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Contribution of Strigolactones to the Inhibition of Tiller Bud Outgrowth under Phosphate Deficiency in Rice

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Strigolactones (SLs) or SL-derived metabolite(s) have recently been shown to act as endogenous inhibitors of axillary bud outgrowth. SLs released from roots induce hyphal branching of arbuscular mycorrhizal (AM) fungi that facilitate the uptake of inorganic nutrients, such as phosphate (Pi) and nitrate, by the host plants. Previous studies have shown that SL levels in root exudates are highly elevated by Pi starvation, which might contribute to successful symbiosis with AM fungi in the rhizosphere. However, how endogenous SL levels elevated by Pi starvation contribute to its hormonal action has been unknown. Here, we show that tiller bud outgrowth in wild-type rice seedlings is inhibited, while root 2′-*epi*-5-deoxystrigol (*epi*-5DS) levels are elevated, in response to decreasing Pi concentrations in the media. However, the suppression of tiller bud outgrowth under Pi deficiency does not occur in the SL-deficient and -insensitive mutants. We also show that the responsiveness to exogenous SL is slightly increased by Pi deficiency. When Pi-starved seedlings are transferred to Pi-sufficient media, tiller bud outgrowth is induced following a decrease in root *epi*-5DS levels. Taken together, these results suggest that elevated SL levels by Pi starvation contribute to the inhibition of tiller bud outgrowth in rice seedlings. We speculate that SL plays a dual role in the adaptation to Pi deficiency; one as a rhizosphere signal to maximize AM fungi symbiosis for improved Pi acquisition and the other as an endogenous hormone or its biosynthetic precursor to optimize shoot branching for efficient Pi utilization.

Keywords: Arbuscular mycorrhizal fungi • *Oryza sativa* • Phosphate • Strigolactone • Tillering.

Abbreviations: AM fungi, arbuscular mycorrhizal fungi; CCD, carotenoid cleavage dioxygenase; *epi*-5DS, 2′-*epi*-5-deoxystrigol; LC-MS/MS, liquid chromatography–tandem mass spectrometry; qRT-PCR, quantitative reverse transcription–PCR; SL, strigolactone; WT, wild type.

Introduction

Strigolactones (SLs) are a group of terpenoid lactones that have been found in root exudates of diverse plant species and were first characterized as seed germination stimulants of root parasitic plants such as *Striga* and *Orobanche* species (Cook et al. 1966, Yokota et al. 1998). SLs were later shown to be root-derived signals that induce hyphal branching of arbuscular mycorrhizal (AM) fungi, which assist the acquisition of inorganic soil nutrients, such as phosphate (Pi) and nitrate, by the host plant (Akiyama et al. 2005). More recently, SLs were shown to act as a new hormone class, or as their biosynthetic precursors, that inhibits shoot branching (Gomez-Roldan et al. 2008, Umehara et al. 2008). Shoot branching involves the formation of axillary buds in the axil of the leaves and subsequent outgrowth of the buds (McSteen and Leyser 2005). SL suppresses shoot branching by inhibiting the outgrowth of axillary buds.

Evidence for this new hormone came from studies using increased branching mutants, including *ramosus* (*rms*) of pea, *decreased apical dominance* of petunia, *more axillary growth* (*max*) of Arabidopsis and particular *dwarf* (*d*) mutants of rice (for reviews, see Ongaro and Leyser 2008, Beveridge and Kyozuka 2010). Grafting experiments suggested that some of these mutants are defective in the biosynthesis of a mobile signal that can move from roots to shoots and suppresses shoot branching, while others are unable to respond to this hormonal signal. Cloning of these genetic loci revealed highly conserved proteins required for the synthesis of or response to this mobile signal.

Two lines of evidence support the idea that RMS/MAX/D proteins are involved in the biosynthesis or signaling of SL (Gomez-Roldan et al. 2008, Umehara et al. 2008). First, SL levels in root exudates (and in roots for rice) were significantly reduced in the putative biosynthesis mutants, such as those defective in carotenoid cleavage dioxygenase 7 (CCD7) (*rms5*/*max3*/*d17*) or CCD8 (*rms1*/*max4*/*d10*) (**Fig. 1**). In contrast, SL levels were not decreased in the *rms4*/*max2*/*d3*

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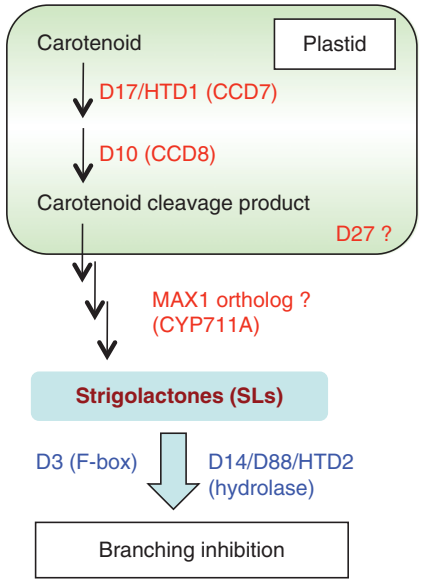

Fig. 1 The SL-dependent branching inhibition pathway in rice. D17/HTD1, D10 and D27 participate in SL synthesis. The role of CYP711A in rice has not been shown, but Arabidopsis CYP711A1/MAX1 has been shown to act downstream of CCDs. D27 is localized to the plastid, but its relative position in the pathway has not been clear. D3 and D14/D88/HTD2 act in a step downstream of SL synthesis. CCD, carotenoid cleavage dioxygenase.

mutants, which are defective in an F-box leucine-rich repeat protein and are thought to be unresponsive to the branch-inhibiting hormone. The second piece of evidence came from SL application experiments. An application of a synthetic SL analog, GR24, or a natural SL inhibited shoot branching of the *ccd7* and *ccd8* mutants, whereas *rms4*/*max2*/*d3* mutant plants were unable to respond to exogenous SL (Gomez-Roldan et al. 2008, Umehara et al. 2008). GR24 treatment also inhibited axillary bud outgrowth of the *max1* mutant (Gomez-Roldan et al. 2008). *MAX1* encodes a cytochrome P450 monooxygenase designated as CYP711A1 (Booker et al. 2005) (**Fig. 1**).

More recent studies using rice *d* mutants have identified new components in the SL pathway. *D27* encodes an iron-containing protein that localizes to the plastid. The *d27* mutant has reduced 2′-*epi*-5-deoxystrigol (*epi*-5DS) content in root exudates and its tiller bud outgrowth is inhibited by GR24 treatment, suggesting that D27 is involved in an early stage of SL biosynthesis in the plastid (**Fig. 1**) (Lin et al. 2009). The *d14* mutant, also reported as *d88* and *htd2*, is an SL-insensitive tillering dwarf mutant that accumulates a higher level of *epi*-5DS than does the wild type (WT) in roots and root exudates (Arite et al. 2009, Gao et al. 2009, Liu et al. 2009). *D14* encodes a protein that belongs to the α/β-fold hydrolase family, and is proposed to function downstream of SL synthesis as a signaling component or as an enzyme that participates in the conversion of SLs to the bioactive branching inhibitor (Arite et al. 2009).

The dual role of SL as a rhizosphere signal as well as being an endogenous hormone (precursor) implies a biological link between AM fungi symbiosis and shoot branching, both of which are regulated by the same chemical signal. Previous studies have demonstrated that SL levels in root exudates (and in roots for some cases) are highly elevated by phosphate (Pi) and/or nitrate starvation in several plant species (Yoneyama et al. 2007a, Yoneyama et al. 2007b, Lopez-Raez et al. 2008, Umehara et al. 2008, Yoneyama et al. 2008). Interestingly, a drastic increase in SL accumulation under mineral nutrient deficiency appears to occur only when the plant depends on the uptake of limited Pi or nitrate by AM fungi symbiosis. For example, in red clover, a leguminous plant capable of symbiotic nitrogen fixation in nodules, increased SL accumulation occurs in response to Pi deficiency, but not to nitrate deficiency (Yoneyama et al. 2007b). In comparison, both Pi and nitrate deficiencies induced SL accumulation in sorghum, which is a host of AM fungi, but cannot perform nitrogen fixation (Yoneyama et al. 2007a). Furthermore, neither Pi nor nitrate deficiency promoted SL production in white lupin (*Lupinus albus*), a non-host of AM fungi (Yoneyama et al. 2008). These observations suggest that the regulation of SL production (exudation) is closely related to the nutrient acquisition strategy of the plants.

How elevated endogenous SL levels under Pi deficiency contribute to its hormonal function has been unknown. Shoot branching is influenced by a number of environmental cues (Cline 1991). Given the discovery of SLs as shoot branching inhibitors and the response of SL levels to low Pi, we speculated in a previous paper that SL might have a function in optimizing the shoot architecture under Pi deficiency in order to utilize the limited resource efficiently in the plant body (Umehara et al. 2008). Previous work has shown that Pi deficiency resulted in reduced shoot growth with reduced tiller growth in rice plants (cv. Nipponbare) (Luquet et al. 2005). However, it has been unknown whether SL plays any role in decreasing the tiller number under low Pi conditions in rice. To address this question, we examined the effect of Pi availability on tiller bud outgrowth and SL levels. We show that the number of outgrowing tillers in WT seedlings decreases, while root SL levels increase, in response to decreasing Pi concentrations in the culture medium. Experiments using *d* mutants illustrate that the inhibition of tiller bud outgrowth under Pi deficiency requires SL biosynthesis and signaling. We also show that tiller bud outgrowth is promoted, while SL levels in roots are decreased, after Pi is supplied to Pi-deficient WT seedlings. Altogether, our results suggest that SL plays a role in inhibiting shoot branching under low Pi conditions in rice.

Results

Effect of Pi on tiller bud outgrowth and SL levels

When WT seedlings were pre-cultured on agar media and then grown hydroponically for 2 weeks (a total of 3 weeks after seed

imbibition), the outgrowth of second and third leaf tillers was reproducibly observed, while the tiller bud at the first leaf remained dormant (**Fig. 2A, B**). We regarded tiller buds >2 mm as growing out, because dormant tiller buds are <2 mm in size in our growth conditions (Umehara et al. 2008). To explore the effect of Pi deficiency on tiller bud outgrowth and SL levels, WT and *d10-1* and *d3-1* mutant seedlings were grown under varying concentrations of Pi during the hydroponic culture for 2 weeks (**Fig. 2A**). When Pi was depleted in the media, tiller bud outgrowth was nearly fully inhibited in WT seedlings, but not in *d3-1* and *d10-1* mutant seedlings (**Fig. 2B, C**). Suppression of tiller bud outgrowth under Pi deficiency accompanied a slight decrease in plant height and shoot and root mass (fresh weight) in WT and *d* mutant seedlings (**Supplementary Table S1**).

To examine the relationships between tiller bud outgrowth and SL accumulation, we determined the levels of *epi*-5DS, a previously identified SL in rice seedlings, in shoots (basal part including both apical and tiller buds and elongation zones of leaf sheaths), roots and root exudates by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using deuterium-labeled *epi*-5DS as an internal standard. *epi*-5DS levels in roots and root exudates of WT seedlings gradually decreased in response to increasing concentrations of Pi in the media (**Fig. 2D**), consistent with previous reports in rice and other plant species (Yoneyama et al. 2007a, Yoneyama et al. 2007b, Lopez-Raez et al. 2008, Umehara et al. 2008, Yoneyama et al. 2008). A negative correlation was evident between the number of outgrowing tillers and *epi*-5DS concentrations in roots and in root exudates in WT seedlings under changing Pi concentrations (**Fig. 2E**). Unlike in WT plants, the number of tillers that grew out in *d3-1* and *d10-1* mutant seedlings was not significantly influenced by Pi concentrations (**Fig. 2C, D**). These results indicate that both SL biosynthesis and D3-dependent SL signaling are required for the suppression of tiller bud outgrowth under low Pi conditions. Although the initiation of tiller outgrowth was persistently observed in *d3-1* and *d10-1* mutant seedlings irrespective of Pi availability, the length of tillers that grew out in these mutants tends to be shorter under Pi deficiency as observed for the length of the main shoot (plant height) (**Supplementary Tables S1, S2**)

Our LC-MS/MS analysis showed that *epi*-5DS levels in the basal part of shoots in WT seedlings were very low; they were determined to be <10 pg g^{-1} FW in all samples. Because of their low abundance, we were unable to quantify *epi*-5DS levels reliably in WT shoot samples, and whether *epi*-5DS levels in WT shoots were also increased under Pi deficiency could not be elucidated. *epi*-5DS levels in *d3-1* mutant shoots were higher than those in WT shoots, presumably as a consequence of feedback regulation in the SL pathway (Foo et al. 2005, Arite et al. 2007), and their reliable quantification was possible (**Supplementary Fig. S1**). As seen for *d3-1* roots, *epi*-5DS levels in *d3-1* shoots were also elevated by Pi starvation, but the difference in *epi*-5DS levels between Pi-deficient and -sufficient conditions were not as large as that observed in roots (**Fig. 2D**, **Supplementary Fig. S1**).

Effect of Pi on SL responsiveness

To determine whether Pi deficiency affects SL responsiveness, we examined the effect of exogenous GR24 (an SL analog) applied to the root on tiller bud outgrowth in *d10-1* mutant seedlings under +Pi and −Pi conditions. Irrespective of the nutrient conditions, a clear inhibitory effect of GR24 on tiller bud outgrowth was detectable in response to as little as 0.1 μM GR24 (**Fig. 3**). GR24 treatment at higher concentrations (1 and 10 μM) was slightly more effective in inhibiting tiller bud outgrowth under Pi deficiency than in Pi-sufficient media. Taken together, our data illustrate that Pi deficiency increases endogenous SL levels and SL responsiveness. These results support the idea that an elevated endogenous SL concentration can contribute to the inhibition of tiller bud outgrowth under Pi deficiency.

Temporary Pi depletion treatment

To investigate further the relationships between tiller bud outgrowth and SL accumulation under Pi deficiency, we examined the effect of temporary depletion of Pi during hydroponic culture. When WT seedlings were exposed to Pi-depleted media only for the second or the third week, the *epi*-5DS levels in root exudates remained low and there was no decrease in the number of outgrowing tillers, unlike in seedlings that stayed in the −Pi media continuously for 2 weeks (**Fig. 4**). These results again indicate a negative correlation between the number of outgrowing tillers and *epi*-5DS accumulation in root exudates.

Time course analysis of SL levels and SL-related gene expression in roots

Our experiments above indicated that tiller bud outgrowth is promoted when WT seedlings in the −Pi media were transferred to the +Pi media during the third week (**Fig. 4A, B**). Time course analysis revealed that the induction of tiller bud outgrowth was not synchronized so well among plants, but it was initially evident in some seedlings 4 d after the supply of Pi (**Fig. 5A, B**). *epi*-5DS levels in roots were drastically decreased within 1 d after Pi was supplied to Pi-starved WT seedlings (**Fig. 5C**). These results indicate that there is a decrease in root *epi*-5DS levels before the initiation of tiller bud outgrowth when Pi-deficient WT seedlings were transferred to Pi-sufficient media.

To investigate which gene(s) in the SL pathway contributed to the Pi-induced decrease in root *epi*-5DS levels, we analyzed their transcript levels by quantitative reverse–transcription PCR (qRT–PCR). *D10*, *D17* and *D27* mRNA levels in roots sharply decreased within 1 d after transfer to the +Pi media, while they increased in the −Pi media (**Fig. 5D**), which resembled the change in *epi*-5DS levels (**Fig. 5C**). In rice, there are five *MAX1*-related genes, *Os01g0700900*, *Os01g0701400*, *Os01g0701500*, *Os02g0221900* and *Os06g0565100*, all of which have been classified as members of the CYP711A family based on their sequence

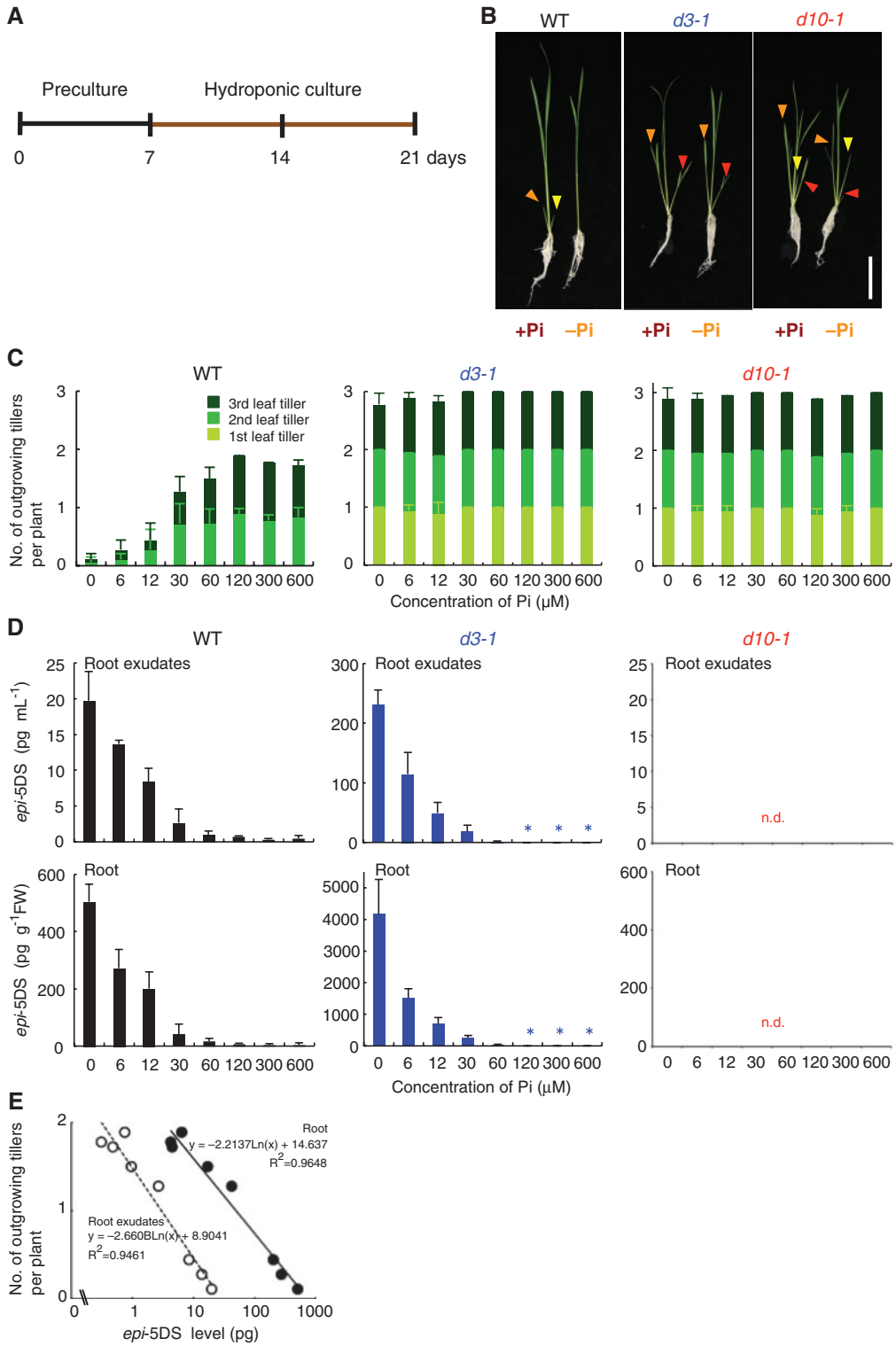

Fig. 2 Effect of Pi on tiller bud outgrowth and SL levels. (A) Schematic diagram showing the experimental conditions. Seven-day-old seedlings grown on agar media were transferred to hydroponic culture media containing various concentrations of Pi. (B) Twenty-one-day-old WT and *d* mutant seedlings grown with or without 600 μM Pi. Red, orange and yellow arrowheads indicate tillers that grew out from the first, second and third leaf, respectively. The scale bar is 5 cm. (C) Number of outgrowing tillers (>2 mm) per plant in six seedlings. (D) SL levels in root exudates and roots. n.d., not detected due to low abundance. Asterisk (*), detected, but could not be quantified reliably due to low abundance. Data are the means ± SD ($n = 3$) for C and D. (E) Correlation analysis between SL levels and the number of outgrowing tillers in the WT. Solid line with filled circles, SL levels in roots (pg g^{-1} FW); dashed line with open circles, SL levels in root exudates (pg ml^{-1}).

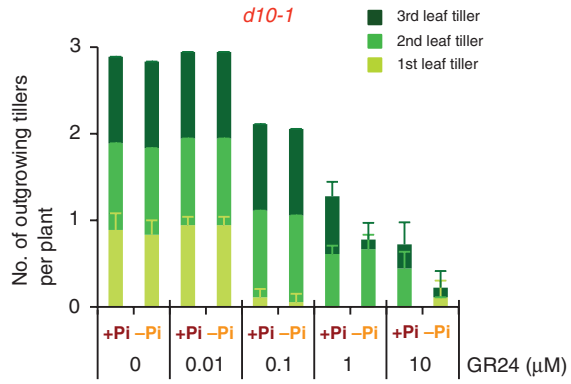

Fig. 3 Effect of Pi on SL responsiveness. *d10-1* mutant seedlings were grown hydroponically with or without Pi as described in **Fig. 2A** in the presence of different concentrations of GR24. GR24 was included in the media during the hydroponic culture. Number of outgrowing tillers (>2 mm) per plant in six seedlings is shown. Data are the means ± SD ($n = 3$). The number of outgrowing tillers was decreased in –Pi relative to +Pi in the following tiller buds: second leaf tiller at 10 μM GR24 ($P = 0.057$); third leaf tiller at 1 μM GR24 ($P = 0.11$).

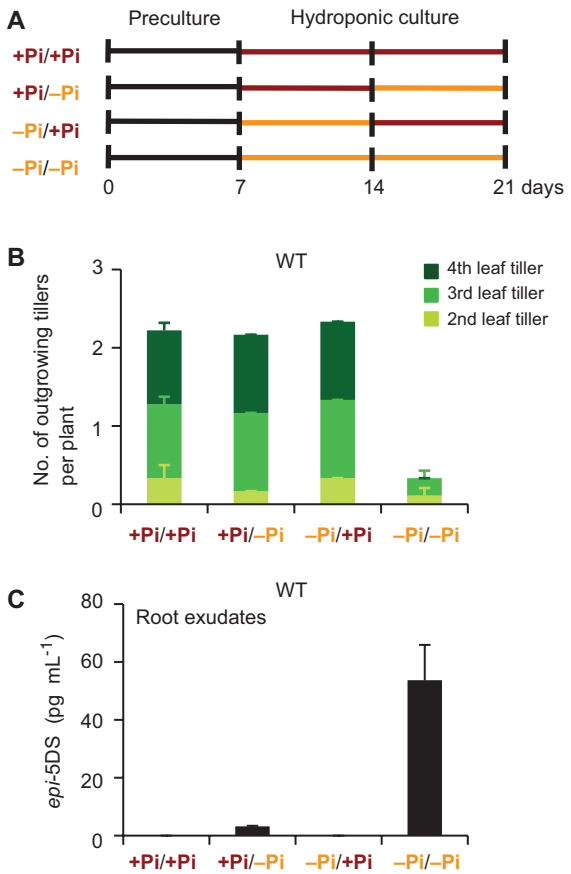

Fig. 4 Effect of temporal Pi depletion on tiller bud outgrowth and SL levels. (A) Schematic diagram showing the experimental conditions. Brown and orange bars indicate a hydroponic culture with and without Pi, respectively. (B) Number of outgrowing tillers (>2 mm) per plant in six 21-day-old WT seedlings. The tiller bud on the first leaf did not grow out in all samples (not shown). (C) SL levels in root exudates. Data are the means ± SD ($n = 3$) for B and C.

similarity to MAX1/CYP711A1 (Nelson et al. 2004). Among them, transcript levels of *Os01g0700900* and *Os02g0221900* decreased within 1 d after the supply of Pi, as observed for *D10*, *D17* and *D27* mRNA abundance. The levels of *Os01g0701500* mRNA in the +Pi media were constant, and were lower than those in the –Pi media. Expression of the other two *MAX1* homologs, *Os01g0701400* and *Os06g0565100*, was not affected by Pi concentrations in our experimental conditions. These results suggest that down-regulation of multiple SL biosynthesis genes by Pi might contribute to a decrease in *epi*-5DS levels in roots. Whether the CYP711A family members in rice are functional orthologs of Arabidopsis *MAX1* needs further studies. Transcript levels of *D14*, which acts in a step downstream of SL synthesis (**Fig. 1**), were not decreased, but rather increased, after transfer to the +Pi media. There was no significant difference in *D3* mRNA levels between +Pi and –Pi conditions (**Fig. 5D**).

Next, we determined transcript levels of SL-related genes in the basal part of shoots in order to deduce whether SL production is also regulated by Pi in the shoot (**Fig. 5D**). Unlike in roots, none of the SL-related transcripts in the shoot drastically decreased in abundance after transfer to the +Pi media. Nevertheless, mRNA levels of *D10*, *D27* and *Os02g0221900* (a *MAX1* homolog) were slightly higher in –Pi than in +Pi at least at one of the time points. These results suggest that Pi might also lower SL levels in the shoot, but that the effect of Pi on SL levels in the shoot was not so great as that in the root. Our qRT–PCR data also showed that most of the SL biosynthetic transcripts were much less abundant in the shoot than in the root, particularly in Pi-deficient conditions (**Fig. 5D**).

Discussion

Phosphorus is a major element required for plant growth. Because Pi is a structural component of nucleic acids and membrane lipids and also takes part in regulatory pathways involving phospholipid-derived signaling molecules or phosphorylation reactions (Amtmann and Armengaud 2009), plant growth is generally inhibited by Pi starvation. Shoot branching is affected by various environmental factors including nutrients (Cline 1991, Waldie et al. 2010). Previous experiments showed that tillering was inhibited by Pi starvation in rice, presumably as a consequence of growth reduction, although the possibility of a specific inhibition of tiller bud outgrowth under low Pi could not be ruled out (Luquet et al. 2005). Here, we showed that a reduction in the number of outgrowing tillers under Pi-deficient conditions correlated well with endogenous *epi*-5DS levels in roots (**Fig. 2**). Importantly, tiller bud outgrowth was not inhibited under Pi deficiency in SL-deficient and -insensitive mutants, while general growth reductions, such as decreased fresh weights of shoots and roots, were observed in these mutants (**Fig. 2**, **Supplementary Table S1**). These results indicate that SL biosynthesis and D3-dependent SL signaling are required for the inhibition of tiller bud outgrowth under Pi deficiency. Our previous results showed that SL application

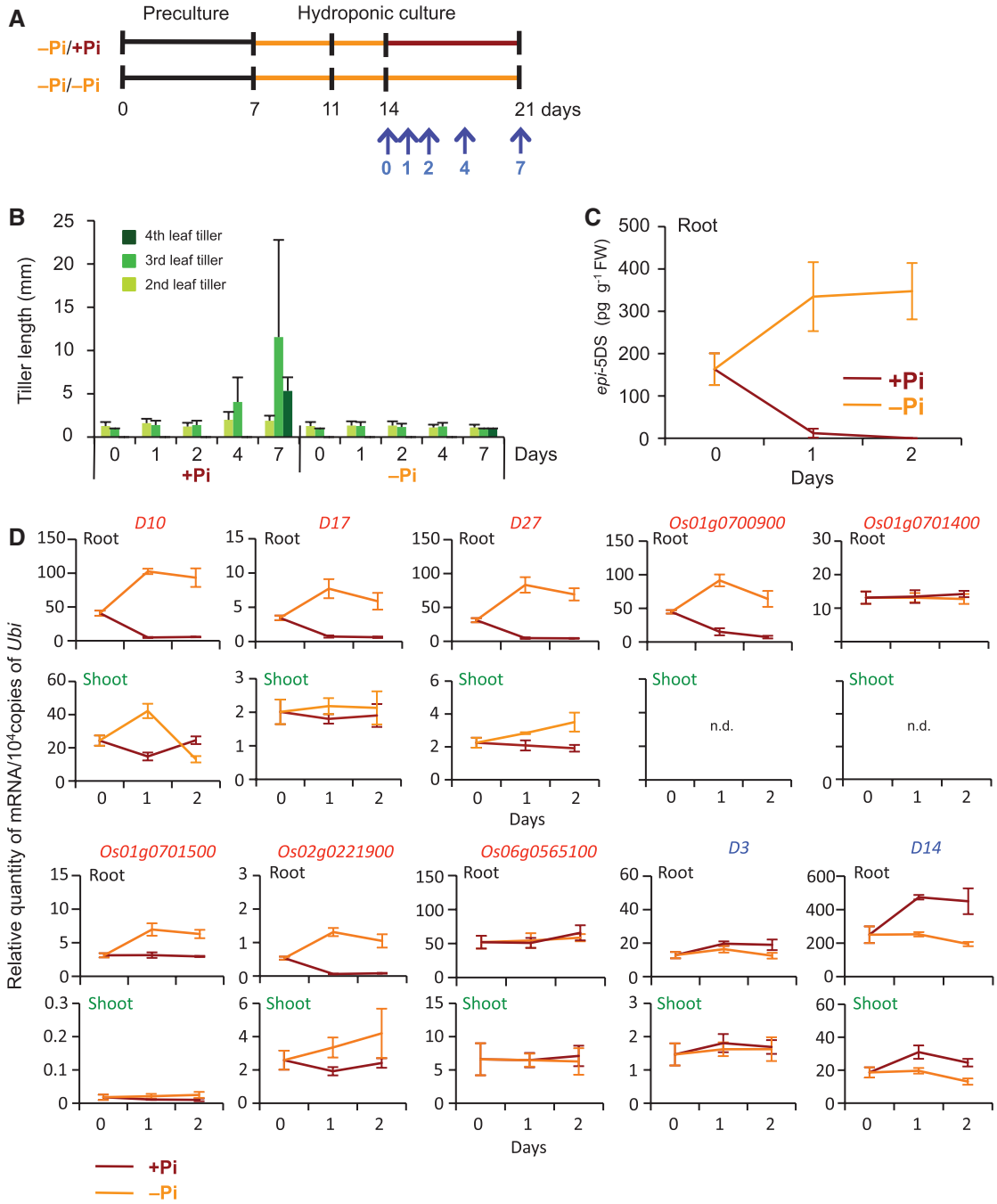

Fig. 5 Time course analysis of tiller outgrowth, SL levels and expression of SL biosynthesis genes after Pi supply. (A) Schematic diagram showing the experimental conditions. Brown and orange bars indicate +Pi and −Pi conditions, respectively. Plants were transferred to a larger vial containing fresh medium on the 11th day to minimize the consumption of other nutrients in the −Pi medium. Plants were then transferred to the +Pi or −Pi media on the 14th day. (B) Tiller length of WT seedlings under +Pi and −Pi conditions. The tiller bud on the first leaf was <1 mm in size in all samples (not shown). Data are the means ± SD ($n = 18$). (C) *epi*-5DS levels in roots. Data are the means ± SD ($n = 3$). (D) Transcript levels of SL-related genes in roots and in the basal part (1.5 cm) of shoots ('shoot') of WT seedlings. RAP IDs (http://rapdb.dna.affrc.go.jp/) are given for *MAX1* homologs. Data are the means ± SD ($n = 3$). n.d., not detected due to low abundance.

to roots (by supplementing hydroponic culture media with 1 or 10 μM GR24) was effective in inhibiting tiller bud outgrowth in WT shoots under Pi-sufficient conditions (Umehara et al. 2008), suggesting that an increased SL production in roots under Pi deficiency could contribute to shoot branching inhibition. An application of SL to roots (5 μM GR24) was also effective in inhibiting axillary bud outgrowth of hydroponically grown Arabidopsis *max3* and *max4* mutants (Umehara et al. 2008).

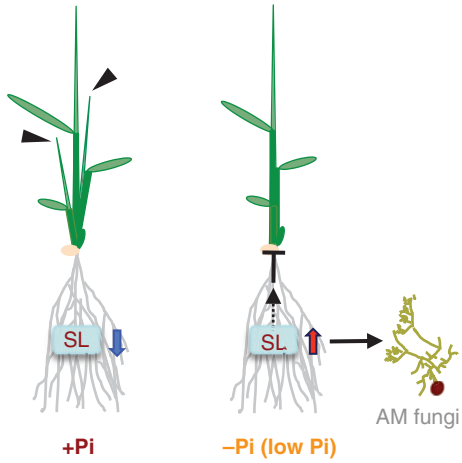

Fig. 6 A model for the role of SL in the adaptation to Pi deficiency. Left: when Pi (and other nutrients) are sufficient, SL levels are low in roots and tiller outgrowth is not inhibited. Right: under low Pi conditions, SL levels in roots are highly elevated and they contribute to the inhibition of tiller bud outgrowth in shoots. Arrowheads highlight outgrowing tillers. Thick arrows depict an increase or a decrease in SL levels. Black arrows indicate the exudation of SL to the rhizosphere and the possible upward movement of SL or SL-derived signal to shoots. The T-bar denotes the inhibitory effect on tiller bud outgrowth. Although only root-derived SL is highlighted here, a contribution of SL synthesis in the shoot to this response cannot be ruled out.

Furthermore, we show in the present study that Pi deficiency slightly increases the responsiveness to SL applied to the roots (**Fig. 3**). Altogether, these results support the idea that endogenous SL levels elevated by Pi starvation in roots contribute to the suppression of tiller bud outgrowth in rice seedlings (**Fig. 6**). However, we cannot rule out the contribution of SL biosynthesis in the shoot to this response, as discussed below.

Pi starvation has been shown to increase SL levels in root exudates (and in roots in some cases) commonly in several mycotrophic plant species (Yoneyama et al. 2007a, Yoneyama et al. 2007b, Lopez-Raez et al. 2008, Umehara et al. 2008, Yoneyama et al. 2008). However, whether any known SL biosynthesis gene contributes to the Pi-dependent change in SL levels has not been clarified. Here, we showed that the supply of Pi negatively regulates the transcript levels of multiple (putative) SL biosynthetic genes in roots, including *D10*, *D17*, *D27* and some *MAX1*-related *CYP711A* genes (**Fig. 5D**), suggesting that these genes may be responsible for the observed changes in *epi-5DS* levels. While we were unable to determine *epi*-5DS levels in WT shoot tissues due to its low abundance, our LC-MS/MS analysis showed that Pi deficiency increased *epi*-5DS content in the basal part of shoots in the *d3-1* mutant, although its induction by Pi deficiency was not as great as that in the root (**Fig. 2**, **Supplementary Fig. S1**). The elevated *epi*-5DS levels in the *d3* mutant shoot could be attributable to an increased supply of *epi*-5DS or its precursor from the root or due to an increase in its local synthesis in the shoot. Our qRT–PCR data showed that transcript levels of several (putative) SL biosynthetic genes were slightly decreased after transfer to the +P media in WT shoots, although the effect of Pi was much stronger in the root (**Fig. 5D**). These results imply that SL levels in the shoot might also be regulated by Pi. Therefore, we cannot exclude the possibility that elevated SL synthesis (though it could be a small induction) in the shoot contributed to the suppression of tiller bud outgrowth under Pi deficiency. Extensive grafting experiments using SL-deficient mutants of pea, petunia and Arabidopsis indicated that SL production in the shoot only is sufficient to inhibit excess branching (i.e. to maintain the number of branches at the non-grafted WT level) (Napoli 1996, Morris et al. 2001, Turnbull et al. 2002, Sorefan et al. 2003), although it is not known whether shoot-derived SL is also sufficient to decrease the number of branches further under certain conditions. Because of technical difficulties in grafting in rice, the effect of SL deficiency in the root only on shoot branching has not been determined.

Previous grafting experiments in dicots also indicated that a product downstream of CCD7/CCD8 enzyme action in the SL biosynthesis pathway (**Fig. 1**) can move from roots to shoots and inhibit branching in *ccd7* and *ccd8* mutant shoots (Booker et al. 2005). Thus, SL can be a potential signal that mediates the detection of nutrient availability by roots and the resulting alterations in shoot architecture. However, it has yet to be clarified whether SL itself is a mobile form in plants. In the current study, we found that *epi*-5DS levels in the basal part of shoots were much lower than those in roots. Survey of natural SLs in root exudates of various plant species revealed highly diverse structures (Yoneyama et al. 2010). It is therefore possible that *epi*-5DS is not a major SL species in the basal part of shoots in rice seedlings. To address these questions, further experiments will be necessary to explore the localization, mobility and metabolism of *epi*-5DS in rice seedlings and to determine whether the levels of any other known SL species and/or unknown SL metabolites are elevated in the shoot by Pi starvation.

In the SL-insensitive *d3* mutant, *epi*-5DS levels in roots and root exudates were much higher than those in WT controls under low Pi conditions (Pi <30 μM), but not under high Pi conditions (Pi >60 μM) (**Fig. 2D**). A similar trend was previously observed for root *epi*-5DS levels in 2-week-old seedlings (Umehara et al. 2008). These results indicate that Pi is a potent negative regulator that limits *epi*-5DS concentrations in roots, and that the lack of D3 function alone is not sufficient to increase *epi*-5DS levels drastically under Pi-rich conditions. As observed for roots, *epi*-5DS in the basal part of shoots was more abundant in the *d3* mutant than in the WT, but the difference in the shoot *epi*-5DS levels between –Pi and +Pi conditions was not as large as that observed in the root (**Supplementary Fig. S1**). These results suggest that the *d3* mutation, rather than Pi availability, may have a greater effect on *epi*-5DS levels in the shoot. This implies that the D3-dependent feedback mechanism normally limits *epi*-5DS to low levels in WT shoots.

Our temporal Pi depletion experiments showed that transfer of WT seedlings from the +Pi media to the −Pi media for 1 week (14–21 d) did not result in a decrease in tiller bud outgrowth or a drastic increase in *epi*-5DS levels, unlike the case where plants were grown in Pi-depleted media continuously for 2 weeks (**Fig. 4**). Because Pi derived from old tissues can support the growth of new and fresh tissues under Pi starvation (Rausch and Bucher 2002), it appears likely that rice seedlings grown in the absence of external Pi for 1 week did not become Pi deficient in our experimental conditions.

Despite some ambiguities discussed above, our current data, together with previous findings, support the idea that SL plays a dual role in the adaptation to Pi deficiency (**Fig. 6**). When Pi and other nutrients are sufficient, SL levels are low in roots and tiller bud outgrowth is not inhibited. In such a nutrient-rich condition, plants would be trophically ready to sustain the subsequent growth and development of new branches. Thus, it makes sense that low SL levels allow plants to initiate bud outgrowth in such conditions. In contrast, under low Pi conditions, plants would need to minimize the production of new shoot branches and save the limited phosphorus resource for existing shoots. An increased production of SL under Pi deficiency would contribute to this response. Under low Pi conditions, SL released from roots plays a role in the acquisition of limited Pi by facilitating the symbiotic interaction with AM fungi (**Fig. 6**). Together, the roles of SL as an endogenous hormone (or its precursor) and an allelochemical in the rhizosphere are likely to be related to the plant adaptive strategy for the acquisition and utilization of mineral nutrient.

Materials and Methods

Plant materials

We used a rice cultivar (*Oryza sativa* L. cv. Shiokari) as the WT and tillering dwarf mutants, *d3-1* and *d10-1*, in the Shiokari background (Ishikawa et al. 2005) in this study.

Growth conditions

Rice seedlings were grown hydroponically as described previously (Umehara et al. 2008). Surface-sterilized rice seeds were incubated in sterile water at 28°C in the dark for 2 d. The germinated seeds were transferred to hydroponic culture medium (Kamachi et al. 1991) solidified with 0.6% agar and cultured at 25°C under fluorescence white light (150–200 μmol m^{-1} s^{-1}) with a 16 h light/8 h dark photoperiod for 5 d. The 1-week-old seedlings were transferred to glass vials containing hydroponic culture media (13 ml) and grown at 25°C for 7 d. The 2-week-old seedlings were then transferred to larger vials containing fresh media (65 ml) at 25°C for another 7 d. All culture media contained 5 mM MES and were adjusted to pH 5.7.

LC-MS/MS analysis

To measure *epi*-5DS released from roots, the hydroponic culture media were collected and extracted with ethyl acetate twice after adding d_1-labeled *epi*-5DS (Umehara et al. 2008) as an internal standard. The ethyl acetate phase was evaporated to dryness under nitrogen gas and dissolved in ethyl acetate : *n*-hexane (15 : 85). The extracts were loaded onto Sep-pak Silica 1 ml cartridges (Waters), washed with ethyl acetate : *n*-hexane (15 : 85) and then eluted with ethyl acetate : *n*-hexane (35 : 65).

Measurements of *epi*-5DS levels in root samples were carried out as described previously with slight modifications (Umehara et al. 2008). The roots (1–1.5 g) were homogenized in 10 ml of acetone containing d_1-*epi*-5DS. The filtrates were evaporated to dryness under nitrogen gas, dissolved in de-ionized water, and extracted with ethyl acetate twice. The ethyl acetate phase was evaporated to dryness under nitrogen gas. The extracts were then dissolved in 20% acetone and loaded onto Oasis HLB 3 ml cartridges (Waters) and eluted with 50% acetone after washing with 20% acetone. The eluates were loaded onto Sep-pak Silica 1 ml cartridges (Waters), washed with ethyl acetate : *n*-hexane (15 : 85) and then eluted with ethyl acetate : *n*-hexane (35 : 65).

Eighteen segments (1.5 cm) of the basal part of shoots were collected and homogenized in acetone containing d_1-*epi*-5DS. The filtrates were evaporated to dryness under nitrogen gas, dissolved in de-ionized water, and adjusted to pH 2–3 using 1 N HCl. After extraction with ethyl acetate twice, the combined ethyl acetate phase was evaporated to dryness under nitrogen gas. The extracts were dissolved in 20% acetone, loaded onto Oasis MAX 3 ml cartridges (Waters) and eluted with 50% acetone after washing with 20% acetone. The eluates were loaded onto Sep-pak Silica 1 ml cartridges (Waters), washed with ethyl acetate : *n*-hexane (15 : 85) and then eluted with ethyl acetate : *n*-hexane (35 : 65).

The purified *epi*-5DS-containing fractions were dissolved in 50% acetonitrile and subjected to LC-MS/MS analysis. LC-MS/MS operation and data analysis were performed according to the method described previously (Umehara et al. 2008).

Gene expression analysis

Total RNA was extracted from roots using an RNeasy Maxi kit (Qiagen) and concentrated using an RNeasy Mini kit (Qiagen). A 2 μg aliquot of total RNA was used for cDNA synthesis using a QuantiTect Reverse Transcription kit (Qiagen). QRT–PCR was performed on an ABI PRISM 7700 sequence detection system using a QuantiTect Probe PCR kit (Qiagen), specific primers and probes listed in **Supplementary Table S3**, according to the manufacturer's instructions. Ubiquitin expression was used as an internal standard.

Supplementary data

Supplementary data are available at PCP online.

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