Cytokinin Receptors Are Involved in Alkamide Regulation of Root and Shoot Development in Arabidopsis

José López-Bucio*, Mayra Millán-Godínez, Alfonso Méndez-Bravo, Alina Morquecho-Contreras, Enrique Ramírez-Chávez, Jorge Molina-Torres, Anahí Pérez-Torres, Masayuki Higuchi, Tatsuo Kakimoto, and Luis Herrera-Estrella

Instituto de Investigaciones Químico-Botánicas, Universidad Michoacana de San Nicolás de Hidalgo, Ciudad Universitaria, CP 58030 Morelia, Michoacan, Mexico (J.L.-B., M.M.-G., A.M.-B., A.M.-C.); Unidad Irapuato, Cinvestav-Guanajuato Km. 9.6 Libramiento Norte, CP 36821 Irapuato, Guanajuato, Mexico (E.R.-C., J.M.-T., A.P.-T.); Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560–0043, Japan (M.H., T.K.); and Laboratorio Nacional de Genómica para la Biodiversidad, Cinvestav-Guanajuato Km. 9.6 Libramiento Norte, CP 36821 Irapuato, Guanajuato, Mexico (L.H.-E.)

Alkamides and N-acylcholaminolamides are a class of lipid compounds related to animal endocannabinoids of wide distribution in plants. We investigated the structural features required for alkamides to regulate plant development by comparing the root responses of Arabidopsis (Arabidopsis thaliana) seedlings to a range of natural and synthetic compounds. The length of the acyl chain and the amide moiety were found to play a crucial role in their biological activity. From the different compounds tested, N-isobutyl decanamide, a small saturated alkamide, was found to be the most active in regulating primary root growth and lateral root formation. Proliferative-promoting activity of alkamide treatment was evidenced by formation of callus-like structures in primary roots, ectopic blades along petioles of rosette leaves, and disorganized tumorous tissue originating from the leaf lamina. Ectopic organ formation by N-isobutyl decanamide treatment was related to altered expression of the cell division marker CycB1/uidA and an enhanced expression of the cytokinin-inducible marker ARR5/uidA both in roots and in shoots. The involvement of cytokinins in mediating the observed activity of alkamides was tested using Arabidopsis mutants lacking one, two, or three of the putative cytokinin receptors CRE1, AHK2, and AHK3. The triple cytokinin receptor mutant was insensitive to N-isobutyl decanamide treatment, showing absence of callus-like structures in roots, the lack of lateral root proliferation, and absence of ectopic outgrowths in leaves under elevated levels of this alkamide. Taken together our results suggest that alkamides and N-acylcholaminolamides may belong to a class of endogenous signaling compounds that interact with a cytokinin-signaling pathway to control meristematic activity and differentiation processes during plant development.

Most organisms are known to contain in their inner and outer membranes amphipatic lipids based on one or two amino acids linked to a fatty acid through an amide bond. Thus, they have structural similarity to ceramides. N-acylcholaminolamides (NAEs) represent compounds with aminooacolcohol linked as an amide to the fatty acid. They were reported as constituents of soy (Glycine max) lecithin and peanut (Arachis hypogaea) meal and as antiinflammatory agents, but now they have been identified in a variety of seeds and plant tissues (Chapman et al., 1999; Chapman, 2004). Anandamide, the main ligand of the cannabinoid receptor in mammals regulates many cellular and developmental responses including the modulation of neurotransmission in the central nervous system, synchronization of embryo development, and vasodilation (for review, see Pertwee, 2006). It appears that NAE signaling has important biological functions in plants, such as germination (Wang et al., 2006; Teaster et al., 2007), defense responses (Chapman et al., 1998), and root development (Blancaflor et al., 2003), but research is still at a relatively early stage.

Alkamides comprise over 200 related compounds that have been found in as many as 10 plant families: Aristolochiaceae, Asteraceae, Brassicaceae, Convallulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Pooaceae, Rutaceae, and Solanaceae. Species containing high levels of alkamides are found in the Asteraceae, Piperaceae, and Rutaceae (Christensen and Lam, 1991; Kashiwada et al., 1997; Parmar et al., 1997). Two reports indicated that alkamides, a class of plant-produced
amino compound-containing lipids, structurally related to NAEs, are also able to play a signaling role in plants. Amidinin, a nonsubstituted alkamide isolated from the actinomycete fungus Amycolatopsis sp., was shown to stimulate growth of rice (Oryza sativa) seedlings (Kanbe et al., 1993) and N-isobutyl-2E,6Z,8E-decatrienamide (affinin), an alkamide present in the roots of Heliopsis longipes was reported to alter the growth and development of the Arabidopsis (Arabidopsis thaliana) root system (Ramirez-Chavez et al., 2004). Developmental alterations induced by affinin included primary root growth inhibition, enhanced formation and elongation of lateral roots, and increased root hair growth. Two affinin-derived alkamides (N-isobutyl-2E-decanamide and N-isobutyl-decanamide) were found to be even more active than affinin in stimulating root hair growth (Ramirez-Chavez et al., 2004). The effects of alkamides on Arabidopsis were found to be independent of auxin signaling as revealed by normal primary root growth response of auxin-resistant mutants to alkamides and because of the inability of alkamides to affect the expression of auxin-inducible gene markers. This information led to the proposal that NAEs and alkamides might belong to a class of endogenous lipid signals that regulate plant development (Lopez-Bucio et al., 2006; Morquecho-Contreras and Lopez-Bucio, 2007). However, it is as yet unknown whether altering the synthesis or signaling pathways of other plant hormones such as cytokinins could mediate the biological activity of alkamides.

In this study, as a first step in exploring the structure-activity relationships of NAEs and alkamides, we quantified the root growth response of Arabidopsis seedlings to natural and synthetic compounds. From a group of similar chain length NAEs and alkamides, we identified N-isobutyl decanamide, an alkamide that is naturally produced in Acmella radicans (Rios-Chavez et al., 2003) and Cissampelos graberrima (Laurerio-Rosario et al., 1996), as the most active compound in inhibiting primary root growth and stimulating lateral root formation. We show that this compound regulates many aspects of plant development by altering cell division and differentiation processes. In leaves, exogenous application of N-isobutyl decanamide was found to alter cell fate determination, leading to the production of ectopic blades along leaf petioles and vigorous outgrowths in the leaf lamina. In the root, these effects were accompanied with developmental transitions from lateral roots to callus-like structures. The involvement of cytokinins in mediating the observed activity of alkamides was tested using a suite of cytokinin-signaling Arabidopsis mutants lacking one, two, or three of the genes encoding the putative cytokinin receptors CYTOKININ RESPONSE1 (CRE1), ARABIDOPSIS HISTIDINE KINASE2 (AHK2), and AHK3. Our results suggest that alkamides may function as partial mimics of some endogenous lipid mediators whose action requires a functional cytokinin-signaling pathway to control meristematic activity and differentiation processes.

**RESULTS**

**Structure-Activity Relationships for Small Chain NAEs and Alkamides with Effects in Root Development**

The mechanism by which NAEs and alkamides exert their developmental effects in plants is unknown but the finding that small chain NAEs and alkamides were active in inhibiting primary root growth and promoting lateral root development suggests a common signaling mechanism for these two classes of compounds (Blancaflor et al., 2003; Ramirez-Chavez et al., 2004). To further investigate the structural features required for an alkamide to regulate root development in Arabidopsis seedlings, three compounds of similar acyl chain length but different saturation degree were evaluated at concentrations ranging from 3.5 to 112 μM, namely affinin, N-isobutyl decanamide, and NAE10:0 (Fig. 1). The three different compounds were found to inhibit primary root growth (Fig. 2A) and to promote lateral root formation (Fig. 2, B and C). Higher concentrations of NAE10:0 were required to inhibit lateral root formation and of affinin to promote lateral root formation. Interestingly, N-isobutyl decanamide was found to be the most active compound, showing a 50% primary root growth inhibition and a 4-fold increase in the number of lateral roots at 14 μM. We also found a dramatic increase in lateral root density (lateral root number per centimeter of primary root) from 7 to 56 μM of this compound.

To determine whether the amino residue and the length of the acyl chain are important for the observed N-isobutyl decanamide activity, we compared the effects of low micromolar concentrations of N-isobutyl decanamide on primary root growth and lateral root
formation with those of octadecanamide, a long chain amide and decanoic acid ethyl ester, a 10 carbon lipid compound lacking the amide moiety (for chemical structures see Fig. 1). Neither octadecanamide nor decanoic acid ethyl ester was found to inhibit primary root growth or to promote lateral root formation as shown for N-isobutyl decanamide (Fig. 3, A and B). These results indicate that both the amino residue and the length of the acyl chain appear to be important for alkamide activity to regulate root system architecture in Arabidopsis seedlings.

Figure 2. Structure activity for different NAE/alkamides with similar chain length. Arabidopsis plants were grown for 12 d on agar plates supplied with the different compounds at the indicated concentrations and the length of the primary root (A), lateral root number per plant (B), and lateral root density (lateral roots/cm; C) determined for a total of 30 seedlings.

Figure 3. Comparative effects of N-isobutyl decanamide, decanoic acid ethyl ester, and octadecanamide in primary and lateral root development. Arabidopsis plants were grown for 12 d on agar plates supplied with the different compounds at the indicated concentrations and the length of the primary root (A) and lateral root number per plant (B) determined for a total of 30 seedlings.
Effect of N-Isobutyl Decanamide on Lateral Root Development

Previous work showed that affinin, the most abundant alkamide present in the roots of *H. longipes* regulates several traits during root system development in Arabidopsis and that N-laurylethanolamine was able to regulate lateral root growth depending on its concentration in the medium (Blancaflor et al., 2003; Ramírez-Chávez et al., 2004). To investigate the effects of N-isobutyl decanamide on lateral root development, Arabidopsis seedlings were treated for 14 d with different concentrations of this molecule and lateral root length and morphology examined. N-isobutyl decanamide concentrations from 3.5 to 28 μM showed a dose-dependent lateral root growth promotion (Fig. 4, A–E). In contrast, 40% to 80% reduction in lateral root elongation was observed at higher concentrations of N-isobutyl decanamide (56–112 μM, see Fig. 4A). Interestingly, N-isobutyl decanamide at 112 μM produced a dramatic morphological effect in which the formation of callus-like structures instead of normal lateral roots was observed (Fig. 4, F and G).

Effects of N-Isobutyl Decanamide on Shoot Development

The formation of callus-like structures in primary roots suggested that N-isobutyl-decanamide could play an important role in cell division. To investigate whether this alkamide could affect shoot development, Arabidopsis Columbia-0 (Col-0) seedlings were grown for 18 d on 0.2× Murashige and Skoog agar medium containing different concentrations of this compound. It was found that Arabidopsis seedlings respond to exogenous N-isobutyl decanamide in a dose-dependent manner. In plants grown on 28 μM, ectopic organogenesis was observed on the adaxial side of petioles of rosette leaves (Fig. 5, A–F). Ectopic organs resembled leaf blades, as revealed by trichome development on their surfaces (Fig. 5, D–F). Plants exposed to 56 μM N-isobutyl decanamide were smaller than solvent treated controls, with shorter, thicker petioles and dark green leaves, which often formed callus-like structures over their surfaces (Fig. 5, G–I). Treatment with 112 μM N-isobutyl decanamide severely impaired growth and plants had a compact rosette with round leaves and short petioles (Fig. 5J). The most severely affected plants showed fleshy and chlorotic cotyledons (Fig. 5K) and occasionally, the entire shoot appeared to be arrested in growth (Fig. 5L). Moreover, a dose-dependent increase in the number of plants with callus-like structures on leaves was evident starting at 14 μM and reaching 100% at 112 μM N-isobutyl decanamide (Fig. 6). None of the described alterations were observed in a large number of solvent-treated control plants or in plants exposed to concentrations of 3.5 to 7 μM N-isobutyl decanamide that were examined during these experiments (Fig. 6).

Effect of N-Isobutyl Decanamide on *CycB1uidA* Expression in Roots and in Shoots

To determine whether the neoplastic effects of N-isobutyl decanamide on shoots were due to an effect of this compound on cell division, we examined the expression of the *CycB1uidA* reporter gene in Arabidopsis seedlings subjected to treatment with different concentrations of N-isobutyl decanamide. The *CycB1uidA* fusion protein is a good cell marker of proliferative activity since it is expressed only in cells in the G2/M phase and is destroyed rapidly when cells passed through mitosis (Colón-Carmona et al., 1999). In the roots of transgenic *CycB1uidA* plants grown in medium lacking N-isobutyl decanamide, cell divisions passed through mitosis (Colo´ n-Carmona et al., 1999). Previous work showed that affinin, the most abundant alkamide present in the roots of *H. longipes* regulates several traits during root system development in Arabidopsis and that N-laurylethanolamine was able to regulate lateral root growth depending on its concentration in the medium (Blancaflor et al., 2003; Ramírez-Chávez et al., 2004). To investigate the effects of N-isobutyl decanamide on lateral root development, Arabidopsis seedlings were treated for 14 d with different concentrations of this molecule and lateral root length and morphology examined. N-isobutyl decanamide concentrations from 3.5 to 28 μM showed a dose-dependent lateral root growth promotion (Fig. 4, A–E). In contrast, 40% to 80% reduction in lateral root elongation was observed at higher concentrations of N-isobutyl decanamide (56–112 μM, see Fig. 4A). Interestingly, N-isobutyl decanamide at 112 μM produced a dramatic morphological effect in which the formation of callus-like structures instead of normal lateral roots was observed (Fig. 4, F and G).

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were clearly visible in the region comprising the root apical meristem (RAM; Fig. 7A). In the aerial part of the plant, cells expressing $\text{CycB1:uidA}$ were restricted to the shoot apical meristem and to the base of developing leaves (Fig. 7B). In young seedlings (7 d after germination) treated with 28 $\mu M$ N-isobutyl decanamide showing an ectopic outgrowth on petiole (arrow; D). Close up of petals with ectopic blades (E and F). Note the presence of trichomes on these blades (arrows). Plants treated with 56 $\mu M$ N-isobutyl decanamide (G–I). Formation of callus-like structures over the surface of leaves (H and I). Plants treated with 112 $\mu M$ N-isobutyl decanamide (J–L). Note the reduction in length of petals and inhibited growth of leaves. Scale bars in A to L = 500 $\mu m$. [See online article for color version of this figure.]

**Figure 5.** Effects of N-isobutyl decanamide on Arabidopsis shoot development. Wild-type Col-0 seedlings were grown for 18 d under varied N-isobutyl decanamide concentrations on vertically oriented agar plates. Control plants at the rosette stage of development (A). Close up of a developing leaf blade showing formation of trichomes (B). Magnification of a petiole (C). Plant treated with 28 $\mu M$ N-isobutyl decanamide showing an ectopic outgrowth on petiole (arrow; D). Close up of petals with ectopic blades (E and F). Note the presence of trichomes on these blades (arrows). Plants treated with 56 $\mu M$ N-isobutyl decanamide (G–I). Formation of callus-like structures over the surface of leaves (H and I). Plants treated with 112 $\mu M$ N-isobutyl decanamide (J–L). Note the reduction in length of petals and inhibited growth of leaves. Scale bars in A to L = 500 $\mu m$. [See online article for color version of this figure.]

**Figure 6.** Effect of N-isobutyl decanamide on callus-like structure formation on leaves. Wild-type (Col-0) seedlings were grown under increasing N-isobutyl decanamide concentrations on vertically oriented agar plates. Eighteen days after germination, the number of plants showing callus-like structures on leaves was scored. Values shown represent the mean of 200 seedlings ± sd.

were clearly visible in the region comprising the root apical meristem (RAM; Fig. 7A). In the aerial part of the plant, cells expressing $\text{CycB1:uidA}$ were restricted to the shoot apical meristem and to the base of developing leaves (Fig. 7B). In young seedlings (7 d after germination) treated with 56 $\mu M$ N-isobutyl decanamide, cell divisions were also mainly confined to the RAM and shoot apical meristem (data not shown). However, later in development (18 d after germination) cell proliferation in the RAM decreased (Fig. 7C) and patches of GUS expression were preferentially seen in the shoot over a wider region in rosette leaves than in control seedlings (Fig. 7D), showing that cells do not exit from the cell cycle with normal developmental timing, resulting in ectopic cell divisions. In callus formed on leaf blades, clusters of cells expressing GUS could be observed, indicating mitotic activity in these tissues (Fig. 7, E and F).

**N-Isobutyl Decanamide Activates Transcription of the Cytokinin Reporter $\text{ARR5:uidA}$**

Cytokinins are a class of phytohormones involved in various physiological responses, including cell division, root hair growth, chloroplast development, and shoot formation (Howell et al., 2003; Kakimoto, 2003). To test the possibility that N-isobutyl decanamide could affect cytokinin signal transduction, we examined transgenic plants harboring a fusion of the $\text{ARR5}$ promoter region to the GUS reporter sequence ($\text{ARR5:uidA}$). This cytokinin-inducible marker has been shown to be sensitive, highly specific for cytokinins, and reflects the expression of the corresponding resident gene (D’Agostino et al., 2000; Romanov et al., 2002). In control plants, the most prominent $\text{ARR5:uidA}$ expression was detected in the shoot meristem region (Fig. 8A) and in the cap of primary roots (Fig. 8B). Moderate staining was also present in the vasculature of the hypocotyl (Fig. 8C), but not in the median
region of the primary root (Fig. 8D). Treatment of 5 μM benzyl aminopurine increased GUS staining in the primary root tip, but did not significantly affect GUS expression in the cotyledons, in the root-shoot junction, or in the median region of the primary root (Fig. 8, E–H). Quantitation of GUS activity by fluorometry showed that ARRKs::uidA plants grown in treatment of 20 μM benzyl aminopurine had an increase of 68.5% in GUS activity when compared to untreated plants (data not shown), whereas plants grown under treatment of 28 μM N-isobutyl decanamide showed a small but statistically significant increase in GUS activity of 15.6% when compared to untreated plants (data not shown). These results suggest a role for decanamide in regulating the localized expression of a cytokinin primary-response gene.

Cell Proliferative Responses to N-Isobutyl Decanamide Are Impaired in Cytokinin-Signaling Mutants

The ARRKs::uidA induction by exogenous N-isobutyl decanamide indicated a possible cross talk between alkamides and cytokinins. To investigate whether alkamide action could involve the cytokinin signaling pathway, we evaluated the effects of exogenously supplied N-isobutyl decanamide on the growth and development of Arabidopsis loss-of-function mutants lacking one, two, or three of the genes encoding cytokinin receptors (CRE1, AHK2, and AHK3; Higuchi et al., 2004; Nishimura et al., 2004). Callus-like structure formation on leaves was chosen as a developmental marker to test the effect of a high concentration (56 μM) of N-isobutyl decanamide on wild type (Col-0) and cytokinin response mutant seedlings. Addition of exogenous alkamide to the growth medium induced the formation of callus-like structures on wild-type plants (Fig. 9, A–C). In contrast, the cre1-12 or ahk3-3 single mutants exhibited 20% to 30% reduced sensitivity to N-isobutyl decanamide in terms of callus formation. Increased resistance to the effect of N-isobutyl decanamide was seen in the ahk2-2 single mutant and in double mutant combinations involving ahk2-2 (Fig. 9A). The triple mutant cre1-12 ahk2-2 ahk3-3 showed the most altered responses in this essay, in which callus formation induced by N-isobutyl decanamide was totally impaired (Fig. 9A). Detailed phenotypical analysis revealed that the extent of callus-like structure proliferation was indeed very reduced in double

Figure 7. Effects of N-isobutyl decanamide on CycB1:uidA expression. In control plants GUS expression is found in the RAM (A) and young leaves (B). In plants grown in 56 μM N-isobutyl decanamide reduced GUS expression is detected in the RAM (C), whereas GUS expression is localized to extended regions in leaves (D) and in callus-like structures (E and F). [See online article for color version of this figure.]
mutants involving ahk2-2, such as ahk2-2 ahk3-3 (Fig. 9, D and E) and in the cre1-12 ahk2-2 ahk3-3 triple mutant (Fig. 9, F and G). In the root, the formation of callus-like structures was also absent in cre1-12 ahk2-2 ahk3-3 triple mutant (Fig. 9, H–K). These results suggest that CRE1, AHK2, and AHK3 have redundant function in callus induction by exogenous N-isobutyl decanamide and that the three cytokinin receptors are required for normal proliferative responses at least in terms of callus-like structure formation.

Effect of N-Isobutyl Decanamide on Primary Root Growth and Lateral Root Formation in Cytokinin Signaling Mutants

The reduction in callus-like structure formation in leaves of the cytokinin receptor mutants in response to N-isobutyl decanamide evidenced the involvement of cytokinin signaling in alkamide responses. To further define whether cytokinin mutants were also less sensitive to N-isobutyl decanamide effects of primary root growth and lateral root formation, the effects of 14 and 28 μM N-isobutyl decanamide on

Figure 8. Effect of N-isobutyl decanamide on cytokinin-regulated gene expression. Six hours of GUS staining of ARR5:uidA seedlings grown for 6 d in medium without cytokinin (A–D), under 5 μM benzyl aminopurine (E–H), under 20 μM benzyl aminopurine (I–L), and with treatment of 28 μM N-isobutyl decanamide (M–P). Photographs are representative individuals of at least 20 plants stained. Scale bars = 300 μm. [See online article for color version of this figure.]

Figure 9. Effects of N-isobutyl decanamide on callus-like structure formation on leaves in wild-type (Col-0) and cytokinin response mutant seedlings. Seedlings were grown for 18 d on nutrient media with or without 56 μM N-isobutyl decanamide on vertically oriented agar plates. Plants showing callus-like structures on leaf surfaces were scored positive (n = 90). The experiment was repeated twice with similar results. [See online article for color version of this figure.]
primary root growth and lateral root density of single, double, and triple cytokinin receptor knockouts were tested. As shown in Figure 10, treatment with 28 μM N-isobutyl decanamide caused 86% inhibition in primary root growth in wild-type (Col-0) plants compared with solvent-treated control plants. In media lacking N-isobutyl decanamide, all seven mutant lines cre1-12, ahk2-2, ahk3-3, cre1-12 ahk2-2, cre1-12 ahk3-3, ahk2-2 ahk3-3, and cre1-12 ahk2-2 ahk3-3 showed smaller primary roots compared to wild-type plants (Fig. 10A). Growth was severely impaired in the triple mutant, giving rise to dwarf plants with small roots. When mutant plants were grown in the presence of 14 or 28 μM N-isobutyl decanamide an inhibition in root elongation was observed depending on the alkamide treatment (Fig. 10A). In medium lacking N-isobutyl decanamide, all single and double cytokinin receptor mutants showed normal lateral root formation, with statistically similar lateral root densities compared to wild-type plants (Fig. 10B). Furthermore, we observed a 4- to 9-fold increase in lateral root density in 14 and 28 μM N-isobutyl decanamide treatments, respectively, in wild-type, single, and double mutants (Fig. 10B). Interestingly, cre1-12 ahk2-2 ahk3-3 mutant plants showed no lateral root induction in N-isobutyl decanamide treatments, indicating an important role for cytokinins in pericycle cell activation in response to this alkamide.

**DISCUSSION**

**N-Isobutyl Decanamide Activates Developmental Transitions in Roots**

The root system originates from a primary root that develops during embryogenesis. This primary root produces lateral roots that increase its exploratory capacity. The root system shares with the shoot the basic body plans and the pathways that are essential for organogenesis and growth (Veit, 2004). Recent studies have addressed the role that hormones, such as auxin and cytokinin, play in root system development. The site of lateral root initiation seems to depend on correct auxin transport to pericycle cells in the primary root (Dubrovsky et al., 2000; López-Bucio et al., 2005), whereas the final architecture of the root is coordinated by hormonally regulated processes that affect cell division and cellular differentiation (Casson and Lindsey, 2003). Recently two groups of single chain amides have been reported to alter several aspects of root development, alkamides, and NAEs (Blancaflor et al., 2003; Ramírez-Chávez et al., 2004). Blancaflor et al. (2003) evaluated the effects of micro-molar concentrations of N-lauroylethanolamine on early root development of Arabidopsis. In young seedlings, a 50 μM concentration of this molecule was found to inhibit root elongation, increase radial swelling of root tips, and alter hair development. Older seedlings showed increased lateral root formation. These developmental effects were related to altered cell division, endomembrane organization, and vesicle trafficking, suggesting that N-lauroylethanolamine may play a role in these fundamental processes (Blancaflor et al., 2003). Similarly, Ramírez-Chávez et al. (2004) reported that affinin, the most abundant alkamide in the roots of *H. longipes*, regulates Arabidopsis root system architecture in a dose-dependent manner. In this work, we extend these previous findings by showing that N-isobutyl decanamide, a saturated alkamide obtained by affinin reduction, alters both primary root growth and lateral root formation. These effects were similar to those for NAE10:0 and affinin (Fig. 2), suggesting that alkamides and NAEs may comprise a
new group of plant growth regulating substances, which may act through common signaling mechanisms to modify root development. Interestingly, both the amide moiety and the length of the acyl chain were found to be essential for biological activity of exogenous applied amides, since octadecanamide and decanoic acid ethyl ester failed to inhibit primary root growth or to stimulate lateral root formation (Fig. 3). We cannot exclude the possibility that the longer acyl chain in octadecanamide results in a lower permeability and that this could partially mask the activity for this compound.

N-Isobutyl Decanamide Regulates Cell Proliferation in Arabidopsis

Leaf initiation at the shoot apical meristem involves a balance between cell proliferation and commitment to make primordia. Arabidopsis has a typical simple leaf, which consists of a petiole and a blade. The developmental control of petioles is presumed to be important in the effective capture of light by ensuring that the leaf blades do not overlap. To produce this leaf shape, the cells on the proximal side of the leaf differentiate into petioles without producing blades or other organs (Ha et al., 2003). One of the most conspicuous effects of N-isobutyl decanamide on shoot development was the ectopic induction of outgrowths along the leaf petioles. These outgrowths resembled a leaf blade in that they showed the presence of trichomes on the adaxial side (Fig. 5). These observations suggest that following treatment with N-isobutyl decanamide, the petiole cells do not undergo correct developmental specification and can be diverted toward other developmental fates or that this alkamide is capable of reprogramming petiole cells to initiate the de novo formation of organs in differentiated cells.

Cell division normally ceases during leaf development (Donnelly et al., 1999; De Veylder et al., 2002) and is not observed in mature organs. Increased concentrations of N-isobutyl decanamide in the growth medium were found to induce the production of callus-like structures in roots (Fig. 4) and on leaves (Fig. 5). Moreover, in the epidermis of mature leaves and in callus-like structures of alkamide-treated plants, clusters of cells expressing CycB1:uidA were observed (Fig. 7), GUS staining being present over a wider region than in nontreated plants, suggesting that cells do not exit from the cell cycle with normal developmental timing, resulting in ectopic cell divisions. Taken together, these results suggest that alkamides alter several aspects of plant morphogenesis through the control of meristematic activity.

Alkamides and NAES: Evidence for Endocannabinoid Signaling in Plant Development?

Alkamides are structurally related to sphingolipids such as ceramide and sphingosine (Ng and Hetherington, 2001; Ramírez-Chávez et al., 2004), and to NAES, of which the latter are likely produced by hydrolysis of the membrane phospholipid N-acylphosphatidylethanolamine by phospholipase D (Chapman, 2000; López-Bucio et al., 2006). In animals, this reaction is part of the endocannabinoid-signaling pathway, which regulates a variety of physiological processes, including cell proliferation, neurotransmission, and embryo development (Howlett and Mukhopadhyay, 2000; Wilson and Nicoll, 2002). In plants, NAES are present in different tissues, being quite abundant in desiccated seeds, where their levels decline during seed imbibition and germination (Chapman et al., 1999). In response to pathogen elicitors, N-acylphosphatidylethanolamine is hydrolized by phospholipase D, and medium chain, saturated NAES are released by plant cells where they act as lipid mediators to modulate ion flux and activate defense gene expression (Chapman et al., 1998). Although the biosynthesis pathway for alkamides is at present unknown, the structural similarity of these molecules with NAES and sphingolipids suggests that molecules derived from the hydrolysis of membrane lipids can act as signaling molecules for plant development. However, it remains to be determined whether NAES and/or sphingolipids can also modify shoot development as shown in this work for N-isobutyl decanamide, and whether alkamides such as affinin and N-isobutyl decanamide can activate defense mechanisms in plant cells as it occurs for NAES and sphingolipids.

N-Isobutyl Decanamide Effects on Cell Proliferation Require Normal Cytokinin Signaling

The ability of alkamides and NAES to regulate organ development in Arabidopsis seedlings is compelling evidence for the possibility that these molecules act as regulators of morphogenesis. Thus, the possibility was raised that alkamides could interact with plant hormones such as auxin or cytokinins to regulate plant cell division.

Our previous work showed that the effects of alkamides on root growth are likely independent on auxin action (Ramírez-Chávez et al., 2004). However, the finding that N-isobutyl decanamide activates the expression of the cytokinin primary-response gene fusion ARR5:uidA suggests that cytokinin signaling is probably involved in the alterations in cell proliferation and differentiation caused by alkamides. Cytokinins are purine derivatives that promote and maintain plant cell division in cultures and are also involved in various differentiation processes including shoot formation, primary root growth, and callus formation (Grayburn et al., 1982; Werner et al., 2001, 2003; Catterou et al., 2002; Higuchi et al., 2004). Three sensor His kinases, CRE1/AHK4/WOL, AHK2, and AHK3 have been shown to act as cytokinin receptors (Kakimoto, 2003). These receptors activate the expression of several response regulators in a cytokinin-dependent manner (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). Further downstream, cytokinin signaling stimulates the G1/S transition of the cell cycle, which
has been proposed to be mediated by the transcriptional induction of the CYCD3 gene that encodes a D-type cyclin (Riou-Khamlichi et al., 1999). We observed that alkamides induce cell proliferation as observed by the formation of callus-like structure in shoots and the increased expression of the CycB1;1uidA gene marker.

When grown on soil, none of the single cytokinin receptor mutants (crel-12, ahk2-2, ahk3-3) exhibited significant defective phenotype. The ahk2-2 ahk3-3 double mutants had smaller leaves and shorter stems than did the wild-type plants. All single and double mutants produced apparently normal flowers that yielded viable seeds. The crel-12 ahk2-2 ahk3-3 triple mutants showed a dwarf phenotype with reduced growth. Occasionally, the triple mutants produced an influorence with nonfunctional flowers, which failed to produce seeds (data not shown). These data suggest that cytokinin receptors play an important role in plant growth and development. The complete lack of N-isobutyl decanamide responses in the triple cytokinin receptor mutant crel-12 ahk2-2 ahk3-3, in terms of lateral roots or callus-like structure proliferation (Figs. 9 and 10) and the absence of ectopic blades on petioles and callus on mature leaves, suggest that cytokinin receptors are necessary for normal cellular responses to alkamides. There are several scenarios that could explain the alkamide-cytokinin interaction; one in which alkamide treatment induces cytokinin biosynthesis and in this way alters plant development. However, since there are no reports that exogenous treatment with cytokinins alone is able to induce lateral roots or callus-like structure formation in leaves in a similar way to N-isobutyl decanamide, makes this possibility unlikely. Another possibility is that the cytokinin receptors are direct targets of alkamides and that this interaction starts the signaling cascade that activates gene expression responsible for cellular responses. A third scenario is that alkamides could alter cytokinin sensitivity in specific tissues by either modulating the level of the cytokinin receptor or their activity by direct or indirect interaction with an as yet undiscovered alkamide receptor. However, since the effects of treatments with N-isobutyl decanamide differ from those produced by exogenous cytokinin, it is likely that alkamide action involves additional signaling pathways yet to be discovered. These different scenarios are currently under investigation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col-0 and the transgenic lines AHRsuidA (D’Agostino et al., 2000) and CycB1uidA (Colón-Carmona et al., 1999) were used for all experiments unless indicated otherwise. The cytokinin receptor mutants crel-12, ahk2-2, ahk3-3, crel-12 ahk2-2, crel-12 ahk3-3, ahk2-2 ahk3-3, and crel-12 ahk2-2 ahk3-3 were previously described (Higuchi et al., 2004; Mähönen et al., 2006).

Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× Murashige and Skoog medium, pH 5.7, 0.5% (w/v) Suc, and 1% (w/v) agar. The basic medium contained 2.0 mM NH4NO3, 1.9 mM KNO3, 0.3 mM CaCl2, 2H2O, 0.15 mM MgSO4·7H2O, 5.0 μM KI, 25 μM H2O2, 0.1 mM MnSO4·H2O, 0.3 mM ZnSO4·7H2O, 1 μM Na2MoO4·2H2O, 0.1 μM CuSO4·5H2O, 0.1 μM CoCl2·6H2O, 0.1 mM FeSO4·7H2O, 0.1 mM NaEDTA·2H2O, insolot (10 mg L−1), and Gly (0.2 mg L−1). Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded growth of the hypocotyl into the air. For plant growth, we used a plant growth cabinet (Percival Scientific) with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 300 μmol m−2 s−1, and temperature of 22°C to 24°C. After growth for different periods as indicated, plants were cleared according to the method by Malamy and Benfey (1997) and lateral roots counted using a 40× objective on a Leica DMR microscope.

Isolation of Crel-12 ahk2-2tk ahk3-3 Triple Mutants

In agar medium homozygous crel-12 ahk2-2tk ahk3-3 triple mutants develop short primary roots and can be easily distinguished from crel-12/crel-12 ahk2-2tk/ahk2-2tk ahk3-3/ahk3-3 (heterozygous for ahk3-3) or crel-12/crel-12 ahk2-2tk/ahk2-2tk ahk3-3. To select for the triple mutants, a pool of seeds produced by a crel-12/crel-12 ahk2-2tk/ahk2-2tk ahk3-3/ahk3-3 plant were sterilized and sown on agar plates. After 10 d, seedlings with short primary roots were selected and transferred to plates with the different decanamide concentrations for a further 10 to 15 d growth period. Separately we have examined the genotypes of at least 50 plants with short roots, and all were confirmed to be triple homoygotes. For triple mutant selection, 500 seeds from this crel-12/crel-12 ahk2-2tk/ahk2-2tk ahk3-3/ahk3-3 segregating population were screened for reduced primary root growth by placing seeds on 100 cm2 nutrient agar plates (20 seeds per plate). The seeds were distributed in two rows on the agar surface at a density of 1 seed/cm, stratified at 4°C for 48 h, and then incubated at 22°C. Putative mutants with short primary roots were selected and transferred to plates with the different N-isobutyl decanamide treatments for a further 10 to 15 d growth period.

Synthesis of Alkamides and NAEs

Affinin was purified from Heliopsis longipes (Gray) Blake (Asteraceae) plants collected at Xichué, Sierra Gorda of Guanajuato State, central México, and N-isobutyl decanamide was obtained from affinin by catalytic reduction as described before (Ramírez-Chávez et al., 2004). Octadecanamide was purchased from Sigma Aldrich. NAE100 was synthesized from the acyl chlorides in ethanolamine (Tripathy et al., 1999), at room temperature, in a reaction mixture of 25 mg of decanoyl chloride, 2.5 mL of dichloromethane, and 2.5 mL of ethanolamine (Sigma-Aldrich) for 15 min with gentle swirling. The reaction was stopped with 50 μL of distilled water and washed with dichloromethane, with an equal volume of bidistilled water (MilliQ® Plus). The NAE was then collected in the organic layer and the dichloromethane was evaporated under N2 gas stream. The product was resuspended in anhydrous ethanol and purity determined by gas chromatography-mass spectroscopy.

Histochemical Analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated at 37°C in a GUS reaction buffer (0.5 mg/mL of 5-bromo-4-chloro-3-indolyl-B-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared by the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 10 transgenic plants were analyzed. A representative plant was chosen for each decanamide or cytokinin treatment and photographed using the Nomarski optics on a Leica DMR microscope.

Data Analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All lateral roots emerged from the primary one and observed at the 3× objective were taken into account for lateral root number data. Primary root length was determined for each root using a ruler. For all experiments, the overall data was statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with a Tukey’s Post Hoc test were used for testing differences in primary root length, lateral root number, and lateral root density under NAE and alkamide treatments in wild-type and mutant plants. Different letters are used to indicate means that differ significantly (P < 0.05).
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