Molecular Genetics of Root Gravitropism and Waving in *Arabidopsis thaliana*

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**ABSTRACT**

When *Arabidopsis thaliana* seedlings grow embedded in an agar-based medium, their roots grow vertically downward. This reflects their ability to sense the gravity vector and to position their tip parallel to it (gravitropism). We have isolated a number of mutations affecting root gravitropism in *Arabidopsis thaliana*. One of these mutations, named *arg1*, affects root and hypocotyl gravitropism without promoting defects in starch content or in the ability of seedlings' organs to respond to plant hormones. The *ARG1* gene was cloned and shown to code for a protein with a J domain at its amino terminus and a second sequence motif found in several cytoskeleton binding proteins. Mutations in the *ARG1* locus promote a strong defect in root gravitropism. Some alleles also confer an increased root resistance to exogenous ethylene and an increased sensitivity to auxin. *ARG1* was cloned and found to encode a putative transmembrane protein which might be involved in polar auxin transport, or in regulating the differential growth response to gravistimulation. When *Arabidopsis* seedlings grow on the surface of agar-based media tilted backward, their roots wave. That wavy pattern of root growth derives from a combined response to gravity, touch and other surface-derived stimuli. It is accompanied by a reversible rotation of the root tip about its axis. A number of mutations affect the presence or the shape of root waves on tilted agar-based surfaces. One of them, *wwe1*, promotes the formation of compressed root waves under these conditions. The physiological and molecular analyses of this mutant suggest that a tryptophan-derived molecule other than IAA might be an important regulator of the curvature responsible for root waving.

**UTILIZATION OF ARABIDOPSIS THALIANA TO STUDY THE PATTERNS OF ROOT GROWTH UNDER DEFINED CONDITIONS**

*Arabidopsis thaliana* as a model for molecular genetic studies in plants

*Arabidopsis thaliana* is a good model system for a molecular genetic study of the mechanisms involved in plant tropisms. Its small size allows the plating of a large number of seedlings in Petri dishes, thereby allowing large-scale screenings of mutagenized populations for defects in tropisms. Also, its short generation time, large seed set produced by self-pollination, small genome, well-defined genetic, RFLP and physical maps, the existence of a genome sequencing project and the efficiency of DNA transformation contribute to the development of *Arabidopsis* as an excellent tool for molecular genetic studies in plants (Somerville and Meyerowitz, 1994).

**PATTERNS OF ARABIDOPSIS ROOT GROWTH IN PETRI DISHES**

Pattern of root growth when seedlings are embedded in an agar-based medium: root gravitropism

When *Arabidopsis thaliana* seedlings are embedded in an agar-based medium in vertical Petri dishes, their hypocotyls grow vertically upward and their roots grow vertically downward, along the gravity vector (Masson et al., 1993; Simmons et al., 1995). Gravitropism defines the mechanisms allowing plant organs to use the gravity vector as a guidance stimulus for growth. Roots usually undergo positive gravitropism, while shoots usually develop a negative gravitropic response. In roots, the columella cells of the root cap perceive the gravity vector through the sedimentation of dense amyloplasts (Sack, 1991; Kuznetsov and Hasenstein, 1997). Somehow, amyloplast sedimentation triggers a signal transduction pathway which results in a redistribution of plant growth regulators (auxin and apoplastic Ca$^{2+}$) across the tip, with accumulation at the bottom of a gravistimulated root. That gradient is then transmitted to the distal and main elongation zones where a differential cellular elongation of opposite root flanks occurs, resulting in the development of a curvature (Figure 1; reviewed in Masson, 1995). Eventually, linear growth resumes after the tip has reached a
Figure 1. The Various Phases of Root Gravitropism, with Arabidopsis Mutations Affecting Them. A. Arabidopsis thaliana seedlings are gravistimulated by a 90° reorientation within the gravity field. B. Consequently, amyloplasts (AM) sediment in the columella cells of the root cap. The nucleus (N) is located at the center of these cells, while the endoplasmic reticulum (ER) is located at their periphery. C. Following amyloplast sedimentation, a gradient of apoplastic Ca²⁺ and auxin is established across the root tip. D. That gradient is transmitted to the distal elongation zone (DEZ) and main elongation zone (MEZ), triggering a differential cellular elongation on both flanks, resulting in the development of a curvature (E). The mature zone (MZ) is not involved in that response. Mutations affecting each phase in Arabidopsis thaliana are represented on the right of the figure. More information on these mutants is provided in Lomax (1997).
position within a set angle from the vertical (gravitational set point angle).

Amyloplast sedimentation is implicated as a gravity susceptor by the fact that growth conditions or mutations which result in alterations of starch content in the root cap also promote gravitropic defects, and by the fact that mutants with intermediate accumulation of starch show intermediate gravitropic responses (Kiss et al., 1996). More importantly, horizontal displacement of amyloplasts by application of a high gradient magnetic field results in the development of a curvature toward the displaced amyloplasts in roots and away from them in shoots (Kuznetsov et al., 1996). Interestingly, neither amyloplast displacement nor curvature develop in starch deficient mutants (Kuznetsov et al., 1996). Furthermore, a curvature in the direction of displaced amyloplasts in shoots was observed when lazy2 tomato mutants were exposed to red light before being assayed (Kuznetsov and Hasenstein, 1997). Taken together, these results strongly argue that sedimentable amyloplasts are the mass that gravity acts upon in gravitropic sensing (Sack, 1991).

How the information derived from amyloplast sedimentation is converted into a physiological output which results in a redistribution of plant growth regulators across the root tip is not understood. Several models attempt to explain this phase of gravitropism. One model proposes that amyloplast sedimentation exerts tension on the cytoskeleton, resulting in a stretching of membranes with concomitant opening of stretch-activated channels (Sievers et al., 1991). This transient opening of stretch-activated channels would allow an increase in cytosolic Ca^{2+} levels, triggering a signal transduction pathway resulting in the development of Ca^{2+} and auxin gradients across the root tip (Sievers et al., 1991; Pont-Lezica et al., 1993; Sack, 1997; Baluska and Hasenstein, 1997).

According to this model, the receptor, or structure that interprets the directional signal derived from amyloplast sedimentation into a physiological output: (Sack, 1991), would be the cytoskeleton. Interestingly, indirect evidence indicates that the columella amyloplasts are entrapped in a dense network of short and intricate microfilaments (Baluska and Hasenstein, 1997). Furthermore, several inhibitors of stretch-activated channels, of calmodulin (CaM) and of Ca^{2+}/CaM dependent protein kinases affect gravitropism. However, there is no convincing evidence yet for the existence of gravitropic-related cytosolic Ca^{2+} changes (Legué et al., 1997).

Another model proposes that the gravity receptor in the columella cells is the endoplasmic reticulum present at the bottom side of these cells, and on which amyloplasts sediment. When a plant is reoriented within the gravity field, the change in position of these amyloplasts might be sensed through its effects on the ER membrane (Sack, 1991).

In any case, amyloplast sedimentation seems to promote a redistribution of plant growth regulators across the root tip, with accumulation at the bottom side (reviewed in Masson, 1995, and Lomax, 1997). Among the redistributed molecules are apoplasic Ca^{2+} and IAA. Both have been shown to negatively regulate root growth. The cross-root gradient is transmitted to the distal and main elongation zones where it promotes a differential growth resulting in the development of a curvature. Interestingly, applications of Ca^{2+} chelators and of inhibitors of polar auxin transport abolish root gravitropism. Also, auxin resistant mutants develop altered root responses to gravistimulation (reviewed in Lomax, 1997).

A large body of research has been and is devoted to the characterization of the molecular and physiological mechanisms involved in auxin transport and response. However, much less research has been aimed at understanding the mechanisms by which amyloplast sedimentation results in a redistribution of auxin and apoplasic Ca^{2+} across the root tip. Similarly, little molecular information is available to explain how that gradient is being transmitted to the distal and main elongation zones where gravity-induced differential growth occurs. My laboratory is studying these early phases of root gravitropism.

Molecular genetics of root gravitropism in Arabidopsis thaliana

Mutants affecting root gravitropism can be identified in populations of mutagenized Arabidopsis seedlings by growing them in vertical agar-based media for a few days, then rotating the plates by 90°. While wild type roots tend to reorient their growth toward the vertical, gravitropic mutant roots curve much slower than wild type, or do not curve at all. Similarly, the growth vectors of mutant roots are more widely distributed around the vertical than those of wild type roots before reorientation (Bullen et al., 1990; Okada and Shimura, 1992; Masson et al., 1993). In an attempt to identify genes involved in gravity sensing, early phases of gravity signal transduction or signal transport to the site of response, we have screened various libraries of T-DNA or Ds-mutagenized Arabidopsis lines for mutants that were affected in root gravitropism but did not develop increased resistance to exogenous auxin (Masson et al., 1993). Indeed, agravitropic mutants with increased resistance to exogenous auxin are likely to be affected in the response phase of gravitropism (Bell and Maher, 1990). Seven mutants falling in that category were identified and initially characterized. Two of them are described further in this review: arg1 and agrl.

arg1. arg1 (altered response to gravity) mutant seedlings are specifically affected in root and hypocotyl gravitropism, and do not display pleiotropic phenotypes. This lack of additional phenotypes in arg1 mutant seedlings suggests that ARGI is involved in gravity perception or in an early phase of gravity signal transduction (Sedbrook and Masson, 1997; Figure 1). Two alleles were identified (arg1-1 and arg1-2); both confer similar phenotypes to mutant seedlings.

Even though arg1-1 and arg1-2 were identified in a collection of T-DNA mutagenized lines, neither was
tagged by a T-DNA insert. Therefore, we relied on map-based strategies to clone the corresponding locus. DNA sequencing of both genomic and cDNA clones revealed that \textit{ARG1} is formed of 11 exons and 10 introns. The 1.9-kb \textit{ARG1} transcript carries an open reading frame which potentially encodes a 410 amino acid protein with interesting structural features (2). Its amino terminus is highly similar to the J domain found in dnaJ-like proteins. Typical dnaJ-like proteins contain 3 conserved domains at their amino end, including the J domain, a glycine-rich domain and a repeated cystein-rich domain, while their carboxy terminus is more divergent (Oh et al., 1993). The J domain is present in \textit{ARG1}; however, the glycine-rich and cystein-rich domains are absent, suggesting a more specific function for the gene. Interestingly, dnaJ-like proteins have been implicated as chaperones in protein folding, in protein compartmentalization within the cell and in specific events in various signal transduction pathways (Blumberg and Silver, 1991; Kimura et al., 1995).

Following the J domain in \textit{ARG1} is a linker region, immediately followed by a putative transmembrane domain, and then a probable coiled coil region with similarity to proteins known to interact with microtubule (e.g. INCENP) or microfilament (e.g. myosin and tropomyosin) components of the cytoskeleton (Sedbrook and Masson, 1997).

The involvement of the cytoskeleton in gravity sensing or response has been highly debated. While the cytoskeleton may play an important role in gravising, recent experiments have shown that neither microtubules nor microfilaments are essential for the differential growth response to gravistimulation (Baluska and Hasenstein, 1997). These observations are compatible with a role for \textit{ARG1} in gravity sensing and/or early phases of gravity signal transduction. Experiments are in progress to determine the subcellular localization of the \textit{ARG1} protein.

Sequence analysis revealed that \textit{argl-1} and \textit{argl-2} carry a single base pair insertion and deletion respectively which result in frameshifts in the middle of the gene for \textit{argl-1}, and at the very beginning of the gene for \textit{argl-2} (Sedbrook and Masson, in preparation). This implies that \textit{argl-2} is probably a null mutation. Yet, \textit{argl-2} roots and hypocotyls still respond partially to gravistimulation. Therefore, \textit{ARG1} function must be redundant with that of another gene, or \textit{ARG1} must facilitate the function of other molecules involved in the transduction of gravity signals in plants. Alternatively, it is also possible that more than one pathway senses and/or transduces the gravity signal into a growth response in plants (Masson, 1995; Barlow, 1995; Sack, 1997).

Even though \textit{argl} mutant seedlings present a specific defect in root and hypocotyl gravitropism, suggesting a specific involvement of \textit{ARG1} in gravitropism, preliminary data suggest that the \textit{ARG1} gene is expressed in all tissues of the plant (Sedbrook and Masson, unpublished data). Additionally, a highly conserved orthologous gene has been identified in \textit{C. elegans}, an organism for which no responses to gravity have been identified yet (Sedbrook and Masson, 1997; and unpublished data). These observations suggest that \textit{ARG1} may have other functions, in addition to its involvement in gravitropism. If such alternative functions exist in plants, we have to postulate that they are masked in \textit{argl} mutant seedlings by functional redundancy, or more simply that the right stimulus has not been tested yet on these plants. Work is in progress to answer these important questions.

\textit{argl} \textit{(aggravitropic)} mutant seedlings are characterized by a rather strong alteration in root gravitropism (Bell and Maher, 1980). Their hypocotyls also develop an abnormal gravitropic response early after germination, but that phenotype is not maintained in older seedlings (Sedbrook, Rosen, Hilson, Chen and Masson, unpublished data). Interestingly, some \textit{argl} alleles confer an increased sensitivity to exogenous auxin (Bell and Maher, 1990). The same strong alleles also confer an increased root resistance to exogenously applied ethylene. In fact, \textit{argl} is allelic to \textit{eir1}, a mutation identified in the Ecker lab conferring root-specific sensitivity to exogenous ethylene (Roman et al., 1995; Hilson, Chen and Masson, in preparation). This interesting combination of phenotypes (increased auxin sensitivity, increased ethylene resistance and altered root gravitropism) is quite unique, and suggests that \textit{ARG1} may be involved in auxin transport. Indeed, auxin efflux during polar auxin transport is ethylene sensitive (Suttle, 1988). Hence, if a mutation affects a gene coding for one of the multiple components of the auxin efflux carrier (Lomax, 1997), one expects that mutant plants will show an increased resistance to ethylene, as well as an increased sensitivity to exogenous auxin. It should however be cautioned that we have not yet eliminated the possibility that \textit{ARG1} might be involved in the regulation of some important aspect of the differential growth response to gravistimulation.

To gain more insight into the function of \textit{ARG1}, we also cloned it by using map-based cloning strategies (Chen, Hilson and Masson, unpublished data). DNA sequencing revealed that \textit{ARG1} potentially encodes a transmembrane protein.

In conclusion, the \textit{ARG1} gene may be involved in the efflux phase of polar auxin transport in the roots. The presence of transmembrane domains in the \textit{ARG1} protein supports that conclusion. Alternatively, it is possible that this gene is involved in some regulatory phase of the differential growth response to gravistimulation (Figure 1). Experiments are in progress to determine the pattern of \textit{ARG1} expression in \textit{Arabidopsis thaliana} root tips, to determine the subcellular localization of the corresponding gene product, and to identify genes coding for proteins which interact with \textit{ARG1} (Chen and Masson, unpublished).

Patterns of \textit{Arabidopsis thaliana} root growth on the surface of vertical agar-based media: root skewing

When wild type \textit{Arabidopsis thaliana} seedlings of most ecotypes are plated on the surface of vertical agar-based media, their roots develop a very different pattern of
growth than anticipated from typical orthogravitropism: while still growing downward, these roots deviate progressively from the vertical (skew), tending to reach a specific angle from the gravity vector (Simmons et al., 1995; Rutherford and Masson, 1996). Amazingly, roots always skew to the same direction on a surface for specific ecotypes, despite an absence of directional cues in that plane (Figure 2). That progressive deviation of root growth from the vertical is accompanied by a rotation of the root tip about its axis, resulting in the development of a rotation of the epidermal cell files on the root surface (Rutherford and Masson, 1996). The rate of cell file rotation (CFR) correlates with the degree of root curvature (Rutherford and Masson, 1996).

Root skewing is probably the result of a combination of two conflicting growth tendencies: gravitropism tending to force the root to grow downward, and root coiling, forcing it to coil right-handedly in a pattern not unlike that expected for a circulation-like process. Indeed, when Arabidopsis seedlings are clinirotated, their roots coil on the agar surface. Furthermore, root coiling also occurs when agravitropic mutants are grown on the surface of vertical agar-based media (Mirza, 1987; Simmons et al., 1995a, 1995b; Rutherford and Masson, 1996). Taken together, these data suggest that gravitropism corrects coiling, the combination of both processes resulting in a skewed pattern of root growth (Rutherford and Masson, 1996).

Mutations were identified that affect root skewing on vertical agar surfaces. These were classified into several categories, depending on their phenotype. Some of these mutations were shown to affect gravitropism, while others did not. Within the latter group, some mutants were shown to develop an exaggerated skewing root growth phenotype on vertical agar surfaces, while others grew vertically, and yet others carried roots that skewed to the left under the same conditions (Rutherford and Masson, 1996). The molecular characterization of these mutants should reveal some important features on the mechanisms involved in that fascinating surface-dependent root growth phenotype (Rutherford, Pearlman, Boonsirichai, Sedbrook, Schulz, Carroll and Masson, unpublished data).

Patterns of Arabidopsis thaliana root growth on tilted agar surfaces

When roots grow in soil, they often encounter obstacles in their path (soil particles, rocks, etc.). Hence, for a plant to survive, a root must be able to sense an obstacle in its path and to respond to it by changing its vector of growth. Therefore, at any time, roots are facing a number of contradicting vectorial stimuli, and they have to decide on a specific—and hopefully optimal—pattern of growth in agreement with the information provided by each one of these stimuli. Hence, signal integration is an important concept to take into consideration when thinking about patterns of root growth in a non-homogenous environment.

An experimental approach to the characterization of coordinated root responses to combinations of environmental stimuli was developed by Charles Darwin in 1880, and modified by Okada and Shimura (1990) for utilization with Arabidopsis thaliana. For that assay, seedlings are germinated and grown on surfaces tilted backward. Gravitropism tends to force roots to grow vertically downward. Hence, the tilted surface constitutes an obstacle in their path. Roots detect both the gravi- and touch stimuli, and respond to that combination of cues by developing a wavy pattern of growth (Figure 2). For Arabidopsis thaliana the tilted surface can be made of medium containing high agar concentrations that the roots cannot penetrate (Okada and Shimura, 1990).

Arabidopsis root waving on tilted agar surfaces is accompanied by a reversible rotation of the root tip about its axis. Hence, it involves a succession of left-handed and right-handed circumnutation-like processes similar to those responsible for root skewing on vertical agar surfaces (Okada and Shimura, 1990; Rutherford and Masson, 1996).

As expected from the data summarized above, all agravitropic mutants identified and tested thus far develop an abnormal root wave phenotype of tilted agar surfaces. Some of these mutants meander on such surfaces, while others coil. This demonstrates the involvement of root gravitropism in the development of a wavy pattern of root growth (Okada and Shimura, 1990; Simmons et al., 1995;
The Tryptophan Biosynthetic Pathway.
Dashed arrows indicate unresolved steps in the pathway (inspired from Niyogi, 1993).

Rutherford and Masson, 1996). On the other hand, the roots of a number of other waving root mutants develop a wild type gravitropic response. This implies that processes other than gravitropism are required for a wild type pattern of root waving. Some of the mutants in this group develop a compressed root wave phenotype, others loop rather than wave, and still others develop no or very dampened waves on tilted agar surfaces (Okada and Shimura, 1990; Simmons et al., 1995; Rutherford et al., 1996, 1997). These mutants are probably affected in the sensing and/or response to the various environmental cues responsible for the phenotype (touch and other surface-derived stimuli), or in structural molecules required for the development of a wild type pattern of root waving under these conditions (Rutherford et al., 1997).

As a first step toward understanding the molecular mechanisms involved in coordinating the plant organs' responses to multiple simultaneous environmental stimuli, we have screened for and identified waving root mutants of Arabidopsis thaliana. One of them, named wvc1 (wavy and compressed root phenotype), will be discussed here.

wvc1 mutant seedlings develop compressed root waves on tilted agar surfaces. The rates of root growth and waving of wvc1 mutant seedlings are similar to those of wild type, suggesting that wvc1 affects simply the shape of the waves (Rutherford et al., 1997). Similarly, wvc1 mutant seedlings develop a wild type response to gravistimulation.

We cloned the WVC1 locus to investigate its function. Results show that wvc1 derives from the insertion of a T-DNA in the coding region of the ASA1 locus. ASA1 codes for the alpha subunit of anthranilate synthase, an enzyme of the tryptophan biosynthetic pathway (Figure 3). That subunit of anthranilate synthase is in fact encoded by 2 genes (ASA1 and ASA2). ASA2 is constitutively expressed at a low level, while ASA1 is expressed at a higher level and is further upregulated in response to wounding and to compatible plant pathogens interactions (Niyogi and Fink, 1992). ASA1 is most strongly expressed in the root tip of Arabidopsis thaliana (Niyogi, 1993).

Because wvc1 is a mutation that affects a gene coding for an enzyme of the TRP biosynthetic pathway, we were able to test the ability of specific intermediates in that pathway to rescue the Wvc1 phenotype. Results indicate that anthranilate, but not chorismate, can rescue the phenotype. Similarly, tryptophan rescues the Wvc1 phenotype when added to the medium at low concentrations, while IAA or various potential IAA precursors in plants do not (Rutherford et al., 1997). Taken together, these data suggest that a TRP derivative, or TRP itself, can regulate the reversible root curvature that develops while roots wave on tilted agar surfaces. Because the rate of root growth is not affected in wvc1 mutant seedlings, we postulated that the phenotype does not derive from a deficiency in protein synthesis caused by a decreased cytoplasmic level of TRP (Rutherford et al., 1997). Interestingly, similar levels of free TRP were found in wild type and mutant seedlings and roots, further reinforcing that conclusion, and suggesting that wvc1 may affect the flow of molecules through TRP in the pathway more than the actual amounts of TRP in the tissues (Rutherford et al., 1997). Experiments are in progress to determine which TRP-derived molecule is responsible for that important regulatory function in root growth.

A number of other root wave mutants have also been identified and are being characterized in our laboratory. Several of these mutants develop compressed root wave phenotypes on tilted agar surfaces (wvc1-wvc26), while others develop no waves under these conditions (wvd1-wvd6). Still other mutants loop rather than wave on tilted agar surfaces. We recently found that several of these mutations are tagged by DNA inserts (T-DNA or transposable elements). Hence, their molecular characterization should be forthcoming, and will provide valuable information on the molecular mechanisms involved in generating and regulating that complex pattern of root growth in response to a combination of environmental cues.

To conclude, our research has focused on characterizing mechanisms by which Arabidopsis thaliana roots utilize complex information in their environment to control the pattern of their growth. Our genetic analysis of root gravitropism has allowed the identification of two loci involved in various phases of the process, and provided
tools that, in the future, could contribute to a better understanding of the involvement of the cytoskeleton in gravity sensing and/or signal transduction, as well as of the processes involved in signal transmission and/or in the response itself. Additionally, we have gained a better understanding of some of the mechanisms underlying the development of more complex growth patterns in response to combinations of environmental cues. In the long term, such studies should contribute to a better understanding of the mechanisms defining complex patterns of root growth in highly heterogeneous environments, such as the soil. Such information is required if one wants to better control that process for better crop productivity in sub-optimal environments such as microgravity.

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ROOT GRAVITROPISM AND WAVING IN A. THALIANA

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