the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2008) **55**, 709–717 doi: 10.1111/j.1365-313X.2008.03543.x

D'orenone blocks polarized tip growth of root hairs by interfering with the PIN2-mediated auxin transport network in the root apex

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Summary

The C_{18} ketone (5*E*,7*E*)-6-methyl-8-(2,6,6-trimethylcyclohex-1-enyl)octa-5,7-dien-2-one (D'orenone) has been postulated to be an early cleavage product of β-carotene en route to trisporic acids; these act as morphogenetic factors during the sexual reproduction of zygomycetes. Here we report that D'orenone blocks the highly polarized tip growth of root hairs, causing tip growth to stop completely within a few minutes. Importantly, external auxin reverses the effects of D'orenone on root hairs. Further analysis revealed that D'orenone lowers the auxin concentration in trichoblasts via PIN2-mediated auxin efflux to below the critical levels essential for root hair growth. D'orenone specifically increases PIN2 protein abundance without affecting PIN2 transcripts, and the PIN2 expression domain enlarges and shifts basipetally, resulting in more active auxin transport. The observation that D'orenone does not interfere with the root hair growth in roots of null mutant lines provides additional evidence that PIN2 is its specific target.

Keywords: auxin transport, PIN2, polarity, root apex, root hair, tip growth.

Introduction

Root hairs are tip-growing tubular outgrowths that emerge from specialized root epidermis cells known as trichoblasts and expand locally at their apical dome (Schiefelbein, 2000). The development of the root hair cell can be divided into three stages: determination of hair and non-hair cellular identity in the rhizodermis (Schiefelbein, 2000), initiation of hair outgrowth, and active tip growth (Baluška *et al.*, 2000).

Root hair formation is influenced by hormone-based signaling pathways, especially of ethylene and auxin (Pitts *et al.*, 1998; Rahman *et al.*, 2002). Once initiated, the tip-focused growth depends on a critical auxin level in trichoblasts (Cho *et al.*, 2008; Lee and Cho, 2006). Auxin export out of trichoblasts is driven by the PIN2 auxin efflux transporter, which is a member of a complex network of PIN transporters (PIN1–4 and 7) that coordinates auxin flow in the root apex (Blilou *et al.*, 2005).

Auxin levels in the trichoblast strongly influence root hair growth. For example, trichoblast-specific over-expression of PINOID and PIN3, resulting in increased auxin efflux from these cells, inhibits root hair growth due to the decrease of cellular auxin levels to below the required threshold value (Lee and Cho, 2006). More recently, trichoblast-specific over-expression of three other efflux transporters (PIN2, PIN4, PGP4) was reported to block root hair tip growth (Cho *et al.*, 2007). Root hair tip growth is restored via exogenous auxin (Cho *et al.*, 2007; Lee and Cho, 2006). Together, these observations imply the existence of an auxin-monitoring system in trichoblasts that prevents tip growth of root hairs when the auxin level is below a critical endogenous level.

Auxin serves as a signaling and networking molecule not only in plants but also in bacteria, fungi and nematodes (Prusty *et al.*, 2004; De Meutter *et al.*, 2005; : Bianco *et al.*, 2006a, b; Curtis, 2007). Auxin is known to mediate inter-organism communication between plant roots and ectomycorrhizal fungi (Ditengou *et al.*, 2003). An early report connecting the actions of fungal apocarotenoids to those

of auxin *in planta* described the inhibitory effect of trisporic acids on the auxin-induced elongation of coleoptiles of *Avena sativa* (Blaydes and Saus, 1978). This observation prompted us to study the effect of trisporoids on root hair growth, focusing on early and late intermediates of the biosynthetic pathway to trisporic acids (Sutter *et al.*, 1996). Here we report that the C_{18} ketone (5*E*,7*E*)-6-methyl-8-(2,6,6-trimethylcyclohex-1-enyl)octa-5,7-dien-2-one (D'orenone, derived from the French word 'd'or' and referring to the golden color of the compound) significantly inhibits the polarized growth of root hairs at nanomolar concentrations by manipulating auxin transport and signaling via its impact on the PIN2 efflux carrier that exports auxin from root cells. As D'orenone is a powerful and readily available molecule that freely passes through membranes, it represents a novel molecular tool to dissect complex signaling networks in root hairs that are linked to the auxin transport network in the root apex.

Results

D'orenone blocks tip growth of root hairs but stimulates root system density

D'orenone rapidly blocked tip growth of root hairs (see Supplementary material). This effect occurs at nanomolar levels (400 nM), with rapid inhibition of the polarized tip growth of existing root hairs and blocking of the formation of new root hairs. Higher concentrations (4–400 μM) completely stopped root hair growth (Figure 1a,b), but the growth of the primary root was not negatively affected in the range 1–40 μM (see below). At higher concentrations, however, the density of lateral roots was slightly increased.

Of all tested compounds (see Experimental procedures), only D'orenone was effective at concentrations as low as 400 nM (Figure 1c). Other apocarotenoids, such as all-*trans*-retinal or all-*trans*-retinoic acid, were much less active or not active at all. For example, retinal (4 μM) affected the relative growth rate of root hairs but required a ten-fold higher concentration to achieve the same effect compared to D'orenone (Figure 1a). Lower concentrations of retinoic acid (1–4 μM), a bioactive signal molecule in animal systems (Chambon, 1996; Liou *et al.*, 2005), had no visible effect on the growth of the root hairs (data not shown). The activity of D'orenone decreased when conjugation of the double bond with the keto group was abolished by reducing the C3=C4 double bond of the side chain (Schachtschabel and Boland, 2007). Activity also decreased upon reduction of the keto group to an alcohol. The resulting 3,4-dihydro- and hydroxy derivatives of D'orenone displayed only weak inhibitory activity when tested on growing root hairs (Figure 1a). Thus, even minor changes in the stereochemistry and polarity of the molecule are sufficient to reduce its biological activity.

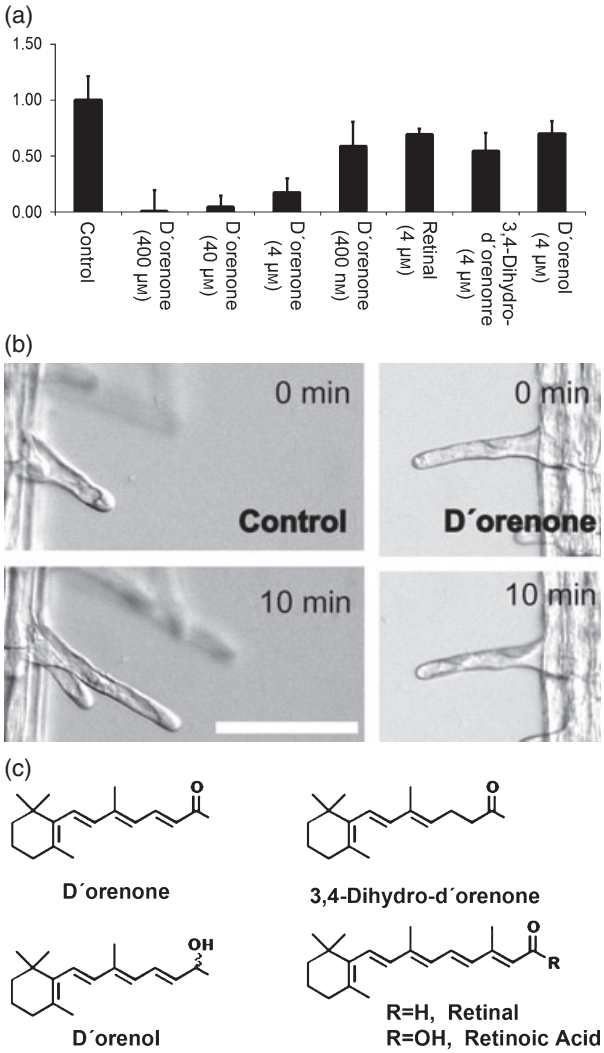


Figure 1. D'orenone blocks tip growth of root hairs but stimulates root system density.
(a) Relative growth rates of root hairs during treatments of short duration with various concentrations of D'orenone. Values are the means of five plants treated for 30 min. Treatments with retinal, D'orenol and 3,4-dihydro-D'orenone using the same protocol show weaker effects than treatment with D'orenone.
(b) Comparison of bright-field pictures from a DMSO mock-treated root hair and a root hair treated with D'orenone (10 μM). Note the prominent cytoplasmic cap at the tip of the fast-growing control root hair. Bar = 100 μm.
(c) Chemical structure of D'orenone and its analogues.

At concentrations higher than 5 μM, D'orenone also transiently affected the root gravity responses of *Arabidopsis thaliana*. Although roots responded to gravi-stimulation, careful analysis of repetitive gravi-stimulation revealed subtle disturbances. For instance, after the third rotation of root apices relative to the gravity vector, a reduction of gravi-oriented growth was seen only in the D'orenone-treated root apices (see Supplementary material). Moreover, D'orenone rescues the root agravitropic phenotype of the PIN2 mutant, and fails to stop completely the root hair tip growth in this

mutant (see below). D'orenone also promoted root system complexity by increasing the lateral root density (see below).

D'orenone increases PIN2 abundance and enlarges the PIN2 expression domain

D'orenone increased the abundance of PIN2 protein in roots (Figures 2 and 3). In order to determine the reason for this effect, GUS lines visualizing both PIN2 transcripts and proteins (Sieberer *et al.*, 2000) were analyzed. Although PIN2 transcript localization was not changed significantly (Figure 2a), PIN2 protein was found in a larger root apex domain (Figure 2b) covering both meristem and transition zone (Verbelen *et al.*, 2006). This expansion of the root apex domain expressing the PIN2 protein was confirmed using the GFP–PIN2 line (Figure 3c). In the D'orenone-treated root apices, the PIN2–GFP signal vanished from the lateral root cap, and a new, rather diffuse, signal appeared in transition zone cells (Figure 3c). Although the overall PIN2 polarity at the cell periphery was maintained at this location, there was an additional signal in small subcellular compartments and the tonoplast (Figure 3e,f). Western blotting showed increased protein levels of PIN2. This high PIN2 level was quite resistant to addition of protein biosynthesis inhibitor cycloheximide (CHX) after the D'orenone treatment (Figure 3g). In contrast to PIN2, the auxin efflux carrier PIN1 was unaffected (Figure 3h,i).

In order to probe auxin signaling, transcription of IAA-inducible genes (RT-PCR for IAA1 and IAA19) and expression of the DR5 reporter gene (Figure 3a) were compared in control and D'orenone-exposed roots. Transcription of IAA1 and IAA19 was not affected (data not shown), but the auxin-responsive reporter $DR5_{rev}$–GFP, which responds to gravi-stimulation (Friml *et al.,* 2003; Ottenschläger *et al.*, 2003), was strongly activated in the root apex after exposure to D'orenone, from 30 min after application (Figure 3a). Experiments with gravi-stimulated roots pre-exposed to D'orenone showed higher expression of the $DR5_{rev}$–GFP reporter on the lower root side, and an unusually high signal for the DR5 reporter was also observed on the upper root side (Figure 3b). Similar responses were reported by Jaillais *et al.* (2006) after treatment with wortmannin, which is a phosphatidylinositol-3-OH kinase inhibitor.

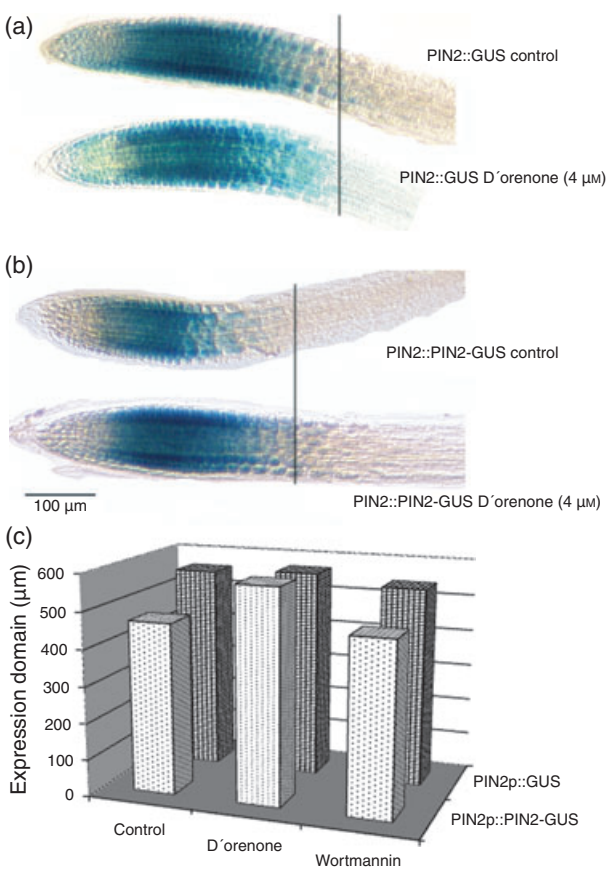


Figure 2. D'orenone affects PIN2 proteins but not transcripts.
(a, b) *PIN2p::GUS* and *PIN2p::PIN2-GUS* expression in root apices. The GUS signal in the apex of control and D'orenone-exposed (4 μM) roots shows no significant responses of the PIN2 promoter transcript localization to D'orenone treatment (a), but PIN2–GUS protein was found in a larger root apex domain after D'orenone treatment (b).
(c) Comparison of the *PIN2p::GUS* (protein) and *PIN2p::PIN2-GUS* (transcript) expression domains in the control root apices and in D'orenone- and wortmannin-treated root apices.

D'orenone increases auxin flux in root apices of Arabidopsis

In vivo analysis of auxin transport in growing root apices (see Mancuso *et al.*, 2005; Bouchard *et al.*, 2006 for details) treated with D'orenone (4 μM) revealed that D'orenone stimulates auxin flux by about 20%, specifically at the transition zone (Figure 3j) that is expressing PIN2 (Figures 2b and 3b). The peak of auxin transport was shifted in the D'orenone-treated roots, resembling the basal shift of the PIN2 domain. These findings support the hypothesis that D'orenone actions are closely related to PIN2-driven auxin transport and auxin signaling.

Auxin rescues the root hair phenotype in D'orenone-treated roots

Exogenous auxin rescues the D'orenone-induced block of root hair tip growth (Figure 4a–c). Root hairs of plants treated simultaneously with auxin (30 nM) and D'orenone (4 μM) showed comparable growth rates to root hairs from controls (Figure 4a,c). Moreover, pre-treatment of roots with external auxin resulted in roots that were resistant to externally applied D'orenone (Figure 4b). It is important to note that external auxin is not able to rescue the wortmannin-induced inhibitory effects on root hairs and root apices (Figure 4a), demonstrating that either the targets or the mode of binding of D'orenone and wortmannin are not identical.

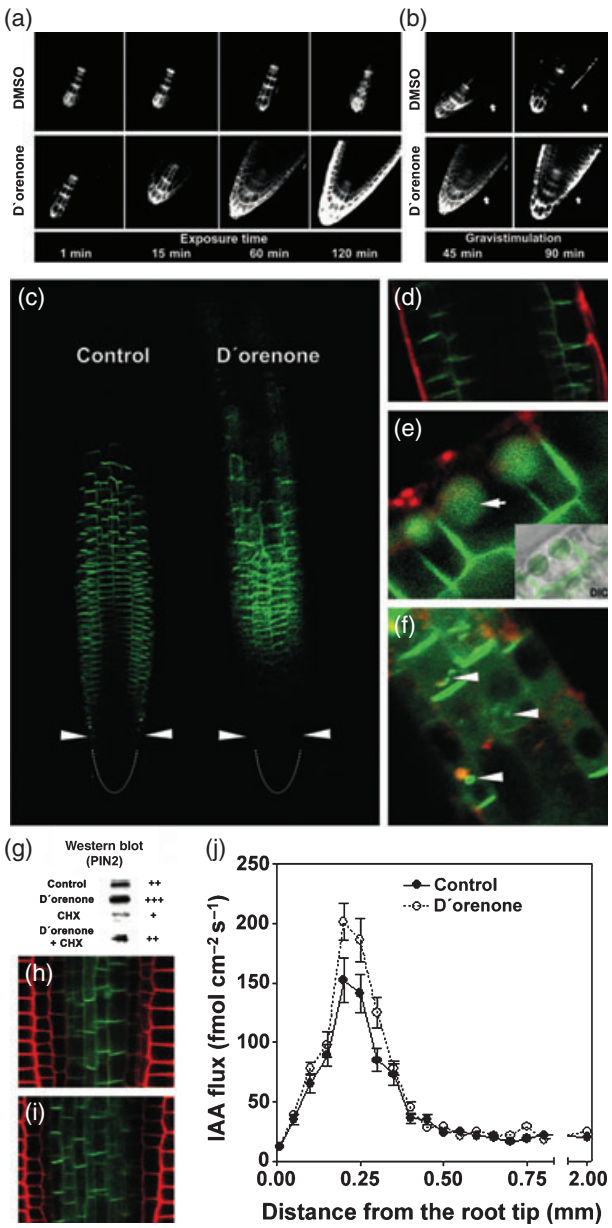


Figure 3. D'orenone enlarges the PIN2 expression domain, stimulates auxin signaling, and promotes auxin transport.
(a, b) $DR5_{rev}$–GFP expression in root tips. GFP signal in the apex of control roots (a, upper panel) and after gravi-stimulation (b) (90 min, arrow indicates the lower part of the root apex). D'orenone (10 μM) activates $DR5_{rev}$–GFP expression in the whole root cap and lateral root cap cells (a, lower panel). Gravi-stimulation of the D'orenone-exposed root apices (10 μM, 90 min, arrow indicates the lower part of the root apex) shows $DR5_{rev}$–GFP signal on the upper side of the root apex also (b).
(c)–(f) PIN2–GFP localization in green and localization of the endocytic tracer FM4-64 in red. (c) DMSO mock-treated control and D'orenone-treated (10 μM) root tips. In the D'orenone-treated root apices, nearly all of the PIN2–GFP signal, which is normally observed only in epidermal cells and cells of the lateral root cap, vanished from the lateral root cap (arrowheads), and new signal for PIN2–GFP appeared in transition zone cells. The root tips of the control show typical polar localization of PIN2–GFP at the plasma membrane of the cross poles (d). In addition to polar localization of PIN2 at the plasma membrane, treatment with D'orenone (10 μM) induced a diffuse cytoplasmic PIN2–GFP signal (f, arrow heads) together with PIN2–GFP-positive tonoplast and vacuoles (e, arrow).
(g) Western blots of root membrane fractions labeled with PIN2 antibody reveal a more distinct band in extracts of D'orenone-treated roots (4 μM for 2h). Treatment with the protein biosynthesis inhibitor cycloheximide (CHX) diminished the signal intensity, and D'orenone partially abolished this CHX effect.
(h, i) PIN1–GFP localization in green and localization of the endocytic tracer FM4-64 in red. PIN1–GFP shows typical polar localization at the plasma membrane. The cross poles of stele cells are clearly labeled (h). D'orenone has no influence on the subcellular localization of PIN1–GFP (i).
(j) The IAA influx profile along the root apex of Arabidopsis. Data shown were collected continuously over a 10 min period and are the means of 10 replicates. Error bars represent SE. Mock-treated controls (black circles) were compared to D'orenone-treated plants (4 μM, white circles). Treated plants show increased influx in cells of the transition zone.

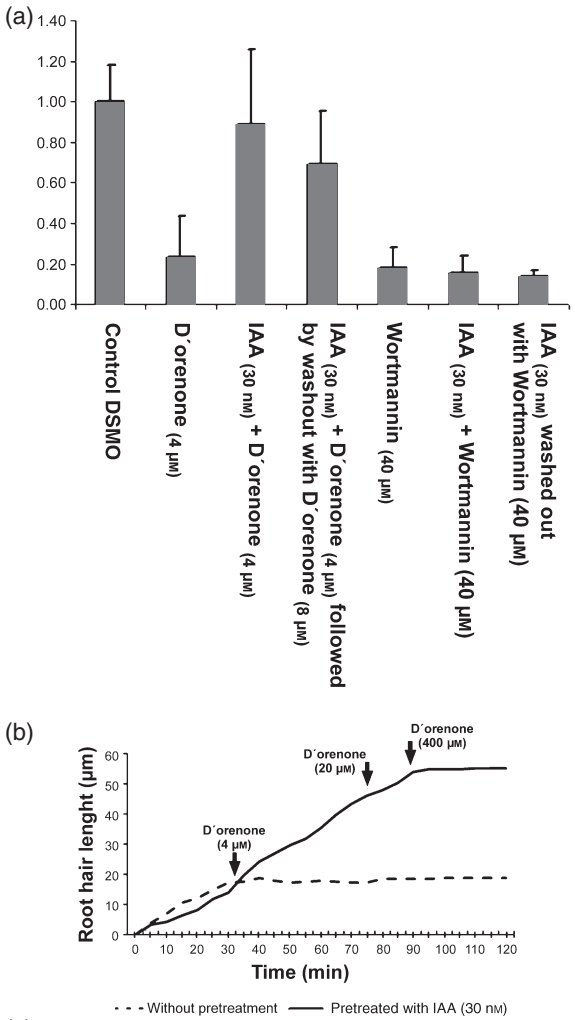
D'orenone effects in PIN2 *mutant roots*

All the above data indicate that D'orenone manipulates polar auxin transport via its effects on PIN2 proteins, which act as auxin efflux transporters responsible for basipetal auxin transport from the tip to the root elongation zone, which is essential for root gravitropism (Chen *et al.*, 1998; Müller *et al.*, 1998; Rashotte *et al.*, 2000; Utsuno *et al.*, 1998). To provide additional genetic evidence for this, we used roots of the *agr1-2* mutant line (Chen *et al.*, 1998), which are agravitropic, and analyzed their gravi-curvature after exposure to D'orenone. D'orenone rescued the agravitropic phenotype of roots in this mutant line (Figure 5). Importantly, D'orenone does not completely block the root hair tip growth in the *agr1-2* mutant line (Figure 5), which is not null line. On the other hand, in the *pin2* null mutant line *eir1-4*, which does not contain any PIN2 protein, root growth is agravitropic after D'orenone treatment. In addition, the cytoarchitecture and growth rates of root hairs are not affected by D'orenone in this line (Figure 6).

Discussion

The highly polarized tip growth of root hairs is relatively well understood and represents one of the best examples of polar cell growth inherently linked to signaling pathways via the dynamic cytoskeleton and endosomal vesicular trafficking (Baluška *et al.*, 2000; Šamaj *et al.*, 2004). Here we show that growth of Arabidopsis root hairs stops within just a few minutes after exposure to D'orenone, which mimics the effects of wortmannin, an inhibitor of phosphatidylinositol-3-OH kinase (PI(3)K) (Jaillais *et al.*, 2006). Nevertheless, the effects of wortmannin differ from those of D'orenone. In contrast to D'orenone, wortmannin unselectively affects the growth of both root hairs and primary roots, and also inhibits root gravitropism (see Jaillais *et al.*, 2006). In addition, as shown here, application of exogenous auxin fully rescues all the effects of D'orenone on root hairs, but not the effects of wortmannin.

At low concentrations (below 5 μM), D'orenone effectively blocked the tip-growing root hairs but did not affect the



(c)

Treatment	Root lenght (mm)	+/–	Lateral root density	+/–
Control (DMSO)	9.40	1.41	0.20	0.15
D´orenone (1 μM)	9.83	1.72	0.21	0.07
D´orenone (10 μM)	8.67	1.58	0.62	0.17
D´orenone (40 μM)	11.00	2.07	1.02	0.21
IAA (30 nM)	1.71	0.49	5.36	0.40
IAA (30 nM) + D´orenone (10 μM)	1.50	0.53	6.22	0.66

Figure 4. Auxin rescues the root hair phenotype in D'orenone-treated roots.
(a) Simultaneous external addition of auxin (IAA, 30 nM) rescues the D'orenone-induced root hair phenotype (D'orenone, 4 μM). Root hairs of double-treated plants show comparable growth rates to control root hairs. External auxin also makes the roots more resistant to additionally applied D'orenone (4 μM, followed by 8 μM). The wortmannin-induced (40 μM) termination of root hair growth is not rescued by externally applied auxin (30 nM). The data are mean values for at least 10 hairs.
(b) Pre-treatment with external auxin (30 nM) makes root hairs more resistant to additionally applied D'orenone (4 μM, followed by 20 and 400 μM).
(c) D'orenone had no significant effects on the growth of primary roots. However, at concentrations of 10 μM or higher, D'orenone slightly increases the number of lateral roots. These data are mean values of at least 25 plants. The root lengths are measured in mm and the lateral root density values are the mean number of lateral roots per mm.

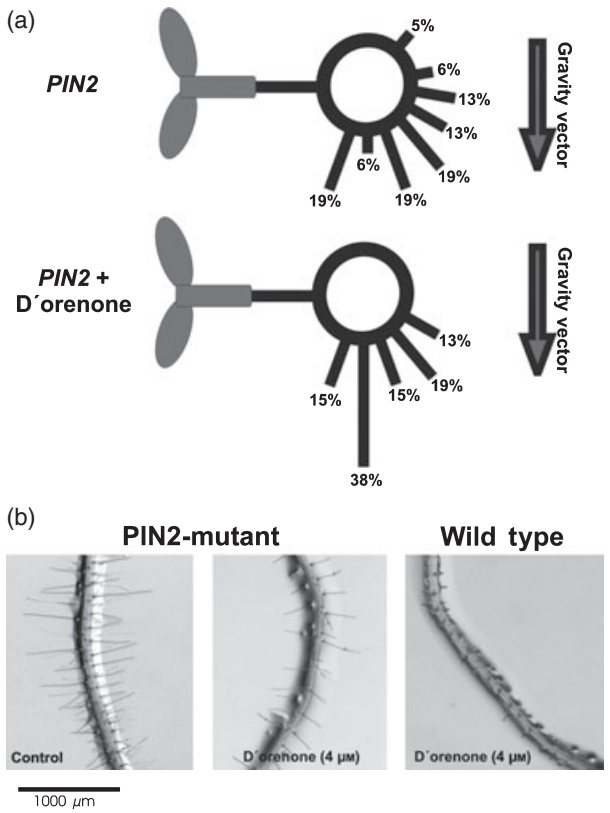


Figure 5. D'orenone rescues the agravitropic phenotype of leaky PIN2 mutant roots.
(a) The leaky *pin2* mutant line (*agr1-2*) with less PIN2 protein than WT has an agravitropic phenotype. D'orenone rescued the agravitropic phenotype of these mutant roots. Data are for 25 plants per experiment.
(b) In contrast to WT roots, D'orenone does not completely block root hair tip growth in the *agr1-2* mutant line.

growth of the main root. Even at concentrations of up to 40 μM, D'orenone did not inhibit primary root growth significantly but increased the number of lateral roots. Moreover, D'orenone rapidly and significantly activates the DR5 promoter, suggesting that this apocarotenoid interacts with auxin signaling at the root apex. As PINs localize dynamically to the plasma membrane and become rapidly internalized, as revealed by treatment with the vesicle-recycling inhibitor brefeldin A (Baluška *et al.*, 2002; Geldner *et al.*, 2001), it is apparent that recycling of PINs is part of the auxin signaling pathway (Baluška *et al.*, 2008; Li *et al.*, 2005; Xue *et al.*, 2007). Moreover, vesicular trafficking of PIN2 is relevant with respect to its proteasomal degradation (Abas *et al.*, 2006; Sieberer *et al.*, 2000), which is accomplished within multivesicular bodies (Jaillais and Gaude, 2007; Jaillais *et al.*, 2006, 2008).

Exogenous auxin fully rescues the D'orenone-inhibited root hairs, resembling the situation in which over-expression of auxin efflux transporters in trichoblasts imposes a block on the tip growth of root hairs that can be over-ridden by exogenous auxin (Cho *et al.*, 2007; Lee and Cho, 2006). All this is in agreement with the model, originally proposed by Lee and Cho (2006), according to which the tip growth of root

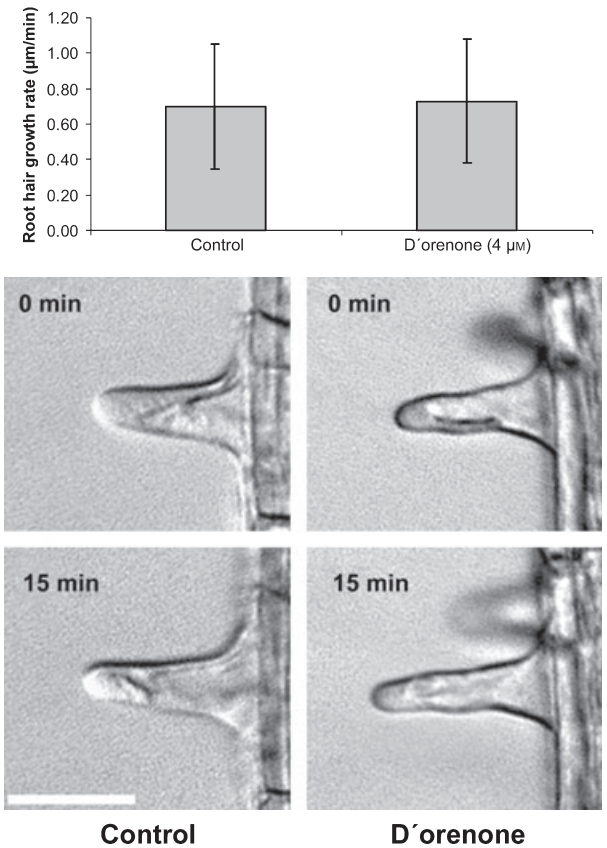


Figure 6. D'orenone has no effect on root hair growth in a PIN2 null mutant. Treatment with D'orenone (4 µM) showed no visible effects on the root hair growth rate and root hair cytoarchitecture in the *pin2* null mutant line (*eir1-4*).

hairs is tightly controlled by critical endogenous levels of auxin within the trichoblasts (Cho *et al.*, 2007; Lee and Cho, 2006). The most critical question with respect to D'orenone action is what the molecular target(s) of this compound is.

The obvious candidate is PIN2, as this auxin efflux transporter is both expressed and active in root hair trichoblasts driving basipetal auxin transport (Chen *et al.*, 1998; Müller *et al.*, 1998; Rashotte *et al.*, 2000; Utsuno *et al.*, 1998) towards the transition zone and distal elongation zone (Verbelen *et al.*, 2006), where the PIN2-driven auxin stream is re-directed from the root periphery towards the central cylinder to join the acropetal auxin stream (Blilou *et al.*, 2005). In support of this scenario, D'orenone significantly increases PIN2 protein abundance and shifts the PIN2 domain into the elongation region. In this respect, D'orenone resembles the effects of brassinosteroids (Li *et al.*, 2005). As PIN2 transcription is not affected, it may be concluded that D'orenone extends the half-life of the PIN2 protein via manipulation of proteasomal degradation (Abas *et al.*, 2006; Sieberer *et al.*, 2000). Interestingly, PIN2 degradation is stimulated at the upper side and slowed down at the lower side of gravi-stimulated root apices (Abas *et al.*, 2006; Jaillais *et al.*, 2006), and over-stabilization of PIN2 results in defective root gravitropism (Abas *et al.*, 2006).

To assist in identification of PIN2 as the potential D'orenone target, we used two *pin2* mutant lines that differ in the stringency of PIN2 removal. The *arg1-2* line is leaky, having a small amount of PIN2 left, and it reacts to D'orenone exposure, but to a lesser extent than wild-type. On the other hand, *eir1* is null mutant completely lacking PIN protein, and this line is not sensitive to D'orenone, providing crucial genetic evidence regarding the PIN2 specificity of D'orenone action on root hairs and root apices.

Our data reveal an increase in PIN2 within vacuole-like compartments of D'orenone-exposed roots, resembling the increased vacuolar localization of excess PIN2 in the PIN2 over-expressing line (Abas *et al.*, 2006). Similar vacuolar-like PIN2 localization was reported after treatment of roots with the phosphatidyl-3-OH kinase inhibitor wortmannin (Jaillais *et al.*, 2006) or the actin polymerization inhibitor latrunculin B (Rahman *et al.*, 2007). This strengthens the hypothesis that D'orenone targets processes that are related to PIN2 degradation, causing slower turnover and increased protein levels of this auxin efflux transporter. As nexin 1-defined endosomes are involved in both recycling and targeting of PIN2 for degradation (Jaillais and Gaude, 2007; Jaillais *et al.*, 2006, 2008), further studies will focus on this critical sorting platform of root cells, which integrates sensory information into adaptive and exploratory behavior of plant roots.

D'orenone has been previously postulated as an early intermediate in the biosynthesis of trisporic acids, which act as chemical signals between the (+)- and (−)-mating types of zygomycetes (Gooday, 1978, 1983; Schachtschabel *et al.*, 2005; Schachtschabel and Boland, 2007). Even slight structural modifications of D'orenone result in a strongly reduced activity of the compound. In particular, the low activity of the 3,4-dihydro derivative is important (Figure 1), as this structural modification separates D'orenone from the structurally related fungal trisporates that act as morphogenetic signals between the mating partners of zygomycetes. It is tempting to speculate that this ketone could resemble or mimic an unknown endogenous retinoid signal that interacts with particular branches of the auxin signaling pathway. D'orenone itself, or a closely related apocarotenoid structure, could be the as yet undefined plant hormone suggested by Bennett *et al.* (2006) and Schwartz *et al.* (2004). This scenario is supported by the fact that primary root growth remains virtually unaffected, whereas root hair growth is clearly inhibited, concomittant with an increased amount of PIN2 protein and an enlarged and shifted PIN2-expressing tissue domain in D'orenone-exposed root apices. It is interesting to note that D'orenone is produced, along with other apocarotenoids, by cyanobacterial enzymes from *Synechocystis* spp. *in vitro*. Even higher plants seem to be able to generate D'orenone from certain apocarotenoids, as has been recently shown for a carotene oxygenase from rice

(Salim Al-Babili, University of Freiburg, Germany, personal communication).

In conclusion, D'orenone might act a hormone-like signaling molecule, closely resembling hypothetical branching factors (Bennett *et al.*, 2006; Sieberer *et al.*, 2006), and/or as an inter-organismic signaling molecule for complex fungal-plant root communication (Prusty *et al.*, 2004; De Meutter *et al.*, 2005; Bianco *et al.*, 2006a, b; Curtis, 2007). D'orenone has the potential to become a valuable tool by which to dissect the integrated processes that underlie sensory-driven PIN2-mediated root growth in general (for salt stress see Sun *et al.*, 2008; Li and Zhang, 2008), and distinguish them from those processes controlling the polarized tip growth of root hairs (Šamaj *et al.*, 2004). In the root apex, D'orenone specifically interacts with PIN2-mediated auxin transport. D'orenone holds the key to understanding how integrated auxin signaling is linked to the complex auxin transport networks of the root apex. As auxin is an ancient signaling molecule that is also active in bacteria, fungi and animals (Prusty *et al.*, 2004; Bianco *et al.*, 2006a, b; Ditengou *et al.*, 2003), a better understanding of this phenomenon will have relevance beyond the plant sciences.

Experimental procedures

Plant material and treatments

Seeds of *Arabidopsis thaliana* (ecotype Columbia) or the *agr1/pin2* mutant (Chen *et al.*, 1998) were surface-sterilized and placed on half-strength MS culture medium (Murashige and Skoog, 1962) without vitamins and containing 1% sucrose, solidified using 0.8% phytagel (Sigma-Aldrich, http://www.sigmaaldrich.com). The plates were store at 4°C for 48 h to break dormancy, and then kept vertically under continuous light for 3–4 days. Alternatively, plants were grown in darkness for 1 week in order to calculate the effects of various concentrations of D'orenone.

Seedlings that were 3–4 days old were transferred to microscopic slides that had been modified into thin chambers using cover slips. The chambers were filled with the same liquid medium without phytagel and placed in sterile glass cuvettes containing medium at a level that reached the open lower edge of the chambers. This allows free exchange of medium between chambers and the cuvette. Seedlings were grown in the vertical position under continuous light for up to 24 h. During this period, root growth stabilized and new root hairs were produced. Inhibitors and chemicals for treatments (retinal, retinol, retinoic acid, wortmannin, IAA, D'orenone and analogues) were added to the culture medium. D'orenone, D'orenol and 3,4-dihydro-D'orenone were synthesized as described previously (Schachtschabel and Boland, 2007). All other inhibitors were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/). Imaging was performed using a confocal laser scanning microscope and binocular microscope (ICS Leica, http://www.leica-microsystems.com) using discus software.

FM4-64 labeling

Roots were incubated for 10 min with 5 μM FM dye at 4°C after various treatments and washed before observation.

Auxin flux measurement

Auxin flux measurements were performed as described previously (Bouchard *et al.*, 2006; Mancuso *et al.*, 2005, 2007). The IAA influx profile along root apices of Arabidopsis was monitored using an IAA-selective microelectrode placed 2 μm from the root surface and used in a self-referencing mode. The sensor was vibrated between two positions 10 μm distant at a rate of 0.1 Hz.

Stable GUS and GFP fusion protein-expressing Arabidopsis lines

PIN2p::PIN2-GFP has been described previously by Shin *et al.* (2005), and *PIN1p::PIN1-GFP* has been described previously by Vieten *et al.* (2005). The auxin-response element $DR5_{rev}$–GFP line was used under the same conditions as described by Schlicht *et al.* (2006). The *PIN2p::GUS* line was characterized by Malenica *et al.* (2007) and Shin *et al.* (2005). The *PIN2p::PIN2-GUS* line used to visualize PIN2 protein in plant tissues has been described previously (Sieberer *et al.*, 2000).

Acknowledgements

Financial support by grants from the Bundesministerium für Wirtschaft und Technologie (BMWi) via Deutsches Zentrum für Luft- und Raumfahrt (DLR, Cologne, Germany; project 50WB 0434), from the European Space Agency (ESA-ESTEC, Noordwijk, The Netherlands; MAP project AO-99-098), and from the Ente Cassa di Risparmio di Firenze (Italy) is gratefully acknowledged. F.B. also receives partial support from the Grant Agency VEGA, Bratislava, Slovakia (project 2/5085/25) and from the Grant Agency APW, Bratislava, Slovakia (project APVV-0432-06). W.B. and D.S. thank the Deutsche Forschungsgemeinschaft for support within the Priority Programme 1152 'Evolution of Metabolic Diversity'. We thank Jiri Friml for the DR5::GFP line, Patrick Masson for the *agr1-2* mutant line, Rujin Chen for the PIN1/2–GFP lines, and Christian Luschnig for the *PIN2p::GUS* line, *PIN2p::PIN2-GUS* line, *pin2* null mutant line (*eir1-4*) and the PIN2 antibody. We also thank Mrs Emily Wheeler for editorial assistance.

Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Effect of D'orenone onGFP-labeled actin bundles in growing root hairs.

Figure S2. Nitroblue tetrazolium staining of mock-treated (control) and D'orenone-treated root hairs showing production of reactive oxygen species, and Fluo 3-AM staining of mock-treated (control) and D'orenone-treated root hairs showing the distribution of cytosolic Ca^{2+}.

Figure S3. 2xFYVE–GFP localization in growing root hairs.

Figure S4. Treatment with the secretion inhibitor brefeldin A.

Figure S5. Gravi-stimulated roots during D'orenone treatment still perceive direction of gravity, but the growth behaviour shows a reduced sensitivity of the root apex.

Appendix S1.

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