Chitinase-like protein CTL1 plays a role in altering root system architecture in response to multiple environmental conditions.

Christian Hermans\textsuperscript{1,2}, Silvana Porco\textsuperscript{1,2}, Nathalie Verbruggen\textsuperscript{2} and Daniel R. Bush\textsuperscript{1,*}

\textsuperscript{1} Department of Biology, Colorado State University, Fort Collins, CO 80523, USA
\textsuperscript{2} Laboratory of Plant Physiology and Molecular Genetics, Université Libre de Bruxelles, Campus Plaine CP242, Bd du Triomphe, B-1050 Brussels, Belgium

\textsuperscript{*}Corresponding author e-mail: dbush@colostate.edu

Running title: CTL1 and root system architecture

Keywords: nitrate, chloride, root architecture, chitinase, development, abiotic factors

Accession numbers: At1g05850
ABSTRACT

Plant root architecture is highly responsive to changes in nutrient availability. However the molecular mechanisms governing the adaptability of root systems to changing environmental conditions is poorly understood. A screen for abnormal root architecture responses to high nitrate in the growth medium was carried out for a population of EMS-mutagenized Arabidopsis thaliana. The growth and root architecture of the arm (anion altered root morphology) mutant described here was similar to wild type plants when grown on low to moderate nitrate concentrations, but on high nitrate arm exhibited reduced primary root elongation, radial swelling, increased numbers of lateral roots and increased root hair density when compared to the wild type control. High concentrations of chloride and sucrose induced the same phenotype. In contrast, hypocotyl elongation in the dark was decreased independently of nitrate availability. Positional cloning identified a point mutation in the AtCTL1 gene that encodes a chitinase-related protein, although molecular and biochemical analysis showed that this protein does not possess chitinase enzymatic activity. CTL1 appears to play two roles in plant growth and development based on the constitutive effect of the arm mutation on primary root growth and its conditional impact on root architecture. We hypothesize that CTL1 plays a role in determining cell wall rigidity and that that activity is differentially regulated by pathways that are triggered by environmental conditions. Moreover, we show that mutants of some subunits of the cellulose synthase complex phenocopy the conditional effect on root architecture under non-permissive conditions, suggesting they are also differentially regulated in response to a changing environment.
INTRODUCTION

Root systems exhibit a high degree of architectural plasticity in response to water and nutrient availability. Root architecture is a genetically defined and environmentally regulated process. In particular, the growth and development of lateral roots (LR) is greatly influenced by environmental factors such as mineral nutrient abundance (Lopez-Bucio et al., 2003; Casimiro et al., 2003; Nibau et al., 2008; Iyer-Pascuzzi et al., 2009; Péret et al., 2009). Nitrate availability is one of the major determinants of root morphology (Zhang and Forde, 2000; Hermans et al., 2006; Gojon et al., 2009). Low nitrate levels in the soil stimulate LR development, which substantially increases the root surface area available for nutrient acquisition. Conversely, high levels of nitrate inhibit LR elongation by preventing LR meristematic activation at post-emergence (Zhang et al., 1999), but generally have no impact on the primary root (PR) growth. Interestingly, when roots of nitrogen deficient plants contact nitrate, LR outgrowth is enhanced within the nitrate rich patch (Zhang and Forde, 1998).

Several sensing and signalling pathways are thought to be involved in root nitrate responses in Arabidopsis thaliana. Lateral root responses to external nitrate abundance have been associated with the MADS-box transcription factor nitrate-regulated1 ANR1 (Zhang and Forde, 1998; Zhang et al., 1999; Zhang and Forde, 2000), and with a systemic signal, possibly glutamine, through a basic leucine zipper (bZIP) and a LIM transcription factor (Tranbarger et al., 2003). Nitrate transporters may also be components of signaling pathways as observed for NRT1.1 and NRT2.1, which have been shown to function as nitrate sensors. Perception of nitrate availability could occur in part through NRT2.1, independent of its nitrate uptake function in the root (Malamy and Ryan, 2001; Little et al., 2005; Miller et al., 2007). NRT2.1 seems to be directly involved in the regulation of LR initiation when nitrate is limiting and sucrose is abundant. It has also been reported that the dual-affinity nitrate transporter AtNRT1.1 acts upstream of ANR1 in mediating the stimulatory effect of a localized nitrate supply on LRs proliferation (Liu et al., 1999; Remans et al., 2006).

MicroRNAs have also been shown to modulate LRs emergence in response to nitrogen (Gifford et al., 2008). The microRNA 167a/b, specifically expressed in pericycle cells and LR cap, was shown to be repressed by increased nitrate supply. Accordingly, this resulted in the induction of one miR167a/b target, the AUXIN RESPONSE FACTOR (ARF8), which in turn reduces emergence of initiated LRs (Gifford et al., 2008; Gojon et al., 2009). In addition to external ion availability, systemic inhibition of LR development has been observed...
when tissue nitrate concentrations are high and this response has been linked to ABA (Signora et al., 2001). Long-distance signals mediating the shoot response to nitrate perception in roots may also involve cytokinins. It is possible that the reduction in cytokinins observed during N-deficiency (Takei et al., 2004) relieves a general inhibition of root growth by this hormone, and that an increase in auxin stimulates cell division and LR development. In the latter case, auxin reprograms cells overlaying LR primordia to facilitate organ emergence and genes encoding several Arabidopsis cell wall remodelling enzymes have been reported to be expressed in these cells (Péret et al. 2009 and references therein). Finally, the impact of the cell wall on cell shape and size is a critical determinate in plant growth and development (Cosgrove, 1999).

In the experiments reported here we screened for root architecture mutants that produce lateral roots in the presence of high nitrate. These conditions repress lateral root growth in wild type seedlings. We describe the physiological and genetic characteristics of the arm (anion altered root morphology) mutant whose phenotype is not only conditional on high, external nitrate, but also on other environmental cues.

RESULTS

Isolation of the arm mutant with altered response to high nitrate

This screen was designed to identify mutants impaired in morphological responses of seedling roots to nitrate abundance. The standard Murashige and Skoog growth medium (Murashige and Skoog, 1962), which is commonly used and adapted in nutritional screens (Hauser et al., 1995; Schneiders et al., 1997; Malamy and Ryan, 2001), was modified by eliminating NH₄NO₃ and limiting KNO₃ to a range of concentrations that bracketed the presence or absence of lateral roots (LRs) in wild type (wt) plants. Thirteen days after germination (dag), wt Columbia (Col-0) seedlings grown on vertical plates with high nitrate (60 mM KNO₃) had a single primary root (PR), and no or very few LRs (Fig. 1A, B; Fig. 2). Growth at 120 mM KNO₃ decreased PR length and completely repressed the lateral branching (Fig. 1A, B). In contrast, plants grown on low (0.6 mM) or moderate (6 mM) KNO₃ had developed many LRs (Fig. 1A, B; Fig. 2). We screened EMS mutagenized Arabidopsis seedlings for the presence of LRs when grown on normally restrictive levels (60 mM) of KNO₃. One mutant showing conspicuous features that were conditional on high nitrate is described here. The phenotype of this mutant grown on 60 mM KNO₃ included: reduced PR length (Fig. 1A), high numbers of LRs (Fig. 1B), radial swelling and increased root hair
length and density (Fig. 1C). At 0.6 and 6 mM nitrate, PR length of mutant seedlings was decreased by one fourth as compared to the wt, but no difference in the number of emerged LRs was observed. High concentrations of KNO₃ inhibited PR elongation by more than one half and induced the emergence of LRs, root hair and radial swelling. Similar observations were apparent after prolonged growth (27 dag) in these restrictive conditions (Fig. 2). Because of these characteristics (and chloride sensitivity described below), the mutant was named arm (anion altered root morphology). It is noteworthy that the total length of LRs elaborated by the arm mutant at 60mM KNO₃ does not compensate for the decrease in PR length compared to wt at high nitrate supply (Supplemental Fig. S1A, B). Yet, because of root cell swelling, the root to shoot dry biomass ratio is not affected by the arm mutation (Supplemental Fig. S1C).

Specificity of the arm phenotype to sugars and minerals

Several studies have shown that sucrose levels can influence morphological responses and in particular to nitrate availability (Zhang et al., 1999, 2000; Malamy and Ryan, 2001; MacGregor et al., 2008). Therefore, we examined root responses to sucrose supplies in the growth medium. Nine combinations of nitrate (0.6; 6 and 60 mM KNO₃) and sucrose (0.1; 1; 5% w/w) were tested. High sucrose supply decreased PR length and increased lateral root density in the arm mutant across all nitrate concentrations (Fig. 3; Supplemental Fig. S2). The combination of high (5%) sucrose and high (60 mM) nitrate had the strongest negative effect, resulting in an 85% reduction of PR length in arm plants compared to wt. Overall, high sucrose induces changes in root morphology that are similar to patterns observed on high nitrate.

Since the mutants identified in this screen exhibited abnormal root architecture in response to high KNO₃ in the growth medium, it was necessary to determine the role of cations, anions, and osmotic potential on the mutant phenotype. Thus, six-day-old seedlings grown on 6mM KNO₃ (considered as the reference substrate) were transferred to fresh media enriched with or depleted of a variety of ions and molecules for an additional six days of growth (Fig. 4). Sixty mM NaNO₃ and 30 mM Ca(NO₃)₂ impacted root morphology to the same extent as 60 mM KNO₃. In contrast, 30 mM K₂SO₄ or MgSO₄ had little effect (Fig. 4A). This shows that the arm mutant is responsive to nitrate rather than the potassium ions of KNO₃. Unfortunately, high phosphate was limited to 15 mM because of precipitation at higher concentrations. Nevertheless, a ten-fold increase of the phosphate concentration had no impact on root architecture. Sixty mM mannitol or sorbitol had no effect, but a slight decrease of PR length was observed at 120 mM sorbitol (Fig. 4B), suggesting this is not an osmotic
effect on root architecture. Chloride salts (30 mM CaCl₂, 60 mM KCl and 60 mM NaCl) induced exactly the same root responses as nitrate salts (Fig. 4B), implicating monovalent anions as the active molecular species since high sulphate and phosphate had little impact and, thus, the rationale behind the mutant’s name (*arm*; anion altered root morphology). We also tested if the anion-responsive root phenotype is caused by pH changes in the agar medium, as a consequence of the high anion influx. Media buffered with 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) resulted in the same mutant phenotype as described before.

**Local mineral sensing and mineral content**

Given the dramatic impact of high nitrate and chloride on the root system, we wanted to determine if this is a localized or systemic response. Seedlings grown on uniformly moderate (6 mM KNO₃) nitrate supply were transferred onto plates divided into regions of high and moderate KNO₃ concentrations (6 and 60 mM) (Fig. 5). When plants were placed such that the root tip zone alone contacted high nitrate, PR growth was completely inhibited and radial swelling of the root tip was apparent in the *arm* mutant after two days of transfer. When the root tip zone was on medium containing moderate nitrate, while a substantial portion of the mature root zone was on high nitrate, no inhibition of PR elongation was observed (Fig. 5A). These observations demonstrate a local response to nitrate ions in the induction of the *arm* root phenotype.

Since the *arm* mutant is responsive to changes in mineral ion availability, we examined the effect of the *arm* mutation on the ionome (Lahner et al., 2003). The mineral content was analyzed in root and shoot of seedlings grown *in vitro* with low, moderate and high KNO₃ conditions. No conspicuous differences in tissue nitrate content that would indicate a severe dysfunction in ion homeostasis was observed between wild type and *arm* (Fig. 6). Likewise, little difference was observed for tissue concentrations of other minerals that included K, Ca, Mg, P, S, and Na. It is noteworthy that the addition of 60 mM KNO₃ in the growth medium resulted in higher K and lower Ca, Mg and Na tissue concentrations in the shoots of both genotypes (Fig. 6).

**Positional cloning of the *arm* mutation**

Prior to physiological and genetic characterization, homozygous M₄ seeds were re-screened for inhibited PR length and increased LR density on high nitrate and then back-crossed five times to wild type Columbia (Col-0) to remove unlinked mutations caused by the
chemical mutagenesis. The mutant phenotype in the F₂ progeny for each back-cross segregated in a 3:1 (wt:mutant) ratio (337:94, \( \chi^2 = 1.75 \) observed for the first cross), demonstrating the recessive monogenic nature of the mutation. A map-based cloning approach was used to identify the mutation responsible for the \textit{arm} phenotype. An F₂ population was generated from a cross between the \textit{arm} mutant in the Col-0 background and wild type Landsberg (Ler-1). The DNA samples of plants showing the mutant phenotype on high nitrate were analyzed. An initial mapping procedure with 22 individuals indicated that the mutation was positioned at the top of chromosome 1 around the single sequence length polymorphism (SSLP) marker F21M12 (Supplemental Fig. S3). Higher resolution mapping with about 500 plants narrowed the region to a 140kb chromosomal section within BAC clones F3F20 and T20M3. That region contains \textit{At}lg05850 (Fig. 7A), which encodes chitinase-like protein 1 (\textit{CTL1}). The first mutant allele \textit{pom1} isolated by Hauser et al. (1995) and other alleles including \textit{elp1} (Zhong et al., 2002) and \textit{hot2-1} & \textit{hot2-2} (Kwon et al., 2007) phenocopy the \textit{arm} mutant phenotype observed at high nitrate (Table I, Fig. 7B). A single nucleotide substitution G\rightarrow T was found in the second exon of \textit{At}lg05850 in the \textit{arm} mutant (Fig. 7A) resulting in a change of S\textsuperscript{168} into I (Fig. 8). Significantly, we could rescue the root phenotype of \textit{elp1} and \textit{hot2} mutant lines on moderate (6 mM) nitrate and 1% sucrose medium (Fig. 7C). If \textit{AtCTL1} is responsible for the \textit{arm} phenotype it should be allelic to \textit{elp1} and \textit{hot2} and indeed, the F₁ progeny of \textit{arm} crossed to either mutant exhibited the \textit{arm} phenotype on high nitrate substrate, indicating that \textit{arm} is allelic to \textit{elp1} and \textit{hot2} (data not shown).

\textbf{Expression profile of \textit{CTL1} gene and chitinase activity test of recombinant \textit{CTL1} protein}

As a first step to dissecting the role of \textit{AtCTL1} in plant development, we analyzed the expression of this gene in response to nitrate availability. RT-PCR was carried with cDNA isolated from root and shoot of wt plants grown on 0.6, 6, and 60 mM \textit{KNO₃} and no significant difference in expression was documented (Fig. 9A). Likewise, no change in message abundance was observed for \textit{CTL1} in the \textit{arm} mutant for all nitrate treatments (results not shown).

\textit{AtCTL1} is designated as chitinase-like protein because it shows significant amino sequence similarity to a group of chitinases (GH19 glycoside hydrolases) (Graham and Sticklen, 1994). However, among class II members of the chitinases family, \textit{AtCTL1} (together with \textit{AtCTL2}) does not possess conserved amino acid residues that are essential for chitinase activity, in particular the H-E-T-T motif which is replaced by S-K-T-S (Passarhino and de Vries, 2002) (Fig. 8). The absence of these essential residues suggests \textit{CTL1} is not a
chitinase. We tested recombinant CTL1 protein purified from *E. Coli* for chitinase activity in a simple plate assay but were unable to demonstrate any activity (Fig. 9B). This result is in agreement with comments from Zhong et al. (2002) who referred to unpublished data yielding a similar outcome.

**Cell elongation defects in ctll mutant hypocotyls are not conditional on nitrate abundance**

Short hypocotyls and cell elongation defects were previously reported in *ctl1* mutant alleles (Reed et al., 1998; Mouille et al., 2003; Hématy et al., 2007; Table I). Given the impact of high nitrate on root cell swelling in the *arm* mutant, we wanted to know if the hypocotyl elongation defect is also conditional on the nitrate supply in the growth medium. To this end, we measured the hypocotyls length of seedlings germinated at three nitrate concentrations (0.6; 6 and 60 mM KNO₃) in the dark. Five days after germination in the dark, wt seedlings had long hypocotyls (Fig. 10). Under the same conditions, the *arm* mutant had significantly shorter hypocotyls than the wt, and the defect was observed across all nitrate concentrations (Fig. 10A, C), whereas inhibition of PR growth was only observed on high nitrate (Fig. 10B). The phenomenon was still observed when dark-grown seedlings were returned to light (Fig. 10D).

**The phenotype of cellulose biosynthesis mutants is conditional on nitrate abundance**

A direct role of CTL1 in cell wall synthesis seems likely because (i) a 75% reduction of cellulose synthesis was observed in *pom1-21* mutant seedlings compared to wt (Mouille et al., 2003), (ii) incomplete cell walls were observed in *elp1* inflorescence stems (Zhong et al., 2002) and (iii) *AtCTL1* expression is highly coordinated with cellulose synthesis in primary walls, based on the expression patterns of cellulose synthase *CESA1, 3, and 6* (Persson et al., 2005). Interestingly, mutations in *CESA3, CESA6* but also in *KORRIGAN I/RADially SWOLLEN 2 (KOR1/RSW2)* encoding a membrane-bound endo-1,4-β-D-glucanase, lead to similar reductions of PR growth and, to some extent, radial swelling as described in the *arm* mutant (Hauser et al., 1995; Fagard et al., 2000; Desprez et al., 2002; Cano-Delgado et al., 2003; Refregier et al., 2004; Hématy et al., 2007). Therefore, we tested the effect of nitrate availability in the growth medium on the *CESA3 (eli1-1), CESA6 (prc1-1)* and *KOR1 (kor1-1)* mutant phenotypes (Fig. 11A, B). At moderate nitrate (6 mM KNO₃) concentration, the mutants displayed slightly shorter PRs. The same conditional effects on root architecture in the presence of high nitrate (60 mM KNO₃) as described for the *arm* mutant was observed in
eli1-1 and prc1-1 mutants (Fig. 11; Supplemental Fig. S4). We note that despite a reduction in PR length, kor1-1 mutants did not elaborate LRs on high nitrate.

Another phenotype of ctl1 mutants was the ectopic deposition of lignin in the pith cells of stems and the root endodermis that would not normally accumulate this polymer (Zhong et al., 2000, 2002; Table I). Interestingly, reduction of cellulose synthesis by inhibitors or in the eli1-1 (cesa3) mutant background resulted in the accumulation of ectopic lignin deposition (Cano-Delgado et al., 2003; Rogers et al., 2005). Thus, we examined the conditional nature of the lignin deposition in roots on the nitrate availability. Lignin accumulation in roots of 13-day-old wild type, arm and cellulose synthesis mutant seedlings was visualized with phloroglucinol, which stains lignin a magenta colour (Fig. 11C). Substantial evidence of lignin deposition was visible in endodermal cells of arm roots grown with high nitrate. However, no staining was observed in wt and arm roots grown at moderate levels of KNO3 (Fig. 11), indicating that lignin deposition is also conditional on the nitrate supply. In the cellulose biosynthesis mutant eli1-1, nitrate-induced lignin deposition was more widespread than observed in the arm mutant (Fig. 11C).

DISCUSSION

Earlier descriptions of allelic mutants of CHITINASE-LIKE 1 and the discovery of permissive growth conditions

Chitin, a polymer of N-acetyl-D-glucosamine (NGlcNAc), is an elicitor of plant defence during fungal infection. In response to fungal attack, the plant activates chitinases that catalyze the hydrolysis of 1,4-β-linkages inside chitin, in order to degrade the fungal cell wall and to limit invasion (Eckart, 2008). At first, the finding in the present screen of CHITINASE LIKE 1 (CTL1) gene involved in root development and nutritional signalling seems to be puzzling. However, numerous reports point to a broader spectrum of roles for GH19 glycoside hydrolase (chitinase) family than just in defence against pathogens. These roles are related to abiotic stress responses, as various as frost, heat, mineral deficiencies, heavy metals or UV exposure (Lima et al., 2002; Kasprzewska, 2003; Bekesiova et al., 2008; Hermans et al., unpublished) but also to developmental processes (Passarhino and de Vries, 2002).

Key features of the root morphology of the arm mutant grown under restrictive conditions as described in this study include: (i) inhibition of primary root growth, (ii) increased lateral branching, (iii) radial swelling of the root tip and elongation zone, and (iv) increased root hair density. Phenotypically, allelic mutants of AtCTL1 have reduced rosette growth and display abnormal patterns of root growth (Hauser et al., 1995; Schneider et al.,
1997; Zhong et al., 2002; Yuen et al., 2005; Kwon et al., 2007; summarized in Table I). The novel insight of the results reported here is for the first time, the finding of permissive growth conditions that allow normal root development of ctl1 mutants (Fig. 2, 7C). It is noteworthy that relatively high nitrate and ammonium concentrations were used in the majority of the previous descriptions of ctl1 mutants (Table I) and those studies using a relatively low nitrate concentration focused primarily on the hypocotyl and not the root phenotype (Mouille et al., 2003; Hématy et al., 2007). In certain cases, the ctl1 mutant phenotype was conditional on high sucrose in the growth medium (Hauser et al., 1995; Supplemental Fig. S2). Low sucrose blocked the abnormal radial expansion of the PR but not completely the inhibition of root length, something that Zhong et al. (2002) reported to some extent with inhibitors of ethylene perception. Here, we showed that several features of ctl1 mutants, such as the promotion of LR proliferation, radial swelling, high root hair density (Fig. 1, 2) and lignin deposition (Fig. 11), are conditional on the environmental conditions (such as high NO₃⁻, Cl⁻, and sucrose). Therefore some earlier conclusions regarding CLT1’s role in cell wall formation and lignin biosynthesis at least in roots (Table I) may need to be revised.

**AtCTL1 plays a role in plant growth and development**

We propose that AtCTL1 plays a role in plant growth and development wherein, (1) it has a constitutive effect on cell wall synthesis and cell elongation and (2) its function is modulated by environmental stimuli that alter organ morphology.

(1) Slight inhibition of PR length under permissive conditions (Fig. 10) implicates CTL1 in primary cell wall synthesis and cell elongation. Hauser et al. (1995) showed that the principal axis of expansion is radial rather than longitudinal in pom1 and that the radial dimension is augmented in epidermis and cortex cells but decreased in endodermis cells (see also Fig. 11). While not as dramatic as ctl1-dependent changes in the presence of high nitrate or chloride, there is a measurable decrease in PR elongation under permissive conditions (Fig. 1, 2). Cell-wall remodeling during elongation requires cell-wall-modifying enzymes, including those involved in the depolymerisation of 1,4-β-linkages in the pre-existing matrix (Cosgrove, 2005; Somerville, 2006). AtCTL1 could play a role in this process if it possesses such a catalytic activity (see later in discussion). Furthermore, CTL1 orthologs in various plant species have been shown to be essential for cell wall structure and cellulose synthesis in both primary and secondary cell walls (Mouille et al., 2003; Aspeborg et al., 2005; Persson et al., 2005; Geisler-Lee et al., 2006). Nevertheless, the exact role of CTL1 in defining the physical properties of the wall remains to be determined.
(2) The *ctl1* mutants described here and in previous screens (Table I) have a conditional impact on root architecture and defects in cell elongation in hypocotyls triggered by darkness. If these were constitutive phenotypes expressed under all conditions and in every organ, it would be easy to ascribe this effect to a direct role in the enzymology of cell wall synthesis or remodelling. However, we have demonstrated here that the dramatic effect on root morphology is inducible by a variety of environmental factors, including high levels of anions and sucrose. One might suggest osmotic potential is the unifying variable, yet significant changes in root morphology are not induced when roots are transferred onto media containing high concentrations of sorbitol or mannitol, where comparable levels of nitrate and chloride still have a striking impact (Fig. 4, 5). It is noteworthy that 30 mM K$_2$SO$_4$ has no effect on root morphology in contrast to decreased root growth in the presence of equivalent concentrations of osmotically active ions in Ca(NO$_3$)$_2$. This observation, in conjunction with the other ion pairs tested in Fig. 4, suggests ion concentrations and electrostatic interactions in the cell wall are not responsible for the conditional phenotype. Multiple environmental variables have been shown to induce the *ctl1* phenotype, including high nitrate and chloride (Fig. 1, 2, 4, Table I), high salt (Kwon et al., 2007) and high temperature (Hong et al., 2003; hot2, perhaps also linked to high chloride). In addition, high sucrose can induce the *ctl1* phenotype (Fig. 3, Supplemental Fig. S2; Hauser et al., 1995). Taken together, these data show that CTL1 is part of multiple response pathways that alter root morphology.

The morphology of wt roots grown under a variety of environmental conditions that induce the *arm* phenotype described here includes a single long PR with the inhibition of LR primordia development at post-emergence (Fig. 2C; Malamy and Ryan, 2001; Signora et al., 2001; Liang and Harris, 2005; Qi et al., 2007). Since the inhibition of LR development under non-permissive conditions does not occur in *ctl1* mutants, even in loss of function alleles (*hot2-2* and *elp1*), we interpret that as a removal of a repressor. In the context of a biochemical activity, non-permissive conditions (such as high nitrate or chloride) in wild type roots might rely on CTL1-dependent stiffening of the cell wall to prevent LR emergence. Likewise in the wt primary root, CTL1-dependent stiffening of specific regions of the cell wall could led to cell elongation versus radial swelling (as seen in the *ctl1* mutants). Thus, the conditional role of CTL1 in changing root architecture is also linked to cell wall synthesis/remodelling and cell elongation. Mutations in some cellulose synthase subunits (*CESA3* and *CESA6*) phenocopy the *arm* mutant under non-permissive conditions, suggesting their activity is also altered in response to environmental conditions. This is not too surprising
assuming regulation of cell wall structure plays a key role in controlling cell shape and, ultimately, root morphology.

**Enzymatic function of CHITINASE-LIKE 1**

A key question regarding CTL1’s role in altering root growth and development in the context of the diversity of mutant phenotypes, e.g. hypersensitivity to salt, decreased thermotolerance, reduced synthesis of cellulose and ectopic deposition of lignin in stems and roots (Table I), is determining the enzymatic function of this protein. On first inspection AtCTL1 belongs to class-II family of chitinases that lack a cysteine-rich lectin domain in the N-terminal region of the protein that has a role in chitin binding (Meins et al., 1994; Passarhino and de Vries, 2002). AtCTL1 also has a putative secretion sequence at the N terminus suggesting that the protein may be secreted into the cell-wall compartment (Zhong et al., 2002; Kwon et al., 2007). According to Hahn et al. (2000), two E residues are required for catalysis in the GH19 family. The first one, within the H-E-T-T active site (E\textsuperscript{127} replaced by K\textsuperscript{127} in AtCTL1), acts as a general acid and the second one (E\textsuperscript{149} in AtCTL1) functions as a base to activate a water molecule for the single-step displacement reaction. The acid moiety of the catalytic site is highly conserved among chitinases between species (Bishop et al., 2000). That residue is replaced by K\textsuperscript{127} in AtCTL1, as well as in all of the CTL1 ortholog proteins (Aspeborg et al., 2005; Fig. 8). Taken together, these data strongly suggest that CTL1 is not a functional chitinase and that conclusion is supported by the absence of measurable chitinase activity in recombinant CTL1 (Fig. 9B). To date, no endogenous substrates have been established for CTL1.

**Conclusion**

In the results reported here we show that CTL1 plays a role in plant growth and development (Fig. 12). It is a constitutive player in cell elongation as demonstrated by a decrease in primary root growth under permissive conditions. Additionally, CTL1 functions as a component of multiple response pathways that control developmental plasticity in root morphology in response to environmental stimuli. The cell elongation defects in hypocotyls (e.g. triggered by darkness) and the conditional impact on root architecture (e.g. in the presence of high concentrations of monovalent anions and sucrose) appear to be driven by CTL1-mediated changes in cell wall structure. Since CTL1 is constitutively expressed across a range of permissive and restrictive conditions, we hypothesize that CTL1-mediated regulation
is not dependent on changes in CTL1 protein abundance and is probably a function of differentially regulated enzymatic activity.

MATERIALS AND METHODS
Mutant isolation and plant growth conditions
M₂ ethyl methanesulfonate-mutagenized seeds (Col-0) were purchased from Lehle Seeds (TX, USA). Seeds were surface sterilized with ethanol 70% (v/v) during 10 min and in a 20% (v/v) HClO during 5 min. They were plated on 1x Murashige and Skoog strength with the nitrogen concentration modified to 60 mM KNO₃, 1% sucrose, 0.8% agar. Seeds were stratified at 4°C for 2 days in the dark, and then incubated vertically in a culture chamber at a temperature of 22°C and a day light regime of 16 h (75 µmol photons m⁻² s⁻¹)/ 8 h darkness. Thirteen-days-old seedlings grown vertically were then screened for the presence of lateral roots at high nitrate concentration.

Genetic Mapping
A total of 480 F₂ from a cross between arm in the Columbia (Col-0) background and wild type Landsberg (Ler-1), and showing the mutant phenotype at high nitrate were used to map the mutation. DNA was isolated from each plant and analysed for recombination events with simple sequence length polymorphism (SSLP) markers and cleaved-amplified polymorphic sequence (CAPS) markers. Primer pairs for newly found SSLP markers between Col-0 and Ler-1 were designed during this mapping process: CSU01: forward, 5'-TTCCAAATACGATTTTCAGGTAG-3'; reverse, 5'-CAAGTGACGGGATCCAAAAT-3'; CSU02: 5'-CTGCTCTCATGCCTCTCTG-3'; reverse, 5'-AATTAACTTTTATGATGTGACAGGAA-3'; CSU03: forward, 5'-TGTTACATGCTTTGTGAG-3'; reverse, 5'-ACAATCCACAGGGAGGATGAG-3'; and CSU04: forward, 5'-ATTGTAAGCAAGCGACTGGA-3'; reverse, 5'-GAATAATCCTTAACACAGTCCTCTGAGATA-3'. The description of other registered markers is available on TAIR (www.arabidopsis.org).

Mineral analysis
Seedlings vertically grown in Petri dishes for 13 days were harvested and dried at 80°C prior to mineral analysis. Nitrate content was measured with a Flow Solution 3000 (OI Analytical, TX, USA) using cadmium reduction. For other mineral analyses, samples were digested with
nitric acid and assayed by inductively coupled plasma mass spectrometry (ICP-MS) at the Purdue Ionomics Information Management Systems, Purdue University (IN, USA).

Cloning of CTL1 gene and expression in E. coli

CTL1 gene (At1g05850) was amplified using the following primers 5’-TAGTTGTAAGCCATGGGAGCAATCAGGAGT-3’ (forward) and 5’-GATATATCATAGGAATCCGAAGAGGAAGA-3’ (reverse), which contained NcoI and BamHI restriction sites (underlined) and Easy A High Fidelity Enzyme (Stratagene) according to manufacturer’s protocol. The 1,100bp fragment was gel purified, digested with NcoI and BamHI and ligated into pET32a(+) vector. In this vector, CTL1 is expressed as a thioredoxin fusion protein. The resulting plasmid was transformed into E. Coli Rosetta2 competent cells (Novagen) to construct recombinant plasmid. For protein expression, one single colony was grown overnight in Luria-Bertani (LB) medium containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) at 37°C with vigorous shaking. The overnight culture was diluted (1:100) into fresh LB containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) and grown at 37°C with vigorous shaking until OD550nm was approximately 0.6-0.8. The recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5mM) for 4h, with vigorous shaking, at room temperature. The cells were harvested by centrifugation at 3,000g for 10min at 4°C and frozen at -80°C.

Purification of recombinant CTL1 protein

The purification of the recombinant protein was as described by Kirubakaran and Sakthivel (2007), except that the dialysis was performed progressively at 6, 4, 2, and 1 M of urea. After renaturation of the protein from inclusion bodies, a batch purification of 6xHis-tagged proteins under native conditions was performed using Ni-NTA agarose (Qiagen) following the manufacturer’s protocol (Qiagen). Protein analysis was performed on 12% sodium dodecyl sulfate-polyacrylamide gel Electrophoresis (SDS-Page; Bio-Rad). Protein quantification was done following the Bradford method (Bradford, 1976).

Chitinase activity assay.

Glycol chitin was obtained by acetylation of glycol chitosan (Sigma-Aldrich, USA) after the method of Trudel and Asselin (1989). Glycol chitin (0.01%) was mixed with melted 0.8% agarose and poured into a petri plate. After solidification, 20µl sterile distilled water, 10µg of chitinase from Streptimyces griceus (Sigma-Aldrich, USA) and 10µg of recombinant CTL1 were pipetted into separate wells in the plate. Plates were incubated at 37°C for 2h, stained with 0.01% of Calcofluor White M2R (Sigma-Aldrich, USA), washed with distilled water and...
visualized under UV light to detected the zone of clearance around the well left by the degradation of chitin by chitinase activity (Kondreddy et al., 2007).

**Acknowledgements**

This work is supported by a grant from the US National Science Foundation to D.R.B., and from the Belgian Science Policy Office (BelSPo project IAPVI/33) to NV. C.H. is currently a postdoctoral fellow of the Fonds National de la Recherche Scientifique (FRNS-FRS) and previously of the Federal Belgian Science Policy (BelSPo, Return grant). S.-W. Hong is thanked for sharing *hot2* seeds, Z.-H. Ye for *elp1* and H. Höfte for *kor, eli1-1* and *e112*. 
References

Aloni R, Langhans M, Aloni E, Dreieicher E, Ullrich C (2005) Root-synthesized cytokinin in Arabidopsis is distributed in the shoot by the transpiration stream J Exp Bot 56: 1535–1544

Anil K, Seshagirirao K, Podile AR (2007) A simple, rapid and yet less expensive method to detect chitinase in agarose plates. J Biophys Biochem Methods 70: 683–684

Aspeborg H, Schrader J, Couthino PM, Stam M, Kallas A, Djerbi S, Nilsson P, Denman S, et al (2005) Carbohydrate-active enzymes involved in the secondary cell wall biogenesis in hybrid aspen. Plant Physiol 137: 983–997

Bekesiova B, Hraska S, Libantova J, Moravcikova J, Matusikova I (2008) Heavy-metal stress induced accumulation of chitinase isoforms in plants. Mol Biol Rep 35: 579–588

Bradford MM (1976) A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting Arabidopsis lateral root development. Trends Plant Sci 8: 165–171

Cano-Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. Plant J 34: 351–362

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Annu Rev of Plant Physiol Plant Mol Biol 50: 391–417

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6: 850–861

Desprez T, Vemhettes S, Fagard M, Refregier G, Desnos T, Aletti E, Py N, Pelletier S, Höfte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiol 128: 482–490
Eckardt NA (2008) Chitin signaling in plants: insights into the perception of fungal pathogens and rhizobacterial symbionts. Plant Cell 20: 241–243

Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S, Höfte H (2000) PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. Plant Cell 12: 2409–2424

Gifford ML, Dean A, Gutiérrez GA, Coruzzi G, Birnbaum K (2008) Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci USA 105: 803–808.

Gojon A, Nacry P, Davidian J-C (2009) Root uptake regulation: a central process for NPS homeostasis in plants. Curr Opin Plant Biol 12: 328-338

Hahn M, Hennig M, Schlesier B, Höhne W (2000) Structure of jack bean chitinase. Biol Crystallogr D56: 1096–1099

Hauser M-T, Morikami A, Benfey P (1995) Conditional root expansion mutants of Arabidopsis. Development 121: 1237–1252

Hématy K, Sado P-E, Van Tuinen A, Rochange S, Desnos T, Balzergue S, Pelletier S, Renou J-P, Höfte H (2007) A receptor-like kinase mediates the response of Arabidopsis cells to the inhibition of cellulose synthesis. Current Biology 17: 922–931

Hermans C, Hammond JP, White PJ, Verbruggen N (2006) How do plants respond to nutrient shortage by biomass allocation? Trends Plant Sci 11: 610–617

Hong S-W, Vierling E (2000) Mutants of Arabidopsis thaliana defective in the acquisition of tolerance to high temperature stress. Proc Natl Acad Sci USA 97: 4392–4397

Hong S-W, Lee U, Vierling E (2003) Arabidopsis hot mutants define multiple functions required for acclimation to high temperature. Plant Physiol 132: 757–767
Iyer-Pascuzzi A, Simpson J, Herrera-Estrella L, Benfey PN (2009) Functional genomics of root growth and development in Arabidopsis. Curr Opin Plant Biol 12: 165–171

Kasprzewska A (2003) Plant chitinases-regulation and function. Cell Mol Biol Lett 8: 809–824

Kwon YR, Kim S-H, Jung M-S, Kim M-S, Oh J-E, Ju H-W, Kim K, Vierling E, Lee H, Hong S-W (2007) Arabidopsis hot2 encodes an endochitinase-like protein that is essential for tolerance to heat salt and drought stresses. Plant J 49: 184–193

Lahner B, Gong J, Mahmoudian M, Smith EL, Abid KB, Rogers EE, Guerinot ML, Harper JF, Ward JM, McIntyre L et al (2003) Genomic scale profiling of nutrient and trace elements in Arabidopsis thaliana. Nat Biotechnol 21: 1215–1221

Isaac Kirubakaran S, Sakthivel N (2007) Cloning and overexpression of antifungal barley chitinase gene in Escherichia coli. Protein Express Purif 52: 159–166

Liang Y, Harris JM (2005) Response of root branching to abscisic acid is correlated with nodule formation both in legumes and nonlegumes. Am J Bot 92: 1675–1683

Lima VM, Magioli C, B de A Gerhardt L, Tarré E, Menezes RMG, Sachetto-Martins G, Margis-Pinheiro M (2002) Bean class IV chitinase promoter is modulated during plant development and under abiotic stress. Physiol Plant 116: 512–521

Little DY, Rao H, Olivia S, Daniel-Vedele F, Krapp A, Malamy JE (2005) The putative high nitrate transporter NRT21 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci USA 102: 13693–13698

Liu KH, Huang CY, Tsay YF (1999) CHL1 is a dual-affinity nitrate transporter of Arabidopsis involved in multiple phases of nitrate uptake. Plant Cell 11: 865–874

López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol 6: 280–287
MacGregor DR, Deak KI, Ingram PA, Malamy JE (2008) Root system architecture in Arabidopsis grown in culture is regulated by sucrose uptake in the aerial tissues. Plant Cell 20: 2643–2660

Malamy JE, Ryan S (2001) Environmental regulation of lateral root initiation in Arabidopsis. Plant Physiol 127: 899–909

Meins F, Fritig B, Linthorst HJM, Mikkelsen JD, Neuhaus J-M, Ryals J (1994) Plant chitinase genes. Plant Mol Biol Rep 12: 22–28

Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM (2007) Nitrate transport and signaling. J Exp Bot 58: 2297–2306

Mouille G, Robin S, Lecomte M, Pagant S, Höfte H (2003) Classification and identification of Arabidopsis cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. Plant J 35: 393–404

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nibau C, Gibbs DJ, Coates JC (2008) Branching out in new directions: the control of root architecture by lateral root formation. New Phytol 179: 595–614

Passarinho PA, de Vries SC (2002) Arabidopsis chitinases: a genomic survey. In The Arabidopsis book Published by the American Society of Plant Biologists doi: 101199/tab0023

Péret B, De Rybel B, Casimiro I, Benkova E, Swarup R, Laplaze L, Beeckman T, Bennett M (2009) Arabidopsis lateral root development: an emerging story. Trends Plant Sci 114: 399-408

Persson S, Wei H, Milne J, Page GP, Sommerville CR (2005) Identification of genes required for cellulose synthesis by regression analysis of public microarrays data sets. Proc Natl Sci USA 102: 8633–8638
Qi X, Wu Z, Li J, Mo X, Wu S, Chu J, Wu P (2007) AtCYT1-INV1 a neutral invertase is involved in osmotic stress-induced inhibition of lateral root growth in *Arabidopsis*. Plant Mol Biol 64: 575–587

Reed JW, Elumalai RP, Chory J (1998) Suppressors of an *Arabidopsis thaliana* phyB mutation identity genes that control light signaling and hypocotyls elongation. Genetics 148: 1295–1310

Refregier G, Pelletier S, Jaillard D, Höfte H (2004) Interaction between wall deposition and cell elongation in dark-grown hypocotyls cells in *Arabidopsis*. Plant Physiol 135: 959–968

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A (2006) A central role for the nitrate transporter NRT21 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. Plant Physiol 140: 909–921

Schneider K, Wells B, Dolan L, Roberts K (1997) Structural and genetic analysis of epidermal cell differentiation in Arabidopsis primary roots. Development 124: 1789–1798

Signora L, De Smet I, Foyer CH, Zhang H (2001) ABA plays a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis. Plant J 28: 655–662

Somerville CR (2006) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 22: 53–78

Takakura Y, Ito T, Saito H, Inoue T, Komari T, Kuwata S (2000) Flower-predominant expression of a gene encoding a novel class I chitinase in rice (*Oryza sativa* L). Plant Mol Biol 42: 883–897

Tranbarger TJ, Al-Ghazi Y, Muller B, Teyssendier de la Serve B, Doumas P, Touraine B (2003) Transcription factor genes with expression correlated to nitrate-related root plasticity of *Arabidopsis thaliana*. Plant Cell Env 26: 459–469
Trudel J, Asselin A (1989) Detection of chitinase activity after gel electrophoresis. Anal Biochem 178: 362–366

Wang C, Li J, Yuan M (2007) Salt Tolerance Requires Cortical Microtubule Reorganization in Arabidopsis. Plant Cell Physiol 48: 1534–1547

Yuen CYL, Sedbrook JC, Perrin RM, Carroll KL, Masson PH (2005) Loss-of-function mutations of ROOT HAIR DEFECTIVE3 suppress root waving skewing and epidermal cell file rotation in Arabidopsis. Plant Physiol 138: 701–714

Zhang D, Hrmova M, Wan C-H, Wu C, Balzen J, Cai W, Wang J, Densmore LD, Fincher GB, Zhang H, Haigler CH (2004) Members of a new group of chitinase-like genes are expressed preferentially in cotton cells with secondary walls. Plant Mol Biol 54: 353–372

Zhang H, Forde BG (1998) An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279: 407–409

Zhang H, Jennings A, Barlow PW, Forde BG (1999) Dual pathways for regulation of root branching by nitrate. Proc Natl Acad Sci USA 96: 6529–6534

Zhang H, Forde BG (2000) Regulation of Arabidopsis root development by nitrate availability. J Exp Bot 51: 51–59

Zhang D, Hrmova M, Wan C-H, Wu C, Balzen J, Cai W, Wang J, Densmore L, Fincher GB, Zhang H, Haigler CH (2004) Members of a new group of chitinase-like genes are expressed preferentially in cotton cells with secondary walls. Plant Mol Biol 54: 353–372

Zhong R, Ripperger A, Ye Z-H (2000) Ectopic deposition of lignin in the pith of stems of two Arabidopsis mutants. Plant Physiol 123: 59–69

Zhong R, Kays SJ, Schroeder BP, Ye Z-H (2002) Mutation of a chitinase-like gene causes ectopic deposition of lignin aberrant cell shapes and overproduction of ethylene. Plant Cell 14: 165–179
Supporting information

Supplemental Figure S1. Physiological characterization of wild type and arm plants

Supplemental Figure S2. Effect of different combinations of nitrate and sucrose on root system architecture of wt and arm mutant

Supplemental Figure S3. Positional cloning of arm mutation

Supplemental Figure S4. Root architecture parameters of wild type, cellulose synthesis mutants and arm plants
Table I: Description of allelic mutants of *AtCTL1* and the nitrate concentrations in the growth medium used in these reports.

| Mutant allele     | Phenotype                                                                                                                                                                                                 | NO₃⁻ concentration | Reference                        |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|----------------------------------|
| *pom1-1*          | Principal axis of root expansion is radial rather than longitudinal. Radial dimensions of epidermis and cortex cells increased.                                                                        | 39 mM               | Hauser et al., 1995              |
| *erh2/pom1-12*    | *ectopic root hair* 2 mutant was isolated in a screen for identifying genes in the specification of epidermal cell fate in PR. More than one third of its root hair in ectopic deposition. Inability to respond to low doses of propyzamide (microtubule-binding drug). | 39 mM               | Schneider et al., 1997           |
| *pom1-14, pom1-15*| Detected as revertants of the phytochrome *phyB-1* mutation, by restoring short length hypocotyls.                                                                                                         | 39 mM               | Reed et al., 1998                |
| *pom1-2, pom1-21* | Shorter hypocotyls, severe reduction in cellulose synthesis. Partial revertant of *thel-3* mutation (mutant allele of *THESEUS1*, a plasma-membrane-bound receptor-like kinase probably acting as a cell-wall-integrity censor). | 9 mM                | Mouille et al., 2003; Hématy et al., 2007 |
| *hot2-1, hot2-2*  | *sensitive to hot temperature* 2 mutants were isolated based on a lesser thermotolerance in a hypocotyl elongation assay. Hypersensitivity to salt.                                                            | 9 mM                | Hong and Vierling, 2000; Hong et al., 2003 |
| *elp1*            | *ectopic deposition of lignin in pith* 1 mutant was identified in a screen for ectopic deposition of lignin in inflorescence stems.                                                                          | 39 mM               | Zhong et al., 2000; 2002         |
| *3G-12/pom1-22*   | Isolated from a screen of callus for enhanced shoot development at sub-optimal concentrations of cytokinin.                                                                                             | 12.5 mM             | Cary et al., 2001                |
| *pom1-26*         | Isolated from a screen for ectopically lignification.                                                                                                                                                  | 39 mM               | Rogers et al., 2005              |
**Figures legends**

**Figure 1.** Effect of nitrate availability in the grow medium on root system architecture of wild type and *arm* plants.
Seedlings were grown vertically on MS medium with increasing nitrate (KNO₃) supply for 13 days. A, Primary root length. B, Lateral root (>1mm) number; n=8 ± SE. open symbol: wild type, closed symbol: *arm* mutant. C, Picture of root tip. Scale bar: 0.5 mm.

**Figure 2.** Conditional phenotypes of wild type and *arm* plants to nitrate supply.
Pictures of seedlings grown vertically on MS medium with 3 nitrate concentrations: 0.6 mM (A, D, G, J); 6 mM (B, E, H, K) and 60 mM (C, F, I, L) KNO₃ for 13 days (A-C, D-E) and 27 days (G-I, J-L). (A-C, G-I) wild type, (D-E, J-L) *arm* mutant. Scale bar: 2 cm.

**Figure 3.** Effect of the C:N ratio on root system architecture of wild type and *arm* plants.
Seedlings were grown at nine different combinations of nitrate (□■: 0.6; △▲: 6; ○●: 60 mM KNO₃) and sucrose (0.1; 1 and 5 % w/w) concentrations. A, Primary root length. B, Lateral root density (number of lateral roots divided by the primary root length between hypocotyl and last observed branching) were measured at day 13. Open symbol: wild type; closed symbol: *arm* mutant. n=8-14 ± SE.

**Figure 4.** Effect of minerals and sugars on primary root elongation of wild type and *arm* plants. Six-days-old seedlings grown on moderate (6 mM KNO₃) medium were transferred to diverse substrates enriched in minerals (A), sugars and chloride salts (B). The relative root growth was calculated by dividing the PR elongation 6 days after transfer on a particular substrate by the average elongation observed on moderate nitrate medium (considered as the reference growth condition). Open bar: wild type; closed bar: *arm* mutant. n=8-14 ± SE. Stars indicate significant differences between genotypes at level <0.05. Scale bar: 0.5 mm

**Figure 5.** Local sensing of nitrate availability in root tip of wild type and *arm* plants.
A, Six-days-old seedlings germinated on moderate (6mM KNO₃) nitrate substrate were transferred onto plates divided by a high (60mM KNO₃) nitrate strip band. Seedlings were transferred so that the older root zone or the root tip alone were in contact with 60mM KNO₃. B-E, Close-ups of root tip: *arm* (B) and wild type (C) contacting high nitrate; *arm* (D) and wt
(E) contacting moderate nitrate. Pictures were taken 2 days after transfer. Bar scale A: 1 cm; B-E: 1 mm. The experiment was repeated with a minimum of 10 seedlings of each genotype.

**Figure 6.** Mineral profile in tissues of wild type and *arm* plants.

Seedlings were grown at three nitrate supplies (0.6; 6 and 60 mM KNO₃). Mineral content in shoot and root was measured 13 days after germination. P and S refer to total phosphorus and sulfur content. Open bars: wild type; closed bars: *arm* mutant. n=5-7 independent biological replicates (20-25 pooled plants) ± SE. Stars indicate significant differences between genotypes at level <0.05.

**Figure 7.** Positional cloning of *arm* mutation and root morphology of mutant alleles of *AtCTL1* as a function of nitrate supply.

A, A single nucleotide mutation in the second exon of *AtCTL1* gene (At1g05850) was found in the *arm* mutant. B-C, Seedlings of wild type, *arm* and previously described *ctl1* alleles that include: *elp1* (Zhong et al., 2000; 2002) and *hot2* (Hong and Vierling, 2000; Kwon et al., 2007) were grown on high nitrate (60 mM KNO₃) over 13 days (C) and moderate nitrate (6 mM KNO₃) over 25 days (C). Scale bar: 2 cm.

**Figure 8.** Multiple alignments of class II chitinases from *Arabidopsis thaliana* and other species

Comparison of AtCTL1-At1g05850 (chosen as consensus sequence), AtCTL2-At3g16920, ABV89660 (*Brassica rapa*), BAAC82645 (*Pisum sativum*), GhCTL1-AAQ56598, GhCTL2-AAQ56599 (*Gossypium hirsutum*), ABN08775 (*Medicago truncatula*) and two other Arabidopsis class II chitinases: At1g02360, At4g01700, using the alignment program VectorNTI. Gaps (-) are introduced to optimize alignment and the degree of shading of boxes represents the level of similarity: identical residues (black), conservative (dark gray), weakly similar (light gray) and different (white). (^) indicate chitinase 19_1 signature C-x(4,5)-F-Y-[ST]-x(3)-[FY]-[LIVMF]-x-A-x(3)-[YF]-x(2)-F-[GSA] and 19_2 signature [LIVM]-[GSA]-F-x-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM] (Passarhino and de Vries, 2002). Residues in bold are essential for catalytic activity and boxed residues putatively bind the substrate in class I chitinases (Hahn et al., 2000; Passarhino and de Vries, 2002). The putative secretion signal in AtCTL1 is underlined. The residue substitutions in *arm* and previously described (*hot2-1, hot2-2, elp1*) mutants are indicated.
**Figure 9. Expression of CTL1 gene and chitinase activity of CTL1 protein**

A. Effect of nitrate supply on the expression of *CTL1* gene in wild type *Arabidopsis thaliana*: RT-PCR analysis of *AtCTL1* transcript abundance in root and shoot tissues. Seedlings were grown at three different nitrate concentrations (0.6; 6 and 60 mM KNO₃) for 13 days prior to harvest. Equal loading of cDNA sample in each lane was verified with the constitutive *At3g62290 (ADP-RIBOSYLATION FACTOR A1E)* gene. The experiment was duplicated and similar results were obtained. B. Detection of chitinase activity in plate. Sterile distilled water, 10 µg chitinase from *Streptimyces griceus* and 10 µg purified of recombinant CTL1 purified from *E. coli* were loaded into an agarose gel containing glycol chitin as substrate. After incubation and staining with calcofluor white M2R, the zone of clearance around the well left by the degradation of chitin by chitinase activity was detected under UV light.

**Figure 10. Effect of nitrate availability on hypocotyl and root length in dark-grown seedlings**

Seedlings were grown vertically at three nitrate (KNO₃) supplies for 5 days in darkness. A, Hypocotyl length. B, Primary root length n>55 ± std. open bar: wild type, closed bar: *arm* mutant. All differences between genotypes are statistically significant at level *P*<0.01. C Picture of seedlings after 5 days growth in darkness. D, Picture of seedlings grown for 5 days in darkness and returned to light for 7 additional days. Scale bars 1cm.

**Figure 11. Effect of nitrate supply on the root morphology and lignin deposition in cellulose synthesis mutants.**

Pictures of whole seedlings (A) and root tip (B) of wild type Col-0, Ws, cellulose biosynthesis mutants (*kor1-1 (KOR1); eli1-1 (CESA3); prc1-1 (CESA6))* and *arm* grown at two different nitrate concentrations (6 and 60 mM KNO₃) for 13 days. All mutants are in the Col-0 background, except *kor1-1* is in Ws. C, Phloroglucinol staining of root tissues (1cm above root tip) of seedlings grown in the same conditions. Bar scales: A, 1cm, B, 0.5 mm, C, 500 µm.

**Figure 12. Proposed model of CTL1 role in plant growth and development**

A, Summary scheme of the *ctl1/arm* phenotype. Mutations in chitinase-like *CTL1* gene in *Arabidopsis* results in cell elongation defects in the hypocotyls (induced by darkness) and in roots (upon restrictive conditions). While the elongation defect in the primary root is constitutive, dramatic changes in root architecture are conditional on anion (nitrate and
chloride) supply, high C/N ratio and sucrose. The conditional root phenotype includes LR elongation, radial swelling and increased root hair density. B, Suggested role of CTL1 in wild type seedlings: (i) it plays a role in cell wall synthesis/structure and cell elongation in various organs, and (ii) it is a component of multiple environmental response pathways (probably involving cellulose synthase subunits CESA3 and CESA6) that influence root morphology by altering cell wall rigidity.
A) Primary root length (cm) vs Sucrose supply (%)

B) Lateral root density (LR number cm⁻¹) vs Sucrose supply (%)

www.plantphysiol.org on August 29, 2017 - Published by Downloaded from Copyright © 2009 American Society of Plant Biologists. All rights reserved.
A  Minerals

B  Osmoticum

Relative growth

wt  arm

Mannitol

Sorbitol

NaCl

KCl

CaCl2

www.plantphysiol.org on August 29, 2017 - Published by Downloaded from

Copyright © 2009 American Society of Plant Biologists. All rights reserved.
Mineral content (mg g$^{-1}$ dw)

KNO$_3$ concentration in growth medium (mM)
Figure 8

AtCTL1

AtCTL2

ABV89660 (Br)

BAC81645 (Ps)

AAQ56598 (Gh)

AAQ56599 (Gh)

ABN08775 (Mt)

At1g02360

At4g01700

hot2-2 → STOP

AtCTL1

AtCTL2

ABV89660 (Br)

BAC81645 (Ps)

AAQ56598 (Gh)

AAQ56599 (Gh)

ABN08775 (Mt)

At1g02360

At4g01700

elpl → STOP

arm S → I

hot2-1 G → R

W

Q

I

19_2

Y

19_1

elpl

arm

hot2-1

19_2
### A

|        | 0.6 | 6   | 60  |
|--------|-----|-----|-----|
| Shoots | 0.6 | 6   | 60  |
| Roots  | 0.6 | 6   | 60  |

mM KNO$_3$

**CTL1**

At3g62290

### B

- **H$_2$O**
- Chitinase from *S. griceus*
- Recombinant AtCTL1 protein purified from *E. coli*
A B

C

D

KNO₃ supply (mM)

Hypocotyl length (mm)

Primary root length (mm)

0.6 6 60 mM KNO₃

wt arm wt arm wt arm

0.6 6 60 mM KNO₃

wt arm wt arm wt arm
6 mM KNO$_3$

60 mM KNO$_3$

Col-0  Ws  kor1-1  eli1-1  pre1-1  arm

Col-0  Ws  kor1-1  eli1-1  pre1-1  arm
Hypocotyl elongation defect (induced by darkness)

Conditional root defect (induced by nutrient cues)

Constitutive root defect

NO$_3^-$, Cl$^-$, C/N, sucrose

Cell elongation

Repulsion of LRs elongation

NO$_3^-$, Cl$^-$, C/N, sucrose