

Interactions of Auxinic Compounds on Ca²⁺ Signaling and Root Growth in *Arabidopsis thaliana*

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Abstract

Auxinic-like compounds have been widely used as weed control agents. Over the years, the modes of action of auxinic herbicides have been elucidated, but most studies thus far have focused on their effects on later stages of plant growth. Here, we show that some select auxins and auxinic-like herbicides trigger a rapid elevation in root cytosolic calcium levels within seconds of application. *Arabidopsis thaliana* plants expressing the Yellow-Cameleon (YC) 3.60 calcium reporter were treated with indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA), and two synthetic herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and mecoprop [2-(4-chloro-2-methylphenoxy) propanoic acid], followed by monitoring cytosolic calcium changes over a 10 minute time course. Seconds after application of compounds to roots, the Ca²⁺ signaling-mediated pathway was triggered, initiating the plant response to these compounds as monitored and recorded using Fluorescence Resonance Energy Transfer (FRET)-sensitized emission imaging. Each compound elicited a specific and unique cytosolic calcium signature. Also primary root development and elongation was greatly reduced or altered when exposed at two concentrations (0.10 and 1.0 μM) of each compound. Within 20 to 25 min after triggering of the Ca²⁺ signal, root growth inhibition could be detected. We speculate that differences in calcium signature among the tested auxins and auxinic herbicides might correlate with their variation and potency with regard to root growth inhibition.

Keywords

Auxinic Compound, *Arabidopsis thaliana*, Herbicide, Calcium Signaling, FRET Imaging

1. Introduction

Indole-3-acetic acid (IAA) was identified as a plant growth hormone in the 1930s [1] [2] and since then has been implicated in virtually all aspects of plant growth and development [3] [4]. IAA is also produced by microorganisms including bacteria and fungi [5] [6]. Indole-3-butyric acid (IBA) (an analog of IAA), was synthesized and found to promote root formation in 1935 [7] and later identified as a naturally occurring auxin in plants [8]. Since then, IBA has been shown to be distributed in a wide variety of plants including *Arabidopsis thaliana* [9] [10]. IBA is synthesized from IAA via a chain elongation reaction similar to those found in fatty acid biosynthesis [3] and peroxisomal β -oxidation can transform IBA into IAA [11]. The IBA synthase purified from maize uses acetyl CoA and ATP as cofactors [12]. The conversion of IBA to IAA has also been reported [13], and it is unclear if IBA is an auxin or if IBA has to be transformed to IAA for the expression of auxin activity.

In 1941, the synthesis of 2,4-dichlorophenoxy acetic acid (2,4-D) was reported [14]. This compound was shown to affect plant growth via auxin-like activity [15], its plant selectivity was examined [16] and its use as a weed control agent under field conditions was established [17]. 2,4-D is currently a widely used herbicide and other synthetic compounds belonging to this herbicide class have been commercialized. Presently, crop seeds (corn, soybean and cotton) with resistance to dicamba (an auxinic herbicide) and 2,4-D are being developed or are commercially available.

Calcium is a well-known cellular messenger in plant and animal systems, and strong correlations have been reported between Ca^{2+} and auxin signaling. In plants, Ca^{2+} is a central second messenger, due to its role in a multitude of essential cellular process (cell division, cell growth/shrinkage, secretion, transcriptional regulation, cellular polarity, etc.), enabling it to influence stomatal aperture regulation, responses to light, responses to biotic and abiotic stresses, immunity and responses to multiple plant hormones, including responses to auxin [18]-[20]. Exogenous application of both synthetic and natural auxins can induce rapid, and transient increases in cytosolic Ca^{2+} concentration in wheat leaf protoplasts [21], maize coleoptile and root cells [22] [23], parsley hypocotyls [22], and *Arabidopsis* roots [24]. In nearly all cases, the increase of cytosolic Ca^{2+} concentration occurred within minutes after auxin application, demonstrating that auxin is a potent inducer of Ca^{2+} signals. One of the important questions that remains is how these auxin-induced Ca^{2+} signals are generated.

Generally, rapid and local Ca^{2+} signals generated in the cytosol depend on a Ca^{2+} current driven by a steep concentration gradient between the cytosol (nanomolar range) and its adjacent organelles or extracellular space (millimolar range). This allows the induction of a rapid and strong Ca^{2+} rise in the cytosol required for immediate activation of an appropriate response by the simple opening of a few Ca^{2+} channels [25] [26]. A calcium signature (characterized by amplitude, duration, frequency and location) can encode a message that contributes to a specific physiological response, after downstream decoding. A large number of calcium sensors in plant cells are known to decode different incoming stimuli [27] [28]. Because Ca^{2+} signals can be regulated by so many different cues and have such a broad impact on cellular processes, it is likely that Ca^{2+} acts to integrate multiple cues in a single output. In the present paper, we focus on the interactions of auxinic compounds on Ca^{2+} signaling to examine: the effects of natural and synthetic auxins on Ca^{2+} signaling (visualized by a YC3.60 calcium reporter and Fluorescence Resonance Energy Transfer (FRET)-based confocal microscopy), and how Ca^{2+} signaling correlates with early short-term growth (min to 17 h) responses and with long-term growth (96 h) in *Arabidopsis thaliana*.

2. Material and Methods

2.1. Chemical Sources and Purity

The auxinic compounds, indole-3-acetic acid, indole-3-butyric acid and 1-naphthalene acetic acid used in these studies were high purity compounds ($\geq 99\%$) obtained from Sigma Chemical Co. (St Louis, MO, USA). The auxinic herbicides (2,4-D and mecoprop) were obtained from Chem Service (West Chester, PA, USA) and were both $>98\%$ pure. Murashige and Skoog (MS) basal salt mixture (containing both macro- and micro-nutrients) was a product of Caisson Laboratories (Logan, UT, USA). Phytigel used as a plant growth media substrate was a Sigma product.

2.2. Preparation of Plant Material

FRET studies: Transgenic *Arabidopsis* (*A. thaliana*; Columbia ecotype) seeds were surface sterilized and planted

as described elsewhere [29]. The seeds were planted on sterile coverslips (48 × 64 mm) coated with 2 mL of 0.5% NuSieve agarose (FMC BioProducts) containing half-strength MS basal salts (Caisson Laboratories) and vitamins (pyridoxine-HCl, thiamine and nicotinic acid). The coverslips were placed inside sterile square plastic petri dishes (9 × 9 cm) and incubated (22°C - 24°C; 40% - 50% RH) under a 16-h light (124 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$) and 8-h dark cycle for 3 to 4 d in an environmental room. The petri dishes were vertically positioned to promote root growth along and against the coverslip [30]. After 3 to 4 days growth under these conditions, the seedlings were used in the FRET studies.

Growth studies: For long term growth studies (96 h) wild type *A. thaliana* seeds were initially surface sterilized and germinated on 0.5× MS-agar petri dishes [29]. After 3 - 4 days growth, uniform seedlings (aver. root length, 5 mm) were chosen and transferred to sterile square plastic petri dishes (9 × 9 cm) containing 0.5× MS-agar with or without the test compound (control). Each test compound was incorporated into warm agar before pouring into the dishes so as to achieve the desired compound concentration in the solidified agar dishes. Four dishes with 7 - 9 seedlings per dish were prepared for each chemical concentration and control treatment. Dishes were incubated (22°C - 24°C; 40% - 50% RH) under continuous light (124 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$) in an environmental chamber. Growth of seedlings was monitored and recorded at 24 h intervals over a 96 h period. In short term growth studies (20 min - 17 h), seedlings were grown as above for 3 - 4 days, and selected seedlings were then exposed to 10 μL of the test compound (1.0 μM) via application to the root tip. Growth (root elongation) was then monitored at 20 min, 90 min and 17 h after application.

2.3. Confocal Laser Microscopy and Measurement of FRET-Sensitized Emission

To visualize these calcium fluxes the YC3.60 calcium reporter which consists of a cyan fluorescent protein (CFP), the C terminus of calmodulin and the calmodulin-binding domain of myosin light chain kinase (M13), and a yellow fluorescent protein (YFP) [31] was used. The binding of calcium to calmodulin triggers a conformational change in the protein that brings CFP and YFP into close proximity. This alters the FRET efficiency between the two fluorophores [31] which correlates with changes in cytosolic $[\text{Ca}^{2+}]$. An inverted Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems) with an argon ion laser and a 63× water-immersion lens (numerical aperture 1.2) or an 20× dry lens (numerical aperture 0.70) was used for imaging. Excitation wavelengths for CFP and YFP were 458 and 514 nm, respectively. Images were acquired at 3 s intervals for 10 min and images were captured at a scanning speed of 200 MHz and a pixel resolution of 256 × 256. Imaging was focused at a depth of ~20 μm below the root epidermal cell layer. At this focal plane, both epidermal and cortical cells in longitudinal view were visible. The sensitized emission was corrected for bleed-through of the emission of the donor (CFP) into the acceptor channel [32] [33]. The Leica application, FRET Wizard (www.leica-microsystems.com/ConfocalMicroscopes), was used to make necessary corrections. *Arabidopsis* roots expressing YC3.60 and either CFP or YFP alone were used for calibration images as previously described [30]. New calibrations were performed on each day of the investigation by acquiring an image of the root zone of interest expressing YC3.60, and optimizing settings (pinhole, laser intensity, and photomultiplier gain) FRET efficiency was calculated by the Leica software as described previously [32].

2.4. Chemical Treatments

Three- to 4-d-old wild-type seedlings expressing YC3.60 were kept in the agarose growth medium on coverslips and carefully placed on the stage of the confocal microscope. Stock solutions of auxinic test compounds were prepared in deionized water. Half-strength MS medium (pH 5.7 adjusted with 1.5 M Tris) without vitamins was used to prepare all treatment solutions. A pipette was used to gently deliver 10 μL of treatment solution onto the surface of the root embedded and growing on the agarose layer. All experiments were replicated and repeated 7 to 10 times.

3. Results

3.1. Effects of Auxinic Compounds on Root Growth and Development

The chemical structures of auxinic compounds studied show structural relationships of the five compounds used in this study (Figure 1). These auxinic compounds caused differential growth effects on *A. thaliana* seedling roots when exposed to two concentrations of each compound under continuous light for 96 h as depicted photo-

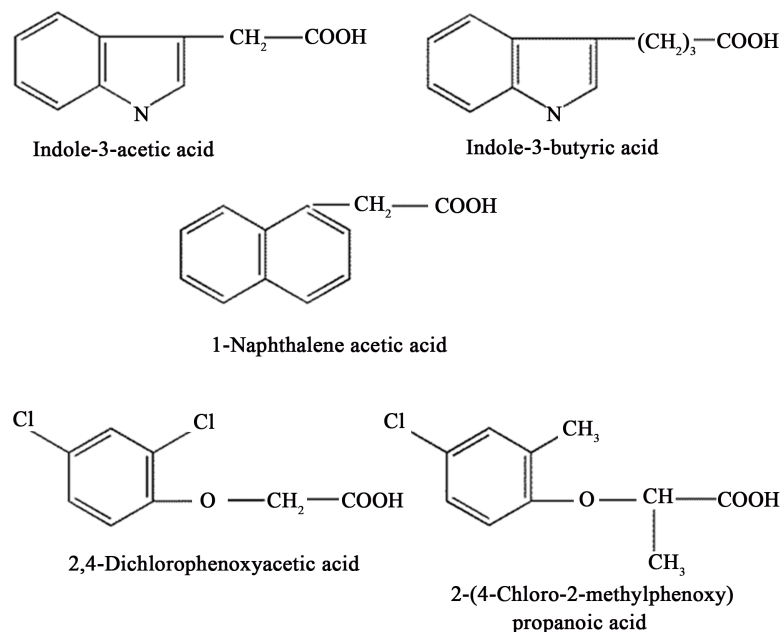


Figure 1. Chemical structures of auxinic compounds used in these studies.

graphically (**Figure 2**). Roots were continuously exposed to the compounds in the MS media. At the low concentrations ($0.10 \mu\text{M}$) of the compound, inhibition of root elongation was in the following order: IBA < IAA < NAA < mecoprop < 2,4-D (**Figure 3**). Exposure to high concentrations ($1.0 \mu\text{M}$) of each compound resulted in a slightly different order of inhibition: IBA < IAA < NAA < 2,4-D < mecoprop. IBA at both concentrations caused the least root growth inhibition and the herbicides (2,4-D and mecoprop) were most inhibitory.

The root zones of *A. thaliana* are illustrated in **Figure 4**. Calcium signaling images were captured in the distal region of the transition zone. Some root growth effects other than that exhibited by the primary root are evident under low magnification, *i.e.*, lateral root formation (**Figure 2**). Higher magnification of the primary root tips revealed differential effects on root structure and development by these compounds (**Figure 5**). Malformations and morphological changes in the root tip were caused by the same chemical treatments that caused inhibition of primary root growth. IBA caused the lowest effect on root growth, with little or no root tip malformation. The most prevalent symptoms were increased root hair formation at the root tip and severe swelling. IAA caused proliferation of root hairs near the root tip at the $1.0 \mu\text{M}$ concentration. NAA caused swelling and increased root hair formation at $1.0 \mu\text{M}$, but especially 2,4-D and mecoprop (**Figure 5**). Root hair formation may be caused by the cessation or reduction of root elongation, while the maturation phase continues, followed by increased root hair formation.

Studies using short-time exposure of auxinic compounds on *A. thaliana* root elongation indicated rapid effects caused by some treatments (**Figure 6**). When compounds were applied (a $10 \mu\text{L}$ drop, $1.0 \mu\text{M}$) to the root tip, no growth occurred by 20 min in the IAA, 2,4-D and mecoprop treatments. Some elongation occurred at 90 min, but little growth occurred during the 90 min to 17 h period in the IAA, 2,4-D and mecoprop treatments.

3.2. FRET Imaging

Calcium signatures elicited by these auxinic compounds were obtained using FRET imaging of the YC3.60 cameleon that detects fluxes of cytosolic calcium levels in *Arabidopsis* root tips (**Figure 7**). NAA- and IAA-treated root tips exhibited distinct biphasic fluxes of cytosolic calcium during a 10 min recording period. Mecoprop, 2,4-D and IBA exhibited rapid initial elevation of cytosolic calcium, followed by a slow decay of signal over time. The maximal FRET efficiency in each of the signatures varied for each compound. Glutamic acid, a known elicitor of cytosolic calcium fluxes in plants [30] was utilized as a standard to verify system accuracy (data not shown). We also used MS media alone as a negative control to confirm a lack of touch-induced calcium fluctuations (**Figure 7(F)**).

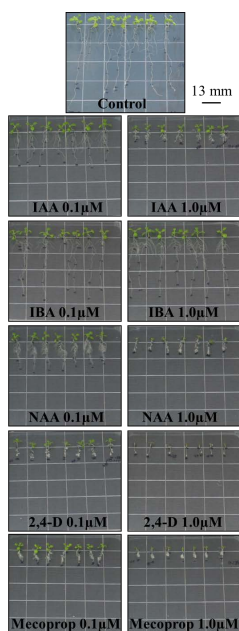


Figure 2. Visual effects of auxinic compounds on growth of *Arabidopsis thaliana* seedlings under continuous light for 96 h. Plants were grown aseptically in square petri plates on 0.5× MS media containing the compounds at 0.10 or 1.0 μM. Black marks on plates were used to record root elongation at specific time intervals after initiation of tests.

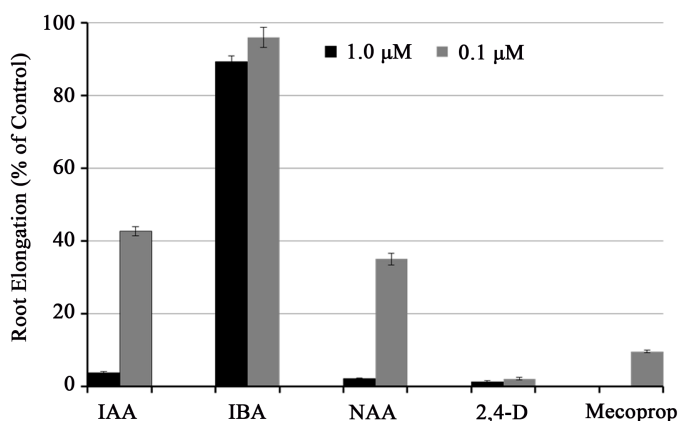


Figure 3. Effects of auxinic compounds on growth of *Arabidopsis thaliana* seedlings under continuous light. Root elongation (histograms) is expressed as percent of untreated control plant roots. Data is the average of duplicate experiments where $n \geq 10$; error bars represent ± 1 SE.

Schematic of *Arabidopsis* root regions

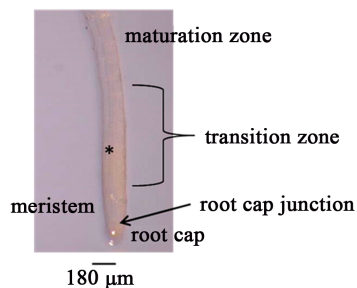


Figure 4. Schematic of *Arabidopsis* root zones. The 10 μL droplet of a test compound covered the entire root tip (maturation zone to root cap); asterisk denotes the region of FRET imaging.

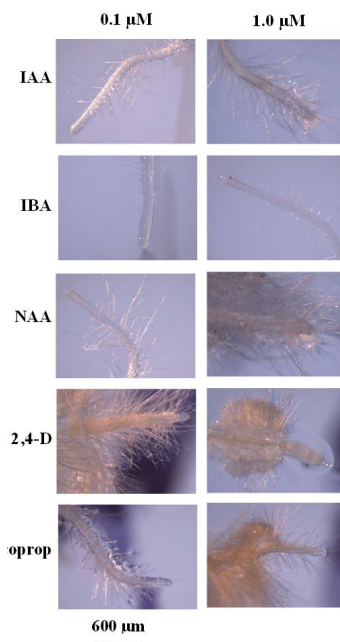


Figure 5. Light microscopic images of the effects of auxinic compounds (applied at two concentrations) on roots of 7-day-old *Arabidopsis* seedlings after continuous exposure (96 h).

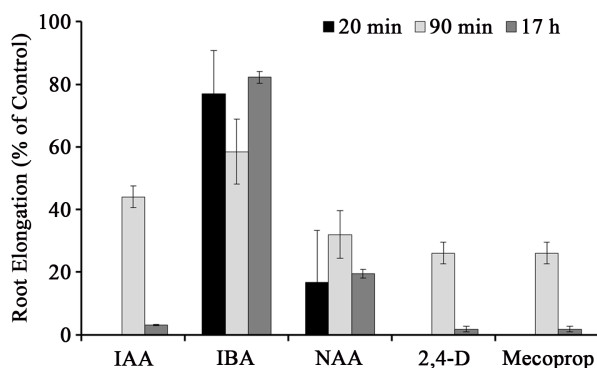


Figure 6. Effects of short-term exposure of various auxinic compounds applied to root tips of *Arabidopsis* seedlings, monitored at several time points. Compounds were applied (1.0 μM) to roots of 4-day-old seedlings and growth (elongation) was measured at 20 min, 90 min and 17 h after application. Data is the average of duplicate experiments where $n \geq 10$; error bars represent ± 1 SE.

The typical calcium signature for 10 μM IAA is biphasic (**Figure 7(A)**). Pseudo-colored images of treated *Arabidopsis* root tips (**Figure 7(G)**, images a-d) during specific time points of a 10 min scan, correspond to pre-IAA application, and various time points after treatment [labeled on the calcium signature (inset 7A)]. These images (a-d) depict the changes in cytosolic calcium levels [hot colors (red), higher cytosolic calcium; cooler colors (blue), lower cytosolic calcium], wherein hot colors correspond to a rise in FRET as cytosolic calcium is elevated and reflected in the signature. IAA and NAA produced similar calcium signatures (**Figure 7**) and similar growth patterns over a longer growth period (**Figure 2** and **Figure 3**).

4. Discussion

Synthetic auxinic herbicides have been used for weed control for about 70 years and numerous synthetic compounds belong to the auxinic family have been developed comprising several chemical classes (benzoic, phenoxyalkanoic, pyridine carboxylic and quinolone carboxylic acids). Despite this wide use over time, the development of weed resistance to these compounds has been relatively low, *i.e.*, 31 reports [34], and the precise

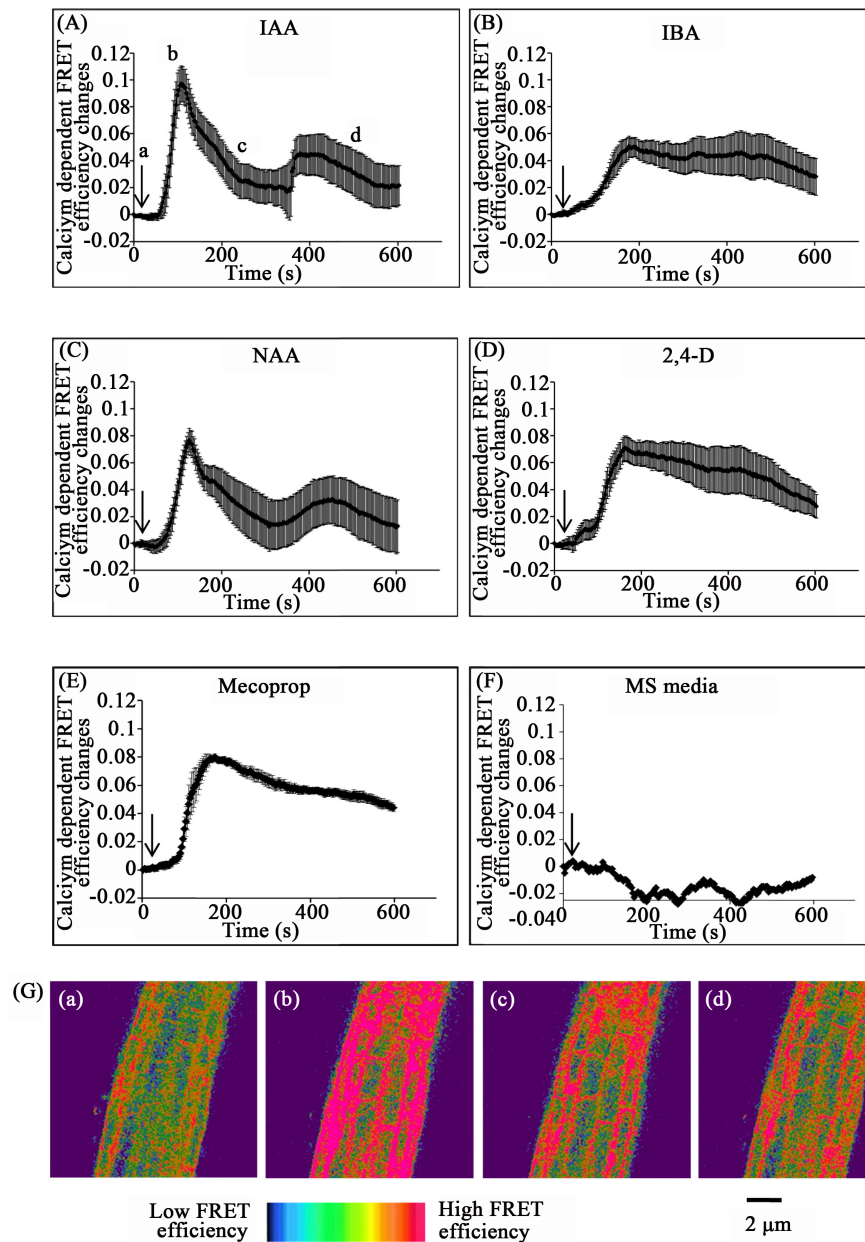


Figure 7. Cytosolic $[Ca^{2+}]$ -dependent FRET efficiency changes in the root transition zone of 72 to 96-h-old *Arabidopsis thaliana* seedlings as affected by several auxinic compounds. Arrows indicate the time of test compound application (10 μ L droplet of a 10 μ M solution). The dark solid line in scans of A through E represents the average of $n \geq 7$; error bars are ± 1 SE. Each scan consists of data (200 data points) gathered at 3 s intervals over a 600 s time course. Inset F is a representative control scan of MS media alone. Inset G (images a-d) depicts the series of pseudo-colored images associated with the changes in cytosolic calcium as a result of exposure to 10 μ M IAA (Inset A): image a) cytosolic calcium levels before IAA application; image b) the sharp rise in cytosolic calcium; image c) gradual decline in cytosolic calcium levels; image d) slight increase of this biphasic scan, followed by a gradual return to near pre-treatment cytosolic calcium levels.

mode of action of these various compounds remains to be explained.

Many reports suggest that calcium acts as a secondary messenger in plants [35] [36]. Furthermore environmental factors (heat and cold shock, wind, light, CO_2 levels, salinity, oxidative stress, wounding, pathogen infectivity and plant hormonal application [37]-[40]) can alter intracellular calcium levels. These results suggest cytosolic Ca^{2+} plays an important role in signaling in response to stresses.

Interaction studies of IAA, auxinic herbicides on isolated protoplasts from auxinic herbicide-resistant and -sensitive wild mustard, suggested that disruption of calcium transport was an integral part of auxinic herbicide action [41]-[43]. Calcium also plays an important role in mediating resistance to auxinic herbicides in intact seedlings [43].

Arabidopsis plants have proven useful as models to investigate and elucidate physiological, molecular biological and genetic aspects of auxinic compound interactions. For example, *Arabidopsis* mutants, selected for resistance to the auxinic herbicide, picloram, but not to 2,4-D or IAA, indicated that picloram acts through a mechanism different than the signaling pathway affected by 2,4-D and IAA [44]. Differential gene expression in *Arabidopsis* treated with IAA, 2,4-D or picloram has also been reported [45]. The major differences in calcium signatures of IAA and 2,4-D (*i.e.*, amplitude and biphasic vs monophasic characteristics) also indicate different mechanisms with regard to calcium signaling of these two compounds in *Arabidopsis* (Figure 7(A) and Figure 7(C)). Likewise, auxinic compounds that elicit similar calcium signatures may act on the same receptor, thus IAA and NAA are similar (biphasic), while IBA, 2,4-D and mecoprop comprise a second group (monophasic), but there are some dissimilarities of the signatures in this latter group (Figure 7(B), Figure 7(D), Figure 7(E)). Recently, another auxinic herbicide (dicamba) was found to elicit many stress-responses and signaling genes in *Arabidopsis* [46]. Although these natural and synthetic compounds possess auxin activity, their chemical structures contain various substituents and ring sizes compared to IAA (see Figure 1); factors that may play an important role in protein binding relative to the binding, transport and regulation of auxin.

The lipophilicity and acidity of these compounds also differ. The pKa values for IAA, IBA and NAA ranged from 4.23 to 4.86, while values for 2,4-D and mecoprop were 2.8 to 3.78. These properties, coupled with the apoplastic and cytoplasmic cell characteristics of the plant root or foliage, also influence the kinetics of cellular uptake and transport of these compounds that pass through plant cell wall membranes into the cytoplasm and organelles. The cytosol is less acidic (pH—7) than the apoplast; (pH—5.5), thus enabling the apoplastic portion (17%) of IAA to remain un-dissociated. The difference in lipophilicity of IAA versus 2,4-D and mecoprop may explain the preference for auxin accumulation in the cytoplasm and thus differences in elicited calcium signatures. The un-dissociated auxin is able to diffuse through the lipophilic lipid bilayer. Once inside the more basic cytosol, un-dissociated auxin dissociates almost completely, to anionic IAA⁻, that is unable to cross the lipophilic lipid bilayer and thus is confined to the cell. Gradient-driven H⁺ symport activity enhances lipophilic diffusion of IAA in apical tissues where concentrations are elevated [47]. *Arabidopsis* AUX1/LAX family of amino acid permease-like proteins are reported to function using this mechanism [48] [49].

Several auxinic compounds including some herbicides that greatly reduced root elongation (exception, IBA only 5% - 10%) (see Figure 2 and Figure 3) also caused dramatic changes in growth morphology of the root tip (Figure 5). Disruption of auxin maxima and gradients in root tissue can interrupt various facets of plant development [50], therefore external application of auxinic test compounds (as in the present studies) interferes with natural auxin gradients. However, such effects are more predominant in plant growth studies rather than in the short-term FRET imaging.

Calcium has been reported to interact in various ways with certain herbicides. The effects of the auxinic herbicides dicamba and mecoprop on auxinic herbicide resistant- and susceptible-wild mustard (*Sinapis arvensis*) was ameliorated by pretreatment of the seedlings with calcium and a calcium ionophore [51]. Auxinic herbicide-resistant seedlings of wild mustard exhibited a wide range of resistance to auxinic herbicides: picloram and dicamba >> 2,4-D and MCPA [4-chloro-2-methylphenoxy]acetic acid] >>> mecoprop and 2,4-DP [2-(2,4-dichlorophenoxy) propionic acid] [52]. The absorption, translocation, and metabolism of foliar-applied 2,4-D, dicamba and picloram did not differ in resistant and susceptible biotypes, but inhibition of IAA binding in preparations of auxin-binding-protein (ABP) by picloram and dicamba was greater in sensitive versus resistant biotype [53]. Mecoprop inhibited IAA binding equally in both biotypes. Similar results were demonstrated in intact seedlings of these biotypes [54].

Auxins are known to stimulate root formation and development, and the compounds used in these experiments demonstrated such effects on *Arabidopsis* roots (Figure 2 and Figure 5). IAA is a key hormone involved in root development, vascular differentiation, gravitropism and initiation of lateral root formation [55]. Based on our results we suggest that the application of these auxin compounds initiate an early signaling cascade, triggered by the fluctuation of cytosolic calcium monitored by the calcium reporter YC3.60. This signaling cascade causes reduction of root elongation within 20 min. of application, later followed by proliferation of elongated root hairs and root malformation and swelling (Figure 5 and Figure 6). Application of exogenous auxinic com-

pounds may disrupt the local auxin gradient and maxima, which in turn can alter the regulation of gene expression that determines cell fate. One process of cell differentiation in roots that is heavily dependent on auxin gradients is root hair development, which was markedly affected in our growth study. Manipulation of polar auxin transport by exogenous compound application or genetic disruption can drastically alter root tip patterning [56], and severe alteration of normal root growth was dramatically displayed in the present study. The rapidity of the inception of growth reduction after compound application (20 - 25 min), correlating with the rapid change in calcium signaling (30 - 45 s), suggests a calcium-mediated signaling cascade initiated by auxinic compound application.

Translating the Ca^{2+} signature into a specific cellular response is governed by several Ca^{2+} -binding proteins (e.g., calreticulin) that act as $[\text{Ca}^{2+}]$ buffers altering both the amplitude and duration of the Ca^{2+} signal, or by Ca^{2+} sensors (e.g., calmodulin) that impact other downstream cellular effectors [18] [57]. Differences in calcium signaling initiated by auxinic compounds in our study indicate that more than one mechanism or signaling pathway may be affected within seconds of compound application to roots. The advantage of using a FRET imaging technique with the YC3.60 calcium reporter enables real time observation of cellular calcium fluxes (Figure 7) which initiate downstream signaling cascades that are expressed over time through alteration of root tip morphology (elongation, secondary root formation and root tip swelling) (Figure 5).

5. Conclusion

Auxinic herbicides induce many morphological changes in plants including: cell elongation, root initiation, epinasty and hypertrophy [58] [59]. These alterations involve consequences of numerous biochemical/physiological changes, including alterations in calcium dynamics, increased protein and DNA/RNA biosynthesis, etc. The elucidation of the molecular mechanisms of action of auxinic herbicides may provide important information about the molecular modes of action of IAA and for the development of herbicide-resistant crops. Further studies utilizing this sensitive tool (YC3.60 calcium reporter) on wild-type and selected auxin perception/signaling mutants expressing the YC3.60 calcium reporter are being undertaken to more fully elucidate the role of calcium signaling and the interactions of these and other auxinic compounds in plants. Information such as this could also be useful in elucidating aspects of auxinic herbicide mode of action, secondary effects of herbicides *in planta*, and of herbicide resistance mechanisms in weeds.

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