Unsaturated amino acids derived from isoleucine trigger early membrane effects on plant cells

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Abstract

Unsaturated amino acids (UnsAA) have been shown to affect the activity of various biological processes. However, their mode of action has been investigated poorly thus far. We show in this work that 2-amino-3-methyl-4-pentenoic acid (C2) and 2-amino-3-methyl-4-pentynoic acid (C3) structurally derived from isoleucine (Ile) exhibited a multisite action on plant cells. For one, C2 and C3 induced early modifications at the plasma membrane level, as shown by the hyperpolarization monitored by microelectrode implantation in the pulvinar cells of *Mimosa pudica*, indicating that these compounds are able to modify ionic fluxes. In particular, proton (H^+) fluxes were modified, as shown by the pH rise monitored in the bathing medium of pulvinar tissues. A component of this effect may be linked to the inhibitory effect observed on the proton pumping and the vanadate-sensitive activity of the plasma membrane H^+ -ATPase monitored in plasma membrane vesicles (PMVs) purified from pulvinar tissues of *M. pudica* and leaf tissues of *Beta vulgaris*. This effect may explain, in part, the inhibitory effect of the compounds on the uptake capacity of sucrose and valine by *B. vulgaris* leaf tissues. In contrast, an unexpected action was observed in cell reactions, implicating ion fluxes and water movement. Indeed, the osmocontractile reactions of pulvini induced either by a mechanical shock in *M. pudica* or by dark and light signals in *Cassia fasciculata* were increased, indicating that, compared to Ile, these compounds may modify in a specific way the plasma membrane permeability to water and ions.

Keywords H^+ -ATPase, Isoleucine analogs, membrane potential, nutrient transport, osmocontractile reaction, unsaturated amino acids

Abbreviations

<i>PMVs</i>	Plasma membrane vesicles
<i>MES</i>	2-(<i>N</i> -morpholino)ethanesulfonic acid
<i>UnsAAs</i>	Unsaturated amino acids
<i>C2</i>	2-amino-3-methyl-4-pentenoic acid
<i>C3</i>	2-amino-3-methyl-4-pentynoic acid
<i>Ile</i>	Isoleucine
<i>Val</i>	Valine
<i>Suc</i>	Sucrose
<i>AA</i>	Amino acid

1. Introduction

Attention towards the “unnatural” amino acids (AAs) has increased since the discovery of their occurrence in organisms. For example, chemical screening of 17 species of *Amanita* resulted in the description of unusual AAs, such as cyclopropyl-derivatives (Yoshimura et al., 1999), chloro-derivatives and unsaturated amino acids (UnsAAs), in particular, 2-amino-5-hexenoic acid and 2-amino-4-hexynoic acid (Li and Oberlies, 2005) and references therein. Different investigations have shown that the UnsAAs exhibit a variety of interesting biological activities, particularly antimicrobial properties (Katagiri et al., 1967; Kirino et al., 1980). For instance, 2-amino-4-methoxy-*trans*-3-butenic acid (AMB) isolated from a fermentation broth of *Pseudomonas aeruginosa* ATCC-27700 inhibited the growth of *Bacillus* spp. 1283 B (Scannell et al., 1972). (Rando, 1974) showed that AMB behaved as an irreversible inhibitor of soluble, pyridoxal-linked aspartate amino transferase. The effect of allylglycine (2-amino-4-pentenoic acid) on particular enzymes also has been the subject of extended studies due to its convulsant effect. Studies on the brain have specified that this compound blocked glutamic acid decarboxylase activity, inhibited γ -aminobutyric acid synthesis (Fisher and Davies, 1976) and increased ornithine decarboxylase activity (Laitinen, 1985).

The synthesis of unnatural amino acids (AAs) has been developing widely in recent years for the consideration of their expected technological advances for use in therapeutic applications in relation to the design of synthetic peptides (Pichereau and Allary, 2005). In addition, due to the presence of characteristic functional groups compared to natural AAs, unnatural AAs can be used as probes to obtain a better understanding of biological processes (Perdih and Dolenc, 2011 and references therein). The synthesis of unnatural AAs has expanded greatly due to technical advances in their incorporation into proteins both *in vivo* and *in vitro* (Noren et al., 1989; Dawson and Kent, 2000; Cropp and Schultz, 2004; Hahn and Muir, 2005). Incorporation of selected AA analogs into proteins made *in vivo* has been in use for many years (Fenster and Anker, 1969; Kiick et al., 2002; Bae et al., 2003). As emphasized by (Mock et al., 2006), the use of monomers other than the 20 natural AAs leads to introduction of new functionalities into proteins bearing novel physical and chemical properties. Thus, this approach has contributed to the elucidation of details of protein structure and function (Dougherty, 2000) and has expanded

96 knowledges concerning protein–protein and protein–nucleic acids interactions
97 (Hohsaka and Sisido, 2002; Johnson et al., 2010).

98 Interestingly, non-coded AAs have been found in naturally occurring peptides.
99 In particular, peptides containing dehydro-AAs have been isolated from bacteria,
100 fungi, marine invertebrates and some plants. These peptides show mainly antibiotic,
101 antifungal, antitumor and phytotoxic activities (Siodlak, 2015 and references
102 therein).

103 The residue Δ -isoleucine (Δ Ile) is found in the compound FR225656 which is
104 produced by the fungi *Helicomyces* spp. (Zenkoh et al., 2003) and behaves as an
105 inhibitor of gluconeogenesis (Ohtsu et al., 2003). Similarly, the linear heptapeptide
106 antrimycin contains four unusual AA residues, including (E)- Δ Ile. Antrimycin,
107 isolated from the bacterium *Streptomyces xanthocidicus* (Morimoto et al., 1981),
108 shows antibiotic activity against *Mycobacterium smegmatis* (Shimada et al., 1981).

109 To our knowledge, as described above, the UnsAAs were used widely either in
110 genetic engineering or as intermediates in the synthesis of materials for medicinal
111 and organic chemistry purposes. The main observations regarding their biological
112 effects are restricted to their inhibitory effects on the growth of various
113 microorganisms. Our attention was paid to UnsAAs derived from Ile, namely 2-
114 amino-3-methyl-4-pentenoic acid (C2) and 2-amino-3-methyl-4-pentynoic acid (C3).
115 C3 inhibited the growth of *Saccharomyces cerevisiae* and *Escherichia coli* (Gershon
116 et al., 1954), and C2 has been shown to be a potent antagonist of Ile and valine (Val)
117 to the growth of *Lactobacillus arabinosus* and *E. coli* (Shive and Skinner, 1963). The
118 antimicrobial properties of both analogs also were confirmed later: C2 and C3 did
119 not support cell growth and were toxic to *E. coli* (Michon et al., 2002). However, the
120 mechanisms of their action on cell machinery have been investigated poorly.
121 Primarily, this work was focused on possible effects induced by UnsAAs on plasma
122 membrane functioning.

123 To achieve our goal, the effects of C2 and C3 were compared to those induced
124 by Ile on bioelectrical membrane potential and on proton fluxes observed in pulvinar
125 cells of *Mimosa pudica*. Furthermore, the effects of C2 and C3 on osmocontractile
126 reactions observed in pulvinar cells of *Mimosa pudica* and *Cassia fasciculata*, which
127 are driven by K^+ and Cl^- fluxes (Samejima and Sibaoka, 1980), may afford
128 interesting data concerning their impact on the ionic migrations taking place at the
129 plasma membrane site. In addition, an investigation into the effects of these

compounds on the uptake of sucrose (Suc) and valine (Val) by *Beta vulgaris* leaf cells was also carried out to enlighten whether C2 and C3 may modify the mode of transport of these types of metabolites, taken up by the cells through a H^+ -substrate co-transport (Delrot et al., 2001). Finally, using plasma membrane vesicles (PMVs), a direct inhibitory action of these compounds has been shown on the activity of the membrane H^+ -ATPase, a key enzyme regulating the studied biological processes.

2. Materials and methods

2.1. Plant material and culture conditions

Seeds of *Mimosa pudica* L., *Cassia fasciculata* Michx. and *Beta vulgaris* L. cv. Aramis were germinated in an organic compost. Seedlings and older plants were grown in this compost watered daily and kept in climate-controlled chambers at $27.5 \pm 0.5^\circ\text{C}$ and $65 \pm 5\%$ relative humidity. Illumination was regulated to give 16 h of light (photophase 06.00 hours-22.00 hours) provided by fluorescent tubes (mixing Osram fluora and Osram day-light types) with a photon fluence rate (400-700 nm) of $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the plant apex.

2.2. Electrophysiological measurements

The experiments were done on primary pulvini from mature leaves of 2-month old plants of *M. pudica*, generally the eighth above the cotyledons. A major advantage of the pulvinus model in electrophysiological assays is linked to its size allowing easy handling and also to its particular anatomy characterized by many layers of parenchyma cells surrounding the central cylinder, insuring therefore impalement of a microelectrode in a well-defined kind of cell. The transmembrane potential was measured by the classical electrophysiological method using microelectrodes (tip diameter $< 1 \mu\text{m}$, tip resistance from 5 to $30 \text{ M}\Omega$). For details, see (Amborabe et al., 2008). Briefly, leaf was excised from the stem and pulvinus fixed to the bottom of a 4 ml plexiglas chamber filled with a buffered medium (10 mM MES/NaOH, pH 5.2) containing 1 mM NaCl, 0.1 mM KCl, and 0.1 mM CaCl_2 (Abe, 1981). The glass microelectrode was impaled into a motor cell of the abaxial (“extensor”) half of the organ. Under these conditions, the resting transmembrane potential (Ψ_0) was in the range of -100 to -150 mV. The assays in which Ψ_0 was out

of the range -110 / -130 mV were discarded since amplitude of the induced electrophysiological modifications may be modulated by the initial resting potential value. Thus, the calculated value from 24 assays was -112 ± 3 mV (SEM).

2.3. Measurement of pH variations

In order to observe H^+ excretion, primary pulvini of *M. pudica* (400 mg) were excised at 10.00 h on 2-month old plants bearing generally 10 leaves. The organs were divided in transverse sections and treated following the same procedure previously described (Amborabe et al., 2008): unbuffered incubation medium composed of 0.50 mM $CaCl_2$, 0.25 mM $MgCl_2$, variations of pH read on a pH-meter provided with combined electrodes (Futura micro-combination, Beckman Coulter) and linked to a potentiometric recorder. Compounds were added as indicated in the figures. In order to quantify the amount of mobilized protons, titration was made 3 h after the application of the compounds on 2 ml of the incubation medium with NaOH or HCl at $5 \cdot 10^{-3}$ N. The experiments were repeated at least 4 times.

2.4. Preparation and use of plasma membrane vesicles

Purified plasma membrane vesicles (PMVs) were prepared by phase partitioning of microsomal fractions from the primary pulvini of *M. pudica* and from leaves of *B. vulgaris* according to (Lemoine et al., 1991) with some minor modifications. After isolation, PMVs were frozen in liquid nitrogen and stored at $-80^\circ C$. They were put in the inside-out configuration by adding 0.05% brij in the assay medium (Vianello et al., 1982). Proton pumping and ATPase activity were measured as described in (Noubhani et al., 1996) with some minor modifications. Proton movement was followed by the decrease in absorbance of 9-aminoacridine absorbance at 495 nm in a medium buffered with 10 mM MES/TRIS at pH 6.5. The assay medium consisted of 50 mM KCl, 10 mM MES/TRIS (pH 6.5), 3 mM ATP, 1 mM dithiothreitol, 1 mg ml⁻¹ bovine serum albumin, 20 μ M acridine orange, 5 mM valinomycin, 300 mM sorbitol, and 75 μ g of protein in a final volume of 1 ml. The reaction was started by addition of 3 mM $MgSO_4$ into the medium. ATPase hydrolysis activity of the PMVs was measured by the phosphate released from ATP by the method of (Ames, 1966) in a medium containing 3 mM $MgSO_4$, 100 mM KCl, 50 mM MES/TRIS (pH 6.5), 3 mM ATP, 250 mM sucrose and 40 μ g of

protein. After 5, 10, 15, and 20 min, 100 μ l aliquots were sampled and mixed with the molybdate reagent which stops the reaction. In treated sets, Ile, C2 and C3 were added at the given concentration 3 min before the beginning of the assay. In the 6 batches of vesicles used in this work, treatment with the plasma membrane H^+ -ATPase inhibitor sodium orthovanadate (Ova) at 0.25 mM showed that about 60-65% of the enzyme activity can be attributed to plasma membrane functioning.

2.5. Experimental procedures for observation of osmocontractile reactions

Seedlings of *M. pudica* bearing the first fully developed leaf constituted the experimental model for the observation of the shock-induced motor reaction, characterized by a leaf drooping in about 2 s and a recovery lasting about 20 min (Saeedi et al., 2013). This type of movement results from a coordinated contraction of the motor cell in response to an excitation of a putative mechanoreceptor (Visnovitz et al., 2007). The seedlings were excised at 10.00 h with a sharp razor blade by cutting the hypocotyl 3 cm below the cotyledons and then dipped in distilled water in small plastic tubes for 4 h to allow recovery from harvesting. At this time, distilled water was replaced by the amino acids and Ova dissolved in a medium buffered with 2.5 mM MES at pH 5.2 for 2 h. Leaf position was determined by measuring the angle formed by the petiole and the line prolonging the hypocotyl by means of a transparent protractor provided with a movable pointer. After measurement of the initial angle (α_i), the pulvini were stimulated by a touch applied on the abaxial half of the organ at 1-h intervals for 4 h. The motor reaction was monitored by measuring the variation of the angle ($\Delta\alpha$) within 10 s after the stimulation. The experiments were carried out on 30 seedlings separated in 3 sets.

In order to observe the dark- and light-induced reactions, the pulvinate leaves of *C. fasciculata* were excised at 09.00 h from plants 3 months old bearing about 15 well-developed leaves. Their cut petioles were dipped in distilled water for 3 h in order to recover from the harvesting shock. The leaves were transferred to test solutions buffered with 2.5 mM MES at pH 5.2 for 1 h and then were either put in the dark in the middle of the photophase or put in the light in the middle of the scotophase. The movements induced by the dark or light signals were monitored by measuring the distance between the leaflets tips with a caliper square, then converted into angular values. For details, see (Saeedi et al., 1984). Curves represent

the mean value obtained from the sum of at least three experiments, so that each experimental point refers to observations on 24 leaves. Each experiment was carried out with a series of eight leaves, so that a plant was represented by one leaf in each series. A dim-green light was used in the dark experimental periods.

2.6. Uptake experiments concerning valine and sucrose

Mature exporting leaves (4-5 weeks old) of sugar beet were used in the experiments described below according to a previously reported method (Sakr et al., 1993), in order to test whether the transport of metabolites may be affected by C2 and C3. Valine was chosen as AA species, because it is a metabolite found in plant exudate (Robinson and Beevers, 1981) and because it appears to share the same transport system as Ile (Li and Bush, 1991). Sucrose is the principal sugar distributed into the plant and is taken up into the cells of beet leaves through a carrier-mediated transport fueled by a sucrose- H^+ symport mechanism. Leaf discs (6 mm diameter) were excised from the leaves after peeling of the lower epidermis, and incubated (peeled face downwards) on a basal medium containing 300 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 20 mM MES (pH 5.2), in presence of Ile, C2 and C3 for 60 min before the addition of the labelled substrates. The incubation with 1 mM [^{14}C] sucrose (final activity 11 kBq ml^{-1}) and 1 mM [3H] valine (final activity 9.25 kBq ml^{-1}) was run for 30 min under mild agitation on a shaker at 25°C. After incubation with the labelled substrate, apoplastic label was removed by three washes of 3 min each on basal medium. The discs were then placed in scintillation vials and digested at 55°C during 24 h in a mixture of perchloric acid (56%), H_2O_2 (27%), and 0.1% Triton X100 (17%). Finally, 4 ml of scintillation liquid (Ecolite TM, ICN) was added to the vials and radioactivity was counted by liquid scintillation spectroscopy (1900 TR Packard)

2.7. Chemicals

The unsaturated derivatives of Ile, namely C2 and C3, structures of which are given in Fig. 1, were synthesized according to Aidene et al. (1997) and were dissolved in the corresponding buffer. All chemicals were purchased from Sigma-Aldrich (France). Stock solution of 1 mM fusicocin (FC) was dissolved in 7% methanol and used at 10 μM final concentration (verification has been made that

the 0.07% final methanol concentration did not interfere with the biological processes). The solutions were generally prepared in a medium buffered with 2.5 mM MES at pH 5.2, since the pulvinar reactions and the uptake of metabolites by the cells are promoted at this acidic pH value close to the apoplastic pH.

2.8. Statistical analyses

As the different studied sets were either too small or did not qualify to use a parametric test, the Kruskal-Wallis test was used to assess statistically significant differences. The test was performed using XLStat v. 2015.1.03 as an add-in for Microsoft Excel v. 14.3.6 running in a Mac OS 10.7.5 environment.

3. Results

3.1 Effect of UnsAAs on bioelectrical membrane potential

The addition of UnsAAs to the bathing medium of *M. pudica* pulvini triggered modifications of the cell membrane potential at concentrations higher than 0.5 mM. Fig. 2 shows the kinetics of the bioelectrical events recorded. Thus, when applied at a final concentration of 2.5 mM, C2 and C3 triggered a hyperpolarization of the membrane potential after a latent time of about 20 s following addition of the compound, peaking some minutes later. This was followed by a phase of depolarization lasting more than 30 min before a slow return to the basal level. In contrast, Ile induced a rapid depolarization after a latent time of about 20 s after addition of the compound, followed by a slow return to the basal level. To ascertain the bioelectrical viability of the impaled cell, the fungal toxin fusicochin, known to trigger membrane hyperpolarization, was added at the end of each experiment. The statistical data concerning these events are given in the Table 1.

3.2. Effect of UnsAAs on proton fluxes and H^+ -ATPase activity

It has been reported previously that tissues of *M. pudica* pulvini induces a spontaneous acidification of the incubation medium as a result of the H^+ -ATPase activity (Amborabé et al., 2008). Fig. 3 shows the time course of the pH variation recorded in the bathing medium of pulvinar sections after addition of the tested compounds. Thus, the additions of C2 and C3 induced a rise in pH that started within

10 min after the addition of the compound at 2.5 mM and lasted about 12 h. Note that the effect induced by C3 was lower than that of C2. In contrast, Ile only stopped durably the spontaneous acidification. Comparatively, fusicoccin has been shown to induce a rapid acidification of the medium in the same experimental conditions (Moyen et al., 2007). The statistical data concerning the corresponding net proton influxes are given in Table 2. It has been verified that the modification in pH value was not due to an increase of the buffer capacity in the incubation medium upon addition of the compounds.

The modification observed in proton fluxes questions whether UnsAAs may act directly on the functioning of the plasma membrane H^+ -ATPase that directs the proton fluxes in plant cells (Serrano, 1989). Regarding this goal, experiments were carried out on inside-out PMVs purified from pulvinar tissues of *M. pudica* and leaf tissue of *Beta vulgaris*. As shown in Table 2, UnsAAs inhibited the proton gradient and the H^+ -ATPase catalytic activity in a dose-dependent manner for concentrations higher than 0.5 mM in both types of plant material. However, it should be stressed that, compared to Ova, with which both components of the enzyme were inhibited to a similar extent (about 60%), C2 and C3 inhibited more dramatically the proton pumping than the catalytic activity in both plant materials. This observation suggests that C2 and C3 likely may exert a protonophore action. Compared to controls, Ile had no significant effect.

3.3. Effect of UnsAAs on osmocontractile reaction of the pulvini

Due to their disturbing action on the H^+ -ATPase activity, which is preponderant in the energetics of membrane functioning, C2 and C3 were expected to affect negatively the ion-driven fluxes that sustain the osmocontractile reactions of pulvinar cells and, consequently, to inhibit these reactions. Surprisingly, C2 and C3 promoted the reactions. Indeed, when used at a 2.5 mM concentration, they slightly increased the $\Delta\alpha$ value, which represents the amplitude of the downward movement of the *M. pudica* leaf. C2 increased the reaction by 22% and C3 by 42%. Ile only increased by 5% the reaction and Ova at 0.5 mM inhibited the reaction by 27 % (Fig. 4A). When applied to leaflets of *C. fasciculata*, the UnsAAs did not modify their initial positions nor their final positions, but they increased the rates of the movements either induced by darkness (Fig. 4B) or by light (Fig. 4C). For example,

in the case of the dark-induced movement, C2 and C3 respectively increased the rates of the movement by 10% and 33%, as calculated 1 h after the dark signal. The effect is more pronounced in the case of the light-induced movement, since the rates of opening of the leaflets respectively increased following treatment with C2 and C3 by 70% and 79%, as calculated 2 h after the light signal. Ile applied at the same concentration did not modify significantly the rate of the movements (-3%) compared to controls.

3.4. Effect of UnsAAs on uptake of radiolabeled Val and Suc

As shown in Fig. 5, Ile inhibited very significantly Val absorption (40%) but also to a weak extent Suc uptake (18%). The treatments with C2 strongly inhibited the uptake of Val and Suc (54% and 46%, respectively). Comparatively, C3 was less efficient (respective inhibitions of 43% and 35%). Compared to the effects of Ile on Val uptake, C2 increased the inhibition by 14% and C3 by 3%. A similar remark on Suc uptake showed that C2 and C3 increased the inhibition produced by Ile respectively by 28% by 17%. This indicated that the effects of the UnsAAs are not due solely to a competitive mechanism.

4. Discussion

In previous works, the compounds studied in this work, namely C2 and C3, which are structurally derived from Ile, have been utilized in the synthesis of compounds useful for medicine and organic chemistry. Thus, C2, C3 and related compounds have been used in the synthesis of isostatine, halipeptin A, eponemycin and manzacidin D (Sugiyama et al., 2013) and references therein. They also have been incorporated into cellular proteins, leading to generation of new materials with useful biological properties. Interestingly, they exhibited inhibitory properties on the growth of the microorganisms when exogenously applied (Gershon et al., 1954; Shive and Skinner, 1963; Michon et al., 2002). However, their impacts at the cell level have been investigated poorly thus far. The comparative data obtained in this work on various plant tissues evidenced that C2 and C3 may act at very precise sites of plant cell functioning, as evidenced by the biological disturbances monitored at the plasma membrane level. These observations led to some general considerations.

4.1. C2 and C3 induced very early modifications on ionic equilibrium

This conclusion is at first sustained by the monitoring of a transient membrane hyperpolarization triggered after a short lag period (within seconds) after the application of the compounds (Fig. 2). This event indicates that ionic fluxes were very early disturbed processes. The hyperpolarization could result either from the exit of a positive charge or the entry of a negative charge. K^+ and Cl^- appear good candidates intervening in the early bioelectrical events triggered by C2 and C3. Indeed, these ionic species have been shown to be involved in the action potential evoked by the pulvinar motor cells (Samejima and Sibaoka, 1980; Abe, 1981; Moran, 2007) and in the wound-induced variation potential in wheat leaves, suggesting that specific ionic channels were activated (Katicheva et al., 2014). The hypothesis of such a mechanism intervening in the mode of action of C2 and C3 should be verified in the future.

Secondly, as shown in Fig. 3, the compounds also modified the spontaneous proton efflux that was monitored in the bathing medium of pulvinar tissues. It should be stressed that the induced proton influx occurred subsequently to the transient hyperpolarization, as deduced from the observed longer lag time (within minutes). By comparing the time course of both processes, one can note that the phase of repolarization coincides with the beginning of the pH increase, suggesting that proton entry into the cells could be the ionic species implicated in this latter event. The time course of pH variation also indicated that proton influx lasted for many hours, suggesting a possible implication in long-term cellular processes. The proton mobilization is a process of importance contributing to the pH homeostasis inside or outside of the cell. Proton fluxes were shown, for example, in the pH changes monitored by pH-sensitive fluorescent probes after the induction of variation potential in pumpkin seedlings (Sherstneva et al., 2015), and also in the determination of apoplast acidification in barley leaves using pH-microelectrode (Visnovitz et al., 2013). The advantage of the method used here was to comparatively quantify the amount of protons mobilized following the different treatments.

4.2. C2 and C3 modified the membrane permeability to water and ions

The data obtained on the osmocontractile reaction of pulvinar cells (Fig. 4) indicated that applications of C2 and C3 modified bulk ionic migrations and associated water movements, by considering the well-documented mechanism driving the seismonastic reaction and the dark-induced movements. Indeed, the coordinated contraction of the motor cells are driven by large effluxes of K^+ and Cl^- (Samejima and Sibaoka, 1980; Satter and Galston, 1981; Moran, 2007), inducing a rapid turgor loss in the cells. These events result in a rapid macroscopic drooping movement of leaves in the case of *M. pudica* (Volkov et al., 2010) and a slow closure of pinnules in *C. fasciculata* (Saeedi and Roblin, 1986). In contrast, light-induced movements are driven by reverse ionic migrations. The ionic fluxes are likely regulated by the activity of K^+ and Cl^- channels and the water movement by the activity of aquaporins localized in motor cell membranes (Fleurat-Lessard et al., 1997; Temmei et al., 2005; Moran, 2007) and references therein. Surprisingly, treatments with C2 and C3 increased seismonastic and dark- and light-induced pulvinar reactions, which do not correspond to responses induced by different previously tested effectors. Indeed, the H^+ -ATPase inhibitor Ova inhibited seismonastic (Fig. 4) and dark- and light-induced reactions (unpublished data). Furthermore, a sole protonophore effect cannot explain the data, since protonophores, such as 2,4-dinitrophenol and carbonylcyanide-*m*-chlorophenylhydrazone, increased the light-induced reaction but inhibited both the seismonastic (Saeedi et al., 2013) and dark-induced reactions (Saeedi and Roblin, 1986). Therefore, one can suppose that C2 and C3 can modify the membrane permeability to ions (in particular the K^+ osmoticum) and water in a specific way. Further research should be addressed to determine the membrane molecular sites that undergo the disturbances, allowing the change of permeability.

4.3. C2 and C3 inhibited the uptake of metabolites

As indicated by the inhibition of Val uptake, C2 and C3 may modify the transport of AAs, at least, in part, through a competitive manner (Fig. 4). However, another component also should be considered. Indeed, C2 and C3 increased the inhibition of Val uptake to a larger extent than would be expected by considering an

Ile-like competitive inhibition. Furthermore, differently than Ile, C2 and C3 inhibited strongly the Suc uptake, suggesting that they acted on a target more general than a specific transporter activity. This behavior is likely explained by the inhibitory effect of these compounds on the H^+ -ATPase activity.

4.4. C2 and C3 showed direct effects on the membrane-bound enzyme H^+ -ATPase

Undoubtedly, C2 and C3 directly inhibited both components of the H^+ -ATPase (i.e., proton pumping and catalytic activity) borne on PMVs (Table 2). Previous observations have shown that UnsAAs might modify the activity of some enzymes. For example, allylglycine can inhibit glutamic acid decarboxylase (Fisher and Davies, 1976) and ornithine decarboxylase (Laitinen, 1985). The inhibition of aspartate amino transferase by AMB has shown that the unusual amino acid plays in this case the role of a suicide inhibitor (Rando, 1974). The H^+ -ATPase in plants is a pivotal enzyme that generates an electrochemical potential across the plasma membrane fueling transmembrane exchanges through a proton motive force (Duby and Boutry, 2009). Due to their inhibitory effect on the H^+ -ATPase activity, acting in this way similarly to sodium Ova, it is expected that C2 and C3 inhibit membrane processes linked to the activity of this enzyme. The observed hindering of the osmocontractile reactions may thus be explained, in part, by the inhibition of H^+ -ATPase. In addition, the collapse of the proton motive force also explains the hindering of the uptake of AAs and Suc which are mediated in plants by a H^+ /substrate co-transport (Delrot et al., 2001).

5. Conclusion and perspectives

As evidenced by the data described in this work, C2 and C3 modify several cell processes very differently from the natural AA Ile and exhibit multisite action in cells. A pivotal action concerns the early disturbances in ionic fluxes through the plasma membrane. Importantly, ionic fluxes through the plasma membrane have been reported to be a prerequisite for many subsequent cell reactions (Colcombet et al., 2009). A second important target concerns the effects of these compounds on the exchange of water, influencing the osmolarity of the cells and the uptake of metabolites essential for normal growth and development. A causal link between these inhibitory effects can be assigned to the observed inhibition of the proton pump

ATPase activity and the protonophore component of these compounds. The disturbances induced, at least at the various cell levels reported here, may explain the long-term effects concerning the growth inhibition of some microorganisms that have been reported above. Note that C2 was more efficient than C3 at inhibiting the uptake of Suc and Val, which is to parallel to a similar observation on the growth inhibition of *E. coli* (Parker et al., 1961).

Future works should be focused on the two main directions opened in the present work. On one hand, investigations have to specify whether C2 and C3 disturb characteristic ion channels and the mechanisms of their actions on these putative targets. On the other hand, a first critical step in the incorporation of UnsAAs in cells is their uptake from the external medium. Additional experiments should be carried out to specify the mechanisms and the characteristics of the intracellular uptake of these types of compounds in order to further observe the disturbances induced by the compounds in other cell compartments and to optimize the incorporation into non-natural mutant proteins in living cells. The analogs may be transported across the plasma membrane either by the amino acid transporter of its counterpart (in our case by the Ile transporter) or by specific machinery.

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Table 1

Effects of isoleucine (Ile) and unsaturated amino acids (2-amino-3-methyl-4-pentenoic acid [C2] and 2-amino-3-methyl-4-pentynoic acid [C3]) applied at 2.5 mM on modifications of transmembrane potential of pulvinar motor cells and on variations of proton (H^+) fluxes monitored in the bathing medium of pulvinar tissues of *Mimosa pudica*. Calculations of $\Delta\Psi$ were made at the optimum, and calculations of net proton influx were made 3 h after addition of the compounds. Superscript letters indicate significant differences at the 5% probability level by Kruskal-Wallis test (*c, d, e* for $\Delta\Psi$ and *g, h, i*, for proton influx) or values not significantly different from the control (C) (*a, b* for $\Delta\Psi$ and *f* for proton influx). +, hyperpolarization; -, depolarization; n, number of assays.

	Compound	C	Ile	C2	C3
Bioelectrical data	n	6	7	6	6
	Latency (sec)	-	22 ± 18^a	28 ± 9^a	18 ± 5^a
	Optimum (min)	-	2.7 ± 1.3^b	4.5 ± 1.4^b	3.5 ± 0.5^b
	$\Delta\Psi \pm SD$ (mV)	-0.2 ± 1.2	-5.4 ± 2.6^c	$+4.7 \pm 2.0^d$	$+10.7 \pm 0.8^e$
Proton flux	n	4	4	4	4
	Latency (min)	-	10.3 ± 2.6^f	10.8 ± 2.8^f	10.5 ± 1.0^f
	Net H^+ influx $\pm$ SD (nmole H^+)	-82.3 ± 18.2	42.1 ± 4.6^g	157.5 ± 14.1^h	120.8 ± 11.2^i

Table 2

Modifications of net proton movement (measured by absorbance variation from the control absorbance at 495 nm) and vanadate-sensitive ATPase activity in plasma membrane vesicles purified from *Mimosa pudica* pulvini and *Beta vulgaris* leaf tissues treated with isoleucine (Ile) and unsaturated amino acids (2-amino-3-methyl-4-pentenoic acid [C2] and 2-amino-3-methyl-4-pentynoic acid [C3]). Values are means $\pm$ SD (number of assays) (percentage of inhibition [-] or activation [+]). The Kruskal-Wallis test was used to assess statistically significant differences in comparison to the control (*p<0.05, **p<0.01, ***p<0.001).

Product	<i>Mimosa pudica</i>				<i>Beta vulgaris</i>	
	Conc.	Absorbance variation	ATPase activity	Absorbance variation	ATPase activity	
					(nmol Pi mg prot ⁻¹ min ⁻¹)	(nmol Pi mg prot ⁻¹ min ⁻¹)
	(mM)	(Unit mg prot ⁻¹ min ⁻¹)	(nmol Pi mg prot ⁻¹ min ⁻¹)	(Unit mg prot ⁻¹ min ⁻¹)		
Control	-	0.34 $\pm$ 0.03 (7)	350 $\pm$ 20 (5)	0.26 $\pm$ 0.02 (18)	179 $\pm$ 15 (14)	
Ile	2.5	0.36 $\pm$ 0.04 (3) [+5]	340 $\pm$ 13 (4) [-3]	0.28 $\pm$ 0.06 (5) [+9]	172 $\pm$ 29 (6) [-4]	
	0.1	0.34 $\pm$ 0.02 (3) [0]	359 $\pm$ 2 (4) [+2]	0.29 $\pm$ 0.04 (4) [+12]	176 $\pm$ 13 (4) [-2]	
C2	0.5	0.31 $\pm$ 0.04 (3) [-7]	300 $\pm$ 6 (4) [-14]	0.23 $\pm$ 0.01 (4) [-10]	170 $\pm$ 21 (4) [-5]	
	1	0.20 $\pm$ 0.04 (4) [-41] *	281 $\pm$ 19 (4) [-20] *	0.14 $\pm$ 0.02 (5) [-45] **	155 $\pm$ 34 (6) [-13]	
	2.5	0.16 $\pm$ 0.02 (3) [-53] **	246 $\pm$ 15 (4) [-30] **	0.09 $\pm$ 0.02 (5) [-64] ***	135 $\pm$ 17 (4) [-24] *	
	0.1	0.37 $\pm$ 0.02 (4) [+10]	357 $\pm$ 11 (4) [+2]	0.23 $\pm$ 0.03 (4) [-10]	167 $\pm$ 5 (6) [-7]	
C3	0.5	0.29 $\pm$ 0.02 (4) [-14]	340 $\pm$ 15 (4) [-3]	0.17 $\pm$ 0.03 (6) [-34] *	149 $\pm$ 27 (6) [-17]	
	1	0.22 $\pm$ 0.03 (4) [-36] *	312 $\pm$ 17 (4) [-11]	0.14 $\pm$ 0.01 (4) [-47] **	128 $\pm$ 21 (6) [-29] **	
	2.5	0.12 $\pm$ 0.03 (3) [-64] **	262 $\pm$ 14 (4) [-25] **	0.09 $\pm$ 0.01 (6) [-66] ***	105 $\pm$ 20 (6) [-41] ***	
Ova	0.25	0.14 $\pm$ 0.06 (5) [-59] ***	141 $\pm$ 16 (5) [-60] ***	0.10 $\pm$ 0.04 (5) [-61] ***	53 $\pm$ 11 (7) [-70] ***

Fig. 1. Structure of Isoleucine (Ile) and unsaturated derivatives C2 and C3

Fig. 2. Typical recordings of transmembrane potential variations in parenchyma cells of the *Mimosa pudica* pulvinus induced by application of buffer (Control, C), isoleucine (Ile), 2-amino-3-methyl-4-pentenoic acid (C2) and 2-amino-3-methyl-4-pentynoic acid (C3). Compounds at 2.5 mM final concentration, prepared as indicated in Materials and Methods, were added at time 0 (black arrow), and 10 μ M fusicoccin (FC) was added at the arrowheads. The numbers at the beginning of the recordings indicate the resting transmembrane potential (Ψ_0 in millivolts). The number of assays is indicated in Table 1.

Fig. 3. Representative time courses of pH variations monitored in the bathing medium of pulvinar tissues of *Mimosa pudica* in control (C) and in presence of isoleucine (Ile), and unsaturated amino acids (2-amino-3-methyl-4-pentenoic acid [C2] and 2-amino-3-methyl-4-pentynoic acid [C3]) applied at 2.5 mM at time 0 (black arrow) . The experiments were conducted at least three times with the same result.

Fig. 4. Effect of isoleucine (Ile), 2-amino-3-methyl-4-pentenoic acid (C2), 2-amino-3-methyl-4-pentynoic acid (C3) applied at 2.5 mM, and sodium orthovanadate (Ova) at 0.5 mM on the osmocontractile reactions of pulvini. (A) Modification of the amplitude of the drooping movement of primary pulvini of *Mimosa pudica* triggered by a shock. The angle variation was calculated at the beginning of the treatment (black columns) and after 2 h of action of the compounds (white columns) on three sets of 10 pulvini. C, controls; vertical bars, SE; $n = 30$. Increase in the rate of the movement of leaflets of *Cassia fasciculata*: (B) closure induced by a dark signal applied in D and (C) opening induced by a light signal applied in L. Vertical bars, SE; $n = 24$. The Kruskal-Wallis test was used to assess statistically significant differences between the different sets at the 1% probability level. A different letter indicate that the sets are not from the same population.

Fig. 5. Effect of isoleucine (Ile) and unsaturated amino acids (2-amino-3-methyl-4-pentenoic acid [C2] and 2-amino-3-methyl-4-pentynoic acid [C3]) applied at 2.5 mM on the absorption of 1 mM valine and 1 mM sucrose by leaf discs of *Beta vulgaris*. Data are means of 60 discs $\pm$ S.E. (from 4 independent experiments). The numbers at the top of the columns indicate the percent inhibition compared to controls. The different letters at the top of each column indicate statistically significant differences by Kruskal-Wallis test for valine uptake (a, b, c, d) or sucrose uptake (e, f, g, h). For valine, p-values < 0.0001 except for comparison between Ile and C3 (p-value = 0,016). For sucrose, p-values < 0.0001 in all cases.