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ORIGINAL RESEARCH

Plant growth promotion by the interaction of a novel synthetic small molecule with GA-DELLA function

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Abstract

Synthesized small molecules are useful as tools to investigate hormonal signaling involved in plant growth and development. They are also important as agrochemicals to promote beneficial properties of crops in the field. We describe here the synthesis and mode of action of a novel growth-promoting chemical, A1. A1 stimulates enhanced growth in both shoot and root tissues of plants, acting by increasing both dry and fresh weight. This suggests that A1 not only promotes uptake of water but also increases production of cellular material. A1 treatment of *Arabidopsis*leads to the degradation of DELLA growth-inhibitory proteins suggesting that A1-mediated growth promotion is dependent upon this mechanism. We performed genetic analysis to confirm this and further dissect the mechanism of A1 action upon growth in *Arabidopsis*. A quintuple *della*mutant was insensitive to A1, confirming that the mode of action was indeed via a DELLA-dependent mechanism. The *ga1-5*gibberellin synthesis mutant was similarly insensitive, suggesting that to promote growth in *Arabidopsis*A1 requires the presence of endogenous gibberellins. This was further suggested by the observation that double mutants of GID1 gibberellin receptor genes were insensitive to A1. Taken together, our data suggest that A1 acts to enhance sensitivity to endogenous gibberellins thus leading to observed enhanced growth via DELLA degradation. A1 and related compounds will be useful to identify novel signaling components involved in plant growth and development, and as agrochemicals suitable for a wide range of crop species.

1 | INTRODUCTION

Plant growth and development involves the integration of many environmental signals and plant hormones (Gray, 2004). Plant hormones including gibberellic acid (GA), abscisic acid (ABA), cytokinin, ethylene, and brassinosteroids regulate many aspects of plant growth and development at relatively low concentrations (Gray, 2004; Rigal et al., 2014). Cytokinin, auxin, GA, and brassinosteroids are considered essential for plant growth, as gauged by the phenotype of mutants with disrupted hormone biosynthesis or perception (Depuydt & Hardtke, 2011). GA specifically promotes important processes in plant growth and development such as seed germination, cell elongation, cell division, as well as floral transition (Richards et al., 2001).

One sentence summary: identification of a novel synthetic small molecule capable of enhancing plant growth through the enhancement of response to endogenous gibberellins.

Bioactive gibberellic acids (GAs) are diterpene phytohormones that modulate plant growth and development throughout the plant life cycle (Sun, 2010). The major function of GAs is to stimulate organ growth through the enhancement of cell elongation and cell division (Gupta & Chakrabarty, 2013; Hedden & Phillips, 2000). The GA receptor was first identified in rice where *OsGID1* gene encodes a protein possessing GA-binding activity, and its mutation results in a severe dwarf phenotype that does not respond to GA in either stem elongation or seed germination (Stepanova, 2008; Stepanova et al., 2005; Tao, 2008). In *Arabidopsis*, there are three homologs of the GA receptor, *AtGID1a*, *AtGID1b* and *AtGID1c* (Nakajima et al., 2006). Single mutation of *GID1a*, *GID1b* and *GID1c* results in the same phenotype as wild type in terms of stem elongation and root length. This suggests that the receptors have a redundant function in *Arabidopsis*; however, the specificity of GID1 homologs function can be observed from double mutants (Iuchi et al., 2007; Suzuki et al., 2009).

In GA signaling, the key mechanism is GA repression of DELLA protein function. DELLA proteins are negative regulators of plant growth that belong to the GRAS protein superfamily of transcriptional regulators. The controlled degradation of these proteins is a major event in plant growth (Hauvermale et al., 2012). There are five DELLA repressor proteins in *Arabidopsis*: REPRESSOR OF GIBBERELLIC ACID (RGA), GA-INSENSITIVE (GAI), RGA-Like Proteins 1,2 and 4 (RGA1, RGA2 and RGA3). Activation of the GA signaling pathway is initiated by the interaction between bioactive GAs and GID1 that promotes a conformational change in the receptor. The formation of a GA-GID1-DELLA complex enables a protein–protein interaction between the DELLA and the F-box protein SLY1 resulting in ubiquitination and degradation of the DELLA protein (Griffiths et al., 2006; Hirano et al., 2010). The degradation of DELLA protein allows for the activation of transcription factors downstream of them to affect the required growth responses.

Growth in etiolated seedlings is regulated by phytochrome interacting factors (PIFs), a subset of basic helix–loop–helix (bHLH) transcription factors (Li et al., 2016). PIFs mediate hypocotyl elongation, and their activity is negatively regulated by the red light photoreceptor PHYB and by DELLA proteins that act to repress the GA signaling pathway (de Lucas et al., 2008). The activation of PHYB by light leads to destabilization of PIFs whilst the accumulation of DELLA proteins block PIF activity by binding the DNA-recognition domain of this factor. In contrast, PIF proteins accumulate and directly regulate genes to maintain skotomorphogenesis in the dark (Li et al., 2016), leading to elongated hypocotyls. For this reason, hypocotyl growth is an often used growth assay to monitor GA-DELLA signaling.

Here, we describe a chemical study of growth in *Arabidopsis* seedlings, which has led to the identification of a compound that promotes growth via the GA-DELLA pathway. Our findings suggest that this chemical acts by enhancing the potency of endogenous GAs, and therefore, this compound and its derivatives have significant potential as probes for plant growth and also as use as agrochemicals.

2 | RESULTS

2.1 | Treatment with A1 results in promotion of root growth in *Arabidopsis*

In our earlier work, a small-scale chemical genetics study was undertaken to explore the role of the known calmodulin inhibitors, N-(6-aminohexyl)-1-naphthalenesulfonamide (W5) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) in plants. W5 and W7 are both naphthalene sulfonamide compounds, with the only difference between them being the C-5 chlorine substituent in W7. Despite their high degree of structural similarity, the activity of these antagonists is significantly different with W5 showing reduced activity as compared with W7 (Gilroy et al., 1987; Kaplan et al., 2006; Sinclair et al., 1996). To attempt to better understand this difference, a small set of structurally related analogues of W5 and W7, chlorinated (AC1, AC2, AC3, AC4) and nonchlorinated (A1, A2, A3, A4), were prepared and monitored for their biological activity (Figure 1a). As expected for calmodulin inhibitors, most compounds inhibited root growth compared with control (DMSO). However, surprisingly, one compound A1 led to enhanced root growth (Figure 2), an observation that merited further study.

2.2 | A1 promotes both fresh and dry weight accumulation in *Arabidopsis*

Having discovered the root growth-promoting properties of A1, we wished to establish if the effects were limited to the roots, or whether A1 could also promote growth of shoot tissue. To investigate this, plants were treated with A1 and then divided into shoot and root material and the fresh and dry weight recorded. As can be seen in Figure 3, the fresh weight of both roots and shoots increased in response to A1. Also in both roots and shoots the dry:fresh weight ratio also increased, demonstrating that the effect of A1 was to enhance true biomass (Figure 3) in all plant tissue, and not just uptake of water.

2.3 | A1 stimulates DELLA degradation in *Arabidopsis*

DELLA proteins are the key negative regulators of plant growth (Hauvermale et al., 2012; Yoshida et al., 2014). DELLA degradation in response to growth signals (such as gibberellins) leads to enhanced growth of plant tissues, including roots (Ubeda-Tomas et al., 2008). The observed promotion of root and shoot growth by A1 (Figures 2 and 3) lead to the hypothesis that A1 might therefore mediate DELLA degradation. To investigate this suggestion, the stability of DELLA proteins upon treatment with A1 was investigated (Figure 4). An assay which allows the visualization of DELLA degradation is to image fluorescent protein fusions to DELLA proteins (Silverstone et al., 2001). Exploiting this technology, *Arabidopsis thaliana* seedlings expressing

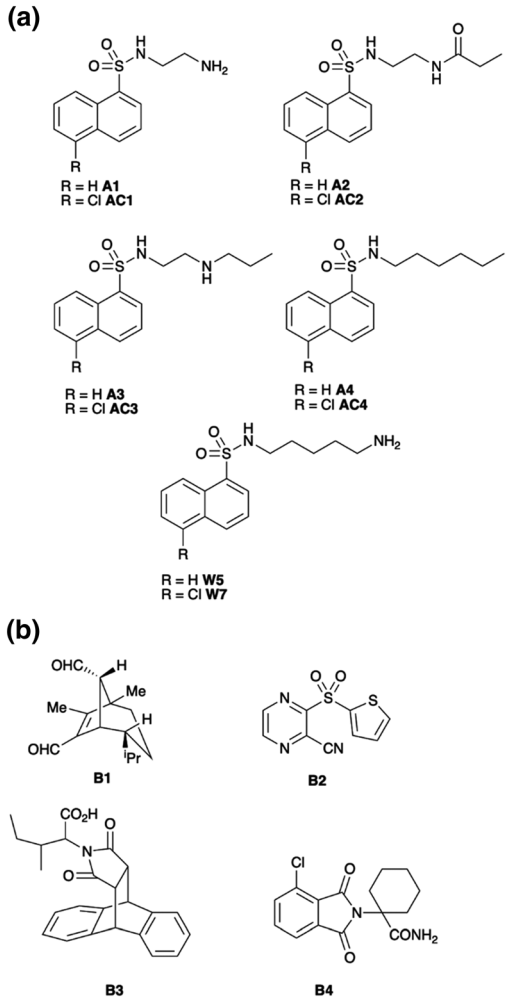

FIGURE 1 (a) Series of new compounds that have been modified from commercial calmodulin inhibitors, W7 and its less active analogue W5. The series are divided into two groups: chlorinated (AC1, AC2, AC3, AC4) and nonchlorinated (A1, A2, A3, A4). (b) Molecular structures of reported modulators of the GA signaling pathway; helminthosporal (B1), thiophenyl sulphone (B2), succinimide (B3), and AC94377 (B4)

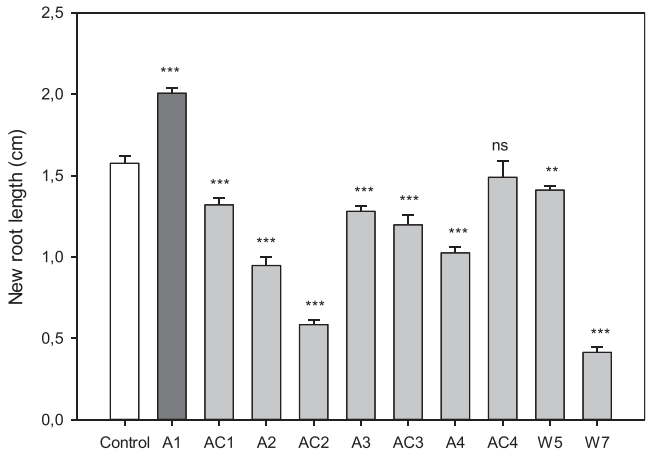

FIGURE 2 New compounds screening to investigate the effect on root elongation; 7 days old *Arabidopsis* (*Col-0*) seedlings where transferred in a medium containing 100 μM of each compound, and after 5 days the length of the new root was measured. Means ($n = 18$) and standard error are reported. Asterisks indicate statistically significant differences (independent *t*-test, $^{**}p < .005$, $^{***}p < .001$; ns: not significant) between DMSO (control: white bar) and chemical treatment (gray bars)

RGA-GFP were treated with A1, with GA acting as a positive control. Roots were then imaged after 2 and 24 h using confocal microscopy (Figure 4). The application of A1 led to reduced GFP fluorescence indicating enhanced degradation of DELLA proteins. The A1 effect on DELLA degradation was as rapid as GA, as the response could be observed after 2 h. Together, these results suggested that A1 promotes plant growth by enhancing DELLA protein degradation and, as such, its action resembles GA.

2.4 | *Arabidopsis della* loss of function mutant is insensitive to A1

The data presented in Figure 4 suggested that A1 acts via DELLA, and we thus hypothesized that A1 action might be mediated through the GA-DELLA signaling pathway. A1 was also observed to have an effect on other GA-dependent processes such as partially reversing ABA-mediated germination inhibition (Figure S1). To determine the mechanism of A1 action via the GA/DELLA signaling pathway, we tested the response of A1 to DELLA and GA synthesis/signaling mutants. For these assays, we focused on hypocotyl growth, because it is well established that DELLA can inhibit the binding of PIFs to their target promoter leading to a reduction in GA-regulated hypocotyl growth (Castillon et al., 2007; de Lucas et al., 2008; Feng et al., 2008). In this way, the hypocotyl assay is more diagnostic for GA than root assays. To establish the use of A1 in this assay, we first tested hypocotyl growth by growing the seedlings on agar plates containing A1, with GA and PAC used as positive and negative controls, respectively, for 3 days in reduced light conditions to achieve a balance between maximum and minimum hypocotyl growth. From these data, it was clear that A1 stimulated hypocotyl growth in a manner very similar to GA (Figure S2). Having established the hypocotyl assay, we then confirmed genetically that the A1 effect upon growth was indeed DELLA-dependent. To test this, a mutant line that lacks all DELLA (GAI, RGA, RGL1, RGL2 and RGL3) function was used. Due to the loss of all DELLA function, this mutant displays a longer hypocotyl phenotype as compared with wild type without treatment (Figure 5). Unlike wild type, no promotion of hypocotyl growth was observed after A1 treatment of this mutant (Figure 5), supporting the suggestion that A1 requires DELLA for its growth-promoting effect.

2.5 | A1 cannot stimulate hypocotyl growth in a GA biosynthesis mutant

The growth effects mediated by A1 via DELLA proteins could be occurring through the action of growth hormones, most notably

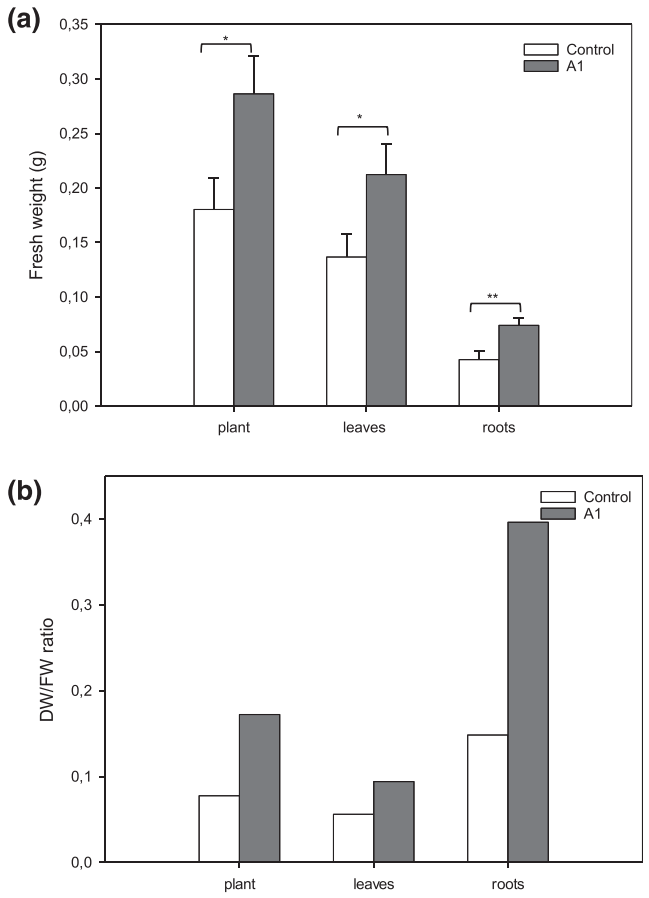

FIGURE 3 (a) Fresh weights of whole plant, leaves and roots of 12 days old seedlings measured after 5 days of chemical treatment with A1 compound (dark gray bars) and control (white bars). Means ($n = 15$) ± SE are shown in the panel. Asterisks indicate statistically significant differences (independent *t*-test, $^*P < .05$, $^{**}P < .005$) between control and A1 treatment. (b) Ratio between means of dry weight and fresh weight is shown

auxins and gibberellins. We discounted a role for auxins as A1 had no effect upon hypocotyl growth in *pif4-101* and *pif4-2* mutants (Figure S3) nor did A1 stimulate the expression of auxin-inducible genes such as *IAA2* and *IAA4* (Figure S3). Therefore, we focused our attention upon gibberellins. As A1 stimulated hypocotyl growth in a very similar fashion to GA, it was possible that A1 was acting as an artificial GA. To determine whether this was the case, hypocotyl growth assays were performed using GA biosynthesis mutants, in order to test if A1 could restore growth. The *ga1-5* mutant contains low levels of bioactive GA, which leads to an increase in DELLA protein and consequent growth inhibition, and hence displays a dwarfed phenotype (Fridborg et al., 1999). As expected, in hypocotyl growth assays, *ga1-5* mutants had shorter hypocotyls than wild type in untreated conditions. The treatment of the seedlings with A1 could not recover hypocotyl elongation (Figure 6). These data suggest that A1 is not simply acting like GA, but it is also possible that A1 requires endogenous GA to obtain its effect on growth promotion, as *ga1-3* with low levels of gibberellins does not respond to A1.

2.6 | A1 requires the GID1 gibberellin receptors to promote growth and acts upstream of them

As it appeared that endogenous biosynthesis of GA might be needed for the A1-mediated increase in hypocotyl growth, we tested the effect of A1 on GA receptor mutants. In *Arabidopsis*, there are three GA receptors named *GID1a*, *GID1b*, and *GID1c*. These three receptors show functional redundancy as there was no phenotype observed in *gid1* single mutants (Griffiths et al., 2006). We therefore tested the effect of A1 on hypocotyl growth in *gid1* double mutants. Without treatment, *gid1a1c* and *gid1b1c* mutants showed a reduced hypocotyl length compared with WT and *gid1a1b*. As shown in Figure 7, A1 treatment increased the hypocotyl length in wild type seedlings; however, in the mutants, this effect was reduced and was not statistically significant. These data suggest that in order to exert its plant growth promotion effect, A1 requires the presence of the GA receptors as well as endogenous suggesting that A1 acts upstream of the GA receptors.

3 | DISCUSSION

The chemical genetic approach employs small molecule compounds to interrogate biological processes due to their ability to selectively modulate protein function (Stockwell, 2000a, 2000b). When compared with classical genetic studies, it can offer a number of advantages. This includes a rapid time scale for activity, the ability to titrate effects, regulated activity (as opposed to constitutive), and in particular an ability to reduce the problem of genetic redundancy which can complicate standard genetic knock out experiments. This is due to the ability of a small molecule to specifically interact with a single protein and act as either an antagonist or agonist, subsequently allowing identification of protein function through a biochemical approach (Toth & van der Hoorn, 2010). Because of these beneficial features of using the chemical genetic approach, we created a series of chemicals based on the calmodulin inhibitors W5 and W7 (Gilroy et al., 1987; Kaplan et al., 2006) in order to investigate calcium signaling in plants. As inhibition of calmodulin has been reported to arrest plant growth (Sinclair et al., 1996), we tested these for efficacy in plants using a simple root growth assay for our screen. Whilst most compounds either inhibited growth or had no effect as expected, surprisingly, one compound A1 actually promoted root growth (Figure 2). Further analyses revealed that A1 promotes not only root growth, but also shoot growth and this effect was associated with increases in both dry and fresh weight (Figure 3). This suggests that A1 stimulates *bona fide* increases in growth including the production of new cellular material, and not just uptake of water.

Central to the regulation of all plant growth are the DELLA proteins (Dill et al., 2001; Hauvermale et al., 2012). These are key negative regulators of plant growth, which limit growth under conditions whereby maximal plant growth is not appropriate, for example, under stress conditions (Achard et al., 2003; Rowe et al., 2016). When growth rate needs to be increased, controlled DELLA protein

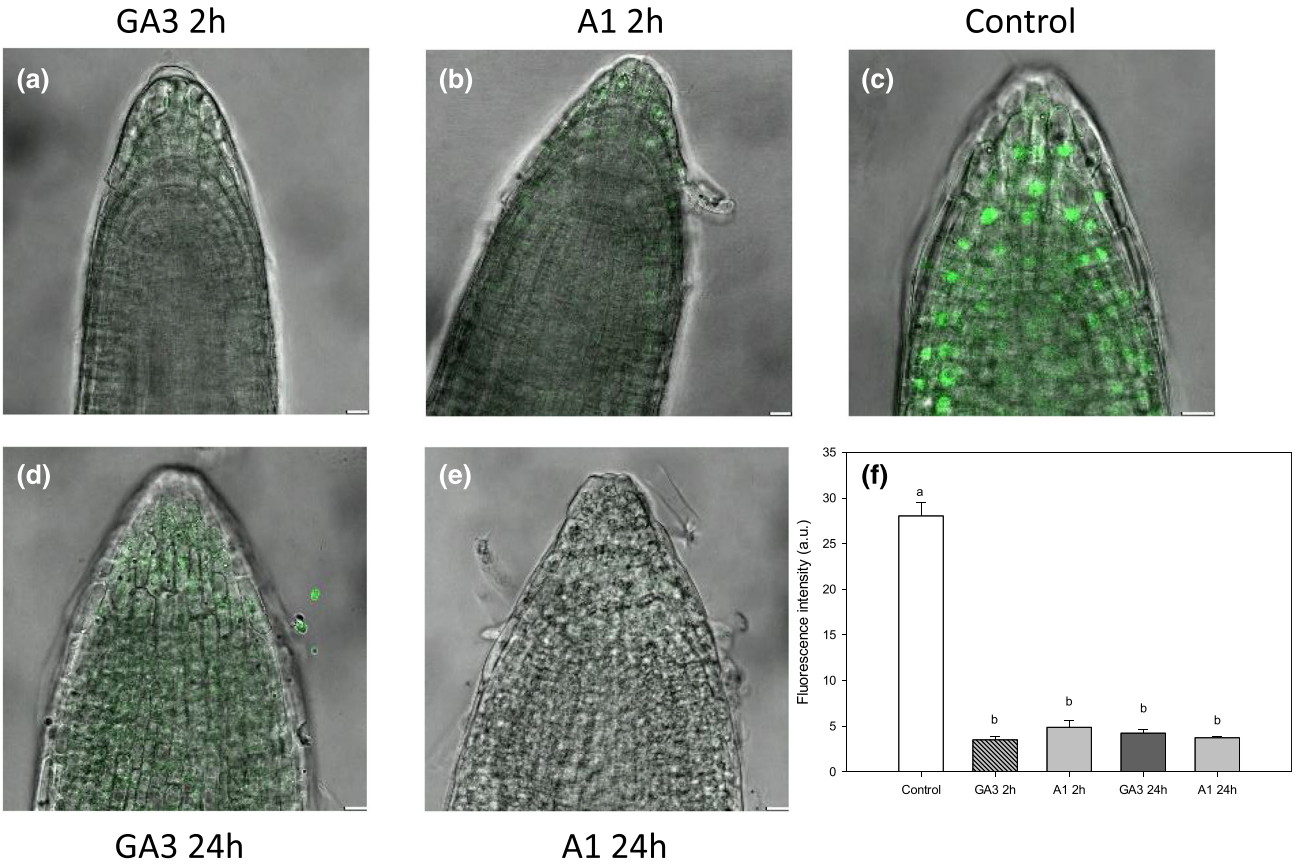

FIGURE 4 Representative confocal images of *Arabidopsis* RGA-GFP root tips in control conditions (c), after 2 and 24 h treatment with GA3 (a,d) and A1 (b,e). GA is used as positive control of DELLA proteins degradation. Images show overlays of fluorescence and bright field. (f) The graph shows the effect of each compound (control: white bar; GA3: gray bar; A1: dark gray bar) on nuclear fluorescence intensity which is inversely correlated with DELLA degradation. Means ($n = 5$) ± SE are shown. A one-way ANOVA followed by Tukey's test was performed to define statistically significant differences among means ($p < .05$). Means not sharing the same letters are statistical significantly different. Scale bars indicate 10 μm

degradation is induced (Dill & Sun, 2001; Sun, 2010). We therefore hypothesized that the enhancement of growth mediated by A1 (Figures 2 and 3) might be occurring through the direct or indirect regulation of DELLA protein degradation. In support of this hypothesis, we found that A1 was as capable as GA at reducing fluorescence in an RGA-GFP fusion line (Figure 4). Moreover, using a hypocotyl growth assay as a more specific marker of GA-DELLA-based signaling, a DELLA quintuple mutant was, in contrast to wild type plants, insensitive to A1 confirming a direct link between A1 and the DELLA proteins (Figure 5).

As we had evidence that A1 was promoting growth through effects on DELLA proteins, we investigated whether the promotion of growth by A1 might involve gibberellins specifically. We therefore tested the effect of A1 upon a *ga1-5* mutant, which is reduced in gibberellin biosynthesis and has lower levels of gibberellins (Fridborg et al., 1999). The *ga1-5* mutant displayed shorter hypocotyls in the absence of any treatment (Figure 6), which is due to reduced levels of endogenous gibberellins as described before (Koornneef & van der Veen, 1980; Fridborg et al., 1999; Sun, 2010). Whilst A1 was capable of stimulating increased hypocotyl growth in wild-type, under the same conditions, it had no effect upon the hypocotyls of *ga1-5* (Figure 6). This finding suggests that the mechanism by which A1 stimulates hypocotyl growth is not through A1 acting as a simple gibberellin substitute. The fact that A1 does not mimic gibberellins is consistent with the literature as whilst there are in excess of one hundred natural GA analogues reported only a very few exhibit significant bioactivity. In contrast to other phytohormones, simple modulators of the GA signaling pathway, such as A1, are relatively rare. Helminthosporal (Figure 1 B1) and related derivatives, first identified by Coombe, promote hypocotyl growth and seed germination, similar to GA (Coombe et al., 1974; Miyazaki et al., 2017; Miyazaki et al., 2018). However, this has an equally complex structure to GA. The thiophenyl sulphone (Figure 1 B2) (Yoon et al., 2013) exhibits GA antagonism whilst succinimide (Figure 1 B3) (Jiang, Shimotakahara, et al., 2017) and AC94377 (Figure 1 B4) (Jiang, Otani, et al., 2017) appear to function as GA mimics. The last two are proposed to function as selective agonists of GID1 leading to DELLA degradation and downregulation of the expression of *GA20ox* genes and the upregulation of *GA2ox* genes. In contrast, A1 had no effect on any of the three possible GID1 double mutants (Figure 7), as determined in the hypocotyl growth assay, nor on the expression of these genes (Figure S4), strongly suggesting a different mode of action. Whilst A1

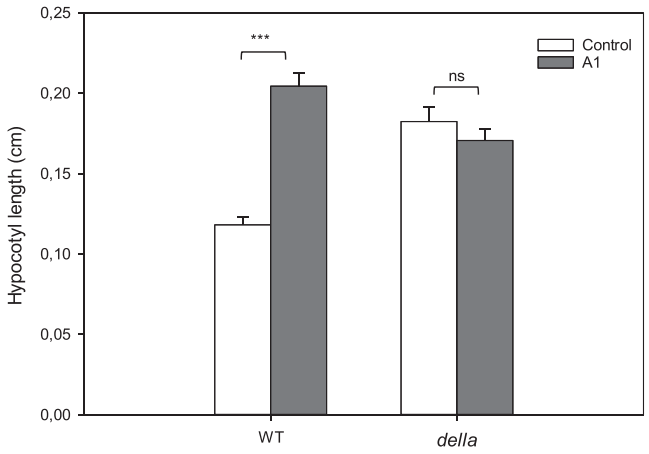

FIGURE 5 Hypocotyl length measured after 3 days treatment with A1 (dark gray bars) and control conditions (white bars) in *Arabidopsis* WT (*Ler-0*) and *DELLA* quintuple mutant which lacks of all DELLA functions. 2 days old *Arabidopsis* seedlings where transferred in a medium containing 100 μM of each compound, and after 3 days the length of hypocotyl was measured. Means ($n = 15$) ± SE are shown and asterisks indicate statistically significant differences (independent *t*-test, *$p < .05$, ***$p < .001$; ns: not significant) between chemical treatment within each genotype

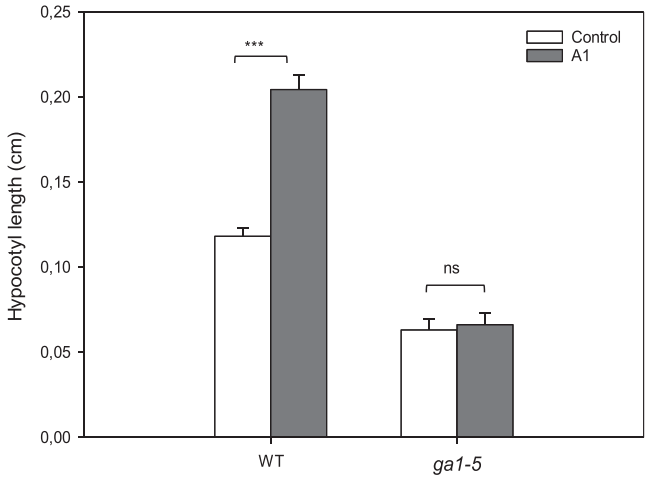

FIGURE 6 Hypocotyl length measured after 3 days treatment with A1 (dark gray bars) and control conditions (white bars) in *Arabidopsis* WT (*Ler-0*) and *ga1-5* mutant which contains low levels of bioactive GA. Means ($n = 15$) ± SE are shown and asterisks indicate statistically significant differences (independent *t*-test, *$p < .05$, ***$p < .001$; ns: not significant) between chemical treatment within each genotype

was capable of stimulating increased hypocotyl growth in wild-type, under the same conditions, it had no effect upon the hypocotyls of a *ga1-5* mutant which is reduced in gibberellins biosynthesis and has lower levels of gibberellins suggesting that the mechanism by which A1 stimulates hypocotyl growth, requires endogenous gibberellins and is not a simple GA receptor agonist.

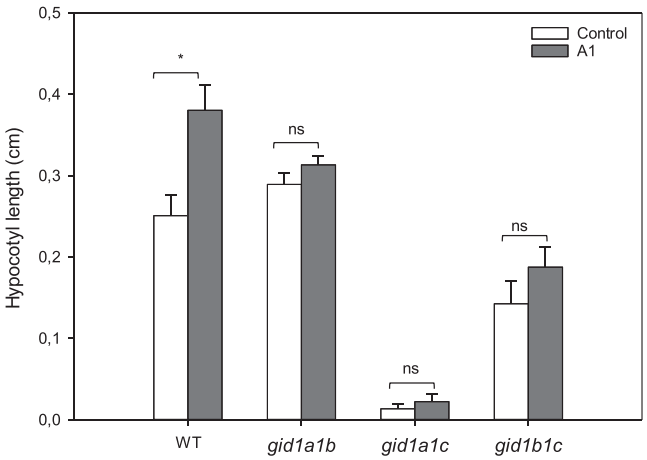

FIGURE 7 Hypocotyl length measured after 3 days treatment with A1 (dark gray bars) and control conditions (white bars) in *Arabidopsis* WT (*Col-0*) and *gid1a1b*, *gid1a1c*, and *gid1b1c* mutants of GA receptors. Means ($n = 30$) ± SE are shown and asterisks indicate statistically significant differences (independent *t*-test, *$p < .05$, ***$p < .001$; ns: not significant) between chemical treatment within each genotype

In summary, we have identified a simple, easy to prepare, small molecule A1 that acts to increase plant growth through the degradation of DELLA proteins in a mechanism that requires the presence, and perception, of endogenous gibberellins. Future work to identify the molecular basis (and target) for potential A1 sensitization of GA perception will provide new insights into GA/DELLA signaling and pave the way to use A1 and related compounds as growth-promoting agrochemicals.

4 | MATERIALS AND METHODS

4.1 | Synthesis of chemicals

Naphthalene sulfonylchloride (1.00 g, 4.41 mmol) was dissolved in 15 ml of dry DCM and added dropwise to a solution of ethylene diamine (5.9 ml, 88.2 mmol, 20 equiv) in 10 ml of dry DCM. After stirring at room temperature for 1 h, the reaction was quenched by addition of 10 ml of H_2O. The mixture was extracted with DCM (3 × 10 ml) and the combined organic layers were dried over $MgSO_4$. The mixture was concentrated to afford a crude product as a light yellow oil (.88 g, 80%). Without further purification, this product (.88 g, 3.5 mmol) was dissolved in 10 ml of dry DCM and added to a solution of di-tert-butyl dicarbonate (1.08 g, 4.94 mmol, 1.4 equiv) in 10 ml of dry DCM. The mixture was stirred at room temperature for 16 h when TLC analysis confirmed complete consumption of the amine. The reaction was then quenched with 10 ml of H_2O and the reaction mixture extracted with DCM (3 × 10 ml). The combined organic layers were dried over $MgSO_4$, concentrated to afford a white powder (1.15 g, 94%). Without further purification, this product (.65 g, 1.8 mmol) was dissolved in 10 ml of dry DCM and HCl

(1 ml of a 4.0 M solution in dioxane [excess]) added. The mixture was then stirred at room temperature for 16 h when TLC analysis (hexane:ethyl acetate, 2:1) showed complete consumption of starting material. After concentrating under vacuum, the solid obtained was washed with diethyl ether, filtered and dried under vacuum overnight to afford the title salt as a white solid (.92 g, 65%). M.p: 178.8–179.3, V_{max} (ATR): (N-H): 3022, 1154, 1130, 1021, 777 cm^{-1}. δ_H (400 MHz, D_2O): 8.55 (d, 1H, $J = 8.0$ Hz, Ar-*H*), 8.28 (d, 1H, $J = 7.8$ Hz, Ar-*H*), 8.25 (d, 1H, $J = 7.8$ Hz, Ar-*H*), 8.13 (d, 1H, $J = 8.0$ Hz, Ar-*H*), 7.81 (t, 1H, $J = 8.0$ Hz, Ar-*H*), 7.74 (t, 1H, $J = 8.0$ Hz, Ar-*H*), 7.68 (t, 1H, $J = 7.8$ Hz, 3-*H*), 3.15 (m, 2H, CH_2), 3.10 (m, 2H,CH_2). δ_C (D_2O, 400 MHz): 135.3 (C-Ar), 134.1 (C-Ar), 132.1 (C-Ar), 129.9 (C-Ar), 129.5 (C-Ar), 128.8 (C-Ar), 127.3 (C-Ar), 127.1 (C-Ar), 124.4 (C-3), 123.2 (C-Ar), 39.8 (CH_2), 39.14 (CH_2). LRMS (ES^+): m/z 251 (M + H), HRMS ($ASAP^+$): Found M + H, 251.0854, $C_{12}H_{15}N_2O_2S$, requires *M* 251.0856. Elemental analysis: Calculated for $C_{12}H_{15}ClN_2O_2S$ C, 50.26; H, 5.27; N, 9.77. Measured C, 50.32; H, 5.27; N, 9.69.

4.2 | Plant materials and growth

A. thaliana wild-type seeds were from laboratory stocks of *Columbia* (*Col-0*) and *Landsberg erecta* (Ler-0) accessions indicated as wild type (WT). The mutants of *gid1* (Griffiths et al., 2006), in Col-0 background, were obtained from Dr. Steve Thomas (Rothamsted Research, UK), whilst *pif 4-101* (Franklin et al., 2011) and *pif 4-2* (Press et al., 2016), in *Col-0* background, and the *della* quintuple mutant (Feng et al., 2008) and *ga1-5* (Fridborg et al., 1999), in *Ler-0* background, were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The *A. thaliana* line expressing RGA-GFP (Silverstone et al., 2001), in *Col-0* background, was obtained from Prof. Keith Lindsey (Durham University, UK).

Seeds were plated out 1× MS medium pH 5.8 (Murashige & Skoog, 1962) in petri dishes with a concentration of either 0.8% or 1.2% (w/v) phytoagar for root and hypocotyl measurements, respectively. After sowing, seeds were stratified on plates at 4°C for a minimum of 48 h to achieve synchronous germination. Seedlings were the grown for either 7 or 2 days for root and hypocotyl measurements, respectively, prior to treatment with chemicals. For hypocotyl measurements, seeds were sown on nylon mesh (acid resistant monofilament nylon filter mesh fabric; GZ, model number H20M). All seedlings were grown in a Percival (CU-36L5D, CLF plant climatics, Emersacker, Germany) with a photoperiod of 16/8 h with a light intensity of either 150 μmol m^2 s^{-1} or 50 μmol m^2 s^{-1} for root and hypocotyl measurements, respectively, and a temperature of 20 ± 1°C.

4.3 | Root and hypocotyl measurements

After growing as described above, seedlings for root assays were transferred to 1.2% (w/v) agar plates containing each chemical at a final concentration of 100 μM (the addition of the chemicals was performed when media had cooled to 50°C after autoclaving), with an equivalent concentration of DMSO as control. At this stage, the positions of the root tips were marked on the petri dishes. The plants were subsequently grown vertically, and after a further 5 days, images of the plates were scanned and the root growth that occurred during the 5 days on chemicals was measured using ImageJ software (18 seedlings were measured for each treatment). For hypocotyl measurements, the assay performed was adapted from de Lucas et al. (2008). After growing seedlings as described above, the nylon mesh was transferred across to the plates containing chemical. The plates were then continued to place in vertical orientation for 3 days under reduced light intensity (by covering plates with two layers of 80 g m^2 white paper) before the plates were scanned. The measurement of hypocotyl was performed using ImageJ software (at least 15 seedlings were measured for each plate).

4.4 | Fresh and dry weight measurements

Leaves and roots fresh weights of 12 days old seedlings, which were subjected to 5 days chemical treatment as described above, were measured. Dry weights were recorded after placing the plant material in oven at 65°C for 3 days. The measurements were performed on 15 seedlings for each treatment.

4.5 | Confocal laser scanning microscopy techniques

Confocal microscopy was performed using a Leica SP5 CLSM FLIM FCCS (Leica Microsystems, Wetzlar, Germany). GFP:RGA seeds were germinated and grown on 1.2% MS vertically for 7 days and then incubated in chemical solution (at the final concentration of 100 μM) for 2 and 24 h before being analyzed. At least five roots were imaged for each time point. The excitation wavelength of the argon laser was 488 nm, and the emission was detected using a bypass filter of 495–550 nm. The fluorescence intensity of the images (1024 × 1024 pixel size) was measured using Leica software, LAS AF Lite.

4.6 | Statistical analysis

Data are shown as means ± standard errors (SEs). A *t*-test was performed to compare the hypocotyl and root length within each genotype between chemical treatment and control conditions (*p value < .05; **p value < .01). The normality of data distribution was tested using the Shapiro–Wilk normality test. Significant differences were analyzed using a one-way analysis of variance (ANOVA). SigmaPlot was used for the analysis (Systat Software Inc., San Jose, USA).

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

M.R.K. and P.G.S. conceived the project and original research plans; M.R.K. and P.G.S. supervised the experiments; M.R.K., N.A.S., P.G.S., and S.P. designed the experiments. N.A.S. performed most of the experiments, and S.P. performed experiments presented in Figure 3; M.R.K. and N.A.S. wrote the article with contributions from all the authors; M.R.K. agrees to serve as the author responsible for contact and ensures communication.

DATA AVAILABILITY STATEMENT

The data and other finding of this study are available from the corresponding author upon reasonable request.

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