

Cannabinoid-Like Signaling and Other New Developmental Pathways in Plants

Alina Morquecho-Contreras • José López-Bucio*

¹ Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria. C. P. 58030 Morelia, Michoacán, México

Corresponding author: * jlbucio@zeus.umich.mx

ABSTRACT

Plant growth and development depend on the interplay of a number of chemical regulators. Traditionally, six groups or classes of plant hormones have received the most attention: auxins, cytokinins, ethylene, abscisic acid, gibberellins and brassinosteroids. Recently, it has become clear that additional signaling molecules are used by plants. The alkamides and *N*-acylethanolamides (NAEs) – a class of small organic compounds related to animal endocannabinoids – appear to play a role in diverse morphogenetic processes including seed germination, alteration of plant architecture and response to pathogen attack. In vertebrates, the endocannabinoid signaling pathway has been found to control cellular and morphogenetic responses including embryo development, cell proliferation, immune responses and apoptosis. The notion that plants use alkamides and NAEs to regulate physiological processes is supported by their occurrence in a wide range of plant species, their selective accumulation and rapid metabolism in response to developmental transitions and by the recent identification of the enzymatic machinery for NAE degradation. Moreover, signal transduction cascades involving glutamate and nitric oxide, partners of NAEs and alkamides in regulating animal physiology, have been found to participate in important developmental processes in plants. This information suggests that endocannabinoid-like compounds and other ancestral signaling pathways might be integral to morphogenetic and adaptive processes in plants.

Keywords: alkamides, *N*-acylethanolamides, endocannabinoids, plant development

Abbreviations: DAF2-DA, 4, 5-diaminofluorescein diacetate; FAAH, fatty acid amide hydrolase; GLR, glutamate receptor; GLU, glutamate; GPCR, G protein coupled receptor; LR, lateral root; NAE, *N*-acylethanolamide; NAPE, *N*-acylphosphatidylethanolamide; NO, nitric oxide; NPA, 1-naphthyl-phtalamic acid; PLD, phospholipase D

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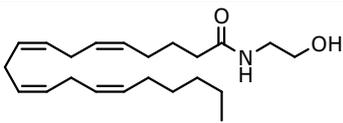
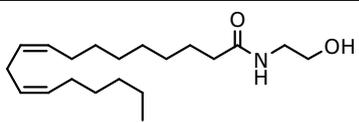
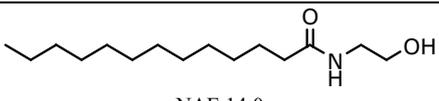
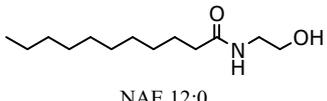
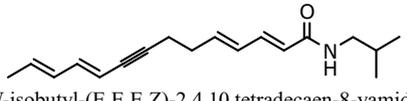
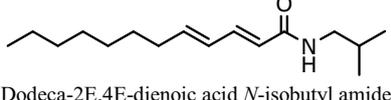
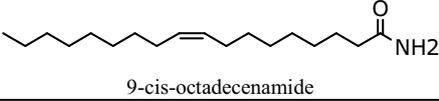
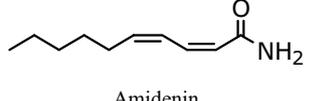
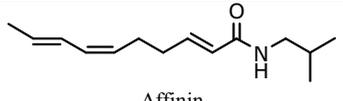
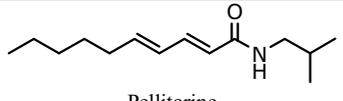
INTRODUCTION

The development of plants is influenced both by internal physiological factors and by environmental signals, which control cell division in meristems, and growth and differentiation in roots and shoots. Plants produce compounds of different chemical identity that mediate a range of cellular functions. Auxins, cytokinins, gibberellins, abscisic acid and brassinosteroids represent different classes of organic molecules, while ethylene is a gas. These compounds are considered as plant hormones because of their widespread occurrence in plants and their diverse physiological roles (Weyers and Paterson 2001). The effects produced by each hormone have been elucidated largely from exogenous applications, by using correlations between hormone levels and growth of defined plant species, or by manipulating hormone levels in mutants and transgenic plants (Weyers

and Paterson 2001; Kakimoto 2003; Woodward and Bartel 2005). Recently, other compounds, namely polyamines, jasmonates, salicylic acid, and the peptide systemin, are being added to the list of potential plant hormones, but whether these have universal effects or act in just a few special cases has not yet been fully determined.

In the past five years, it has become evident that plants use – along with the above mentioned classical hormones – a diverse array of small molecules for extra- and intracellular signaling. Of particular interest are the plant lipids because, like their mammalian counterparts, they act not only as structural components of membranes but also as signaling molecules that regulate developmental and adaptive responses to environmental stimuli (Cruz-Ramírez *et al.* 2004; Wang 2004). *N*-acylethanolamides (NAEs) comprise a group of lipids, which are produced from the hydrolysis of *N*-acylphosphatidylethanolamide (NAPE), a minor lipid

Table 1 Characteristics and effects of NAEs and alkamides.

Molecule (with example)	General characteristics	Biological effects
 Anandamide	<ul style="list-style-type: none"> • Located in all mammalian cells as a trace compound, but principally in brain where it is synthesized from a phospholipid precursor (NAPE). • Metabolized by a FAAH. • Activate CB receptors. 	<ul style="list-style-type: none"> • Modulation of neurotransmission in central nervous system. • Synchronization of embryo development. • Nociception. • Brain development. • Memory processes. • Cardiovascular and immune regulation. • Cell proliferation.
 NAE 18:2	<ul style="list-style-type: none"> • Predominant NAE in desiccated seeds. • Metabolized by plant lipoxygenases <i>in vitro</i>. 	<ul style="list-style-type: none"> • Plays a role in regulating seed germination.
 NAE 14:0	<ul style="list-style-type: none"> • Activation of PAL gene expression. 	<ul style="list-style-type: none"> • Perception of pathogen elicitors. • Activation of defense responses.
 NAE 12:0	<ul style="list-style-type: none"> • Produced from hydrolysis of NAPE by PLD. • Hydrolyzed by AtFAAH. • Rapid metabolism during seed germination. 	<ul style="list-style-type: none"> • Regulation of seed germination and normal seedling development. • Regulation of cell division and expansion. • Regulation of root architecture. • Control of cytoskeletal structure.
 <i>N</i> -isobutyl-(E,E,E,Z)-2,4,10,tetradecaen-8-ylamide	<ul style="list-style-type: none"> • Present in <i>Chrysanthemum</i> plants. 	<ul style="list-style-type: none"> • Provides host-plant resistance to <i>Frankliniella occidentalis</i> (western flower thrips).
 Dodeca-2E,4E-dienoic acid <i>N</i> -isobutyl amide	<ul style="list-style-type: none"> • Isolated from <i>Echinacea</i>. 	<ul style="list-style-type: none"> • Binds to de human CB2-receptor.
 9-cis-octadecenamide	<ul style="list-style-type: none"> • Isolated from the lichen <i>Stereocaulon alpinum</i>. 	<ul style="list-style-type: none"> • Inhibits the formation of cyclooxygenase products in sheep seminal vesicle microsomes.
 Amidenin	<ul style="list-style-type: none"> • Isolated from <i>Amycolaptosis</i> sp. 	<ul style="list-style-type: none"> • Growth-regulating activity in rice seedlings. Plant growth promotion effects at low concentrations (low μM range).
 Affinin	<ul style="list-style-type: none"> • Isolated from <i>Heliopsis longipes</i> roots. 	<ul style="list-style-type: none"> • Growth-promoting effect at low concentrations (10^{-8} - 10^{-5} M). • Regulates root hair and lateral root formation. • Regulates cell division and differentiation processes. • Fungistatic and bacteriostatic activities.
 Pellitorine	<ul style="list-style-type: none"> • Isolated from <i>Stauranthus perforatus</i>. 	<ul style="list-style-type: none"> • Allelochemical effect on <i>Amaranthus hypochondriacus</i> and <i>Echinochloa crus-galli</i> seedlings. • Insecticidal activity.

constituent of cell membranes, by phospholipase D (PLD) (Schmid *et al.* 1996). In mammals, anandamide (NAE 20:4) acts as an endogenous ligand for cannabinoid receptors and has a number of physiological roles including the modulation of neurotransmission in the central nervous system (Wilson and Nicoll 2002), synchronization of embryo development (Paria and Dey 2000) and vasodilation (Kunos *et al.* 2000). In plants, there is information indicating that NAEs might be involved in diverse physiological processes, including seed germination, pathogenesis, regulation of root architecture and response to herbivory. Alkamides (*N*-alkyl amides) are compounds structurally related to NAEs, which have been found to alter root development and to regulate cell division and differentiation processes in *Arabidopsis thaliana*. In this review, we summarize recent research into the role of NAEs and alkamides in plant development and discuss the possibility that NAEs and alkamides could act in a common signaling pathway similar to the endocannabinoid system.

ENDOCANNABINOID SIGNALING

Plants produce compounds of different chemical nature that modulate physiological responses in animals (Table 1). *Cannabis sativa* extracts have been in use for centuries by different civilizations for medicinal and recreational purposes (Zlas *et al.* 1993). The demonstration that many of the actions of Δ -9-tetrahydrocannabinol (THC), the main bioactive compound of *Cannabis sativa* in mammals occur by activation of membrane cannabinoid receptors (CB1 and CB2) led to the search for endogenous cannabinoids, also termed 'endocannabinoids'. Endocannabinoids are defined as cellular compounds capable of functionally activating one or both of the established CB receptors (Di Marzo 1998). The first endocannabinoid identified was a *N*-acyl ethanolamide, anandamide (arachidonoyl-ethanolamide or NAE 20:4) (Table 1). This compound was initially isolated from pig brain and found to activate CB receptors (Devane *et al.* 1992).

Table 2 Signaling pathways modulated by endocannabinoids, NAEs and alkamides.

Signaling molecule	Pathways involved
Animal endocannabinoids (anandamide)	Inhibition of adenylyl cyclase. Inhibition of Ca ²⁺ channels. Inhibition of gap junction-mediated and glutamate-triggered Ca ²⁺ waves. Activation of inwardly rectifying K ⁺ channels. Activation of the MAPK pathway. Activation of cytosolic phospholipase A ₂ . Activation of NOS.
Alkamides in animals	Elevation of intracellular Ca ²⁺ . Modulation of cAMP.
NAEs/alkamides in plants	Modulation of PLD. Activation of defense responses. Activation of NOS? Interaction with glutamate signaling?

Other endocannabinoids, either ethanolamide analogs of NAE 20:4, or alternatively other arachidonic acid derivatives (i.e. 2-arachidonoylglycerol) have been isolated also from pig brain (Hanus *et al.* 1993, 2001).

Endocannabinoids exert their regulatory functions via the cannabinoid receptors CB1 and CB2 and multiple signal transduction pathways (Table 2). CB1 receptors have a widespread distribution in the brain (Herkenham *et al.* 1990). CB2 receptors are predominantly localized to cells of the immune system and to a lesser extent, in the brain (Griffin *et al.* 1999; van Sickle *et al.* 2005). Binding of endocannabinoids to CB receptors requires that the endocannabinoid should have an aliphatic chain of 20-22 carbons, with at least three non conjugated *cis* double bonds with a saturated tail of at least the last five carbons (Reggio and Traore 2000). CB1 receptors are primarily coupled to heterotrimeric G proteins, the stimulation of which leads to inhibition of adenylyl cyclase, inhibition of N- and P/Q-type calcium channels, activation of potassium channels, induction of nitric oxide synthase (NOS), activation of cytosolic phospholipase A and induction of mitogen-activated protein kinases (Table 2) (For Review see Macarrone and Finazzi 2003; Gertsch *et al.* 2006; Pertwee 2006). Recent information indicates that plants produce endocannabinoid-like compounds. For instance, *Echinacea* alkamides show strong affinity to CB2 receptors, which regulate multiple signal transduction pathways including increased cAMP, p38/MAPK signaling and ATF-2/CREB-1 activation (Gertsch *et al.* 2004; Raduner *et al.* 2006). These results highlight the role of alkamides as potent immunomodulators and ligands for CB2 receptors.

STRUCTURE AND DISTRIBUTION OF NAEs AND ALKAMIDES

Although anandamide is perhaps the most thoroughly studied of the NAE class of compounds, other naturally occurring NAEs have been known for almost 50 years (For review see Fowler 2003). NAEs with pharmacological effects in animals include palmitoylethanolamide (PEA, C16:0), stearoylethanolamide (SEA, C18:0) and oleoylethanolamide (OEA, C18:1). Interestingly, SEA, like PEA, has no affinity towards either CB1 or CB2 receptors, whereas OEA has been found to reduce the binding of anandamide to those receptors (Jonsson *et al.* 2001). These results suggest that NAE compounds might act either as ligands or as inhibitors of CB receptors and that different signaling pathways might be involved in physiological responses to each compound.

In plants, NAEs have been found in seeds of some Angiosperms such as cotton, corn, *Arabidopsis*, soybean, peanut, okra, tomato and pea, with a total content ranging from 500 to 1600 ng/g in fresh weight, and acyl chain length ranging from 12 to 20 carbon atoms (Table 1) (Chapman 2004). Gas chromatography-mass spectroscopy (GC-MS) measurements have shown NAEs containing 16C and 18C

to be the most abundant NAE compounds in dry seeds. Total NAE concentrations drop drastically following imbibition in seeds of pea, cotton, peanut and *Arabidopsis* and the decline is more pronounced for NAE 18:2 (Chapman *et al.* 1999; Wang *et al.* 2006). In desiccated *Arabidopsis* seeds, total NAE content was of 2000 ng/g, and this concentration declined to 500 ng/g 96 h after sowing (Wang *et al.* 2006). Decreased NAE concentration in seeds after imbibition and during early plant growth is consistent with the notion that NAE catabolism is a prerequisite for germination and post-embryonic growth.

The vegetative tissues of plants have lower NAE contents than seeds and their NAE profile also differs from that in seeds in that NAE 12:0 and NAE 14:0 appear to predominate (Chapman 2004). There is evidence that in stem and leaves medium chain NAEs are synthesized on demand rather than stored. For instance, in tobacco leaves the concentration of NAE 14:0 is of approximately 5 ng/g in a fresh weight basis. Interestingly, a 10 min treatment with nanomolar concentrations of two protein elicitors, xylanase and cryptogin, caused a 10 and a 50-fold increase in NAE 14:0 content, respectively (Tripathy *et al.* 1999). Exogenously supplied synthetic NAE 14:0 at concentration of 1 μM induced a 4-fold increase in phenylalanine ammonia lyase (PAL) expression in a manner similar to that elicited by xylanase and cryptogin in both cell suspensions and leaves of tobacco (Tripathy *et al.* 1999). These results suggest that NAE14:0 may participate in the signal transduction events leading to plant defense responses.

Similar to NAEs, alkamides are a class of lipid molecules with different chain length and saturation grade (Table 1). These compounds are relatively common in plants, being present from lichens to Angiosperms. In angiosperms, they have been found in a variety of families, including the Asteraceae, Brassicaceae, Leguminosae, Piperaceae and Rutaceae (Gertsch *et al.* 2006; López Bucio *et al.* 2006).

Natural alkamides are often insecticidal (Tsao *et al.* 2005). The western flower thrips *Frankliniella occidentalis* (Pergande) is one of the major insect pests of greenhouse floriculture. In addition to feeding on leaves and flowers, thrips can vector diseases such as tomato spotted virus, which affects a wide range of plants. Host-plant resistance to *F. occidentalis* is of potential to reduce the use of pesticides for control of this pest. Chrysanthemum cultivars show a wide variation in degree of host-plant resistance to *F. occidentalis*. Evidence for an alkamide associated with host-plant resistance to *F. occidentalis* was presented by Tsao *et al.* (2005). The authors conducted bioassay-directed fractionation of extracts of chrysanthemum leaves to isolate compounds that exhibited repellent activity against thrips. Analysis of a particular fraction that correlated with the degree of resistance of chrysanthemum to *F. occidentalis* revealed the presence of an unsaturated isobutylamide, *N*-isobutyl-(*E*, *E*, *Z*)-2,4,10,12-tetradecatetraen-8-ynamide (Table 1). This work presents compelling evidence that plant produced alkamides account for natural host-resistance to thrips and open the possibility that managing alkamide production in crops might represent a valuable target to control insect pests in agriculture.

The participation of alkamides in host-resistance to insects can be due to their role as elicitors of plant defense responses. For instance, it has been reported that linolenoyl-L-glutamine, an amide produced in oral secretions of caterpillars is able to induce the production of volatile chemicals from plants that attract predators and parasites of the caterpillar while it feeds (Lait *et al.* 2003).

Recent information suggest that alkamides can regulate microbial growth. In addition they can represent a new class of allelochemical compounds. The alkamide affinin (Table 1) from *Heliopsis longipes* and its reduced amides show fungistatic and bacteriostatic activities. Affinin was effective in inhibiting the mycelial growth of *Sclerotium cepivorum* and *Rhizoctonia solani* at concentration of 75 μg/ml and repressing growth of *Escherichia coli* and *Bacillus subtilis* at a concentration of 150 μg/ml. The bacteriostatic

effect was more clearly observed for *N*-isobutyl-2*E*-decenamide, an alkamide derivated from affinin, which inhibited bacterial growth at concentration of 5 µg/ml, a concentration 30-fold lower than that of affinin (Molina-Torres *et al.* 2004). On the other hand, Anaya and coworkers (2005) showed that pellitorin, an alkamide from *Stauranthus perforatus*, a rutaceous tree, has a strong allelochemical effect on the growth of weeds. Pellitorine in concentration of 100 µg/ml supplied to the culture medium *in vitro* caused a 45 and 80% inhibition in root growth of *Amaranthus hypochondriacus* and *Echinocloa crus-galli*, respectively. In a greenhouse experiment, the decomposition of leaves and roots of *S. perforatus* incorporated as green manures (2%) to the soil had a significant inhibitory effect in the growth of weeds. The allelopathic action of decomposition of leaves was comparable to that of DPCA (dimethyl tetrachloroterephthalate), a commercial herbicide (Anaya *et al.* 2005). Taking into account the above presented information, it is tempting to speculate that alkamide production in plants not only provides an adaptive advantage in response to pathogens and pests, but also might represent a strategy for competition.

Occasionally, alkamide accumulating plants can occur in different plant families (Molina-Torres *et al.* 1996). Certain medicinal plants such as *Echinacea angustifolia*, *Echinacea purpurea* and *Heliopsis longipes* accumulate alkamides in certain plant tissues (Bauer and Remiger 1989; Molina-Torres *et al.* 1996). In *H. longipes*, affinin accumulates especially in roots, where it is present in as high as 1% (w/w) on a fresh weight basis (Molina-Torres *et al.* 1996). Currently, no information is available about the ecological or physiological relevance of this alkamide accumulation, or about the cellular state where affinin accumulates. However, recent experiments indicate that alkamide accumulating plants such as *H. longipes* and *Acmella radicans* are resistant to the inhibitory effect of alkamides in root growth *in vitro* (López-Bucio J and Ramírez-Chávez E, unpublished information). This suggests that alkamide accumulating plants have evolved mechanisms to tolerate high concentrations of alkamides in their tissues.

In *E. purpurea*, alkamides accumulate preferentially in flower heads and in roots. Their levels are low at the beginning of vegetative growth and increase at the flowering stage (Letchamo *et al.* 1999; Qu *et al.* 2005). For total alkamides, concentrations varied from 5.0 to 27.6 mg/g in roots and from 0.22 to 5.3 mg/g in vegetative tissues (Qu *et al.* 2005). In *Piper cenocladum*, alkamide accumulation has been found to be dependent of resources such as light and the general availability of nutrients. Alkamide levels are higher in plants grown under balanced resource conditions than in poor soils, and their levels decrease with increased light intensity (Dyer *et al.* 2004). The optimal conditions for production of amides (2.8% dry weight) were fertilized soils, and the conditions associated with the lowest levels of amides (0.34% dry weight) were poor soils. To date, no information is available about the effects of individual nutrients such as nitrogen, phosphorus or potassium on alkamide accumulation. The reason why certain plants accumulate alkamides is not clear, however it could be an evolutionary advantage to cope with biotic and abiotic factors. Alternatively, alkamide accumulation might represent a mechanism to adjust their growth and development, as their importance in cellular processes is increasingly being appreciated (see below).

BIOSYNTHESIS AND METABOLISM OF NAEs AND ALKAMIDES

NAEs are produced from the PLD-mediated hydrolysis of NAPE, a minor lipid constituent of cellular membranes (Schmid *et al.* 1996). In mammals, levels of NAPE and NAE in cultured cortical neurons are greatly increased following treatment with the Ca²⁺ ionophore ionomycin, by K⁺ and by activation of glutamate receptors. Interestingly, both NAPE and NAE concentrations increased by 2 to 3-fold

after 5-10 d in the presence of glutamate, indicating that neuronal activity regulates NAE production (Hansen *et al.* 1995, 1997).

Anandamide plays an important signaling role in cellular and developmental responses acting as a ligand for cannabinoid receptors, which belong to the class A rhodopsin-like G protein coupled receptor (GPCR) family (Gertsch *et al.* 2006). The activity of anandamide is rapidly terminated through a two-step process consisting of a putative carrier-mediated transport followed by hydrolysis by a serine hydrolase, referred to as fatty acid amide hydrolase (FAAH) (Fig. 1) (Cravatt *et al.* 2004). The importance of this enzyme in animal physiology was evident after the cloning of the mice FAAH gene. For instance, the target disruption of this gene resulted in mice with hypersensitivity to exogenous anandamide and a 10-fold elevation of endogenous brain anandamide levels (Cravatt *et al.* 2001). Interestingly, there are some similarities between plants and animals in the metabolism of NAEs. For example, a functional homologue of the mammalian FAAH has been identified in plants by a combination of bioinformatics, molecular and biochemical approaches (Shresta *et al.* 2003). A full-length alignment of *Arabidopsis* FAAH amino acid sequence with rat FAAH showed that these proteins share an amidase region typical of a family of proteins that include amidase or amidohydrolase enzymes. The amidase region of these proteins consists of about 125 amino acids. There is 18.5% identity between the *Arabidopsis* FAAH and rat FAAH when compared over the entire length of the proteins, whereas there is 37% identity within the amidase region. Functional homologues of the *A. thaliana* FAAH (*AtFAAH*) were also identified in *Oryza sativa* and *Medicago truncatula*, supporting a common mechanism for the regulation of NAE hydrolysis in diverse plant species (Shresta *et al.* 2006).

Important information about the *in vivo* role of *AtFAAH* in NAE metabolism in plants came from its manipulated expression in *A. thaliana*. In this way, Wang and coworkers (2006) reported that *AtFAAH* expression and FAAH catalytic activity increased during seed germination and seedling growth, consistent with the timing of NAE depletion during seedling establishment reported previously by the same group (Chapman 2004). Moreover, the authors identified T-DNA mutants of *A. thaliana* and generated transgenic plants overexpressing *AtFAAH*. They found that seeds of *AtFAAH* mutants had elevated levels of endogenous NAEs, and seedling growth was hypersensitive to exogenously applied NAE 12:0. In contrast, seeds and seedlings of *AtFAAH* overexpressing plants had lower endogenous NAE content, and seedlings were less sensitive to exogenous NAE (Wang *et al.* 2006). These results suggest that *AtFAAH* is a modulator of endogenous NAE levels in plants, and that NAE depletion might be a prerequisite for certain plant developmental transitions.

Alkamides and NAEs are structurally related to sphingolipids. The possibility that a breakdown product of ceramide or other sphingolipids may result in metabolites with the observed function of alkamides has been recently considered (Ramírez-Chávez *et al.* 2004). Whether alkamides and sphingolipids have similar biological activities or act through common signaling pathways remains to be determined.

REGULATION OF PLANT DEVELOPMENT BY NAEs AND ALKAMIDES

Several key observations suggest that NAEs and alkamides function as regulators of processes associated with cell division, expansion and seedling growth. First, seed germination and seedling growth depend upon synchronized cell division in the meristems and cell expansion. In *A. thaliana*, the increase in *AtFAAH* expression and NAE hydrolase activity during seed germination and early plant growth correlates with the depletion of endogenous NAEs (Wang *et al.* 2006). Second, micromolar concentrations of NAE 12:0 and NAE 18:2 supplied to *A. thaliana* seedlings grown in agar

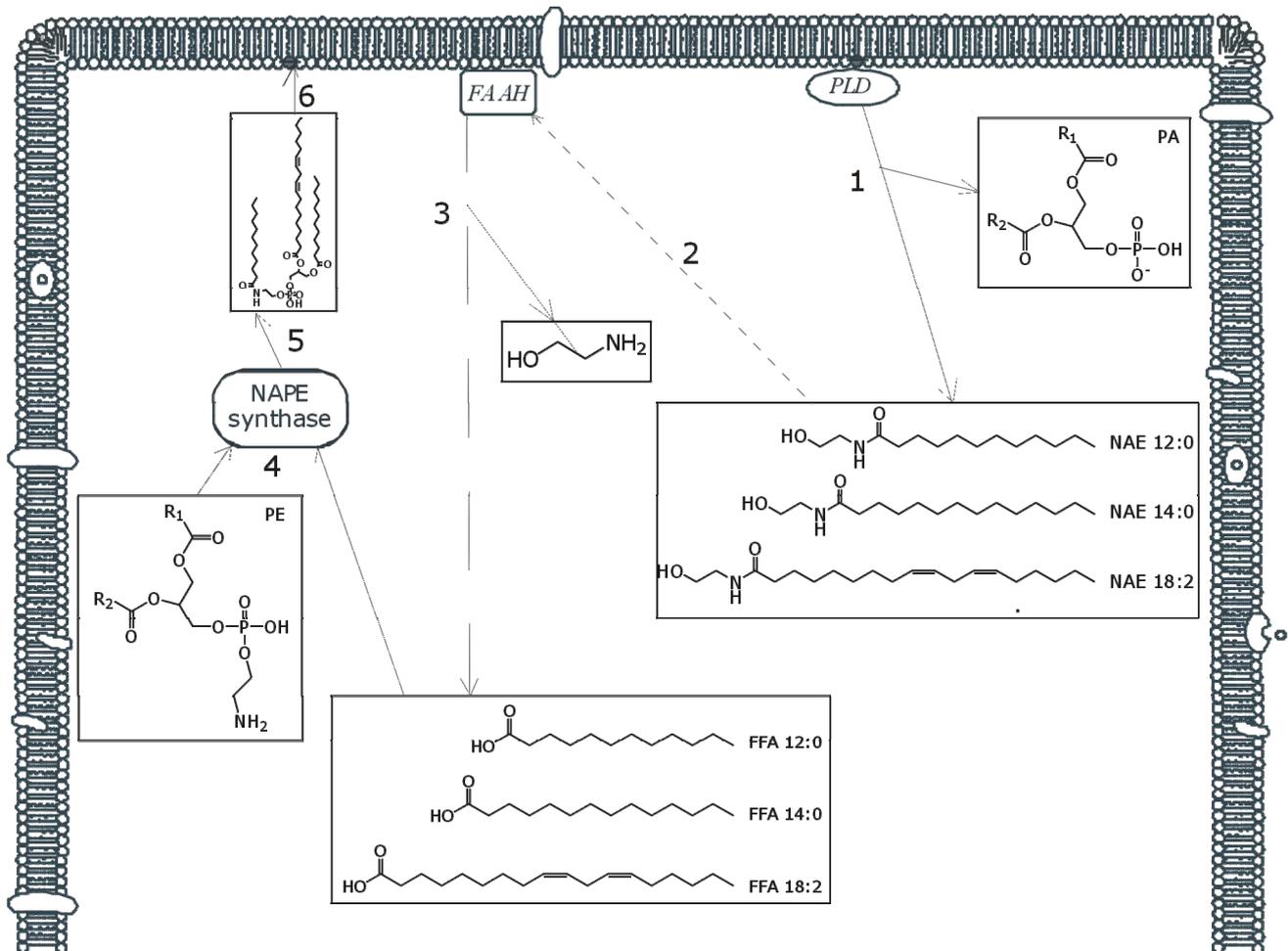


Fig. 1 NAE metabolism in plant tissues. NAEs and alkamides are probably derived from a minor membrane lipid constituent, the *N*-acylated-phosphatidylethanolamine (NAPE) (on gray in scheme), hydrolyzed by a phospholipase D (PLD) (1). This reaction produces a corresponding NAE and a molecule of phosphatidic acid (PA) (2). NAEs are hydrolyzed by a fatty acid amide hydrolase (FAAH) (3) producing a free fatty acid (FFA) and ethanolamine (4). FFA can be utilized to acylate the amino head group of phosphatidylethanolamine (PE) through an enzyme designated NAPE synthase (5) producing NAPE (6), which is integrated into the cell membrane.

plates inhibited root development and disrupted normal cell growth in a dose-dependent and selective manner (Blancaflor *et al.* 2003; Motes *et al.* 2005; Wang *et al.* 2006). Third, constitutive overexpression of *AtFAAH* led to plants with reduced NAE content in their seeds and seedlings. This reduction correlated with accelerated seedling growth and the formation of organs that were significantly larger in size (Wang *et al.* 2006). These results suggest that NAEs might be acting as repressors of morphogenetic processes and that their degradation is a prerequisite to normal plant development. However, the precise mechanism underlying NAE metabolism and plant growth may involve a more complex signaling network, because there are multiple types of NAEs in plants and because their structurally related alkamides have found to promote growth and regulate plant architecture.

Two reports indicate that alkamides may act as plant growth regulating substances. Kanbe and coworkers (1993) showed that amidinin (**Table 1**), a non-substituted alkamide isolated from the actinomycete *Amycolatopsis* sp., promoted the growth of rice (*Oryza sativa*) plants at concentrations of 0.6 and 1.8×10^{-5} M and inhibited growth at concentration of 6×10^{-5} M. More recently, our group evaluated the effects of affinin and reduced amides in the growth and development of *A. thaliana* seedlings (Ramírez-Chávez *et al.* 2004). Together with a general plant growth promoting effect, affinin showed a dose-dependent effect in root system architecture that included increased growth of root hairs (**Fig. 2A-D**), and lateral roots at concentration of 2.8×10^{-5} M (**Fig. 2E-G**), and decreased primary root growth at higher concentrations (**Fig. 2G**). Similar to NAEs,

the repressing effect of affinin on primary root growth correlated with an inhibition in proliferative activity in the primary root meristem and reduced lateral root elongation (**Fig. 2G**-inset), suggesting that NAEs and alkamides may share common signaling mechanisms in the regulation of cellular processes.

SIGNALING MOLECULES POTENTIALLY INVOLVED IN NAE AND ALKAMIDE REGULATION OF PLANT DEVELOPMENT

The effects of NAE 12:0 and affinin in root development suggest that phytohormones could be involved in the response of plants to alkamides. Auxin is involved in altering primary root growth and in promoting root hair and lateral root formation. It has been shown that application of natural and synthetic auxins increases lateral root formation, whereas auxin transport inhibitors reduce lateral root numbers (Casimiro *et al.* 2001; Himanen *et al.* 2002; López-Bucio *et al.* 2005; Woodward and Bartel 2005). Two lines of evidence indicate that the effects of affinin on the *Arabidopsis* root system architecture are likely independent of auxin signaling. First, the examination of primary root growth of auxin-resistant mutants *aux1-7*, *eir1*, and *axr4-2* in response to a high affinin concentration revealed a primary root growth inhibition similar to WT plants. Second, alkamides failed to activate the expression of the auxin-inducible gene markers *DR5:uidA* and *BA3:uidA* in primary roots (Ramírez-Chávez *et al.* 2004). This information suggests that alkamides regulate root development by an auxin independent signaling mechanism. The potential of emer-

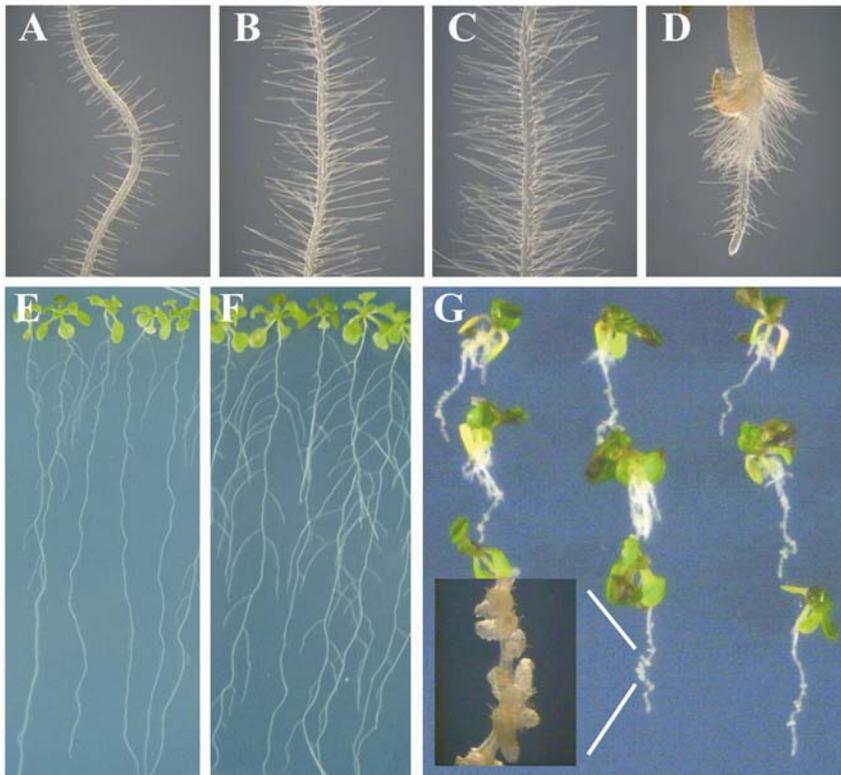


Fig. 2 Regulation of plant growth by affinin.

Wildtype *A. thaliana* (Col-0) seedlings were grown for (A-D) 6 or (E-G) 10 d under 0 (A, E), 3.2 (B, F), 12.5 (C) or 25 (D, G) mg/l affinin concentrations on vertically oriented agar plates. At d 6, light microscope images of control (A) and affinin induced-root hairs (B-D) were taken. The promoting effects of affinin in lateral root development are appreciated at d 10 (E-G). Primary root growth inhibition by high concentrations of affinin correlates with the transformation of lateral roots into nodule-like structures (G-inset).

ging signaling molecules in plants such as GLU and NO as alkamide interacting partners in plant growth regulation is further considered, since in animals GLU activates the biosynthesis of NAEs during neuronal activity (Hansen *et al.* 1995, 1997), and because NAEs have been found to activate NO biosynthesis through regulation of NO synthase (Maccarrone and Finazzi-Agró 2003).

GLUTAMATE

Mineral nutrients such as N, P and Fe are critical factors for plant growth and development that act as signals to modify physiological responses (López-Bucio *et al.* 2003). Plants can obtain N from nitrate, the major form of N in soil, or from aminoacid pools present in the soil as organic matter is decomposed by microorganisms. Given the importance of aminoacids for plant N nutrition, until recently, very little work had been done to evaluate potential regulatory interactions between aminoacids and plant development. Glutamate is an aminoacid critical for N metabolism, being the product of the GOGAT cycle and an amino-N donor for synthesis of other aminoacids. Recent information indicates that GLU can act as a signal to regulate cellular and morphogenetic processes in plants (Sivaguru 2003; Walch-Liu *et al.* 2006). Treatments with millimolar concentrations of L-GLU inhibited mitotic activity in the root apical meristem and increased radial expansion of root cells, giving rise to the formation of short primary roots. Interestingly, GLU did not interfere with lateral root initiation or outgrowth. Only some time after emergence, lateral roots acquired GLU sensitivity, indicating that their ability to respond to GLU is developmentally regulated (Walch-Liu *et al.* 2006). GLU is thought to act by its recognition by a family of ionotropic GLU receptors (GLRs). GLRs are involved in excitatory neurotransmission in mammalian synapses and have been found to be phylogenetically conserved in bacteria, plants and animals (Chiu *et al.* 2002). In *A. thaliana*, there is a family of 20 genes that encode homologs to animal GLU receptors (Lam *et al.* 1998; Lacombe *et al.* 2001). Members of this family have been proposed to be the best candidates for the role of GLU sensors in root tips (Walch-Liu *et al.* 2006).

The observation that both NAEs and GLU inhibit primary root growth and stimulate radial expansion, effects

related to microtubule depolymerization (Blancaflor *et al.* 2003; Sivaguru *et al.* 2003; Walch-Liu *et al.* 2006), suggests that these compounds may interact in the signaling cascades that modify root development. It has been recently reported that the short root phenotype of a rice mutant defective in the expression of the *OsGLR3.1* gene is due to disruption of proliferative activity in the root apex (Li *et al.* 2006), suggesting that this *GLR* gene has an essential role in cell division. Further insight into potential interactions between NAEs and GLU in plant development may come from the analysis of other members of the *GLR* family from *A. thaliana* and *O. sativa* and from NAE determinations following GLU treatment.

NITRIC OXIDE

Nitric oxide (NO) is a diffusible molecule involved in diverse physiological processes in phylogenetically distant species (Gow and Ischiropoulos 2001). It was first described in mammals, where it plays variable functions ranging from vasodilation to neurotransmission and immune responses (Gow and Ischiropoulos 2001). NO has been quantified in plants and novel and important roles are being attributed to this molecule, from pathogen defense to growth and development (Lamattina *et al.* 2003; Neill *et al.* 2003). With regard to plant architecture, increasing information has been accumulating pointing out to a critical role for NO in adventitious and lateral root formation. Lateral roots (LRs) and adventitious roots (ARs) are important in determining the success of plants by allowing the construction of branched root systems, which are more efficiently in exploring the soil layers to extract water and mineral nutrients. LRs originate from the root pericycle, in which individual quiescent cells are stimulated to dedifferentiate and proliferate to form a LR primordium. Cells in the LR primordium differentiate and grow, causing the LR to emerge through the primary root (Laskowsky *et al.* 1995; Dubrovsky *et al.* 2001). Pagnussat and associates were the first to demonstrate that NO participates in the auxin-regulated process of adventitious root formation in plants (Pagnussat *et al.* 2002, 2003). They showed that in cucumber (*Cucumis sativus*) plants, the NO-donor sodium nitroprusside promotes adventitious rooting in auxin-depleted plants treated with 1-naphthyl-phtalamic acid (NPA), an auxin transport inhibitor.

On the contrary, the specific NO scavenger cPTIO prevented the effect of sodium nitroprusside (Pagnussat *et al.* 2003). Later, it was confirmed that NO also plays a central role in lateral root formation in tomato (Correa-Aragunde *et al.* 2004). Interestingly, detection of endogenous NO by the specific probe 4,5-diaminofluorescein diacetate (DAF-2 DA) revealed that the NO signal was specifically located in LR and AR primordia (Correa-Aragunde *et al.* 2004; Xiaoping and Ai-Xia 2004), suggesting a role for NO both for LRP initiation and outgrow. These results are similar to those reported by Ramírez-Chávez *et al.* (2004), which showed a stimulatory effect of auxin in several stages of LRP development, although this effect was found to be independent of auxin. Recent findings showing that alkaloids promote adventitious root formation in a dose dependent way (M. Millán-Godínez, A. Méndez-Bravo, J. López-Bucio, unpublished results), allow us to speculate that NO could play a role in the alkaloid-induced lateral root formation. A common role of NO as a second messenger in mediating the pericycle responses to auxin and alkaloids may explain why the two regulators exert an independent but similar stimulatory effect in lateral root formation.

CONCLUDING REMARKS

In the last five years, tremendous strides have been made in our understanding of plant signaling and gene regulation. A novel group of plant signaling molecules, NAEs and the related alkaloids, which are probably derived from membrane lipids by the action of PLDs, have been found to regulate many aspects of plant growth and development. NAEs share structural similarity with anandamide, an endocannabinoid from mammals. This observation, together with the finding that certain alkaloids isolated from *Echinacea angustifolia*, are able to interact with mammalian cannabinoid receptors (Woelkart *et al.* 2005; Raduner *et al.* 2006), led to the suggestion that components of an endocannabinoid signaling system may be conserved in animals and plants (López-Bucio *et al.* 2006). Although anandamide itself has never been isolated from plant sources, it has been suggested that plant alkaloids may be regulating cellular responses through novel signaling mechanisms that probably involve endogenous lipid signals (Ramírez-Chávez *et al.* 2004). Although the effects of alkaloids on root hair and primary root growth are somewhat independent from the canonical auxin pathway, the possible cross-talk between these regulators is just emerging since alkaloids have been found to elicit lateral and adventitious root initiation and growth. There may even be some link with the gibberellic acid and abscisic acid pathways as suggested by the activation of FAAH during germination (Wang *et al.* 2006). In this regard, our group has achieved an important goal in isolating alkaloid oversensitive and resistant *Arabidopsis* mutants that show altered germination and post-embryonic growth, highlighting the importance of this pathway in plant development (Pelagio-Flores R and López-Bucio J, unpublished information).

GLU and NO, two signaling molecules with novel signaling roles in plants, have been found to modify root system architecture in a similar way to NAEs and alkaloids. Elucidating how alkaloids interact with GLU and NO to regulate root system architecture and other aspects of plant development remains a major challenge. The adoption of an integrated experimental approach that combines molecular genetics, cell biology and analytical chemistry could be an effective strategy to increase our understanding on the biological roles of NAEs and alkaloids in plants and their signaling mechanisms.

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