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Yoneyama, K, Xie, X, Nomura, T et al. (2 more authors) (2022) Supra-organismal regulation of strigolactone exudation and plant development in response to rhizospheric cues in rice. Current Biology, 32 (16). pp. 3601-3608. ISSN 0960-9822

https://doi.org/10.1016/j.cub.2022.06.047

Supra-organismal regulation of strigolactone exudation and plant development in response to rhizospheric cues in rice

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Keywords

Strigolactones, neighbour detection, root exudates, rice, plant-plant interactions

SUMMARY

Plants have evolved elaborate mechanisms to detect neighbouring plants, which typically involve the perception of 'cues' inadvertently produced by the neighbour.[1] Strigolactones are hormonal signalling molecules[2,3] that are also exuded into the rhizosphere by most flowering plant species to promote arbuscular mycorrhizal symbioses.[4] Since flowering plants have an endogenous perception system for strigolactones,[5] strigolactones are obvious candidates to act as a cue for neighbour presence, but have not been shown to act as such. To test this hypothesis in rice plants, we quantified two major strigolactones of rice plants, orobanchol and 4-deoxyorobanchol, in root exudates by using LC-MS/MS (MRM), and examined feedback-regulation of strigolactone biosynthesis and changes in shoot branching phenotypes in rice plants grown at different densities in hydroponics and soil culture. We show that the presence of neighbouring plants, or greater root volume, results in rapidly-induced changes in strigolactone biosynthesis, sensitivity, and exudation, and the subsequent longer-term changes in shoot architecture. These changes require intact strigolactone biosynthesis in neighbouring plants and intact strigolactone signalling in focal plants. These results suggest that strigolactone biosynthesis and exudation in rice plants are driven by supra-organismal environmental strigolactone levels. Strigolactones thus act as a cue for neighbour presence in rice plants, but also seem to act as a more general root density sensing mechanism in flowering plants, that integrates soil volume and neighbour density, and allows plants to adapt to the limitations of the rhizosphere.

RESULTS

Crowding induces decreased strigolactone biosynthesis and exudation

To understand the effects of plant density-sensing on strigolactone biology, we used a hydroponic system to grow rice plants (*Oryza sativa* cv. Nipponbare) under phosphate (P) deficiency. Many soils are P-deficient, and even in fertile soils phosphorus concentration is lower than 10 µM.[6] In addition, ca. 80% of applied phosphorus may be fixed in the soil through the interaction with other ions and unavailable to plants.[7] For example, when rice, maize, pea, and tomato plants were grown under 'normal' nutrient conditions (160 µM P in our test) in hydroponics, strigolactone levels in the growth media were quite low and often below detection limits.[8,9] However, we could detect and quantify strigolactones from plants grown under 'normal' nutrient conditions in soils, indicating that the plants were subjected to some level of P deficiency in particular in the rhizosphere where P can be rapidly depleted by plant root uptake.[10] Therefore, using P-depletion in our hydroponic work is likely more relevant to 'normal' soil conditions, than a 'normal' hydroponic condition, at least in terms of strigolactone exudation. After a 4-day period of P-depletion, 16-day old seedlings were placed into either a 1-, 2- or 3-plant culture in the same volume of hydroponate (180 mL). After 72 h, we measured levels of 4-deoxyorobanchol (4DO) and orobanchol (OB) in the hydroponate. Remarkably,

we found that levels of both 4DO and OB were essentially identical irrespective of the number of plants in the system; thus per plant exudation in a 2-plant culture was half that of a 1-plant culture, while exudation in a 3-plant culture was a third of that level (Figure 1A), indicating that the net strigolactone exudation in 1, 2- and 3-plant culture is constant (Figure S1A). As root growth biomass was not yet affected by density (Figure S1C, D), these results strongly suggest that plants can detect other plants and homeostatically adjust their strigolactone exudation before physiological responses including root growth become apparent. We measured levels of 4DO in the roots of the plants, and found no corresponding decrease in 4DO in the roots; if anything, there is a slight but non-significant increase (Figure 1B, Figure S1B). OB levels in root tissues were below the detection limit of our set-up. We examined expression of strigolactone biosynthesis genes in the roots by qRT-PCR, and found that expression of *D27*, *D17*, *D10* and *Os01g0701400* was reduced by 50–70% in plants grown in 3-plant culture compared to those in 1-plant culture (Figure 1C, D). However, expression of *Os01g070900* involving the conversion of carlactone to 4DO via carlactonoic acid (Figure 1D) was not significantly changed (Figure 1C). The decreased expression in strigolactone biosynthetic genes was accompanied by 3-fold increased expression of the *D14* SL receptor in plants grown in 3-plant culture, though there was no increased expression of the *D3* F-box or the *D53* target proteins (Figure 1C, D). Thus, our results indicate that short exposure to neighbouring plants triggers a density-dependent reduction in strigolactone biosynthesis and exudation, with a balancing increase in strigolactone sensitivity.

To assess how quickly plants respond to neighbouring plants, we repeated the experiment with a 7-day P-depletion period prior to transfer to either 1- or 3-plant cultures, and then sampled strigolactone levels every 24 h after transfer (Figure S1E, F). We found that per-plant exudation of 4DO was already significantly decreased by 24 h, attaining the characteristic 3-fold reduction by 48 h (Figure S1E), while OB levels were not reduced at 24 h, and did not attain the 3-fold reduction until 72 h after transfer (Figure S1F). Thus, changes in strigolactone biosynthesis and exudation begin almost immediately after exposure to neighbouring plants, but take some time to equilibrate, and there are some differences in timescale on which this occurs for different strigolactone species.

Our results raise the interesting question of whether changes in strigolactone exudation occur in response to the presence of neighbouring plants *per se* or in response to the overall density of roots (including their own) in the environment. To distinguish between these possibilities, we grew rice plants in our previously described hydroponic system, and after 7-day P-depletion transferred them to 1-plant cultures in either 180 mL or 360 mL hydroponic vessels. We observed that 4DO levels after 24 h were 2-fold higher in the 2-fold larger vessel (Figure S2A), strongly suggesting that plants exude strigolactone until a particular concentration in the growth media is reached. Therefore, plants appear to modulate strigolactone exudation in response to the density of roots, rather than presence of neighbours *per se*. Consistent with our previous results, 4DO levels in the roots of the plants were

not altered (Figure S2B), and the increase in strigolactone exudation in the 360 mL vessel was accompanied by a comparable increase in expression of core strigolactone biosynthesis genes (Figure S2C).

Density-dependent changes in strigolactone exudation require strigolactone perception

Given that these strigolactone biosynthesis responses can be triggered in the absence of neighbouring plants, an intriguing possibility is that changes in strigolactone exudation associated with changes in root density are actually driven by the concentration of strigolactone in the environment, and not by a separate cue. To test this possibility, we tested the ability of *d14* strigolactone receptor mutants to respond to changes in plant density using our hydroponic system. We found that, unlike in wild-type plants, there was no statistically significant reduction in per-plant 4DO or OB exudation in either 2- or 3-plant cultures relative to 1-plant cultures (Figure 2A). Thus, total strigolactone levels in the system were much higher in this experiment in the 2- and 3-plant cultures. Consistent with this, we observed a small increase in the levels of 4DO present in the root tissues of plants grown in 2- or 3-plants cultures (Figure 2B). In accordance with the failure to downregulate strigolactone exudation, we observed no reduction in the expression of core strigolactone biosynthesis genes in *d14* mutants (Figure 2C).

Strigolactone biosynthesis and exudation is homeostatically regulated in response to environmental strigolactone

To test whether environmental strigolactones, including those produced by other plants, drive changes in strigolactone biosynthesis and exudation, we utilized combinatorial experiments in which we grew wild-type plants together with *d17* (CCD7) strigolactone biosynthesis mutants in 2-plant cultures (Figure 3A). Consistent with previous results, wild-type plants grown in 2-plant culture with other wild-type plants reduced their exudation of 4DO by 2-fold, 24 h after transfer (Figure 3B). However, wild-type plants grown in 2-plant culture with *d17* mutants did not significantly reduce their exudation of 4DO in the same time frame. This indicates that the lack of strigolactone exudation from *d17* mutants leads to a failure to down-regulate 4DO exudation in the wild-type plants, despite the increase in root density. We also utilized this set-up to demonstrate that plants can indeed take-up and perceive environmental strigolactone. As anticipated, we found no 4DO present in the roots of *d17* plants grown in 1-plant culture; however, 4DO was detected in the roots of *d17* plants co-cultured with wild-type plants (Figure 3C, Figure S3). 4DO levels in roots were not different between WT plants grown in 1-plant culture and WT plants co-cultured with *d17* mutants (Figure 3C). In accordance with these results, expression of strigolactone biosynthesis gene *D27* was higher in WT plants co-cultured with *d17* mutants than in WT plants co-cultured with other WT plants. By contrast, expression of *D27* gene was downregulated in WT plants co-cultured with *d14* mutants (Figure S4C). In general, rice *d14* mutants produce and exude more SLs than WT plants as they lack feedback

inhibition[11-13] (Figure S4A, B), and therefore it seems that strigolactones exuded by *d14* mutants negatively affect strigolactone biosynthesis in WT plants. Collectively these results show that rice plants can detect and respond to environmental strigolactone by changes in strigolactone biosynthesis and exudation, and that *d17* mutants are unable to trigger these changes in other plants because they cannot exude strigolactones. Furthermore, the expression of some genes especially *D3* and *D14* upregulated in *d17* mutants growing together with WT plants, though there were no statistically significant differences (Figure S4D), suggested that the shoot branching of SL biosynthesis mutants would be suppressed by co-culture with neighbouring plants exude strigolactones.

Crowding in soil also reduces strigolactone exudation and affects the shoot branching in WT plants

Finally, we wanted to assess whether the detection of environmental strigolactone influenced the growth and development of plants, and also their relevance for soil-grown plants. The results were consistent with those observed in the hydroponic system, with a slight but not statistically significant reduction in per-plant exudation in the 3-plant culture of both 4DO and OB in WT plants (Shiokari background, which in general exude more strigolactones than the Nipponbare background WT plants), but not in *d14* and *d 53* mutants (Figure 4A). Furthermore, we observed that shoot branching levels in 3-plant cultures tended to reduce compared to 1-plant cultures in WT plants, but not in strigolactone biosynthesis or perception mutants (Figure 4B). These differences in wild-type were not statistically significant due to low sample size, but in a companion manuscript, we show statistically significant differences in pea plant growth using a similar experimental design.[14] To confirm that these changes in shoot branching are mediated by the perception of strigolactone exuded by neighbouring plants, we grew combinations of *d10* (SL deficient) and *d14* (SL insensitive) plants together with *d17* or WT plants in soil and determined the number of shoot branches (Figure 4C). We found that shoot branching of *d10* mutants was suppressed when they were grown together with WT plants, but not *d17* plants; conversely, *d14* showed no suppression when grown either WT or *d17* plants. Our data thus show that detection of environmental strigolactone can modulate plant development.

It is intriguing to notice that the rice plants in 3-plant soil culture exhibit less shoot branching as compared to that in 1-plant soil culture (Figure 4B) despite producing less SLs, 4DO and OB (Figure 4A). These results are consistent with recent findings that canonical and non-canonical strigolactones are not interchangeable and perform different roles. For example, Ito et al. demonstrated that shoot branching phenotype of *Os900* knockout rice mutant which produces neither 4DO nor OB is the same to WT plants, suggesting 4DO and OB are not endogenous shoot branching inhibitors.[15] Mashiguchi et al. also show that these canonical strigolactones are not shoot branching inhibitors in rice plants.[16] Other reports suggest that non-canonical strigolactones are true hormones though their identities have not yet been clarified.[17,18]

DISCUSSION

Plant-plant interactions are non-transient in nature; plants cannot choose or change their neighbours, and as such may end up in extraordinarily long associations with members of other species.[19] Territorial conflicts between plants are not easily 'winnable', and although neighbouring plants may directly compete with each other for resources, these interactions may often be mutually detrimental to both parties.[20] The mechanisms by which plants detect each other typically rely on the presence of 'cues'; information inadvertently revealed by neighbouring plants. The most obvious example of this is the change in the abundance of red:far red wavelengths in light reflected by plants, which permits their detection by neighbouring plants.[21] The reflected light can trigger a range of growth responses in neighbouring plants, most notably the shade avoidance response.[22] Plants can also respond to the touch of neighbouring plants,[23] and to a range of chemicals present in the atmosphere (volatiles) and rhizosphere (exudates), which their neighbours release for other, functional reasons.[24,25]

Where tested, all groups of land plants (though not necessarily every species) exude strigolactones into the substrate.[26] Since exuded strigolactones promote the formation of associations with arbuscular mycorrhizal fungi (AMF), it is likely that this is their ancestral function.[27] Mosses do not form associations with AMF, but still exude strigolactones.[28] This allows neighbouring colonies to detect each other, and to avoid competition for space.[28] Thus, in mosses, strigolactones may act as active signals to neighbouring plants, in the absence of any other known functional role. In flowering plants, most species exude strigolactones to promote associations with AMF, and thus strigolactones could also act cues for the presence of neighbouring plants. In this study, we aimed to test the hypothesis that flowering plants use 'environmental' strigolactones as a cue for the presence of neighbouring plants. Collectively, our data suggest that plants can take up environmental strigolactones produced by other plants, and use this to regulate their own physiology and development as a homeostatic response to the environment. Strigolactone biosynthesis in the root system is under strong homeostatic control by strigolactone signalling,[3,4,11-13] and therefore offers a simple read-out of a plant's response to strigolactones. Our data thus show that strigolactones are a key molecule in neighbour detection and plant-to-plant communication in flowering plants, echoing previous work in the moss *Physcomitrella patens.*[28] This suggests that in flowering plants, strigolactones act as a 'cue' for neighbour detection; a functional molecule that inadvertently reveals the presence of a plant to its neighbours. In this context, the homeostatic downregulation of strigolactone biosynthesis in response to environmental strigolactones makes much sense. If the concentration of rhizospheric strigolactone is already high, this provides that plants with information that neighbouring plants are present, and increasing strigolactone exudation is unlikely to result in the recruitment more mycorrhizal partners, nor the capture of a higher

proportion of soil resources. Thus, individual plants can reduce their 'expenditure' in line with their likely reduced 'profit' from strigolactone exudation.

Our data also suggest a further possibility – that in addition their role in mycorrhizal signaling, strigolactones are also exuded for adaptive purposes relating to the sensing of soil volume. Plants show strong, pro-active responses to limitations in soil, and recent work suggests that plants measure the concentration of at least two different chemicals in the soil as a proxy for both available volume and neighbour density.[29] Our data show that plants can detect both their root volume and neighbour density, and precisely adjust their strigolactone biosynthesis exudation in response. Given our other data, the most obvious explanation is that they detect root density through strigolactone concentration: in a smaller volume strigolactone concentration rises more quickly, leading to earlier downregulation of exudation. Again, this simple mechanism would prevent 'over-expenditure' by the plant relative to the potential nutrient 'profit' that can be obtained from a smaller root volume. This suggests that strigolactone exudation may be doubly-functional, acting in both mycorrhizal recruitment and in root density sensing. In the context of root density sensing, it is likely that plants will derive benefit from detecting and responding to root density, *irrespective* of whose roots they are. In this sense, strigolactones are neither exactly a cue nor a signal, but rather a 'universal density signal'. In essence, all plants will benefit from both exuding strigolactones and to responding to environmental strigolactones – whether it is a plant detecting its own roots, or its neighbours' roots, or advertising its roots to its neighbours – because environmental strigolactones levels provide important information about overall root density in the environment. Strigolactones are chemically unstable and rapidly hydrolyzed especially in alkaline solutions. However, strigolactones have longer half-lives in the slightly acidic rhizosphere than in bulk soils, certainly long enough to exhibit their biological activities in the context of plant-plant interactions,[18] and as is indeed already demonstrated by the role of strigolactones in communication between plants and root parasitic weeds and AM fungi. Our work thus suggests that strigolactone exudation is an important tool for plants to adapt to the limitations of their soil environment, and to avoid unwinnable competitions in a crowded world.

ACKNOWLEDGEMENTS

We would like to appreciate Profs. Jyunko Kyozuka and Tadao Asami for kindly providing the rice *dwarf* mutants, and d_6-4DO, respectively. We also would like to thank Noriko Haruna and Mika Kaneta for technical assistance. KaY is supported by the JST Agency PRESTO (JPMJPR17QA) and the Japan Society for the promotion of Sciences (KAKENHI 19K05757 and 19KK0395). TB is supported by BBSRC (BB/R00398X/1).

AUTHOR CONTRIBUTIONS

KaY, XX, and TN designed the study. KaY planned and carried out experiments and analyzed data. KaY, KoY and TB wrote the manuscript.

DECARATION OF INTERESTS

The authors declare they have no competing interests.

MAIN FIGURE TITLES AND LEGENDS

Figure 1. Crowding alters strigolactone biosynthesis and exudation

(A) Quantification of 4-deoxyorobanchol (4DO) and orobanchol (OB) present in the hydroponate in 1-, 2- or 3-plant cultures 72 h after transfer from 4-day P-depletion, expressed as pg/plant. Graph shows all individual data points. n = 7–9 biologically independent samples. The different letters denote a statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$). See also Figure S1 and S2A.

(B) Quantification of 4DO present in the roots of plants in 1-, 2- or 3-plant cultures 72 h after transfer from 4-day P-starvation, expressed as pg/g fresh weight. Graph shows all individual data points. n = 3–9 biologically independent samples. The same letter denotes no statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$). See also Figure S1 and S2B.

(C) Quantification of strigolactone-related gene expression in the roots of plants in 1-, 2- or 3-plant cultures 72 h after transfer from 4-day P-depletion. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. n = 3 biologically independent samples. Expression levels for each gene normalized to 1 plant culture (=1). Asterisks indicate a statistically significant difference relative to 1-plant culture at the same time point (Student's t-test, $p < 0.05$). See also Figure 2C.

(D) Diagram showing the biosynthesis and signaling pathways for strigolactone.

Figure 2. Strigolactone perception is needed for altered exudation responses

(A) Quantification of 4-deoxyorobanchol (4DO) and orobanchol (OB) present in the hydroponate in 1-, 2- or 3-plant cultures of *d14* mutants 72 h after transfer from 4-day P-depletion, expressed as pg/plant. Graph shows all individual data points. n = 3 biologically independent samples. The same letter denotes no statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$).

(B) Quantification of 4-deoxyorobanchol (4DO) present in the roots of plants in 1-, 2- or 3-plant cultures of *d14* mutants 72 h after transfer from 4-day P-starvation, expressed as pg/g fresh weight. Graph shows all individual data points. n = 3 biologically independent samples. The same letter denotes no statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$).

(C) Quantification of strigolactone-related gene expression in the roots of plants in 1-, 2- or 3-plant cultures of *d14* mutants 72 h after transfer from 4-day P-starvation. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. n = 3 biologically independent samples. Expression levels for each gene normalized to 1 plant culture (=1). No asterisk indicates no statistically significant difference relative to 1-plant culture at the same time point (Student's t-test, $p < 0.05$).

Figure 3. Strigolactone exudation is homeostatically regulated by environmental strigolactones

(A) Cartoon showing experimental set-up.

(B) Quantification of 4-deoxyorobanchol (4DO) and orobanchol (OB) present in the hydroponate in 1-plant cultures of WT rice, or 2-plant cultures of WT + WT or WT + *d17* mutants 24 h after transfer from 7-day P-depletion, expressed as pg per WT plant. Graph shows all individual data points. n = 3 biologically independent samples. The different letters denote a statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$). See also Figure S3.

(C) Quantification of 4DO present in the roots of WT or *d17* mutants grown either alone in 1-plant cultures, or in 2-plant cultures of WT + *d17*, 24 h after transfer from 7-day P-depletion, expressed as pg/g fresh weight. Graph shows all individual data points. n = 3 biologically independent samples. The different letters denote a statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$). See also Figure S4.

Figure 4. Crowding in soil also reduces strigolactone exudation and inhibits shoot branching

(A) Quantification of 4-deoxyorobanchol (4DO) and orobanchol (OB) present in the soil of 1- or 3-plant cultures after 10 days of P-depletion, expressed as pg/plant (4DO) or MS-peak area/plant (OB). No asterisk indicates no statistically significant difference relative to 1-plant culture at the same time point (Student's *t*-test, $p < 0.05$).

(B, C) Determination of shoot branching of plants grown in soil after transferred to each condition for 10 days. The error bars indicate the SEM (n = 3–8 biologically independent samples). No asterisk indicates no statistically significant difference, and an asterisk indicates a statistically significant difference (Student's *t*-test, $p < 0.05$).

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kaori Yoneyama (yoneyama.kaori.wx@ehime-u.ac.jp).

Material availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.

● Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rice seeds (*Oryza sativa* cv. Nipponbare) were obtained from a local supplier. Seeds of rice *dwarf* mutants (*d10-2, d14-2, d17-2*) in the cv Nipponbare background, *O. sativa* cv. Shiokari, Norin 8, and *d53* in the cv Norin 8 background were provided from Prof. Junko Kyozuka (Tohoku University), as detailed in the Key resource table. Plants were grown in controlled growth conditions, as described in Method details.

METHOD DETAILS

Hydroponic experiment

Rice seeds were surface-sterilized in 70% ethanol for 3 min and thoroughly rinsed with sterile distilled water. Then, the seeds were germinated in sterile distilled water for 2 days at 25–28°C in the dark. Germinated seeds were sown in a strainer (28 x 23 x 9 cm, W x L x H) lined with a sheet of gauze moistened by placing it on a slightly larger container (28.5 x 23.5 x 11 cm, W x L x H) containing tap water for 7 days, 1/2 Tadano-Tanaka (TT) media[30] (1.43 mM NH_4NO_3, 1 mM $NaNO_3$, 0.16 mM NaH_2PO_4, 1 mM K_2SO_4, 1 mM $CaCl_2$, 1mM $MgSO_4$, 20 mM $MnSO_4$, 50 mM H_3BO_3, 3 mM $ZnSO_4$, 0.2 mM $CuSO_4$, 0.05 mM $(NH_4)_6/Mo_7O_{24}$, 40 mM $FeSO_4$) for 7 days, and phosphate free 1/2 TT media for 10 days in a plant growth shelf (NK system, #LS-1520PFD-4-10L) under 16-h light (28°C, approximately 240 μmol m^{-2} s^{-1})/8-h dark (25°C) regime. One μM 2-(*N*-morpholino)ethanesulfonate (MES) was used as a buffer in the hydroponic media and pH of media was set to 6.0. Media was disposed of and replaced every 2–3 days. For test experiments, the equal sized plants were selected and transferred into plastic cups (9.5 cm in diameter, 17 cm deep, ca. 200 ml in volume or 19 cm in diameter, 17 cm deep, ca. 400 ml in volume) containing 180 or 360 mL of distilled water to remove the effects of nutrients depending on plant number or volume of media. To float plants, disk shaped styrofoam (1.8 cm thick) having holes were put on the cup and plants were set in the hole with sponge belt (1cm cube).[31]

Soil experiment

To examine shoot branching phenotype and SL exudation from plants grown in soils, rice plants were first grown hydroponically as in the hydroponic experiment. After growing in 1/2 TT media for 8 days, seedlings were transferred to each condition (1 seedling /pot or 3 seedlings/pot, co-culture with WT or *d* mutant) in pots (i.d. 10 cm, 10 cm deep) filled with vermiculite (300 g) and watered twice a week with 150 mL/pot of a mixture of 1/2 TT media lacking phosphate and distilled water for 10 days. To provide each rice seedling with similar amounts of available nutrients per plant, mixtures

of 1/2 TT and distilled water were applied at 50 mL and 100 mL, 100 mL and 50 mL, and 150 mL and 0 mL to pots containing 1, 2, and 3 seedlings, respectively. Plants were grown under the same environmental conditions of hydroponics. The number of shoot branching was counted as one which the tiller was over 1 cm.

Strigolactone extraction

The root exudates released into hydroponic media (ca. 100 mL) were collected, 500 pg of d_6-4DO dissolved in acetonitrile was added as an internal standard, and then immediately extracted with 100 mL of ethyl acetate by using separatory funnel (200 mL). In the case of soil culture, 200 mL of tap water was poured onto the soil surface evenly and root exudates (ca. 200 mL) eluted from the holes of pot bottom were collected, which were then extracted with 200 mL of ethyl acetate. The upper phase (ethyl acetate solution) was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. For extracting strigolactones from root tissues, the harvested fresh root tissues (ca. 1 g) were soaked in ethyl acetate in the dark at 4°C for 2 days. After removal of plant residues by filtration, ethyl acetate extract was washed with 0.2 M K_2HOPO_4 by using separatory funnel, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. These samples were transferred to vials and kept at 4°C until analysis.[31]

Strigolactone analysis

Rice (*O. sativa* cv Nipponbare) plants were shown to produce and exude mainly orobanchol and 4-deoxyorobanchol among known strigolactones,[32] thus we analyzed these two strigolactones by using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). For this, the Acquity UPLC System (Waters) coupled to a Xevo TQD triple-quadrupole mass spectrometer (Waters MS Technologies) with electrospray (ESI) interface was used. Chromatographic separation was achieved using an ODS column (ACQUITY UPLC, BEH C_{18}, 2.1 × 100 mm, 1.7 µm; Waters) with a water-methanol gradient containing 4% 50 mM ammonium acetate to promote ionization. Separation started at 35% methanol, followed by a 2 min gradient to 55% methanol, followed by a 13 min gradient to 95%, kept 96% methanol for 2 min to wash column and then back to 35% methanol for 3 min. The column was equilibrated at this solvent composition for 5 min before the next run. Total runs time was 25 min. The column oven temperature was maintained at 40°C with a flow-rate of 0.2 mL min^{-1} (sample injection volume of 1 µL).

For mass spectrometry, the nebulizer and desolvation gas flows were 50 L h^{-1} and 1200 L h^{-1}, respectively. The capillary voltage was set at 0.5 kV, the source temperature at 120°C and the desolvation has temperature at 550°C. Fragmentation was performed by collision induced dissociation with argon at 3~5 x 10^{-3} mbar. The collision energy was optimized for each compound. Multiple reaction monitoring (MRM) was used to detect each strigolactone. MRM transitions for orobanchol eluting 4.8 min were monitored for *m/z* 347/97 at a collision energy (CE) of 22 V and *m/z* 347/233 at CE of 10 V with a cone voltage of 30 V. The MRM transitions of *m/z* 331/97 at a CE

of 20 V and *m/z* 331/217 at 22 V with a cone voltage of 25 V were used for the detection of 4DO eluting at 8.0 min. The MRM transition of *m/z* 353/97 at a CE of 20 V with a cone voltage of 25 V was used for d_6-4DO eluting at 7.9 min. Data acquisition and analysis were performed using MASSLYNX 4.1 software (Waters).

Gene expression analyses

Total RNA was extracted from the roots (>100 mg) of RNeay Plant Mini Kit (QIAGEN) according to manufacturer's instructions and quantified with a spectrophotometer Nano Drop One C (Thermo Fisher Scientific, #ND-ONEC-W). One μg of total RNA was used to synthesize the cDNA with the PrimeScript RT Reagent Kit with gDNA eraser (Takara Bio, Japan). Real-time PCR was performed by ΔΔCT method on a StepOnePlus real-time PCR system (Thermo Fisher Scientific, #StepOnePlus-01) with THUNDERBIRD SYBR qPCR kit (Toyobo, Japan). The PCR program was as following: an initial DNA denaturation at 95°C for 20 s; 40 cycles including a denaturation step at 95°C for 3 s, an annealing step at 60°C for 30 s and an extension step at 95°C for 15 s; and a melting curve from 60°C to 95°C. *OsTFIIE* was selected as an internal reference gene in this study, based on its use in previous studies.[32] The specific primers used for qRT-PCR are listed in Supplementary Table 1.

QUANTIFICATION AND STATICTICAL ANALYSIS

Initial data was stored and organized in Microsoft Excel, and then subjected to ANOVA utilizing JMP software, version 5.0 (SAS Institute INC., Cary, NC, USA).

SUPPLEMENTAL ITEM TITLES

Table S1. List of primer for real-time PCR, related to STAR Methods.

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Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Oryza sativa (japonica, c. v. Nipponbare)	Nouken, Japan	N/A
Chemicals, peptides, and recombinant proteins		
2-(*N*-morpholino)ethanesulfonate (MES)	TCI	4432-31-9
NH_4NO_3	FUJIFILM Wako	017-03235
$NaNO_3$	FUJIFILM Wako	195-02545
$NaH_2PO_4/2H_2O$	FUJIFILM Wako	192-02815
K_2SO_4	FUJIFILM Wako	169-04485
$CaCl_2/2H_2O$	FUJIFILM Wako	033-25035
$MgSO_4/7H_2O$	FUJIFILM Wako	131-00405
$MnSO_4/5H_2O$	FUJIFILM Wako	130-13182
H_3BO_3	FUJIFILM Wako	021-02195
$ZnSO_4/7H_2O$	FUJIFILM Wako	264-00402
$CuSO_4/5H_2O$	FUJIFILM Wako	039-04412
$(NH_4)_6/Mo_7O_{24}/4H_2O$	FUJIFILM Wako	019-03212
$FeSO_4/7H_2O$	FUJIFILM Wako	094-01082
Ethyl acetate	FUJIFILM Wako	056-00367
Anhydrous $MgSO_4$	FUJIFILM Wako	132-00435
Methanol	FUJIFILM Wako	134-14523
Ammonium acetate	Millipore Sigma	73594-25G-F
d_6-4-deoxyorobanchol	Provided from Prof. Tadao Asami (Tokyo Univ., Japan)[33]	N/A
Critical commercial assays		
RNAeasy Plant Mini Kit	QIAGEN	# 74904
PrimeScript RT Reagent Kit with gDNA eraser	Takara Bio	# RR047B
THUNDERBIRD SYBR qPCR kit	Toyobo	# QPS-201
Experimental models: Organisms/strains		
Oryza sativa: *dwarf 10-2*	[34]	N/A
Oryza sativa: *dwarf 14-2*	[35]	N/A
Oryza sativa: *dwarf 17-2*	[36]	N/A
Oryza sativa: Shiokari	Rice Genome Resource Center (RGRC), Tsukuba, Japan	N/A
Oryza sativa: Norin 8	Rice Genome Resource Center (RGRC), Tsukuba, Japan	N/A
Oryza sativa: *dwarf 53*	[37]	N/A
Oligonucleotides		
Real-time PCR primer sets	See Table S1	

Figure 1

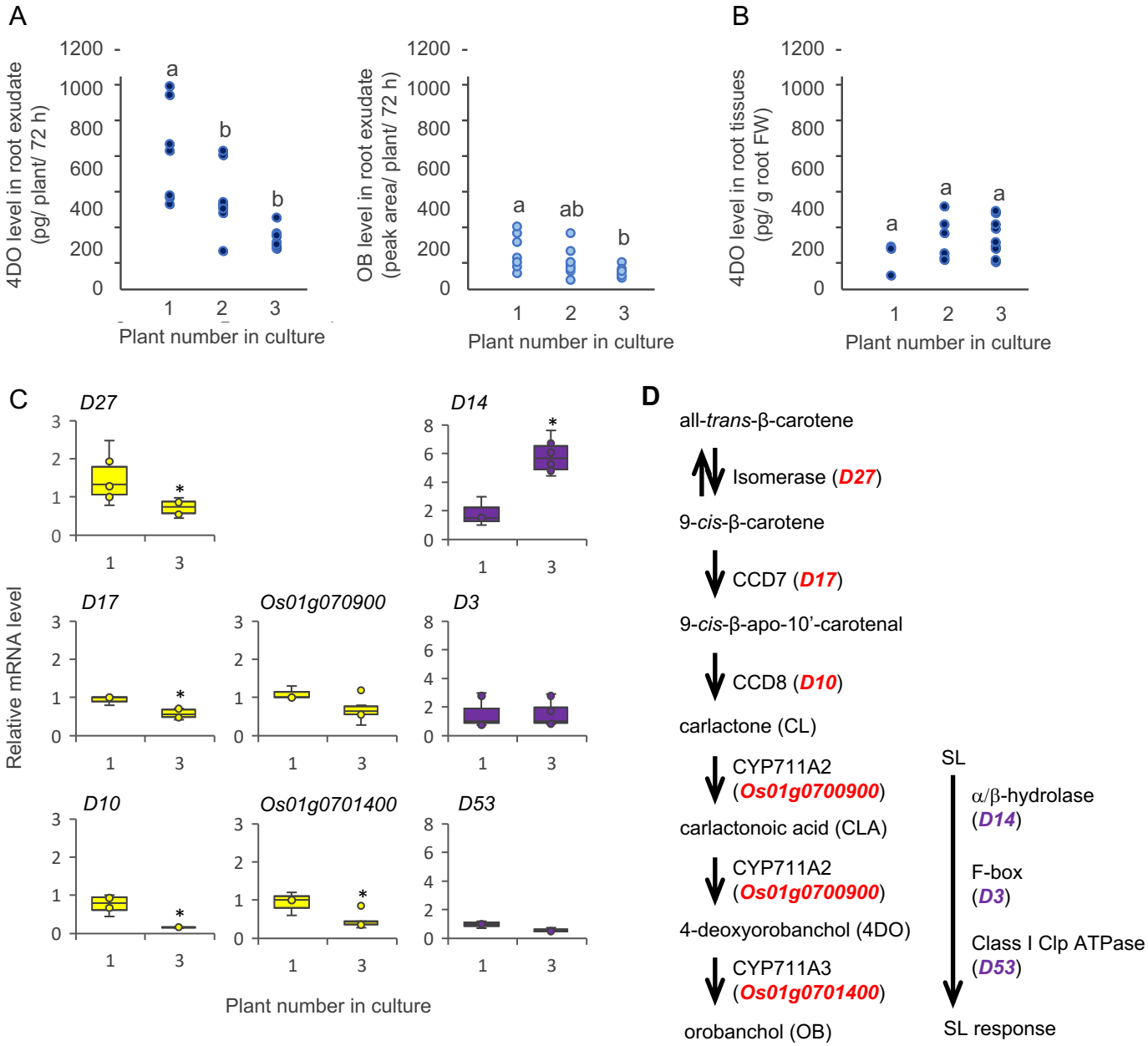

A
4DO level in root exudate (pg/ plant/ 72 h)
OB level in root exudate (peak area/ plant/ 72 h)
Plant number in culture
B
4DO level in root tissues (pg/ g root FW)
Plant number in culture
C
D27
D14
D17
Os01g070900
D3
D10
Os01g0701400
D53
Relative mRNA level
Plant number in culture
D
all-trans-β-carotene
Isomerase (D27)
9-cis-β-carotene
CCD7 (D17)
9-cis-β-apo-10'-carotenal
CCD8 (D10)
carlactone (CL)
CYP711A2 (Os01g0700900)
carlactonoic acid (CLA)
CYP711A2 (Os01g0700900)
4-deoxyorobanchol (4DO)
CYP711A3 (Os01g0701400)
orobanchol (OB)
SL
α/β-hydrolase (D14)
F-box (D3)
Class I Clp ATPase (D53)
SL response

Figure 2

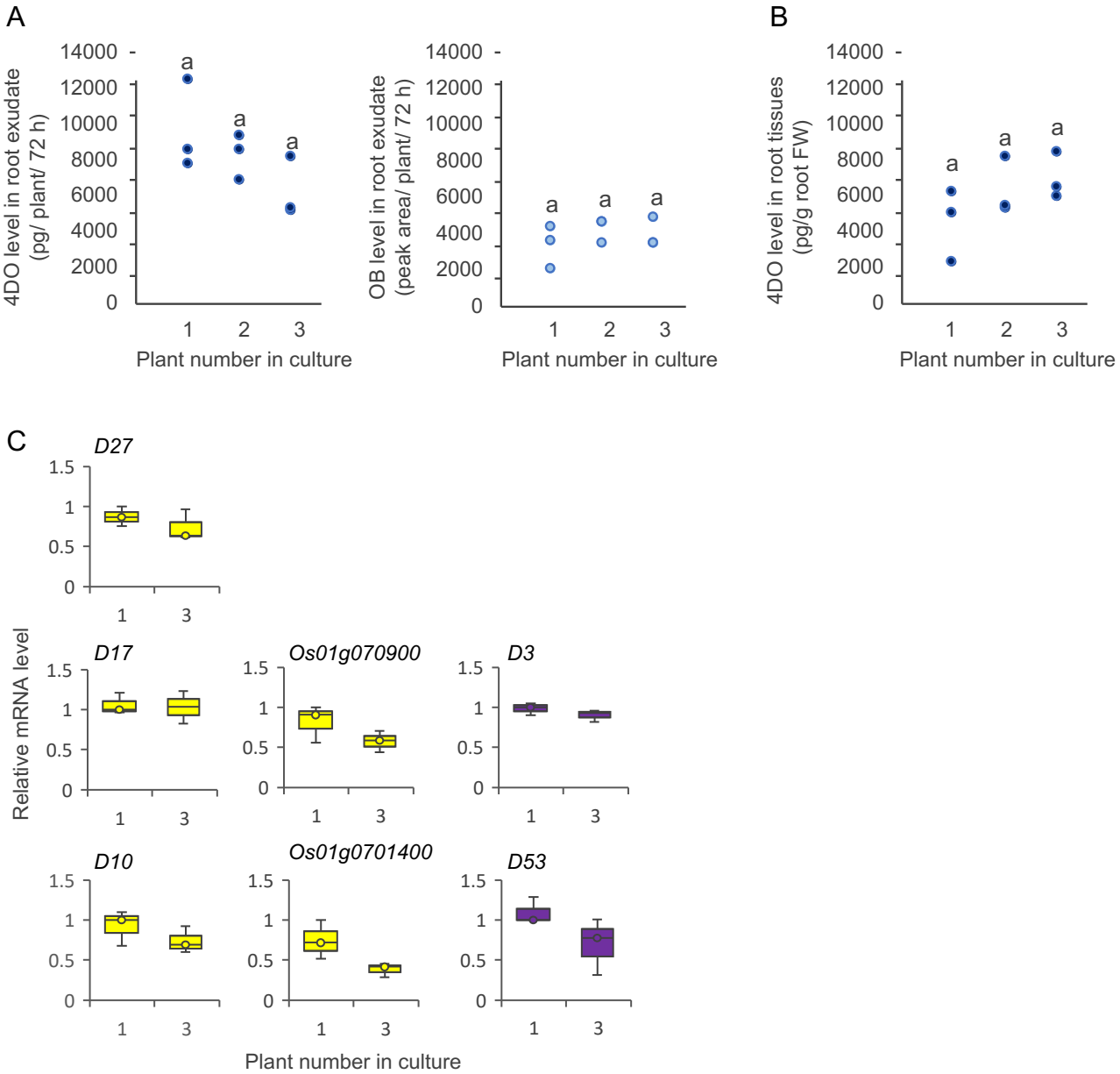

A
4DO level in root exudate (pg/ plant/ 72 h)
14000
12000
10000
8000
6000
4000
2000
0
a
a
a
1
2
3
Plant number in culture
OB level in root exudate (peak area/ plant/ 72 h)
14000
12000
10000
8000
6000
4000
2000
0
a
a
a
1
2
3
Plant number in culture
B
4DO level in root tissues (pg/g root FW)
14000
12000
10000
8000
6000
4000
2000
0
a
a
a
1
2
3
Plant number in culture
C
Relative mRNA level
D27
D17
Os01g070900
D3
D10
Os01g0701400
D53
1.5
1
0.5
0
1
3
Plant number in culture

Figure 3

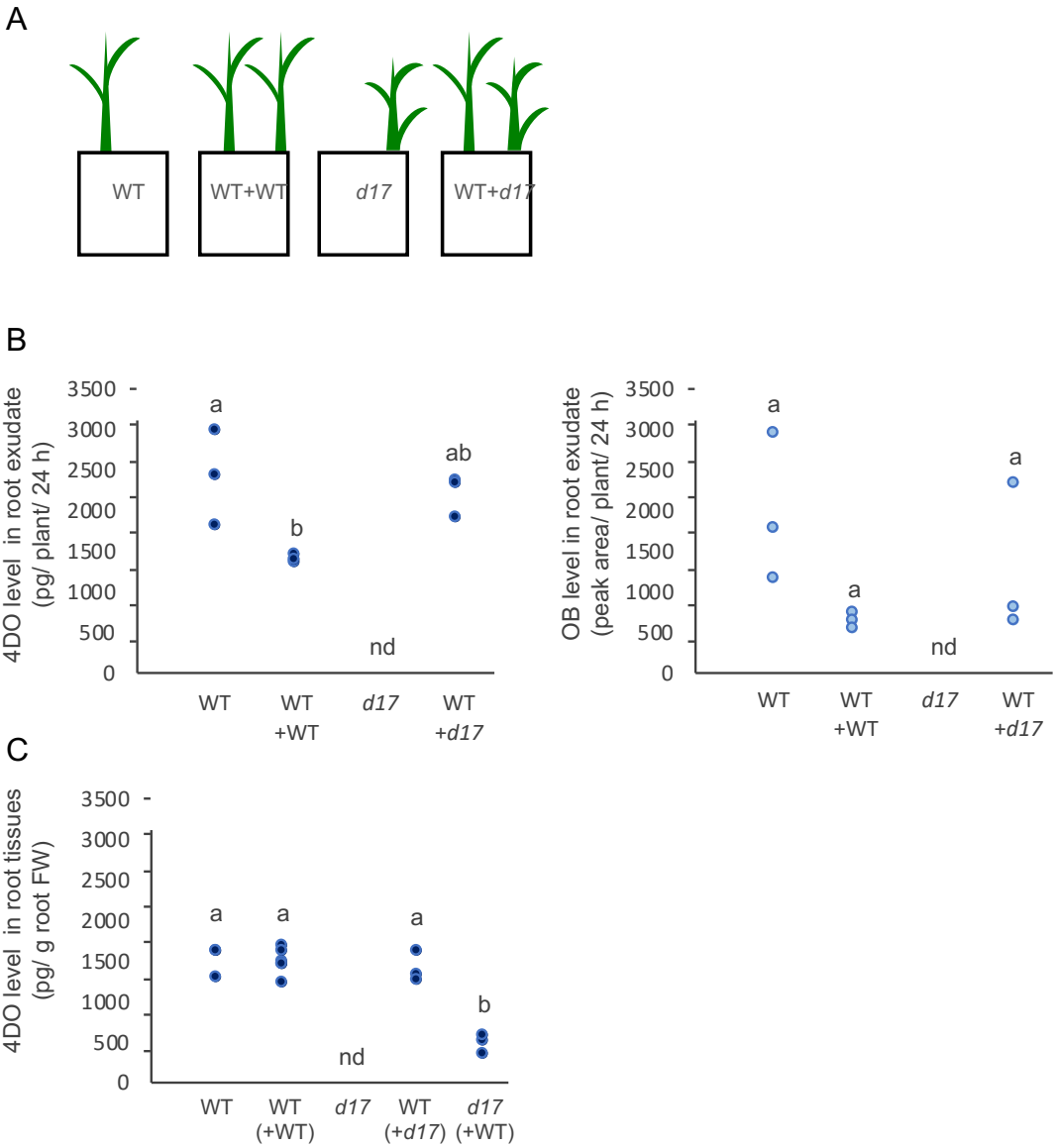
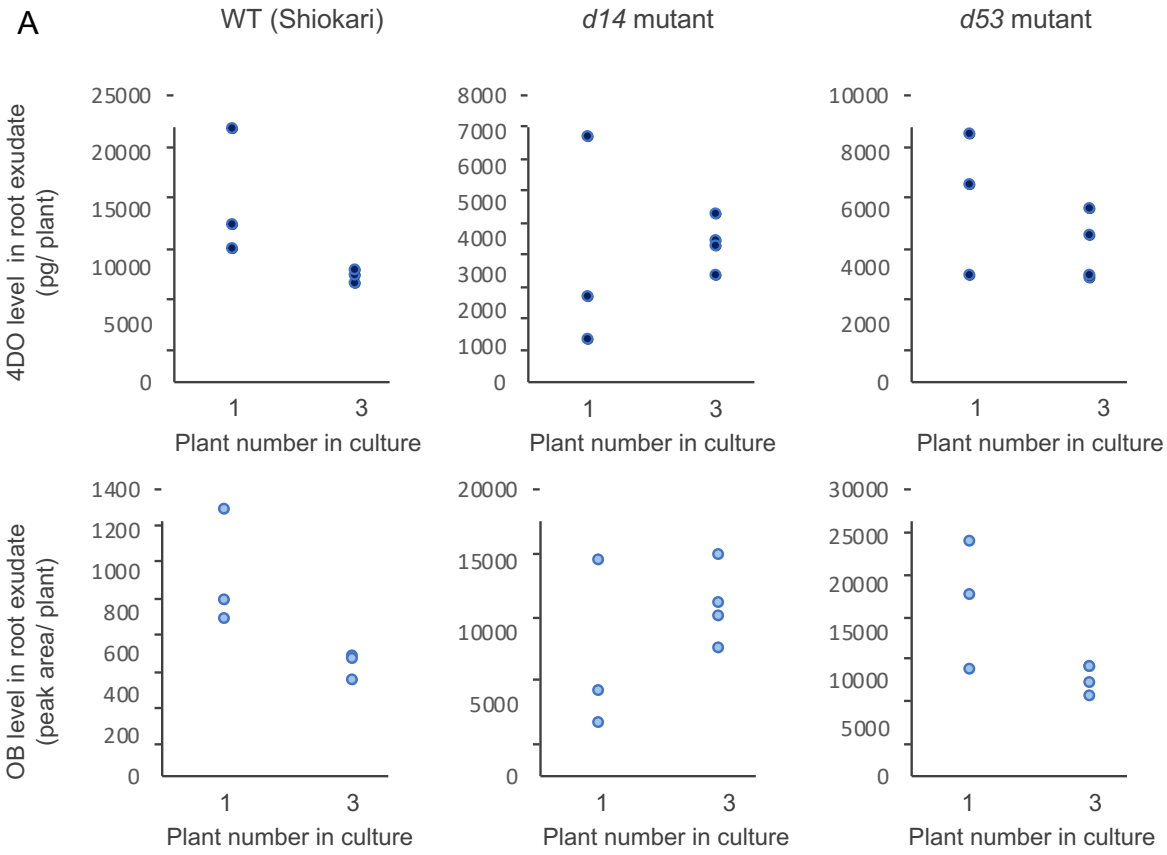
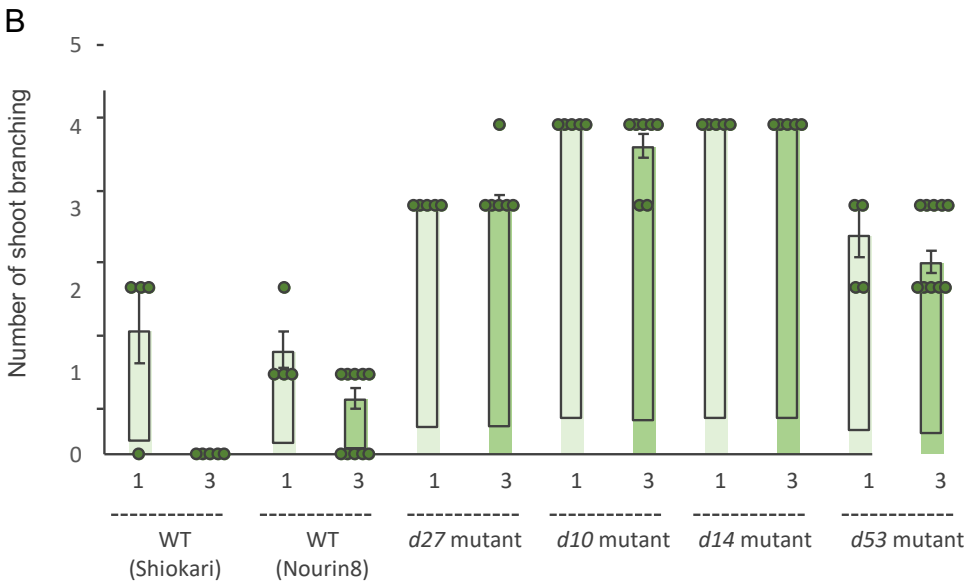

A
WT
WT+WT
d17
WT+d17
B
4DO level in root exudate (pg/ plant/ 24 h)
3500
3000
2500
2000
1500
1000
500
0
a
b
ab
nd
WT
WT +WT
d17
WT +d17
OB level in root exudate (peak area/ plant/ 24 h)
a
a
a
nd
C
4DO level in root tissues (pg/ g root FW)
a
a
a
b
nd
WT
WT (+WT)
d17
WT (+d17)
d17 (+WT)

Figure 4


A
WT (Shiokari)
d14 mutant
d53 mutant
4DO level in root exudate (pg/ plant)
OB level in root exudate (peak area/ plant)
Plant number in culture


B
Number of shoot branching
WT (Shiokari)
WT (Nourin8)
d27 mutant
d10 mutant
d14 mutant
d53 mutant

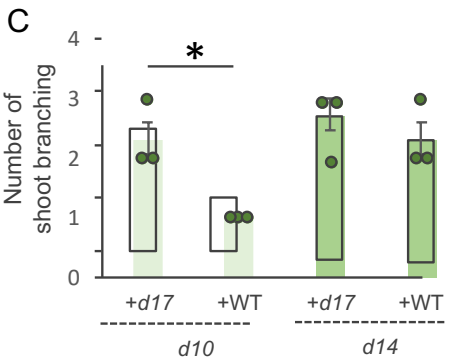

C
Number of shoot branching
*
+d17
+WT
d10
d14

Figure 1

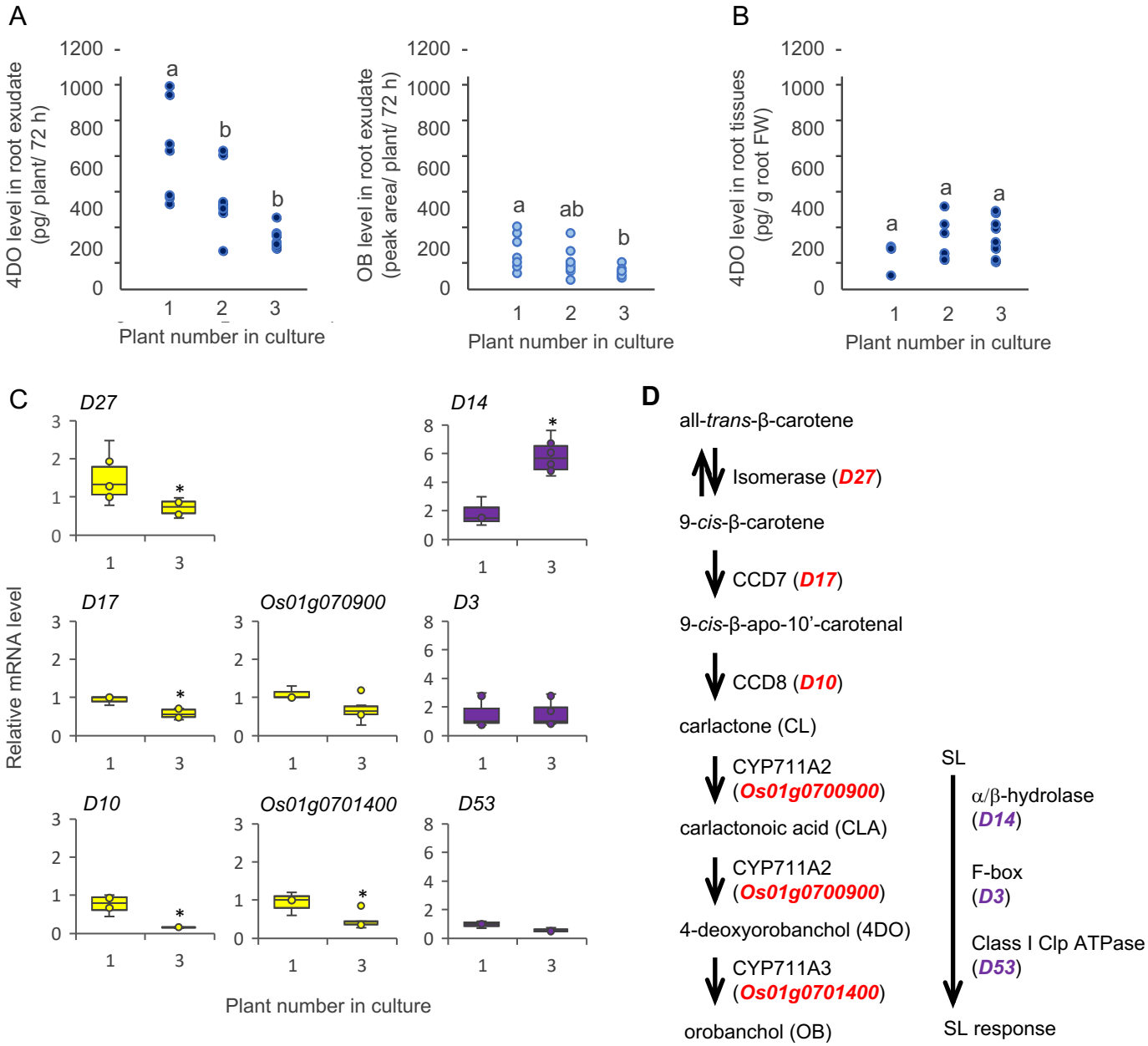

A
4DO level in root exudate (pg/ plant/ 72 h)
OB level in root exudate (peak area/ plant/ 72 h)
Plant number in culture
B
4DO level in root tissues (pg/ g root FW)
Plant number in culture
C
D27
D14
D17
Os01g070900
D3
D10
Os01g0701400
D53
Relative mRNA level
Plant number in culture
D
all-trans-β-carotene
Isomerase (D27)
9-cis-β-carotene
CCD7 (D17)
9-cis-β-apo-10'-carotenal
CCD8 (D10)
carlactone (CL)
CYP711A2 (Os01g0700900)
carlactonoic acid (CLA)
CYP711A2 (Os01g0700900)
4-deoxyorobanchol (4DO)
CYP711A3 (Os01g0701400)
orobanchol (OB)
SL
α/β-hydrolase (D14)
F-box (D3)
Class I Clp ATPase (D53)
SL response

Figure 2

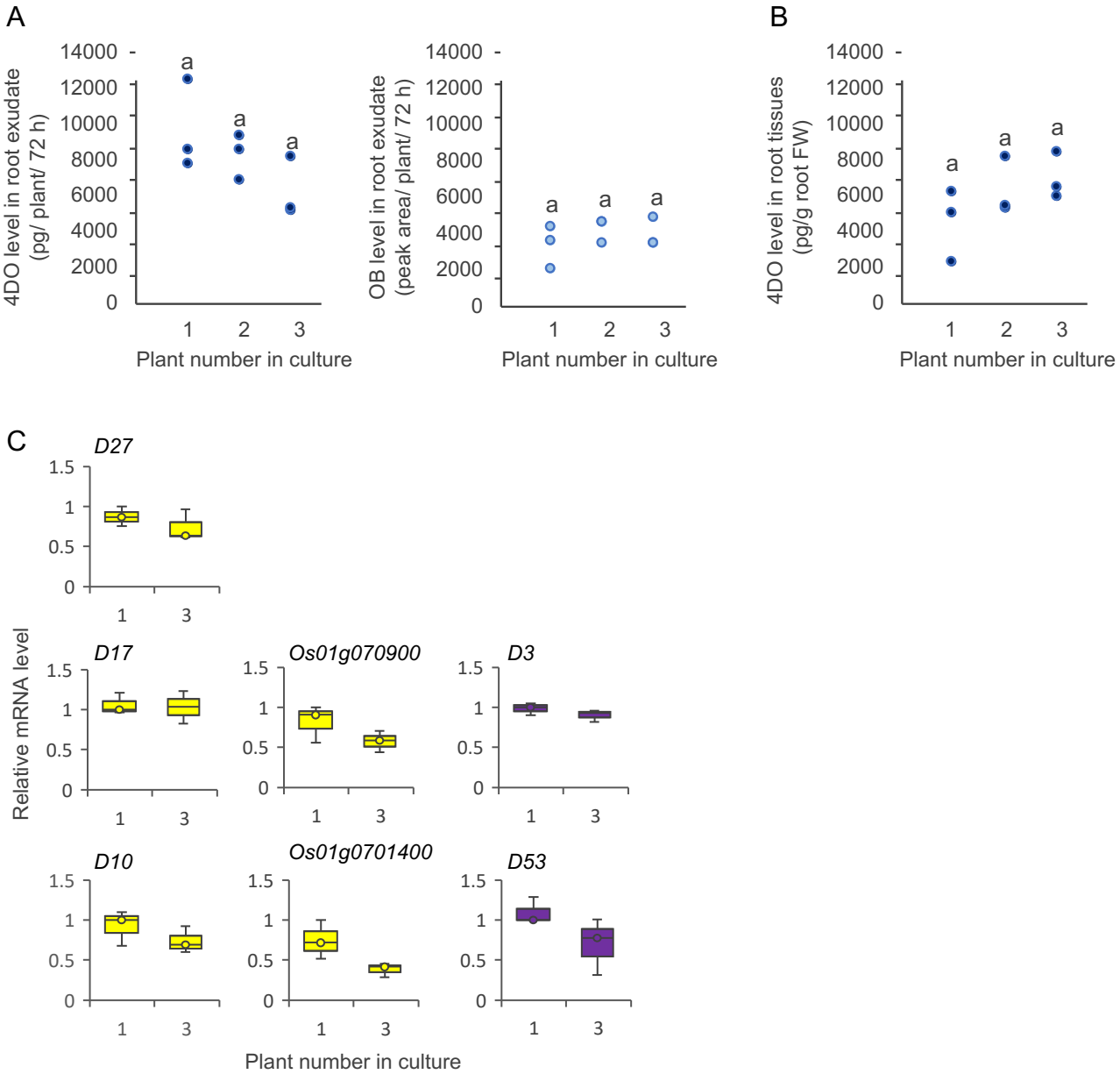

A
4DO level in root exudate (pg/ plant/ 72 h)
14000
12000
10000
8000
6000
4000
2000
0
a
a
a
1
2
3
Plant number in culture
OB level in root exudate (peak area/ plant/ 72 h)
14000
12000
10000
8000
6000
4000
2000
0
a
a
a
1
2
3
Plant number in culture
B
4DO level in root tissues (pg/g root FW)
14000
12000
10000
8000
6000
4000
2000
0
a
a
a
1
2
3
Plant number in culture
C
Relative mRNA level
D27
D17
Os01g070900
D3
D10
Os01g0701400
D53
1.5
1
0.5
0
1
3
Plant number in culture

Figure 3

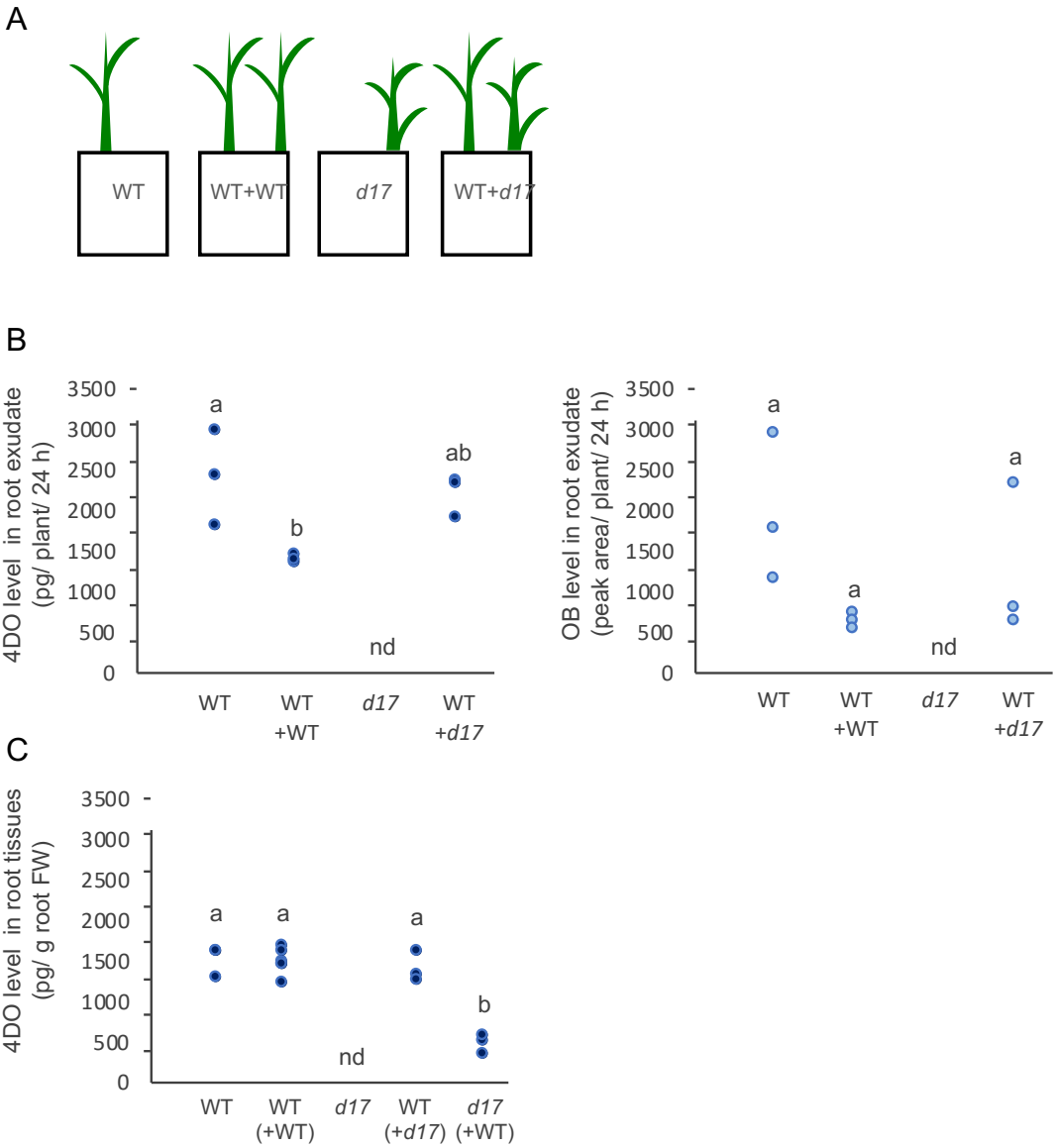
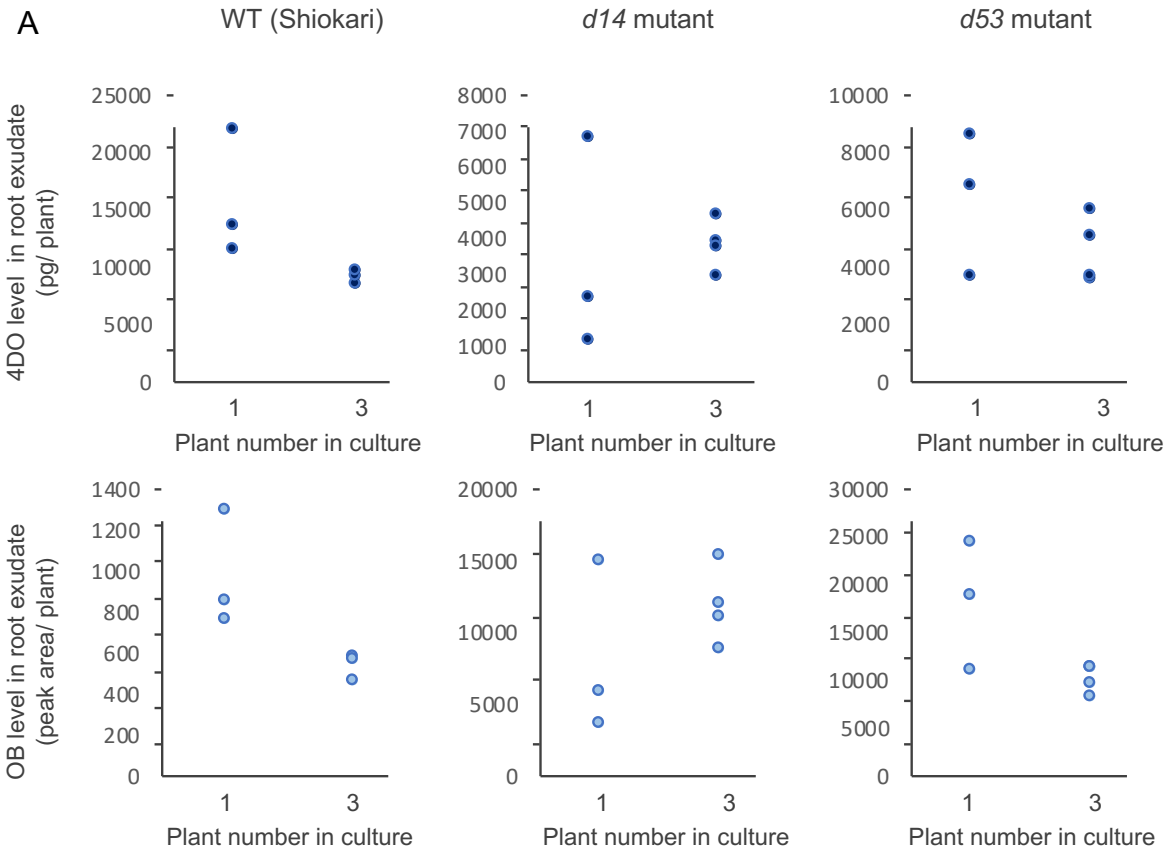
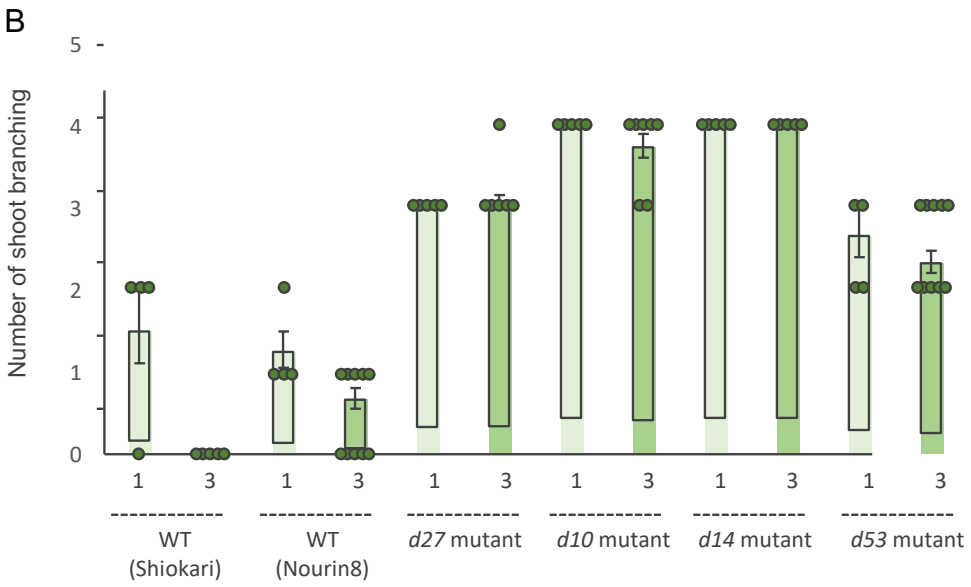
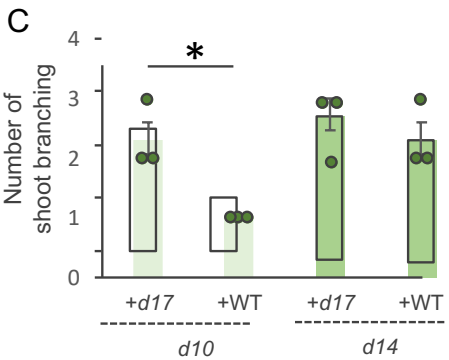

A
WT
WT+WT
d17
WT+d17
B
4DO level in root exudate (pg/ plant/ 24 h)
3500
3000
2500
2000
1500
1000
500
0
a
b
ab
nd
WT
WT +WT
d17
WT +d17
OB level in root exudate (peak area/ plant/ 24 h)
a
a
a
nd
C
4DO level in root tissues (pg/ g root FW)
a
a
a
b
nd
WT
WT (+WT)
d17
WT (+d17)
d17 (+WT)

Figure 4


A
WT (Shiokari)
d14 mutant
d53 mutant
4DO level in root exudate (pg/ plant)
OB level in root exudate (peak area/ plant)
Plant number in culture


B
Number of shoot branching
WT (Shiokari)
WT (Nourin8)
d27 mutant
d10 mutant
d14 mutant
d53 mutant


C
Number of shoot branching
*
+d17
+WT
d10
d14

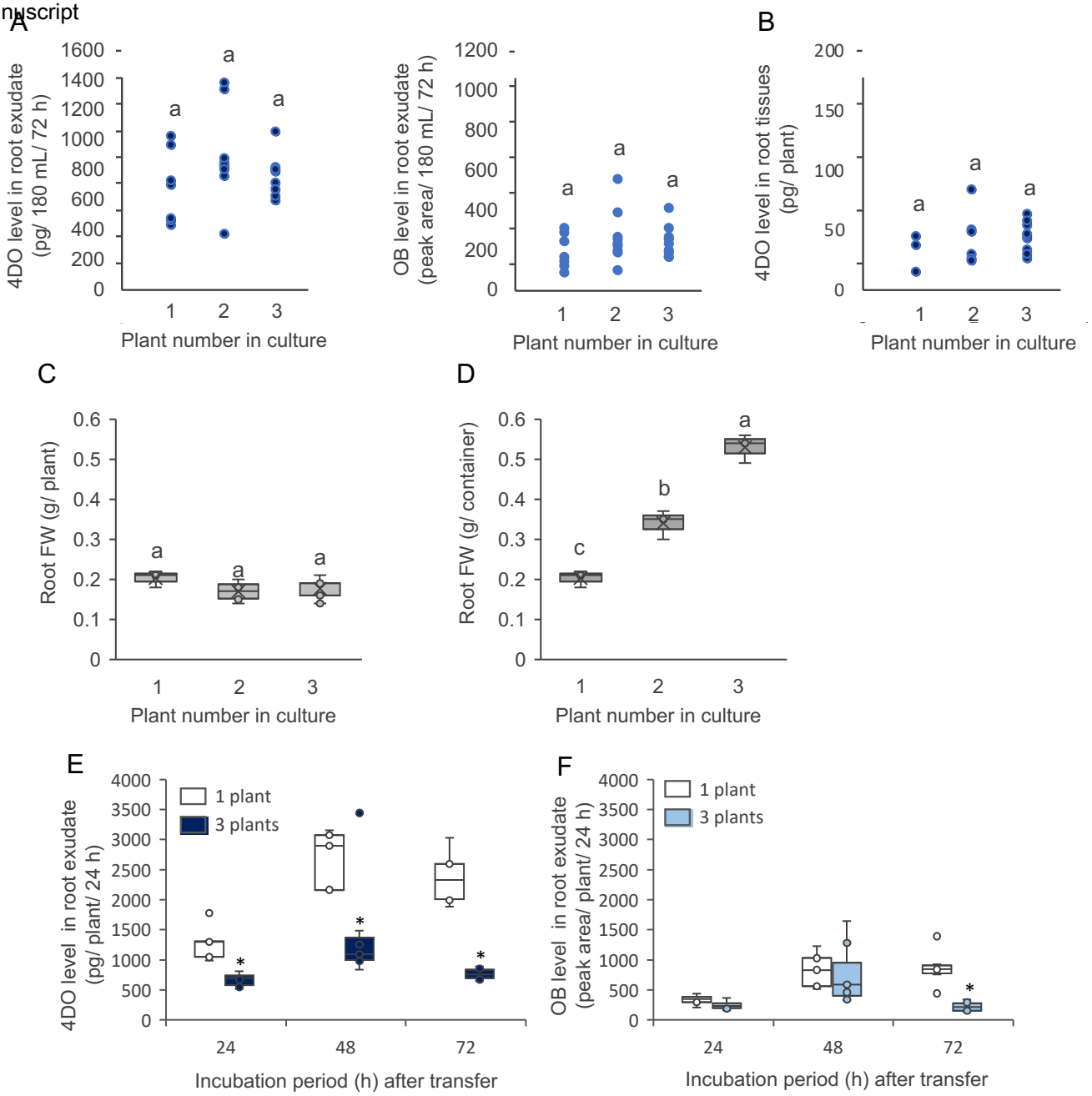


Figure S1. The net strigolactone exudation in 1, 2- and 3-plant culture is constant and changes in strigolactone exudation in response to neighbouring plants occur rapidly, related to Figure 1.

(A) Quantification of 4-deoxyorobanchol (4DO) and orobanchol (OB) present in the hydroponate in 1- or 3-plant cultures 72 h after transfer from 4-day P-depletion, expressed as pg/180 mL of media. Graph shows all individual data points. *n* = 7-9 biologically independent samples. The same letter denotes no statistically significant different (ANOVA, Tukey-Kramer HSD test, $p < 0.05$).

(B) Quantification of 4DO present in the roots of plants in 1-, 2- or 3-plant cultures 72 h after transfer from 4-day P-depletion, expressed as pg/plant. Graph shows all individual data points. *n* = 3-9 biologically independent samples. The same letter denotes no statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$).

(C, D) Measurements of root fresh weights expressed as g of plants or container. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. *n* = 3 biologically independent samples. The same letter denotes no statistically significant difference and different letters denote a statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p<0.05$).

(E, F) Quantification of 4DO and OB present in the hydroponate in 1- or 3-plant cultures 24, 48 and 72 h after transfer from 7-day P-starvation, expressed as pg/plant (4DO) or MS-peak area/plant (OB). The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. *n* = 5-7 biologically independent samples. Asterisks indicate a statistically significant difference relative to 1-plant culture at the same time point (Student's *t*-test, $p<0.05$).

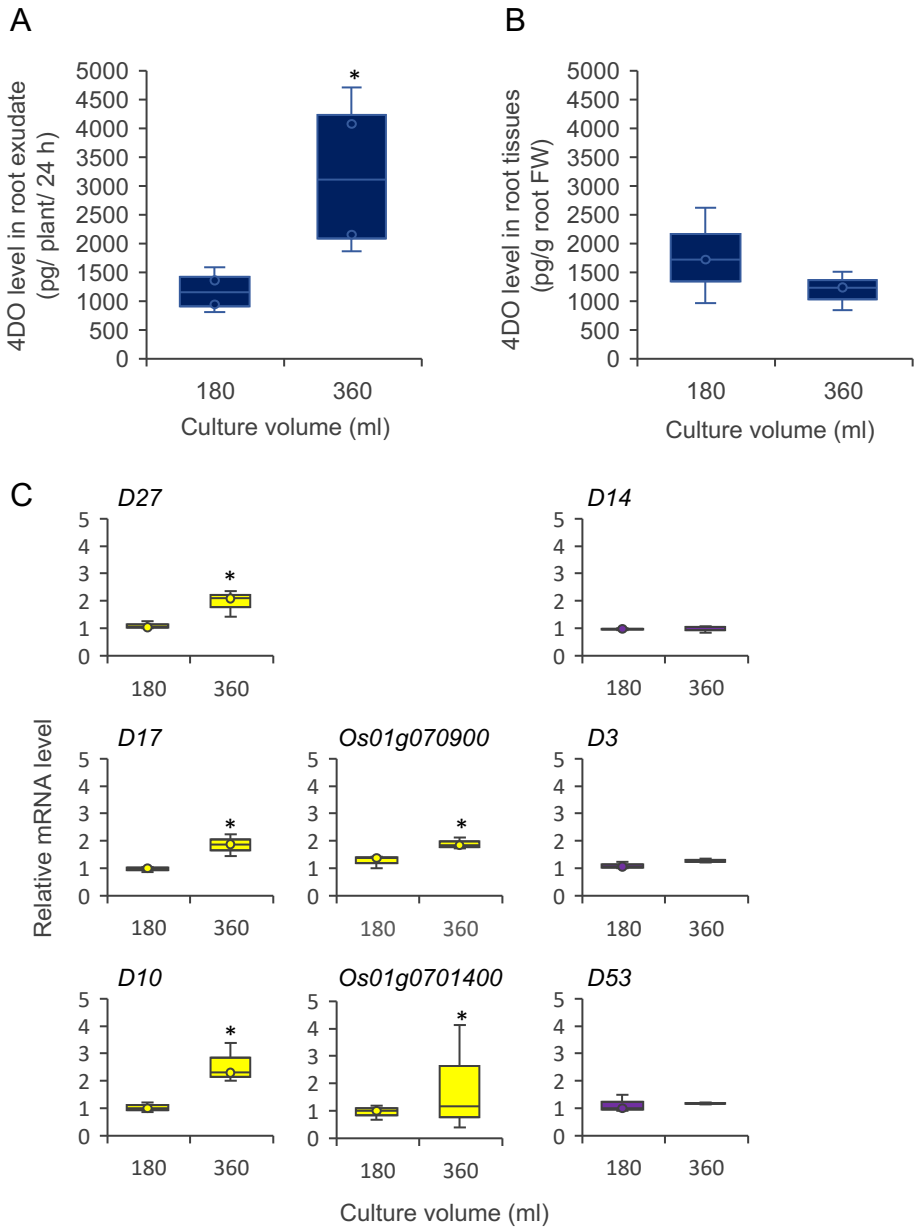


Figure S2. Strigolactone exudation responds to root density, related to Figure 1.

(A) Quantification of 4-deoxyorobanchol (4DO) present in the hydroponate in 1-plant cultures 24 h after transfer from 7-day P-depletion to 180 mL or 360 mL hydroponic vessels, expressed as pg/plant. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. n = 4 biologically independent samples. Asterisks indicate a statistically significant difference relative to 180 mL culture at the same time point (Student's t-test, $p < 0.05$).

(B) Quantification of 4DO present in the roots of plants 1-plant cultures 24 h after transfer from 7-day P-depletion to 180 mL or 360 mL hydroponic vessels, expressed as pg/g fresh weight. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. n = 3 biologically independent samples. No asterisk indicates no statistically significant difference relative to 180 mL culture at the same time point (Student's t-test, $p < 0.05$).

(C) Quantification of strigolactone-related gene expression in the roots of plants in 1-plant cultures 24 h after transfer from 7-day P-starvation to 180 mL or 360 mL hydroponic vessels. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. n = 3 biologically independent samples. Asterisks indicate a statistically significant difference relative to 180 mL culture at the same time point (Student's t-test, $p < 0.05$).

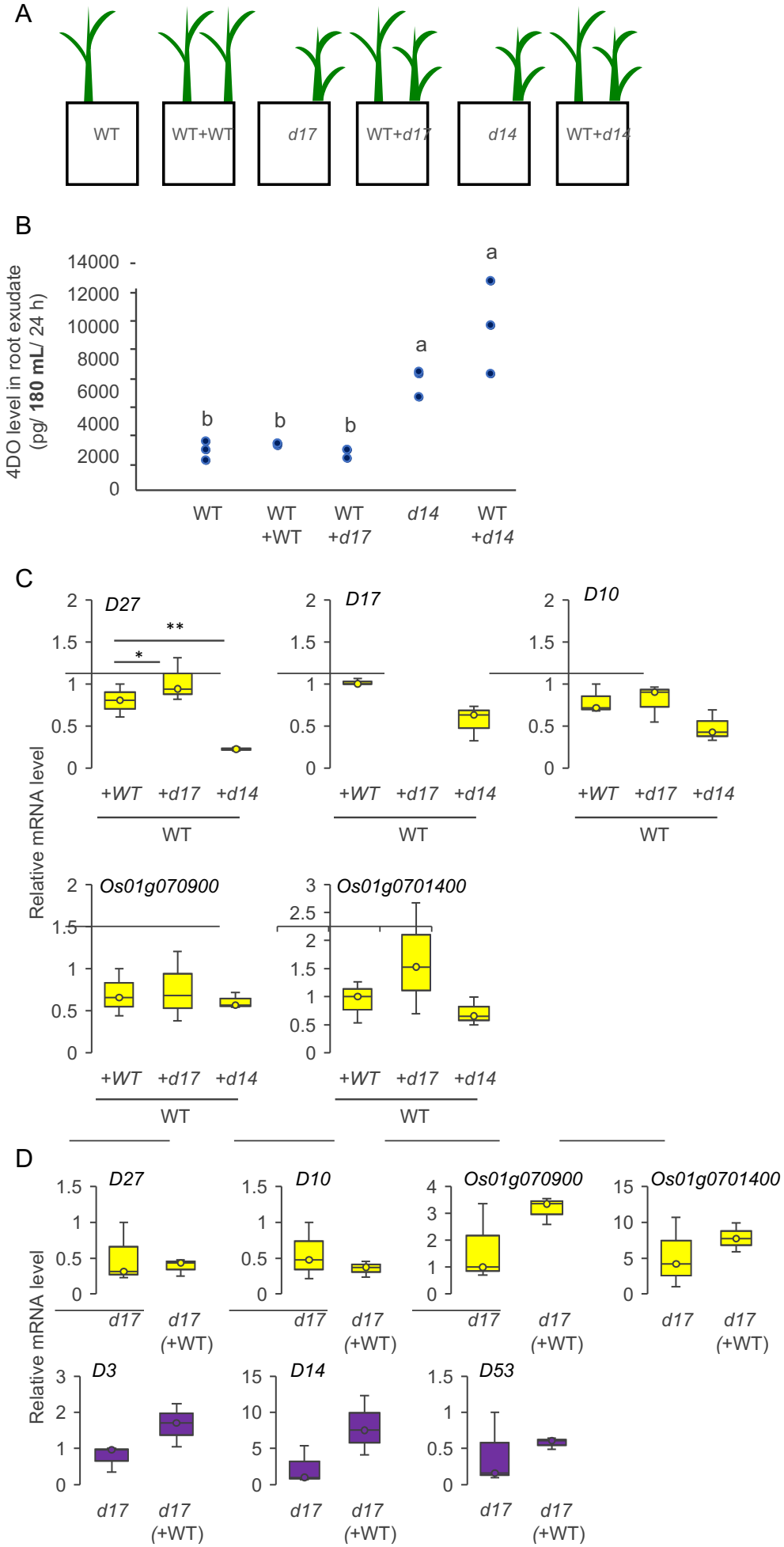

A
WT
WT+WT
d17
WT+d17
d14
WT+d14
B
4DO level in root exudate (pg/ 180 mL/ 24 h)
14000
12000
10000
8000
6000
4000
2000
0
a
a
b
b
b
WT
WT +WT
WT +d17
d14
WT +d14
C
Relative mRNA level
D27
D17
D10
Os01g070900
Os01g0701400
**
*
+WT
+d17
+d14
WT
D
D27
D10
Os01g070900
Os01g0701400
D3
D14
D53
d17
d17 (+WT)

Figure S3. Strigolactone exudation is homeostatically regulated by environmental strigolactones, related to Figure 3.

(A) Cartoon showing experimental set-up.

(B) Quantification of 4-deoxyorobanchol (4DO) present in the hydroponate in 1-plant cultures of WT or *d14*, 2-plant cultures of WT+WT, WT+*d17* mutants, or WT+*d14* mutants 24 h after transfer from 7-day P-depletion, expressed as pg per 180 mL media. Graph shows all individual data points. $n = 3$ biologically independent samples. The different letters denote statistically significant difference (ANOVA, Tukey-Kramer HSD test, $p < 0.05$).

(C, D) Quantification of strigolactone-related gene expression in the roots of WT plants grown in 2-plant cultures with either WT, *d17,* or *d14* plants, and 1-plant cultures of *d17* plants or *d17* plants co-cultured with WT plants 24 h after transfer from 7-day P-depletion. The upper and lower confines of the box indicate the interquartile range, the central line indicates the median, and the whiskers represent the maximum and minimum values. $n = 3$ biologically independent samples. Asterisks indicate a statistically significant difference (Student's *t*-test, $p < 0.05$).

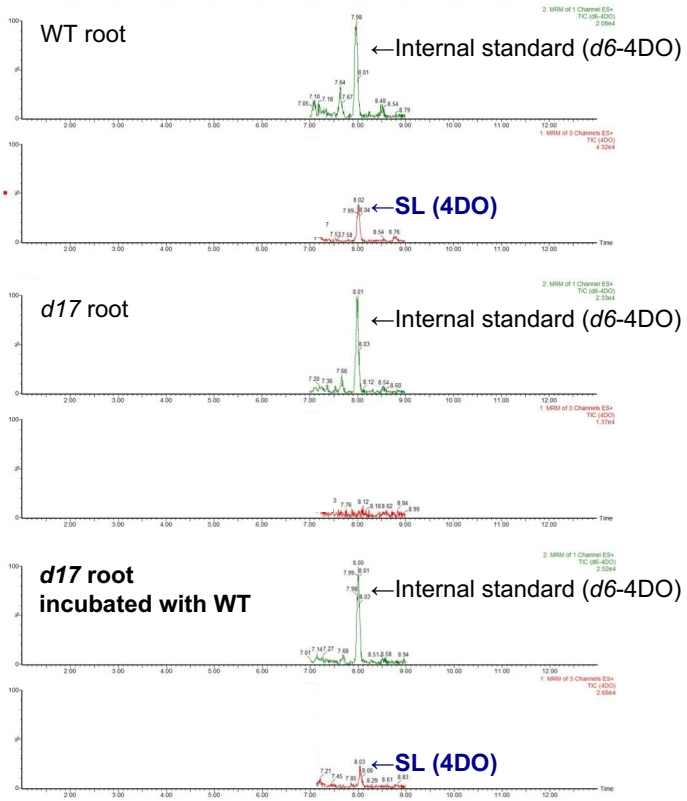


Figure S4. Strigolactone was detected from *d17* roots co-cultured with WT, related to Figure 3.
Detection of d_6-4-deoxyorobanchol (d_6-4DO, internal standard) and 4-deoxyorobanchol (4DO) in root tissues of WT, *d17,* and *d17* grown with WT. Multiple reaction monitoring chromatograms of d_6-4DO (green) and 4DO (red) by LC-MS/MS are shown.

Primer name	5' to 3' sequence
D27-F	AGATGACCCTGCATTGAAGCA
D27-R	GCAATTCACACCATGTTCTGC
D10-F	CTGTACAAGTTCGAGTGGCACC
D10-R	CCTGTCCGTCTCCTCGTAC
D17-F	CCTCGTCCAGAAGCGTGAG
D17-R	TAGTGGGTGTCGGTGAAGGC
CYP711A2-F	CGTGAACCTCACGCTCGG
CYP711A2-R	TTCATTGCAGCCGTCCG
CYP711A3-F	TGCATTGAGTGCGTGTCCA
CYP711A3-R	GAAGCCGAGAGCGAGATCG
D3-F	CCCAACCTCCGCAAGCT
D3-R	GACGCAATCGCTGAACCG
D14-F	GCCTCTCCCCGGTTCTTG
D14-R	TGCTGTATCTCCTCCAGCTCG
D53-F	CCAAGCAGTTTGAAGCGAC
D53-R	CCGCAAGTTTATCAAAGTCAA
OsTFIIE-F	GTGCAGCCCAAGGCTAAG
OsTFIIE-R	CGTCGAATAAGCGTAGAGCA

Table S1. List of primer for real-time PCR, related to STAR Methods.