h.

The effect of light on the secretion of phytosiderophore was investigated by shading Fe-deficient plants with a light-proof box. Shading treatment

commenced the night before collection of the root exudates. Root exudates were collected every 3 h from 6:00 to 18:00, from plants with or without shading treatment, and under the same air and water temperatures in a temperature-controlled greenhouse (15°C). The plants were subjected to Fe-deficiency for 18 days (*L. perenne*) or 22 day (*P. pratensis*) prior to the day of collection. All above experiments were conducted under natural lighting.

Quantitative determination of phytosiderophores in root exudates

Hydroponic media containing the roots exudates were passed through cation-exchange columns (Amberlite IR-120B, H^+ form) immediately after collection. The cation exchange columns were eluted with 2 M NH_4OH, and the eluates were concentrated in a rotary evaporator at 40°C. The residues were dissolved with 1 ml distilled water, and the sample was used for quantitative analysis of phytosiderophores.

The amount of phytosiderophore was determined by measuring its Fe-solubilizing capacity according to Takagi (1976), with some modifications. Briefly, 1.5 ml distilled water and 0.1 ml 0.5 M Na-acetate buffer (pH5.6) were added to a 0.5 ml sample solution in a glass test tube. After addition of 0.2 ml 5.0 mM $Fe(OH)_3$ suspension, the tubes were incubated in a water bath at 55°C for 2 h, with occasional mixing. The reaction mixture was then centrifuged at 500×g for 5 min at room temperature and the dissolved Fe in the supernatant was measured by the *o*-phenanthroline method (Takagi 1976).

Results

Secretion time differs with plant species

We first performed a rough screening for secretion time (only two time points) among ten cultivars of perennial grasses and found that the secretion time only differed with species, but not cultivars (data not shown). We then further characterized two cultivars from *L. perenne* and *P. pratensis*. When grown under the same conditions, both *L. perenne* (cv. 'Tove') and *P. pratensis* showed a diurnal rhythm in the secretion of phytosiderophores (Fig. 1); however, the time of maximal secretion differed between the two species.

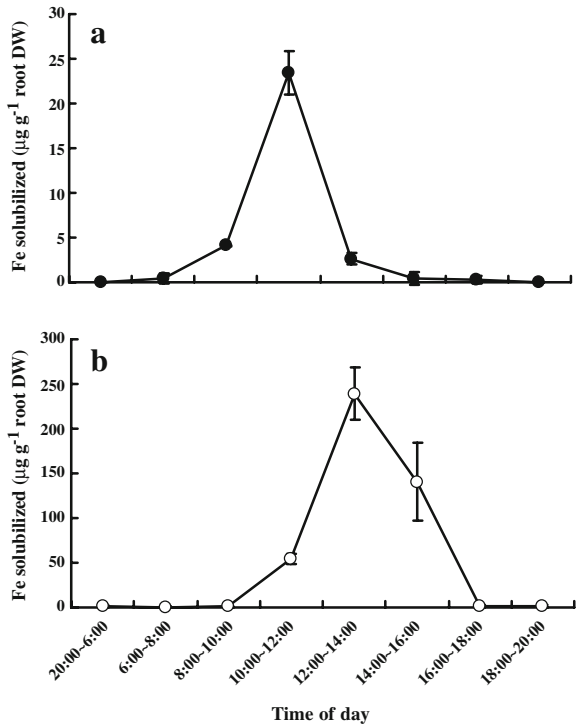

Fig. 1 Diurnal changes of phytosiderophore secretion in Fe-deficient *L. perenne* (cv. 'Tove') (**a**) and *P. pratensis* (cv. 'Baron') (**b**). The collection of root exudates was conducted every 2 h from 6:00 to 20:00 h at 20°C. Sunrise was at 5:30. Error bars represent±SD (n=3)

The secretion peak was observed between 10:00 and 12:00 h in *L. perenne* (sunrise 5:30) (Fig. 1a), but 2 h later (between 12:00 and 14:00) in *P. pratensis* (Fig. 1b). Phytosiderophores were not secreted at all during the night period (20:00~6:00) in both species (Fig. 1). Therefore, there was a 2-h difference in secretion time between *L. perenne* and *P. pratensis*.

Effect of temperature and light on the secretion time of phytosiderophore

To investigate the temperature on the secretion time of phytosiderophore, Fe-deficient plants pre-cultured under the same conditions were placed at different temperatures for 2 days before collection of phytosiderophore. As a result, the secretion peaks shifted with temperatures in both plant species (Fig. 2). In *L. perenne*, the secretion peak was observed between 8:00 and 12:00 h at 25°C, between 10:00 and 12:00 h at 15°C, and between 12:00 and 14:00 h at 10°C, respectively (Fig. 2a). In *P. pratensis*, the secretion

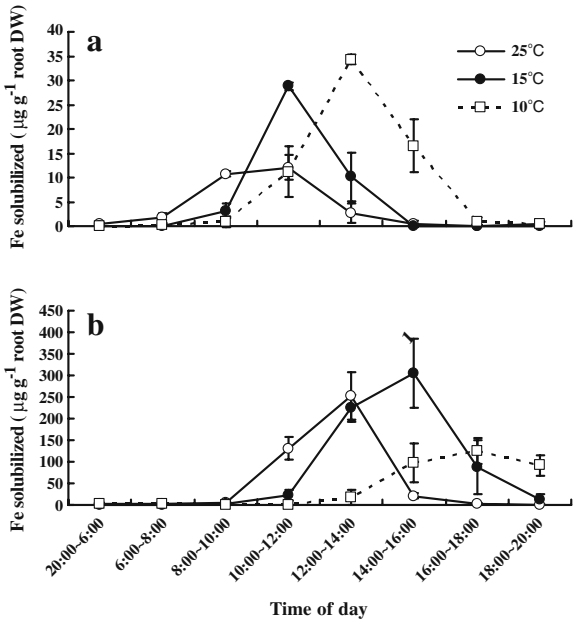

Fig. 2 Effect of temperature on the secretion time in *L. perenne* (cv. 'Tove') (**a**) and *P. pratensis* (cv. 'Baron') (**b**). Fe-deficient plants were placed in a greenhouse at 10, 15, or 25°C 2 days before the collection of root exudates. The collection was conducted every 2 h from 6:00 to 20:00 h. Sunrise was at 5:30. Error bars represent±SD (n=3)

peak was between 12:00 and 14:00 h at 25°C, 14:00–16:00 h at 15°C, and 16:00–18:00 h at 10°C (Fig. 2b). These results indicate that a higher temperature results in earlier secretion, whereas a lower temperature delays the secretion in both species (Fig. 2).

To further investigate the sensing organ (roots vs. shoots) of the temperature for the secretion, collection of root exudates was conducted under the same air temperature (15°C, temperature around the shoots), but different water temperatures (25°C, 15°C, and 10°C, root-zone). Decreasing temperature in the rooting zone generally delayed the time of peak phytosiderophore secretion for both species. In *L. perenne*, peak phytosiderophore secretion occurred from 6:00–9:00 at 25°C, 9:00–12:00 at 15°C and 12:00–15:00 at 10°C (Fig. 3a). A similar peak secretion time vs. temperature pattern was observed in *P. pratensis* (Fig. 3b), although the secretion peak times were approximately 3 h later for all temperatures relative to *L. perenne*. The air and water temperature was almost constant during the collection of phytosiderophores (Fig. 3c). These results indicate that the secretion time of phytosiderophores is controlled by the temperature around the root-zone in both species.

The effect of light on the secretion phytosiderophores was also investigated in both species. To avoid the effect of light on the biosynthesis of phytosiderophore during the day, shading treatment started from the previous night before collection of phytosiderophores and continued till the end of collection. Shading and non-shading treatments during the collection of root exudates showed the same pattern of secretion in both *P. pratensis* and *L. perenne*

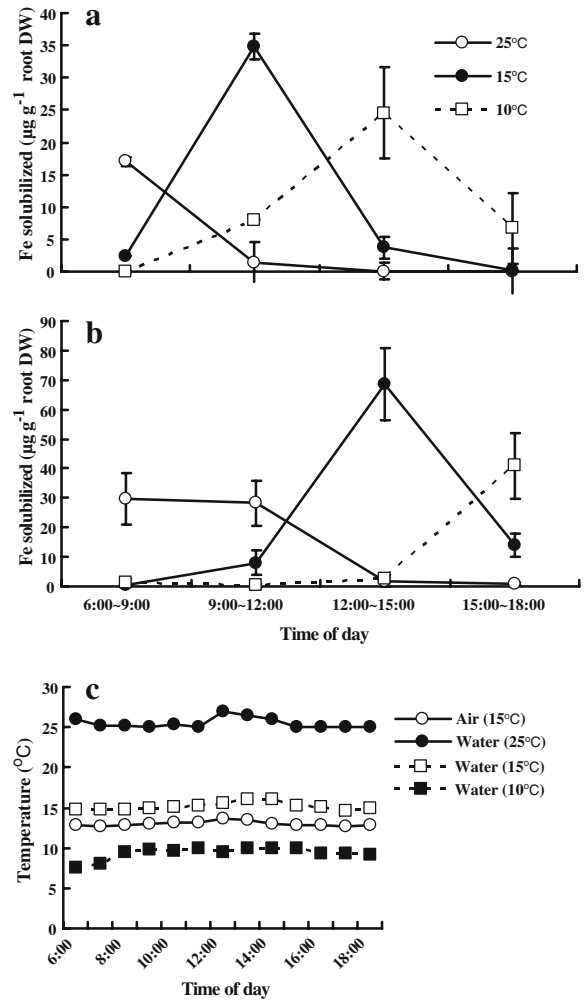

Fig. 3 Effect of root-zone temperature on the secretion time in *L. perenne* (cv. 'Tove') (**a**) and *P. pratensis* (cv. 'Baron') (**b**). Roots of Fe-deficient plants were exposed to water pre-cooled or pre-warmed to 10, 15, or 25°C under the same air temperature at 15°C. The temperature in water and air was shown in (**c**). The collection was conducted every 3 h from 6:00 to 18:00 h. Sunrise was at 5:00. Error bars represent±SD (n=3)

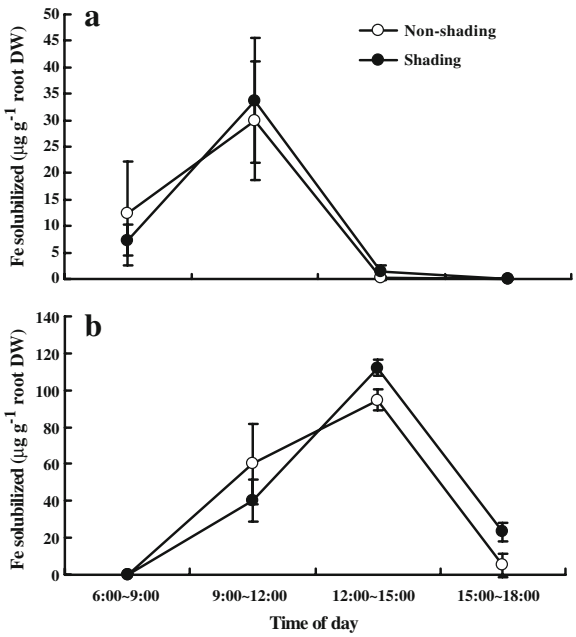

Fig. 4 Effect of short-term shading on the secretion of phytosiderophores in *L. perenne* (cv. 'Tove') (**a**) and *P. pratensis* (cv. 'Baron') (**b**). Root exudates were collected every 3 h from plants which had been pre-treated with Fe-deficiency at 15°C. Short term shading was achieved by covering plants with a light-proof box in the night before collection of the root exudates. Sunrise was at 5:00. Error bars represent±SD (n=3)

(Fig. 4), suggesting that light does not directly influence the secretion time of phytosiderophores.

Discussion

Among graminaceous plants examined, there are two patterns for the secretion of phytosiderophores. A distinct diurnal rhythm in secretion has been reported in barley (Takagi et al. 1984), wheat (Zhang et al. 1991), *Hordelymus europaeus* (Gries and Runge 1992), and *Festuca rubra* (Ma et al. 2003). In other species, such as maize (Yehuda et al. 1996) and rice (Inoue et al. 2009), there is no diurnal rhythm in the secretion. Species with diurnal secretion patterns have different times of peak secretion rate. For example, in barley and wheat, maximum secretion rates occurs 4 h after the onset of light period (Takagi et al. 1984; Zhang et al. 1991). On the other hand, the secretion peak occurred at 5.5 h in *Hordelymus europaeus* (Gries and Runge 1992), and between 2 and 5 h in *Festuca rubra* (Ma et al. 2003). Since the growth conditions are different in these previous studies, it is difficult to conclude whether the differences in the secretion time results from the growth conditions or from species itself. In the present study, we compared the secretion pattern between *P. pratensis* and *L. perenne* under the same growth conditions.

P. pratensis and *L. perenne* secrete different kinds of phytosiderophores in response to Fe-deficiency; *P. pratensis* secretes DMA, AVA and HAVA, while *L. perenne* secretes. DMA, HDMA and epiHDMA (Ueno et al. 2007). The amount of these phytosiderophore secreted is higher in *P. pratensis* than in *L. perenne*, but the secretion amount increased with the progression of Fe-deficiency in both species (Ueno et al. 2007). Since the growth rate was different between the two species tested and the experiments were conducted in different seasons, we had to use plants of different ages and with different lengths of Fe-deficiency duration in order to obtain sufficient amounts of phytosiderophores for quantitative determination. However, *P. pratensis* and *L. perenne* showed distinct diurnal rhythms in the secretion of phytosiderophores irrespective of plant age and the duration of Fe-deficiency, with a difference of 2-3 h in the secretion peak between the two species (Figs. 1 and 3). Furthermore, in our preliminary experiment comparing ten cultivars of perennial grasses but with fewer collection times, we found that the secretion pattern differed between the species, but not cultivars (data not shown) These results indicate that the secretion time differ consistently between *P. pratensis* and *L. perenne*.

The diurnal rhythm in the secretion of phytosiderophore has been suggested to be affected by both temperature and light (Ma et al. 2003; Reichman and Parker 2007). In the present study, earlier secretion was correlated with increased temperature (Fig. 3); however, shading experiments revealed that phytosiderophore secretion is not triggered by light in both perennial grass species (Fig. 4). Furthermore, the temperature of the rooting zone, but not the air temperature controls secretion time (Fig. 3). These results support the idea that the initiation of phytosiderophore secretion is triggered by the temperature around the roots. Our results are in agreement with temperature-sensitive secretion patterns found in *Festuca rubra* (Ma et al. 2003), but do not correspond with light-sensitive secretion patterns found in wheat (Reichman and Parker 2007). By using a square-wave

light regime, Reichman and Parker (2007) concluded that the secretion of phytosiderophores in wheat is mainly mediated by changes in light rather than temperature. However, in their study, plants were shaded for a longer time, which may have affected phytosiderophore biosynthesis. Therefore, the lack of secretion of phytosiderophores under darkness may be the result of decreased synthesis. In our experiment, we started the shading treatment from only the previous night, which should have little effect on phytosiderophore biosynthesis. Another possibility is that phytosiderophore secretion time is controlled differently between wheat and perennial grasses.

The mechanism responsible for diurnal rhythm of phytosiderophore secretion is still unknown. Recently, diurnal changes were reported in the expression of some genes involved in biosynthesis of phytosiderophores in rice (Nozoye et al. 2004) and uptake of Fe (III)-phytosiderophore complex in rice and barley (Inoue et al. 2009; Nagasaka et al. 2009). Some elements associated to the diurnal change have been proposed to be present in the promoter region of these genes. Since the gene responsible for the secretion of phytosiderophore has not been cloned yet, it remains to be examined whether similar elements are involved in the diurnal rhythm of phytosiderophore secretion, or the secretion is regulated independent of biosynthesis and uptake.

Phytosiderophore secretion by grasses may increase Fe availability for coexisting species. Recently, it was reported that citrus can utilize Fe effectively from Fe(III)-phytosiderophore complex secreted from Poa (Cesco et al. 2006). Moreover, the combination of three perennial grasses, *F. rubra, L. perenne* and *P. pratensis*, has been shown to prevent Fe-deficiency more effectively compared to single species (Rombolá, personal communication). The secretion peak time of *F. rubra* is between those of *L. perenne* and *P. pratensis* (Ma et al. 2003). When these perennial grass species are grown together in an orchard, the combined effect of different secretion peak times may maintain Fe availability for orchard trees for longer period of time relative to single grass species. The characterization of phytosiderophore secretion patterns of perennial grasses provides important information for designing an optimal combination of species that can be used for effective correction of Fe-induced chlorosis of crops grown on calcareous soils.

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