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Inhibition of auxin transport and auxin signaling and treatment with far red light induces root coiling in the phospholipase-A mutant *ppla-I-*1. Significance for surface penetration?

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ABSTRACT

When grown on a non-penetretable at a surface angle of 45°, Arabidopsis roots form wave-like structures and, in wild type rarely, but in certain mutants the tip root even may form circles. These circles are called coils. The formation of coils depends on the complex interaction of circumnutation, gravitropism and negative thigmotropism where – at least – gravitropism is intimately linked to auxin transport and signaling. The knockout mutant of patatin-related phospholipase-AI-1 (*pplaI-1*) is an auxin-signaling mutant which forms moderately increased numbers of coils on tilted agar plates. We tested the effects of the auxin efflux transport inhibitor NPA (1-naphthylphtalamic acid) and of the influx transport inhibitor 1-NOA (1-naphthoxyacetic acid) which both further increased root coil formation. The pPLAI-1 inhibitors HELSS (haloenol lactone suicide substrate = E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one) and ETYA (eicosatetraynoic acid) which are auxin signaling inhibitors also increased coil formation. In addition, far red light treatment increased coil formation. The results point out that a disturbance of auxin transport and signaling is one potential cause for root coils. As we show that the mutant *pplaI-1* penetrates horizontal agar plates better than wild type plants root movements may help penetrating the soil.

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1. Introduction

At first glance root tips growing full circles, so-called coils, and roots growing in a sinusoidal form (waves), seem to be ephemeral, baroque and superfluous phenomena in primary roots. Formation of coils depends on circumnutation, gravitropism and negative thigmotropism (Migliaccio and Piconese, 2001; Migliaccio et al., 2013) and occur when roots grow on a non-penetrable surface (hard agar) on a usually 60°–45° tilted surface where upright is 90°. Tilting also induces roots to grow in a wavy fashion. Roots, like shoots, move their tips in a circle-like movement when unrestricted by soil or other obstacles (Okada and Shimura, 1990; Mullen et al., 1998; Kim et al., 2015). On 90° upright surfaces the circumnutation

of the root tip is down-regulated by gravitropism such that the root tip becomes straightened out and the root shows no or a very weak waving pattern (Thompson and Holbrook, 2004). On an impenetrable surface tilting of the root tip induces substantial movement to either side which, becomes "frozen" as a wave-like sinusoidal pattern with the tip moving generally down. Obviously, at some points in the wave the root tip does not perfectly point down but more or less sideways or even up. Gravitropism recurrently will correct this to growth downward. Especially in an agravitropic mutant, when gravitropic sensing is decreased, the tip may continue to grow upward and eventually form a full circle, called coil.

Negative thigmotropism is the phenomenon that a root retracts slightly from a hard and impenetrable surface when growing down and, instead, grows sideward (Massa and Gilroy, 2003). On a surface, circumnutation movement periodically tries to press the tip onto the surface, leading to a negative thigmotropic response, as well as to lift it slightly in a periodic pattern. It was speculated that, when looking down the root, the negative thigmotropic response happens preferentially only on one side and the lifting on the other

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so that, in summing up many such movements, an asymmetry in the overall direction of root growing down will be induced. This way, asymmetric growth is assumed to induce the so-called slanting angle which is usually a deviation to the left from the plumb line when looking onto the plant and the surface (Migliaccio and Piconese, 2001; Migliaccio et al., 2013). Likely in conjunction with clockwise circumnutation, the root coils in the wild type grow clockwise so that decreased gravitropic control of downward growth on a tilted surface also is asymmetric with preference of one side i.e. to the left.

The first six waving and coiling mutants were first described by Okada and Shimura (1990). Although probably often not systematically collected, many mutants of root growth patterns were described thereafter (Sedbrook and Kaloriti, 2008; Migliaccio et al., 2013). Out of the waving mutants *wav1* to *wav6* two genes were identified as belonging to auxin physiology, the influx transport protein *AUX1* as *WAV5*, the efflux transporter gene *PIN2/EIR1* as *WAV6*, and WAV2 belongs to the BUD EMERGENCE 46 gene family (Mochizuki et al., 2005) which is thought to inhibit root bending. Other genes affecting waving and coiling and functioning in auxin physiology are *RCN1*, a protein phosphatase subunit regulating PIN protein activity (Garbers et al., 1996), *AXR4*, a regulator of AUX1 (Dharmasiri et al., 2006), and *WAG1* and *WAG2* code for protein kinases that are related to kinases which regulate PIN protein activity (Santner and Watson, 2006). The coils producing mutants *mlo4* and *mlo11* code for receptors in pathogen perception and were identified as being involved in thigmotropism and gravitropism regulation (Chen et al., 2009; Bidzinski et al., 2014). We described the coil producing phospholipase A mutant *pplaI-1* as agravitropic and disturbed in red light physiology (Effendi et al., 2014). During this work, we realized that *phyB-9* mutant roots are agravitropic and also produce strongly increased coil numbers. It was shown in a space experiment that *pplaI-1* forms increased coil numbers in microgravity (Scherer and Pietrzyk, 2014). In a different group of waving and coiling mutants tubulin and tubulin-associated proteins are mutated (Sedbrook and Kaloriti, 2008; Migliaccio et al., 2013). They show as a phenotype right-handed slanting or counterclockwise coiling. Of many known root coiling or waving mutants the genes are not yet identified and probably many coiling or slanting/waving mutants were identified serendipitiously, not by systematic screening of, for instance, auxinic mutants.

The known mutant collection of coiling mutants suggested testing tools that were known from in auxin physiology and to also test them in their capacity to induce or decrease coils. The phospholipase A mutant *pplaI-1* was a suitable object because it belongs to the group of auxin signaling mutants and shows weak defects in gravitropism, phototropism and red light physiology and shows moderately increased coil numbers on tilted hard agar (Effendi et al., 2014) so that increases still can be monitored. Therefore, we tested with this mutant the influence of auxin efflux and influx inhibitors, pPLA inhibitors, and red/far red light as coil-inducing agents. As it is conceivable that root growth movements are integrated in the process of soil penetration (Inoue et al., 1999; Minorsky, 2003) we investigated this and found increased surface penetration rates in the *pplaI-1* mutant. Taken together, the results point out auxin transport and auxin signaling as important factors in root coil formation and surface penetration.

2. Materials and methods

Seeds for most experiments were stratified for 4 d and plated on ½ MS medium (2% (w/v) Bacto-agar) supplemented with 1% (w/v) sucrose and appropriate amounts of inhibitors in DMSO or mock. Inhibitors used were HELSS (haloenol lactone suicide substrate: E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one), 1-NOA (1-naphthoxyacetic acid), 2-NOA (2-naphthoxyacetic acid), 1-NPA (1-naphthylphtalamic acid) and ETYA (eicosatetraynoic acid). Plants were grown on petri dishes standing upright for 3 days in the light to orient the roots and for nine additional days or as indicated tilted to 45° where upright is corresponding to 90° (16 h light: 8 h dark; 50 μmol/m^{-2} × s^{-1}; fluorescent tubes) at 22.5 °C. After scanning (CanonScan 8800F; resolution 600 dot per inch) coils were counted as percent per plate. Red and far red light experiments were done with 100% MS medium and 2% (w/v) sucrose. The higher osmotic concentration of sucrose induces more coils (Effendi et al., 2014). For three days plants were kept upright in white light (16 h light: 8 h dark; 50 μmol/m^{-2} × s^{-1}), after that tilted to 45° and kept for additional six days in red or far red (350 μmoles/m^{-2} × s^{-1}) which was applied in an LED box at 22.5 °C (CLF, Plant Climatics) for another 6 days at 22.5 °C. For surface penetration experiments ½ MS medium was used and the agar concentrations as indicated. Surface penetration was counted by careful visual inspection of each plate separately. Parallel white light assays were kept in laboratory light as above. For statistics, percent values for one plate each were taken. Three independent experiments were made for each inhibitor concentration or irradiance with together 36–48 individuals. Error bars represent standard error.

To make a time laps movie plants on the plates were photographed with a Canon EOS D30 camera with macro lens every 20 min and pictures were combined to a movie by the program moviemaker®. Photos were taken from the backside of the plates so that in the movie sides are switched in comparison to other figures and influence of background light is noticeable. From right to left 8 seedlings can be seen, starting rightmost with a Ws wild type plant, then a *pplaI-1* plant, etc. alternating. Only *pplaI-1* seedlings formed coils. In the dark period a flashlight was used. Days are indicated by the circadian cotyledon movements and elongation pulses in the early morning time. The 2% agar plates (1/2 MS) were kept at 45° after three days upright growth and then kept 45° tilted with a day-night cycle in white light (16 h/8 h) for 6 days.

3. Results

Naphthylpthalamic acid (NPA) was found as an inhibitor of gravitropism (Geissler et al., 1985) and later identified as an inhibitor of PIN protein activity (Bailly et al., 2012). When we added increasing concentrations of NPA to wild type (wt Wassilewskia) and to *pplaI-1* at 1 μM NPA coil formation rose from about 10% (mock) to more than 40% in *pplaI-1* seedlings (Fig. 1). Induction of coils in the wt was not apparent, only at 0.1 μM NPA a small increase to about 7% was found. In all mock treatments in the wt taken together no coils were observed.

The root slanting angle was also markedly increased by NPA and stronger in the *pplaI-1* mutant than in the wt. Despite an increased slanting angle in the wt, however, no coils were observed in the wt as were in the *pplaI-1* mutant. This argues for an only loose correlation between c slanting angle and coil formation. At >0.5 μM NPA roots became increasingly diagravitopic which were clearly apparent at 1 μM NPA. At 5 μM NPA root directions became irregular but only incomplete coils were observed (not shown). Root growth was inhibited above 1 μM NPA as well as hypocotyl growth in both lines.

1-Naphthyloxyacetic acid (1-NOA) is an auxin influx transport inhibitor and 2-NOA is a weaker analogon (Imhoff et al., 2000; Parry et al., 2001). 1-NOA inhibits influx transport at the auxin transporter AUX1 which is expressed in the root tip and other tissues (Swarup et al., 2001). Addition of 1-NOA induced coils already at 0.1 μM strongly in the mutant *pplaI-1*. In the presence of 3 μM 1-NOA the increase was 63% in *pplaI-1* but none in the wt (Fig. 2A and B).
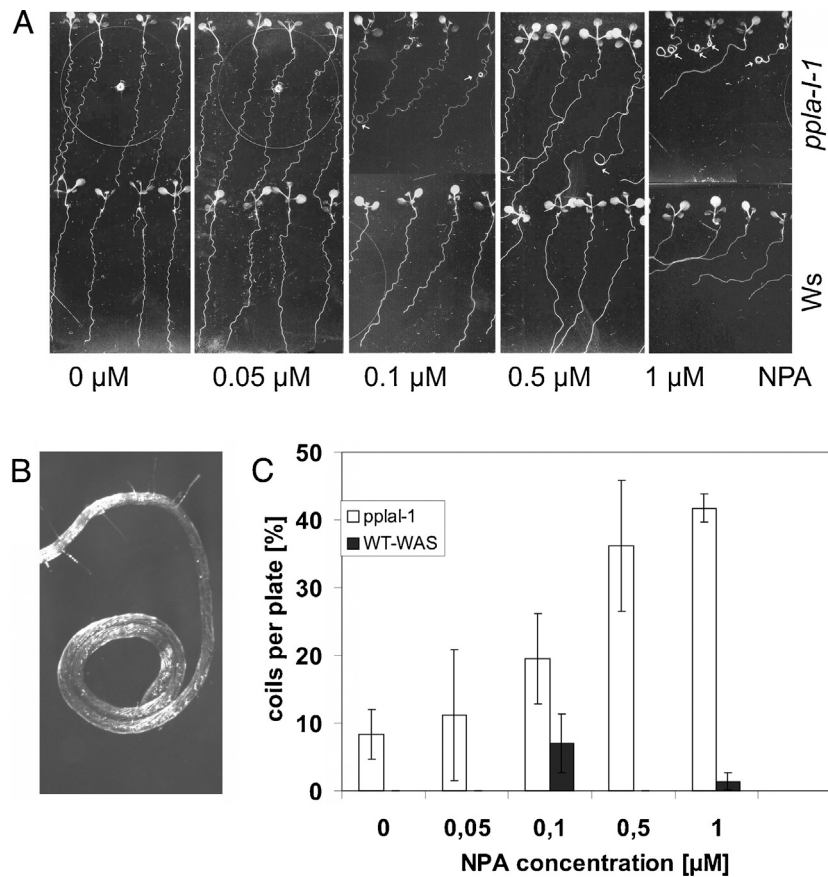
Fig. 1. Root coil formation in response to the auxin efflux inhibitor NPA. (A) Scans of seedlings. Arrows indicate coils. (B) Single coil from the mutant *pplaI-1*. (C) Quantification of coils. After three days of growth on upright plates coiling was induced by inclining the plates back by 45° relative to the horizontal surface and then keeping them 10 further days supplemented with NPA as indicated. Values are averages of six plates with 36–48 seedlings per concentration; S.E.

At 1 μM 1-NOA treatment by 2-NOA much weaker coil formation was observed. This reached about 40% in *pplaI-1* and was almost absent (about 5%) in the wt (Fig. 2C and D). Similarly as for NPA, root slanting angles increased in the presence of either inhibitor (Fig. 2C and D). Again in the wt, despite an increase in slanting angles no coil formation was observed in the wt, only in the mutant *pplaI-1*. Both inhibitors inhibited root and hypocotyl growth in both lines.

Auxin activates pPLA activity within about 2 min (Paul et al., 1998) and pPLA inhibitors inhibit auxin-induced elongation and gene expression (Scherer and Arnold, 1997; Scherer et al., 2007). Therefore, we tested the pPLA inhibitors HELSS and ETYA in our coiling tests (Fig. 3). Coiling increased from 0% in the mock assay to 25% at 20 μM HELSS in the wt and from 23% in the mock assay to 57% at 20 μM HELSS in the mutant *pplaI-1*. In the wt the pPLA inhibitor ETYA did neither induce coils in mock assays nor at 1 μM but in *pplaI-1* plants 31% coiling in mock controls and 55% coiling with 1 μM ETYA were found (Fig. 3C). The slanting angles increased at and above 5 μM HELSS in *pplaI-1* plants, a concentration at which coiling rates increased in *pplaI-1* as well but despite some apparent increase of slanting angles in the wt no coil formation was found in the wt. Also, growth inhibition of roots and hypocotyls was small so that again, the slanting angles or root growth (inhibition) provide no explanation for the coil formation despite similar trends in both parameters. In the patatin-related phospholipase A gene *pPLAI* is one in a family of ten genes (Scherer et al., 2010) and the results with pPLA inhibitors with *pplaI-1* indicated that *pPLA* genes other than *pplaI-1* participate in coil formation. So we tested knockouts of all other nine genes (Labusch et al., 2013) in the coil test (online resource Fig. S1 in the online version at DOI: 10.1016/ j.jplph.2016.03.010). Several of these knockouts indeed showed increased coil numbers. Genetic redundancy in the *pPLA* gene family for the property of coil formation is indicated by the results.

We had tested blue and red light receptor mutants for their ability to form coils in white light and only in the *phyB-9* mutant we found a strong increase in coil formation, not in *phyA-211, cry1, cry2, phot1* or *phot2* (Effendi et al., 2014). Therefore, we applied red, far red or white light to wt and *pplaI-1* seedlings. Coils were induced by red weakly and by far red light strongly in *pplaI-1* (Fig. 4). In the wt this response was much weaker.

It has been suggested that root movements may help to penetrate the soil (Inoue et al., 1999; Minorsky, 2003). Since root coiling may be viewed as a kind of mobility we wanted to test the penetrating ability of the wt and *pplaI-1* mutants on horizontal agar plates depending on the hardness of this surface. We found that the *pplaI-1* mutant at 1.0%–1.6% agar achieved about 15–20% higher surface penetration rate as compared to the wt (Fig. 5). The root tips were in the inside of coils and so the downward gravitropic movement of the tip may have been guided by this behaviour which may have increased the probability of surface penetration despite a slightly weaker gravitropic response of the mutant roots (Scherer and Pietrzyk, 2014). In a movie that we made on seedlings growing on 45° tilted agar plates (see online resource supplemental movie) several mutant seedlings forming coils can be viewed which continue to form coils inside the first coil and the root tips of *pplaI-1* seedlings. This keeps the root tip for several turns inside the coils. Wild type seedlings formed waves only. This exemplifies that the probability of forming a surface penetration attempt can be higher for the tip already being inside a coil.
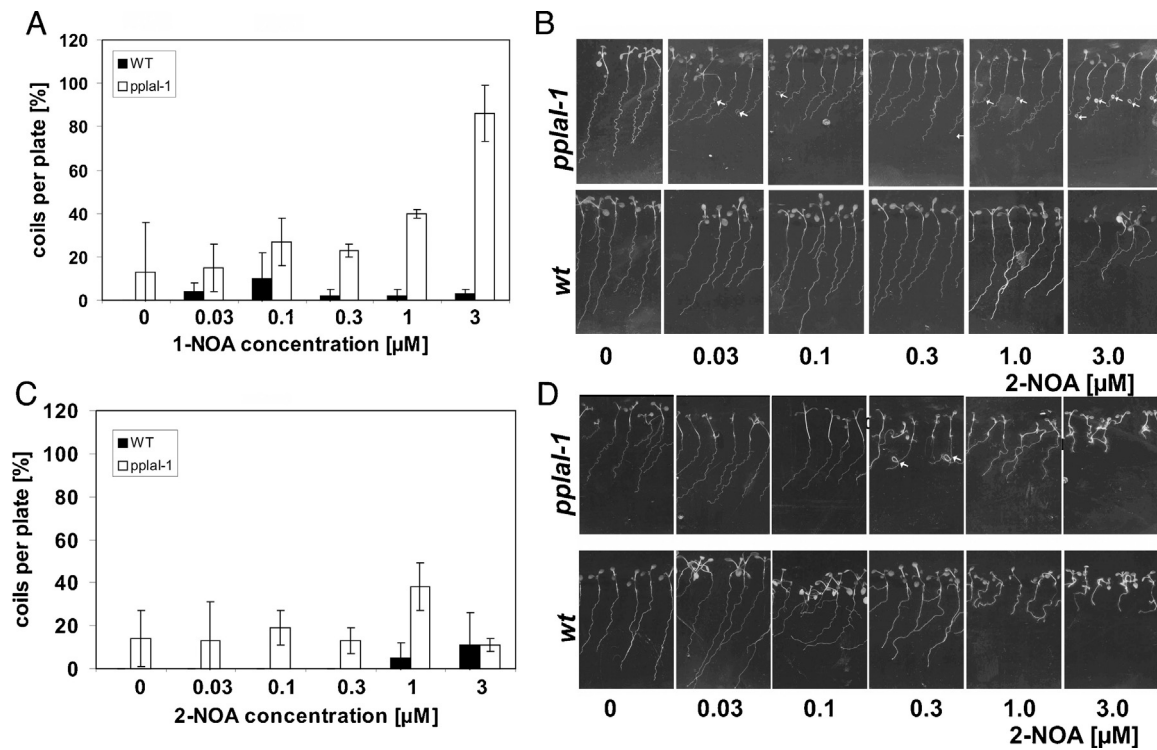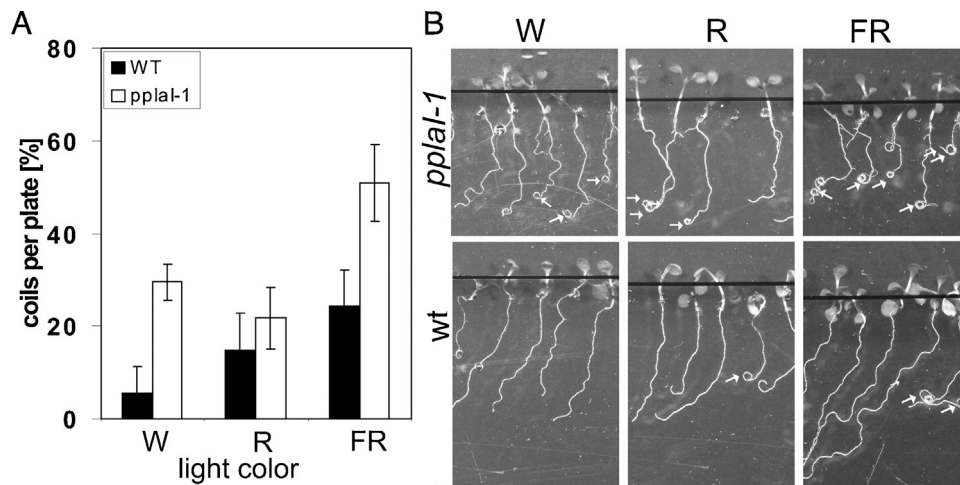
Fig. 2. Root coil formation in response to the auxin influx inhibitor 1-NOA and the analogon 2-NOA. (A, C) Quantification of coils. (B, D) Scans of seedlings. Arrows indicate coils. After three days of growth on upright plates coiling was induced by inclining the plates back by 45° relative to the horizontal surface and then keeping them 10 further days supplemented with inhibitors as indicated. Values are averages of six plates with 36–48 seedlings per concentration; S.E.
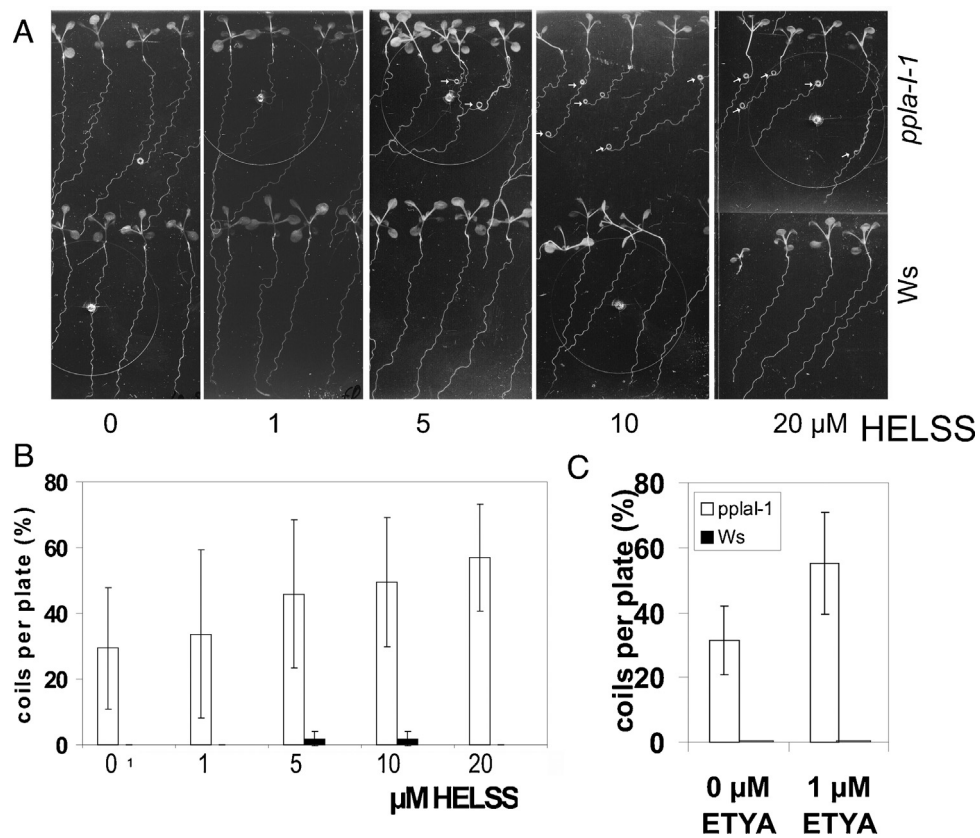

Fig. 3. Root coil formation in response to the auxin and phospholipase inhibitors HELSS and EYTA. (A) Scans of seedlings. Arrows indicate coils. (B, C) Quantification of coils. After three days of growth on upright plates coiling was induced by inclining the plates back by 45° relative to the horizontal surface and then keeping them 10 further days supplemented with inhibitors as indicated. Values are averages of six plates with 36–48 seedlings per concentration; S.E.

Fig. 4. Root coil formation in response to the red (3 μmol/m^{-2} × s^{-1} R), far red (3 μmol/m^{-2} × s^{-1} FR) and white (50 μmol/m^{-2} × s^{-1} W) light. (A) Quantification of coils. (B) Scans of seedlings. Arrows indicate coils. After three days of growth on upright plates coiling was induced by inclining the plates back by 45° relative to the horizontal surface and then keeping them 10 further days in different light conditions as indicated. Values are averages of six plates with 36–48 seedlings per concentration; S.E.
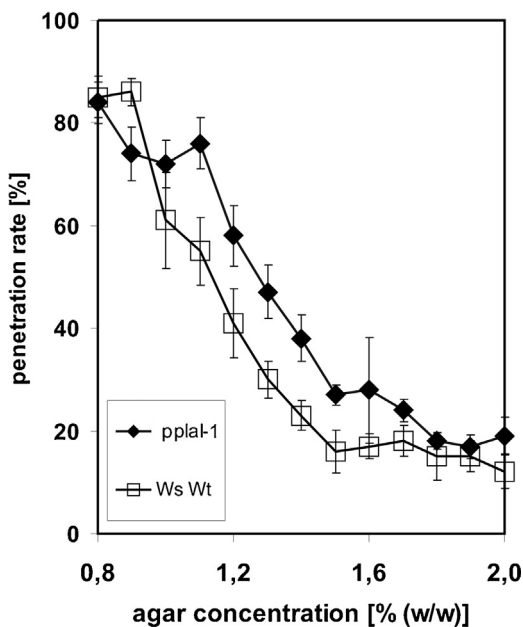
Fig. 5. Dependence of the surface penetration of agar surfaces by wt and *pplaI-1* roots on the agar concentration. Seedlings were grown for 10 days in white light on horizontal plates supplemented with ½ MS medium and increasing agar concentrations. Percentage of surface penetration was counted by visual inspection. (n = 40–45 per plate; 7 plates per average value; S.E.).

4. Discussion

Chemicals like NPA (Geissler et al., 1985) and 1-NOA (Imhoff et al., 2000; Parry et al., 2001) affect auxin transport and affect coiling. Inhibitors of pPLA (HELSS and ETYA) inhibit auxin signal transduction and elongation (Scherer and Arnold, 1997; Scherer et al., 2007) and all these relevant inhibitors increased coil formation. Far red light (Liu et al., 2011) inhibits auxin transport and induced coiling. Rapid regulation of auxin transport is a hallmark of root gravitropism (Friml et al., 2002; Ottenschläger et al., 2003) and regulation of auxin-dependent elongation is equally important in gravitropism. The pPLA inhibitor experiments with the mutant *pplaI-1* show that more pPLA's than only pPLAI participate in coil formation. Thus, our physiological results are in line with the knowledge about many known mutants of waving and coiling most of which are affected in gravitropism and auxin transport (Bennett et al., 1996; Müller et al., 1998) or are modifiers of auxin transport (Hobbie and Estelle, 1995; Garbers et al., 1996; Dharmasiri et al., 2006; Chen et al., 2009; Effendi et al., 2014) or of auxin signal transduction (Effendi et al., 2014).

Circumnutation, negative thigmotropism and gravitropism are postulated to make waving and coiling as root growth patterns (Migliaccio and Piconese, 2001; Migliaccio et al., 2013). Another explanation of root movements is provided by Tan et al. (2015) who describe them as responses to cues outside of the root. This description, however, does not include circumnutation which is autonomous. Waving and coiling can be induced in Arabidopsis by tilting agar plates from a 90° angle (with respect to the horizontal support) to a 60°–45° (Simmons et al., 1995). Without additional stimuli e.g. high solute concentration (Buer et al., 2000) usually only mutants form coils but waves originate on a tilted surface in wt plants (Thompson and Holbrook, 2004) but not on a vertical 90° surface.

On a 45° tilted surface the root tip will sense the impenetrable surface as an obstacle leading to a negative thigmotropic reaction (Massa and Gilroy, 2003). Negative thigmotropism leads the root tips to move sideward until it can grow down freely again. Though an agar surface at 45°–60 is an obstacle to a downward growing root tip the thigmotaxis experiment by Massa and Gilroy cannot suggest a simple rule for the formation of root growth patterns like coils. Circumnutation is thought to occur also on a 45°–60° surface but its consequences for root growth patterns are not known in detail. It should periodically lead to stronger contact with the surface and is probably the main component to induce slanting. Gravitropism guides the root down.

Though these three responses circumnutation, negative thigmotropism and gravitropism, are taken as a quite plausible explanation for coiling (Migliaccio and Piconese, 2001), the interactions of all three factors are not clear in detail. Potentially, a strong pressing of the root tip to the surface by circumnutation could lead to sideward growth as in negative thigmotropism. Adding circumnutation and thigmotropism up in several periodic events this could lead to asymmetrical growth to the left as it is observed when waves and a slanting angle of the root are formed. Slanting increased with all inhibitors and in red light in the wt and thi response was always stronger in the mutant but did not lead to coil induction the wt (compare Fig. 2B and D) so that the correlation of slanting - which could be based on circumnutation - and coil induction remained unclear.

Root circumnutation is correlated with periodic changes in H^+ and Ca^{2+} activity in the root (Shabala and Newman, 1997) and activation of the proton pump is a hallmark of auxin action (Hager 2003; Takahashi et al., 2012) whereas the link of auxin to calcium physiology is much less clear (Di et al., 2015). Circumnutation of the shoot is enhanced by gravity as experiments in space showed (Brown et al., 1990; Johnson, 1997; Hatakeda et al., 2003) and, in the root, gravitropism is linked to circumnutation (Mullen et al., 1998; Minorsky, 2003; Kim et al., 2015). The clockwise tip growth direction is enhanced when gravity, by positive gravitropism, and light from above, by negative phototropism, direct the root the same direction. Clock-wise direction is decreased when light is applied form below so that both direct the root into an opposing direction (Mirza, 1987) which, however, was not tested here. The similarity in the frequencies of the waving pattern and the circumnutation pattern of roots suggested that circumnutation may cause the thigmotropic force when tactile stimulation starts (Mullen et al., 1998). In agreement with observations on shoots (Johnson, 1997) differences in growth rate did not correlate with the period of the oscillations. However, recently the *ageotropum* pea mutant was identified also as a modifier of circumnutation (Kim et al., 2015) but other agravitropic mutants were not investigated under the viewpoint as potential circumnutation mutants so that circumnutation mutations or negative thigmotropism mutants are not known. Thus, the quantitative impact of these two parameters on coiling remains unclear.

After a period of sideward growth of the tip, gravitropism will lead the root tip downward so that this would shape periodically the downward growing part of the waves (see only resource Supplemetary Video S1 in the online version at DOI: 10.1016/j.jplph.2016.03.010). Waving periodicity and shape are genetically determined i.e. were changed in several mutants. Four of these six genes were identified in waving mutants (Okada and Shimura, 1990). Two of those, *AUX1* and *PIN2*, affect gravitropism (Bennett et al., 1996; Friml et al., 2002), one is an E3 ligase with unknown function, and one belongs to the BUD EMERGENCE 46 gene family (Mochizuki et al., 2005). Reduced gravisensing is clearly correlated with coil formation, e.g. in the mutants *pin2, aux1*, and *pplaI-1*. In conclusion, the choices of our inhibitors and far red light experiments find a clear correlation in the properties of most of the known coiling/waving mutants as auxinic mutants related to auxin signal transduction, auxin transport and gravitropism.

Root tip mobility could be related to soil penetration of the tip. Literature on root tip mobility or movements and soil penetration is scarce. Deep water rice seedling roots forming large spiral angles are more effective in colonization of soil than those with smaller ones. They make 2–3.5 rotations per day. Faster seedlings are not so efficient (Inoue et al., 1999). However, the conditions in water and a soft soil may be different from our conditions with hard agar and a comparatively dry surface. Our coiling experiments (Fig. 4) point out a role for far red light. Red light inhibits auxin transport and far red light decreases this effect (Liu et al., 2011) which could provide the link between red light and root movements, inducing coil formation as a consequence of disturbances in auxin transport. Our far red experiments, the high coiling rate of *phyB-9,* and the increased surface penetration of *pplaI-1* (which is a coiling and red light signaling mutant (Effendi et al., 2014)) suggest as a testable speculation that a seedling shaded by green plants might bury the root tip faster in the soil than in white light.

5. Conclusion

In the pplaI-1 mutant disturbing auxin signaling and transport by inhibitors and by far red light are potential causes for root coils. Increased root movements may help penetrating the soil.

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