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Genetic responses to alkamides in Arabidopsis

*Author to whom correspondence should be addressed:
Name: José López-Bucio
Address: Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México.
Telephone: 5 443 3265788, Fax: (443) 3265788
E-mail: jbucio@zeus.umich.mx.

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Characterization of *drrl*, an alkamide resistant mutant of Arabidopsis reveals an important role for small lipid amides in lateral root development and plant senescence.

Full names of authors:
Alina Morquecho-Contreras, Alfonso Méndez-Bravo, Ramón Pelagio-Flores, Javier Raya-González, Randy Ortiz-Castro, and José López-Bucio*
Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México.
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*Corresponding author:
Name: José López-Bucio.
Address: Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México.
Telephone: 5 443 3265788, Fax: (443) 3265788
E-mail: jbucio@zeus.umich.mx.
Alkamides belong to a class of small lipid signals of wide distribution in plants, which are structurally related to the bacterial quorum-sensing (QS) signals N-acyl-L-homoserine lactones (AHLs). Arabidopsis (Arabidopsis thaliana) seedlings display a number of root developmental responses to alkamides, including primary root growth inhibition and greater formation of lateral roots. To gain insight into the regulatory mechanisms by which these compounds alter plant development, we performed a mutant screen for identifying Arabidopsis mutants that fail to inhibit primary root growth when grown under a high concentration of N-isobutyl decanamide. A recessive N-isobutyl decanamide-resistant mutant (decanamide resistant root-drr1) was isolated because of its continued primary root growth and reduced lateral root formation in response to this alkamide. Detailed characterization of lateral root primordia (LRP) development in wild-type and drr1 mutants revealed that DRR1 is required at an early stage of pericycle cell activation to form LRP in response to both N-isobutyl decanamide and N-decanoyl-L-homoserine lactone, a highly active bacterial QS signal. Exogenously supplied auxin similarly inhibited primary root growth and promoted lateral root formation in wild-type and drr1 seedlings, suggesting that alkamides and auxin act by different mechanisms to alter root system architecture. When grown both in vitro and in soil drr1 mutants showed dramatically increased longevity and reduced hormone and age dependent senescence, which were related to reduced lateral root formation when exposed to stimulatory concentrations of jasmonic acid (JA). Taken together, our results provide genetic evidence indicating that alkamides and AHLs can be perceived by plants to modulate root architecture and senescence-related processes possibly by interacting with JA signaling.
INTRODUCTION

Plant growth and development require the integration of a variety of environmental and endogenous signals, which together with the intrinsic genetic program, determine plant form and longevity. Lipids have long been recognized as signals that have the capacity to trigger profound physiological responses. In animals, ceramides and sphingosines are lipids that have pro-apoptotic and antiproliferative actions (Wymann and Schneiter, 2008). In plants, ceramides, sphingosines and phosphatidic acid are involved in mediating plant growth, development and responses to environmental stimuli (Wang, 2004; Worrall et al., 2003).

In the past few years, additional small lipids have been found to acts as plant signals, including alkamides and N-acyl-etanolamines (NAEs). Alkamides comprise at least 200 amides with varied acyl chain length and saturation grade (reviewed by López-Bucio et al., 2006; Morquecho-Contreras and López-Bucio, 2007). These compounds have been found to alter root and shoot system architecture in Arabidopsis (Ramírez-Chávez et al., 2004, Campos-Cuevas et al., 2008). NAEs represent compounds with aminoalcohol linked as an amide to the fatty acid. They are likely produced from the hydrolysis of N-acyl phosphatidylethanolamines (NAPEs), a minor constituent of cell membranes by phospholipase D (Chapman, 2004). NAEs have been found to accumulate in seeds of some higher plants, including cotton, corn, Arabidopsis, soybean tomato and pea and their levels decline during germination (Wang et al., 2006).

Many Gram negative bacteria produce alkamide-related substances termed N-acyl-L-homoserine lactones (AHLs). These compounds participate in cell-to-cell signaling that is usually referred to as quorum-sensing (Pearson et al., 1994). The AHL signals contain a conserved homoserine lactone (HL) ring and an amide (N)-linked acyl side chain. The acyl-chain moiety of naturally occurring AHLs can differ in length and substitution at position C3, which is either unmodified or carries an oxo-or hydroxyl group (Pearson et al., 1994; Parsek et al., 1999). These molecules are freely diffused through the bacterial membrane, which is to some extent dependent upon the length of the acyl side chain and the nature of any C3 substitutions and distribute within the rhizosphere (Pearson et al., 1999; Scott et al., 2006; Schuhegger et al., 2006). Evidence has accumulated indicating that plants are able to perceive AHLs. The application of AHLs to Medicago truncatula and Arabidopsis thaliana plants resulted in differential transcriptional changes in roots and shoots, affecting expression of genes potentially involved in...
development (Mathesius et al., 2003; Von Rad et al., 2008). Ortíz-Castro et al., (2008) evaluated *Arabidopsis thaliana* growth responses to a variety of saturated AHLs ranging from 4 to 14 carbons in length, focusing on alterations in post-embryonic root development. The compounds affected primary root growth, lateral root formation and root hair development. While this information clearly indicates that plants are able to sense a variety of small lipid signals including alkamides, NAEs and AHLs, which modulate root architecture, the genetic mechanisms involved in signal perception to these compounds are unknown.

The Arabidopsis root system is an excellent model to dissect the genetic and developmental processes that determine plant architecture. It mainly consists of an embryonic primary root and post-embryonic developed lateral roots (LRs) (López-Bucio et al., 2005). LR formation is influenced by a wide range of environmental cues, such as nutrients or water availability in the soil (López-Bucio et al., 2003; Malamy, 2005; Nibau et al., 2008). The plasticity of LR formation is of critical importance, allowing plants to compete for resources and adapt to constantly changing growth conditions. LRs originate from pericycle founder cells located opposite to xylem poles, which undergo several rounds of anticlinal divisions to create a single layered primordium composed of up to ten small cells of equal length (termed stage 1) (Dolan et al., 1993; Malamy and Benfey, 1997; Dubrovsky et al., 2001). Further anticlinal and periclinal divisions create a dome-shaped primordium (spanning stages III-VII), which eventually emerges from the parental root (Malamy and Benfey, 1997; Casimiro et al., 2003; Péret et al., 2009).

The phytohormone auxin (indole-3-acetic acid, IAA) plays an important role during each stage of LR formation (De Smet et al., 2006; Fukaki et al., 2007; Dubrovsky et al., 2008; Fukaki and Tasaka, 2009). Application of IAA or synthetic auxins such as 2, 4-D or naphthaleneacetic acid (NAA) stimulates LR formation (Celenza et al., 1995; Woodward and Bartel, 2005), whereas polar auxin transport inhibitors such as *N*- (1-napthyl)-phtalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) prevent LR formation (Casimiro et al., 2001; Himanen et al., 2002). Consistently, Arabidopsis mutants with increased auxin levels such as *rooty* and its alleles *alfl* and *surl* have increased numbers of LRs (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants with defective auxin transport, perception or signaling including *auxl*, *axrl* and *tir3/doc1/big* show reduced LR formation (Swarup et al., 2001; Gil et al., 2001; Lincoln et al., 1990). In contrast to auxin, less is known about the action of
alkamides, AHLs and other related small lipid signals on LR formation and whole plant development.

To identify the genetic components responsible for the root architectural responses to alkamides, we performed a visual screening for Arabidopsis mutants that under high N-isobutyl decanamide concentration do not manifest primary root growth reduction. We isolated an N-isobutyl decanamide-resistant mutant (decanamide resistant root-drr1) defective on a single recessive trait. Detailed cellular and developmental studies of WT and drr1 plants indicate that drr1 mutants show resistance to primary root growth inhibition and lateral root growth promotion induced by both an alkamide (N-isobutyl decanamide) and a bacterial QS signal (N-decanoyl homoserine lactone, C10-AHL). We further show that DRR1 is a crucial component of regulation of plant senescence, which likely links alkamide and jasmonic acid in modulating plant longevity and LR development.

RESULTS

Isolation of drr1, an Arabidopsis mutant with altered primary root growth response to N-isobutyl decanamide

From a group of similar chain length alkamides and NAEs, López-Bucio and co-workers (2007) identified N-isobutyl decanamide, a C10 saturated alkamide that is naturally produced in *Acmella radicans* (Ríos-Chávez et al., 2003) and *Cissampelos glaberrima* (Laurerio-Rosario et al., 1996), as the most active compound in inhibiting primary root growth and stimulating lateral root formation in Arabidopsis.

To investigate the genetic basis of plant responses to alkamides, we screened 25,000 lines from T-DNA insertion mutant collections (Krysan et al., 1999) by inspecting the root architecture of plants grown over the surface of 0.2x Murashige and Skoog agar plates supplied with 30 µM N-isobutyl decanamide. A mutant line was isolated that, in contrast to the wild-type, was able to sustain primary root growth under this inhibitory concentration of the alkamide (Fig. 1A). The mutant was backcrossed to WT plants (Wassilewskija, Ws ecotype) three times prior to detailed phenotypical analysis. In F2 progeny from these crosses, in plants grown in medium supplied with 30 µM N-isobutyl decanamide, the line segregated the mutant phenotype in a 1:3 ratio (Table 1). These results indicate that the primary root growth resistance to the alkamide resulted from a
recessive single-gene mutation. We named this locus decanamide resistant root 1 (drr1). To further study the developmental alterations induced by N-isobutyl decanamide in WT and drr1 mutants, we grew ecotype Wassileskija (Ws) and drr1 plants side by side in vertically oriented agar plates with varied alkamide content. WT plants grown in 0.2x Murashige and Skoog agar medium without N-isobutyl decanamide show a typical root system consisting of a long primary root with many lateral roots forming in a gradient from the root/shoot junction to the primary root tip (Fig. 1B). In the same medium, drr1 mutants developed a long primary root albeit lacking visible lateral roots (Fig. 1B), thus indicating that DRR1 is important for normal lateral root development under normal growth conditions. In WT plants treated with 20, 25 or 30 µM N-isobutyl decanamide, there was a dose-dependent inhibitory effect of the alkamide on primary root growth, which correlates with an increase in lateral root formation. In these plants, multiple lateral roots developed, giving rise to a highly exploratory root system with different architecture to that observed in plants grown in medium without N-isobutyl decanamide (Fig. 1C-E). In contrast, alkamide-treated drr1 mutants showed longer primary roots and reduced lateral root formation when compared to WT plants in most concentrations of N-isobutyl decanamide tested (Fig. 1C-E).

drr1 mediates the root architecture responses of Arabidopsis to N-isobutyl decanamide

To more clearly define the alterations in the root architectural response to N-isobutyl decanamide caused by mutation in DRR1, we performed temporal and single-point measurements of primary root length, lateral root number per plant and lateral root density in WT and drr1 mutants treated with varied concentrations of N-isobutyl decanamide. Primary root growth was similar in WT and drr1 plants in concentrations of up to 15 µM N-isobutyl decanamide, while in concentrations of 20, 25 and 30 µM of this compound, drr1 primary roots were significantly longer that WT plants (Fig. 2A). N-isobutyl decanamide increased the number of emerged lateral roots in WT plants, while drr1 plants were resistant to this effect (Fig. 2B). The density of emerged lateral roots dramatically increased in response to alkamide treatment in WT plants, but the mutants showed again reduced responses. The most contrasting responses between WT and drr1 plants were observed in 25 µM N-isobutyl decanamide, in which WT plants
showed a highly branched root system harboring second-and third-order lateral roots (Fig. 1C-E), with a 6-fold increased density of lateral roots (Fig. 2C). In this alkamide concentration, *drr1* mutant plants produced less than 15% of the lateral roots observed in WT plants. Interestingly, although *drr1* mutants produced consistently fewer lateral roots compared to WT in most *N*-isobutyl decanamide treatments, exposure to 30 µM *N*-isobutyl decanamide caused a 8-fold increase in lateral root number and 2-fold increase in lateral root density (Figs. 2B and C), indicating that the mutants are not completely insensitive to the alkamide.

**drr1** mutants are resistant to inhibitory effects of *N*-isobutyl decanamide on cell division in primary root meristems

An important factor determining primary root growth reduction in WT seedlings grown in high *N*-isobutyl decanamide concentrations is the reduction in cell division in the root meristem (López-Bucio et al., 2007). To analyze the cell division responses of *drr1* mutants to alkamide treatment, we crossed *drr1* with a transgenic plant harboring the *CycB1:uidA* construct, which is expressed only in cells in the G2/M phase of the cell cycle and is a marker of mitotic activity (Colón-Carmona et al., 1999). *CycB1:uidA* seedlings and *drr1* mutant seedlings were grown in 0.2x MS agar media supplied with the solvent or with 20, 25 and 30 µM *N*-isobutyl decanamide. In both WT *CycB1:uidA* and *drr1* mutant seedlings supplied with the solvent only, a patchy pattern of single cells expressing *CycB1:uidA* was observed in the primary root meristem (Fig. 3A and B). In WT plants subjected to treatment with 30 µM *N*-isobutyl decanamide, GUS expression in the primary root tip decreased and root hairs were formed in close proximity to the root meristem (Fig. 3C). Interestingly, *CycB1:uidA* expression in the primary root apex of *drr1* seedlings treated with the alkamide was not as much inhibited as in WT and their root meristems were anatomically similar to those of solvent only-treated seedlings (Fig. 3D). Root hair formation close to the root meristem was not observed in *drrl* seedlings treated with the alkamide (Fig. 3D). Next, we quantified the length of the primary root meristems in WT and *drr1* plants at 4 and 12 d after germination. At these developmental stages, increased concentrations of *N*-isobutyl decanamide decreased the length of the meristem in WT plants, while *drr1* mutants were resistant to this effect (Fig. 3E).
**drr1 is defective on N-isobutyl decanamide-induced lateral root primordia development**

Lateral root formation is a major determinant of root system architecture. Next, we investigated the effects of N-isobutyl decanamide on lateral root primordia (LRP) development and lateral root emergence in WT and *drr1* plants. LRP originating in the primary root, and emerged lateral roots were counted at 6 d after germination in plants grown in 0.2x MS agar medium supplied with the solvent only or with 30 µM N-isobutyl decanamide. The developmental stage of each LRP was classified according to Malamy and Benfey (1997) (see "Materials and Methods"). In solvent-treated WT plants, most LRP remained at an early developmental stage (stage 1). Interestingly, *N*-isobutyl decanamide treatment both increased the number of LRP at stage 1, and the density of emerged lateral roots (Fig. 4A and B). Solvent-treated *drr1* mutants showed similar LRP density to WT plants (Fig. 4A), but dramatically decreased density of emerged lateral roots (Fig. 4B), indicating that the mutant is not inherently defective on LRP initiation, but rather shows a retardation in the maturation of LRP. When treated with *N*-isobutyl decanamide, *drr1* mutant seedlings did not show an increase in stage I LRP or in LRP emergence observed in WT plants (Fig. 4A and B), indicating the *DRR1* locus is involved in alkamide responses in the pericycle and during LRP development. Although alkamide treatment significantly increased the density of emerged lateral roots in *drr1* mutants, *drr1* always showed lower lateral root density than WT plants in the different growth conditions (Fig. 4B). These results indicate that *N*-isobutyl decanamide modify root system architecture both by inducing more pericycle cells to form stage I LRP and by accelerating the emergence of LRP from the primary root to form mature lateral roots. Mutations in *drr1* interfere with both of these processes.

**drr1 is defective in root architectural responses to N-decanoyl-L-homoserine lactone, a quorum-sensing signal from bacteria**

Previous studies documented that *N*-acyl-L-homoserine lactones (AHLs), a class of alkamide-related QS signals from bacteria modulate root system architecture in Arabidopsis (Ortíz-Castro et al., 2008; Von Rad et al., 2008). To determine if *DRR1* is involved in AHL responses, we tested the primary root growth and lateral root response of *drr1* seedlings to *N*-decanoyl-L-homoserine lactone (C10-AHL) over a range of concentrations.
concentrations of this compound as compared to WT plants. *drr1* had a level of resistance to primary root growth inhibition by C10-AHL over most concentrations tested (Fig. 5A). At 30 µM C10-AHL in WT plants, about 60% inhibition of growth occurred, whereas in the mutant it was of about 30%. As previously reported (Ortíz-Castro et al., 2008), C10-AHL stimulated lateral root formation (Fig. 5B). A dose-dependent effect increasing lateral root density was observed (Fig. 5C), confirming the positive role of AHLs in lateral root induction. In contrast, *drr1* mutants showed reduced lateral root formation when compared to WT seedlings over most concentrations of C10-AHL tested (Fig. 5B and C).

*drr1* shows normal auxin responses

Several auxin-related mutants have been characterized in screens for primary root growth resistance to inhibitory amounts of IAA, which display alterations in LR formation (Swarup et al., 2001; Rogg et al., 2001; Fukaki et al., 2002). To determine if *drr1* operates in a genetically defined auxin pathway, WT Arabidopsis seedlings (Col-0 and Ws), *drr1* seedlings and the auxin-related mutants *aux1-7* and *axr2* were evaluated in primary root growth response assays to IAA. Firstly, to confirm auxin-resistance of auxin-related mutant lines, homozygous *aux1-7* and *axr2-1* seedlings were screened for resistance to IAA based on primary root growth. In these experiments, *aux1-7* and *axr2* were resistant to the inhibition of primary root elongation by IAA when compared to WT Col-0 seedlings (Fig. 6A). These mutants also failed to form abundant root hairs at the root tip region in response to increasing IAA concentration in the medium, a phenotype associated with increased auxin resistance (Fig. 6B). In contrast, the auxin response in *drr1* mutants was equally sensitive to IAA than as the WT (Ws ecotype) both in primary root growth assays (Fig. 6A), and towards induction of root hair formation close to the root tip (Fig. 6B). Because *drr1* mutants showed normal root responses to IAA, we conclude that auxin signaling is unaffected in the mutant.

To better understand the role played by auxin in lateral root formation in WT and *drr1* plants, we tested the effects of NAA to activate lateral root formation in a transfer assay. In these experiments, WT and *drr1* plants were first germinated and grown for 7 d in 0.2x MS agar medium. At day 7 after germination, plants were transferred to 0.2x MS liquid media supplied with the solvent or varied concentrations of NAA for an additional 4 d period. At this stage, the number and density of lateral roots were
determined. As shown in Fig. 7, NAA treatment caused a dose-response effect in lateral root formation (Fig. 7A), which was similar between WT and *drr1* plants. Both WT and *drr1* plants produced highly branched root systems with normal lateral root growth (Fig. 7B-I). These results indicate that *drr1* seedlings are not inherently defective in pericycle cell activation to form LRs and are able to correctly sense and respond to auxins.

**drr1** mutants show extended longevity

To study the role of *DRRI* in plant growth and development, we compared the phenotype of WT and homozygous *drr1* plants of the same age that were first germinated and grown for 10 d on 0.2x MS agar medium and then transferred to soil. WT and *drr1* plants were grown side by side during their entire life cycle. The young and adult phenotypes of plants are shown in Fig. 8. During the first 28 d after transfer, it was observed a general delay in growth of *drr1* mutants as illustrated by their delay in stem formation (Fig. 8A and B; Fig. 9A), and significantly decreased rosette size during early stages of vegetative growth (Fig. 9B). At 28 days after transfer, the rosette leaves of WT plants had already turned yellow and stem growth ceased, but *drr1* leaves remained green and 7 d later, the stems just started to be formed (Fig. 8A-C, Fig. 9A and B). At 35 days after transfer, WT leaves had turned completely yellow and showed signs of death with drying (Fig. 8C). In contrast, the *drr1* mutant leaves retained a significant amount of chlorophyll and maintained the integrity of the leaf shape (Fig. 8C). The extension of leaf longevity at a whole plant level dramatically increased in *drr1* mutants with time. Delayed flowering was accompanied by the generation of new leaves, increased rosette size and greater stem length in *drr1* mutants when compared to WT plants (Fig. 8D and E; Fig. 9A-C). *drr1* sustained chlorophyll production for a longer time period (Fig. 9D). In addition, the shoot architecture of *drr1* mutants was different than that observed in WT seedlings, producing only one primary stem with reduced branches, which suggest increased apical dominance in the mutants (Fig. 9E and F). Aside from the delayed senescence and altered shoot architecture, *drr1* mutants produced fertile flowers that yielded fruits with fully viable seed (Fig. 8D). We determined that the longevity in *drr1* mutants was extended by approximately 2-fold when compared to WT plants, which correlates with a 3-to-4 fold increase in the number of visible leaves and overall increased plant size (Fig. 9A-C).
The *drr1* mutant shows delayed senescence symptoms in JA and alkamide induced senescence

Leaf senescence is modulated by JA (Schommer et al., 2008). Therefore, the possibility was open that the *drr1* mutant could be deficient in the JA-induced senescence program. We compared the effects of JA and *N*-isobutyl decanamide in WT and *drr1* plants in a senescence induced assay for detached leaves (Fig. 10). In this assay, after 6 days of incubation in water, WT detached leaves gradually lost chlorophyll content (Fig. 10A and C). A deficient senescence program for detached *drr1* plants incubated in water was evident (Fig. 10B and D). In response to treatments with JA and *N*-isobutyl decanamide, WT leaves showed severely senescence symptoms that were reduced in *drr1* mutants (Fig. 10E-I). Taking together the increased *drr1* longevity in soil and the delaying response to hormone-induced senescence, we conclude that *DRR1* plays an important role in the senescence process modulated by JA and *N*-isobutyl decanamide as well as in age-dependent senescence.

*drr1* is altered in jasmonate-mediated lateral root induction

Jasmonates are signals involved in root system architecture modulation (Wasternack, 2007). The inhibitory effect of methyl jasmonate (MeJA) on primary root growth has been well recognized and widely employed as a useful trait to identify jasmonate-related mutants in Arabidopsis. Recently, it has been reported that MeJA also promotes lateral root formation (Sun et al., 2009). The MeJA dose-response of *drr1* in primary root growth and lateral root formation was compared with *jar1*, a MeJA-insensitive mutant and WT seedlings of the Ws and Col-0 ecotypes, the Col-0 ecotype provided the genetic background for *jar1* and therefore included as an additional control. When compared to Ws and Col-0 plants, the *jar1* mutant showed strong resistance to MeJA-induced primary root growth inhibition over most concentrations tested (Fig. 11A). The primary root growth inhibition in *drr1* was essentially the same as in Ws seedlings (Fig. 11A). Interestingly, MeJA, at concentrations of 30 to 45 µM increased emerged lateral roots in WT seedlings and in *jar1* mutants by 70 to 150% (Fig. 11B; Supplemental Figure S1). In the absence of MeJA, LR formation in *drr1* was significantly reduced compared with WT and *jar1* plants. However, *drr1* mutants failed to produce increased numbers of lateral roots when grown on medium containing a range of concentrations of
MeJA (Fig. 11B; Supplemental Figure S1). Our data reveals that **drr1** encodes a novel genetic locus modulating the effects of MeJA on LR formation.

**DISCUSSION**

**drr1** mutants define a locus involved in root architectural responses to both alkamides and AHLs

This report describes the identification and characterization of an Arabidopsis mutant that was defective in its root response to *N*-isobutyl decanamide but with additional characteristics, which suggest that alkamides play a role in plant longevity. Our characterization of root architectural responses in WT and *drr1* to *N*-isobutyl decanamide provided insights into the genetic mechanisms mediating the responses to alkamides. While *N*-isobutyl decanamide inhibited primary root growth and promoted lateral root formation in WT seedlings, resistance to the repressive effect of this alkamide on primary root growth and the failure to increase lateral root formation typified the *drr1* phenotype (Figs. 1 and 2).

Detailed cellular analysis of WT and *drr1* plants showed that the mutants sustained almost normal root meristematic activity when grown under inhibitory concentrations of *N*-isobutyl decanamide as revealed by cell counts and *CycB1:uidA* expression in the primary root meristem (Fig. 3). Interestingly, the typical increase in lateral root primordia initiation and LR emergence observed in WT plants treated with the alkamide was reduced in *drr1* (Fig. 4). Several types of reported experimental evidence suggested that conditions that reduce primary root meristematic activity, including destruction of the primary root meristem by cell ablation or physical decapitation of the root tip, elicit an increase in lateral root number (Tsugeki and Fedoroff, 1999). Our findings that *drr1* mutants grown in medium lacking alkamides sustain normal primary root growth but reduced lateral root formation (Figs. 1 and 2) indicates that LR proliferation in response to *N*-isobutyl decanamide is not a direct consequence of primary root growth inhibition, but rather suggest a positive effect of the alkamide on pericycle cells to produce more LRP (Fig. 4).

Many bacterial species use small molecule signaling to communicate with each other and to coordinate their growth activities, a process commonly referred to as quorum-sensing (Taga and Bassler, 2003; Reading and Sperandio, 2006). Diverse Gram-
negative bacteria produce N-acyl-homoserine lactones (AHLs), these compounds contain a conserved homoserine lactone (HL) ring and an amide (N)-linked acyl side chain. Our previous work indicated that saturated medium (C8-C14)-chained AHL compounds showed a dose-dependent effect on root architecture, inhibiting primary root growth, and promoting lateral root formation (Ortíz-Castro et al., 2008). In this work, we used the  
\[ \text{drr1} \]
mutant to determine whether alkamides and AHLs could be perceived by similar genetic mechanisms. We show that N-decanoyl-L-homoserine lactone (C10-AHL) inhibited primary root growth and promoted lateral root formation in Arabidopsis WT seedlings (Fig. 5).  
\[ \text{drr1} \]
mutants showed reduced sensitivity to both N-isobutyl decanamide and N-decanoyl-L-homoserine lactone, indicating a potential genetic interaction in plant responses to alkamides and AHLs in roots. These results also suggest that plants have evolved the capacity to sense AHLs in order to activate developmental responses.

Several reports indicate that bacteria commonly associated to plants are capable to produce a variety of AHLs (Cha et al., 1998; Elasri et al., 2001; Khmel et al., 2002; D’Angelo-Picard et al., 2005). Several strains of  
\[ \text{Pseudomonas} \]
have been studied for their ability to colonize plant-related niches, such as the rhizosphere (i.e.  
\[ \text{P. aeruginosa}, \text{P. fluorescens} \]
and  
\[ \text{P. putida} \]), where they can act as plant growth promoting rhizobacteria by antagonizing plant-deleterious microorganisms and through the production of traits that directly influence plant disease resistance and growth (Venturi, 2006). The rhizospheric  
\[ \text{P. putida} \]
plant beneficial strains WCS358 and IsoF produce 3-oxo-C\(_{12}\)-AHL, whereas in the rhizosphere-colonizing biocontrol  
\[ \text{P. fluorescens} \]
strain F113 it has been reported the production of three AHL molecules, including C10-AHL (Laue et al., 2000; Venturi, 2006). Interestingly, C10-AHL and C12-AHL seem to be also produced in the nitrogen-fixing bacterial symbiont  
\[ \text{Sinorhizobium meliloti} \] (Teplitski et al., 2003; Marketon et al., 2002). The marked resistance of  
\[ \text{drr1} \] to C10-AHL on root development opens new possibilities to identify novel genetic determinants involved in plant-bacteria interactions. Furthermore, the  
\[ \text{drr1} \] mutant can be used as a tool to identify novel plant growth-promoting bacterial strains, which could modulate root system architecture through AHL production.

Two recent reports suggested that auxin signaling might be involved in plant responses to AHLs (Mathesius et al., 2003; Von Rad et al., 2008). Auxins are signaling molecules that regulate the asymmetric pericycle cell divisions and thereby influence the patterning of newly initiated LRP. Thus the possibility was open that altered auxin
responses could be responsible of reduced LR formation in \textit{drrl} mutants. Our results, however, showed that \textit{drrl} mutants are not resistant to IAA or NAA treatments in terms of primary root growth inhibition or lateral root formation (Figs. 6 and 7), indicating that \textit{DRR1} might not be directly connected to the auxin response pathway to modulate plant growth and development. These results are in agreement with our previous research showing that both alkamides and AHLs modulate root system architecture likely through auxin-independent signaling mechanisms (Ramírez-Chávez et al., 2004; Campos-Cuevas et al., 2008; Ortíz-Castro et al., 2008).

\textit{DRR1} plays a role in senescence-related processes

Senescence is a developmental process that limits the longevity of an organism. Genetic studies of longevity mutants have also suggested that some common mechanisms, such as alterations in energy metabolism and oxidative damage, might play a role in determining life-span in animals as divergent as nematodes, Drosophila and mammals (Lin et al., 1998; Parkes et al., 1998). Plants also undergo a distinctive senescence process at an organ and/or organism level. A number of studies have provided evidence suggesting that leaf senescence is an active process controlled by a genetic program (Woo et al., 2001; Woo et al., 2002, Schommer et al., 2008). However, our understanding of how senescence and longevity is controlled at the whole plant level remains quite limited. Our results suggest that lateral root development and age-dependent plant senescence are directly connected through \textit{DRR1}. Obvious alterations were seen in \textit{drrl} plants grown in soil under long days (16h light/8h dark conditions). In \textit{drrl} plants, leaf senescence was delayed by about four-to-five weeks when compared to WT plants (Figs. 8 and 9). The extended longevity of leaves was related to an extended growth period as well as slower onset and/or progression of senescence after the maturation stage. The reduced growth observed at early stages of development in \textit{drrl} mutants opens the possibility that it may contribute to extended longevity. Consistent with this hypothesis, we observed that reduced lateral root formation was not caused by the failure of the pericycle to produce these structures, but to a retarded development of LRP to emerge from the primary root (Fig. 4). In this way, the \textit{drrl} mutation shows a senescence character that differs from the delayed leaf senescence phenotype observed in the \textit{oresara (ore)} or \textit{teosinte branched/cycloidea/PCF (tcp)} mutants previously described (Woo et al., 2001; Woo et al., 2002; Schommer et al., 2008). In \textit{ore} mutants,
the retarded senescence phenotype seems to be specifically observed in leaves. For instance, the leaf longevity in ore9-1 was extended only by about 27%, without affecting other developmental traits such as flowering time and/or plant size (Woo et al., 2001). To our knowledge, no lateral root phenotypes have been reported for leaf senescence mutants such as the ore or tcp lines. Interestingly, the drrl mutants also show that plants that bolt and senesce late produce more leaves and increase in size (Figs. 8 and 9), which could lead to potential agricultural applications. Together, our findings suggest that DRR1 may function normally as a positive regulator of senescence in Arabidopsis, limiting the longevity at the whole plant level. Because the drrl mutation affects a wide variety of age-dependent developmental and senescence responses (Fig. 10), DRR1 may function upstream in the regulatory cascade of senescence pathways.

**drrl mutants reveal a crosstalk between alkamides and jasmonate in LR formation**

Cross-resistance of mutants to multiple hormones is well documented (Wilson et al., 1990; Hobbie and Estelle, 1994; Tiryaki and Staswick, 2002), and suggests that the action of hormones is coordinated by common intermediates or modulators. Several phytohormones are involved in leaf senescence, including ethylene, cytokinin and jasmonic acid (Schommer et al., 2008). High concentrations of \( N \)-isobutyl decanamide have been found to induce callus formation in leaves and in roots (López-Bucio et al., 2007). Although not explicitly tested here, preliminary information shows that drrl plants are also resistant to callus formation (data not shown). The proliferative growth activity elicited by \( N \)-isobutyl decanamide on callus formation in leaves and lateral root formation in roots was previously shown to be decreased or even absent in Arabidopsis mutants lacking one, two, or three of the putative cytokinin receptors CRE1, AHK2, and AHK3 (López-Bucio et al., 2007). The triple cytokinin receptor mutant crel-12/ahk2-2/ahk3-3 was particularly insensitive to high alkamide concentrations in terms of developmental alterations indicating that \( N \)-isobutyl decanamide requires, at least in part, a functional cytokinin-signaling pathway to control meristematic activity and differentiation processes. However, the primary root growth response of the drrl mutants to kinetin, a highly active cytokinin in modulating root development, was similar to that observed in WT plants (Supplemental Fig. 2A), indicating that drrl is not resistant to root inhibition by cytokinin. However, we can not exclude the possibility
that crosstalk between alkamide and cytokinin responses may account for the increased longevity and/or reduced senescence of *drr1* mutants. Abscisic acid (ABA) and ethylene are two growth regulators also involved in senescence, *DRR1* mutation renders the *drr1* seedlings more sensitive to the primary root growth inhibitory effect of low ABA concentrations than WT seedlings (Supplemental Fig. 2B), whereas the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) similarly inhibited growth (Supplemental Fig. 2C).

The plant hormone jasmonic acid (JA) plays a key role in the environmental stress responses and developmental processes of plants. A recent report has revealed an important role of JA in lateral root development. In such work (Sun et al., 2009), it was shown that exogenous MeJA promotes LR formation in Arabidopsis WT plants but not in *anthranilate synthase 1* (*asal*) mutants, leading to the proposal that localized auxin biosynthesis in response to jasmonate could be important for fine-tuned modulation of LR formation. Our detailed morphological comparison among WT, *drr1* and *jar1* plants indicated that, when grown on JA-free medium, LR development in *drr1* was significantly lower than in WT or *jar1* plants. Interestingly, while JA application led to increased LR numbers in WT and *jar1* plants, it failed to activate LR formation in *drr1* (Fig. 11; Supplemental Fig. S1). Comparison of the primary root response to JA also show that *drr1* behave essentially different to *jar1*, which was very insensitive to primary root growth inhibition by JA but responded similarly to WT plants in lateral root induction by this compound. Therefore, *drr1* shows alkamide resistance in terms of primary and lateral root growth, whereas it has resistance to jasmonate in LR formation only. Taking into account these results, it is tempting to speculate that further crosstalk of alkamide signaling with phytohormones such as cytokinins or jasmonates might vary in different tissues or in a developmental context, possibly explaining why *drr1* mutants exhibit no defects in primary root growth inhibition assays to these phytohormones.

In summary, we have provided evidence that alkamide and AHL signaling are under genetic control in Arabidopsis and that normal responses to these signals are important for plant development. Elucidation of the genetic identity of the *DRR1* product is critical to understand the molecular mechanisms underlying the distinct effects of these and other small lipid signals on root architecture adjustment and their role in plant longevity.
MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis thaliana* WT plants (Col-0 and/or Ws ecotypes), the transgenic line *CyCB1::uidA* (Colón-Carmona et al., 1999), and the mutant lines *jar1* (Tiryaki and Staswick, 2002), *axr2-1* (Timppe et al., 1994) and *aux1-7* (Picket et al., 1990) were used for all experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes with sterile distilled water, seeds were germinated and grown on agar plates containing 0.2x MS medium (Murashige and Skoog, 1962). MS medium (Murashige and Skoog basal salts mixture, Cat. M5524) was purchased from Sigma (St. Louis, MO, USA). The suggested formulation is 4.3 g L$^{-1}$ of salts for a 1x concentration of medium; we used 0.9 g L$^{-1}$, which we consider and refer to as MS 0.2x. This medium lacks of amino acids and vitamins. Phytagar (micropropagation grade) was purchased from Phytotechnology (Shawnee Mission, KS, USA). Plants were placed in a plant grown chamber (Percival Scientific AR-95L) with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 100 µmol m$^{-2}$s$^{-1}$ and temperature of 22 °C.

Mutant isolation procedure

T-DNA lines (Ws; Krysan et al., 1999) were provided by the Ohio Arabidopsis Seed Stock Center. Seeds were surface sterilized and plated on 0.2x Murashige and Skoog Medium supplied with 30 µM *N*-isobutyl decanamide. A number of approximately 25,000 T-DNA lines were screened for reduced lateral root formation by placing seeds on nutrient agar plates (20-to-25 seeds/plate). The seeds were distributed in two rows on the agar surface at a density of 1 seed / centimeter, stratified at 4ºC for 48 h, and then incubated at 22ºC. Fourteen days after germination, *N*-isobutyl decanamide-treated plants have a short primary root and a large number of lateral roots are formed. Putative mutants with long primary roots and reduced number of lateral roots were selected, transferred to soil, and allowed to self-fertilize. Homozigous M$_3$ seeds were rescreened for sustained primary root growth in medium supplied with 30 µM *N*-isobutyl decanamide, transferred to soil and back-crossed three times to WT (Wassilewskija-Ws ecotype) to remove unlinked mutations.
Genetic analysis of *drr1* mutants

To determine the segregation pattern of the *drr1* phenotype, 990 F2 seedlings derived from a cross between *drr1* x Ws were analyzed in MS 0.2x-agar medium supplied with 30 µM *N*-isobutyl decanamide. A typical 3:1 recessive segregation was observed for the wild-type/*drr1* phenotype. Co-segregation of primary root growth resistance and increased longevity was further confirmed in *drr1* seedlings grown in soil.

**Hormone treatments**

For all experiments, Murashige and Skoog (MS) 0.2x nutrient media were supplemented with *N*-isobutyl decanamide, *N*-decanoyl-L-homoserine lactone, or the indicated phytohormones. Ethanol-dissolved compounds were added to cooled (50 ºC) molten medium and poured into plates. Control plates were supplied with the greatest concentration of solvent used in the treatments. For hormone-induced senescence, leaves at 22 days after leaf emergence were detached and floated on sterilized water in the presence or absence of 35 µM jasmonic acid or 35 µM *N*-isobutyl decanamide for 6 days. All treatments were performed at 22 ºC under dark conditions. Chemicals were purchased from Sigma Chemical Co. (St. Louis).

**Analysis of growth and statistical analysis**

Growth of primary roots was registered using a rule. Lateral root number and lateral root density were determined by counting the lateral roots present in the primary root from the tip to the root/stem transition. Lateral root density was determined by dividing the lateral root number by the primary root length and expressed as LRD cm⁻¹. The length of the meristem was determined as the distance between the quiescent center to the cell file where cells started to elongate. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS, Chicago, IL, USA). Univariate and multivariate analyses with a Tukey’s post hoc test were used for testing differences in growth and root developmental responses in WT and mutant lines. Different letters are used to indicate means that differ significantly (*P* < 0.05).
Determination of developmental stages of LRP

LRP were quantified at day 4 after germination. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are: Stage I. LRP initiation. In the longitudinal plane, approximately 8-10 ‘short’ pericycle cells are formed. Stage II. The formed lateral root primordium is divided into two layers by a periclinal division. Stage III. The outer layer of the primordium divides periclinally, generating a three layer primordium. Stage IV. LRP with four cell layers. Stage V. The LRP is midway through the parent cortex. Stage VI. The LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII. The LRP appears to be just about to emerge from the parent root.

Chlorophyll determination

We used leaves from wild-type (Ws) and *drr1* plants germinated and grown on 0.2x MS medium and then transferred to soil for 35 days. WT leaves were yellowed as a result of age-dependent senescence; *drr1* leaves remained green at this stage. We used a hand-held chlorophyll meter CCM-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA), to calculate a chlorophyll content index (CCI) based on absorbance measurements at 660 and 940 nm on 15 independent leaves. Five separate measurements with the hand-held meter were made on each leaf. Chlorophyll content was finally determined as previously described (Richardson et al., 2002).

Histochemical analysis of GUS activity

Transgenic plants that express the *uidA* reporter gene (Jefferson et al., 1987) were stained in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl, β-D-glucuronide) in phosphate buffer (NaH$_2$PO$_4$ and Na$_2$HPO$_4$, 0.1 M, pH7) with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide, for 12 h at 37 °C. Plants were cleared and fixed as previously described by Malamy and Benfey (1997). The processed roots were included in glass slips and sealed with commercial nail varnish. For each marker line and for each treatment, at least 10 transgenic plants were analyzed.
Microscopy

The *Arabidopsis thaliana* root system was analyzed with a stereoscopic microscope (Leica MZ6, Leica Microsystems, Wetzlar, Germany). Total lateral roots were counted at 30x magnification. Primary root meristems were analyzed in semi-permanent preparations of cleared roots using a composed microscope (Axiostar Zeiss Plus, Carl Zeiss, Göttingen, Germany) at 100X or 400X magnifications. Images were captured with a Sony Cyber-shot DSC-S75 digital camera (Sony Electonics Inc., Oradell, NJ, USA) adapted to the microscope and processed with the Zeiss Axio Vision 4AC software (Carl Zeiss).

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LITERATURE CITED


Table 1. Segregation ratio of progeny resulting from crosses between *drr1* mutant and wild-type (WT) seedlings

<table>
<thead>
<tr>
<th>Generation</th>
<th>Phenotype of progeny</th>
<th>Ratio obtained</th>
<th>Ratio tested</th>
<th>( \chi^2 ) ( a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Many lateral roots (WT)</td>
<td>Few lateral roots (<em>drr1</em>)</td>
<td>Wt:<em>drr1</em></td>
<td>Wt:Mutant</td>
</tr>
<tr>
<td><strong>F₁</strong></td>
<td>128</td>
<td>0</td>
<td>2.81:1</td>
<td>3:1</td>
</tr>
<tr>
<td><strong>F₂</strong></td>
<td>730</td>
<td>260</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \)With one degree of freedom and a critical value of 5%, the hypothesis is accepted if the \( \chi^2 \) is smaller than 3.841
FIGURE LEGENDS

Figure 1. Genetic screen and phenotypic characterization of *drrl* mutants. (A), Photograph of an agar plate supplied with 30 µM N-isobutyl decanamide showing a putative *drrl* mutant with long primary root. (B), Five 14-d-old wild-type (Ws) and *drrl* seedlings grown side by side on the surface of agar plates containing 0.2x Murashige and Skoog (MS) medium lacking N-isobutyl decanamide. (C, D and E), Photographs of agar plates supplied with 20, 25 or 30 µM N-isobutyl decanamide, respectively, showing five WT (left) and *drrl* (right) plants grown side by side. Photographs from B-to-C are representative individuals of four plates per treatment. Scale bar = 1 cm.

Figure 2. Effects of N-isobutyl decanamide on the root system architecture of wild-type (Ws) and *drrl* plants. (A), Primary root length. (B), Number of emerged lateral roots per plant. (C), Lateral root density expressed as the number of lateral roots per centimeter. Data were recorded at 12 days after germination. Values shown are the mean ± SD, (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

Figure 3. *CycB1:uidA* expression in transgenic wild-type and *drrl* seedlings. Twelve hour GUS staining of *CycB1:uidA* primary roots in WT and *drrl* Arabidopsis seedlings grown on agar-solidified 0.2x MS medium with or without N-isobutyl decanamide. (A and B), solvent-treated seedlings. (C and D), Plants supplied with 30 µM N-isobutyl decanamide. (E) Meristem length. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Scale bar = 100 µm.

Figure 4. Effects of N-isobutyl decanamide on wild-type (Ws) and *drrl* lateral root development. (A), Lateral root primordia (LRP) stage distribution in 6-d-old primary roots grown on media supplied with the solvent only or with 30 µM N-isobutyl decanamide (indicated in the figure as decanamide). (B), Emerged lateral root density in the same experiment. WT and *drrl* seedlings were cleared and the number and stage of LRP recorded according to Malamy and Benfey (1997). Values shown are the mean ±
SD, (n = 15). Different letters represent means statistically different at the 0.05 level. This analysis was repeated twice with similar results.

**Figure 5.** Effects of N-decanoyl-L-homoserine lactone on the root system architecture of wild-type (Ws) and *drrl* plants. (A), Primary root length. (B), Number of emerged lateral roots per plant. (C), Lateral root density expressed as the number of lateral roots per centimeter. Data were recorded at 14 days after germination. Values shown are the mean ± SD, (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

**Figure 6.** Auxin responses in WT and *drrl* seedling roots. (A) Primary root growth in 12-d-old primary roots of WT (Col-0 and Ws), *axr2-1* and *aux1-7* auxin resistant mutants and in *drrl* Arabidopsis mutants grown on media supplied with the solvent only or with varied IAA concentrations. (B) Morphology of root tips of wild-type and mutant lines exposed to IAA. Seedlings were photographed at 7 days after germination using a digital camera connected to a dissecting microscope. Values shown in (A) are the mean ± SD, (n = 30). The experiment was repeated twice with similar results.

**Figure 7.** Auxin restoration of lateral root development in *drrl* plants. (A), Total lateral root number per plant in 11-d-old WT (Ws) and *drrl* mutants that were first grown for 7 d in 0.2x MS agar medium and then transferred to 0.2x MS liquid medium supplied with the solvent or with varied concentrations of NAA for an additional 4 d growth period. (B-I), Representative photographs of WT and *drrl* lateral roots in plants exposed to NAA. Values shown in A are the mean ± SD, (n = 15). Different letters represent means statistically different at the 0.05 level. This analysis was repeated twice with similar results.

**Figure 8.** Phenotypes of wild-type and *drrl* plants grown in soil. (A-D) Phenotypes of wild-type (Ws) (left) and *drrl* plants (right) grown side by side at 14, 28, 56 or 84 days after transfer to soil. (E) Close-up of rosette leaves at 84 days after transfer. Plants were grown with a 16 h light/8 h dark cycle at 22ºC in a growth chamber. The retarded leaf senescence in *drrl* was related to retarded emergence of floral stems and flowering time.
**Figure 9.** Age-dependent senescence symptoms and other developmental traits of wild-type (Ws) and $drr1$ plants grown in soil. (A), Age-dependent stem size. (B), Rosette diameter. (C), Number of visible rosette leaves (D), Chlorophyll content in rosette leaves at 28 days after transfer to soil. (E), Stem number. (F), Number of stem branches. Plants were grown with a 16 h light/8 h dark cycle at 22°C in a growth chamber and developmental traits monitored during their entire life cycle. Values shown are the mean ± SD, (n = 18). Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

**Figure 10.** Hormonal-dependent senescence symptoms in the $drr1$ mutant. Detached leaves of WT (Ws ecotype) and $drr1$ plants were incubated in 2 ml water solutions supplied with the indicated concentrations of compounds. The plates were included in a growth chamber (Percival ARR95L) under darkness conditions and representative photographs of leaves subjected to the different treatments were taken 6 d later (A-H), and chlorophyll determination performed (I). The experiment included at least three independent samples of 5 leaves each and was replicated three times with similar results.

**Figure 11.** Effects of JA on primary root growth and lateral root development in wild-type (Ws, Col-0) and mutant lines ($drr1$, $jar1$). (A), Primary root length. (B), Number of emerged lateral roots per plant. Data were recorded at 12 days after germination. Values shown are the mean ± SD, (n = 20). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

**Supplemental figure S1.** $drr1$ shows defective LR formation in response to JA treatment. Photographs of agar plates supplied with the solvent or with varied concentrations of JA. Note the stimulatory effect of JA on LR formation in WT Ws and Col-0 seedlings and in $jar1$ but not in $drr1$.

**Supplemental figure S2.** Effect of kinetin, abscisic acid and ethylene on primary root growth in WT and $drr1$ plants. Data were recorded at 14 days after germination. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was used as an ethylene donor. Values shown are the mean ± SD, (n = 20). Different letters represent
means statistically different at the 0.05 level. The experiments were repeated twice with similar results.
Control  30 μM decanamide

Cyc B1

A  C

B  D

drr1/Cyc B1

E

Meristem length (μm)

Solvent  20  25  30 μM

CycB1:uidA  drr1/CycB1:uidA  CycB1:uidA  drr1/CycB1:uidA

4 d.a.g.  12 d.a.g.
Solvent                0.1                0.2             0.4 uM NAA

Ws

B

D

F

H

drr1

C

E

G

I
Water 6d

35 µM N-isobutyl decanamide

**t0**  
A  
B

Ws

drr1

Water 6d  
C  
D

35 µM JA

E  
F

G  
H

I

Chlorophyll content (µg/cm²)

Water t0  
Water 6d  
35 µM JA  
35 µM N-isobutyl decanamide

Ws  
drr1

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