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TECHNICAL ADVANCE

Ratiometric gibberellin biosensors for the analysis of signaling dynamics and metabolism in plant protoplasts

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SUMMARY

Gibberellins (GAs) are major regulators of developmental and growth processes in plants. Using the degradation-based signaling mechanism of GAs, we have built transcriptional regulator (DELLA)-based, genetically encoded ratiometric biosensors as proxies for hormone quantification at high temporal resolution and sensitivity that allow dynamic, rapid and simple analysis in a plant cell system, i.e. Arabidopsis protoplasts. These ratiometric biosensors incorporate a DELLA protein as a degradation target fused to a firefly luciferase connected via a 2A peptide to a renilla luciferase as a co-expressed normalization element. We have implemented these biosensors for all five Arabidopsis DELLA proteins, GA-INSENSITIVE, GAI; REPRESSOR-of-ga1-3, RGA; RGA-like1, RGL1; RGL2 and RGL3, by applying a modular design. The sensors are highly sensitive (in the low pM range), specific and dynamic. As a proof of concept, we have tested the applicability in three domains: the study of substrate specificity and activity of putative GA-oxidases, the characterization of GA transporters, and the use as a discrimination platform coupled to a GA agonists' chemical screening. This work demonstrates the development of a genetically encoded quantitative biosensor complementary to existing tools that allow the visualization of GA *in planta*.

Keywords: gibberellin, gibberellin metabolism, phytohormone signaling, plant protoplasts, quantitative ratiometric biosensors, technical advance.

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INTRODUCTION

The phytohormone gibberellin (GA) plays a major role in a plethora of developmental and growth processes such as seed germination, vegetative growth and flowering (Davière & Achard, 2013). In plants, there is a great number of non-bioactive GAs, including catabolites or intermediates, which are transported and converted into only a few bioactive GAs (Yamaguchi, 2008). The biosynthesis of the bioactive gibberellins GA_1, GA_3, GA_4 and GA_7 is a complex, multi-stepped process involving different enzymes, including various GA-oxidases (Hedden & Phillips, 2000; Yamaguchi & Kamiya, 2000) (Figure 1a).

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Perception of bioactive GAs triggers a signal relay eventually leading to changes in gene expression. Three key components are involved in GA perception and signaling: (i) the GA receptor GA INSENSITIVE DWARF1 (GID1), with three family members in *Arabidopsis thaliana* (GID1a, b and c) (Nakajima et al., 2006; Ueguchi-Tanaka et al., 2005), (ii) an F-Box protein constituent of an SCF E3 ubiquitin-ligase complex, encoded by two genes in Arabidopsis (*SLEEPY1* [*SLY1*] and *SNEEZY*) (Ariizumi et al., 2011; McGinnis et al., 2003) and (iii) the transcriptional regulator DELLA, composed by a family of five members in Arabidopsis (GA-INSENSITIVE,GAI; REPRESSOR-of-ga1-3, RGA; RGA-like1, RGL1; RGL2 and RGL3) (Achard & Genschik, 2009; Davière & Achard, 2013; Peng et al., 1997; Silverstone et al., 2001). The binding of GAs to the receptor leads to the formation of a complex between GA-GID1, DELLA and SLY1 which causes the polyubiquitylation and proteasome-mediated degradation of the DELLA protein, thereby triggering GA downstream signaling responses (Ariizumi et al., 2008; Murase et al., 2008) (Figure 1b).

To date, GA levels are typically quantified by analytical methods, e.g. mass spectrometry, which require the disruption of the tissue and does not allow dynamic analysis (Okamoto et al., 2009; Urbanová et al., 2013). Although a first set of GA-sensitive molecular devices have been developed to monitor *in vivo* bioactive GA distribution in plants, e.g., a Förster Resonance Energy Transfer (FRET) biosensor (Rizza et al., 2017), or to manipulate cellular processes using GAs via hormone activated Cas9-based repressors (Khakhar et al., 2018), other *in vivo* GA-related studies are hindered by the lack of reliable, versatile, and easy-to-use biosensors in plant cells. Such studies include the analysis of the temporal dynamics of bioactive and non-bioactive GA compounds, the characterization of GA-related metabolic activities, or the identification of physiologically relevant GA transporters. For instance, putative GA transporter activities have been mainly studied in *Xenopus* oocytes or the yeast *Saccharomyces cerevisiae*, which have the limitations of being non-plant systems (Corratgé-Faillie & Lacombe, 2017).

Using the degradation-based signaling mechanism described above, we built genetically encoded ratiometric biosensors engineered from each of the five different DELLAs by applying a modular design. We have implemented the biosensors in Arabidopsis protoplasts and tested them in various contexts to perform a thorough analysis of specificity, sensitivity and dynamics of each one towards different bioactive GAs and known precursors, and profit from the high sensitivity (up to low pM-range) to answer questions on GA metabolism, transport and signaling. These include the involvement of certain GA-oxidases in the GA biosynthesis process, as well as the potential use as a quantitative chemical screening platform in protoplasts.

RESULTS

Design of five genetically encoded distinct GA quantitative biosensors

To provide a quantitative proxy of the variation of hormone levels at high sensitivity and temporal resolution and to allow dynamic analyses in a plant cell system, we designed five degradation-based ratiometric GA biosensors using the intrinsic perception machinery for GAs of the DELLAs (Figure 1b,c). We followed the molecular engineering principles described previously (Samodelov et al., 2016; Wend et al., 2013). For this, we employed the full-length cDNA of the five different DELLAs of *Arabidopsis thaliana*, GAI/RGA/RGL1/RGL2/RGL3, as sensor modules (SM) and fused them to the firefly (FF) luciferase to monitor their degradation. A renilla (REN) luciferase was utilized as a normalization element and connected via a 2A peptide to the SM-FF fusion which enables their stoichiometric co-expression and leads to a decrease in FF luminescence relative to REN luminescence upon hormone induction (Figure 1c). The separation of SM-FF and REN by a self-processing 2A peptide allows for co-translational cleavage and the expression of all sensor elements from the same transcript (Ryan & Drew, 1994; Wend et al., 2013). In addition, two controls were used, replacing the SM by: (i) a short repetitive sequence, GAGAGAGAGA GAGA, that is not degraded in the presence of the hormone and termed CtrlQuant (Samodelov et al., 2016) and (ii) a RGA version with a 17 amino acid deletion in its N-terminal region (RGAΔ17) (Dill et al., 2001; Murase et al., 2008), which has been demonstrated to impair its interaction with GID1 and therefore the GA-induced degradation *in vivo*.

Figure 1. Biosynthesis and deactivation of bioactive gibberellins (GAs) *in planta*, GA perception mechanism in *Arabidopsis thaliana* and GA biosensor design.
(a) The bioactive gibberellins GA_1, GA_3, GA_4 and GA_7 are synthesized from their precursors, GA_{20} or GA_9, in a single-step or multi-step conversions mediated by GA 3-oxidases. GA 2-oxidases catalyze the catabolic deactivation of GA_1 and GA_4.
(b) Schematic overview of the GA perception mechanism in *A. thaliana*. Upon binding of GAs to the co-receptor GID1, DELLAs associate with the SLY1 and SKP1/CUL1/F-box E3-ubiquitin ligase complex (SCF^{SLY1}) and become polyubiquitylated (U), and targeted for degradation by the 26S proteasome. As a consequence, DELLA-bound transcription factors (TFs) are released and are able to bind to specific promoter regions thereby regulating gene expression (only the sequestration mechanism is shown for simplicity). goi, gene of interest.
(c) The five GA biosensor constructs contain one of each DELLA as a sensor module (SM) fused to a firefly luciferase (FF). A 2A peptide connects a renilla luciferase (REN) as a normalization element with the DELLA-FF fusion which leads to stoichiometric co-expression of both polypeptides. As a consequence of GA induction, DELLA-FF becomes ubiquitylated and consequently degraded, whereas REN levels remain constant, which leads to a decrease in FF/REN ratio.

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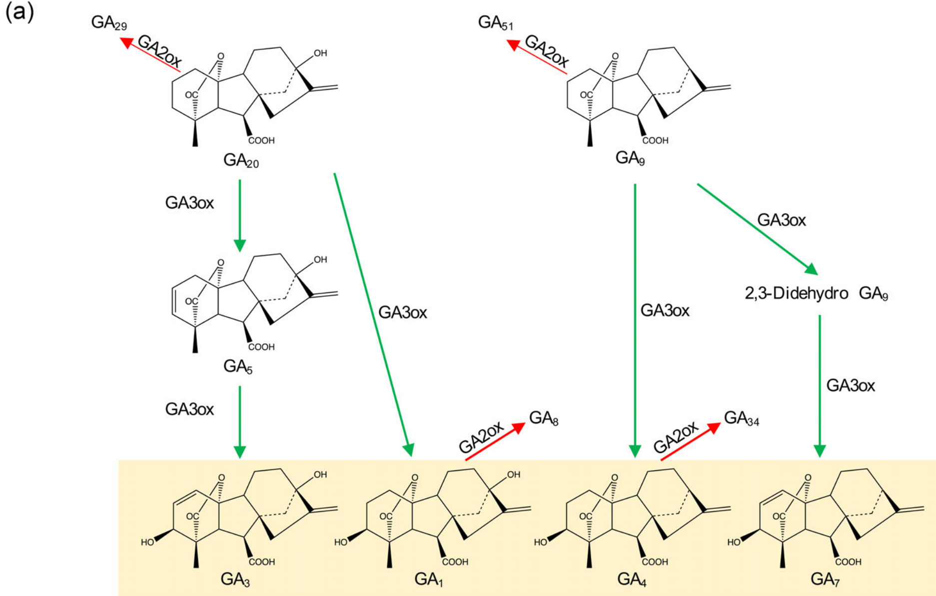
(a)



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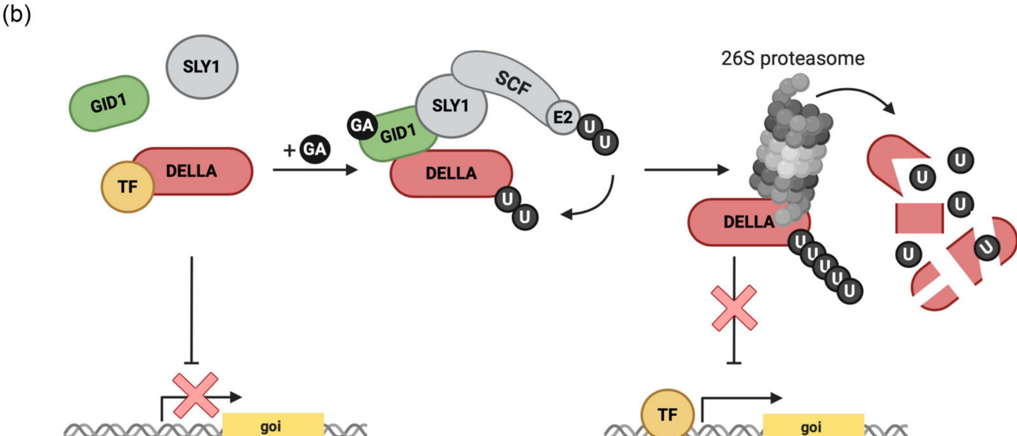

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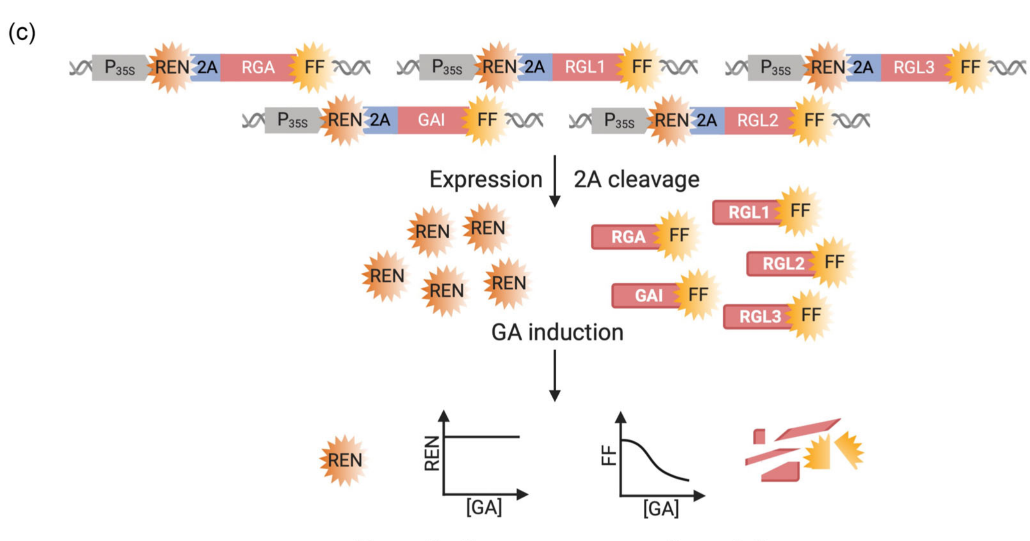

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Sensitivity and specificity analysis of the different DELLA-based biosensors towards bioactive GAs in Arabidopsis protoplasts

To analyze the specificity and sensitivity of the DELLAs towards the different bioactive GAs, the GA biosensors were expressed in Arabidopsis wild-type protoplasts. As a control, we used the GA-insensitive version RGAΔ17. After 5 h incubation with increasing concentrations of GAs (from 10 pM to 10 μM), the firefly and renilla luciferase activities were determined, and the FF/REN ratios were analyzed.

All DELLAs showed a decrease in FF/REN ratio with increasing concentrations of all four GAs tested, meaning that all five biosensors were responsive to these GA species (Figure 2). The CtrlQuant sensor (Figure S1) and the RGAΔ17 biosensor showed no degradation (Figure 2; Figure S3). The various DELLA-based gibberellin biosensors showed high sensitivity, although with differential behavior towards distinct GAs, especially at lower hormone concentrations, up to the low pM range. As a comparison, already existing sensor systems in *Xenopus* oocytes, the yeast *S. cerevisiae* or *in planta* have a working range in the nM to μM concentrations (Corratgé-Faillie & Lacombe, 2017; Rizza et al., 2017). All DELLAs (except RGL3) showed significant reductions in the pM range of GA_4 and GA_7 which is in agreement with the role of GA_4 being the major bioactive GA in Arabidopsis (Yamaguchi & Kamiya, 2000). Induction with GA_1 and GA_3 resulted in lower degradation rates (mostly in the nM range) (Figure 2).

The RGA sensor showed the highest sensitivity towards the different GAs with significant reductions starting at 10 pM for GA_4 or GA_7, and over 50% degradation at low nM concentrations for GA_4.

There were also large differences in maximum degradation among the different DELLAs, from 40% for the RGL3 biosensor to almost 70% degradation of the RGA biosensor when incubated with 1–10 μM GA_4 for 5 h. Additional experiments with the proteasomal inhibitor MG132 showed the degradation dependency on the 26S proteasome (Figure S2). To evaluate whether the presence of endogenous GAs might interfere with sensor activity and detection thresholds, we incubated the protoplasts with the GA biosynthesis inhibitor paclobutrazol (PAC). We observed no difference in basal sensor activity (Figure S4) therefore the effects observed after GA addition will depend on a previously established steady-state level of the biosensors. Thus, the high sensitivity of the RGA biosensor, in comparison with the other four DELLA-based sensors and also to other previously reported GA biosensors, coupled to a very high reproducibility of the assays, makes this particular biosensor a very valuable tool for further GA studies in protoplasts.

Characterization of the dynamic behavior of the RGA biosensor

To further characterize the most sensitive GA biosensor, namely the RGA-based SM, time-response analyses were performed. For this, Arabidopsis wild-type protoplasts were transformed with the RGA biosensor and, after 20 h, incubated with increasing concentrations of GA_4 (from 100 pM to 1 μM). Luciferase activity was then determined by taking samples at 30, 60, 120, 240 and 480 min of incubation with the hormone (Figure 3). After 30 min, a 20% degradation at high GA_4 concentration (μM range) was observed. Significant degradation of the RGA-FF fusion at low GA_4 concentrations (pM range) started after 240 min. Finally, after 480 min, a significant decrease (20%) in the FF/REN ratio at low GA_4 concentrations (pM range) occurs. Additionally, the maximum RGA-FF degradation was reached, ca. 60%, starting at low nM GA_4 concentrations. The characterization of the system's time dependence demonstrated that RGA not only displays high sensitivity and specificity towards GA_4, but also a fast degradation response. For all these reasons, we set out to test its possible uses in the context of biological questions in proof of principle experiments using plant protoplasts.

Use of the GA biosensor to test the activity and fate of GA precursors

It has been previously shown by surface plasmon resonance studies that OsGID1 (the rice GID1 receptor) largely discriminates between different GA compounds. For example, its specificity is 100-fold higher towards the bioactive GA_1 or GA_4 than towards the GA_9 precursor (Yoshida et al., 2018). Following this, we reasoned that the high sensitivity of the sensor should allow to test the capacity of protoplasts to metabolize GA_9 and other precursors to bioactive GAs such as GA_4: degradation of the sensor upon incubation with the precursors would necessarily inform on how they have been converted into bioactive GAs. Therefore, we tested two different known GA precursors, namely GA_{20} and GA_9, which are catalytically transformed into GA_1 and GA_4, respectively (Hedden & Thomas, 2012) (Figure 1a). Arabidopsis wild-type protoplasts were transformed with the DELLA biosensors and then incubated with increasing concentrations of the two GA precursors. The FF/REN ratios were determined after 5 h of incubation. All five DELLAs showed a decrease in the FF/REN ratio when incubated with GA_9, although large differences in sensitivity were observed (Figure 4). The RGA biosensor showed again the highest sensitivity towards GA_9 with significant reduction at low nM levels and 50% degradation at high GA_9 concentrations (100 nM), whereas RGL2 showed the lowest sensitivity with only 30% degradation at high GA_9 concentrations (1 μM). The incubation with the GA_1 precursor GA_{20} resulted in only a reduced degradation of

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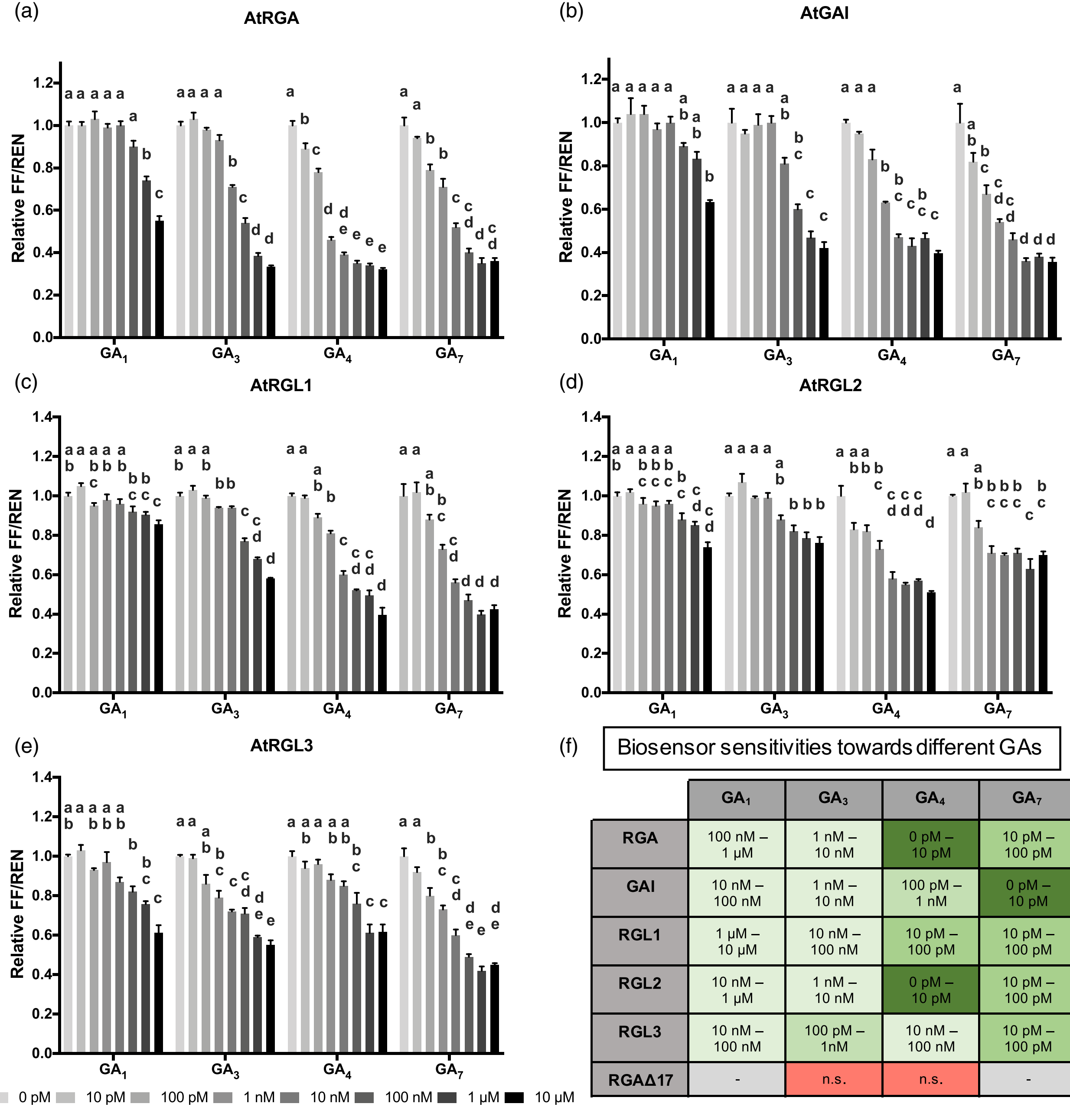

Figure 2. Sensitivity and specificity of RGA-, GAI-, RGL1-, RGL2- and RGL3-based biosensors towards bioactive gibberellins.
Arabidopsis thaliana wild-type protoplasts were transformed with the different sensor constructs comprising either RGA (A), GAI (B), RGL1 (C), RGL2 (D) or RGL3 (E) as a sensor module (SM). Twenty hours after transformation, the protoplasts were supplemented with serial dilutions ranging from 10 pM to 10 µM of either GA_1, GA_3, GA_4 or GA_7, for 5 h. Afterwards, the luciferase activity was determined. The error bars represent the SEM (n = 6). One-way analysis of variance (ANOVA) was performed with $P < 0.05$ (for RGL1, 2 and 3 with GA_1) or $P < 0.01$.
(F) Color-coded table summarizing the biosensor sensitivities towards the different bioactive gibberellins (dark green: sensitivity lower than 10 pM, green: sensitivity between 10 and 100 pM, lime-green: sensitivity between 100 pM and 1 nM, lime-green shade: sensitivity higher than 1 nM, red: not significant). The range in the cells is determined considering the two lowest concentration values between which there are statistically significant differences (from a to b in the ANOVA) in the degradation of the sensor. The table includes also the RGAΔ17 sensor as a control.

the DELLAs. Only RGA, GAI and RGL3 showed a significant decrease in the FF/REN ratio at high GA_{20} concentrations (100 nM–1 µM), whereas RGL1 and RGL2 showed only minor degradation at µM concentrations only. These results imply that the precursors are indeed converted into their bioactive products during the GA incubation time, and the comparison between the sensor activity towards the precursors and towards the bioactive GAs indicates that GA_9

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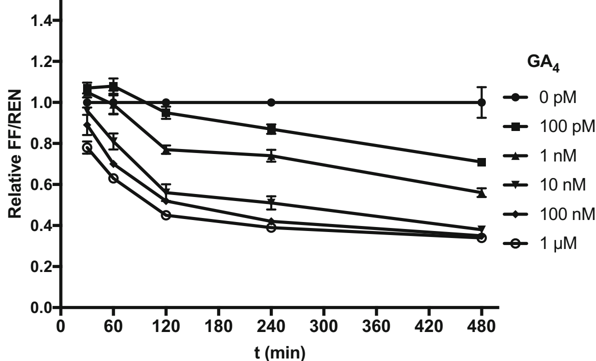

Figure 3. RGA biosensor kinetic analysis.
Arabidopsis thaliana wild-type protoplasts were transformed with the RGA biosensor construct. Twenty hours after transformation, the protoplasts were supplemented with serial dilutions ranging from 100 pM up to 1 μM of GA_4 for 30, 60, 120, 240 and 480 min before luciferase activity determination. The error bars represent the SEM for $n = 6$ replicates.

is more readily metabolized into GA_4, than GA_{20} into GA_1 in plant protoplasts provided that both precursors are transported at similar rates into the cell.

Fast characterization of GA 2-oxidases-dependent gibberellin metabolism

GA 2-oxidases (GA2ox) are major inactivation enzymes for bioactive GAs (Rieu et al., 2008). In Arabidopsis, GA2ox1,2,3,4 and 6 have been shown to act specifically against C19-GAs including GA_1 and GA_4 (Rieu et al., 2008; Thomas et al., 1999) (Figure 1a). They catalyze the 2-β-hydroxylation of GA_4 to GA_{34}, and of GA_1 to GA_8. GA2ox7 and 8, on the other hand, catalyze the 2-β-hydroxylation of C20-GAs, such as the common precursor GA_{12} (Schomburg et al., 2003).

We applied the biosensor to study the specificity and activity of three different GA2ox. Arabidopsis protoplasts were transformed with the RGA-based SM construct and a construct for the overexpression of either GA2ox1, GA2ox2 or GA2ox8, and incubated 20 h post transformation with GA_1 and GA_4 at a concentration range between 1 nM and 10 μM. As a control, the RGA sensor construct was co-transformed with a control plasmid (not coding for any oxidase). As depicted in Figure 5(A), the FF/REN ratio when incubating with a range of GA_1 concentrations showed no significant difference in the degradation of RGA between the control condition and GA2ox1, GA2ox2 and GA2ox8, indicating that the sensor is not detecting a relevant difference that might point towards the oxidases acting on GA_1 (i.e. catabolizing it to a non-bioactive GA).

The broad dynamic range and high sensitivity of the RGA sensor towards GA_4 allowed us to analyze the effect of GA2ox enzymes also at lower and higher concentrations. By overexpressing either GA2ox1 or GA2ox2, we observed a reduced degradation of the sensor compared to the control. In particular, GA2ox2 seems to have a higher capacity to act on GA_4. This effect was still visible even at 100 nM–10 μM concentrations, whereas GA2ox1 activity was overcome at 10–100 nM GA_4 (Figure 5B). We could show in this protoplast system that GA2ox1 and GA2ox2 inactivate the bioactive C19-GA GA_4 and convert it to non-bioactive catabolites which are no longer able to induce the degradation of RGA. GA2ox8, which should act on C20 GAs only (Schomburg et al., 2003), did indeed not have a direct effect on GA_1 or GA_4. In summary, this illustrates how GA biosensors can be utilized to test the activity of GA-deactivating enzymes, therefore opening up the possibility to distinguish the specificity of naturally occurring or engineered GA 2-oxidases.

Use of the GA sensor to characterize putative GA analogs

Chemical screenings have opened the possibility to find novel hormone-like compounds with applications both in research and in agriculture (Dejonghe & Russinova, 2017; Hicks & Raikhel, 2012; Rigal et al., 2014). Among the compounds found to date, different classes have been described: some act as hormone analogs that interact with the receptors (He et al., 2011; Park et al., 2009), while others interfere with the stability or the activity of components of the signaling pathway (De Rybel et al., 2009; Ye et al., 2016). Distinguishing between these and other possibilities is a limiting stage in the characterization of the compounds, to which our sensor system can contribute.

Reduction of GA biosynthesis using PAC has been shown to promote photomorphogenic development in seedlings growing in darkness, which then display unfolded cotyledons and hypocotyl stunting (Alabadí et al., 2004, 2009). This defect can be reverted by the application of GA_3, and we screened 10 000 compounds of the DIVERSet™ collection searching for those that could mimic the effect of GA_3. Eight compounds were found to consistently restore hypocotyl elongation at a concentration of 20 μM, in the presence of 0.4 μM PAC (Figure 6A; Table S1). The rescue was also observed at concentrations as low as 1 μM, however none of the compounds matched the efficiency of 20 μM GA_3 at any concentration (Figure 6B–D).

To determine if the compounds behaved as GA analogs, we tested if they were able to trigger DELLA degradation. For this, we compared the analysis of GFP-RGA stability in seedlings by Western blot, to the use of the GA sensor developed here. First, 7-day-old GFP-RGA seedlings grown on 5 μM PAC were treated with 20 μM of each compound or GA_3 for 4 h, and the levels of GFP-RGA were compared with those of untreated seedlings. We observed that only GA_3 caused a reduction of GFP-RGA (Figure 6E). Second, Arabidopsis protoplasts were transformed with the RGA biosensor and, 20 h after transformation, the protoplasts were supplemented with 1 μM GA_4 or each of the compounds for 4 h, and the FF/REN ratio was determined.

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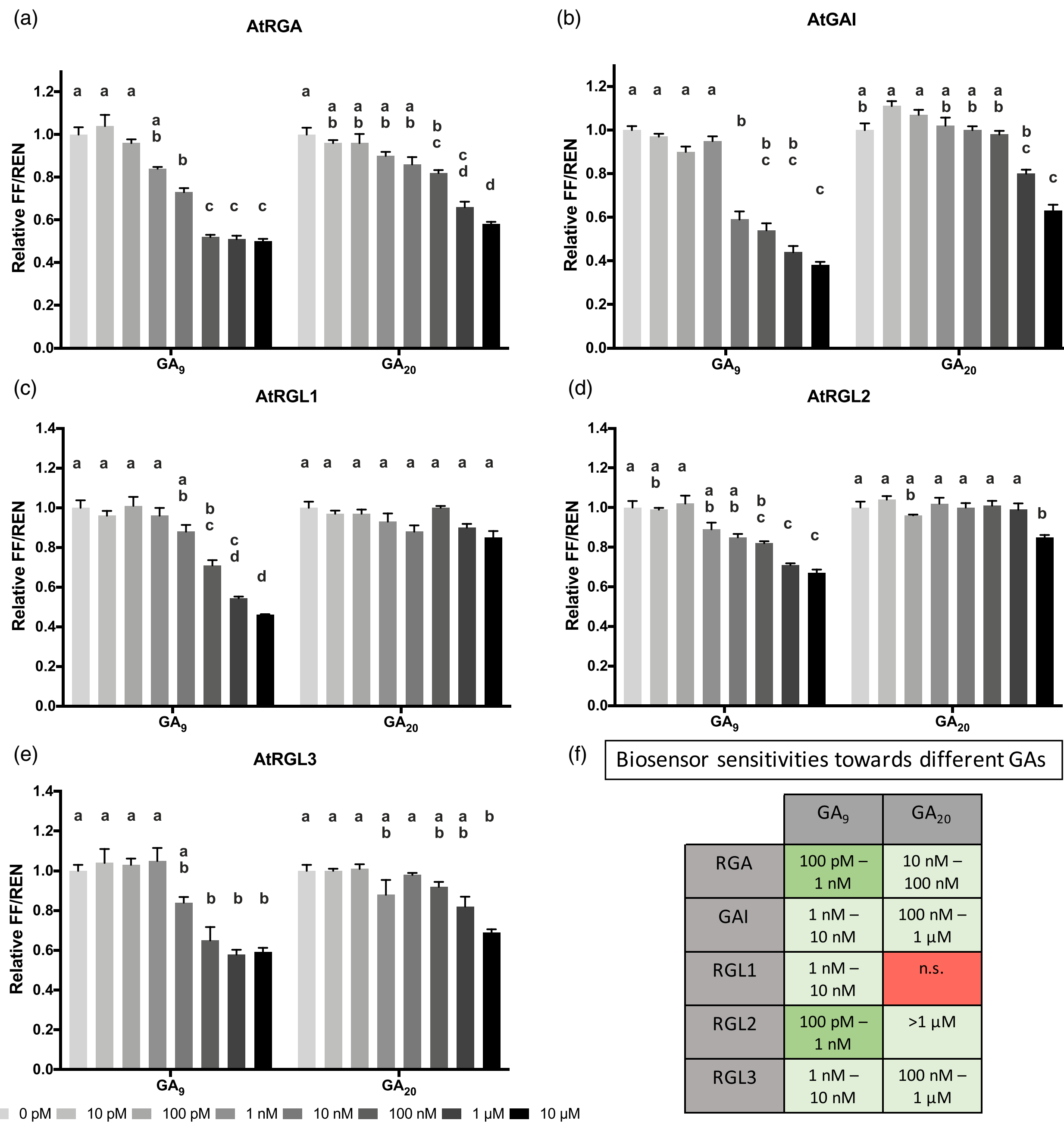

	GA_9	GA_{20}
RGA	100 pM – 1 nM	10 nM – 100 nM
GAI	1 nM – 10 nM	100 nM – 1 µM
RGL1	1 nM – 10 nM	n.s.
RGL2	100 pM – 1 nM	>1 µM
RGL3	1 nM – 10 nM	100 nM – 1 µM

Figure 4. Sensitivity and specificity of RGA, GAI, RGL1, RGL2 and RGL3 upon two known precursors of bioactive GAs, GA_9 and GA_{20}.
Arabidopsis thaliana wild-type protoplasts were transformed with the different sensor constructs comprising either RGA (A), GAI (B), RGL1 (C), RGL2 (D) or RGL3 (E) as a sensor module (SM). Twenty hours after transformation, the protoplasts were supplemented with serial dilutions ranging from 10 pM to 10 µM of either GA_9 or GA_{20} for 5 h. Afterwards, the luciferase activity was determined. The error bars represent the SEM (n = 6). One-way analysis of variance (ANOVA) was performed with $P < 0.05$ (for GAI with GA_{20} and RGL2 with GA_9) or $P < 0.01$.
(F) Table summarizing the biosensor sensitivities when incubated with the different bioactive gibberellins (green: sensitivity between 10 and 100 pM, lime-green: sensitivity between 100 pM and 1 nM, lime-green shade: sensitivity higher than 1 nM; red: not significant). The range in the cells is determined considering the two lowest concentration values between which there are statistically significant differences (from a to b in the ANOVA) in the degradation of the sensor.

In agreement with the whole seedling assays, only GA_4 triggered degradation of the sensor (Figure 6F). These results suggest that the compounds promote hypocotyl elongation through a GA-independent pathway or downstream of DELLA activity. More importantly, they indicate that the sensor system can be used as a streamlined alternative for the time-consuming and less sensitive western blot analyses of DELLA protein stability.

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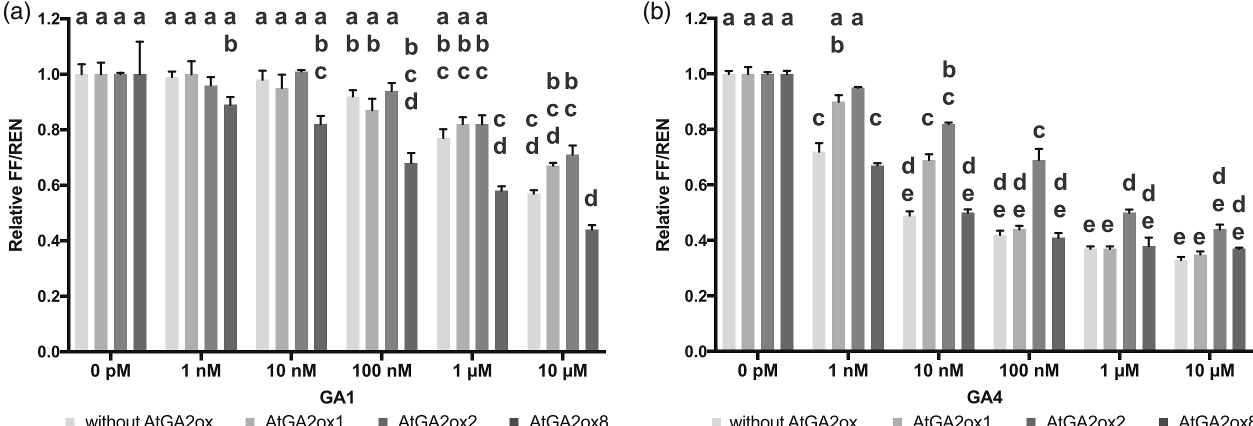

Figure 5. RGA biosensor as a tool to study the activity and specificity of GA oxidases in plant cells.
Arabidopsis thaliana wild-type protoplasts were transformed with the RGA biosensor construct and an additional GA 2-oxidase (either GA2ox1, GA2ox2, GA2ox8 or a control). Twenty hours after transformation, the protoplasts were supplemented with serial dilutions from 1 nM to 10 µM of GA_1 (A) or GA_4 (B) for 4 h. Afterwards, luciferase activity was measured. The error bars represent the SEM ($n = 6$). One-way analysis of variance (ANOVA) and multiple comparisons for statistical significance were performed with $P < 0.05$.

GA transporter activity studies in plant cells

The importance of GA transport across membranes (either in short or long distances) has become undisputed in recent years (Binenbaum et al., 2018). In general, two systems are extensively used to analyze transporter activities and substrates: *Xenopus* oocytes and the yeast *S. cerevisiae* (Corratgé-Faillie & Lacombe, 2017). We set here to test whether the GA biosensors developed are applicable to characterize the activity, specificity and directionality of flow, of putative GA transporters in protoplasts. The first GA transporter identified through a direct genetic screening, NPF3, belongs to the large NRT1/PTR FAMILY (NPF) and it has already been shown to transport GA_3 and GA_4 (among other substrates) (Chiba et al., 2015; David et al., 2016; Tal et al., 2016). However, these tests have not been performed in plant cells or at physiologically relevant GA levels yet. We therefore co-transformed protoplasts with the RGA biosensor and either NPF3 or a control without transporter activity, and incubated the cells with a range of GA_3 and GA_4 concentrations for 2 and 4 h. Afterwards, the luciferase activity was determined. No qualitative differences were observed between the two incubation times, indicating that the equilibrium between external and internal GAs had been reached already at 2 h (Figure 7; Figure S5). The results also suggest that GA_3 is transported by NPF3 into the protoplasts (Figure 7A), and this activity is detectable at low and high GA_3 concentrations (range of 100 pM to 100 nM). At high pM/low nM concentrations, there was a ca. 40% stronger decrease in FF/REN ratio in the presence of the NPF3 transporter. For GA_4, no difference in sensor degradation was observed in the presence of NPF3 (Figure 7B) suggesting that the transport of GA_3 into plant protoplasts via NPF3 is a limiting step.

DISCUSSION

The quantitative monitoring and study of the dynamic analysis of intracellular GA levels is still challenging with current analytical and molecular biology tools. The use of such measurements is not only the *in situ* observation of the variation of endogenous GA concentrations in intact plants, which can be done with existing tools (Khakhar et al., 2018; Walia et al., 2018). Another important application that cannot be achieved with existing reporters is the investigation in plant cells of GA metabolism and signaling events that indirectly require large-scale quantitative GA monitoring. To overcome the limitations of existing tools, we built five genetically encoded ratiometric GA biosensors based on the intrinsic GA-induced DELLA proteasomal-mediated degradation mechanism which is the first molecular event caused by the interaction between the hormone and its receptor. Compared with alternative tools (Khakhar et al., 2018; Walia et al., 2018), this biosensor allows dynamic visualization of the direct effects caused by GA perception in plant cells with the ease of transient expression assays, scaling up compatibility, as well as the highly sensitive analysis (low pM range) of intracellular changes upon exogenous application of GAs within plant cells.

This system has the advantage of providing a convenient platform for fast experimental testing of hypotheses related to GA metabolism, transport and signaling, and therefore is complementary to biosensors aimed at the quantification of endogenous hormone levels in intact tissue based on FRET approaches. As shown here, these GA biosensors can be utilized as quantitative molecular proxies to investigate, among other questions, (i) the specificity of GA-inactivating enzymes with respect to different

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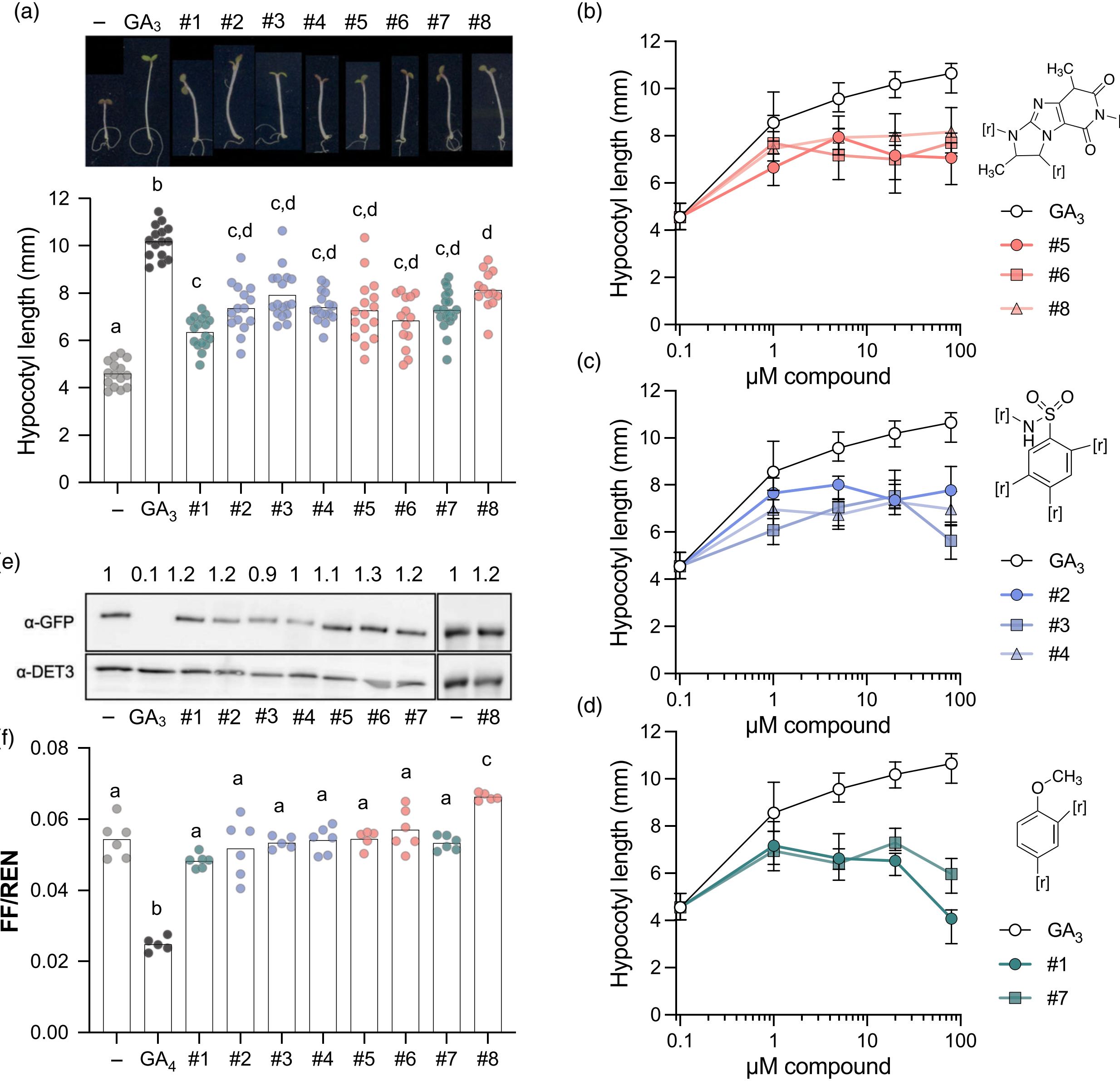

Figure 6. Analysis of putative GA analogs.
(A) Representative images and hypocotyl lengths of seedlings grown in the light for 4 days, followed by 4 days in darkness with 0.4 μM PAC and either mock, or 20 μM GA_3, or 20 μM of the indicated compounds (see Table S1 for identification). Error bars represent SEM ($n > 12$).
(B–D) Dose–response curves for hypocotyl elongation in 8-day-old seedlings grown in darkness with 0.4 μM PAC and the indicated concentrations of the compounds. Error bars represent SEM ($n > 12$).
(E) Western blot analysis of 8-day-old transgenic seedlings expressing pRGA::GFP-RGA grown in 5 μM PAC and treated with either mock, or 20 μM of GA_3 or the indicated compounds. Numbers indicate the GFP/DET3 signal ratio normalized to the mock control.
(F) *A. thaliana* wild-type protoplasts were transformed with the RGA biosensor and 20 h after transformation, the protoplasts were supplemented with 1 μM of GA_4, or the indicated compounds. Error bars represent SEM ($n = 6$). The compounds have been grouped based on the identical core of their chemical structure, which is shown on panels (B–D) (i.e.: the structures do not correspond to "selected compounds" but to the core of their structures). The differential features are depicted as R1, R2, etc, and the actual structure of every compound is shown in Table S1.

bioactive GAs; (ii) the ability of different GA molecules or potential synthetic and natural analogs to trigger GA perception events or (iii) the function of putative GA transporters. Beyond the proof-of-principle experiments shown here, further immediate applications can be easily implemented. For instance, the protoplast system is optimal for large-scale screening of compound libraries targeted at the identification of receptor agonists or antagonists, or compounds that interfere with other downstream stages of GA signaling. The conservation of the GA perception system among vascular plants (Hernández-García et al., 2020) also suggests that the GA biosensor can be used to study

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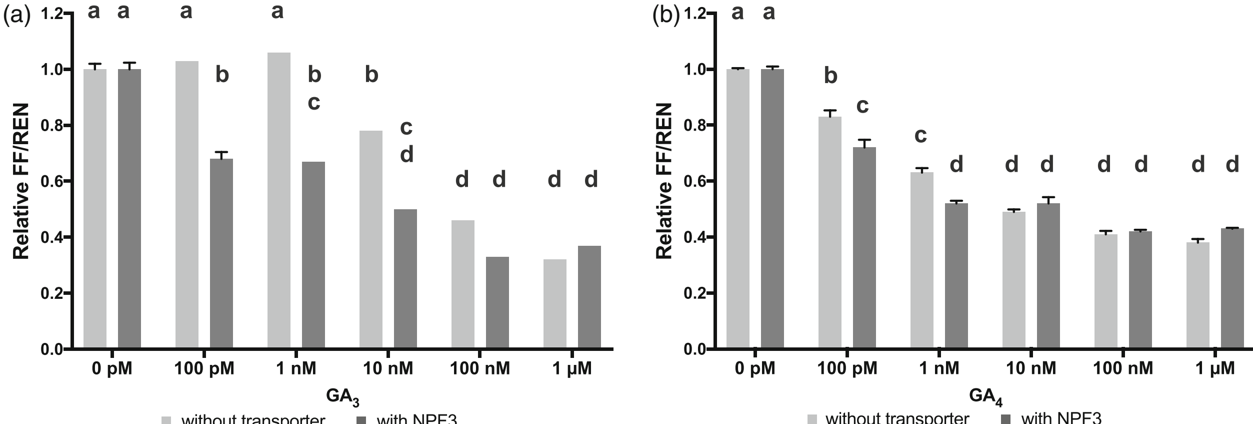

Figure 7. RGA biosensor as a tool to study GA transport processes in a plant system.
Arabidopsis thaliana wild-type protoplasts were co-transformed with the RGA biosensor construct and the NPF3 GA transporter, or a control. Twenty hours after transformation, the protoplasts were supplemented for 2 h with concentrations of GA_3 (A) or GA_4 (B) ranging from 100 pM to 1 μM. Afterwards, luciferase activity was measured. The error bars represent the SEM (n = 6).

elements from many other species (including enzymes, transporters or signaling components) in Arabidopsis protoplasts. And hypothesis testing can be strengthened by introducing this GA biosensor in protoplasts of different mutant backgrounds to study potential regulation of GA perception by other signaling pathways.

Apart from illustrating different applications of the GA biosensor, the assays described here have provided information on GA biology. It has been proposed that the three Arabidopsis GID1 receptors have different affinities for each of the five DELLAs (Suzuki et al., 2009). Our results show that the five biosensors display different sensitivities, and that these differences are not necessarily maintained in response to every bioactive GA molecule (e.g. RGL3 is the most sensitive sensor towards GA_3 and the least sensitive one to GA_4, whereas RGA is the most sensitive towards GA_4). Although there are several possible explanations (competition with the endogenous DELLAs, differential accumulation of each of the GID1 receptors in these cells, differential affinity, etc.), our system allows to describe the global behavior of the cells in terms of the intensity of the GA signal being triggered.

The fact that known GA precursors like GA_9 and GA_{20} induce a DELLA-FF degradation, implies that either these precursors are metabolized into their bioactive products in our plant cell system or alternatively directly detected by the sensors. However, we favored the former based on previous reports. By comparing lycophyte and angiosperm GA receptor affinities, it has been proposed that GID1 has evolved a stronger capacity to discriminate bioactive GAs from precursors or inactive GAs (Yoshida et al., 2018), so it is relatively safe to interpret the results with GA_9 and GA_{20} in terms of their conversion into GA_4 and GA_1, respectively. Thus, it is not surprising that GA_9 application is more effective than that of GA_{20}.

Using this sensor system, we have also provided alternative proof of the strong specificity of GA2ox1 and -2 on C19 GAs such as GA_4, whereas GA2ox8 had no influence. This had already been shown *in vitro* and inferred from Arabidopsis mutant analysis, GC/MS or expression analysis via RT–PCR (Rieu et al., 2008; Schomburg et al., 2003; Thomas et al., 1999), albeit the latter being either non/semi-quantitative or demand the disruption of the plant tissue.

We have also shown that our system is potentially useful to generate information about GA transporters. The observation that expression of NPF3 increases the sensitivity of the biosensor towards GA_3, but not GA_4, has two implications. First, our data show that Arabidopsis protoplasts have active GA transport capacity that is not limiting for GA_4, given that expression of NPF3 does not improve the perception of exogenous GA_4. This could be due to NPF3 and other NPF-family members with demonstrated GA transport ability (Chiba et al., 2015) or to SWEET13/14 (Kanno et al., 2016). Second, our data are in agreement with previous reports that support a much higher transport activity of NPF3 for GA_4, compared with GA_3 (Tal et al., 2016), indicating that the intrinsic capacity of Arabidopsis protoplasts to transport GA_3 is limited. This example highlights the potential applicability of the system to analyze the behavior of transporters towards different GA species, the direction of transport (import/export), and in addition provides a putative strategy for biotechnological improvement of GA action.

Finally, eight new compounds have been identified that mimic the GA-induced growth promotion of GAs, but that are not acting as GA analogs. In other words, they circumvent the growth arrest imposed by DELLA accumulation acting either downstream or in parallel to DELLAs. Several post-translational modifications have been shown

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to alter DELLA activity in a GID1-independent manner, such as *O*-fucosylation and *O*-GlcNAcylation (Zentella et al., 2016, 2017), and chemical interference with these processes might affect the activity, but not the stability of DELLAs. Alternatively, the activity of specific growth-related transcription factors or their interaction with DELLAs could be modulated by these chemical compounds, similar to what has been already extensively documented in other systems (Chen & Koehler, 2020).

In summary, although the conventional methods for analyzing GA contents need the disruption of tissues or demand complex and expensive preparation procedures, the protoplast system in combination with the sensors introduced here is relatively cheap and technically simple. In combination with other methods, such as genetic analyses, our new system depicts a useful completion for quantitative investigations of GA signaling and metabolic analyses. For instance, GA signaling components could be analyzed in mutant protoplasts as it was already done for strigolactone signaling (Samodelov et al., 2016). Future perspectives could be the expansion of this principle to the implementation of engineered fluorescence sensors in plants and luminescence sensors in an orthogonal system like mammalian cells (Wend et al., 2013), or the implementation of high-throughput platforms for screening purposes, including novel synthetic analogs or inhibitors.

MATERIALS AND METHODS

Plasmid construction

The expression vectors and cloning strategies are described in Tables S2 and S3.

Plant material, protoplast isolation and transformation

The seeding of the *A. thaliana* Col-0 seeds as well as the protoplast isolation were performed as previously described (Samodelov et al., 2016).

For the protoplast transformation, 30 μg of the sensor construct were adjusted to a volume of 20 μl with MMM Medium (MES, mannitol, and magnesium; 15 mM $MgCl_2$, 5 mM MES, 0.465 M mannitol [pH 5.8]). For the GA-oxidase or transporter studies, 15 μg of the GAoxidase, the transporter or a control plasmid were added to the sensor construct and then adjusted with MMM Medium to a volume of 20 μl.

The DNA was carefully mixed with 500 000 protoplasts in 100 μl of MMM solution and incubated for 5 min.

Afterwards, 120 μl of polyethylene glycol (PEG) solution (2.5 ml 0.8 M mannitol, 1 ml 1 M $CaCl_2$, 4 g PEG4000 (Sigma-Aldrich, Darmstadt, Germany), and 3 ml H_2O, prepared fresh for each experiment) were added in a dropwise manner. Finally, 120 μl MMM were supplemented, overlaid to a final volume of 1.8 ml per reaction with PCA (protoplast culture *Arabidopsis*, 0.32% [w/v] Gamborg B5 basal salt powder with vitamins (bioWORLD, US), 2 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, 0.342 mM L-glutamine, 58.4 mM sucrose, 550 mOsm with glucose, 4.2 μM Ca-pantothenate, 2% [v/v] biotin from a biotin solution of 0.02% [w/v] in H_2O, 0.1% [v/v] Gamborg B5 Vitamin Mix [pH 5.8], and 1:2000 ampicillin [stock solution: 1 mg ml^{-1}]). In this manner, multiple transformations were performed together and pooled before hormone induction.

Treatment with GA and luminescence analysis

The inducer substrates GA_1, GA_3, GA_4, GA_7, GA_9 and GA_{20} were obtained from OlChemim Ltd. (Olomouc, Czech Republic) and prepared as a 10 mM stock solution in ethanol. The proteasomal inhibitor MG132 (Sigma-Aldrich) was prepared as a 40 mM stock solution in dimethyl sulfoxide (DMSO) and added directly to the protoplasts 2 h before induction with GA at the final concentrations indicated. PAC (Duchefa, Haarlem, Netherlands) was prepared as a 10 mM stock in DMSO and added directly to protoplasts after transformation at a final concentration of 1 μM.

The general treatment with GAs and the luminescence analysis were performed as described in Samodelov et al. (2016) for strigolactones. Briefly, 20–24 h after transformation, the transformation replicates were pooled together and for each concentration of the inducer substrate and for each measuring time point, 960 μl protoplast solution were pipetted into a 2 ml deep-well storage plate (Corning). Serial dilutions of the inducer substrate GA_1, GA_3, GA_4, GA_7, GA_9 or GA_{20} were prepared in PCA at a 11-fold concentration of the desired final experimental concentration and 96 μl were mixed with 960 μl protoplast solution. The duration of the following GA incubation step depended on the type of analysis: 5 h for selectivity/specificity analysis towards different GAs, 4 h for transporter and GA2oxidase analysis and 30 min, 1, 2, 4 and 8 h for dynamic analysis of the RGA sensor.

For the luminescence determination, 80 μl of the induced protoplasts were pipetted into two separate white 96-well assay plates in order that firefly and renilla luminescence could be determined simultaneously in two plate readers. Before the measurement, 20 μl of firefly substrate (0.47 mM D-luciferin [Biosynth AG, Eching, Germany], 20 mM tricine, 2.67 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA·$2H_2O$, 33.3 mM dithiothreitol, 0.52 mM adenosine 5′-triphosphate, 0.27 mM acetyl–coenzyme A, 5 mM NaOH, 0.26 mM $MgCO_3 \cdot 5H_2O$, in H_2O) or coelenterazine (472 mM coelenterazine stock solution in methanol, diluted directly before use 1:15 in phosphate-buffered saline) were added to the samples. Firefly luminescence was determined in a Berthold Technologies Centro XS^3 LB960 Microplate luminometer, whereas renilla luminescence was determined in a Berthold technologies $Tristar^2S$ LB942 Multimode Plate Reader.

Chemical screening

Arabidopsis thaliana L*er* seeds were surface-sterilized and sown at a density of two seeds per well in 96-well microtiter plates filled with 100 μl of MS medium supplemented with 0.4 μM PAC, and either 0.2% DMSO or 20 μM of each compound from Chembridge's 10K DiverSET™ collection (San Diego, CA, USA). Plates were sealed and incubated for 4 days in continuous white light and 4 days in darkness, at 75% humidity. After that time, compounds that caused a hypocotyl size increase of around 2-fold were identified. Of the 256 compounds selected in the first round, only eight behaved consistently in the following selection rounds (Table S1).

Western blot analysis

Seven-day-old transgenic seedlings expressing pRGA::GFP-RGA (Silverstone et al., 2001) grown on MS medium in continuous light at 21°C for 5 days and MS medium supplemented with 5 μM PAC for two more days were treated with 20 μM of each compound or GA_3 for 4 h. Whole seedlings were collected and the tissue was

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flash-frozen in liquid nitrogen. Protein extracts and western blots were done as previously described (Blanco-Touriñán et al., 2020), using anti-GFP antibody (JL-8, 1:5000; Clontech-Takara, Saint-Germain-en-Laye, France) and anti-DET3 (1:10 000; provided by K. Schumacher, University of Heidelberg, Heidelberg, Germany) as normalization control.

Statistical analysis

Ordinary one-way ANOVA and multiple comparisons for statistical significance were performed with GraphPad Prism 7 for Mac Os X version 10.13.1.

AUTHOR CONTRIBUTIONS

JA, LJS, SLS and MDZ designed and/or cloned the constructs. LJS, JB, SLS, TB and RO-F performed preliminary tests, and JA conducted all Arabidopsis protoplast experiments. FG-E conducted the chemical screenings in Arabidopsis plants. WW, SA-B, DA, MAB and MDZ supervised the research. JA, FG-E, DA, MAB and MDZ analyzed the data and discussed results. DA, MAB and MDZ planned and directed the research. JA, FG-E, DA, MAB and MDZ wrote the initial manuscript with input from the authors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. CtrlQuant activity upon induction with GA_3 and GA_4.

Figure S2. CtrlQuant GA-dependent AtRGA degradation is mediated by the 26S proteasome.

Figure S3. CtrlQuant GA-dependent AtRGA degradation is mediated by the DELLA domain.

Figure S4. CtrlQuant Endogenous GAs do not interfere with the activity of the sensor.

Figure S5. CtrlQuant RGA biosensor as a tool to study GA transporter in a plant system.

Table S1. Compounds tested as putative GA analogs.

Table S2. Expression vectors used in this study.

Table S3. Oligonucleotides used in this study.

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