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Evidence that L-Glutamate Can Act as an Exogenous Signal to Modulate Root Growth and Branching in *Arabidopsis thaliana*

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The roots of many plant species are known to use inorganic nitrogen, in the form of NO_3^-, as a cue to initiate localized root proliferation within nutrient-rich patches of soil. We report here that, at micromolar concentrations and in a genotype-dependent manner, exogenous L-glutamate is also able to elicit complex changes in *Arabidopsis* root development. L-Glutamate is perceived specifically at the primary root tip and inhibits mitotic activity in the root apical meristem, but does not interfere with lateral root initiation or outgrowth. Only some time after emergence do lateral roots acquire L-glutamate sensitivity, indicating that their ability to respond to L-glutamate is developmentally regulated. Comparisons between different *Arabidopsis* ecotypes revealed a remarkable degree of natural variation in L-glutamate sensitivity, with C24 being the most sensitive. The *aux1-7* auxin transport mutant had reduced L-glutamate sensitivity, suggesting a possible interaction between L-glutamate and auxin signaling. Surprisingly, two loss-of-function mutants at the *AXR1* locus (*axr1-3* and *axr1-12*) were hypersensitive to L-glutamate. A pharmacological approach, using agonists and antagonists of mammalian ionotropic glutamate receptors, was unable to provide evidence of a role for their plant homologs in sensing exogenous glutamate. We discuss the mechanism of L-glutamate sensing and the possible ecological significance of the observed L-glutamate-elicited changes in root architecture.

Keyword: *Arabidopsis thaliana* L. — Auxin — Lateral roots — Mutants — Natural variation — Root apical meristem.

Abbreviations: BMAA, β-methylamino-L-alanine; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GFP, green fluorescent protein; GluR, glutamate receptor; GUS, β-glucuronidase.

Introduction

One of the primary functions of a plant's root system is to capture water and mineral nutrients from the soil. To enable roots to explore the soil volume more effectively, they are equipped with sophisticated mechanisms for sensing and responding to a range of environmental stimuli, including light, gravity, moisture, touch and nutrients (Porterfield 2002). The ability to convert these environmental stimuli into appropriate developmental responses accounts for the high degree of morphological plasticity that is a feature of plant root systems (Bradshaw 1965). Many plant species respond to localized supplies of certain nutrients (such as nitrate and phosphate) with a localized proliferation of lateral roots within the nutrient-rich zone, a foraging response that serves to increase the precision of root placement within the soil (Hutchings and John 2004).

Although nitrate is the major source of N for plants growing in aerobic soils, it is now recognized that organic forms of N can sometimes contribute to plant nutrition (Ohlund and Nasholm 2004, Jones et al. 2005a, Weigelt et al. 2005). Amino acids represent the largest fraction of low molecular weight dissolved organic N in the soil (Jones et al. 2005b), and plant roots are equipped with a complex set of amino acid uptake systems, including some with high affinity for their substrates (Fischer et al. 1998). While plants must compete with microorganisms for soil amino acid pools (Hodge et al. 2000), there is nevertheless evidence that amino acids can be a significant source of N for plant growth in boreal, alpine and other ecosystems (Nasholm et al. 1998, Lipson and Nasholm 2001, Jones et al. 2005a).

Given the importance of amino acid N for plant nutrition, and the potential value of amino acids as cues for the location of an organic N-rich patch of soil, it is surprising that there are few reports of regulatory interactions between amino acids and root development. Studies with excised roots reported both positive and negative effects of glycine, glutamate and other amino acids on root growth and lateral root development that appeared to be independent of any nutritional role (Skinner and Street 1953). High exogenous concentrations (>1 mM) of aspartate and other branched chain amino acids inhibited root growth in barley (Rognes et al. 1986), while tryptophan

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stimulated or inhibited root growth depending on its concentration (Katonoguchi et al. 1994, Barazani and Friedman 2000). A recent report indicated that cell elongation in *Arabidopsis* roots was rapidly inhibited by millimolar concentrations of glutamate (Sivaguru et al. 2003). Here we report a study of the effects of low concentrations of amino acids on *Arabidopsis* root growth. Our data show that external L-glutamate, at micromolar concentrations, acts in a stereospecific manner to elicit complex changes in the pattern of root growth and branching that can significantly alter root architecture. A preliminary report on part of this work has appeared (Filleur et al. 2005).

Results

Effect of low concentrations of glutamate and other amino acids on primary root growth

To investigate the possible interactions between amino acids and root development, we tested the effect of 21 amino acids at low concentration (50 μM) on root growth in aseptically grown *Arabidopsis* seedlings (ecotype C24) (Fig. 1). Some amino acids were tested in mixtures and, where an effect was observed in preliminary experiments, the individual amino acids were then applied separately. Of the amino acids examined, only two (glutamate and tryptophan) were found to have a significant effect on root growth at this concentration (Fig. 1A). Glutamate's effect was the strongest and was highly specific since only the L-stereoisomer and not the D-form disrupted root growth (Fig. 1B). Tryptophan was not only less inhibitory but, as discussed below, its effects on other aspects of root development were quite distinct from those of glutamate.

The dose–response curve in Fig. 1C shows the effect of a range of L-glutamate concentrations on primary root growth in C24. Significant growth inhibition (~30%) was already evident at 20 μM L-glutamate, and 100 μM was sufficient for maximal inhibition (~80%). Increasing the concentration 10-fold to 1 mM had little additional effect on primary root growth (or other aspects of the root phenotype, as seen in Fig. 2 below), and even at this concentration there was no visible evidence of toxicity to the plant.

Because 0.5 mM glutamine was routinely included as the background N source, its effect on root growth and its possible interaction with L-glutamate were tested in a separate experiment. When 4-day-old seedlings were transferred to medium with or without glutamine there was no difference in their growth over the following 6 d (Fig. 1D). In other experiments we have found that even millimolar concentrations of glutamine have no detrimental effect on root growth (data not shown). Significantly, the presence or absence of the 10-fold excess of glutamine also had no effect on the root's sensitivity to L-glutamate (Fig. 1D).

Effect of applying L-glutamate to different root zones on primary root growth and root branching

Glutamate treatment of *Arabidopsis* root cells has been shown to elicit rapid changes in cytoplasmic Ca^{2+} concentrations and transient membrane depolarizations (Dennison and Spalding 2000, Dubos et al. 2003, Demidchik et al. 2004), implying the existence of glutamate-gated ion channels and a potential role for glutamate as a signal molecule in roots. Therefore, to investigate the possibility that the observed effects on root growth were due to the perception of exogenous L-glutamate rather than a consequence of systemic changes in N metabolism (and to identify which part of the root was perceiving the L-glutamate), we devised an experiment in which the L-glutamate was applied to different root zones of 4-day-old seedlings. Using segmented agar plates, it was possible either to restrict the L-glutamate treatment to the primary root tip (when L-glutamate was present only in the bottom segment) or to treat the entire root system except for the primary root tip (when the L-glutamate was present only in the top segment; see Fig. 2). Two different concentrations of L-glutamate were used: 50 μM and 1 mM.

We reasoned that if 50 μM L-glutamate were exerting its effect through changes in plant metabolism, a 50 μM L-glutamate treatment that was applied to the entire root system except for the root tip should be sufficient to inhibit root growth. Likewise, a 50 μM L-glutamate treatment that was applied only to the root tip would have a much weaker effect on plant metabolism than the same treatment applied to the whole root system, and so should have a similarly weaker effect on root growth. Fig. 2A and D shows that neither of these predictions held true. When 50 μM L-glutamate was applied to the length of the primary root except for the root tip, there was no effect on primary root growth. Furthermore, even when the L-glutamate concentration was increased to 1 mM, only a small decrease in primary root growth occurred. On the other hand, exposing just the primary root tip to 50 μM L-glutamate was sufficient to elicit the maximum inhibitory effect seen when 50 μM (or 1 mM) was applied to the whole root system (Fig. 2A, D). These results appear inconsistent with an effect on plant metabolism and point to the root tip as the site of L-glutamate perception.

To investigate the effect of exogenous L-glutamate on root branching, we measured lateral root lengths and densities in the top and bottom zones of the segmented agar plates. In seedlings where just the primary root tip was exposed to 50 μM L-glutamate, mean lateral root length and lateral root density in the apical (glutamate-treated) zone were each stimulated >2-fold (Fig. 2B, C). Surprisingly, the

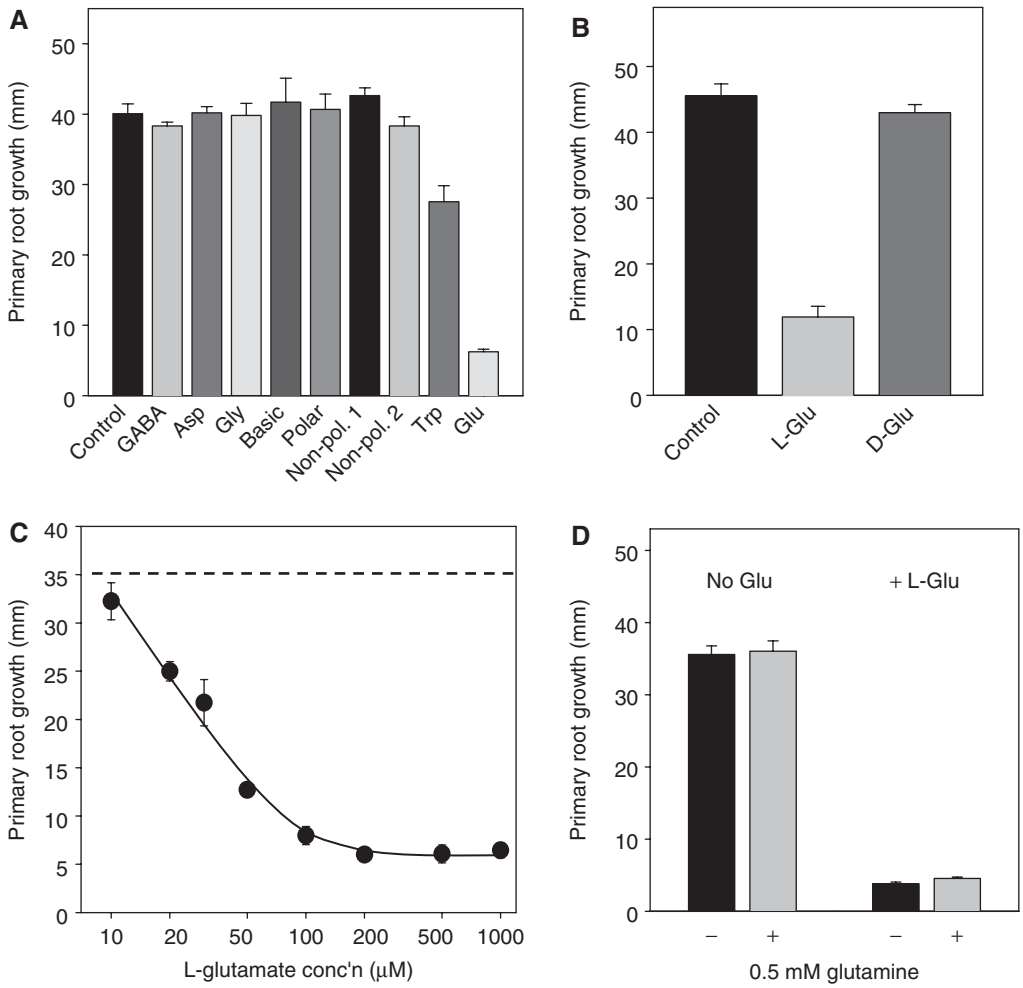

Fig. 1 Exogenously applied L-glutamate acts as a stereospecific inhibitor of *Arabidopsis* root growth. *Arabidopsis* seedlings (ecotype C24) were germinated on vertical agar plates and treatments were initiated by transferring selected 4-day-old seedlings to fresh plates containing appropriate media. Unless otherwise stated, all media contained 0.5 mM glutamine as the background N source. (A) Effect of different amino acids on primary root growth. Seedlings were transferred to vertical agar plates containing different amino acids or pooled groups of amino acids (at 50 μM each) and growth was measured over a 6 d period (±SEM; $n = 6$). The pooled groups were: basic (lysine, arginine, histidine), polar (alanine, valine, leucine, isoleucine), non-polar 1 (serine, tyrosine, asparagine, cysteine) and non-polar 2 (threonine, proline, methionine, phenylalanine). (B) Effect of L- or D-glutamate (50 μM) on primary root growth (±SEM; $n = 9$). (C) Effect of a range of L-glutamate concentrations on primary root growth. Growth was measured over a 6 d period after transfer to the treatment plates (±SEM; $n = 6$). The dotted line indicates the extent of primary root growth on control plates without L-glutamate (34.9 ± 1.3 mm). Note the log scale on the *x*-axis. (D) Effect of 50 μM L-glutamate on primary root growth in the presence (+) or absence (−) of 0.5 mM glutamine (±SEM; $n = 8$–9).

presence of L-glutamate in the upper segment had no direct effect on lateral root production in the exposed part of the root (Fig. 2C, D). However, the same treatment did lead to a reduction in lateral root length in the exposed region (Fig. 2B), showing that laterals do acquire L-glutamate sensitivity later in their development. We have observed that cessation of growth generally occurs when the L-glutamate-treated laterals are 5–10 mm long (Fig. 2D; P.W.-L. and B.G.F., unpublished results). Tryptophan had a very different effect on root branching, increasing lateral root density throughout the length of the primary root and blocking lateral root growth just after emergence (data not shown).

L-Glutamate sensitivity in different Arabidopsis ecotypes

We have compared a number of different accessions of *Arabidopsis* for their sensitivity to 50 μM and 1 mM L-glutamate (Fig. 3). The most striking difference was between C24 (the ecotype used initially) which was 80% inhibited by 50 μM L-glutamate, and RLD1, which was almost insensitive at this concentration. Niederzenz, L*er*

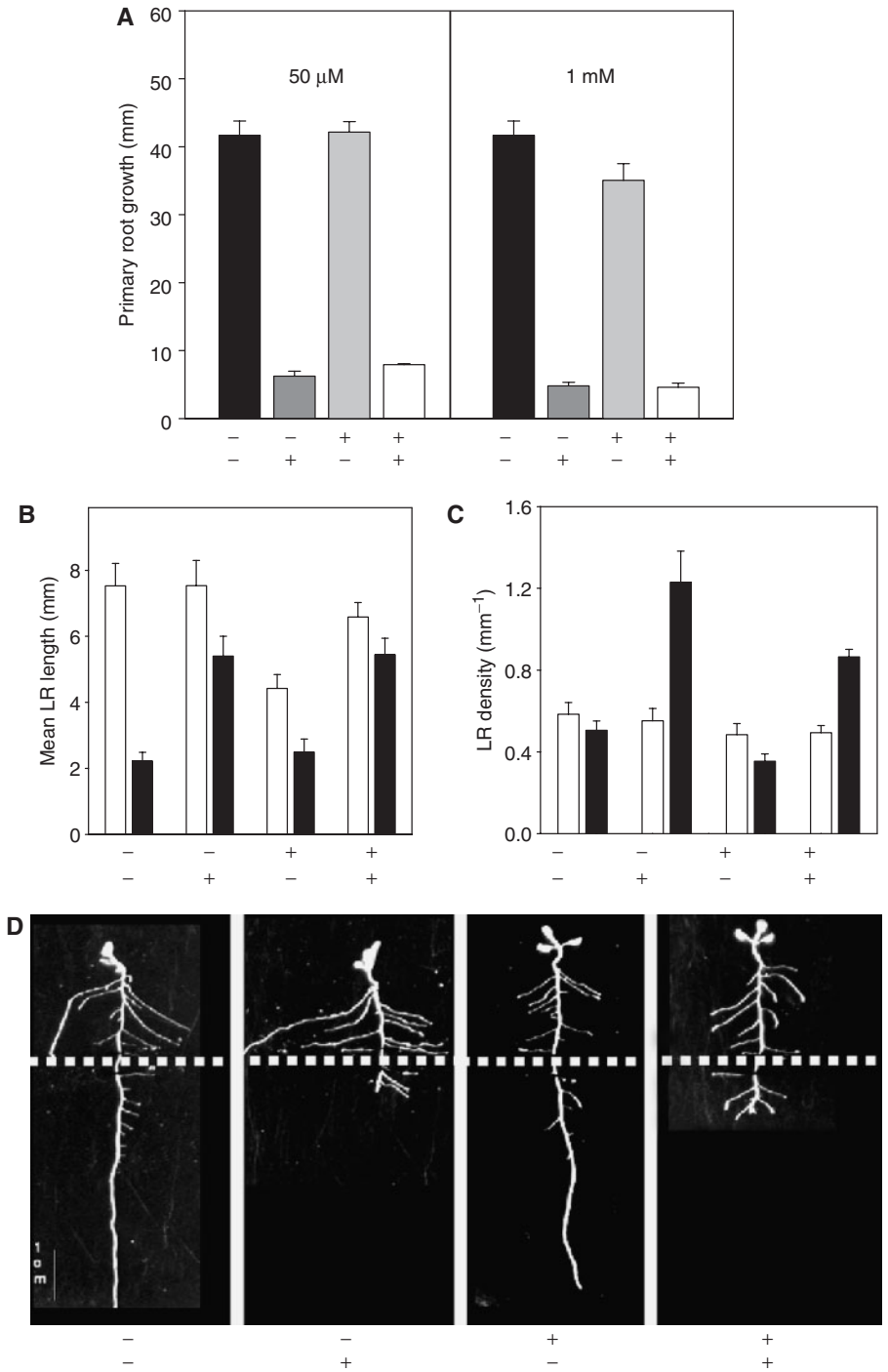

Fig. 2 Exogenous L-glutamate is sensed at the primary root tip and the response includes an increase in root branching in the apical zone. Four-day-old C24 seedlings were transferred to segmented agar plates in which the two halves of the plate (separated by a 3 mm gap to prevent diffusion) contained different concentrations of L-glutamate. The presence (+) or absence (−) of L-glutamate in the upper and lower segments is indicated. At the time of transfer, only the tip of the primary root was in contact with the lower zone. Root measurements were made 6 d after transfer. (A) Effect on primary root growth of exposing different parts of the root system to 1 mM or 50 μM L-glutamate (±SEM; $n = 5$–6). (B) Effect on mean lateral root length in each segment. Open bars, upper segment; filled bars, lower segment (±SEM; $n = 5$–6). (C) Effect on lateral root density in each segment. Only visible laterals were counted. In the lower segment, the data refer to the branching zone (from the first emerged lateral). Open bars, upper segment; filled bars, lower segment (±SEM; $n = 5$–6). (D) Root architecture of seedlings subjected to the different L-glutamate treatments. (Note that lateral roots have been spread out for display purposes.) Dotted lines indicate the boundary between the upper and lower segments (bar = 1 cm).

and Col-0 had intermediate levels of sensitivity. However, even RLD1 was partially sensitive to 1 mM L-glutamate (~30% inhibition) (Fig. 3). Furthermore, even though higher concentrations are required to produce a response, the changes in root architecture in the less sensitive ecotypes are similar to those seen in C24, i.e. increased lateral root lengths and densities near the root apex (data not shown). We have also surveyed 16 other ecotypes (Aua/Rhön, Bensheim, Cvi-0, Col-4, Col-3, Col-PRL, Dijon G, Estland, Gre-0, Kin-0, Mh-0, No-0, RLD, S96, Turk Lake and WS) without finding any that were as glutamate sensitive as C24 (data not shown).

Effects of L-glutamate on the morphology of the root tip and its ability to recover from growth inhibition

Fig. 4A shows a low magnification image of the primary root tip of an L-glutamate-inhibited C24 seedling, taken 4 d after transfer. Compared with the control, the inhibited root tip is seen to be thickened and slightly distorted, which we found to be characteristic features associated with the most strongly inhibited roots. Consistent with the slowing of root growth, lateral roots can be seen emerging within a few millimeters from the root tip in the L-glutamate-inhibited roots (Fig. 4A), and the distance from the root apex to the first root hair was also markedly reduced (Fig. 4A, B), indicating that the combined size of the meristem and the elongation zone has dramatically decreased (see below).

We have examined the time course of glutamate-elicited changes in primary root growth rate and the ability of the root to recover from the L-glutamate inhibition (Fig. 5). Four-day-old seedlings were transferred to plates containing 1 mM L-glutamate, and after 2, 3 or 4 d treatment, they were removed to plates without L-glutamate. During the first 24 h on L-glutamate, there was very little effect on primary root growth (~14% inhibition), but by day 2 growth was inhibited on average by 52% and by day 3 growth had ceased. The same time course of changes in growth rate was seen in C24 roots exposed to 50 μM L-glutamate (data not shown). Roots that were removed

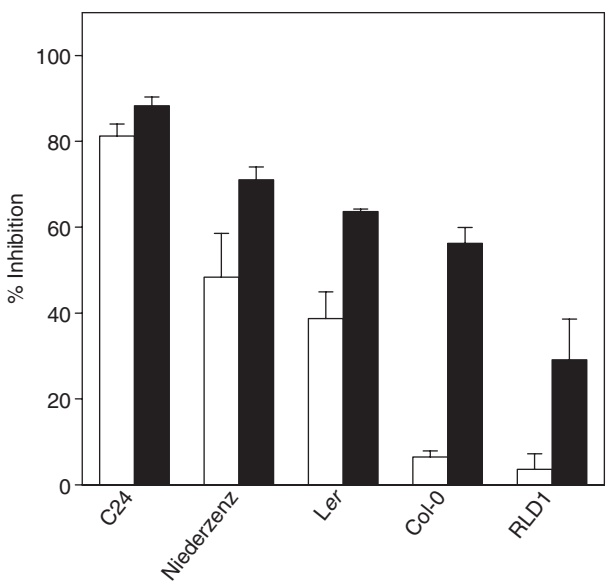

Fig. 3 Natural variation in L-glutamate sensitivity between *Arabidopsis* ecotypes. The percentage inhibition of root growth by 50 μM (open bars) or 1 mM L-glutamate (filled bars) was determined for each ecotype over a 6 d period. Data are means of three independent experiments (±SEM).

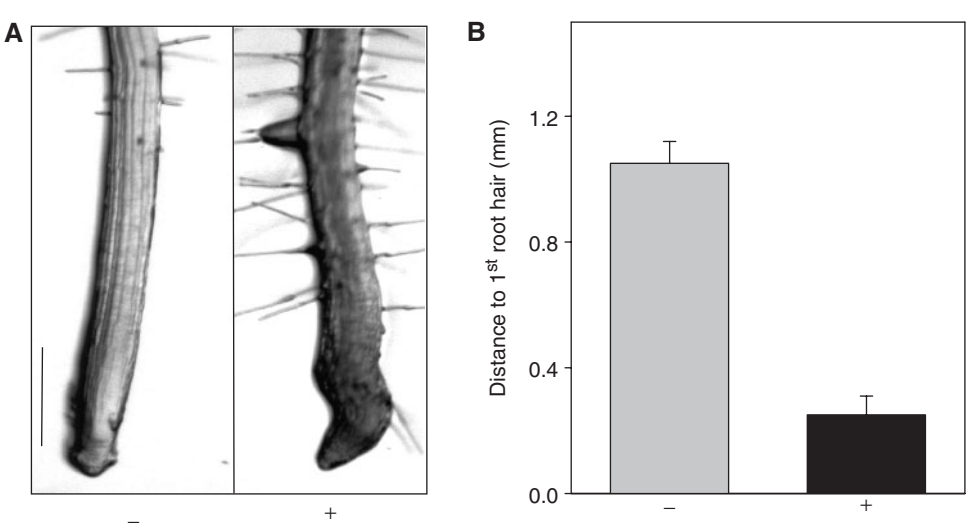

Fig. 4 Effect of L-glutamate on root tip morphology. Seedlings (C24) were transferred for 4 d to plates with (+) or without (–) 50 μM L-glutamate. (A) Primary root tips were imaged in situ on the surface of the agar plate (bar = 250 μm). (B) Distance from the root apex to the first root hair as estimated from the images (± SEM; $n = 6$).

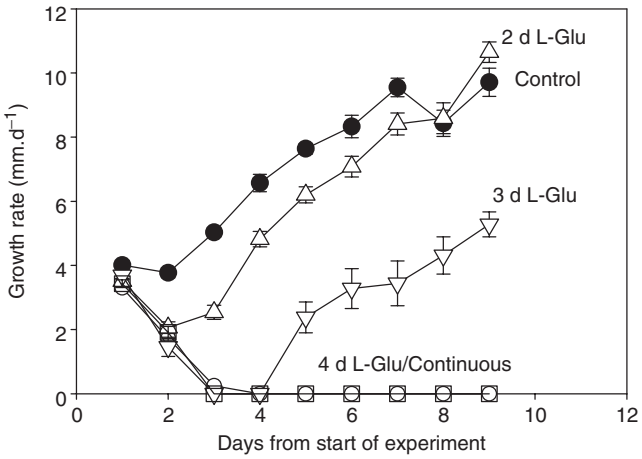

Fig. 5 Time course of changes in primary root growth rate during exposure to L-glutamate and during recovery from L-glutamate treatment. At the start of the experiment, 45 C24 seedlings were transferred to plates containing 1 mM L-glutamate, or to plates without L-glutamate (control, filled circles). One subset of 18 seedlings was maintained continuously on L-glutamate (open circles), while other subsets of nine seedlings each were transferred to glutamate-free medium after 2 d (open triangles), 3 d (open inverted triangles) or 4 d (open squares). Growth rates in each 24 h period after the start of the experiment were estimated from root length measurements made at daily intervals (±SEM; $n = 6$). Note that only four of the nine seedlings maintained for 3 d on L-glutamate recovered their primary root growth after withdrawal of the L-glutamate treatment, and the data shown are from these seedlings only.

from L-glutamate at the end of day 2 recovered their growth quite quickly, and 6 d later were growing as fast as the controls (note that the growth rate of the primary root is accelerating during this phase of *Arabidopsis* development; Beemster and Baskin 1998). However, by day 3 (when the roots had stopped growing), only about 50% of the roots that were removed to glutamate-free medium recovered their growth, and the re-growth of those that did recover was delayed by 24 h. None of the seedlings that were transferred after 4 d on L-glutamate recovered. Thus there was a critical point, around 3–4 d after exposure to L-glutamate, after which the root tip was unable to recover.

Cytological studies of the effect of L-glutamate on the primary root tip

To investigate the effect of L-glutamate on mitotic activity and cell elongation in the root tip, we have conducted a cytological investigation using C24 and a cyclin::β-glucuronidase (cyclin::GUS) marker line that allows changes in mitotic activity to be readily monitored (Hauser and Bauer 2000). Because the cyclin::GUS line is in the less glutamate-sensitive Col-0 background, 2 mM L-glutamate was used to obtain an inhibitory effect comparable with that of 50 μM on C24. As seen in Fig. 6A, during the first 24 h of treatment the primary roots of the cyclin::GUS line were inhibited by 25% and those of C24 by 27%; during the second 24 h they were inhibited by 68 and 77%, respectively. The effect of 2 mM L-glutamate on other aspects of the Col-0 root phenotype, including root tip curvature (Fig. 6G) and root branching (data not shown), were also comparable with those observed in C24 on 50 μM L-glutamate. Finally, the similarity between the cytological data obtained with C24 and the cyclin::GUS line (Fig. 6B–F) confirms that the phenomenon we were observing in the two lines is the same, despite the difference in the L-glutamate concentration required to elicit it.

Fig. 6B shows that there was only a minor reduction in cell length in the newly differentiated zone in both genotypes during the first 24 h. From the growth rate and the length of the mature cells, it can be estimated that 40–50 new mature cells must have been added to each cell file in the first 24 h of the L-glutamate treatment. Since this is roughly equal to the total number of cells in a single cell file in the meristem and the elongation zones combined, we conclude that the newly differentiated cells we were measuring at 24 h would have been located in the most apical part of the meristem at the time that the treatment began. These cells were able to complete their cell divisions and elongation essentially unaffected by the L-glutamate treatment. In cytological terms, this explains the relatively minor effect that L-glutamate has on root growth during the first 24 h period.

Measurements of the extent of the GUS-stained region of the root tip indicate that the length of the mitotically active zone decreased by 36% during the first 24 h of treatment, and had decreased by 60% at 48 h (Fig. 6G, H). Consistent with the latter observation, there was also a decrease in the number of cells in the meristematic zone during this period (by 17 and 50%, respectively; Fig. 6E). We conclude that mitotic activity in the apical meristem is a primary target of L-glutamate in the early stages of treatment.

During the first 24 h of treatment, there were no consistent effects on cell number in the elongation zone (Fig. 6C), nor in cell length in the meristematic zone (Fig. 6F). However there was a significant effect on mean cell length in the elongation zone (reduced by 38 and 30% in Col-0 and C24, respectively; Fig. 6D) and this is manifested by the end of day 2 in a decrease in mature cell size. It is not clear whether L-glutamate's effect on mean cell length in the elongation zone is attributable to a direct but delayed effect on cell elongation. It might alternatively be a secondary consequence of the reduction in the size or activity of the meristem, perhaps mediated through changes in auxin metabolism or transport (Blilou et al. 2005).

To investigate possible effects on auxin distribution in the root tip that might play a role in the process of growth inhibition, we have used a *DR5rev::GFP* (green fluorescent

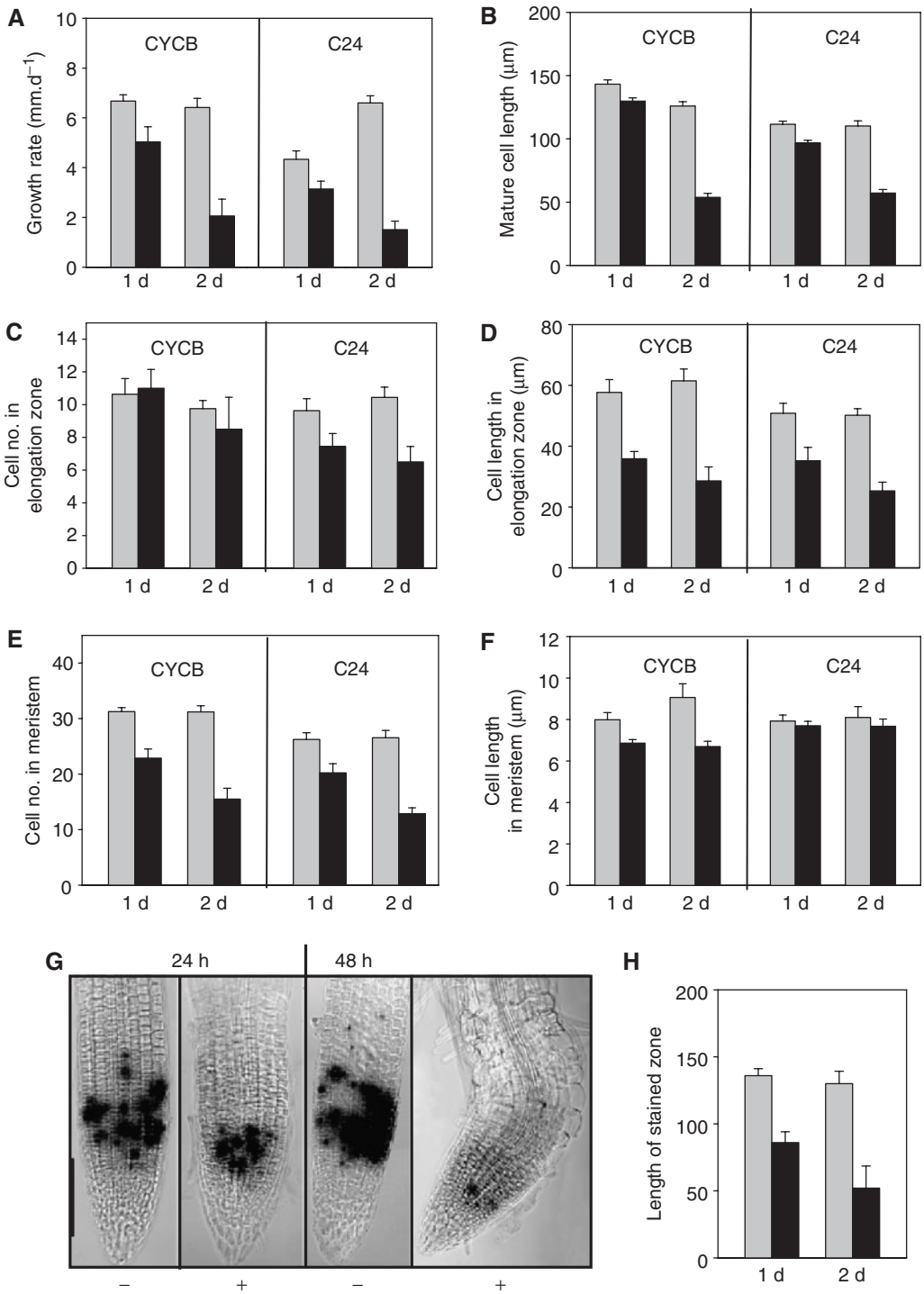

Fig. 6 Cytological changes in L-glutamate-treated roots of C24 and a cyclin::GUS marker line (Col-0 background). Four-day-old seedlings of C24 and a cyclin::GUS marker line (CYCAT1:CDB:GUS) (Hauser and Bauer 2000) were transferred to control plates (without glutamate; gray bars) or to plates containing L-glutamate (black bars) either at 50 μM (for C24) or at 2 mM (for the cyclin::GUS line). Measurements were made 1 and 2 d after transfer. (A) Growth rate of the primary roots; (B) epidermal cell length in the newly differentiated zone; (C) cell number in the elongation zone; (D) cell length in the elongation zone; (E) cell number in the meristem; (F) cell length in the meristem; (G) primary root tips of the cyclin::GUS marker line treated for 1 or 2 d with 2 mM L-glutamate (+) or without L-glutamate (−) and stained for 2 h for GUS activity (Jefferson et al. 1987) (bar = 100 μm); (H) mean lengths of the mitotically active zone as estimated from the length of the GUS-stained region in cyclin::GUS seedlings (±SEM; $n = 6–9$).

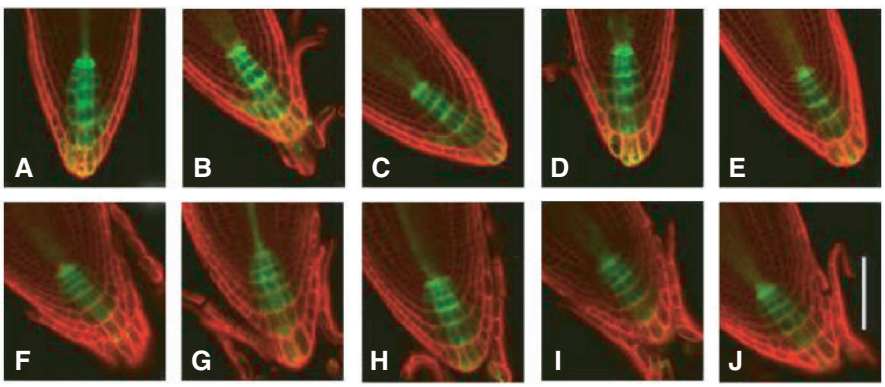

Fig. 7 Use of a *DR5rev::GFP* line to study changes in the auxin maximum in the root tip during L-glutamate treatment. Four-day-old seedlings of the *DR5rev::GFP* line (Col-0 background) were transferred to control (glutamate-free) plates (A–E) or to plates containing 2 mM L-glutamate (F–J). After 2 d, roots were counterstained with propidium iodide (red) and examined by confocal microscopy (bar = 75 μm).

protein) marker line. This line carries the *GFP* gene under the control of a synthetic auxin-inducible promoter and provides a means of monitoring changes in auxin distribution in plant tissues (Friml et al. 2003). As reported previously, *DR5rev::GFP* expression is highly localized to the quiescent center, columella initials and mature columella (Fig. 7A–E), coinciding with the 'auxin maximum' in the root tip (Friml et al. 2003, Ottenschlager et al. 2003). No changes in the pattern of *DR5rev::GFP* expression were observed after 2 d treatment with 1 mM L-glutamate, demonstrating the absence of any major effects on auxin distribution in this part of the root tip. However, the intensity of GFP fluorescence was consistently reduced in the glutamate-treated roots (Fig. 7F–J), indicating a diminution in the auxin maximum at this stage in the process of growth inhibition.

Effect of alterations in auxin transport and signaling on L-glutamate sensitivity

To investigate further the role of auxin in the L-glutamate response, we tested the effect of L-glutamate on primary root growth in a number of auxin response mutants (Fig. 8A, B). The mutants were in the less glutamate-sensitive Col-0 background, and the concentration of L-glutamate used (0.5 mM) was chosen as being at the lower end of the concentration range required to inhibit root growth strongly in this ecotype (data not shown). In this way, we aimed to maximize the chances of detecting either increased or decreased L-glutamate sensitivity. One auxin mutant, *aux1-7* (Maher and Martindale 1980), was found to be partially resistant to L-glutamate (Fig. 8A), while two mutants at the *AXR1* locus (*axr1-3* and *axr1-12*) (Lincoln et al. 1990) were hypersensitive (Fig. 8B). None of the other auxin response mutants tested (*axr2-1*, *axr4-2* and *tir1-1*) were altered in their L-glutamate sensitivity (Fig. 8A). The TIR1 auxin receptor is encoded

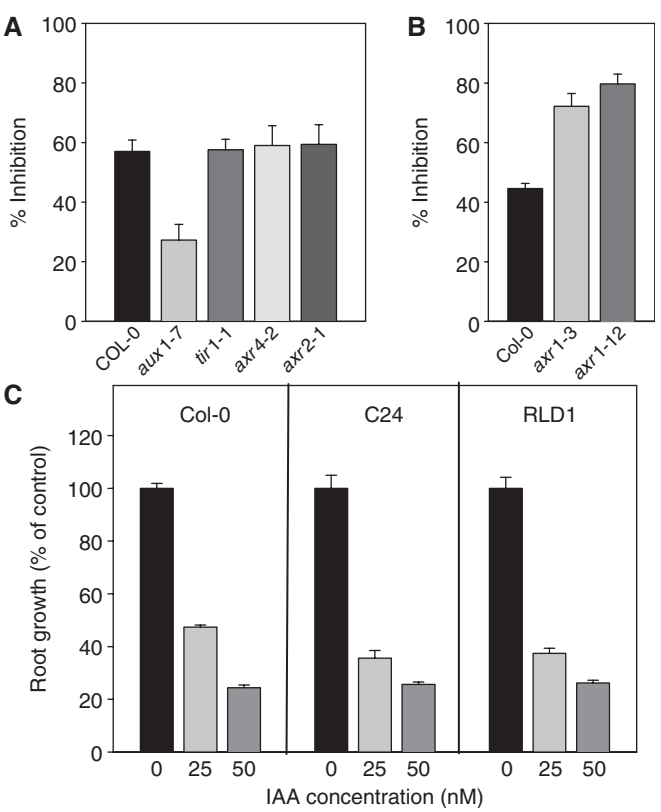

Fig. 8 Glutamate sensitivity of auxin mutants and auxin sensitivity of ecotypes differing in glutamate sensitivity. (A, B) Seedlings of six auxin-insensitive mutants and the parental line (Col-0) were transferred to medium containing 0.5 mM L-glutamate, primary root growth over the following 6 d period was measured and data are expressed as percentage inhibition compared with the same line without glutamate (±SEM; $n = 4$–18). The *axr1* mutants were analyzed in a separate experiment from the other auxin mutants. (C) The three ecotypes were treated with different concentrations of IAA for 6 d and the percentage inhibition of primary root growth was determined by comparison with the no IAA controls (±SEM; $n = 8$–12).

by a member of a small gene family (Woodward and Bartel 2005) so, at least in this case, redundancy of function could account for the lack of a phenotype.

The finding that some auxin response mutants had altered sensitivity to L-glutamate suggested the possibility that the observed natural variation in glutamate sensitivity (Fig. 3) could be linked to ecotype-to-ecotype differences in auxin sensitivity. However, when we compared the IAA sensitivity of three ecotypes differing markedly in their glutamate sensitivity (Col-0, C24 and RLD1), no significant differences were observed (Fig. 8C). Furthermore, unlike the *aux1* and *axr1* mutants, none of these ecotypes displayed any obvious defects in gravitropism (data not shown).

Pharmacological studies to investigate the mechanism of L-glutamate perception

We have used a pharmacological approach to look for evidence of a role for glutamate receptor-like proteins (GluRs) in L-glutamate sensing at the root tip. β-Methylamino-L-alanine (BMAA), a cycad-derived agonist of mammalian GluRs, was previously shown to inhibit *Arabidopsis* root growth and cotyledon opening, and to stimulate elongation of light-grown hypocotyls, in a glutamate-reversible manner (Brenner et al. 2000). We found that when seedlings were transferred to medium containing 50 μM BMAA, both primary root growth and lateral root growth (but not emergence) were rapidly inhibited. Shoot growth was also very severely inhibited, something not seen with L-glutamate, even at millimolar concentrations. When the BMAA concentration was reduced to 10 μM, inhibition of root and shoot growth was less severe. However, at neither BMAA concentration was there any evidence of the positive effect on root branching that accompanies glutamate inhibition of root growth. Furthermore, C24 (glutamate hypersensitive) and RLD1 (glutamate hyposensitive) did not differ in their sensitivity to BMAA (data not shown). We therefore obtained no evidence that a BMAA-sensitive glutamate receptor is involved in perception of the L-glutamate signal that leads to inhibition of root growth.

We also tested three known antagonists of mammalian GluRs, 6,7-dinitroquinoxaline-2,3-dione (DNQX) (at 1 mM), MK801 (at 200 μM) and 2-amino-5-phosphonopentanoate (AP-5) (at 200 μM), again without obtaining evidence of an involvement of GluR-like receptors. None of the antagonists affected primary root growth in C24 when applied on their own, as measured over a 6 d period, and none antagonized the inhibitory effect of 50 μM L-glutamate (data not shown). While these compounds have been extensively used as tools to obtain supporting evidence for the existence of functional glutamate receptors in plants (Lam et al. 1998, Dubos et al. 2003, Kang and Turano 2003, Sivaguru et al. 2003, Kang et al. 2004), their physiological targets in plant cells have not been identified. It is also worth noting that in experiments where aequorin was used as a chemiluminescent Ca^{2+} indicator protein (Dubos et al. 2003), it was found that DNQX was able to block glutamate-mediated changes in cytosolic [Ca^{2+}] in cotyledons but not in root tips, suggesting that glutamate-gated channels in root tips are insensitive to DNQX.

Discussion

Exogenous L-glutamate acts as a highly specific signal to modify root architecture

We have found that when L-glutamate is applied exogenously to *Arabidopsis* roots it inhibits primary root growth and stimulates root branching in the apical region of the primary root. There were major differences between ecotypes in their L-glutamate sensitivity, but in the most sensitive ecotype (C24) the response was evident at concentrations as low as 20 μM. The remarkable specificity of the effect is shown by the root's insensitivity to the structurally related amino acids aspartate and γ-aminobutyric acid (GABA), even when these were supplied at 1 mM (data not shown), and by the root's insensitivity to D-glutamate. Tryptophan was the only other amino acid to which roots had a comparable sensitivity, but its effects on root architecture were quite distinct from those of L-glutamate and were more characteristic of an auxin effect. This response is consistent with tryptophan's known role as a precursor of IAA (Muller et al. 1998).

Glutamate occupies a strategic position in plant N metabolism, being the product of the GOGAT cycle and an amino-N donor for synthesis of other amino acids (Stitt et al. 2002). Although *Arabidopsis* roots have a high-affinity L-glutamate influx system (K_m 14–15 μM; W. Koch and W. Frommer, personal communication), there are several lines of evidence indicating that the L-glutamate effect is not attributable to any impact that the exogenous amino acid may have on plant metabolism. First, we were able to show using ecotype C24 that the root's sensitivity to 50 μM L-glutamate was not influenced by the presence or absence of a 10-fold excess of glutamine (Fig. 1D). Thus the effect is unrelated to the glutamine-reversible phenomenon of 'general amino acid inhibition' seen when plant tissue cultures are exposed to high concentrations (>1 mM) of amino acids (Bonner and Jensen 1997). Secondly, the fact that root growth was not inhibited by much higher concentrations of glutamine (0.5 mM) suggests that the root's response to exogenous L-glutamate does not arise through changes in the endogenous glutamate pool. Glutamate and glutamine are readily interconverted in vivo (through the GOGAT cycle) and it has been noted previously that the endogenous glutamate pool in the root

is relatively unaffected by exogenously applied glutamate (Stitt et al. 2002, Beuve et al. 2004). Indeed, a glutamine treatment was more effective than a glutamate treatment at increasing tissue glutamate concentrations in *Brassica napus* roots (Beuve et al. 2004). Thirdly, the results of applying L-glutamate to different zones of the root showed that for the primary root tip to be inhibited it must be directly exposed to the L-glutamate. Even when the primary root was treated with 1 mM L-glutamate (a 10-fold higher concentration than is needed for maximal inhibition), so long as the root tip itself did not come into direct contact with the L-glutamate, its growth was not significantly inhibited. The systemic effect that would be expected if root growth were responding to changes in plant N metabolism was therefore not observed. Furthermore, the finding that applying L-glutamate locally to the primary root tip is sufficient to mimic the effect on primary root growth of a whole root treatment confirms that the root tip itself is the site of L-glutamate perception. Given the specificity of the effect and the responsiveness of the root to low external L-glutamate concentrations, it seems most likely that the root tip is responding to localized changes in the apoplastic L-glutamate concentration.

L-Glutamate inhibits mitotic activity in the root apical meristem, but not in developing lateral roots

Cytological studies indicate that mitotic activity in the root apical meristem is a primary target for L-glutamate. Using a cyclin::GUS marker line, we were able to show that there was a significant reduction in the length of the mitotically active zone during the first 24 h after L-glutamate treatment, when little effect on root growth was observed. The lag in the onset of growth inhibition appears to be linked to the lack of any early effect on cell elongation. These observations contrast with a previous report that high concentrations of glutamate (5 mM) inhibited primary root growth in *Arabidopsis* (Columbia ecotype) within minutes, apparently due to disruption of cell elongation in the distal elongation zone (Sivaguru et al. 2003).

Recovery experiments, where C24 roots were removed to glutamate-free plates at intervals after the start of L-glutamate treatment, revealed that at around the time that growth ceases (after ~3 d on L-glutamate), the root tip loses its ability to resume growth, suggesting that the meristem had been lost. Similar results were obtained in a study of the onset of determinate root growth in Arabidopsis seedlings germinated under phosphate-limiting conditions (Sanchez-Calderon et al. 2005). It was found that the time when mitotic activity in the root apical meristem ceased (10 d after germination) approximately coincided with the time when roots transferred to high phosphate medium could no longer recover their growth. However, it appears that loss of the meristem is not an inevitable consequence of L-glutamate inhibition. When roots were less strongly inhibited (e.g. in less sensitive ecotypes), they continued to grow at a reduced rate for at least 6 d from the start of the L-glutamate treatment (data not shown).

While the inhibitory effect of L-glutamate on primary roots was particularly striking, there were also distinct and complex effects on root branching. The most obvious was an increase in the mean lateral root length in the apical region of the primary root, indicating that outgrowth of the lateral roots behind the growth-inhibited primary root tip was stimulated. It is commonly observed that inhibition of primary root growth is accompanied by a compensatory increase in root branching (e.g. Reed et al. 1998, Tsugeki and Fedoroff 1999), which is thought to be triggered by disruption of auxin and/or cytokinin fluxes at the root tip (Casson and Lindsey 2003). The enhanced growth of laterals near the glutamate-inhibited tip is likely to be a related phenomenon. More intriguing, given the early effect of L-glutamate on mitotic activity in the primary root meristem, is the evident insensitivity of mitotic activity in the developing lateral root. It is this early insensitivity that allows the lateral roots to proliferate when the primary root tip is inhibited by L-glutamate. However, as is apparent from Fig. 2B and D (compare lateral root lengths in the top segment with and without L-glutamate), lateral root growth is ultimately inhibited by L-glutamate, indicating that the laterals acquire L-glutamate sensitivity later in their development.

A possible role for auxin in the response to L-glutamate

Possible interactions between auxin signaling and glutamate signaling were investigated by examining the L-glutamate sensitivity of a number of auxin response mutants. While the majority of auxin mutants tested were unaffected, the *aux1-7* mutant was partially insensitive to L-glutamate, while two mutants at the AXR1 locus were hypersensitive. AUX1 is an auxin influx facilitator participating in both acropetal and basipetal auxin transport at the root tip (Swarup et al. 2001). The partial L-glutamate resistance of the *aux1-7* mutant therefore suggests that auxin transport within the root tip may have a positive role in mediating the L-glutamate effect. It is known that changes in auxin distribution in the root tip can affect meristematic activity (Blilou et al. 2005) and experiments in which the *DR5rev::GFP* line was used to monitor changes in auxin distribution in L-glutamate-treated roots indicated a reduction in the intensity of the auxin maximum at the root tip. However, we cannot discount the possibility that the reduction in the auxin maximum is a result (rather than a cause) of the decline in meristematic activity in L-glutamate-treated roots.

Like *AUX1*, *AXR1* is a positive regulator of the auxin response, so the L-glutamate hypersensitivity of the *axr1*

mutants was unexpected. It is possible that *AXR1* plays a role in glutamate signaling that is independent of (or additional to) its role in auxin signaling. *AXR1* encodes a protein related to the ubiquitin-activating enzyme E1 (Leyser et al. 1993) and, because the ubiquitin/26S proteasome pathway appears to impinge on almost every aspect of plant biology (Vierstra 2003), mutations in the *AXR1* gene could well have pleiotropic effects. Indeed, *axr1* mutants have been reported to have diminished responses to a number of signals in addition to auxin, including jasmonate, ethylene, cytokinin, light and cold (Timpte et al. 1995, Schwechheimer et al. 2002, Tiryaki and Staswick 2002). However, to our knowledge, this is the first instance where loss-of-function *axr1* mutations have resulted in sensitization to a signal.

Mechanism of L-glutamate sensing

A family of ionotropic glutamate receptors (iGluRs) involved in excitatory neurotransmission in mammalian synapses is phylogenetically conserved in bacteria, plants and animals (Chiu et al. 2002). Glutamate-gated ion channel activity has been demonstrated in roots (Dennison and Spalding 2000, Dubos et al. 2003. Demidchik et al. 2004), but a link between these channels and the 20 GluR-like (*AtGLR*) genes in *Arabidopsis* has still not been established. Specific *AtGLR* genes have been implicated in a diverse set of physiological processes, including Ca^{2+} allocation within the plant (Kim et al. 2001), abscisic acid biosynthesis and signaling (Kang et al. 2004), and the regulation of C and N metabolism (Kang and Turano 2003, Dubos et al. 2005), but without a clear understanding of their mode of action.

Pharmacological experiments reported here, using known agonists and antagonists of mammalian iGluRs, failed to confirm a role for iGluR-type receptors in the L-glutamate effect. However, in view of uncertainties surrounding the targets of these reagents in plant tissues, members of the *AtGLR* family still seem to be the best available candidates for the role of L-glutamate sensors in the root tip. It has recently been reported that the short-root phenotype of a rice mutant defective in the expression of the *OsGLR3.1* gene is due to disruption of meristematic activity in the root apex (Li et al. 2006), suggesting that this *GLR* gene has an essential role in the maintenance of the root meristem. We are currently analyzing the L-glutamate sensitivity and root phenotypes of a collection of *Arabidopsis* mutants with T-DNA insertions in each of the 20 *AtGLR* genes to establish whether any are involved in the perception of exogenous glutamate.

Functional significance of L-glutamate sensitivity

Tests on a selection of other plant species have found similar effects of L-glutamate on root growth in a significant proportion of cases, including two *Arabidopsis* relatives (*Thlaspi caerulescens* and *Thellungiella halophila*), wild poppy and tomato (Walch-Liu et al. 2006; P.W.-L. and B.G.F., unpublished results). It is therefore intriguing to speculate on the possible functional significance of L-glutamate's ability to modulate root growth and branching. It has been proposed that glutamate may be an endogenously generated intercellular signal, its exudation from root cells being triggered by intrinsic or extrinsic factors such as Al^{3+} toxicity (Dennison and Spalding 2000, Sivaguru et al. 2003). However, an alternative possibility is that the phenomenon may have an ecological significance, an idea supported by the high degree of natural variation in L-glutamate sensitivity we observed between *Arabidopsis* accessions.

For L-glutamate to be considered a realistic environmental signal for plant roots, it would have to occur naturally in soils at concentrations sufficiently high to elicit a response (i.e. $\geq$20 μM for the more sensitive ecotypes). While L-glutamate concentrations in bulk soil solution may normally be low (<10 μM) (Jones et al. 2005a), concentrations in excess of those needed to affect root growth are likely to occur routinely within patches of decomposing organic matter: plant and animal tissues contain free L-glutamate at millimolar concentrations (Joy et al. 1992, Young and Ajami 2000) and an even larger pool of L-glutamate is available for proteolytic release in the protein fraction.

The ability of roots to respond to concentrations of L-glutamate occurring within organic N-rich patches could be highly significant. There is an increasing appreciation of the importance of amino acids as an N source for plants (Nasholm et al. 1998, Lipson and Nasholm 2001, Jones et al. 2005a, Weigelt et al. 2005). It has been argued that organic-rich patches are precisely the places where plants should be able to compete most effectively with microorganisms for amino acid N (Jones et al. 2005a). Indeed, species whose roots proliferate more abundantly within organic N-rich patches have been shown to be more successful at N capture (Hodge 2004). It is therefore possible that the locally increased root proliferation that we observe in the apical region of a primary root encountering a source of L-glutamate (e.g. Fig. 2D) could serve as a novel foraging strategy that enables the plant to exploit more efficiently the nutrients contained within organic N-rich patches.

The opportunity to test this hypothesis will come with the availability of mutants and near-isogenic lines that differ in their L-glutamate sensitivity. Other important questions for the future concern the nature and identity of the receptor(s) responsible for initiating the L-glutamate response and the molecular basis of the natural variation in root tip sensitivity to L-glutamate.

Materials and Methods

Plant material

Arabidopsis thaliana L. (Heynh.) ecotypes were originally sourced from Lehle Seeds (Round Rock, TX, USA) and, unless otherwise stated, ecotype C24 was used. The cyclin::GUS marker line, CYCAT1:CDB:GUS, was generated by J. Celenza (Boston University, MA, USA) (Hauser and Bauer 2000). The *DR5rev::GFP* auxin reporter line (Friml et al. 2003) was obtained from the European Arabidopsis Stock Centre (catalog no. N9361). The *aux1-7* and *axr4-2* mutants were gifts from Malcolm Bennett (Nottingham, UK) and Ottoline Leyser (York, UK), respectively. Other mutants were supplied by the European Arabidopsis Stock Centre.

Culture conditions

The growth medium, based on a 50-fold dilution of Gamborg's B5 medium (Gamborg et al. 1968), was as previously described (Zhang and Forde 1998) except that 1% Phytagel^{TM} was used and 1 mM each of $MgCl_2$ and $CaCl_2$ was added to aid solidification. Amino acids in solution were filter-sterilized and added to the growth medium after autoclaving. No inorganic N sources were used and, unless otherwise stated, all media contained 0.5 mM glutamine as the background N source. Seeds were surface sterilized and germinated on solidified growth medium in 90 mm Petri dishes. After stratification in the dark for 2 d at 4°C, the plates were transferred to a growth room and held vertically at 22–24°C with a 16/8 h photoperiod at ~120 μmol m^{-2} s^{-1}. After 4 d, a homogeneous subset of the seedlings was transferred to treatment plates (three per plate) and cultured for up to 6 d. Note that in experiments where it was necessary to omit the glutamine from some of the treatment plates (see Fig. 1D), growth of the primary root was unaffected for up to 6 d, presumably because the seedlings had accumulated sufficient N reserves.

When L-glutamate was to be applied independently to different parts of the primary root, segmented plates were prepared in which the upper and lower halves of the agar were isolated by a 3 mm gap. To initiate the treatment, 4-day-old seedlings were transferred to the segmented plates such that only the tip of the primary root was in contact with the lower zone.

Roots were imaged using a GelDoc gel documentation system (BioRad, Hercules, CA, USA) or a digital scanner. Root lengths were determined from the digital images using ScionImage (Scion Corporation, Frederick, MD, USA) or Optimas Image Analysis software (Version 6.1, Media Cybernetics Inc., Silver Spring, MD, USA). All experiments were performed at least twice with similar results; quantitative variations in glutamate's inhibitory effect were sometimes observed, apparently due to interactions with as yet unspecified environmental factors.

Cytological measurements

Roots of the cyclin::GUS marker line were stained as previously described (Jefferson et al. 1987). Root tips were imaged using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) at 10× and 40× magnification, a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Simple PCI software (Compix Inc., Cranberry Township, PA, USA). Overlapping images were merged using Canon PhotoStitch software (Canon Inc., Toyko, Japan) to visualize the whole root tip. These images were used to quantify cell number and cell lengths using Optimas Image Analysis software.

Confocal microscopy

Roots of the *DR5rev::GFP* line were stained briefly with 10 μM propidium iodide before microscopy. Fluorescent signal detection of GFP and propidium iodide was performed using a Leica SP2-AOBS confocal laser scanning microscope. The GFP and propidium iodide images were electronically superimposed using LCS Lite software (Leica).

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