WHEN CHEMISTRY MEETS BIOLOGY - GENERATING INNOVATIVE CONCEPTS, METHODS AND TOOLS FOR SCIENTIFIC DISCOVERY IN THE PLANT SCIENCES

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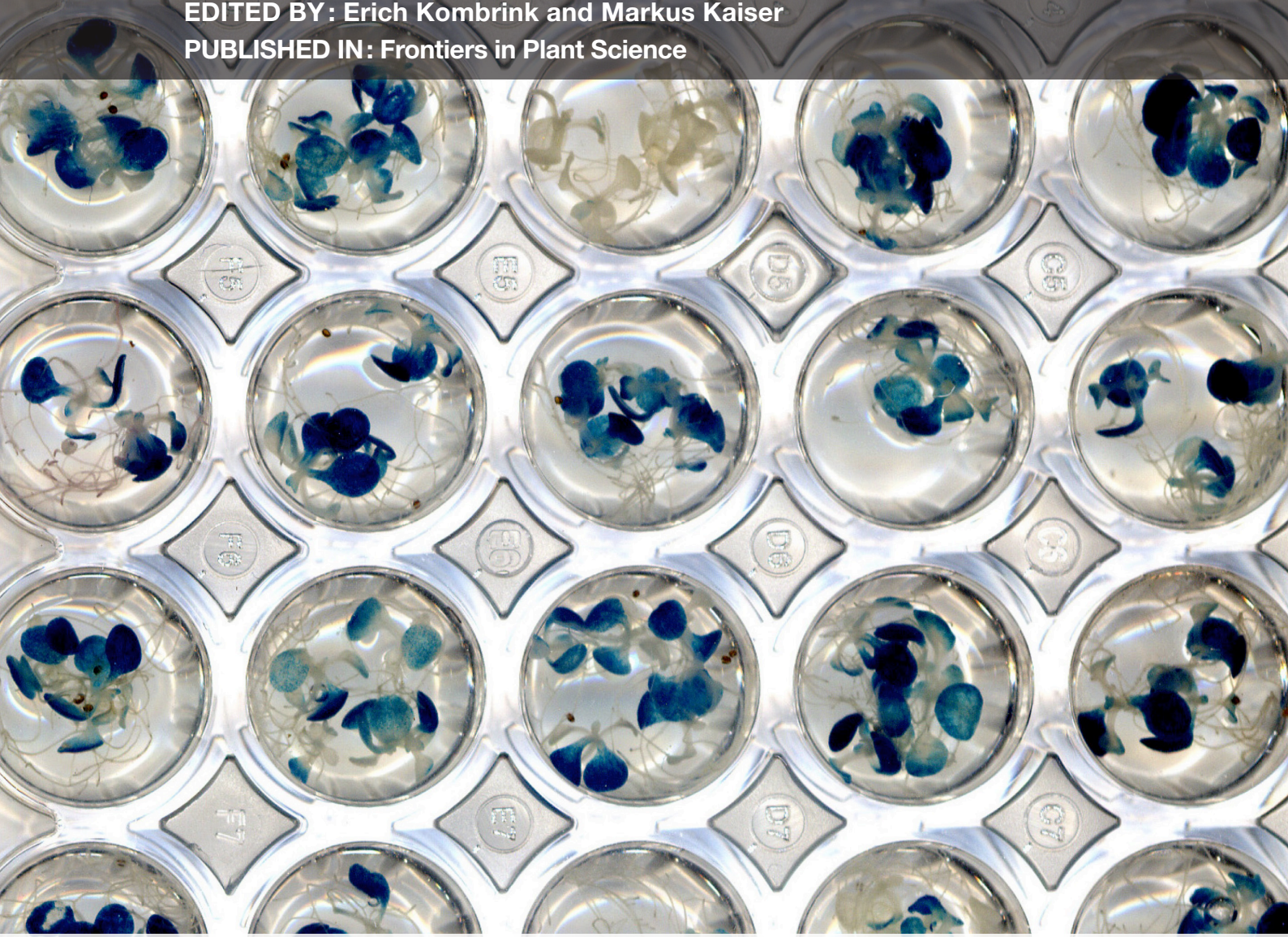

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WHEN CHEMISTRY MEETS BIOLOGY - GENERATING INNOVATIVE CONCEPTS, METHODS AND TOOLS FOR SCIENTIFIC DISCOVERY IN THE PLANT SCIENCES

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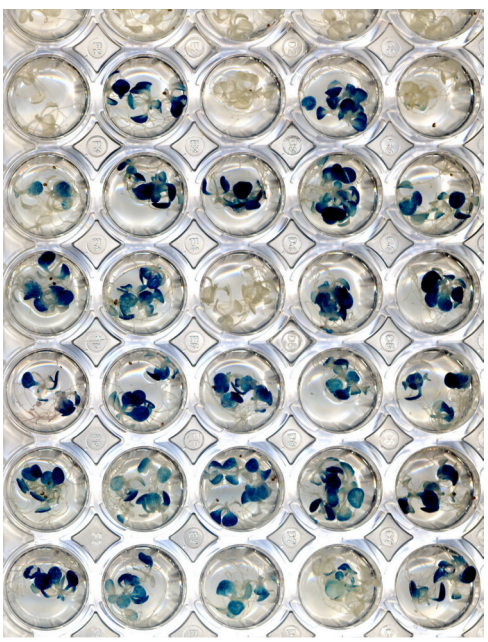

The identification of novel bioactive small molecules is commonly achieved by phenotypic screening of chemical libraries. The model plant Arabidopsis thaliana is particularly suited for this approach as it is small and can easily be grown in microplates. Also, the availability of a large number of mutants and reporter lines, harboring marker genes such as ß-glucuronidase (GUS; mediating blue tissue coloration if expressed) under the control of specific plant promoters, allows selection of chemicals that target (activate or inhibit) particular signaling pathways. The figure presents an example of this general screening approach by showing expression of the jasmonate-responsive reporter, LOX2p::GUS, under the influence of diverse chemicals.

Biologically active small molecules have increasingly been applied in plant biology to dissect and understand biological systems. This is evident from the frequent use of potent and selective inhibitors of enzymes or other biological processes such as transcription, translation, or protein degradation. In contrast to animal systems, which are nurtured from drug research, the systematic development of novel bioactive small molecules as research tools for plant systems is a largely underexplored research area. This is surprising since bioactive small molecules bear great potential for generating new, powerful tools for dissecting diverse biological processes. In particular, when small molecules are integrated into genetic strategies (thereby defining "chemical genetics"), they may help to circumvent inherent problems of classical (forward) genetics. There are now clear examples of important, fundamental discoveries originating from plant chemical genetics that demonstrate the power, but not yet fully exploited potential, of this experimental approach. These include the unraveling of molecular mechanisms and critical steps in hormone signaling, activation of defense reactions and dynamic intracellular processes.

The intention of this Research Topic of Frontiers in Plant Physiology is to summarize the current status of research at the interface between chemistry and biology and to identify future research challenges. The research topic covers diverse aspects of plant chemical biology, including the identification of bioactive small molecules through screening processes from chemical libraries and natural sources, which rely on robust and quantitative high-throughput bioassays, the critical evaluation and characterization of the compound's activity (selectivity) and, ultimately, the identification of its protein target(s) and mode-of-action, which is yet the biggest challenge of all. Such well-characterized, selective chemicals are attractive tools for basic research, allowing the functional dissection of plant signaling processes, or for applied purposes, if designed for protection of crop plants from disease. New methods and data mining tools for assessing the bioactivity profile of compounds, exploring the chemical space for structure–function relationships, and comprehensive chemical fingerprinting (metabolomics) are also important strategies in plant chemical biology. In addition, there is a continuing need for diverse target-specific bioprobes that help profiling enzymatic activities or selectively label protein complexes or cellular compartments. To achieve these goals and to add suitable probes and methods to the experimental toolbox, plant biologists need to closely cooperate with synthetic chemists. The development of such tailored chemicals that beyond application in basic research can modify traits of crop plants or target specific classes of weeds or pests by collaboration of applied and academic research groups may provide a bright future for plant chemical biology. The current research topic covers the breadth of the field by presenting original research articles, methods papers, reviews, perspectives and opinions.

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EDITORIAL
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Editorial: When Chemistry Meets Biology – Generating Innovative Concepts, Methods and Tools for Scientific Discovery in the Plant Sciences

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Keywords: agricultural biotechnology, bioactive small molecule, chemical biology, chemical genetics, high-throughput screening, phytohormones, plant defense, plant–pathogen interactions

The Editorial on the Research Topic

When Chemistry Meets Biology — Generating Innovative Concepts, Methods and Tools for Scientific Discovery in the Plant Sciences

The term "chemical biology" is commonly associated with research at the interface of chemistry and biology, as revealed by a recent census amongst scientists working in the field (Anonymous, 2015). Distinctively, as a truly interdisciplinary science, chemical biology combines scientific concepts and experimental approaches from both of its parent fields to understand molecular mechanisms in complex biological systems. New chemical tools and technologies are developed to dissect, visualize and manipulate biological processes or pathways and, conversely, studying biological systems may foster the development of new chemical principles. Although chemical biology has so far found most applications in pharmaceutical drug discovery, the search for novel bioactive small molecules that produce phenotypes in plants is also an established strategy in the agrichemical industry. In these approaches, the primary focus was laid on the discovery and improvement of herbicides, pesticides and other agriculturally useful compounds.

Upon recognition of the opportunities that bioactive small molecules may provide to basic plant biology research, in particular in combination with genetic strategies, the research field of plant "chemical genetics" emerged and has grown substantially over the past decade. Essentially, small molecules are utilized to generate recognizable phenotypes in a manner that is analogous to forward mutation genetics. However, chemical genetics has the potential to circumvent inherent problems of classical, forward genetics, such as lethality, pleiotropy or redundancy of gene functions because small molecules can be applied in a conditional, dose-dependent and reversible manner. The persistent challenge remains to identify the cognate target of such bioactive chemicals to discover the genes, proteins or pathways that are responsible for a given phenotype. Likewise, chemicals can be combined with other profiling technologies, such as genomics, proteomics or metabolomics. There are now clear examples of important, fundamental discoveries originating from plant chemical biology/genetics that demonstrate the power of this experimental approach.

The current issue on plant chemical biology provides a snapshot of the field, comprising review articles, perspectives and original research articles that both novices and experts may find useful. In their perspective article, Hicks and Raikhel focus on successful applications and the current challenges that the field of plant chemical biology/genetics faces as it matures. The discovery of novel bioactive chemicals is generally

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achieved by screening of compound collections (so-called chemical libraries), using robust and reliable phenotypes. The design and execution of such screening campaigns is outlined in a review article by Serrano et al. Here, in particular the newcomer will find useful recommendations that will help to avoid common pitfalls. In addition, recent success stories of plant chemical biology are highlighted, which may serve as teaching examples for implementation of future chemical biology projects. Along the same lines, in an original research article, Halder and Kombrink describe a facile bioassay for quantifying β-glucuronidase (GUS) activity *in situ*, which may turn out as a useful screening tool. Importantly, the methodology can be adopted for any transgenic *Arabidopsis* line harboring an inducible (or repressible) GUS reporter, and such lines are available for numerous developmental stages or signaling pathways. Once a chemical screening campaign has provided a new bioactive compound, the ultimate goal is to identify its mode of action and molecular target (or targets). Dejonghe and Russinova discuss in their review article different strategies for direct target identification, the current ones as well as the emerging ones, which have not yet found broad application in plant biology. Despite all recent progress, this still remains the most challenging, laborious and time-consuming step of chemical biology projects.

A recurring theme of plant chemical biology has been the search for, and application of bioactive small molecules in plant hormone signaling. In this sector a large assortment of chemicals has been identified and used as agonists or antagonists of phytohormones and thereby provided new insights into plant hormone biology. Two review articles by Rigal et al. and Fonseca et al. summarize some prominent examples of using chemical biology/genetic strategies in plant hormone research. Redundancy is largely avoided, as the former article focuses on signaling mediated by abscisic acid (ABA), salicylic acid (SA), auxin (IAA), cytokinin (CK), and brassinosteroids (BR), while the latter covers jasmonate (JA) signaling as well as phytohormone homeostasis, transport and hormonal crosstalk. In addition, Nakamura and Asami provide a focused review on chemical regulation of strigolactone (SL) signaling. Strigolactones have recently attracted much attention, as they are multifunctional molecules that not only act as phytohormones, inhibiting shoot branching, but also serve as rhizospheric communication signals between plant and symbiotic fungi and/or parasitic plants from the *Striga* and *Orobanche* genera. Identification or design of inhibitors of SL biosynthesis or SL receptors is a potential method to control these devastating and agronomically important root parasites. Finally, Almeida-Trapp et al. describe in an original research article the development and validation of an analytic method for quantification of six phytohormones that are frequently associated with stress responses, IAA, ABA, SA, JA, jasmonoyl-isoleucine (JA-Ile), and 12-oxo-phytodienoic acid (OPDA). Such a critically evaluated and validated method is obviously important for all plant hormone related work allowing direct comparison of hormone levels established in different laboratories.

A second favorite topic of plant chemical biology research has been plant–pathogen interactions and plant immune responses. Naturally occurring small molecules such as toxins produced by pathogens or phytoalexins produced by plants upon infection serve to intercept with growth and development of plants and pathogens, respectively. Other, not-so-small molecules (i.e., peptides) are involved in the initial perception of pathogen invasion by specific plant receptor-like kinases. Mott et al. discuss in their review the diverse roles of apoplastic molecules (peptides and small molecules) in modulating plant–pathogen interactions. A different view on such interactions is provided by Bektas and Eulgem who discuss in their review the function of synthetic elicitors that induce plant defense responses, but are distinct from known natural elicitors of plant immunity. A large variety of such compounds has been identified through screening efforts or targeted synthesis as analogs of natural compounds such as salicylic acid. They are attractive for basic research, allowing functional dissection of the plant immune system, as well as for applied purposes, as they can protect crop plants from diseases. Stokes and McCourt develop the applied aspect of chemicals in biological systems (here chemistry and agricultural biotechnology) a step further by predicting a future trend toward "personalized" agriculture, which essentially means to develop highly selective and species-specific herbicides and growth regulators. Indeed, recent success stories in plant chemical biology demonstrate that the corresponding technologies and tools are available. Thus, the development of tailored chemicals that modify traits of crop species or target specific classes of weeds or pests by collaboration of applied and academic research groups (in analogy to the current drug discovery process) may provide a bright future for plant chemical biology.

AUTHOR CONTRIBUTIONS

EK served as editor of the research topic "When Chemistry Meets Biology – Generating Innovative Concepts, Methods and Tools for Scientific Discovery in the Plant Sciences" and wrote this editorial. MK served as editor of the research topic "When Chemistry Meets Biology – Generating Innovative Concepts, Methods and Tools for Scientific Discovery in the Plant Sciences" and wrote this editorial.

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PERSPECTIVE ARTICLE
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Plant chemical biology: are we meeting the promise?

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As an early adopter of plant chemical genetics to the study of endomembrane trafficking, we have observed the growth of small molecule approaches. Within the field, we often describe the strengths of the approach in a broad, generic manner, such as the ability to address redundancy and lethality. But, we are now in a much better position to evaluate the demonstrated value of the approach based on examples. In this perspective, we offer an assessment of chemical genetics in plants and where its applications may be of particular utility from the perspective of the cell biologist. Beyond this, we suggest areas to be addressed to provide broader access and enhance the effectiveness of small molecule approaches in plant biology.

Keywords: chemical biology, chemical proteomics, small molecule, auxin, abscisic acid (ABA), jasmonate, endomembrane, vesicle

INTRODUCTION

The collaboration between plant biologists and chemists aimed at discovering new genes and protein functions has been accelerating over the past decade. In particular, the application of synthetic small molecules for basic discovery in plant systems has been growing and is becoming more sophisticated among basic researchers. There is now a modest number of plant-related research centers in the US, Belgium, Germany, and Sweden among other locations which house diverse and focused chemical collections, instruments, and expertise in screening for bioactive molecules and identification of their cognate targets. Such centers provide expertise and collaborative opportunities for plant biologists with varied interests who may not have access to chemical collections and other infrastructure. This is an important achievement given the broad range of plant biology, genomics, analytical and synthetic chemistry, proteomics, microscopy, and bioinformatics that may be required depending upon the specific project.

Our laboratory was an early adaptor of chemical genetics (i.e., the screening of small molecules and cognate target discovery) with an emphasis on the cell biology of plants. Along with our domestic and international colleagues, we have learned much about the benefits and challenges of the approach as a means to understand biological systems through the use of small bioactive molecules and other tools including "omics" approaches and biochemistry (i.e., chemical biology). As such we would like to offer several practical perspectives on the field from the viewpoint of cell biologists who have come to appreciate and value the power of multiple disciplines in solving problems. The concepts of plant chemical genetics have been reviewed previously by us and colleagues (Blackwell and Zhao, 2003; Kaschani and van der Hoorn, 2007; Walsh, 2007; De Rybel et al., 2009a,b; Hicks and Raikhel, 2009; Robert et al., 2009; Toth and van der Hoorn, 2009; Hicks and Raikhel, 2010, 2012; Ma and Robert, 2014). In addition, there are recent practical volumes covering many aspects of chemical biology in plant systems (Audenaert and Overvoorde, 2014; Hicks and Robert, 2014). The objective here is not to review the overall field; rather the focus will be on successful applications and the perspectives they offer at a practical level. We will then turn our focus to current challenges.

WHAT IS THE VALUE OF CHEMICAL GENETICS TO PLANT BIOLOGY?

At a fundamental level, the key to chemical genetics as with conventional genetics is in generating recognizable phenotypes at the whole plant, organ, cell or subcellular level. Whereas genetics generates phenotypes based on mutations that, in turn, perturb protein expression or function, chemical genetics approaches generate phenotypes for the most-part by altering protein function directly. Given the variations possible within proteins in terms of amino acid number and sequence, post-translational modification, and secondary and tertiary structure, the potential target space for small molecules is vast. From the perspective of an academic plant biologist, however, the ability to access this chemical space is limited at a practical level by the availability of compounds and the resources to screen them. There are novel aspects to using a chemical approach. This includes access to an increased range of phenotypes compared to genetics alone since small molecules in principle are able to target multiple members of a protein family or essential proteins when applied at sub-lethal concentrations. From the cell biology perspective, the ability of reversible drug-like molecules to effectively relate cellular phenotypes to that at the organism level is very powerful as is the ability to study rapid cellular processes such as endomembrane trafficking or hormonal signaling (Hicks and Raikhel, 2012; Ma and Robert, 2014). One frequent claim is that chemical genetics approaches can permit the identification of genetically lethal single genes as well as functionally overlapping members of protein families. This is stated in the introductions of many manuscripts and reviews. Excluding industrial screens for pesticides, as a basic research community, we now approaching a decade of experience in plant chemical screening,

target identification and the resulting biological knowledge. What do the results tell us?

SOME SUCCESS STORIES

Although there have been chemical screens reported in the literature covering many areas of plant biology including endomembrane trafficking, hormone signaling, immunity, cell walls, and small RNAs (Hicks and Raikhel, 2012) with others appearing frequently [for example, see recently (Park et al., 2014; Paudyal et al., 2014; Xia et al., 2014)], we will focus in this review only on select molecules for which the cognate targets have been identified as these are the most informative for discussion here. Even within this limitation, the overall impact of chemical genetics in basic plant research has been widespread with some impressive successes (**Table 1**).

There are multiple examples in hormone signaling. The transcriptional repression of auxin responses requires either TIR1 or one of its five homologs. By screening for resistance to a specific and highly potent picolinate-type auxin, DAS534, the homolog ABF5 was identified as a target (Walsh et al., 2006). ABF5 was not identified previously in mutant screens for 2,4D or IAA auxin resistance. The selectivity of DAS534 for ABF5 compared to other previously used auxins resulted in distinct plant phenotypes and permitted its identification as a target. Brassinosteroid perception and signaling occur via the plasma membrane BRI receptor system in which downstream signaling is activated by members of the GSK3-like kinase family of proteins. The compound bikinin was found to target a subset of seven of these kinases of which four were not implicated previously in brassinosteroid signaling (De Rybel et al., 2009b). In addition, the use of a brassinosteroid synthesis inhibitor, brassinazole, resulted in the identification of BIL4, a transmembrane protein among a family of five in *Arabidopsis* that may be involved in the control of cell elongation (Asami et al., 2003; Yamagami et al., 2009).

Perhaps the best example of groundbreaking work with small molecules was the use of a novel abscisic acid (ABA) agonist, pyrabactin, to identify the ABA receptor family (Park et al., 2009). Mutants resistant to pyrabactin were not resistant to ABA due to functional redundancy within the ABA receptor family. ABA insensitivity could only be achieved via multiple loss-of-function mutations within the so-call PYR/PYL receptors that belong to a large protein family known as the steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain proteins. The receptors interact with PP2Cs releasing the negative regulation of Snf2-related kinase 2, activating ABA-responsive gene transcription. What quickly followed initial reports were crystallography studies detailing the molecular mechanisms of receptor binding and function (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009). This was made possible by the ability to induce a detectable phenotype through the specific activity of pyrabactin for a PYR receptor among 14 within the family.

In the area of auxin biology, several molecules have been found to target auxin transporters. Gravicin is a chemical that inhibits gravitropism in *Arabidopsis* roots. The compound was subsequently found to target PGP19, a member of the super-family of ABC transporters with as many as129 members (Sanchez-Fernandez et al., 2001; Rea, 2007), which is involved in auxin transport. PGP19 also interacts with PIN auxin transporters (Rojas-Pierce et al., 2007). Another molecule (BUM) appears to target the PGP1 auxin transporter (Kim et al., 2010). Another example yet to be published is the identification of a specific exocyst component involved in recycling of PINs and other plasma membrane proteins. Very recently, there are exciting reports of specific protein family members involved in jasmonic acid-isoleucine conjugation and signaling (Meesters et al., 2014) as well as rationally designed jasmonate antagonists (Monte et al., 2014). Cognate targets for other molecules have been published for example in cell wall biosynthesis where certain members of the cellulose synthase family of proteins are targeted by isoxaben (Desprez et al., 2002; Somerville, 2006). While there may be additional examples of known small molecule targets, the examples cited here offer a perspective on plant chemical genetics. Namely, successful target identification is more likely to lead to results that are biologically meaningful which is essential goal of chemical biology. Anything short of this is ultimately a technical exercise.

WHAT WORKS? WHAT DOES NOT?

What is clear from these examples is that success with small molecules generally lies in their ability to target one or more members of protein families. In such cases conventional loss-of-function mutants may not generate a detectable phenotype.

Table 1 | Published small molecules with identified targets within gene families.

Small molecule	Target gene famliy	Function	Genes in family	Reference
Pyrabactin	PYR/PYRL	ABA preception	14	Park et al. (2009)
DAS 534	TIR/ABF	Auxin perception	5	Walsh et al. (2006)
Bikinin	GSK-3 kinase	Brassinosteroid signaling	10	De Rybel et al. (2009b)
Brassinazole	BIL4/BIL4-like	Possibly BR signaling	5	Yamagami et al. (2009)
Isoxaben	CESA	Cellulose biosynthesis	10	Desprez et al. (2002)
Gravicin	PGP/ABC transporter	Membrane transport	Up to 129	Rojas-Pierce et al. (2007)
BUM	PGP/ABC transporter	Membrane transport	Up to 129	Kim et al. (2010)
Jarin-1	GH3 amino acid conjugating enzymes	JA-Ile synthesis and signaling	19	Meesters et al. (2014)

Interestingly, in our examples the small molecules did not target all members of a conserved family. In the cases of auxin (Walsh et al., 2006) and ABA (Park et al., 2009) perception for which there are known ligands, the small molecules displayed altered target selectivity for receptors compared to known synthetic or native ligands. In other cases, inhibition of a subset of enzymes within a family (Asami et al., 2003; De Rybel et al., 2009b; Meesters et al., 2014) or inhibition of a class of transporters (Rojas-Pierce et al., 2007; Kim et al., 2010) resulted in distinct phenotypes. In some cases the phenotypes were scored in genetic screens for resistance to identify the cognate targets. So it seems that compounds should be promiscuous across a protein family in the interest of generating phenotypes, but not too much so. One hypothesis for this may be that small molecules acting too broadly may generate generalized growth phenotypes that confound genetic screens for targets. For example, perhaps such compounds are more likely to have off-target effects outside of a specific protein family making it difficult to identify the cognate target. But overall, the trend indicates that the power of chemical biology as practiced lies in the ability to generate phenotypes among protein families. This is highly significant since in *Arabidopsis* one third or more of the genes are in families (Hicks and Raikhel, 2012).

What about essential single copy genes? In principle, small molecules should be able to target essential proteins/genes in a dose-dependent manner, in other words by treatment at sub-lethal concentrations. So why is this not included in our examples from plants? The answer may be in the approaches used to identify targets. The most reliable approach used to identify cognate targets in plants is to screen for altered sensitivity to compounds. This takes advantage of EMS-induced mutations (or T-DNA loss-of-function insertions) and the well-developed genetics and genomics available in *Arabidopsis*. The ability to sequence whole genomes from pooled mapping populations has greatly increased the speed of EMS mutation identification (Schneeberger et al., 2009; Austin et al., 2011; Hartwig et al., 2012). Among our examples, with the exception of cases where the targets were deduced based on activity within known pathways (De Rybel et al., 2009b; Meesters et al., 2014) or molecules were rationally designed to target a receptor complex (Monte et al., 2014), forward EMS screens were used to identify targets. The dilemma of using forward EMS mutant screens for resistance is that recessive loss-of-function mutations in essential genes will not be represented or recoverable from a screening population. Thus, unless one is fortunate to identify a relatively rare gain-of-function (dominant) mutant displaying small molecule resistance, the probability of identifying an essential gene as a cognate target is relatively small. Thus, the recoverable targets favor members of protein families from which one or few members targeted by a small molecule result in a phenotype that is not lethal and can be scored for mapping. It cannot be determined from the literature how many reported compounds were pursued for targets genetically, or, of those attempted, how many mutant screens did not result in targets. But this may explain, at least in part, why in addition to the effort required a relative minority of novel compounds shown to be bioactive in plants have reported targets. Overall then, the power of small molecules when combined with EMS mutants appear to be in identifying the functions of protein families and subsets of their members.

THE CASE FOR CHEMICAL PROTEOMICS

For cases outside of protein families these observations would argue for alternative approaches for the identification of targets that are less biased against essential genes. These alternative methods are based on the affinity of small molecules for their protein targets [discussed in (Hicks and Raikhel, 2010)]. This set of approaches is also known as chemical proteomics (Futamura et al., 2013). In principle, coupling bioactive molecules to a solid matrix or bead permits direct affinity purification of potential targets. Such methods have been in use for drug target discovery for some time (Rix and Superti-Furga, 2009), yet have not gained much traction among plant biologists. This may be due to several factors including ready collaborations with synthetic chemists capable of producing tagged molecules. Contributors to the uncertainty of these approaches include (1) the feasibility of producing appropriate tagged bioactive molecules based on structure-activity relationships (SAR), (2) the often unknown affinity of proteins for their small molecule ligand and their abundance, (3) the unknown intracellular or organellar location of the target, (4) whether the cognate target will bind to the ligand in protein extracts *in vitro* due to ionic strength and pH for example, and (5) whether a protein complex is required for binding. An additional important reason that alternative approaches may have not been explored fully is that most plant biologists are trained as geneticists who are comfortable especially with *Arabidopsis* resistance screens and mutation mapping. A shift to other approaches requires additional training and collaboration. These issues require a careful initial characterization of compound potency and the analysis of structural analogs, including those with moieties such as amines that are suitable for coupling to biotin or other tags without greatly diminishing activity. This requires early collaboration with synthetic chemists before focusing exclusively on a small molecule.

On the optimistic side, mass spectroscopy instruments have greatly increased in sensitivity making feasible the association between compounds and protein targets even at extremely low levels. This in itself addresses many of the issues cited above. More recently, we have utilized an approach known as drug affinity responsive target stability (DARTS; Lomenick et al., 2009, 2011a,b). The approach is based on the principle that small molecule binding to a target site can stabilize a protein thereby decreasing its sensitivity to protease digestion. The small molecule-protein interaction can then be detected using mass spectroscopy (i.e., chemical proteomics). The advantage of the approach is that is not dependent upon producing a modified ligand. Rather the active compound can be used. Based upon several factors cited above, this approach will not work in all cases or even most cases, but it is simple to establish the assay and is therefore a simple first approach to target identification. Another important aspect of chemical proteomics approaches is the ability to detect the interactions of many proteins with a small molecule (Rix and Superti-Furga, 2009; Futamura et al., 2013). This can be used to detect the range of desired targets as well as off-target effects and should be applicable to plant systems. This has proven useful especially among pharmaceuticals where a profile of targets can

address drug specificity and off-target effects. Even within large gene families chemical proteomics has been used to profile drug selectivity (for recent example, see Ku et al., 2014). A group of compounds could be examined rather quickly for interactions, with the most promising warranting further investigation. If there is an antibody to a suspected target then the approach can be made more focused, increasing the probability of success. Other possible methods not requiring tagged molecules include methods designed to co-elute proteins with active small molecules (Chan et al., 2012). Although still being developed and adopted for plants, these approaches to assay for direct protein-ligand interaction are an important option prior to or in combination with genetics for target identification and should provide broader access to essential genes.

FINAL PERSPECTIVES

Briefly, there are several other noteworthy areas that have gained from chemical biology from the cell biology perspective. One is the realization that with screens that are rapid and simple, it has been possible to find small molecules that directly perturb vesicle trafficking (Drakakaki et al., 2011) which impacts other processes such a cell wall biosynthesis (Park et al., 2014). As it turns out, virtually all such compounds result in scorable growth or developmental phenotypes to identify cognate targets. Furthermore, rapid mapping by sequencing requires the scoring of far fewer recombinants than conventional mapping, so it is now much more feasible to map mutations by scoring at the microscopy level. A perhaps overlooked, but powerful benefit of chemical biology is the ability to score for intracellular phenotypes directly then link the phenotypes directly to their corresponding developmental consequences. Given the available large selection of fluorescent protein-tagged intracellular markers, it is efficient to score small molecules across a selection of intracellular markers. It is then possible to focus on desired intracellular and plant developmental phenotypes. The contrasting option is to use genetics. This would require generating and screening mutagenized populations across the same selected marker lines followed by screening each population for intracellular defects, a daunting task even assuming that the same range of phenotypes could be obtained by the two approaches. Another area that we have noted previously is the need for continued enhancement in plants of automated screening utilizing image and video analysis (Hicks and Raikhel, 2009). This includes the tracking of plant development at macro and micro levels [for several recent examples, see (Tisne et al., 2013; Sozzani et al., 2014)], vesicle movement related to basic mechanisms, plant immunity and development within tissues such as the meristem (Salomon et al., 2010; Tataw et al., 2013; Ung et al., 2013; Beck et al., 2014). In quantifying aspects of vesicle or organelle diameter, velocity, and directionality we can learn about the regulation of dynamic subcellular compartments in real time. In this manner small molecules can be used to directly modulate intracellular trafficking in a quantifiable manner to dissect pathway function and regulation.

Our final perspective on chemical genetics is that as the field is maturing, it is becoming critical for labs to focus on the biology and what can be learned using this approach. That is, the focus should be increasingly on the biology aspects of chemical biology. It is not enough to simply find new compounds with interesting bioactivities. Rather, we have to push harder to demonstrate that biological insight has been gained in each study. This is a characteristic of each successful example published in high profile journals and what we should strive for as a community.

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REVIEW
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Considerations for designing chemical screening strategies in plant biology

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Traditionally, biologists regularly used classical genetic approaches to characterize and dissect plant processes. However, this strategy is often impaired by redundancy, lethality or pleiotropy of gene functions, which prevent the isolation of viable mutants. The chemical genetic approach has been recognized as an alternative experimental strategy, which has the potential to circumvent these problems. It relies on the capacity of small molecules to modify biological processes by specific binding to protein target(s), thereby conditionally modifying protein function(s), which phenotypically resemble mutation(s) of the encoding gene(s). A successful chemical screening campaign comprises three equally important elements: (1) a reliable, robust, and quantitative bioassay, which allows to distinguish between potent and less potent compounds, (2) a rigorous validation process for candidate compounds to establish their selectivity, and (3) an experimental strategy for elucidating a compound's mode of action and molecular target. In this review we will discuss details of this general strategy and additional aspects that deserve consideration in order to take full advantage of the power provided by the chemical approach to plant biology. In addition, we will highlight some success stories of recent chemical screenings in plant systems, which may serve as teaching examples for the implementation of future chemical biology projects.

Keywords: *Arabidopsis thaliana*, bioactive small molecules, chemical genetics, chemical libraries, high-throughput screening, structure–activity relationship, target identification

Introduction

Forward genetic screenings have been widely used to identify the genetic elements behind biological traits. The isolation of mutants with particular phenotypes from a randomly mutagenized population is an unbiased process with the obvious advantage of targeting genes without prior knowledge of their functions. Traditionally, the identification of the responsible gene by mapping *via* experimental crosses was the most tedious and time-consuming step in this process. The advent of next-generation sequencing greatly facilitated this process, allowing genetic mapping and gene identification in relatively short time (Prioul et al., 1997; Miki and Mchugh, 2004; Schneeberger et al., 2009; Austin et al., 2011; Nordström et al., 2013). However, forward genetic screening approaches will reach their limits under three unfavorable circumstances: (1) when multiple genes are responsible for one single trait (i.e., redundancy of gene function), (2) when a gene product is crucial for survival of an organism (i.e., lethality due to loss of gene function),

or (3) when a single gene is responsible for multiple phenotypes (i.e., pleiotropy of gene function).

It has been suggested and eventually demonstrated that these limitations can be circumvented by chemical genetic approaches (Schreiber, 1998; Stockwell, 2000; Blackwell and Zhao, 2003). This method relies on small bioactive molecules that modulate protein function, either by acting as agonist or antagonist thereby mimicking modification of the encoding gene products. In case of redundancy of gene function, the advantage is that a chemical compound (e.g., inhibitor) may target several proteins with identical or similar function (e.g., isoenzymes) if corresponding ligand binding sites are present. Such chemicals can be applied to plants with different genetic backgrounds or to different plant species to phenocopy genetic mutations (e.g., creating chemical instead of genetic knock-outs). Correspondingly, in cases of mutant lethality, application of a chemical (e.g., inhibitor) may be delayed to developmental stages, when the corresponding gene function is no longer essential. Since chemicals can be applied not only at different stages, but also at different concentrations, dosage-dependent phenotypes could be created, and the chemical phenotype could even be reversed (i.e., back to wild type) if a soluble compound is washed out again, thereby extending the experimental repertoire for circumventing mutant lethality.

Already characterized compounds are well-accepted as chemical tool, such as the phosphoinositide 3-kinase inhibitor wortmannin, the inhibitor of vesicular transport brefeldin A, the bacterial phytotoxin coronatine or variations of the protease inhibitor E-64 (Murphy et al., 2005; Samaj et al., 2006; Kolodziejek and Van Der Hoorn, 2010; Wasternack and Kombrink, 2010). Of course, many more such selective compounds exist. For example herbicides, which usually target primary metabolic processes that are necessary for growth and development of plants, played fundamental roles in understanding aspects of plant processes, such as photosynthesis, cell wall physiology or function of microtubules (Dayan et al., 2010). However, by using already existing chemical tools, plant biologists depend on discoveries from pharmacological screenings (Grozinger et al., 2001; Zhao et al., 2003) or random findings and are limited in case no chemical tool is available for a particular research area. Therefore, the challenge is to find novel compounds by using plant systems for chemical screening to expand the repertoire of chemical tools that target a large diversity of biological functions (Walsh, 2007; Hicks and Raikhel, 2012; Dayan and Duke, 2014).

Similar to genetic screenings, which can be carried out in forward and reverse direction, one can distinguish between forward and reverse screening strategies in chemical genetics (**Figure 1**). Commonly, phenotypic or forward screening approaches aim at dissecting a biological process in animal or plant systems *via* identification of novel bioactive small molecules that selectively modulate any of the molecular components contributing to the phenotype. This approach aims at similar components as forward genetics and is unbiased with respect to the chemical's target and thus well-suited for basic research (Hicks and Raikhel, 2012). By contrast, a target-based or reverse screening approach aims at identifying chemicals that selectively interfere with a defined target. This strategy is often applied in pharmaceutical research when novel agonists or antagonists of drug targets that have been recognized as important are wanted. Such screening can be based on any protein-mediated phenotype such as enzymatic activity, protein-protein interactions or transcription factor binding (Subramaniam et al., 2001; Jung et al., 2005; Zabotina et al., 2008). The importance of target-based screenings in pharmaceutical research is reflected by the fact that half of the experimental and marketed drugs target only five protein families: G protein-coupled receptors, protein kinases, proteases, nuclear receptors, and ion channels (Inglese et al., 2007). Such limitation to few targets seems reasonable for applied research, but less suited for basic research, because it does not allow exploration of new phenotypes and new areas of biology with chemical tools (Eggert, 2013).

For the purpose of this review, we primarily use the term "chemical biology" to refer to the overall strategy of identifying and applying chemical tools for dissecting biological systems, whereas "chemical genetics" more specifically refers to combinations of chemicals with genetic approaches. In our view, a chemical biology approach comprises the following three essential elements: (1) a robust, reliable and quantitative readout to screen for small bioactive molecules, (2) a rigorous validation process to characterize selected candidate compounds, and (3) a strategy for target identification, which can be dismissed in the target-based approach. However, these three components are not sufficient for chemical screening projects, since additional elements and details need to be considered. In the following first part of this review, we will outline and discuss the general strategy of chemical biology projects, thereby providing guidelines for designing successful screenings, for hit selection and validation, and for identification of targets and modes of action. In the second part, we will describe selected examples of chemical biology projects in plant biology to highlight some characteristics of success stories of plant chemical screenings.

Strategy to Identify Chemical Tools

When conventional genetic methods fail to answer a biological question, a chemical biology approach should be considered. It is clear that not each genetic project can easily be adapted to a chemical biology approach, because this requires different resources, experimental methodology and experience. This may be one of the reasons, why the potential of plant chemical biology has not yet been fully exploited, despite the fact that plants are attractive and well-suited for such an alternative approach. For example, the model plant *Arabidopsis thaliana* is small and can easily be grown in microplates. With its flexible culture conditions and the abundance of mutants, including a large number of reporter lines expressing diverse marker genes, it allows for dissection of virtually every signaling pathway or biological response provided it can be analyzed at the seedling stage (Hicks and Raikhel, 2012). Alternatively, cultured cells derived from *Arabidopsis* or other non-model plants are likewise amenable for facile chemical manipulation in microplates. Thus, there are ample opportunities for applying chemical screens and an enormous potential for new discoveries in the plant sciences. The general strategy to identify new chemical tools is fairly simple and in the end little specialized equipment is required, such as a versatile

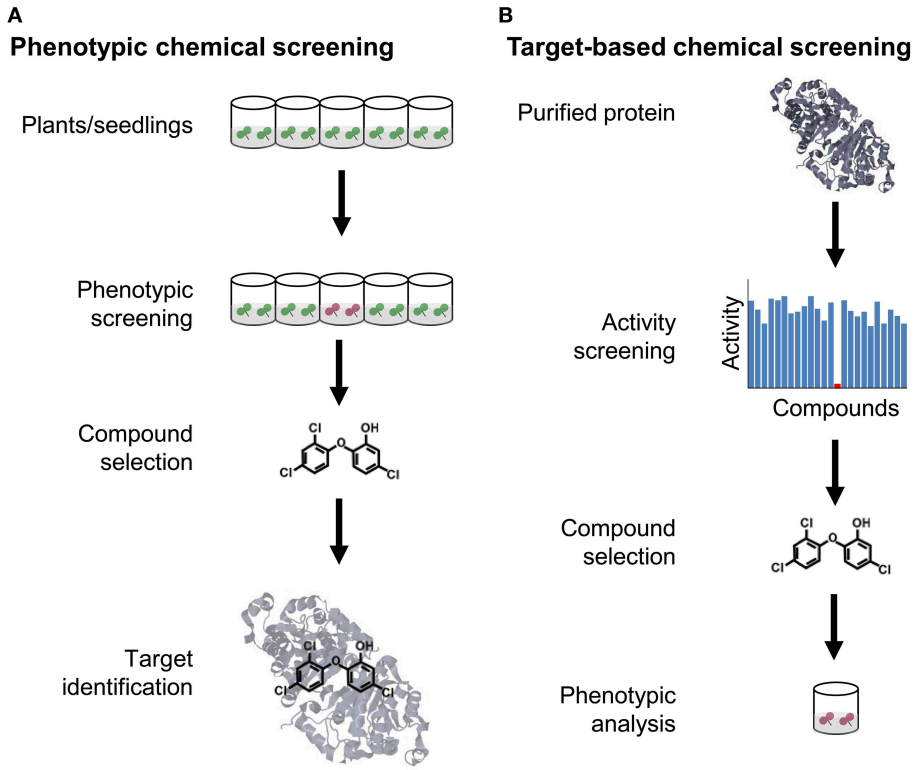

FIGURE 1 | Comparison of forward and reverse chemical screening. **(A)** The goal of phenotypic or forward chemical screening is to identify from an arrayed library of chemicals a (selective) bioactive compound causing a phenotypic alteration, usually in a microplate format. Once a selective compound is found, the molecular target is identified, either by a genetic approach or some type of biochemical purification strategy. **(B)** The goal of target-based or reverse chemical screening is to identify a compound that modulates the activity of a selected protein. Subsequently, the chemical is used to determine the phenotypic consequences when applied to plants.

microplate reader. However, particular attention should be paid to the screening methodology, which includes careful design and critical assessment of the bioassay used for the primary screening, careful planning of subsequent secondary assays for validation of selected hit compounds and principle considerations concerning target identification strategies (**Figure 2**). Thus, it is important to see the primary screening only as the first step in a composite process leading to the development and application of new chemical tools.

The Design of a Chemical Screening Campaign

Entering a chemical screening campaign requires enduring commitment and appropriate resources (e.g., chemical library, multimode microplate reader, or other monitoring device). Thus, careful strategic planning will help to avoid pitfalls and maximize useful outputs.

Assay Development

An important step before starting a chemical screening is to invest into assay development. It is imperative that screening is based on a reliable, reproducible, and robust bioassay. First, it needs to be considered, whether the phenotype is suitable for scoring in the microplate format, which is inevitable for the screening process, in particular when large numbers of chemicals are involved and cumulating in high-throughput screening (HTS). For target-based approaches, such as *in vitro* enzyme assays, it is easy to use microplates with 384 or 1536 wells, but for growing single seedlings, plates with at maximum 96 wells are required. However, single-plant measurements may compromise reproducibility and in order to increase the confidence and robustness of the readout it may be beneficial to grow multiple plants in larger wells (48- or 24-well plates). In general, any phenotype that can be recorded in the microplate format is suitable for chemical screenings. However, an important consideration is to design assay conditions that allow acquisition of quantitative data during the screening, preferably in an automated fashion. Clearly, quantitative screening data will allow the application of statistical procedures and automatic, unbiased hit selection by setting threshold values. Furthermore, quantitative screening data permit to distinguish compounds with strong or weak activities, which may be useful to have for identification of new bioactive chemical scaffolds. Among quantitative readouts, fluorescence provides very strong signal intensity and is therefore the most widely used detection method in HTS in the animal field, allowing direct visualization in the tissue (Fan and Wood, 2007). In contrast, the signal strength of luminescence is significantly lower compared to fluorescence, but it exhibits an enormous dynamic range, which is mainly due to almost complete

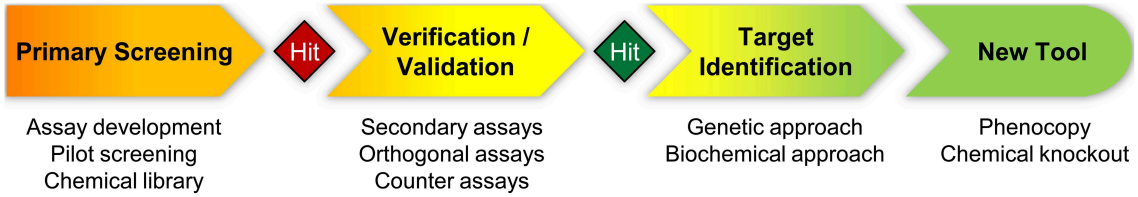

FIGURE 2 | Workflow of chemical biology screening strategy.

absence of background signal. Therefore, bioluminescence is an emerging method in HTS (Fan and Wood, 2007).

Acquisition of quantitative data in plant chemical biology can easily be achieved by automatic multimode microplate readers capable of recording luminescence, fluorescence and/or absorbance as generated by reporters such as luciferases, β-glucuronidase (GUS) or fluorescent proteins (Stewart, 2001; Ruijter et al., 2003). In addition, numerous biosensors exist that allow detection and quantification of intracellular concentrations of particular small molecules, including calcium ions (using aequorin), phosphate (using rhodamin-labeled phosphate binding protein), nitric oxide (using the fluorescent indicator 4,5-diaminofluorescein-2 diacetate (DAF-2DA)) and many others (Okumoto et al., 2012). For selection of an appropriate reporter system it is obvious that interference with fluorescence of chlorophyll, cell walls, and other cellular components should be avoided (Ruijter et al., 2003). Despite the obvious advantages, only few chemical screenings in plant science were based on quantitative data that were collected from diverse systems such as cultured cells, isolated membrane fractions, excised maize coleoptiles, or *Arabidopsis* seedlings analyzing absorption after quantitative staining, radioactivity of radiolabeled UDP-glucose, plant extracts *via* HPLC, or luminescence of a luciferase reporter line (Zabotina et al., 2008; Nishimura et al., 2012; Noutoshi et al., 2012; Tóth et al., 2012; Meesters et al., 2014). Remarkably, most chemical screenings with microplate-grown seedlings have assessed visible phenotypes, which can only be scored with less ease and reliability (see Supplementary Table 1 for a list of plant chemical screenings). These phenotypes include inhibition of germination, growth expansion of tissues (e.g., roots, hypocotyls), bleaching of seedlings, accumulation of secondary products (e.g., flavonoids), changes in gravitropic response or chromogenic staining using the GUS reporter. Automated image-based screenings using enhanced microscopy methods and image processing software to record phenotypes at a cellular level will be good options to enable quantification of such phenotypes and to extend the phenotypes available to HTS in plant sciences (Hicks and Raikhel, 2009). *Arabidopsis* is by far the most frequently employed plant and only few screenings have used alternative systems such as cultured tobacco cells, *in vitro* germination and growth of pollen tubes or non-plant systems such as yeast (Zouhar et al., 2004; Yoneda et al., 2007; Robert et al., 2008; Drakakaki et al., 2011; Noutoshi et al., 2012) (cf. Supplementary Table 1).

Finally, the reliability and robustness of the assay for screening purposes needs to be validated. Therefore, it is crucial to test both positive and negative controls in order to assess the dynamic range and signal variation for the experimental setup and to determine the reproducibility. The actual screening should also include both controls; thereby it can be estimated, whether candidate hits can be identified with a high degree of confidence. Acquisition of quantitative data also allows statistical analysis as determination of the screening window coefficient, called Z' factor, which is a common quality metric for evaluation and validation of HTS assays, reflecting signal dynamic range, and the data variation (Zhang, 1999). The Z' factor is defined in terms of four parameters: the means of both the positive (μ_{pc}) and negative controls (μ_{nc}) and their respective standard deviations (σ_{pc}, σ_{nc}) (see Formula 1).

$$Z' \text{ factor} = 1 - \frac{(3\sigma_{pc} + 3\sigma_{nc})}{|\mu_{pc} - \mu_{nc}|} \quad (1)$$

The Z' factor ranges from negative infinity to 1, and a high value (>0.5) defines an excellent assay, a low value (>0) an acceptable assay and a negative value (<0) an ineffective assay with too much overlap between the positive and negative controls for the assay to be useful (**Figure 3**). However, it is fair to mention that this stringent statistical parameter was developed to evaluate and validate HTS assays, and although in principle useful, it may be too rigorous for application to bioassays in complex plant systems such as whole seedlings, which are prone to variability. Particular care is required when the assay requires scoring of a qualitative phenotype. Under these circumstances, measures have to be installed that generate reliable and comparable data sets from which hits can be extracted with high confidence. This may include direct application of a second readout during the screening process or subjecting only selected positive hits to a useful alternative bioassay as previously demonstrated for a number of screening campaigns (Gendron et al., 2008; De Rybel et al., 2009; Forde et al., 2013; Hu et al., 2014).

Hit Selection

Chemical screenings can be performed in different ways, with single or replicate measurements. The use of replicates allows a minimum of statistical analysis and thereby gives improved confidence in hit selection by reducing the number of false positive or negative hits. With small chemical libraries (<500 compounds) it is feasible and convenient to screen with replicates, but when large chemical libraries (>2000 compounds) are used, it is worthwhile to consider time, labor and costs, as these will proportionally increase. Therefore, current practice in drug discovery is

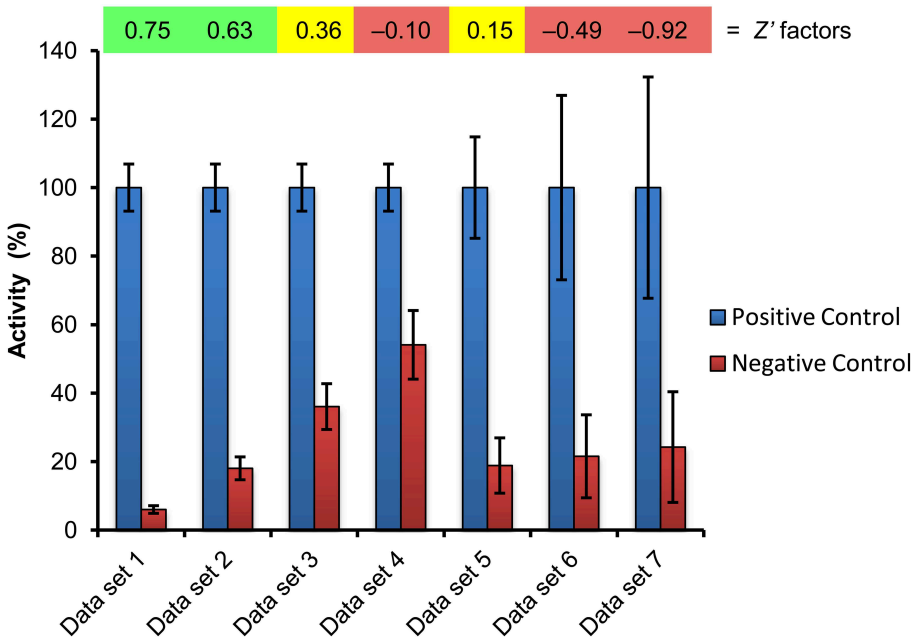

FIGURE 3 | Estimation of assay quality by *Z′* factor determination. The positive and negative controls included with a screening plate (cf. **Figure 4**) were used to calculate the *Z′* factor, which is shown above the corresponding data set. The values of data set 1 are from a real experiment recently published (Halder and Kombrink, 2015), and the resulting *Z′* factor of 0.75 indicates that this is an excellent assay for screening (quantification of GUS activity). Gradual, hypothetical increase of the negative control value (data sets 2–4) reduces the screening window and correspondingly the *Z′* factor, leading to marginal ($Z' = 0.36$) and unacceptable ($Z' = -0.10$) assay quality for screening purposes. Likewise, increasing variability of assay data leads to decreasing *Z′* factors and assay quality (data sets 5–7). Green, yellow, red indicate excellent, marginal, and inacceptable assay quality, respectively.

to omit replicates (such screenings may involve >100,000 compounds), which requires very robust and reliable bioassays (Malo et al., 2006). However, for non-commercial screening projects in plant science, with libraries rarely exceeding 10,000 compounds (Supplementary Table 1) the advantages of replicate measurements prevail the drawbacks. In conjunction with replicate measurements, two additional points deserve consideration: (1) The library size should be related to the number of expected (and finally uncovered) hit compounds, as these will subsequently require thorough characterization. The expected hit rate increases with the target space (number of potential targets) and will be higher with readouts that are dependent on a large network (e.g., hormone signaling), whereas the target space is limited in case of short signal transduction chains comprising only few components. It is difficult to put numbers on the expected hit rate because, irrespective of theoretical considerations, it will largely depend on the stringency of hit selection. Based on our own experience using quantitative and qualitative screenings, hit rates vary between less than one and up to few percent (Serrano et al., 2007, 2010; Meesters et al., 2014). (2) The question of how to design the microplate setup should be answered for any library screening. As mentioned earlier, it is useful to include control treatments on each plate. The problem of potential plate-to-plate variation should not be underestimated, especially when a screening campaign extends over longer time periods, and appropriate controls help to normalize and better compare quantitative readouts and to identify outliers and deviating plates. Because of possible positional effects, the controls should ideally be randomly distributed across the plate, which is of course not very convenient. However, chemical libraries are often delivered in microplates with the first and last columns left empty, which can be used for respective controls. An efficient way for arranging the controls is to use alternate wells for positive and negative controls along these two columns (**Figure 4**) (Malo et al., 2006).

Selection of hits in qualitative screenings can result in subjective and arbitrary decisions. Such bias can be avoided by selecting hits on the basis of quantitative, normalized data. Many different methods have been developed to normalize quantitative data (for review see Malo et al., 2006). Common normalization approaches include "factor or percent of control" (FOC, POC) and "factor or percent of sample," which are easy to calculate and interpret (**Figure 4**). However, the first method requires a large number of controls to provide an adequate estimation of their mean, whereas the latter method omits controls altogether and instead relates each sample values to the mean of all samples on the plate, which is a valid assumption provided that most compounds on a plate are inactive and thus can serve as controls. Similarly, the classical *Z* score or *Z* transformation—not to be confused with the *Z′* factor mentioned above—also excludes control measurements but incorporates the sample variation and relating it to within-plate variation of all samples. Specifically, the Z score is calculated by subtracting from each sample value (x_i) the mean

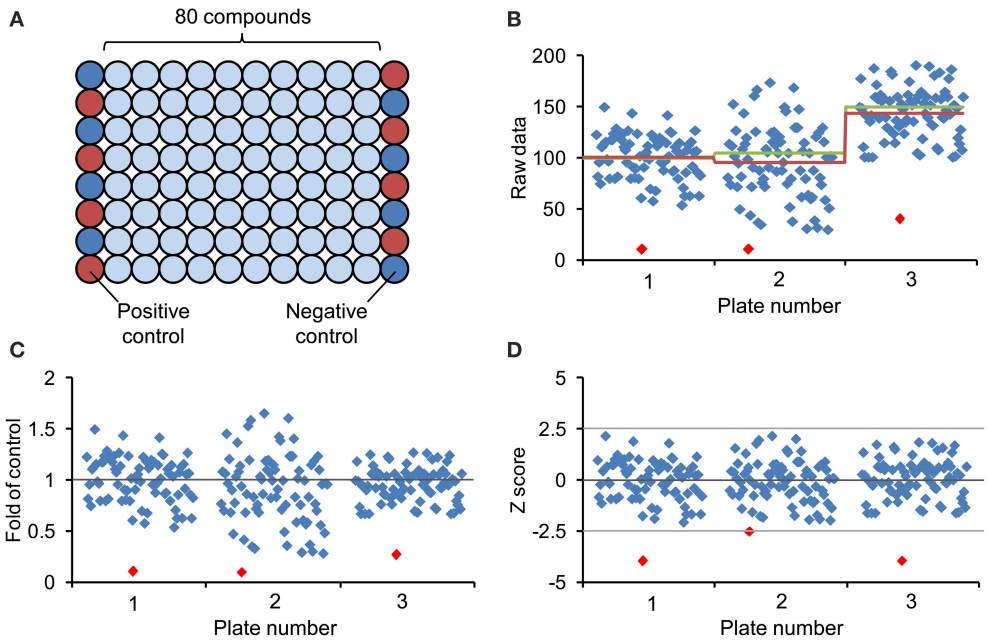

FIGURE 4 | Design of chemical screening plate and methods for data normalization and visualization. (A) Generally, commercial arrayed chemical libraries are provided with 80 different compounds stored in the middle of 96-well plates and the first and last columns are left empty. Correspondingly, column 1 and column 12 are available for controls and to minimize edge-related bias, the eight positive controls (red circles) and the eight negative controls (blue circles) are distributed across these columns in alternating order. **(B)** Scatter plot of hypothetical screening data showing three different plates. The red line represents the corresponding plate average, the green line the mean of assumed control value (included on each of the plates), which are not necessarily identical with the plate average. **(C)** Representation of screening data after normalization to plate average (fold of control). **(D)** Screening data after normalization by *Z* score transformation (see text). The *Z* score dampens the plate-to-plate variation and increases confidence in hit selection by introduction of a common threshold value. Notice that the lowest value of each plate (red diamond) may not fulfill the cut-off criteria after *Z* score transformation.

of all plate values ($\bar{x}$) and dividing this difference by the standard deviation of all measurements (σ_x) (see Formula 2).

$$Z\ score = \frac{x_i - \bar{x}}{\sigma_x} \quad (2)$$

All normalization procedures described above can only account for systematic plate-to-plate variation but not for within-plate systematic effects, such as extreme edge or row effects or other indicators of technical problems. To cope with these, the *B* score and other statistical methods are available that make minimum assumption about positional effects and may be applied to remove systematic row, column or well-effects. However, since these calculations are based on an iterative algorithm and since complex biological systems such as plant seedlings provide rather variable data, it is not easy and may not be appropriate or possible to estimate the *B* score. Based on these considerations, we recommend applying the *Z* score to chemical screening data in plant systems. Following *Z* transformation of the raw data, the mean of all measurements is represented by zero (0) in the plate-well scatter plot (**Figure 4**). The highly variable values from a hypothetical screen of three 96-well plates cover the range from +2.5 to -2.5 standard deviations around the mean and by defining an appropriate threshold value (e.g., −2.5 standard deviation), the *Z* score allows objective selection of hit compounds (**Figure 4D**). Of course, the *Z* score can also be based on control values rather than the plate average (provided it is based on sufficient data). In fact, such added controls may serve to verify the assumption for using the plate average and may also help to identify unexpected problems such as an unusual high number of positive hits. Since the *Z* score, when based on the arithmetic mean, is sensitive to statistical outliners, the substitution of the mean and standard deviation by the outlier-insensitive median and median absolute deviation results in a robust *Z* score.

Eventually, the identification of "hits" or "screening positives" is the goal of any screening campaign and it is essential to subject only the most promising compounds to the subsequent work flow (**Figure 2**). Thus, hit selection is the critical process of deciding which sample values differ meaningfully from the controls. For screens based on qualitative data, the selection might be biased by the wish to not miss potentially valuable hits. Therefore, phenotypic screening may be prone to high rates of false positive hits. It may be useful to develop a rating system for phenotypical strength or to select only a limited percentage of compounds showing the highest scores. By contrast, quantitative screening data are less prone to biased hit selection, but of course, the hit rate is affected by the setting of the corresponding threshold value. Such variable adjustment will allow subjecting as many compounds as feasible and convenient to confirmatory re-screening. Of note, less stringent selection criteria will increase

the number of false positive hits and correspondingly reduce the number of false negative hits (Malo et al., 2006).

Finally, to ensure a successful screening, data acquisition and analysis should go hand in hand, i.e., data should be analyzed while the screen is in progress, to allow the identification of problems as they occur. It is important to visualize the raw data as well as the transformed and normalized values because these might indicate different technical problems (**Figure 4**) (Birmingham et al., 2009). Alternatively, it is advisable to perform a pilot screening using a small number of selected molecules with defined bioactivity or a small chemical library (see next section). Such pilot screening gives an impression about the variability of the assay under screening conditions and may indicate the expected hit rate, which should be considered in selecting the size of a chemical library.

Chemical Libraries and Screening Concentration

A collection of small molecules—commonly referred to as chemical library—is the starting point for performing the actual chemical screening. Ideally, the compounds of such library should have general properties that allow for high selective bioactivity, such as low molecular weight, the capacity to pass through membranes and strong and effective interaction with their targets (Smukste and Stockwell, 2005). The bioavailability of a chemical in a biological system depends on its solubility, uptake, distribution and metabolism within the organism. In pharmaceutical drug research, the Lipinski's rule of five (RO5) describes molecular properties for orally administered human drugs that would make it likely to be taken up into cells (Lipinski et al., 1997). The parameters include the molecular mass (<500 dalton), the octanol-water partition coefficient ($\log P < 5$), number of hydrogen bond donors (N-H and O-H bonds <5) and hydrogen acceptors (N and O atoms <10). Note that all numbers are multiples of five, which is the origin of the rule's name. However, the rule does not predict if a compound is pharmacologically active, it rather describes physicochemical properties that from experience are favorable for drugs and, correspondingly, violation of at least one of these criteria generally makes a compound less suitable as a drug. As a rule of thumb, there are many exceptions to Lipinski's rule. For example, it was shown that sets of herbicides and insecticides do not comply with the RO5 (Tice, 2001, 2002), indicating that bioavailability of small molecules may significantly differ between organisms or their particular mode of action. Therefore, not only compounds in compliance with RO5 may be of interest, in particular when screening in plant systems.

The success of a chemical screening campaign is intimately connected not only with the assay and screening design, but also with the selection of the appropriate chemical library. Numerous chemical libraries are commercially available, which differ in size, composition and chemical diversity. Since these collections are usually designed for drug research, they mostly comprise RO5 compliant compounds (Shelat and Guy, 2007). In industrial research settings, HTS of very large libraries (>100,000 compounds) is facilitated by automation, using one or more robots for sample handling and data collection. By contrast, in initial screenings in plant systems that were carried out in academic environments, less than 100 molecules were analyzed (Min et al., 1999; Hayashi et al., 2001). More recently, plant chemical screening projects have also successfully employed more than 40,000 compounds (see Supplementary Table 1). However, when considering library size, it has to be balanced with screening effort and cost as well as with the expected hit rate. It needs to be critically assessed, how many candidate compounds identified in a primary screening can eventually be carried through all subsequent characterization and selection steps. There is a number of examples that valuable compounds were identified from relatively small compound collections (Min et al., 1999; Hayashi et al., 2001; Serrano et al., 2007; He et al., 2011; Tóth et al., 2012; Meesters et al., 2014).

Chemical libraries not only differ in size, but also in composition and the nature of compounds, which may affect the screening strategy and the outcome of a screening project as discussed in detail (for drug screening) elsewhere (Shelat and Guy, 2007). Here it suffices to briefly describe five relevant categories of libraries to provide a basis for general considerations: (1) Bioactive collections (libraries of bioactive compounds) contain compounds with well-characterized biological activities (e.g., protein kinase inhibitors). Such libraries (usually smaller in size) are useful because they facilitate narrowing down or even identifying molecular targets. (2) Natural product libraries are assembled from compounds isolated from various organisms. They are considered to provide higher hit rates, because they comprise compounds that are synthesized and transported in biological systems and might therefore bind to related protein scaffolds in a heterologous system (Koehn and Carter, 2005; Li and Vederas, 2009). (3) RO5 libraries represent the majority of screening collections. They are typically derived from chemical synthesis and may suffer from limited structural diversity when containing multiple derivatives of certain templates. (4) To enhance the structural complexity of chemical libraries, diversity-oriented synthesis (DOS), and biology-oriented synthesis (BIOS) strategies have been developed, aiming at novel chemotypes with high complexities that resemble natural products (Schreiber, 2000; Shelat and Guy, 2007; Kaiser et al., 2008). (5) Fragment libraries represent another special case of compound collections that is being used for certain screening strategies that aim at identifying only substructures (fragments) of bioactive molecules that are subsequently optimized by chemical modification (Carr et al., 2005). Relevant for plant screening projects have so far been only compound collections of categories 1 through 3 and combinations thereof.

A special collection of bioactive compounds of interest for the plant research community is the Library of AcTive Compounds on Arabidopsis (LATCA) that Sean Cutler and colleagues assembled from diverse chemical libraries, such as LOPAC (Sigma-Aldrich), and Spectrum (Microsource) and other screening collections (Chembridge, Maybridge), as well as common inhibitors, herbicides, plant hormones and research chemicals (http://cutlerlab.blogspot.de/2008/05/latca.html; accessed December 2014). The selection was based on activity in various phenotypic screens of *Arabidopsis* seedlings monitoring hypocotyl length (Zhao et al., 2007). Thus, this collection of about 3600 compounds with proven activity in plant systems is a good starting point for screening projects and hits can potentially be

associated with known pathways or target proteins. However, on the downside, by design this library excludes compounds not causing the selected growth-related phenotypes but nonetheless may impair novel and/or important functions.

Closely related to selecting the chemical library is the question about the concentration to be used in the screening. There is no general answer to this question, but a few things need to be considered. Most commonly, chemical libraries are provided as 10 mM stock solutions solved in DMSO. For most bioassays performed with *Arabidopsis* seedlings, final DMSO concentrations of 1–5% can be tolerated, which puts an upper limit to the screening concentration at 100–500 μM. However, at high concentrations many chemicals may be toxic or cause stress responses thereby increasing the risk of generating many false positive (or false negative) hits. Although performing the screening at various concentrations would be the ideal solution, this approach requires additional effort, time, and costs. Typically, this is affordable only in commercial research programs employing robotic systems for handling microplates, dispensing fluids and determining activity in very robust and reliable bioassays, with the advantage of directly generating the half maximum effective concentration (EC_{50}) values from a chemical screen (Miller et al., 2012). In plant research, as in other systems, the initial screening is carried out at a fixed concentration, which largely depends on the type of bioassay and the chemical library. HTS in drug discovery usually use low concentrations in the micromolar or nanomolar range, since high concentrations generate more hits, which require more effort for validation and effective compounds that are active at low concentrations are more desirable. In addition, compounds with high activity represent useful lead structures that could be used for chemical optimization and synthesis of more effective drugs (Landro et al., 2000). With respect to the bioassay, it is worthwhile to consider that in target based screening approaches carried out *in vitro*, the compounds typically show higher potency because they have direct access to the target without restriction by membranes or other barriers. On the other hand, phenotypic screening *in vivo*, employing cells or whole organism often require higher concentrations because the chemicals have to cross membranes or other barriers and might require transport to different organs or cellular compartments for activity. Another issue not to be neglected is the stability of compounds and metabolic conversion to active (or inactive) products, which is more likely to occur in complex systems such as cell-based assays. Chemical screenings performed in plant systems have employed a wide range of concentrations (2-200 micromolar) with the majority of screenings restricting the range to 20–50 micromolar (see Supplementary Table 1). One should not get too excited about hits that require high concentrations of the compound, such as 200 micromolar or more. As already stated by Paracelsus (1493–1541), the founder of modern toxicology and medicinal chemistry, "the dose makes the poison" (Borzelleca, 2000) and hence using relatively high concentrations bears the risk of obtaining false positive or negative hits (depending on the type of assay), as a result of stress responses to inappropriate cytotoxic concentrations. Thus, especially libraries that are enriched in bioactive compounds (e.g., Bioactive, Natural Product, and DOS/BIOS collections) can be used even at lower concentrations (10-25 micromolar) to avoid numerous unspecific hits. Conversely, libraries of high chemical diversity (RO5 and fragment collections) can potentially be screened at higher concentrations (∼100 micromolar). Compounds with weak activity identified from such screenings, can often be converted to more active derivatives by chemical modification, yielding valuable information about the structure–activity relationship (SAR).

An interesting approach for reducing time and effort that is needed for library screening is to use pools of compounds (Devlin et al., 1996). Individual chemicals are combined in such a way that each is contained twice in unique compound pools. Screening of these pools creates unique distribution patterns for each component of the pools, which allows identification of an active compound by its pattern without the need to re-analyze each member individually. Obviously, this strategy relies on the assumption that the majority of compounds is inactive in a given bioassay that is sufficiently robust and sensitive. However, there are also certain caveats associated with this approach: (1) The combination of compounds may eventually lead to lower applicable concentrations (considering an upper limit of solvent that can be applied), which may only allow the identification of potent compounds; (2) molecular interaction between compounds may affect their stability and their activity (Hann et al., 1999); (3) false positive or negative hits may originate from additive or opposite biological activity of compounds in the same well. Although compound pooling has been successfully applied for chemical screening in a plant system (Tsuchiya et al., 2010), it has to be carefully considered whether or not it offers a true advantage.

Verification and Validation of Hits

After hits have been selected from the primary screening, the next essential step is to rigorously validate the compound's biological activity and establish whether or not they selectively impair only one particular phenotypic readout (**Figure 2**). The first step is to repeat the screening assay with the selected hits to eliminate false positives. False negatives can only be avoided by screening in replicates. Missing an active compound may be annoying, but may be irrelevant if a sufficient number of positive hits has been identified. Again, a robust bioassay and application of stringent selection criteria are key to identifying strong candidate compounds. It is long been known that the dosage of a chemical affects the quantity of a response (Hill, 1910). Therefore, determination of rough pharmacodynamics by using various concentrations should at least be considered to re-evaluate the selected primary hits that would convey information about dose dependency and increase confidence in hit selection.

In order to establish reliable dose-response relationships, it is necessary to have a quantitative readout. However, even if a non-quantitative phenotypic readout is used for screening, it may be quantifiable in subsequent, individual bioassays. For example, hypocotyl length of seedlings visually inspected in chemical HTS can be quantified for individual compounds (Gendron et al., 2008; Savaldi-Goldstein et al., 2008; De Rybel et al., 2009; Lin et al., 2010; He et al., 2011). Likewise, GUS activity in HTS by staining, can be quantified *in vitro* by enzymatic conversion of the substrate 4-methylumbelliferyl-β-D-glucuronide to

fluorescent 4-methylumbelliferone (Armstrong et al., 2004; Serrano et al., 2007; Knoth et al., 2009). An important parameter for evaluating a drug or chemical is the half-maximum effective concentration (EC_{50}), or for inhibitors, the half-maximum inhibitory concentration (IC_{50}) (Holford and Sheiner, 1981). For accurate EC_{50}/IC_{50} calculation, it is essential to include sufficient assay concentrations to accurately determine both the maximal and minimal effective concentration (Sebaugh, 2011). Once the EC_{50}/IC_{50} value is established, subsequent experiments for characterization of a compound can be carried out at a defined EC_{50}/IC_{50}, avoiding adverse effects at unnecessarily high concentrations at which the compound may be toxic or impinge on unrelated biological readouts.

The second step in validation of primary hits should be an independent bioassay from the same signaling pathway to confirm the chemical's biological activity by an alternative readout, e.g., a different reporter or quantifying endogenous gene expression. Such secondary assays are also referred to as orthogonal assay (Malo et al., 2006) and depending on the screening design and library size, it could be directly integrated into the primary screening, which is then performed with two different readouts in parallel or one after the other (Gendron et al., 2008; Tsuchiya et al., 2010; Nishimura et al., 2012, 2014; Hu et al., 2014). The toxicity of chemicals is also an issue that should not be neglected. To exclude that induced cell death interferes with the biological readout, cell death should be monitored upon chemical treatment separately or, if the bioassay allows, as integral part during the recorded readout (Noutoshi et al., 2012). In reporter-based screenings, the potential interference of a chemical with the reporter activity also needs to be considered. For example, 2-3 percent of a chemical library typically interfere with luciferase activity and in addition, two percent of the same library usually exhibit fluorescence at a similar wavelength as 4-methylumbelliferone, which is the frequently used substrate for quantitative measurement of GUS activity (Inglese et al., 2007). Correspondingly, reporter-based screening results need to be verified by appropriate counter assays to eliminate false positives. Dual or single reporter lines harboring different reporters under the control of the same promoter represent excellent tools, but any other control is also appropriate, such as monitoring endogenous gene expression (Meesters et al., 2014).

Another important step during characterization of a bioactive agent is to evaluate the compound's selectivity. The ideal chemical tool affects only a single target, which is an essential component of the studied biological process; it does not interact with secondary sites, so-called off-targets and thus has no side effects. In pharmacology, such selectivity is highly desirable because it facilitates registration and marketing of a drug. Early stage identification of possible off-targets can reduce time and costs and an extensive characterization may prevent drugs from been withdrawn from the market (MacDonald et al., 2006; Hughes et al., 2011). Of course, basic research is not restricted by such regulation, but generally, target-selective small molecules are superior chemical tools.

To establish selectivity of a candidate compound, its impact on numerous independent biological readouts needs to be tested. Such counter assays can easily be performed with transgenic reporter lines that respond to different stimuli. But in fact, any assay that is independent of the screened phenotype would be suitable. However, it is also important to bear in mind, that some signaling pathways share similarities in their perception and signaling mechanisms or cross-talk with each other, as recently demonstrated for the plant hormones auxin, gibberellin, jasmonate and salicylic acid (SA) (Katsir et al., 2008; Pieterse et al., 2009; Santner and Estelle, 2009; Vlot et al., 2009; Lumba et al., 2010). Thus, also the selection of bioassays to be used for counter screening needs careful consideration to avoid pitfalls. Although selective chemicals are preferable, even non-selective compounds may be of value. For example, three non-selective and mechanistically distinct inhibitors of germination (cycloheximide, methotrexate, and 2,4-dinitrophenol) were applied in a comparative microarray study to uncover the common genes that are exclusively involved in germination (Bassel et al., 2008).

Consulting chemical databases (e.g., ChEMBL, PubChem) for retrieving information about primary hits may facilitate the validation process considerably. Much of this information originates from other screening campaigns, predominantly from animal systems and drug discovery programs, but still, this information may point to potential targets and indicate whether a compound is selective or affects various processes. Along the same lines, it should be considered to use the same chemical library for multiple screenings with different biological readouts, therefore enabling easy validation by comparing the results of independent screening campaigns. Such parallel independent screenings provide the instant possibility to filter out the compounds with unique or common activity profiles, which eventually may save efforts and costs for subsequent compound validation. Another advantage of chemical databases is the possibility to search for structural derivatives and their bioactivities. Such derivatives of a candidate compound are important for studying the SAR, which may lead to a panel of compounds with different specific activities. The knowledge of the SAR may also be crucial for subsequent target identification strategies, because it may identify the site(s) of a molecule that tolerates modifications without loss of activity and inactive analogs may serve as useful control in biochemical target identification strategies (Meesters et al., 2014).

Finally, it should not be ignored that validation of a bioactive compound identified from a chemical library should always include verification of its chemical identity and purity. Eventually, it may be necessary to re-synthesize the compound if no alternative source or provider can be identified.

Target Identification

Once a small molecule has been selected from the chemical screening, the molecular target needs to be identified in order to fully understand the compound's effect on the biological system. However, target identification is usually the limiting step of a chemical genetic project. This is mainly due to three limitations, but not restricted to these: (1) Weak and reversible interaction between the small ligand and its protein target (i.e., low binding affinity); (2) low abundance of the target (or multiple targets); (3) adverse, intrinsic properties of the small molecule, e.g., lack of suitable functional groups preventing appropriate chemical modification (i.e., introduction of a tag) or impaired activity

after such modification (Burdine and Kodadek, 2004; Zheng et al., 2004; Walsh and Chang, 2006; Terstappen et al., 2007). In chemical biological research, different technological approaches have been successfully applied to identify small molecule targets. In this section, we will briefly describe few examples and provide an overview of possible target identification strategies, which include genetic screening, biochemical affinity purification, proteomic methods, and DNA-based approaches. For a more detailed discussion, we refer to review articles focusing on this topic (Tashiro and Imoto, 2012; Schenone et al., 2013; Ziegler et al., 2013; Dejonghe and Russinova, 2014).

A generally applicable target identification methodology is forward genetic screening; in fact, the integration of small molecules into genetic strategies specifically defines "chemical genetics." Essentially, the genetic screening part aims at identification of mutants that escape from the chemically induced phenotype. Such mutants that are either insensitive or hypersensitive to previously identified compounds are used for genetic mapping, because the corresponding locus (or a closely associated component) is likely a direct target. In the past, physical mapping of a mutation was time-consuming and labor-intensive. However, with the advent of new sequencing technologies such as next-generation sequencing (NGS) the rapid and cost-effective identification of mutations by whole-genome sequencing has been made possible (Schneeberger et al., 2009). With millions of short reads that are generated from F2 mapping populations using NGS platforms (e.g., Ilumina Genome Analyzer) the distribution of the single nucleotide polymorphism (SNPs) between the reference (i.e., the corresponding wild-type) and mutant genomes are analyzed. Using this methodology the number of candidate genes causing the mutant phenotype can be narrowed down in a rather short time period (Schneeberger et al., 2009; Austin et al., 2011).

Forward genetic screenings have been successfully used in plant chemical biology. For example, glutamine phosphoribosylamidotransferase (AtGRAT2) has been identified as target of the novel herbicide DAS734, a phenyltriazole acetic acid derivative (**1**) (**Figure 5**), thereby establishing its utility as a new and specific inhibitor of plant purin biosynthesis (Walsh et al., 2007). Similarly, P-glycoprotein19 (PGP19), a member of the superfamily of ATP-binding cassette (ABC) transporters was shown to bind gravicin (**2**), which was identified in a chemical screen for inhibitors of gravitropism and functions by selectively impairing auxin transport activity of PGPs but not that of other auxin transporters such as PIN proteins (Rojas-Pierce et al., 2007). Most remarkable, however, is the identification of the abscisic acid (ABA) receptor by combined chemical and genetic approaches (Park et al., 2009). From a chemical screening for seed germination inhibitors, the small molecule pyrabactin (**3**) (**Figure 5**) was identified, which induced phenotypes resembling ABA treatment (e.g., activation of ABA-responsive genes), thus acting as an ABA agonist (Zhao et al., 2007; Park et al., 2009). However, mutants isolated by genetic screening for pyrabactin insensitivity were not resistant to ABA. The identified causal gene *PYRABACTIN RESISTANCE 1* (*PYR1*), encodes a member of the superfamily of proteins containing the so-called START domain, which is important for binding and transfer of lipids; other members of this superfamily, referred to as PYR1-LIKE (PYL) or REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) were identified as interactors of *ABA-INSENSITIVE 1/2* (*ABI1/2*) encoding type 2C protein phosphatases (PP2Cs), which function as negative regulators of ABA signaling (Ma et al., 2009; Park et al., 2009). Importantly, PYR1/PYL and PP2Cs act as a family of ABA co-receptors forming a ternary complex with ABA, which results in inhibition of PP2C activity and initiation (de-repression) of downstream responses, including activation of ABA responsive genes (Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Park et al., 2009; Cutler et al., 2010). The identification of the long-sought ABA receptor is an outstanding example, among others (Hicks and Raikhel, 2014), demonstrating the power of chemical genetics to circumvent gene redundancy as pyrabactin selectively activates only one out of 14 PYR1/PYL proteins, a property that is distinctly different from ABA (Cutler et al., 2010).

Biochemical *in vitro* purification methods using labeled small molecules are the traditional and direct approaches for target identification (Schenone et al., 2013; Ziegler et al., 2013). However, this methodology suffers from severe limitations. While target identification of chemically reactive small molecules *via* affinity purification and proteomics has become routine (Wang et al., 2008; Kaschani et al., 2009), target identification of relatively inert small molecules (i.e., non-covalent binding ligands) remains challenging. Introduction of tags for photoaffinity cross-linking, immobilization on a solid support or radio-labeling requires prior knowledge of SAR to retain biological activity and, of course, the presence of suitable functional groups. In addition, these modifications (as well as the subsequent purification steps) may be labor-intensive and time-consuming. Further difficulties are encountered when targets are present in low abundance, as is often the case for membrane-localized receptors, or the ligand shows only low binding affinity (Burdine and Kodadek, 2004; Terstappen et al., 2007). To circumvent these and related problems, alternative profiling and target identification strategies have been invented, many of which are sophisticated and/or technically challenging (Lomenick et al., 2009, 2011; Rix and Superti-Furga, 2009; Schenone et al., 2013).

A new profiling technique to identify the protein target (or targets) at the proteome scale without the necessity to modify the corresponding small molecule is the drug affinity responsive target stability (DARTS) method (Lomenick et al., 2009; Aghajan et al., 2010). The DARTS method is based on the thermodynamic stabilization of the protein target upon binding of the small molecule, which renders the protein less prone to degradation by proteases in comparison to non-bound proteins. The advantage of this approach is that it can be performed in crude extracts without prior protein purification and that target identification is label free. Although the DARTS method is restricted to abundant targets, signal loss is limited as no washing steps are required. Alternatively, target deconvolution can be achieved by RNA profiling technologies (DNA-microarray analysis) or other genomic approaches (Terstappen et al., 2007; Schenone et al., 2013; Ziegler et al., 2013). Analyzing transcriptional changes in response to a chemical, using DNA microarrays or RNA-seq, allows identification of a molecule's molecular signature, which can be compared with preexistent transcriptional profiles of collections of mutants

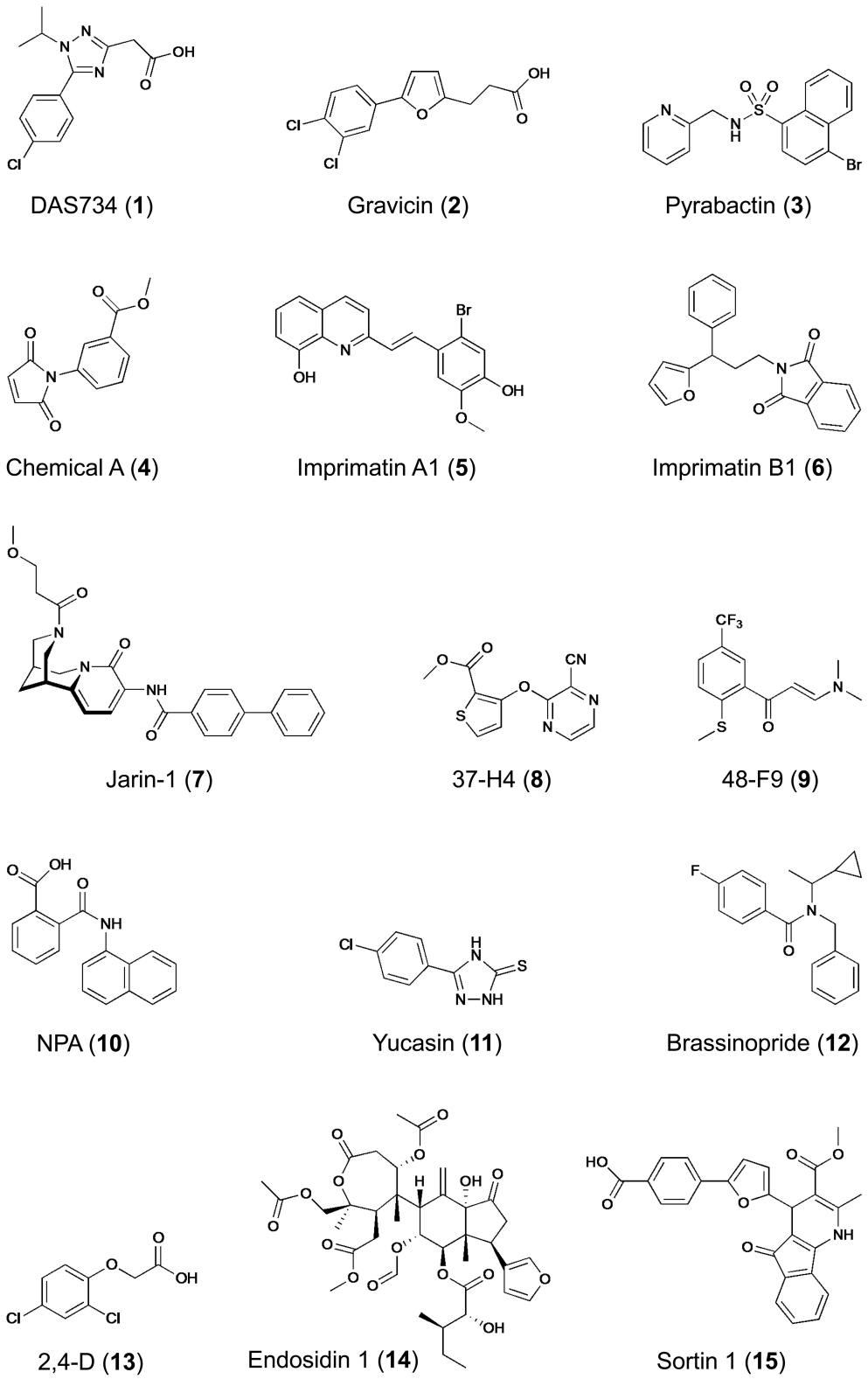

FIGURE 5 | Structures of bioactive compounds identified in different chemical screening campaigns. Examples refer to compounds mentioned in this paper.

or caused by other compounds. However, this approach has several limitations: (1) The target needs to mediate a transcriptional output, (2) it requires the prior existence of such molecular signatures (e.g., for drug discovery such profiles may be available from databases), and (3) it does not relieve from extensive characterization of target candidates by ligand binding assays (Stockwell, 2000; Walsh and Chang, 2006).

Another set of methods have in common that identification of a small molecule target is combined with cloning of its cDNA (Terstappen et al., 2007; Schenone et al., 2013). Such expression cloning technologies, including the yeast three-hybrid (Y3H) system, phage display and mRNA display, artificially increase the abundance of the target by expressing it as recombinant fusion protein, which may have properties that are different from the native original, in particular, when post-translational modifications are involved. Among these techniques, the Y3H system is particularly appealing because it not only offers direct access to the genes encoding target proteins, but it also relies on small molecule–protein interactions in living cells rather than *in vitro* and it permits scanning of whole proteomes for targets (Kley, 2004; Terstappen et al., 2007; Cottier et al., 2011). Importantly, this approach is not restricted to model organisms. The Y3H technology, originally developed by Licitra and Liu (1996), is an extension of the commonly used yeast two-hybrid system by introducing a third hybrid component, the small molecule of interest linked to another ligand, usually methotrexate or dexamethasone (Cottier et al., 2011). The functional output of the small molecule (as part of the hybrid ligand) binding to its cDNA encoded protein target is growth of the corresponding transformed yeast cell to a colony, which will serve to directly identify the binding protein by cDNA sequencing. Of course, this promising technology also suffers from limitations: (1) The functional readout of the system is gene activation and therefore only soluble proteins that are translocated to the yeast nucleus are detectable (e.g., excluding membrane-localized receptors), (2) identification of multimeric protein complexes is not possible because only single cDNAs are expressed in individual yeast cells, and (3) uptake of hybrid ligands (as they are relatively large molecules) may be impaired or excluded as yeast has efficient drug extrusion systems.

Another novel and sensitive technology to determine ligand–target interaction is the analysis of a protein microarray using the surface plasmon resonance (SPR) imaging (SPRi). The SPR technology records changes in light refraction on sensor chip surfaces that occur upon interaction between two (or more) binding partners, one of which is covalently linked to the sensor chip surface. SPRi is the current leading technology for label-free detection of protein interactions and a powerful tool for affinity-based biosensors in high throughput screens (Hall et al., 2007; Ray et al., 2010). Instead of linking only one particular protein to the sensor chip, proteins originating from cDNA libraries ideally representing the whole proteome (Yamada et al., 2003; Gong et al., 2004) could be spotted onto the protein microarray to detect the interaction partner of the compound of interest. Additionally, the recent development of nanohole arrays increases spatial resolution, facilitating the development of protein arrays (De Leebeeck et al., 2007). Advantages of SPRi-protein array analysis are that even natural low abundant proteins are detectable and that it enables kinetic characterization of the protein–ligand interaction (Rich et al., 2002). In addition, it can be used to identify not only the main target of a small molecule, but also off-targets with weaker interaction (Lomenick et al., 2011). However, identification of small molecule targets using proteome arrays is an unexplored field in plant sciences.

Chemical Screenings in Plant Biology

In the past decade, plant chemical biology has seen substantial progress with more than thirty performed chemical screenings analyzing various biological processes (see Supplementary Table 1). After having discussed the conditions and recommendations for performing such chemical screenings, we will now present a few striking examples and highlight their distinct characteristics.

Target-Based Approaches

In plant chemical biology, there are only two examples of target-based chemical screenings. In the first example Yoshitani and colleagues were interested in finding specific ligands of an *Arabidopsis* protein in order to unravel its unknown function (Yoshitani et al., 2005). This study combined *in silico* screening based on the protein's three-dimensional structure with subsequent evaluation of candidate compounds using immobilized recombinant protein in a SPR assay. From a chemical database, 103,773 compounds were taken for *in silico* screening. Two rounds of molecular docking to a predicted ligand-binding site identified 10,000 and 300 top scoring compounds, respectively. Out of the best scores, 69 compounds were subsequently analyzed for their binding properties at the molecular level. Four compounds showed weak interactions with the recombinant protein and all shared common structural features, suggesting that these determine their affinity to the target protein. However, the protein function remains elusive, but the compound's common structure can serve as a lead for the development of specific inhibitors or may provide important clues toward elucidation of the protein function. Essentially, this is a proof of concept that computational screening in combination with SPR-based experimental evaluation can discover candidate ligands or substrates. Clearly, this approach depends on the correct prediction of a potential binding site; it cannot be applied for proteins undergoing structural changes upon ligand binding. There are several studies with non-plant proteins that provide evidence for virtual screening as effective tool for identifying protein function (Kalyanaraman et al., 2005; Hermann et al., 2007; Song et al., 2007; Mallipeddi et al., 2012). The advantage of combining *in silico* with experimental screening is that the virtual pre-selection of compounds can dramatically reduce time, effort and expenses associated with experimental screening.

The second example of target-based screening relates to the biosynthesis of plant cell wall polysaccharides (Zabotina et al., 2008). The synthesis of highly complex polysaccharides constituting the plant cell wall is thought to involve at least 1000 genes and biochemical changes caused by mutations create only weak phenotypes difficult to discern (Somerville et al.,

2004). Therefore, a chemical biology approach seemed appropriate. Most enzymes involved in the synthesis of extracellular polysaccharides are located in the Golgi apparatus and therefore, Zabotina and colleagues monitored the conversion of radiolabeled UDP-glucose in isolated pea stem microsomal fractions. This quantitative *in vitro* screening led to identification of ten compounds (out of 4800 screened) that inhibited the incorporation of glucose into cell wall carbohydrates. Remarkably, chemical A (**4**) (**Figure 5**) not only inhibited Golgi-localized glucosyltransferase activity, but also modified cell wall composition *in planta* and activated plasmamembrane-bound callose synthase without affecting the endomembrane morphology (Zabotina et al., 2008). Chemical A represents a novel drug with great potential for the study of the mechanisms of Golgi and plasmamembrane-bound glucosyltransferases and a useful tool for identification of additional enzymes involved in polysaccharide biosynthesis. Despite the presence of additional enzymes in the assay that could be molecular targets, one can classify this screening as target-based due to the fact that a specific substrate was used, which drove the assay toward identification of effectors of proteins capable of using this particular nucleotide sugar as substrate.

Phenotypic Approaches

As mentioned earlier, the majority of the chemical screenings performed in plant systems are forward or phenotypic screenings using a qualitative readout. Despite the obvious advantages of quantitative screening assays, only few examples exist for this superior strategy (Supplementary Table 1). Noutoshi and colleagues performed such a quantitative chemical screening with cultured *Arabidopsis* cells aiming at the identification of compounds that enhance disease resistance by specifically potentiating pathogen-activated cell death (Noutoshi et al., 2012). This study was inspired by the fact that exogenous application of SA (and related compounds that even have practical applications) confers disease resistance to plants (Kessmann et al., 1994; Schreiber and Desveaux, 2008; Bektas and Eulgem, 2014). Out of 10,000 diverse chemicals, five compounds were identified that increased cell death upon challenge with pathogenic *Pseudomonas* bacteria but that were not toxic by themselves (up to concentrations of 100 μM). Importantly, *Arabidopsis* cell death was quantified by Evans blue staining in three replicates and selected candidates were subjected to a dose–response analysis, which provided a high confidence of hit selection. The identified compounds represented two distinct molecular structural backbones, which were designated imprimatins A (**5**) and B (**6**) (**Figure 5**) for immune-priming chemicals. Remarkably, the immune-priming effect was also effective in *Arabidopsis* seedlings as treatment with imprimatins enhanced resistance to bacterial infection. Further characterization of the compounds revealed that pretreatment with imprimatins increased the accumulation of endogenous SA, whereas its metabolite, SA-*O*-β-D-glucoside, was reduced. This is the result of the selective inhibition of two SA glucosyltransferases (SAGTs) as demonstrated by *in vitro* enzyme assays. In addition, loss of function mutants of these two SAGTs phenocopied the effect of imprimatins, indicating that SAGTs are involved in immune priming by modulating the pool of free SA. Considering potential application, the results of this study demonstrate that manipulation of the active free SA pool *via* SA-inactivating enzymes could be a useful strategy for fortifying plant disease resistance and may lead to novel and useful crop protectants. However, whether the protection conferred by these compounds is as durable as that of other plant activators remains to be established (Noutoshi et al., 2012; Bektas and Eulgem, 2014).

Another example of employing a quantitative chemical screening strategy has recently led to the identification of a selective inhibitor of jasmonate signaling (Meesters et al., 2014). *Arabidopsis* seedlings harboring a jasmonate-inducible luciferase-based reporter system allowed facile screening for inhibitors of jasmonate-induced gene expression by *in vivo* monitoring of luciferase activity. Although the quantified *in vivo* luciferase luminescence showed considerable variation resulting from differences in seedling size and orientation in microplate wells, the method impresses by its simplicity and yielded several candidate inhibitors from a small library of approximately 1700 compounds of natural and semi-synthetic origin. Rigorous validation of the identified candidates by orthogonal and counter assays uncovered jarin-1 (**7**) (**Figure 5**) as selective inhibitor of different jasmonate-dependent phenotypes (Meesters et al., 2014). The cognate target of jarin-1 was identified by systematic scanning of all known components participating in jasmonate biosynthesis and signaling, eventually establishing that jarin-1 binds to and inhibits the activity of jasmonoyl-L-isoleucine synthetase, encoded by *JASMONATE RESISTANT 1* (*JAR1*), which catalyzes the conjugation of jasmonic acid (JA) with L-isoleucine to the bioactive form of the hormone, (+)-7-*iso*-JA-L-Ile. Notably, JAR1 is the only member of the large family of adenylate-forming enzymes, conjugating several plant hormones (e.g., auxin, SA, JA) with amino acids, that is impaired by jarin-1 (Meesters et al., 2014). As this inhibition is effective not only in *Arabidopsis* but also in other plants, jarin-1 could prove a useful chemical tool for jasmonate research. Collectively, this study provides an outstanding example of a complete chemical genetic procedure, including hit selection by quantitative screening, verification and validation of primary hits by orthogonal and counter assays, SAR studies, and finally identification and characterization of the selective compound's molecular target.

In contrast to quantitative screenings, qualitative screenings may lead to biased hit selection, as phenotype evaluation is then prone to subjective decisions. To increase the confidence in hit selection, one possibility is to use multiple readouts. Essentially, this approach combines primary screening with first hit validation in one step, thereby helping to eliminate compounds that have pleiotropic effects. Plant hormones participate in multiple biologic processes and to circumvent their pleiotropic responses, several chemical screenings focusing on responses caused by plant hormones (e.g., auxin, strigolactone, or ethylene) have utilized such multiple readouts (Tsuchiya et al., 2010; Nishimura et al., 2012, 2014; Hu et al., 2014). For example, in search for new auxin transport inhibitors two parallel screenings were applied to the same chemical library of 10,000 compounds: (1) monitoring the gravitropic curvature of maize coleoptiles, and (2)

determination of indole-3-acetic acid (IAA) transport in coleoptile segments (Nishimura et al., 2012). Further characterization of eight candidate compounds originating from both screens eventually led to the identification of two new inhibitors of IAA transport [e.g., 37-H4 (**8**) and 48-F9 (**9**) (**Figure 5**)] that are structurally different to the known auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA, **10**), and therefore represent novel tools for dissecting the mechanism of auxin transport in plants. In a follow-up analysis, the same screening approach was used to identify inhibitors of IAA biosynthesis (Nishimura et al., 2014). As three selected compounds shared structural features with methimazole, an artificial substrate for flavin-containing mono-oxygenase (FMO), it was postulated that they may target YUCCA (YUC), a plant FMO (or FMO-like) protein that participates in IAA biosynthesis by catalyzing the hydroxylation of the amino group of tryptamine (Dai et al., 2013). The most potent inhibitor, yucasin (**11**) (**Figure 5**), was confirmed to impair the activity of recombinant *Arabidopsis* YUC1 protein *in vitro* and was further shown to suppress the high-auxin phenotype of plants overexpressing *YUC1*. However, yucasin did not affect IAA-dependent gene expression or auxin signaling after exogenous application of IAA (Nishimura et al., 2014). Thus, yucasin was shown to be a potent inhibitor of YUC enzymes *in vitro* and *in planta* and a useful tool in the quest for missing components of auxin biosynthesis and signaling.

Similarly, sequential screening for two different phenotypes was also successfully applied to find new inhibitors of brassinosteroid (BR) action (Gendron et al., 2008). Several chemical inhibitors of BR synthesis had previously been identified (Izumi et al., 1985; Asami et al., 2000, 2003, 2004; Sekimata et al., 2001, 2002) and their application in suppressor screens uncovered novel components of BR signaling (Wang et al., 2002; Yin et al., 2002, 2005; He et al., 2005). In search for novel inhibitors of BR signaling/synthesis, the retarded hypocotyl-length of dark-grown *Arabidopsis* seedlings served as first selection criterion, as inhibition of BR action causes dwarfism. Seedlings of a transgenic *Arabidopsis* line harboring the BR-repressed *CPD::GUS* reporter showing short hypocotyls upon treatment with chemicals were subsequently monitored for *GUS* expression as second indicator of reduced endogenous BR levels (Gendron et al., 2008). By this approach, chemicals impairing growth either directly or indirectly (e.g., by affecting other hormonal pathways) were easily eliminated. As result of this stringent selection scheme, only one unique inhibitor of BR biosynthesis, brassinopride (**12**) (**Figure 5**), was identified from a library of 10,000 diverse chemicals. The structure of brassinopride is quite different from other known BR inhibitors and physiological experiments further showed that it not only affected BR biosynthesis but also activated the ethylene signaling pathway (Gendron et al., 2008). Although this study did not uncover a direct target of brassinopride, it provided new insight into BR and ethylene cross-talk in seedling development. Another chemical screen monitoring also hypocotyl length aimed at identification of growth promoting compounds (Savaldi-Goldstein et al., 2008). Taking advantage of a BR-deficient *Arabidopsis* dwarf mutant, thereby facilitating the phenotypic analysis, 100 out of 10,000 compounds screened were found to promote hypocotyl length (Savaldi-Goldstein et al., 2008). Rather than performing extensive verification and validation of all compounds, the authors chose to search for common structural features and identified several compounds that share high similarity to the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D, **13**) (**Figure 5**). Remarkably, auxin had not been previously reported to directly affect hypocotyl length of light-grown seedlings. The effect of these synthetic proauxins on hypocotyl length was explained by efficient absorption and diffusion into this organ, where they undergo cleavage to functional auxins. Indeed, the compounds satisfied the Lipinski's RO5, they have a high probability of facile diffusion across cell membranes, and when incubated with seedlings, they liberated auxin- and 2,4-D-like molecules. Thus, the chemical biological approach has led to the discovery of novel proauxin analogs with selective activity in specific plant tissues (Savaldi-Goldstein et al., 2008). This example illustrates the need to consider various aspects associated with a compound's bioactivity, including uptake (facilitated for RO5 compounds), translocation or chemical modification (metabolism, detoxification) as it may occur within the cells/organism.

The commonly used plant systems for chemical screenings are seedlings or cultured cells, but particular biological processes may require other systems that suit better the needs for studying the process of interest. For example, proteins are delivered to and recycled from the plasmamembrane *via* endosomes, but the process and pathways of vesicle and cargo sorting is poorly understood and chemical modulators of vesicle trafficking are therefore desirable. The process of unidirectional (or polar) cellular growth involves intense vesicle trafficking and in plants this is obvious especially in root hairs and pollen tubes (Cole and Fowler, 2006). To identify chemicals affecting essential steps in plasmamembrane–endosome trafficking, Robert and colleagues designed an automated image-based screening with tobacco pollen by microscopic monitoring germination and tube morphology, which are both dependent on vesicle transport (Robert et al., 2008). Although only 2016 chemicals were screened, several bioactive compounds were identified, including cantharidin, a protein phosphatase inhibitor previously shown to affect the localization of auxin transporters (thus providing a proof of concept for the screen), and endosidin1 (ES1, **14**) (**Figure 5**), which interfered selectively with endocytosis not only in pollen but also *Arabidopsis* seedlings. In fact, ES1 treatment blocked the endocytosis of several auxin transporters (PIN2, AUX1), which are known to recycle in *Arabidopsis* roots, as well as the brassinosteroid receptor BRI1, leading to a brassinosteroid-insensitive phenotype, thereby demonstrating that all three plasmamembrane-resident proteins share overlapping endocytic pathways (Robert et al., 2008). Two additional findings are important in this context. First, the automated image-based phenotyping is suitable for high-throughput screening, as demonstrated by a subsequent report extending the approach to high-content intracellular image analysis using more than 46,000 compounds (Drakakaki et al., 2011). Second, an independent chemical screening for effectors of the circadian clock in *Arabidopsis* seedlings also identified ES1 (**14**), and subsequent work showed that ES1 treatment stabilized the actin cytoskeleton *in vivo*, which caused

changes in vesicle trafficking (Tóth et al., 2012). The identification of the actin-stabilizing effect was facilitated by comparing the effect of the compound on plant development to mutant phenotypes and to other drug treatments. Remarkably, ES1 also affected microfilaments in mammalian cells, indicating that its target is highly conserved. Thus, ES1 affects rhythms (i.e. period length of the clock) and endosome trafficking by altering the actin network. As it differs from previously described inhibitors, it may be a useful tool for studying actin-related processes.

For studying fundamental processes in plants, it may be useful to initiate work in a different simplified biological system. Trafficking of endomembranes is evolutionarily conserved and a cell autonomous process and therefore the unicellular eukaryote yeast, *Saccharomyces cerevisiae*, was employed as a substitute for a plant-based system to identify chemicals affecting the endomembrane system (Zouhar et al., 2004). A further rational for this approach lies in the fact that vacuolar biogenesis is an essential process in plants and mutants lacking proper vacuole development are embryo lethal (Rojo et al., 2001). Therefore, using yeast grown in 96-well microplates, a library comprising 4800 diverse chemicals was screened for compounds that caused secretion of carboxypeptidase Y (CPY), which is normally targeted to the vacuole (Zouhar et al., 2004). One of several identified protein-sorting inhibitors, named sortin1 (**15**) (**Figure 5**), was also active in *Arabidopsis* seedlings, causing reversible root growth inhibition and secretion of the plant CPY. Remarkably, sortin1-hypersensitive *Arabidopsis* mutants exhibited severe vacuolar morphology phenotypes and also showed defects in flavonoid accumulation (Rosado et al., 2011). Although the cognate target of sortin1 is not yet known and the mechanism of transport and vacuolar accumulation of flavonoids likewise remains unclear, sortin1-hypersensitive mutants and sortin1, as well as structural derivatives, will be useful tools to shed more light on vacuolar biogenesis and flavonoid transport in *Arabidopsis*. Again, these results clearly demonstrate the power of the chemical screening approach for identifying novel plant-active compounds affecting the endomembrane system in plants, which has proven difficult to dissect by conventional genetics.

Exploring New Experimental Systems

The central feature of all chemical screening projects is a miniaturization bioassay that is suitable for automated HTS. Most chemical screenings in plant systems have so far been conducted with *Arabidopsis* seedlings grown in microplates. Other systems such as cultured cells, pollen tubes germinated *in vitro*, or yeast cells (as heterologous substitute) have also been applied successfully, but not every pertinent biological question can be adapted to the microplate format. For example, automated systems for the analysis of root architecture have been reported (Armengaud et al., 2009; Ingram et al., 2012; Wells et al., 2012). These systems are not miniaturized and therefore chemical treatment would be difficult and expensive to perform. However, with a special effort Forde and colleagues developed a customized microplate system for high-content automatic image analysis of root architecture in *Arabidopsis* seedlings, which can be combined with chemical treatment (Forde et al., 2013). This provides a good example that even uncharted biological territory can be made accessible to interrogation by chemical biology. But there are still numerous plant processes that are recalcitrant to exploitation by the potential of chemical biology such as flowering, which is commonly associated with mature and large-size plants that cannot be hosted in microplates. As a substitute, duckweeds (Lemneae and Wolffieae sp.), which include the smallest flowering plants known, can easily be grown in liquid medium in microplates and were previously suggested to serve as model systems for studying flowering even before the emergence of *Arabidopsis* as model plant (Maheshwari and Chauhan, 1963; Kandeler, 1984). Indeed, it was shown that flowering of this aquatic plant can be controlled by application of chemicals such as SA, nitric oxide (NO) or cytokinin (Maheshwari and Venkataraman, 1966; Venkatar et al., 1970; Khurana and Maheshwari, 1983; Khurana et al., 2011). Despite apparent differences in NO-mediated induction of flowering in the monocotyledonous plant *Lemna aequinoctialis* and the dicot *Arabidopsis thaliana* (Khurana et al., 2011), the small aquatic duckweeds bear great potential for serving as powerful model systems for diverse chemical screening projects ranging from microscopic to macroscopic phenotypes such as endomembrane trafficking and flowering control, respectively.

Conclusions and Perspectives

Research in plant chemical biology has gained enormous momentum during the past 10 years with more than 30 diverse chemical screening campaigns being published that resulted in the identification of a large number of novel bioactive small molecules representing useful chemical tools for further dissecting biological processes (Supplementary Table 1). So far, there is a certain bias for analyzing synthesis and signaling pathways related to phytohormones, which may be related to the fact that these are bioactive small molecules mediating drastic phenotypic alterations (Fonseca et al., 2014; Rigal et al., 2014). Conversely, this also indicates that there is still enormous scope for extending chemical screening projects into yet unexplored areas of biology. As noted previously, one such area is cell biology with the need to score for intracellular phenotypes such as membrane trafficking, which requires establishment of automated screening systems for image and video analysis (Hicks and Raikhel, 2009, 2014). Likewise, application of biosensors, capable of monitoring intracellular concentrations of small molecules, and selective dyes for staining subcellular structures should be part of this development (Mur et al., 2011; Swanson et al., 2011; Okumoto et al., 2012). Given the availability of large collections of fluorescent-tagged intracellular marker proteins as well as the abundance of miscellaneous reporter lines and mutants, *Arabidopsis* will remain the prevailing experimental system for plant chemical biology. Thus, a steady and extensive application of chemical genetic approaches can therefore be expected. However, a continuous challenge is to develop screening methods that are rapid, simple, and robust (Zhang, 1999; Halder and Kombrink, 2015). In addition, the full potential of quantitative data acquisition thereby allowing rigorous application of statistical tools

for hit selection and validation has not yet been realized in the plant sciences, whereas this approach is routine in drug discovery programs (Malo et al., 2006, 2010; Swinney and Anthony, 2011). Finally, target identification remains the biggest challenge in all chemical biology projects and yet this step is indispensible for understanding a chemical's mode of action. Correspondingly, it is not sufficient to simply find new compounds with interesting bioactivities; rather we have to push harder to gain insight into the biological systems under investigation by application of chemical tools.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00131/abstract

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Facile high-throughput forward chemical genetic screening by *in situ* monitoring of glucuronidase-based reporter gene expression in *Arabidopsis thaliana*

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The use of biologically active small molecules to perturb biological functions holds enormous potential for investigating complex signaling networks. However, in contrast to animal systems, the search for and application of chemical tools for basic discovery in the plant sciences, generally referred to as "chemical genetics," has only recently gained momentum. In addition to cultured cells, the well-characterized, small-sized model plant *Arabidopsis thaliana* is suitable for cultivation in microplates, which allows employing diverse cell- or phenotype-based chemical screens. In such screens, a chemical's bioactivity is typically assessed either through scoring its impact on morphological traits or quantifying molecular attributes such as enzyme or reporter activities. Here, we describe a facile forward chemical screening methodology for intact *Arabidopsis* seedlings harboring the β-glucuronidase (GUS) reporter by directly quantifying GUS activity *in situ* with 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as substrate. The quantitative nature of this screening assay has an obvious advantage over the also convenient histochemical GUS staining method, as it allows application of statistical procedures and unbiased hit selection based on threshold values as well as distinction between compounds with strong or weak bioactivity. At the same time, the *in situ* bioassay is very convenient requiring less effort and time for sample handling in comparison to the conventional quantitative *in vitro* GUS assay using 4-MUG, as validated with several *Arabidopsis* lines harboring different GUS reporter constructs. To demonstrate that the developed assays is particularly suitable for large-scale screening projects, we performed a pilot screen for chemical activators or inhibitors of salicylic acid-mediated defense signaling using the *Arabidopsis PR1p::GUS* line. Importantly, the screening methodology provided here can be adopted for any inducible GUS reporter line.

Keywords: chemical screening, chemical genetics, high-throughput screening, bioactive small molecules, β-glucuronidase activity, reporter gene expression, salicylic acid

INTRODUCTION

In search for new tools that aid the dissection of complex biological processes, chemical genetics has been recognized as alternative experimental strategy to classical genetics approaches. Its strength lies in the potential to circumvent problems that are commonly encountered in classical genetics, such as redundancy, lethality, or pleiotropy of gene functions (Blackwell and Zhao, 2003; Stockwell, 2004; Hicks and Raikhel, 2012). For example, small molecules can in principle target multiple members of a protein family or, alternatively, the effects they exert can be temporally controlled and possibly reversed by withdrawing the chemical from the system. However, in contrast to animal systems, which are nurtured from drug discovery programs and cancer research, the application of chemical genetics in basic plant research stands quite in contrast to industrial applications such as pesticide (herbicide and fungicide) discovery and has only recently found broader application as documented in a number of reviews (Blackwell and Zhao, 2003; Raikhel and Pirrung, 2005; Kaschani and van der Hoorn, 2007; Hicks and Raikhel, 2009, 2012, 2014; Tóth and van der Hoorn, 2010).

Fundamentally, the key similar feature between chemical genetics and classical genetics is the generation of recognizable phenotypes at the whole plant, organ, cell, or subcellular level. While in genetic approaches phenotypes are created by mutations that result in altered protein expression or function, chemicals mostly interfere with protein functions directly, but when this alteration affects transcription factors or upstream components it may also result in modified gene expression. Correspondingly, numerous screenable phenotypes can be used for chemical interference and the model plant *Arabidopsis thaliana* is particularly suitable for such approaches. This is not only because of its small size, permitting easy cultivation in 96-well microplate format either on agar or in liquid medium, but also because large collections of mutants and transgenic lines are available, allowing to perform a diversity of phenotypic and reporter-based chemical screening strategies. Likewise, cultured cells are a prime

choice for chemical screens. However, screening at the whole plant level offers its own advantages to monitor morphological responses that are dependent on multicellular structures such as root growth, cell-wall formation, seed germination, hypocotyl elongation and other developmental processes, as well as organ- and cell-type-specific gene expression *via* selective reporter readouts. In recent years, numerous chemical screens covering many areas of plant biology have demonstrated the increasing impact of chemical genetics on basic plant research, including some impressive success stories in which for selected small molecules the cognate targets have been identified (Hicks and Raikhel, 2014). There are multiple examples addressing questions related to plant hormone signaling, i.e., responses to auxin, abscisic acid (ABA), jasmonic acid (JA), or brassinosteroids (Hayashi et al., 2003, 2008; Zhao et al., 2003; Armstrong et al., 2004; Walsh et al., 2006; Gendron et al., 2008; De Rybel et al., 2009; Park et al., 2009; Meesters et al., 2014), endomembrane trafficking (Zouhar et al., 2004; Surpin et al., 2005; DeBolt et al., 2007; Rojas-Pierce et al., 2007; Kim et al., 2010), plant pathogen interactions and plant immune responses (Serrano et al., 2007, 2010; Schreiber et al., 2008; Knoth et al., 2009; Noutoshi et al., 2012), and cellulose biosynthesis resp. cell wall formation (Desprez et al., 2002; Yoneda et al., 2007; Park et al., 2014). However, the most impressive example of groundbreaking work with small molecules was the identification and use of a novel ABA agonist, pyrabactin, that led to the identification of the long-searched-for ABA receptor (Melcher et al., 2009; Park et al., 2009; Santiago et al., 2009; Cutler et al., 2010).

In plant chemical genetic screens, the GUS reporter system has frequently been used. The simplicity and easiness of the histochemical GUS staining method, which relies on cleavage of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and formation of a blue-colored precipitate, made this approach a suitable and preferred choice for monitoring activity (phenotypic evaluation) in large-scale chemical screening approaches (Hayashi et al., 2003; Armstrong et al., 2004; Serrano et al., 2007; Gendron et al., 2008; Knoth et al., 2009). However, on the down side, this method provides only qualitative data, which are prone to subjective decisions and biased hit selection. Alternatively, GUS activity can be quantitatively determined by spectrophotometrical or fluorimetrical assays monitoring the cleavage of p-nitrophenyl-β-D-glucuronide or 4-methylumbelliferyl-β-D-glucuronide (4-MUG), respectively (Jefferson et al., 1987). Although reliable and robust, the shortcomings of these assays are that they are labor-intensive and time-consuming, as they require tissue homogenization and protein extraction, which renders these assays unsuitable for screening of large libraries. Alternatively, luciferase- or GFP-based reporter systems, allowing monitoring of true *in vivo* activities, are also suitable for chemical screening, but as these systems are less abundant than GUS-based reporters, there are only few documented applications (Yoneda et al., 2007; Tóth et al., 2012; Forde et al., 2013; Motte et al., 2013; Meesters et al., 2014).

Since GUS is the prevailing reporter system in plants, we wanted to combine the best out of both outlined approaches of GUS activity determination for a screening platform, and thus we explored whether the ease of the histochemical GUS staining method could be merged with the advantages of quantitative enzyme assays. To this end, we have established a simple chemical screening methodology, which is based on detergent-facilitated infusion of 4-MUG substrate through any GUS expressing plant tissue and direct quantification of fluorescence emitted by the released 4-methylumbelliferone (4-MU) in the same solution (Blázquez, 2007). Importantly, this assay is not only fast, robust and reliable, but also provides quantitative (or semi-quantitative) data directly *in situ*, thereby minimizing sample handling and allowing unbiased identification of hits *via* numeric threshold values derived from statistical procedures (Malo et al., 2006; Birmingham et al., 2009). To demonstrate the potential and superiority of our screening methodology, we used the transgenic *A. thaliana* line harboring the salicylic acid (SA)-responsive *PR1p::GUS* reporter to screen separately for both activators and inhibitors of SA signaling. *PATHOGENESIS-RELATED 1* (*PR1*) is as a canonical SA marker gene, regulated by multiple transcription factors, such as TGAs and WRKYs, and it is robustly up-regulated upon plant infection with biotrophic pathogens and during the systemic immune response (Vlot et al., 2009; Tsuda et al., 2013). In this small pilot experiment, we faithfully identified the known strong activator acetylsalicylic acid (ASA) and the translation inhibitor cycloheximide (CHX), but additional modulators of *PR1* gene expression that exert only weak effects were also captured. Thus, as expected from a quantitative assay, our method enables facile, automatic data acquisition and can also reliably distinguish between compounds with high and low potency. With this facile method at hand, large-scale screening campaigns using any GUS-expressing *Arabidopsis* line can be carried out in a time-, labor-, and cost-effective manner.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

In this study we used *A. thaliana* Columbia-0 (Col-0) transgenic lines carrying the following reporter genes in the Col-0 (or Col-5) genomic background: *PR1p::GUS* (Shapiro and Zhang, 2001), *DR5::GUS* (Ulmasov et al., 1997), *WRKY29p::GUS* (Serrano et al., 2007), and *DC3::GUS* (Chak et al., 2000). *Arabidopsis* seeds were surface-sterilized and seedlings grown hydroponically in 96-well microplates (PerkinElmer Inc., Germany) containing 0.2 ml of half-strength MS basal salt medium (Murashige and Skoog, 1962) supplemented with 0.5% sucrose. After stratification for 2 days at 4°C in the dark, plates were placed for 12 days in a growth chamber at a day/night cycle of 16/8 h at 21/19°C, respectively.

ANALYSIS OF GENE EXPRESSION IN GUS REPORTER LINES

Gene expression of β-glucuronidase (GUS) reporter lines was induced by treatment with the appropriate phytohormones as previously reported to yield maximum activity, i.e., *PR1p::GUS* was treated with 200 μM SA for 24 h, *DR5p::GUS* with 5 μM indole 3-acetic acid (IAA) for 4 h, *DC3p::GUS* with 100 μM ABA for 24 h, and *WRKY29p::GUS* with 1 μM peptide epitope of bacterial flagellin (flg22) for 4 h. Following this treatment, the medium was removed by aspiration and seedlings were used immediately (or stored at −80°C) for quantification of

GUS activity by *in situ* or *in vitro* assays. To reveal the organ- and cell-type-specific expression patterns of reporter genes, histochemical GUS staining was performed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) as previously described (Ancillo et al., 2003) using 12-day-old seedlings after treatment as specified above.

Quantification of GUS activity in vitro

The quantitative GUS assay was carried out as previously described (Sprenger-Haussels and Weisshaar, 2000). In brief, tissue samples (1–4 seedlings corresponding to 20–100 mg) were transferred to microtubes, homogenized in extraction buffer (100 mM potassium phosphate, 1 mM DTT, pH 7.5) and debris removed by centrifugation (30 min, 13,000 g, 4°C). The clear supernatant (50 μL) was mixed with GUS assay buffer (50 μL) containing 2 mM 4-methylumbelliferyl-β-D-glucuronide (4-MUG), 50 mM Na-phosphate pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 10 mM β-mercaptoethanol. Aliquots (20 μL) were sampled after 0, 30, and 60 min incubation at 37°C (unless otherwise stated), mixed with 0.2 mL 0.2 M Na_2CO_3 and 4-MU fluorescence was determined in a microplate reader (FluoroCount, Packard Bioscience, Meriden, Connecticut) using an excitation/emission wavelength of 365/455 nm. GUS activity was calculated using the ΔE_{455} increments (0–30 and 30–60 min) and appropriate 4-MU standards (50–5000 pmol). Specific activities were related to the protein concentration determined according to Bradford (Bradford, 1976) with bovine serum albumin as standard. All reported values are the mean (±SD) of at least four biological replicates.

Quantification of GUS activity in intact seedlings (in situ)

To adjust the quantitative GUS assay for large-scale screening applications, we optimized a previously reported method (Blázquez, 2007) by minimizing handling time and effort. In brief, single 12-day-old seedlings grown in 96-well microplates were incubated with 150 μL lysis buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100) containing 1 mM 4-MUG at 37°C for 90 min, unless otherwise stated. Of note, seedlings should be completely submerged in lysis buffer to allow ubiquitous substrate supply. At the end of the incubation period, 50 μL 1 M Na_2CO_3 (stop solution) was added to each well and 4-MU fluorescence directly determined in a microplate reader as before (excitation/emission wavelength of 365/455 nm). Activity is either directly expressed as relative light units (RLU per assay or seedling) or was converted to molar units using a standard curve (150 μL 50–1000 μM 4-MU in lysis buffer, plus 50 μL stop solution). All results are typically the mean (±SD) of at least four biological replicates.

CHEMICAL LIBRARY SCREENING

A small compound library, comprising 40 hand-picked chemicals (1 mM dissolved in DMSO), was used for screening. *Arabidopsis* seedlings harboring the *PR1p::GUS* reporter were grown in 96-well microplates for 12 days and before chemical treatment, growth medium was removed and replaced by fresh half-strength MS medium. To conditionally modulate SA signaling, seedlings were pretreated with chemicals (dissolved in DMSO) at a final concentration of 20 μM for 1 h before addition of 200 μM SA (dissolved in DMSO) to induce *PR1p::GUS* expression and subsequent incubation for 24 h unless otherwise stated (screening for inhibitors). Alternatively, omission of SA allowed screening for activators of *PR1p::GUS* expression. All chemicals were analyzed in two replicates and their activity normalized to control samples (without added chemical) that were contained on the same microplate (first and last column). The organization of samples in 96-well microplates is shown in **Figure 1**.

STATISTICAL ANALYSIS

The quantitative data analysis was performed in Excel spreadsheets with the embedded basic statistical functions (mean, standard deviation, Student's *t*-test, r.m.s. linear regression).

A common quality metric for evaluation and validation of high-throughput screening assays are the *Z* and *Z*' factors (Zhang et al., 1999; Birmingham et al., 2009). The *Z*' factor, often used during assay optimization, relies on high-value (positive) and low-value (negative) controls and is calculated by Equation (1), with μ representing the mean and σ the standard deviation of the high-value (subscript "hc") and low-value (subscript "lc") controls, respectively.

$$Z' \text{ factor} = 1 - \frac{(3\sigma_{hc} + 3\sigma_{lc})}{|\mu_{hc} - \mu_{lc}|} \quad (1)$$

The *Z*' factor ranges from negative infinity to 1, with values >0.5 indicating an excellent assay, >0 an acceptable assays and <0 an unacceptable assay. Correspondingly, the *Z* factor may be calculated using actual screening data (high values) instead of separate positive control values and thus serves to directly assess performance of the screen (Zhang et al., 1999; Birmingham et al., 2009).

The *Z* score, not to be confused with the *Z* and *Z*' factors, representing the number of standard deviations from the mean, is frequently used to normalize screening data such that individual

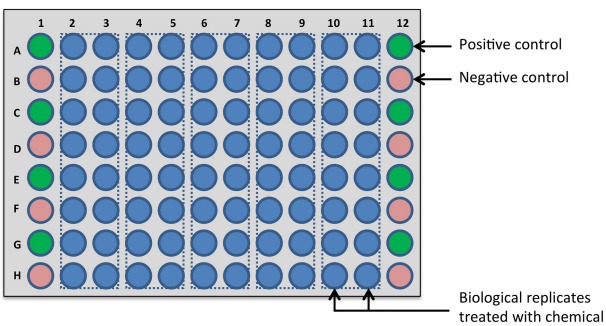

FIGURE 1 | Design of chemical screening plate. In the screens described here, each chemical is tested in two biological replicates in the central wells of a 96-well microplate (blue circles), allowing 40 chemicals to be analyzed. This design is recommended, because in commercial compound libraries, 80 different compounds are generally stored in the middle of 96-well plates and the first and last columns are left empty. Correspondingly, column 1 and column 12 are available for controls and to minimize edge-related bias, the eight positive controls (green circles) and the eight negative controls (red circles) are distributed across these columns in alternating order.

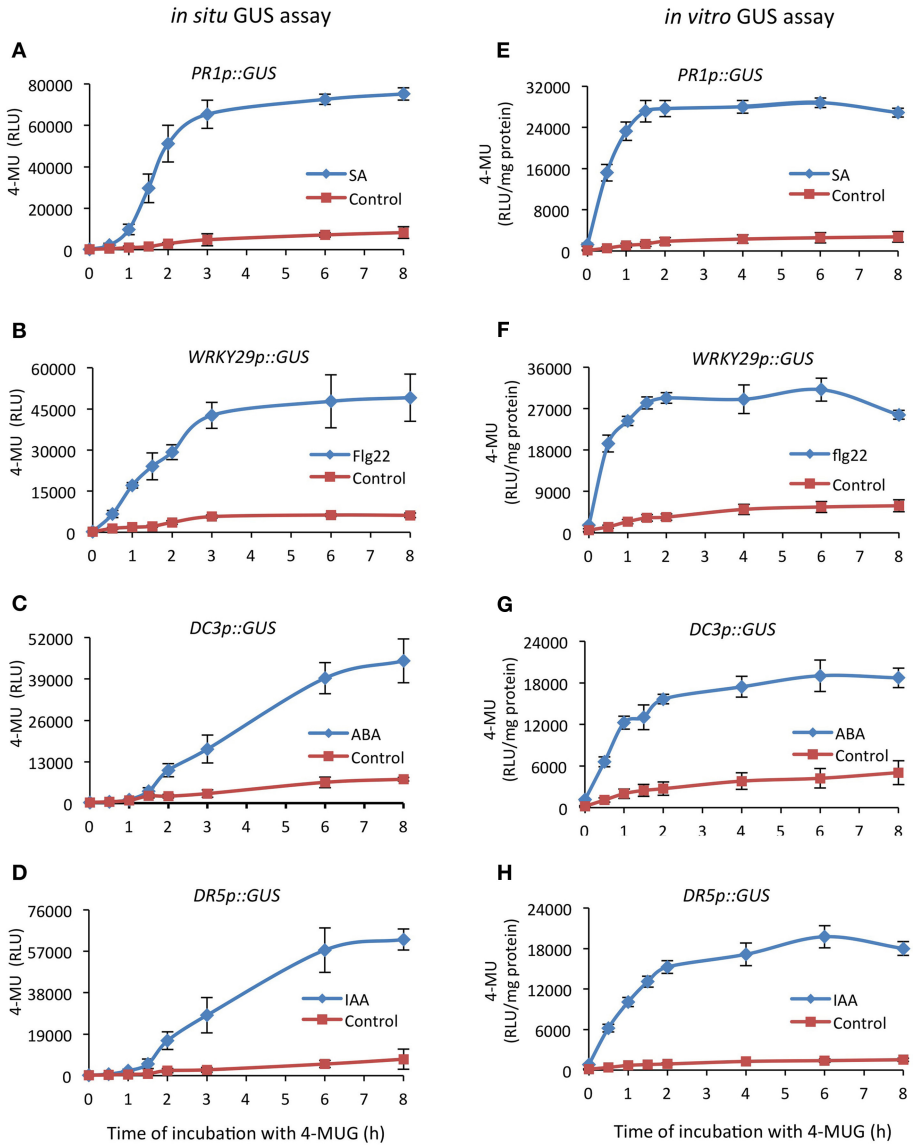

FIGURE 2 | Comparison of GUS activity determined in whole seedlings (*in situ*) and in protein extracts (*in vitro*). Seedlings of transgenic *Arabidopsis thaliana* lines harboring different inducible promoter–GUS fusions were grown for 12 days hydroponically in microplates and then treated with the respective inducer (or solvent as control) for an appropriate time period to obtain high expression levels of the reporters. **(A,E)** *PR1p::GUS* seedlings received 200 μM SA for 24 h, **(B,F)** *WRKY29p::GUS* received 1 μM flg22 for 4 h, **(C,G)** *DC3p::GUS* received 100 μM ABA for 24 h and **(D,H)** *DR5p::GUS* received 5 μM IAA for 4 h. Following this treatment, the medium was removed and for monitoring GUS activity *in situ* **(A–D)**, seedlings were incubated with the substrate 4-MUG (1 mM) for the indicated time periods before the reaction was terminated by addition of stop solution (Na_2CO_3). The released reaction product, 4-MU, was directly quantified by its fluorescence in a microplate reader. For quantifying GUS activity *in vitro* **(E–H)**, seedlings were homogenized and conversion of the substrate 4-MUG (2 mM) in clarified protein extracts was determined as described in the Materials and Methods Section. 4-MU release is given in relative light units (RLU) emitted from the whole *in situ* assay **(A–D)** or normalized to the protein concentration for the *in vitro* assay **(E–H)**. All values represent the mean (±SD) of four biological replicates.

measurements are rescaled relative to the whole-plate variation (Malo et al., 2006; Birmingham et al., 2009). The Z score was calculated by Equation (2), with x_i representing the raw value of the individual compound i, μ and σ are the mean and standard deviation, respectively, of all values within a plate.

$$Z \text{ score} = \frac{x_i - \mu}{\sigma} \quad (2)$$

RESULTS

DIRECT QUANTIFICATION OF GUS ACTIVITY IN INTACT *ARABIDOPSIS* SEEDLINGS

We wanted to establish a facile GUS assay that does not require tissue homogenization and yet provides a reliable, quantitative output that is suitable for large-scale chemical library screening. Therefore, we used *Arabidopsis* seedlings harboring different inducible GUS reporter constructs, which were grown

hydroponically in 96-well microplates and treated accordingly to provide high GUS activity. Such seedlings were then directly incubated with GUS assay buffer, which was supplemented with Triton X-100 to enhance the permeability of both the substrate 4-MUG and the product 4-MU throughout the tissue. The release of the product (4-MU, monitored by its fluorescence) occurred with a delay of 20–60 min, followed by a linear increase for about 2 h until the substrate was depleted (**Figure 2**). Apparently, the delay of product release is inversely correlated with total GUS activity; strong promoters, such as *PR1* or *WRKY29* (**Figures 2A,B**), providing high levels of expression (and enzyme activity) showed shorter delays of substrate release in comparison to DC3 or DR5 (**Figures 2C,D**), which yield lower expression levels and extended delays.

To confirm that the *in situ* GUS assay faithfully records activity, we also determined rates of substrate conversion *in vitro* by a conventional GUS activity assay (Sprenger-Haussels and Weisshaar, 2000), using seedlings that were subjected to the same treatments. As expected, in protein extracts the release of the product (4-MU) occurred instantaneously but otherwise followed a similar time course, as in intact seedlings (**Figures 2E–H**). Next, we directly compared the specific GUS activity profiles in biological samples, i.e., transgenic *Arabidopsis* lines harboring different reporter constructs, that were treated accordingly to provide high expression levels of the respective reporter gene. As apparent from **Figure 3**, our *in situ* method and the established *in vitro* GUS assay generally recorded nearly identical induction of activity in response to specific treatments in all tested reporter lines, ranging between 15-fold for *PR1p::GUS* (SA responsive) and 5-fold for *DC3p::GUS* (ABA responsive) when comparing positive and negative controls. Of note, the *in situ* GUS activity in this experiment was determined from a fixed incubation period of 2 h for all samples, whereas the *in vitro* activity assay recorded initial rates over maximally 1 h (cf **Figure 2**). Therefore, as result of delayed substrate release, the *in situ* method had a tendency to provide lower values, ranging from a maximum deviation of -30% (*DR5p::GUS*, **Figure 3D**) to virtually identical values (*PR1p::GUS*, **Figure 3A**). From this we conclude that GUS activity can be directly and reliably estimated in intact seedlings, but the conditions need to be adjusted to each particular reporter lines such that product release remains in the linear range (or near linear range) and not all 4-MUG has been consumed. For the *PR1p::GUS* line, we selected an incubation time of 90 min for all subsequent experiments (cf **Figure 2A**).

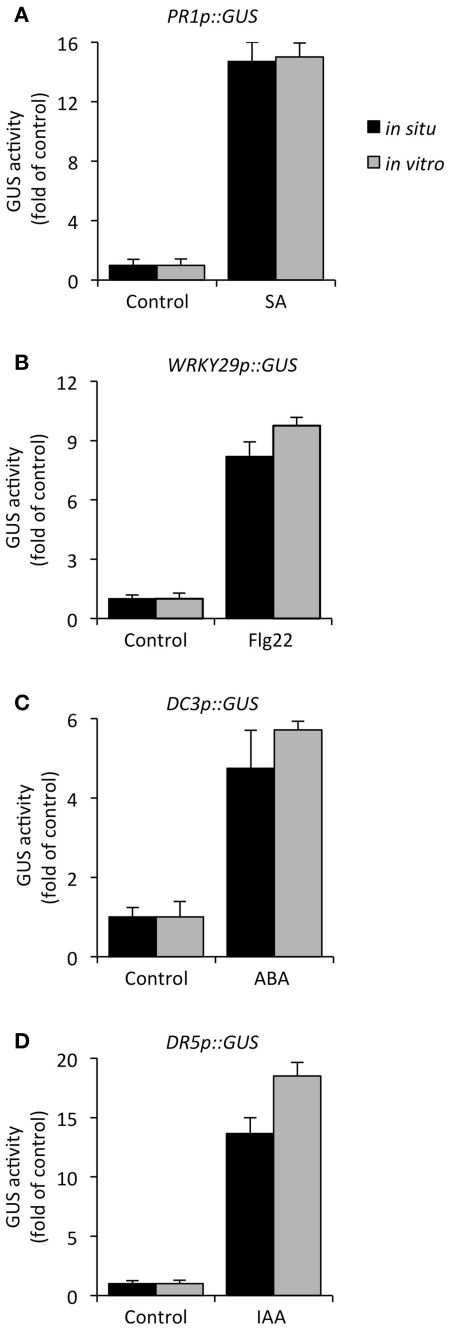

FIGURE 3 | Comparison of induced expression of diverse promoter–GUS reporter genes as determined by *in situ* and *in vitro* GUS assays. Twelve-day-old transgenic *Arabidopsis* seedlings were appropriately treated to obtain high reporter gene expression: **(A)** *PR1p::GUS* (200 μM SA, 24 h), **(B)** *WRKY29p::GUS* (1 μM flg22, 4 h), **(C)** *DC3p::GUS* (100 μM ABA, 24 h), and **(D)** *DR5p::GUS* (5 μM IAA, 4 h). GUS activity *in situ* (black bars) was determined after incubation of whole seedlings with the substrate 4-MUG (1 mM) for 2 h and it is compared to GUS activity (initial rate) determined *in vitro* (gray bars) using protein extracts prepared from seedlings that were treated identically. For better comparison, the resulting activities *in situ* [relative light units (RLU) per assay] and *in vitro* (pmol min^{-1} mg^{-1} protein) are normalized to untreated control samples, thus showing fold of induction in response to treatment. All values represent the mean (±SD) of four biological replicates.

ROBUST AND RELIABLE GUS QUANTIFICATION IN FRESH AND FROZEN *ARABIDOPSIS* SEEDLINGS

To further validate the reliability and robustness of GUS activity quantification in intact seedlings, we applied the *in situ* GUS assay to analyze the time course of *PR1p::GUS* expression upon treatment with SA. Here, a standard curve with known 4-MU concentrations was used to normalize the activity, i.e., the emitted fluorescence, which was again compared to the GUS activity determined *in vitro*. As shown in **Figure 4**, both assays provide a similar result (i.e., GUS activity profiles), demonstrating that *PR1* gene expression is rapidly up-regulated, reaching a maximum at 12 h and slowly declining thereafter. In control seedlings,

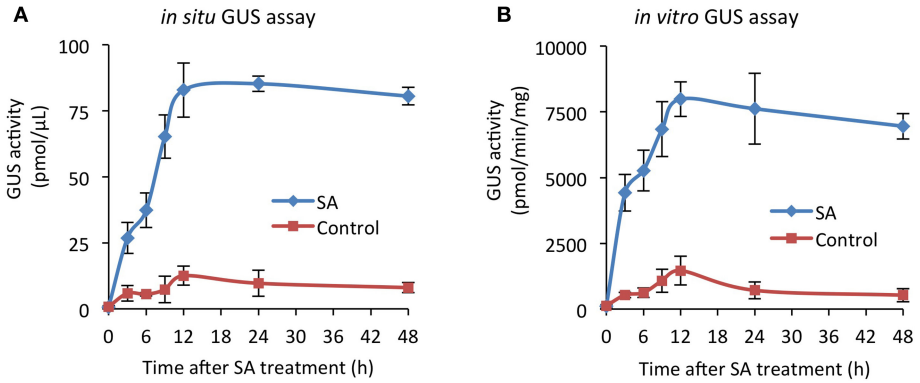

FIGURE 4 | Time course of *PR1p::GUS* expression upon treatment with SA. *Arabidopsis* seedlings harboring the SA-responsive *PR1p::GUS* reporter gene, grown for 12 days in liquid culture, were treated with 200 µM SA (or 0.2% DMSO as control) for the indicated time periods. **(A)** GUS activity was determined with intact seedlings (*in situ*) and **(B)** in total extracts (*in vitro*) derived from seedlings of the same experiment. Specific activities are derived from 4-MU standard curves and are normalized to assay volume **(A)** or total extractable protein **(B)**. All values represent the mean (±SD) of four biological replicates.

treated with solvent (DMSO), only a low activity increase occurred.

For many biological applications it is necessary or useful to freeze samples for subsequent bioassays. We therefore explored whether the new GUS assay can also be performed with frozen seedlings without loss in performance. Therefore, *PR1p::GUS* seedlings were treated with SA (200 µM) as before and at the end of the incubation period (24 h) half of the samples were used to quantify GUS activity immediately. The other half was transferred to Eppendorf tubes, frozen in liquid nitrogen and stored at −80°C for 4 weeks. (Of note, for short-term storage samples can also be frozen directly in closed microplates). Without much thawing, seedlings were provided with substrate-containing lysis buffer and activity was recorded as before. The GUS activity determined in fresh and frozen seedling diverged by maximally 20% in both SA-treated and control samples (**Figure 5**).

We conclude, the described GUS activity assay for application with intact seedlings is robust and reliable and the facile acquisition of quantitative data makes it particularly suitable for application in large-scale screening programs.

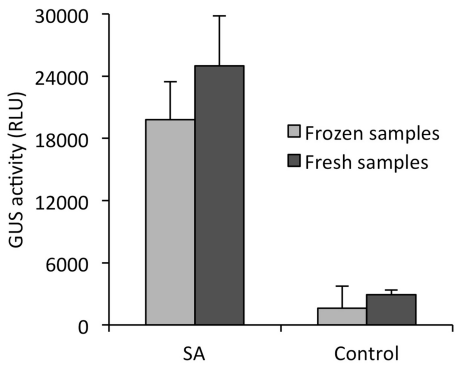

FIGURE 5 | Comparison of *in situ* GUS activity in fresh and frozen seedlings. *Arabidopsis* seedlings harboring the SA-responsive *PR1p::GUS* reporter gene, grown for 12 days in liquid culture, were treated with 200 µM SA or 0.2% DMSO (control) for 24 h. Half of the samples served for instant determination of GUS activity *in situ* (black bars) as described in Materials and Methods. The other half was frozen and stored at −80°C for 4 weeks. For determining GUS activity, frozen seedlings were transferred to microplate wells prefilled with assay buffer containing 1 mM 4-MUG and incubated for 90 min before quantifying 4-MU fluorescence. Activity is given in relative light units (RLU) emitted from total assays and all values represent the mean (±SD) of four biological replicates.

THE GUS PRODUCT 4-MU IS READILY RELEASED FROM THE PLANT TISSUE

The functionality of the GUS assay with intact seedlings relies on the included detergents, Triton X-100, which facilitates penetration of substrate and product throughout the seedlings (Blázquez, 2007). To demonstrate that this is a valid assumption, we monitored whether the product of the reaction, 4-MU, indeed leaks out of the seedlings or stays within. To this end, we treated *PR1p::GUS* seedlings with various SA concentrations and after 24 h determined GUS activity (**Figure 6A**). From the results it is apparent that increasing SA caused higher *PR1* gene expression, reaching a maximum at 200–300 µM as previously reported (Bartsch et al., 2010). Higher SA concentrations were toxic and therefore no gene expression (GUS activity) was detectable. When from the same experiment, the seedlings were removed from the assay buffer, transferred to new microplates, and the fluorescence emanating from the seedlings and the assay buffer was separately recorded, we observed that the entire signal was almost exclusively associated with the solution (**Figure 6B**). This indicates that the enzyme's product, 4-MU, is readily released from the plant tissue and collected in the medium.

CHEMICAL LIBRARY SCREENING WITH GUS ASSAY IN INTACT SEEDLINGS

To demonstrate the general suitability of the new GUS assay methodology for chemical library screening with intact seedlings harboring inducible GUS reporter constructs, we performed a pilot screen with just 40 selected compounds, which fit in one 96-well microplate when assayed in duplicates. The general design

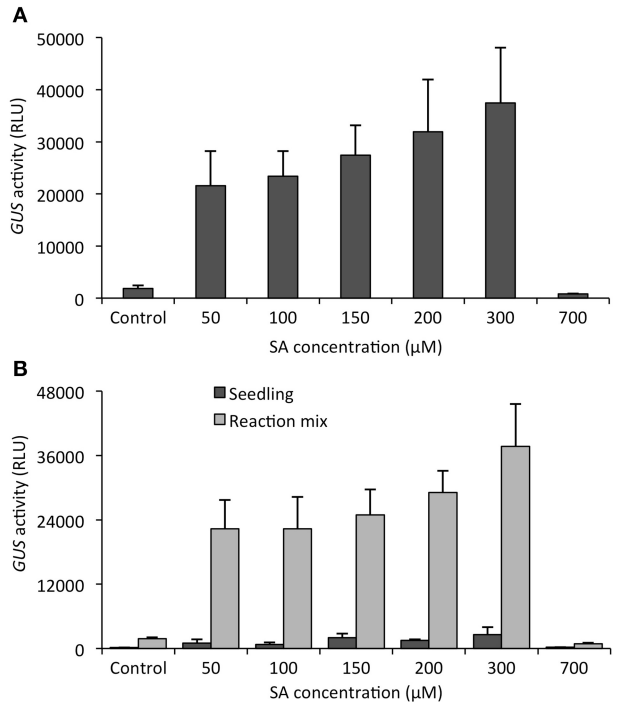

FIGURE 6 | The reaction product of the *in situ* GUS assay accumulates in the medium. *Arabidopsis* seedlings harboring the SA-responsive *PR1p::GUS* reporter gene, grown for 12 days in liquid culture, were treated with increasing concentrations of SA (or 0.2% DMSO as control) for 24 h. **(A)** GUS activity of whole seedlings (*in situ*) was quantified as before (see Materials and Methods). **(B)** Following 4-MU quantification, seedlings were removed from first assay mixture, transferred to new microplates and 4-MU fluorescence emitted from seedlings only (black bars) or the reaction mixture devoid of seedlings (gray bars) quantified. The reaction product, 4-MU, is almost exclusively localized in the medium. All values represent the mean (±SD) of four biological replicates.

of the screening plate, which should also be adopted for large-scale screening campaigns comprising several thousand chemicals, is shown in **Figure 1**; it includes positive (SA treatment) and negative (DMSO) controls alternating in the first and last column. Since we used an inducible GUS reporter system, it could be applied for bidirectional screening for either activators of gene expression or inhibitors that impair induced gene expression.

However, before proceeding directly to screening data analysis, we first assessed the quality of our assay conditions to ensure that the resulting data meet the minimum standards and permit legitimate conclusions. Therefore, we calculated the *Z*' factor, which is a common quality metric for evaluation and validation of high-throughput screening assays (Zhang et al., 1999; Birmingham et al., 2009), using the eight positive and eight negative control values included in each of the two screening plates (cf. **Figure 1**). The high-value (SA treated) control (RLU = 42,826 ± 5342 and 37,266 ± 2480) and low-value (DMSO treated) control (RLU = 1243 ± 459 and 2294 ± 711) represent the screening window (Supplementary Figures 1A,B) and yielded *Z*' factors of 0.58 and 0.73, respectively. By exceeding the value of 0.5, this clearly defines the SA-induced *PR1p::GUS* expression as an excellent assay for chemical screening purposes, when using the established conditions for *in situ* quantification of GUS activity.

In the screen for **activators** of *PR1p::GUS* expression, 12-day-old seedlings were treated with chemicals at 20 μM for 24 h followed by instant quantification of GUS activity. Only one constituent of the library, which was identified as acetylsalicylic acid (ASA, **32**), also named aspirin, caused an appreciable increase in GUS activity (**Figure 7A**). ASA has previously been demonstrated to activate plant defense responses, similar to SA (White, 1979; Spoel et al., 2003; Loake and Grant, 2007). Importantly, the recorded activity was about 8-fold higher than the negative control values (RLU = 1243 ± 459) and about 25% of the positive control values obtained with 200 μM SA (RLU = 42,826 ± 5342) (**Figure 7A** and Supplementary Figure 1A). To gain further confidence in our hit selection, we also calculated the *Z* score, which serves to normalize the data and also provides explicit information on the variation in sample and control measurements (Malo et al., 2006; Birmingham et al., 2009). Hit compounds are selected on the basis of a threshold value, which is typically set to a *Z* score of 2–3, i.e., SD above or below the normalized mean (*Z* score = 0). With a *Z* score > 5, ASA can be classified as strong hit, whereas weak candidates [e.g., compound **34** (cycloheximide, CHX) with a *Z score* ≈ 1] would require confirmation by additional experiments (**Figure 7B**).

In the screen for **inhibitors** of *PR1p::GUS* expression, seedlings were pre-incubated with the library constituents for 1 h before addition of 200 μM SA and quantifying GUS activity after 24 h as before. From the raw data it appears as if the variation of induced activity is relatively high (**Figure 8A**); however, the coefficient of variation ($Cv = \sigma/\mu$) is only 0.15 when calculated across the whole screening plate, which compares favorably with the corresponding *Cv* value of 0.25 for non-induced activities (e.g., screening plate for activators, cf. **Figure 7A**). Irrespectively, the translational inhibitor CHX **34** was clearly identified as a strong hit, as also apparent after *Z* score transformation of the activity data, which yields a value <-2 (**Figure 8B**). By contrast, the mycotoxin neosolaniol **23**, which also impairs protein translation (Serrano et al., 2010), and thiomersal **37**, an antiseptic and antifungal agent, showed up as relatively weak inhibitors. This is also apparent from their *Z* scores of approximately −1 (**Figure 8B**). Again, the validation of such weak inhibitors would require additional experiments, such as determination of concentration dependency, bioavailability and/or stability, which is beyond the scope of this paper. The structures of all the compounds acting as activators or inhibitors of *PR1* expression identified in this small pilot screen are shown in **Figure 9**.

As a final step to further characterize the outlined screening methodology, we generated a replicate correlation plot to visualize the overall reproducibility (**Figure 10**). The calculated Pearson's correlation coefficient ($r = 0.94$) for both primary screens is a quality metric and demonstrates a good overall reproducibility and reliability of replicates. From this we conclude that the GUS activity assay with intact seedlings provides quantitative data of sufficient robustness and accuracy to allow confident hit identification in chemical screening campaigns.

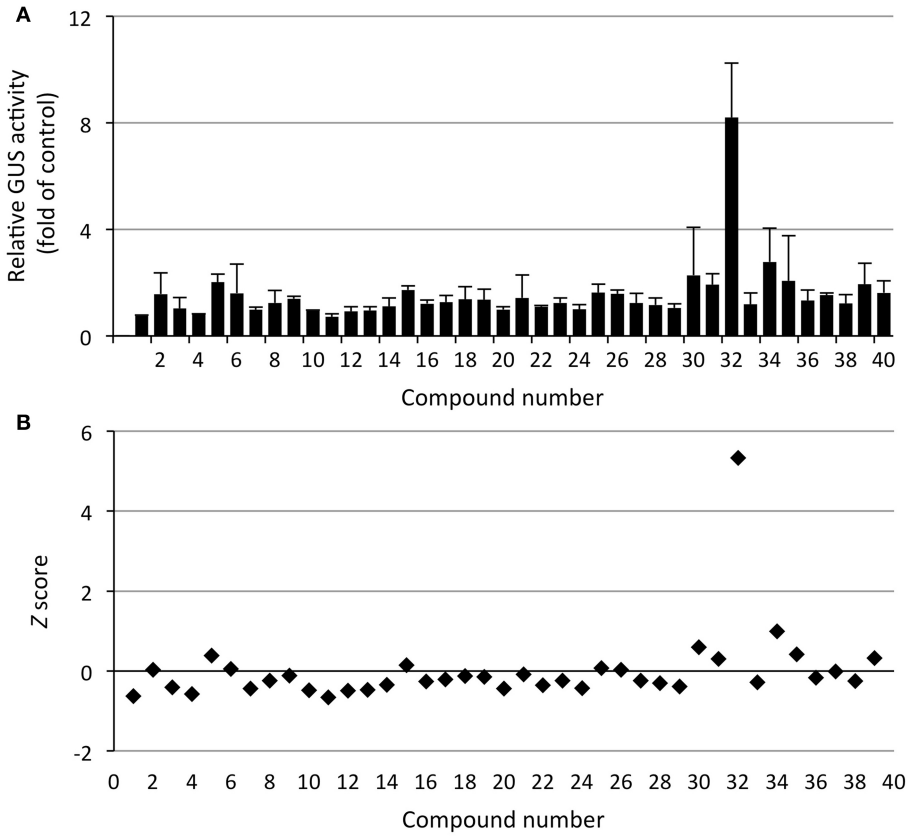

FIGURE 7 | Screening for activators of SA signaling. *Arabidopsis* seedlings harboring the SA-responsive *PR1p::GUS* reporter gene, grown for 12 days in liquid culture, were treated with 40 diverse chemicals (20 μM) for 24 h. **(A)** GUS activity of whole seedlings (*in situ*) was quantified by incubation with 4-MUG (1 mM) for 90 min (see Materials and Methods) and normalized to the control samples (DMSO treated). Values represent the mean of duplicate samples and the error bars indicate the corresponding high and low values. One compound (**32**, acetylsalicylic acid) appeared as strong activator of reporter gene expression, causing 8-fold induction. **(B)** *Z* score transformation of the screening data (see Materials and Methods) likewise identifies compound **32** as strong hit ($Z > 5$), whereas compound **34** (cycloheximide) is a marginal hit ($Z \approx 1$), which requires confirmation and further validation. The raw activity data of this screen are presented in Supplementary Figure 1A.

DISCUSSION

Here, we have established and validated a new forward chemical genetic screening method using intact *A. thaliana* seedlings harboring diverse GUS reporter constructs for direct quantification of GUS activity. Its direct application in the microplate format used for seedling growth requires only a minimum of sample handling and allows automatic acquisition of quantitative data, which are a prerequisite for unbiased identification of hits *via* numeric threshold values derived from statistical procedures (Malo et al., 2006; Birmingham et al., 2009). Clearly, this approach is superior over frequently used qualitative screening approaches that are based on visual evaluation of GUS stained tissue, which is prone to biased hit selection (Hayashi et al., 2003; Armstrong et al., 2004; Serrano et al., 2007; Gendron et al., 2008; Knoth et al., 2009; Kim et al., 2011). Likewise, the outlined procedure is superior to other quantitative GUS assays carried out *in vitro*, which rely on tissue extraction and, although accurate, are much more labor-intensive and time-consuming. The screening methodology we describe is facile, accurate, reliable, and robust and therefore suitable for high-throughput screening projects. Although this method monitors activity only *in situ* (rather than *in vivo*) it compares well with the luciferase reporter system, which allows true activity recording *in vivo* and therefore represents the most frequently used screening tool in drug discovery programs (Inglese et al., 2007). However, in plants, including *Arabidopsis*, GUS is still the prevailing reporter system in use and therefore the outlined procedure may find frequent application.

To demonstrate the reliability and robustness of the *in situ* GUS quantification with intact seedlings, we directly compared it to the conventional, frequently used quantitative *in vitro* GUS assay. Using different inducible GUS reporter lines, we observed similar patterns of substrate conversion in both assays. However, the GUS activity recorded *in situ* cannot easily be normalized to protein content or fresh weight without compromising on its ease and simplicity, but molar conversion rates can be obtained from the emitted RLU by its relation to a standard curve with known product (4-MU) concentrations. Although signal intensity is affected by seedling size, the observed variability of recorded GUS activity in replicate samples is not exceeding that of the normalized GUS activity determined *in vitro* (cf. **Figures 1**, **2**). The

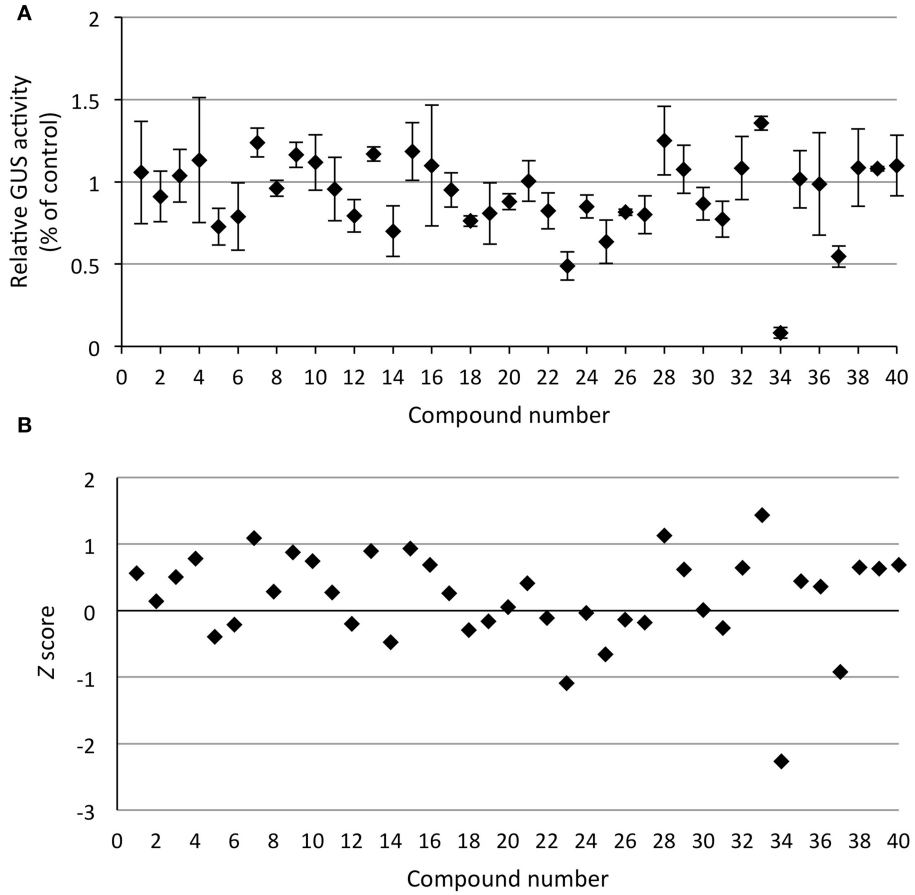

FIGURE 8 | Screening for inhibitors of SA signaling. *Arabidopsis* seedlings harboring the SA-responsive *PR1p::GUS* reporter gene, grown for 12 days in liquid culture, were treated with 40 diverse chemicals (20 μM) for 1 h prior to addition of SA (200 μM) to induce reporter gene expression. **(A)** GUS activity of whole seedlings (*in situ*) was quantified by incubation with 4-MUG (1 mM) for 90 min (see Materials and Methods) and normalized to the SA-treated control samples. Values represent the mean of duplicate samples and the error bars indicate the corresponding high and low values. One compound (**34**, cycloheximide) appeared as strong inhibitor of reporter gene expression. **(B)** *Z* score transformation of the screening data (see Materials and Methods) likewise identifies compound **34** as strong hit ($Z < -2$), whereas compounds **23** (neosolaniol) and **37** (thiomersal) are marginal hits ($Z \approx -1$), which require confirmation and further validation. The raw activity data of this screen are presented in Supplementary Figure 1B.

same conclusion is derived from the high correlation coefficient ($r = 0.94$) of replicate samples, demonstrating high accuracy and reproducibility of GUS activity quantification. Furthermore, the *in situ* GUS assay is suitable for application to a large variety of GUS reporter lines, irrespective of their particular cell-type and organ-specific expression patterns and modes of regulation. This is not only true for the four reporter lines used in this study (Supplementary Figure 2), but also for several additional lines that we currently apply in various experiments.

To further affirm the suitability of the described GUS assay for chemical screening projects, we employed it in a small pilot screen using seedlings of the *PR1p::GUS* reporter line in search for modulators of SA signaling. Both a strong activator, ASA, and a strong inhibitor, CHX, of reporter gene expression were identified with high confidence *via* their modulation of GUS activity (**Figures 7**, **8**). The bioactivity of both types of compound has previously been described (White, 1979; Spoel et al., 2003; Loake and Grant, 2007; Serrano et al., 2010; Meesters et al., 2014), here they served as positive and negative controls, respectively. The major advantage of the method, however, lies in the acquisition of quantitative expression data, which allows application of statistical tools for unbiased hit selection (Malo et al., 2006; Birmingham et al., 2009). In addition, quantitative screening data permit to distinguish between compounds with high and low potency, which may be useful for subsequent experimental strategies aiming at the discovery of new bioactive scaffolds. However, such weak activities as uncovered here need further critical evaluation.

In conclusion, we provided an efficient, facile, reliable and robust screening methodology, based on quantitative estimation of GUS activity in intact *Arabidopsis* seedlings, which can easily be adopted for any transgenic line harboring the GUS reporter. The acquisition of quantitative data in combination with the ease of sample and assay handling compare favorably with the convenience of truly *in vivo* activity monitoring systems such as luciferase or fluorescent proteins (GFP, RFP, etc.) and therefore

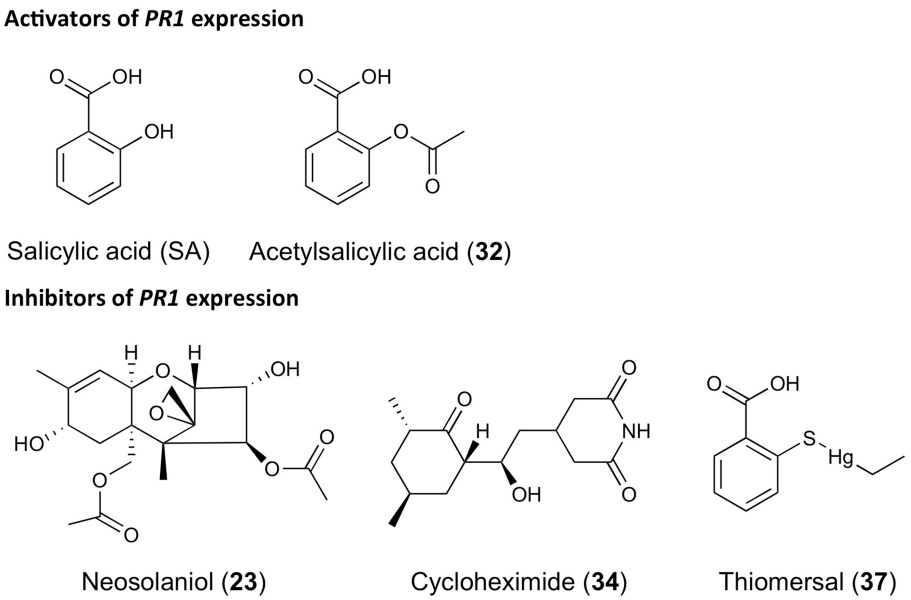

FIGURE 9 | Structures of bioactive compounds modulating *PR1* gene expression. Examples refer to compounds mentioned in this paper that were identified in the small pilot screen described.

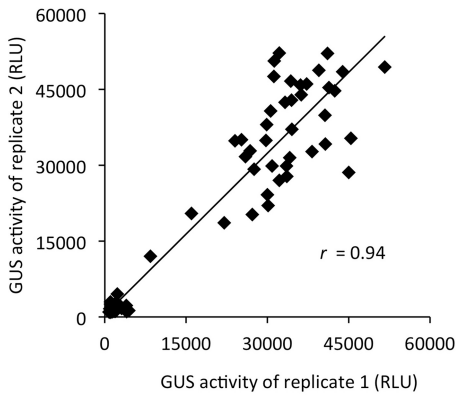

FIGURE 10 | Replicate correlation plot of screening data. The raw activity values, replicate 1 and 2, of the two pilot screening plates for activators and inhibitors of *PR1p::GUS* expression were plotted against each other. The high value of Pearson's correlation coefficient ($r = 0.94$) indicates that the *in situ* GUS assay is robust, reliable and provides reproducible screening data.

the outlined methodology has great potential for broad application particularly in time- and labor-intensive large-scale chemical screening campaigns.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00013/abstract

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Target identification strategies in plant chemical biology

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The current needs to understand gene function in plant biology increasingly require more dynamic and conditional approaches opposed to classic genetic strategies. Gene redundancy and lethality can substantially complicate research, which might be solved by applying a chemical genetics approach. Now understood as the study of small molecules and their effect on biological systems with subsequent target identification, chemical genetics is a fast developing field with a strong history in pharmaceutical research and drug discovery. In plant biology however, chemical genetics is still largely in the starting blocks, with most studies relying on forward genetics and phenotypic analysis for target identification, whereas studies including direct target identification are limited. Here, we provide an overview of recent advances in chemical genetics in plant biology with a focus on target identification. Furthermore, we discuss different strategies for direct target identification and the possibilities and challenges for plant biology.

Keywords: small molecule, target identification, plant biology, chemical genetics, *Arabidopsis thaliana*

INTRODUCTION

A proven way to study how something works is to perturb the process of interest in a well-defined and controlled manner. In biology, this is often accomplished by introducing alterations into the genome of an organism, such as mutations or ectopic expression. A major disadvantage of working at the gene level is that the resulting organism will live in a steady state with the induced genetic change. Additionally, perturbations of essential gene functions will lead to lethality, unless conditional, and perturbations of a gene member of a large gene family might have no effect due to redundancy. In order to address gene redundancy and lethality problems, together with the possibility to perturb a system in a more dynamic manner, chemical biology approaches can be used. In chemical biology, typically small molecules are applied to a biological system, altering the process of interest by binding target molecules. A key feature of chemical biology is its conditional nature. Small molecules can be used for any desired time and concentration, and in most cases can be washed out of the system of choice, making them an ideal tool to study dynamic processes for a certain period of time. Crucially, small molecules will not alter an organism over generations and are not restricted to bind only proteins, but can modulate a biological system by binding lipids or nucleic acids (Ziegler et al., 2013). Finally, using different approaches, the target of small molecules needs to be identified to get a better understanding of the affected process.

Chemical genetics strategies in plant biology lag behind the animal field, in which drug development provided a plethora of different target identification strategies. In plant biology, most target identification strategies consist of a phenotyping approach or a forward genetics strategy based on small molecule resistance screens. A few examples exist of strategies, such as affinity purification, that were successfully applied (Tresch, 2013).

An important aspect of chemical biology is linking the induced phenotype to one or more targets (**Figure 1**). Usually, only the relevant target, or target with the highest affinity for the small molecule, is identified and validated, although so-called "off-targets" might contribute substantially to the overall phenotype. Therefore, it has become increasingly important to understand and generate the small molecule interactome, in order to explain the observed phenotypes (Lounkine et al., 2012). This aspect is especially important for small molecules with a commercial application in healthcare or agriculture.

Several reviews have addressed the chemical genetics approaches in plant biology and the challenges and opportunities that lay ahead (Tóth and van der Hoorn, 2010; Kumari and van der Hoorn, 2011; Hicks and Raikhel, 2012; Xuan et al., 2013). Other recent reviews include a comprehensive overview of target identification strategies in mostly animal systems (Ziegler et al., 2013), and a thorough overview of small molecules with known targets and mode of action in plant biology (Tresch, 2013). Given that plant biological research uses limited target identification approaches, this review will briefly discuss the current ones, and will mostly focus on emerging new strategies, which have not found a broad application in plant biology yet (**Table 1**). Where applicable, examples from plant biology will be given, and benefits and shortcomings will be discussed. The ultimate aim of this review is to convince the reader to look further than the established target identification strategies when a chemical genetics approach is considered.

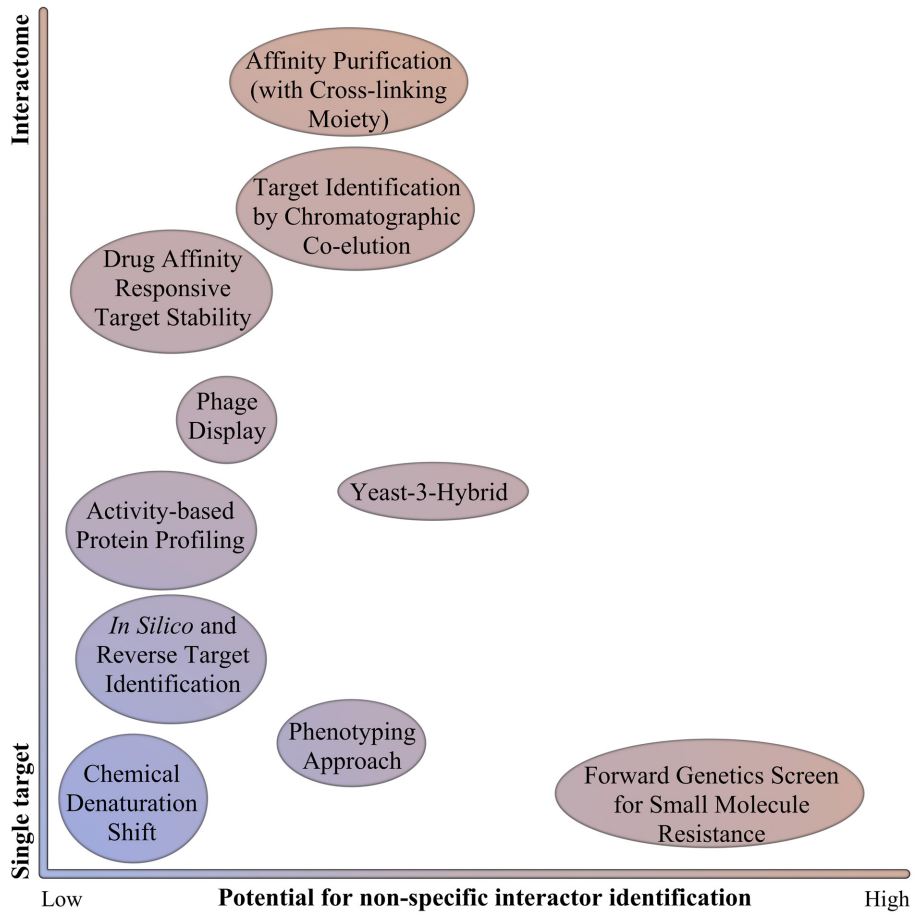

FIGURE 1 | Schematic representation of target identification strategies. Target identification strategies are represented in function of their ability to identify only one target or several targets (interactome), and the potential to identify non-specific interactors (such as proteins that will confer resistance or induce the appropriate readout without actually binding specifically the small molecule).

COMMONLY USED TARGET IDENTIFICATION STRATEGIES IN PLANT CHEMICAL BIOLOGY

FORWARD GENETIC SCREEN FOR COMPOUND RESISTANCE

Forward genetic screen for compound resistance is a commonly used target identification strategy in plant chemical biology in which a mutant population is grown in the presence of small molecules and screened for resistance. Selected resistant individuals are subsequently characterized in terms of their mutations. A major disadvantage is the inherent selection against targets with an essential gene product, provided that the induced mutation causes a knock-out or renders the protein inactive. Essential gene targets might still be selected when the mutation allows proper protein function, but inhibits the small molecule from binding. Additionally, gene redundancy can prevent identification of the target, and certain mutations might make plant resistance to compound treatment, without affecting the true target. A small molecule called "non-auxin-like lateral root inducer" or naxillin illustrates the latter scenario. Identified from a screen for small molecules able to enhance lateral root development, naxillin was found to affect lateral root development more specifically than auxin. The only identified resistant mutant from an ethylmethane sulfonate (EMS)-mutagenized *Arabidopsis* population, *naxillin resistant 1* (*nar1*), proved to be affected in the *INDOLE-3-BUTYRIC ACID RESPONSE 3* (*IBR3*) gene, which is involved in the conversion of indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA). The characterization of *nar1* helped to reveal that naxillin acts upstream of auxin signaling by positively affecting the IBA to IAA conversion at specific sites in the root, thereby inducing lateral root development, but failed to identify the true target of naxillin (De Rybel et al., 2012). Major advantages of the forward genetic screen approach are the straightforward experimental setup and the availability of high-throughput next-generation sequencing techniques, which allow relatively quick target identification once the resistant individuals are isolated.

Whether or not mutants are more sensitive to the compound depends on the nature of the mutation. Resistant mutants might arise from mutations affecting the small molecule binding, or from the absence of the target protein, although the latter is not applicable to proteins with essential function. Alternatively, less of the protein target might result in hypersensitivity, as less small molecule is required to exert the same phenotypic effect.

Table 1 | Overview of the target identification strategies.

Target identification strategy	Examples[a]	Modified small molecule	References
ESTABLISHED STRATEGIES			
Forward genetics screen for small molecule resistance	Pyrabactin, gravacin, DAS734	No	Rojas-Pierce et al., 2007; Walsh et al., 2007; Park et al., 2009
Phenotyping approach	Bikinin, kynurenine, imprimatins	No	De Rybel et al., 2009; He et al., 2011; Noutoshi et al., 2012
In silico and reverse target identification	IGPD inhibitors, galvestine1 and galvestine2	No	Schweitzer et al., 2002; Botté et al., 2011
EMERGING STRATEGIES			
Activity-based protein profiling	Bicyclic hydantoin, serine hydrolases	Yes	Kaschani et al., 2012a,b
Yeast-3-Hybrid	Jasmonic acid, abscisic acid, compound 8, cucurbic acid, cucurbic acid methylester, 2,6 dihydroxybenzoic acid	Yes	Cottier et al., 2011
Affinity purification with cross linking moiety	Atrazine, jasmonate glucosate, castasterone	Yes	Pfister et al., 1981; Kinoshita et al., 2005; Nakamura et al., 2008
Phage display	Brz2001	Yes	Takakusagi et al., 2013
PROMISING STRATEGIES			
Affinity purification	None yet	Yes	Ziegler et al., 2013
Chemical denaturation shift	None yet	No	Schön et al., 2013
Target identification by chromatographic co-elution	None yet	No	Chan et al., 2012
Drug affinity responsive target stability	None yet	No	Lomenick et al., 2009

[a] *The examples correspond with those given in the text.*

Besides, resistance to small molecules might also be caused by the overexpression of the target protein.

Examples of such an approach are the identification of targets for pyrabactin, gravacin and DAS734. The synthetic seed germination inhibitor pyrabactin was shown to act as a specific agonist of abscisic acid (ABA) because transcriptional responses of seeds growth in presence of ABA compared to pyrabactin were highly correlated, whereas this was not the case in seedlings (Park et al., 2009). Pyrabactin allowed the identification of the PYR/PYLs (for "pyrabactin resistance" and "PYR-like"), members of the ligand binding cyclase subfamily of the START protein superfamily, through a forward genetics screen for compound resistance (Park et al., 2009). This protein family was independently identified as RCAR (for "regulatory component of ABA receptor") (Ma et al., 2009). The PYR/PYL/RCARs were shown to be ABA receptors (Park et al., 2009), which after perception bind to type 2C protein phosphatases, thereby inactivating them. The role as ABA receptor for the PYR/PYL/RCAR protein family was later confirmed by crystallographic data (Santiago et al., 2009).

Gravacin was identified as an inhibitor of the gravitropic response in *Arabidopsis* seedlings (Surpin et al., 2005). A population of 220,000 EMS-mutagenized F2 seeds were screened for a gravitropic response when grown on gravacin, identifying through a map-based cloning approach an E to K substitution in the gene coding for P-GLYCOPROTEIN 19 (PGP19) (Rojas-Pierce et al., 2007). Several different mutant alleles for PGP19 showed resistance to gravacin, confirming the identified mutation as the cause of gravacin resistance. Furthermore, gravacin binding to PGP19-containing microsomes was severely reduced in *pgp19* mutants compared to wild type controls (Rojas-Pierce et al., 2007).

A phenyltriazole acetic acid compound, DAS734, was identified as a potent bleaching agent of developing leaves. Addition of adenine could alleviate the effects, hinting toward a target in the purine biosynthesis pathway (Walsh et al., 2007). A screen for DAS734 resistance of 480,000 EMS-mutagenized *Arabidopsis* ecotype Col-0 seedlings resulted in several resistant lines, some of which had the same mutation. Map-based cloning identified *GLUTAMINE PHOSPHORIBOSYLAMIDOTRANSFERASE 2* (*GPRAT2*) as the gene containing all mutations (Walsh et al., 2007). Expression of *AtGPRAT2* in *Escherichia coli* allowed the purification of the protein and the evaluation of its activity in the presence of DAS734. The small molecule was able to potently inhibit GPRAT2 activity in a slow, but reversible manner. In addition, expression of the mutant *GPRAT2* gene in *E. coli*, isolated in the forward genetics screen, revealed an increase in the inhibitory concentration (IC_{50}) of more than 500 times, indicating a strong resistance to DAS734, and confirming GPRAT2 as its target (Walsh et al., 2007).

PHENOTYPING APPROACH

Opposed to a forward genetics approach, the phenotyping approach usually starts from a screen of small molecules against an appropriate readout for the biological process of interest, followed by further tests that narrow down the possible target proteins. Biochemical validation is used to confirm the

hypothetical target protein. The main drawback is the requirement for proper readouts that is applicable for known signaling pathways and enzymes involved in primary and secondary metabolism. Processes of highly organized, rapid and dynamic nature, such as endomembrane trafficking, will be much harder to characterize with such a strategy. As one searches specifically within a process of interest, this approach usually will yield only one target, or target family, but will not provide an overall picture of small molecule interactors. The latter implies, however, that target identification can be fairly straightforward because the search is directed. Some recent examples are the identification of targets for bikinin, kynurenine and imprimatins.

The small molecule bikinin was discovered in a screen for molecules able to induce phenotypes similar to those caused by the application of the most active brassinosteroid (BR), brassinolide, in young *Arabidopsis* seedlings (De Rybel et al., 2009). The target of bikinin was identified through comparative phenotypic analysis of different BR-related mutants grown on bikinin. As bikinin was able to rescue a gain-of-function *bin2-1* mutant to wild type, it was hypothesized that the GSK3-like kinase BIN2 is the direct target. This hypothesis was confirmed by *in vitro* binding studies. In addition, the list of bikinin targets was expanded to other *BIN2* homologs and a competition with ATP was suggested as the mode of compound action.

The selective aminotransferase inhibitor, affecting local auxin biosynthesis, L-kynurenine (Kyn), was identified in a screen for suppressors of the constitutive ethylene response (He et al., 2011). Although Kyn did not rescue the constitutive ethylene response phenotypes of the *eto1-2* and *ctr1-1* mutants and wild type plants treated with the synthetic ethylene precursor 1-aminocyclopropane-1-carboxylic acid, it rescued the shortened root phenotype at submicromolar concentrations. Kyn was shown to inhibit ETHYLENE INSENSITIVE 3 (EIN3) accumulation in *Arabidopsis* roots, which led to a reduction in local auxin responses. As active ethylene signaling increased the reduction of local auxin responses in the presence of Kyn, it was concluded that Kyn represses ethylene-mediated auxin responses (He et al., 2011). Further unraveling of auxin responses led to the hypothesis that Kyn might inhibit TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1). Enzymatic activity tests on purified TAA1 confirmed that Kyn is a competitive and potent inhibitor, inhibiting the conversion of tryptophan to indole-3-pyruvic acid. Computational modeling validated Kyn as a competitive inhibitor of TAA1, outcompeting tryptophan. In addition, when tryptophan was applied in excess, it reversed the inhibitory effects of Kyn (He et al., 2011).

The third example comes from a screen for small molecules affecting disease resistance in plants that identified five small molecules belonging to two different structural groups, named imprimatins (Noutoshi et al., 2012). The authors showed an increase in salicylic acid (SA) in treated plants, but unlike control plants, after pathogen infection imprimatins did not accumulate the inactive form of SA, SA-2-O-β-D-glucoside (SAG), which usually increases in parallel with an increase in SA. An enzymatic test on UGT74F1 and UGT76B1, two enzymes that convert SA to its inactive form SAG, confirmed imprimatins as inhibitors of these enzymes. Thus, the increased *Pst-avrRpm1*-induced cell death after imprimatin treatment is due to an inhibition of the SA-to-SAG conversion (Noutoshi et al., 2012).

IN SILICO-BASED TARGET IDENTIFICATION STRATEGIES

Not only forward, but also reverse and *in silico* design strategies have been successfully used. The starting point is a protein of interest, or a small molecule scaffold. Screening of additional small molecules aims at finding a specific interactor for the protein of interest, or at improving binding characteristics for an existing small molecule. A validation step *in vivo* confirms the findings of the reverse or *in silico* strategy.

A first example concerns a study of more than a decade ago in search of novel inhibitors of imidazole glycerol phosphate dehydratase (IGPD), an attractive herbicide target (Schweitzer et al., 2002). Based on previously identified IGPD triazole inhibitors (Mori et al., 1995), a pharmacophore model was developed to search available 3D-databases (Schweitzer et al., 2002). A pharmacophore model contains spatial information on functional groups essential for small molecule action. The model was used to search commercial databases of about 370,000 small molecules in total. From the approximately 1200 hits, small molecules, which were too high in molecular weight or too expensive, were excluded. From the resulting 140 hits, a group of bispyrroles proved to be interesting from a chemistry perspective and was chosen to perform a substructure search on about 600,000 small molecules. Finally a group of monopyrrole aldehydes was selected as a new class of IGPD inhibitors with activity in the low micromolar range. As this group does not fit the original pharmacophore model perfectly, it might be possible that this new group acts through a different mechanism as the original triazole inhibitors (Schweitzer et al., 2002).

A second example illustrates a screen for inhibitors of monogalactosyldiacylglycerol (MGDG) synthesis in *Arabidopsis* that used *E. coli* lipid vesicles containing recombinant MGD1 and a small molecule library with a little less than 24,000 entries. After initial screening, a new set of small molecules was put together based on chemical similarities with the hits from the first screen, which led to a selection of two small molecules: galvestine1 and galvestine2, two competitive inhibitors relative to diacylglycerol (DAG) of MGD1, MDG2, and MDG3 (Botté et al., 2011).

EMERGING TARGET IDENTIFICATION STRATEGIES IN PLANT CHEMICAL BIOLOGY

ACTIVITY-BASED PROTEIN PROFILING (ABPP)

The activity-based protein profiling (ABPP) target identification strategy relies on small molecules with a so-called "warhead," which react with residues in the active site of enzymes in an irreversible manner (van der Hoorn et al., 2011). The small molecules are attached via a linker to a functionality, such as biotin for affinity purification, or to a fluorophore for visualization. As the small molecules react with their respective target proteins to form a covalent bond, no additional cross-linking is required for further affinity purification. However, not every small molecule is capable of reacting with its target protein, therefore ABPP is only applicable for small molecules able to react with their target protein. Equally, not every protein will react with a small molecule to form a covalent bond, and thus ABPP results in a substantially

less complex proteome, which facilitates a more straightforward analysis. Importantly, ABPP enables to assign activity to certain proteins, not only within the entire proteome, but also within a protein family thereby creating activity-based sub-classes. Recent examples of the use of ABPP are illustrated by studying the mode of action of the bicyclic hydantoin and several serine hydrolases (SHs) inhibitors in *Arabidopsis*.

The bicyclic hydantoin sparked the attention when it was found as a side product from synthesis efforts for syringolins (Kaschani et al., 2012a). To identify a molecular target from *Arabidopsis* cell cultures, the bicyclic hydantoin was labeled with biotin and rhodamine, and both versions were applied to either detect or pull down the protein target. An affinity purification coupled with a mass spectrometry (MS) was used to identify the glyceraldehyde 3-phosphate dehydrogenase GAPC1 and GAPC2 as targets of the bicyclic hydantoin. Both GAPC1 and GAPC2 were heterologously expressed in *E. coli*, and shown to bind the rhodamine-tagged bicyclic hydantoin in an activity-dependent manner (Kaschani et al., 2012a).

The second example employed a competitive ABPP approach to evaluate the effect of different putative SH inhibitors in *Arabidopsis* (Kaschani et al., 2012b). Competitive ABPP assesses the ability of small molecules to compete with ABPP probes. A reduced labeling by the probe in the presence of the small molecule indicates binding of the small molecule to the protein(s) under investigation. A rhodamine-tagged fluorophosphonate (FP) and a trifunctional nitrophenol phosphonate (TriNP) tagged with both rhodamine and biotin were used as ABPP probes. The main finding of the study was a differential sensitivity of different *Arabidopsis* SHs to the SH inhibitors tested (Kaschani et al., 2012b). An additional study on SHs reports the development of a paraoxon-like para-nitrophenol phosphonate activity-based probe predominantly labeling carboxylesterase12 in *Arabidopsis* (Nickel et al., 2012).

YEAST 3-HYBRID

The yeast 3-hybrid (Y3H) approach relies on the principles of the yeast 2-hybrid (Y2H) technology, but uses a modified small molecule of interest to allow interaction between the DNA-binding domain and the transcriptional activator (**Figure 2**). Initially (Licitra and Liu, 1996), Y3H was based on the Y2H system using the LexA DNA-binding domain and the transactivation domain from the bacterial protein B42 (Gyuris et al., 1993). The so-called "hook" consisted of the LexA DNA-binding domain fused to the hormone-binding domain of the rat glucocorticoid receptor. The latter binds to dexamethasone, which is part of the hybrid small molecule comprising dexamethasone and FK506, or the "bait." Finally, the "fish" consisted of human FKBP12 fused to the transcriptional activator B42. For screening purposes, FKBP12 represents any cDNA library of choice, whereas FK506 represents the small molecule of interest.

To date, Y3H in plant chemical biology was used in an attempt to identify targets for small molecules with implications in plant defense responses (Cottier et al., 2011). The Y3H system used to this end was based on the LexA DNA-binding domain fused to dihydrofolate reductase (DHFR), which binds with high affinity to methotrexate (Mtx), and had been used previously with success (Becker et al., 2004). The hybrid ligand was composed of Mtx fused via a polyethylene glycol (PEG) linker to several small molecules, namely jasmonic acid (JA), ABA, compound 8 (cpd 8), cucurbic acid (CA), cucurbic acid methylester (CAMe) and 2,6 dihydroxybenzoic acid (6OH-SA). The "fish" was a collection of cDNA libraries from wounded or pathogen infected leaves and inflorescence from *Arabidopsis*, fused to the GAL4 transcriptional activator (Cottier et al., 2011). Although no targets were identified for Mtx-ABA and Mtx-JA, and no interaction could be shown when the known ABA and JA receptors were expressed as "fish," potential target proteins were identified for the other small molecules. This study validates Y3H as a target identification strategy for plant chemical biology.

The Y3H technique has a few notable advantages because it allows screening for small molecule-protein interactions *in vivo*, correction for low abundant proteins, easy identification of the target protein(s) or even interacting protein domains and, additionally, detection of essential gene products, and even straightforward characterization of an entire protein family as the target of a small molecule. However, although Y3H is an *in vivo* method, the ability of the protein to bind the small molecule of interest is assessed out of its biological context and one protein at a time, and therefore is less suited when small molecules require more than one protein to bind or the proper biological context. Moreover, proteins not able to translocate to the yeast nucleus, such as transmembrane or membrane-associated proteins cannot be screened; also the fact that the small molecules need to be modified, and the occurrence of multi-drug resistance in yeast can pose a problem. Variants of the Y3H system have been described (Ziegler et al., 2013), but have not found an implementation in plant chemical biology yet.

An improved version of Y3H screening is based on covalent labeling of SNAP-tag fusion proteins (Chidley et al., 2011). The SNAP-tag is based on the human O^6-alkylguanine-DNA alkyltransferase that will covalently attach the alkyl group of its substrate to one of its cysteine residues. As its substrate specificity is not so high, it can also accept O^6-benzylguanine (BG) as a substrate (Keppler et al., 2003). The Y3H is modified in such a way that the DNA-binding domain LexA is fused to a SNAP-tag, and the small molecule of interest is derivatized with BG (Chidley et al., 2011). The improved Y3H approach includes first, the use of a triple mutant for broad-spectrum drug transporters. Second, false-positives are eliminated by a negative selection using 5-fluoroorotic acid in the absence of the modified small molecule, and later colonies are grown both in the presence and absence of the modified compound to score for specific interactions. Additionally, growth of colonies in the presence of the "bait" and an excess of the free, unmodified small molecule could potentially pinpoint colonies expressing a specific target, because the free small molecule will out-compete the "bait," thereby preventing further growth or reporter expression (Licitra and Liu, 1996). The SNAP-tag can also be combined with a GST-tag and thus, the GST-SNAP-tagged fusion protein can readily be used in affinity purification approaches with the same modified small molecules (Chidley et al., 2011).

Concerning synthesis of the modified small molecule for Y3H, a recent study evaluated the length and nature of the linker

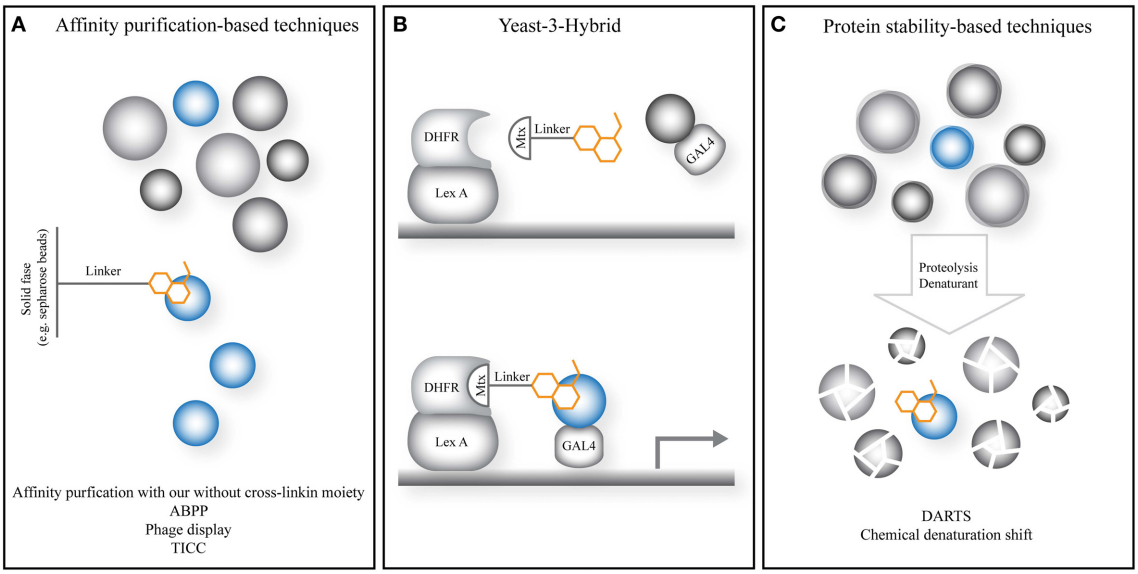

FIGURE 2 | General principle of emerging and novel target identification strategies in plant chemical biology. (A) Strategies relying on the affinity of the small molecule to isolate the target protein from a complex mixture such as a lysate or cellular environment. **(B)** The yeast-3-hybrid approach uses the activation of a transcriptional response by bringing together a DNA-binding domain and transcriptional activator via a fusion of the small molecule of interest and a known small molecule with high affinity for a known protein target. The latter is fused to the DNA-binding domain. The small molecule probes a cDNA library fused to the transcriptional activator. **(C)** Strategies relying on increased protein stability utilize small molecules to stabilize the increased dynamics, instability and degradation upon treatments such as denaturants or proteases, preventing or slowing down target protein degradation. Gray spheres: non-target proteins; blue spheres: target protein; orange cartoon: small molecule of interest; Lex A, Lex A DNA-binding domain; Mtx, methotrexate; DHFR, dihydrofolate reductase, target protein of Mtx; GAL4, GAL4 transcriptional activator.

(Tran et al., 2013). An important conclusion of the use of a triazole-containing linker opposed to a PEG linker was the lower background growth of yeast in the presence of a negative control, and thus a decreased amount of possible false positives.

AFFINITY-BASED TECHNOLOGIES

A much less explored target identification strategy in plant chemical biology is affinity purification. Typically, a derivatized small molecule is generated consisting of a selectivity function, which is the small molecule of interest, bound via a linker moiety to a tag such as biotin, which allows target isolation from a complex mixture. Incubation of an appropriate lysate with the modified compound, immobilization on a solid support, and subsequent washing of unbound proteins enable isolation of target proteins for liquid chromatography and MS analysis (**Figure 2**). As such, the general principle of this approach is shared with ABPP.

Crucial for derivatization is structure activity relation (SAR) analysis. SAR analysis involves testing of a collection of analogs of the original small molecule to assess which functional groups and moieties are essential for its activity. SAR analysis is not only important for derivatization, but can also provide crucial information about the mode of action of small molecules. A good illustration of the latter is sirtinol, identified as an inhibitor of sirtuin deacetylases in yeast and human cells (Grozinger et al., 2001). Sirtinol in *Arabidopsis* was characterized as an enhancer of auxin signaling, and was found to bind and inhibit SIRTINOL RESISTANT1 (SIR1), a negative regulator of auxin signaling upstream of the *Aux/IAA* genes. *SIR1* and other *SIR* genes encode proteins involved in molybdopterin biosynthesis, and the incorporation of molybdenum to form molybdenum cofactor (moco), an essential cofactor in for example aldehyde oxidases (Zhao et al., 2003). SAR analysis predicted that sirtinol can be hydrolyzed into 2-hydroxy-1-naphthaldehyde (HNA). HNA is subsequently converted by a moco-containing aldehyde oxidase into 2-hydroxy-1-naphtoic acid (HNC). The latter is an active auxin analog, hence explaining the sirtinol-induced phenotypes (Dai et al., 2005).

An important advantage of affinity purification is the ability to probe for target proteins of any molecular process of interest, in other words, any small molecule can be potentially used, and does not require activity toward the target in contrast to ABPP. It provides the possibility to uncover the small molecule interactome, thus not only the main target, but also "off-targets," might contribute to the observed phenotype. The latter implies that specific readouts should be available to distinguish the target of interest from off-targets in the validation procedure. Additionally, affinity purification yields data on potential targets as well as biochemical proof of binding, provided proper controls are included.

A variant of affinity purification consists of the incorporation of a cross-linking moiety, thus giving rise to a tri-functional probe. These so-called "capture compounds" consist of a selectivity function, which is the small molecule of interest, a reactivity function, which is the cross-linking moiety, and a sorting function (such as biotin) (Köster et al., 2007; Fischer et al., 2010, 2011). The cross-linking moiety usually is activated by UV-light, thereby forming covalent bonds with proteins in close proximity. As the small molecule-target interaction is secured by a covalent

bond, washing can be stringent, removing unspecific binders; hence, incorporating a cross-linking moiety might solve problems with weak interactions and low abundant or less accessible protein targets. Crucial for both affinity purifications and procedures involving cross-linking moieties are proper controls to distinguish "true" targets from non-specific interactors. Preferably an inactive analog of the small molecule modified in the same way is used, but it should be noted that inactivity *in vivo* does not necessarily mean it will not bind the target protein in a lysate, because inactivity might be due to altered uptake. In addition, the "interactome" for the solid phase used (such as streptavidin-coated beads), or the linker with biotin alone might serve as an essential background list. Equally, multiple repeats with different probes can distinguish targets from background signals in a statistical manner. Competition experiments with unmodified compounds may reveal specific binders from non-specific binders, and serve as an essential control. However, in order to detect a competition with the unmodified small molecule, quantification of eluted proteins is required. To this end, stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002, 2009) can be used or other forms of differential labeling of peptides or proteins (Gant-Branum et al., 2009; Collier and Muddiman, 2012). In short, two samples are prepared and differentially labeled (e.g., heavy and light) according to the SILAC protocol. Both samples are differentially treated: one with the probe only, the other with the probe and free small molecule in competition. When the concentration of the free small molecule is high enough, and when the affinity toward the target is higher than that of the modified small molecule, specific targets should not be retained after pull-down. Subsequent combination of both samples in a 1:1 ratio and MS analysis results in a distinguishable peptide pair originating from the different samples because they only differ by their isotopic mass difference. Non-specific binders should be represented by peptides of equal intensity for both samples, opposed to peptides representing specific binders, because their intensity should be much lower due to the imposed competition with the free small molecule (Ong et al., 2009). Additional issues might arise when the target protein is low abundant or of hydrophobic nature.

One of the first examples of compound photo-affinity labeling in plant research concerns the modification of atrazine with a photo-reactive azido group. The subsequent azido-atrazine was radioactively labeled to allow detection of covalently bound polypeptides on a polyacrylamide gel (Pfister et al., 1981). Although atrazine is a well-characterized inhibitor of photosystem II reactions, and azido-atrazine was shown to act in a very similar way, target identification stops at the level of radioactively labeled polypeptides of 32–34 kDa on a polyacrylamide gel, be it from purified chloroplast thylakoids.

A second example is given by efforts to identify the molecular target of the small molecule responsible for nyctinastic leaf movement of *Albizzia saman* (Nakamura et al., 2008). The trifunctional probe consisted of the small molecule of interest, a jasmonate glucoside, benzophenone as the reactivity function and biotin as the selectivity function. Additionally, an inactive enantiomeric analog was modified in the same way, serving as a control (Nakamura et al., 2008). Both probes were activated at 365 nm, and SDS-PAGE analysis revealed a differential band, which disappeared using the unmodified small molecule as competitor.

A third example concerns the biotin-labeled photoaffinity castasterone (BPCS) (Kinoshita et al., 2005). This report shows the ability of castasterone, an active BR (Vriet et al., 2013), to bind the BR receptor BR-INSENSITIVE1 (BRI1). The binding site is mapped to an island domain in between leucine-rich repeat 21 (LRR21) and LRR22 of the extracellular domain of BRI1. This observation was later confirmed by structural data of BRI1 in complex with brassinolide (Hothorn et al., 2011; She et al., 2011). Until now to our knowledge no examples exist in plant research of an affinity purification approach without covalent binding to the target protein.

In order to circumvent the bio-availability problems that are likely to arise with biotin tags or fluorophores attached to the small molecule, a two-step labeling of small molecules was optimized in *Arabidopsis* using so-called mini-tags, based on azide and alkyn functional groups (Kaschani et al., 2009). The well-known cysteine-protease inhibitor E64 was used to study and establish the two-step labeling technique. Because E64 is a covalent inhibitor of its targets, the two-step labeling consists of a first modification of E64 with a mini-tag, minimizing interference of the tag on E64 activity and bioavailability. After incubation of the sample with modified E64, a second step attaches biotin modified with the appropriate mini-tag in a click-chemistry reaction, allowing subsequent purification and detection of the target protein (Kaschani et al., 2009). One of the main advantages is the ability to label *in vivo*, which, in the explained setup is not possible for small molecules that do not bind covalently to their target protein. This problem can be solved by introducing a photo-activatable group together with a mini-tag, which allows cross-linking *in vivo*, with subsequent preparation of the lysate and attaching biotin for affinity purification.

Although *in situ* proteome profiling with a small molecule modified for photo-cross-linking has the advantage of identifying target proteins in the proper biological context, care should be taken with possible effects of UV irradiation on the proteome. Certainly when exposure lasts for several minutes, damage might be induced, which eventually might compromise the final protein target list.

In order to perform *in situ* proteome profiling, the small molecule of choice should be modified with a photo-cross-linking group, together with a group that allows additional bioorthogonal modification, usually done with a clickable group. In this way, after cross-linking in live cells, a group for affinity purification or visualization can be added afterward. The latter option allows the usage of a technique called fluorescence difference in two-dimensional gel electrophoresis (FITGE). This method uses two samples, one labeled with the active small molecule, the other with an inactive form or other control, and labels both samples with a different fluorophore. This differential labeling allows detection of both samples together on a 2D SDS-PAGE gel. Spots that are labeled by both fluorophores are probable because of unspecific binding events, whereas spots only labeled with the fluorophore attached to the active small molecule are potential hits that can be identified by subsequent MS approaches (Park et al., 2012). Differences between lysates and live cells as starting

material were reported, in which live cells might likely provide more reliable target identification and can be even a requirement to detect the main target.

PHAGE DISPLAY

The phage display strategy relies on whole, fragmented cDNA or random peptide sequences translationally fused to the phage coat protein, so that the peptides are displayed on the outside. An immobilized small molecule can retain the peptides that bind to the small molecule. A subsequent bacterial infection allows the identification of selected peptides. A recent example in plants used a quartz-crystal microbalance (QCM) biosensor in combination with T7 phage display and the receptor-ligand contacts (RELIC) bioinformatics server to identify binding sites for the BR biosynthesis inhibitor brassinazole (Brz2001) in the cytochrome P450 enzyme DWARF4 (DWF4) that catalyzes the rate-limiting hydroxylation of the C22 position in the BR biosynthesis (Asami et al., 2000; Sekimata et al., 2001; Takakusagi et al., 2013; Vriet et al., 2013). The QCM measures voltage-induced crystal vibrations on a gold electrode, which will decrease as the overall mass on the gold electrode increases. Takakusagi et al. (2013) used a modified version of Brz2001, which forms a monolayer on the gold electrode. The QCM-measured vibrations will decrease as peptides bind the immobilized small molecule. A random 15-mer peptide library was incubated with the Brz2001-covered gold electrode, which resulted in the identification of 34 peptides. Subsequent use of the RELIC bioinformatics platform (Mandava et al., 2004) detected within the 34 selected peptides a subset of amino acids potentially involved in small molecule binding that map to a potential disordered loop of DWF4 (Takakusagi et al., 2013).

Given the possibility to modify the small molecule of interest, and the ability to coat the gold electrode, this technique allows a quick assessment of possible target proteins. It allows a coverage of the proteome without the possibility of missing out on low abundant proteins due to the easy amplification of the signal by bacterial infection. It is less suited for interactions requiring post-translational modifications, very hydrophobic peptides, and protein-small molecule interactions, for which several amino acid residues involved in binding are scattered across the protein primary sequence. When the small molecule only binds the appropriate amino acid residues form a binding pocket after protein folding, phage display with a random small peptide library will likely not work. Phage display with entire proteins might solve the problem, but will select any hydrophobic protein, besides the possibility that the protein might not fold properly.

LABEL-FREE COMPOUND-BASED TECHNOLOGIES

An obvious drawback of affinity purification is the requirement for "taggable" positions on the small molecule of interest. In addition, the small molecule should still be active with at least part of the intended modification (such as the linker), because the modification might hinder proper binding of the small molecule to its targets. Efficient isolation of the target is also dependent on the affinity of the small molecule, because low-affinity interactions might be lost during washing. Therefore, rather gentle washing conditions should be used, which have the disadvantage of generating extensive lists of possible target proteins. In an effort to solve these problems, approaches that do not require labeled small molecules are being developed.

CHEMICAL DENATURATION SHIFT

One way of testing ligand interactions is by measuring protein stability, which depends on a number of factors, including temperature, denaturants and ligand binding. An increase in protein stability, and thus denaturing conditions, to higher temperatures is indicative of ligand binding. Classically, this is measured by fluorescence or differential scanning calorimetry (Straume and Freire, 1992; Lo et al., 2004). However, because estimations on binding affinities require prior knowledge on enthalpy and heat capacity of protein denaturation and ligand binding, such an approach is not suited for high throughput screening, because binding thermodynamics are yet unknown for small molecules in a library. Additionally, the rank order given to small molecule interactors based on the induced shift in protein denaturation temperature (T_m) is not necessarily the same at the physiological temperature and the measured T_m. An alternative approach was proposed based on a chemical denaturation shift (Schön et al., 2013), in which instead of measuring T_m, the increase in concentration of a denaturant is measured required to denature the protein in the presence of its ligand. Although the study is an optimization and proof of concept of the chemical denaturation shift approach, it illustrates the usability to provide proof of ligand binding, and is additionally suited for high-throughput screening setups in a reverse chemical genetics strategy.

TARGET IDENTIFICATION BY CHROMATOGRAPHIC CO-ELUTION (TICC)

The main disadvantage of labeling small molecules with any tag, is the possibility of selecting against small molecules or natural products that do not allow any modification (i.e., they lose biological activity altogether). To this end, target identification strategies using unmodified small molecules are being developed. One example of such a strategy is the TICC technology (Chan et al., 2012). The idea is to look for a shift in the retention time of the small molecule of interest in a complex protein mixture compared to the small molecule alone during non-denaturing high-performance liquid chromatography. The shift in retention time would be indicative of binding to a particular protein target, which can be identified by further deconvolving the fraction in which the small molecule elutes by additional complementing and orthogonal fractionations. Key to the success of this approach is the ability to separate free from protein-bound ligands (Chan et al., 2012).

DRUG AFFINITY-RESPONSIVE TARGET STABILITY (DARTS)

The DARTS approach takes advantage of the stabilization of a protein target upon small molecule binding, thereby rendering the protein less susceptible to proteolytic digestion (Lomenick et al., 2009, 2011). This idea also formed the basis for the chemical denaturation shift approach (Pace and McGrath, 1980). DARTS can be used to confirm a certain small molecule-protein interaction by specifically evaluating proteolytic digestion via western blotting, but equally can be used to evaluate possible new small molecule-protein interactions by looking at entire lysates.

Although the latter situation might result in visibly stabilized targets when high abundant, low abundant target proteins might not be readily visible on gel (Lomenick et al., 2009).

Both DARTS and TICC share some important advantages. First they are label free, require no derivatization and use the original small molecule. This is not only important in terms of small molecule tolerance toward modification, but also saves time, as SAR analysis can be limited. A second important advantage is their independency of any protein nature, mode of action or model system. Both techniques solely rely on affinity of the small molecule for its target protein. The latter also dictates an inherent weakness: interactions of lower affinity might be missed. Both techniques have their specific weaknesses too. Whereas membrane proteins remain challenging for TICC, DARTS is not applicable to any protein, because some proteins are more resistant to digestion and might be missed. In addition, the small molecule might interact in such a way that digestion of the protein is not, or hardly affected.

FUTURE PERSPECTIVES

Traditionally, chemical biology approaches have a strong background in pharmaceutical and agricultural fields, whereas basic research lags behind, certainly in plant biology. Over the recent years though, plant biology has witnessed an increasing interest in chemical biology approaches for processes such as endomembrane trafficking, hormonal signaling and primary and secondary metabolism (Hicks and Raikhel, 2012; Mishev et al., 2013; Tresch, 2013; Ma and Robert, 2014). Depending on which aspect of plant biology the small molecule of choice is affecting, and what the intended use will be, knowledge on the interactome of the small molecule might be essential. As the induced phenotype is only the sum of the individual targets affected, a deconvolution of the phenotype toward the individual contributions of the affected targets is of paramount importance. To this end, current commonly used target identification strategies in plant biology fall short. Therefore, an evolution toward more biochemical and alternative strategies is required. Surprisingly, one of the most successful target identification strategies in animal research is much less used in plant research: affinity purification. Although the technique has important shortcomings such as the need for small molecule modification and the preference for abundant soluble protein targets, it is one of the few strategies capable of revealing the small molecule interactome. Several variants exist of the basic affinity-based pull-down principle to accommodate for shortcomings of the technique, and their implementation in fundamental plant biology research as well as in a more commercial research environment should spur our understanding of dynamic cellular mechanisms. In addition, development of affinity purification-based approaches in combination with other well-established techniques might provide additional dimensions to the interactome resulting from classic affinity-based setups. One such example is Chem-seq, in which a combination of affinity purification and chromatin immunoprecipitation (ChIP) technology can provide new insights into the role of small molecules at a genome-wide level (Anders et al., 2014).

Other approaches that are more established in other systems than in plants, besides the ones mentioned in this review, might find their way into plant biology as well. A possible example could be multi-copy suppression profiling, in which the central idea relies on increased tolerance toward the small molecule when the target protein is present in higher copy numbers (Ziegler et al., 2013). Similarly to EMS screens, overexpression line collections such as activation-tag collections could be screened for more tolerance toward small molecules. Problems with gene redundancy and lethality would be overcome, and identification of the potential target should be simple. Equally, one overexpression line could be used to screen an entire collection of small molecules for more tolerance. In addition, adaptation of the Cellular Thermal Shift Assay (CETSA) (Martinez Molina et al., 2013) for target identification purposes might be the onset toward a relatively easy strategy to identify possible target proteins without the need of small molecule modification. The technique relies on increased stability of the target protein in the presence of the small molecule at higher temperatures, according to a similar principle as DARTS and the chemical denaturation shift. Moreover, this approach has proved to be successful at the cellular and even tissue level (Martinez Molina et al., 2013).

Finally, the choice for a particular target identification strategy greatly depends on the aim of the study and available resources, still considering that several complementary approaches to prove protein target binding will only make the study stronger. Although initial efforts to setup an affinity purification target identification approach are greater compared to for example resistance screens, affinity purification has important advances over the well-established target identification strategies in plant biology. Therefore, plant biology can only benefit from adapting affinity-based target identification approaches in future chemical biology projects.

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Towards personalized agriculture: what chemical genomics can bring to plant biotechnology

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In contrast to the dominant drug paradigm in which compounds were developed to "fit all," new models focused around personalized medicine are appearing in which treatments are developed and customized for individual patients. The agricultural biotechnology industry (Ag-biotech) should also think about these new personalized models. For example, most common herbicides are generic in action, which led to the development of genetically modified crops to add specificity. The ease and accessibility of modern genomic analysis, when wedded to accessible large chemical space, should facilitate the discovery of chemicals that are more selective in their utility. Is it possible to develop species-selective herbicides and growth regulators? More generally put, is plant research at a stage where chemicals can be developed that streamline plant development and growth to various environments? We believe the advent of chemical genomics now opens up these and other opportunities to "personalize" agriculture. Furthermore, chemical genomics does not necessarily require genetically tractable plant models, which in principle should allow quick translation to practical applications. For this to happen, however, will require collaboration between the Ag-biotech industry and academic labs for early stage research and development, a situation that has proven very fruitful for Big Pharma.

Keywords: herbicides, chemical genetics, agricultural biotechnology, growth regulators, chemical screening, genomics

INTRODUCTION

Historically, the pharmaceutical industry has developed drug treatments that target the widest segment of the population. Although this business model has been very successful, there is a need to update this "one size fits all" approach to drug development. Genetic variability in the human population renders some individuals less responsive to certain therapies (McDonald et al., 2009). More importantly, an individuals' genetic makeup can make them susceptible to dangerous side effects from the medication (Daly et al., 2009). This has led to suggestions that drug treatments need to take into account a patients' genome, hence the development of the field of pharmacogenomics (Weinshilboum and Wang, 2006; Wang et al., 2011). By tailoring drugs regimens to the needs of the individual based on their unique set of alleles, more effective and safer therapies can be prescribed (Ginsburg and Willard, 2009).

Understanding the molecular basis of disease is fundamental to designing selective drug treatments. For example, over 1500 different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene have been identified in cystic fibrosis (CF) patients[1]. Although 90% of CF patients have an in-frame deletion that results in the mislocalization of the CFTR gene product, a small fraction of CF patients (∼5%) have a missense mutation G551D-CFTR that has correct CFTR localization but reduced chloride channel activity (Van Goor et al., 2011). Using this allelic information, researchers identified compounds that specifically rectify the perturbation caused by each CFTR allele (Ramsey et al., 2011; Van Goor et al., 2011). For example, the drug Ivacaftor binds the ion channel to promote chloride transport in patients harboring the G551D-CFTR allele (Yu et al., 2012; McPhail and Clancy, 2013). Ivacaftor has been developed into a clinically effective therapeutic under the trade name Kalydeco (Whiting et al., 2014). During the development of novel CF therapeutics, genetics informed the drug discovery process and enabled high-throughput screening to identify compounds that selectively targeted each allele.

The parallels between the pharmaceutical and agricultural chemical industry are striking. As with many pharmaceuticals, the foundation of the Ag-chemical industry is the identification of chemicals that have generalized benefits to a wide variety of crops. Popular herbicides kill plants by targeting vital processes conserved across plant biology but not found in mammals, such as photosynthesis or amino acid biosynthesis (**Table 1**; Shaner, 2004), however, a broad-spectrum herbicide that targets a common process in plants may not prove beneficial to a farmer that is trying to selectively kill one type of plant while preserving another. To overcome this issue, inventive Ag-biotech companies deal with the indiscriminate action of these compounds by engineering transgenic crops (GMOs) for herbicide resistance (Mazur and Falco, 1989; Funke et al., 2006; Pollegioni et al., 2011). This approach worked famously well for Monsanto in the development of *Roundup Ready* crops that have been engineered for resistance to glyphosate, the active ingredient in the herbicide *Roundup* (Padgette et al., 1995). Glyphosate binds and

[1]http://www.genet.sickkids.on.ca/cftr

Table 1 | Herbicide mode-of-action and chemical targets.

Mode of action	Site of action	Chemical family	Resistant weed species (U.S.)
Lipid synthesis	Acetyl CoA carboxylase (ACCase)	Arloxyphenoxy propionate	15
		Cyclohexanedion	
Amino acid synthesis	Acetolactate synthease (ALS)	Sulfonylurea	38
	5-enolpyruvyl-shikimate-3-phosphate synthase (EPSP)	Glycine	7
Growth regulators	Auxin receptor	Phenoxy-carboxylic acid	7
	Auxin transport	Benzoic acid Semicarbazone	
Photosynthesis	Photosystem II electron transport	Triazine, trazinone,	22
		Nitrile,	1
		Benzothiadiazole,	7
		Ureas	
	Photosystem 1 electron transport	Bipyridilium	
Nitrogen metabolism	Glutamine synthase	Phosphonic acid	0
Pigment inhibitors	Diterpene synthase	Isoxazolidinone	0
	Hydroxyphenylpyruvate dioxygenase	Isoxazole, triketone	0
Cell membrane disruptor	PPO inhibitors	Diethylether,	2
		N-phenylphthalimide,	4
		Thiadiazole	
Seedling root growth	Microtubule inhibitors	Dinitroaniline	6
Seedling shoot growth	Lipid synthesis (non-ACCase)	Thiocarbamate	5
	Long chain fatty acid inhibit	Chloroacetamide	1

Broad-spectrum herbicides target a wide range of plant-specific processes, including photosynthesis and amino acid biosynthesis. The widespread use of herbicides has selected for resistance in some common weed species, necessitating the development of novel pest control measures.

inhibits the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme, the penultimate step in the shikimate biosynthesis (Padgette et al., 1995; Funke et al., 2006). *Roundup Ready* plants express a microbial EPSP synthase that does not bind glyphosate, and are therefore resistant to the inhibitory effect of the herbicide (Padgette et al., 1995; Funke et al., 2006). In this way, spraying herbicides over engineered crops enables farmers to inhibit all plant growth aside from the desired resistant plants (Padgette et al., 1995).

DECONSTRUCTING THE HERBICIDE-GMO INDUSTRIAL COMPLEX

In the 1960s then U.S. President Dwight Eisenhower warned of the developing military-industrial complex that had formed which resulted in the arms industry influencing military decisions and vice versa. Facetiously, this argument could be applied to a modern view of herbicides and GMO technologies. In some sense, industry wants the user to buy the herbicide resistant crop so that they buy the company's favorite herbicide. In other words the two technologies are inextricably linked. Though the application of broad-spectrum herbicides in combination with engineered resistant crops has proven commercially successful, this model has led to a lack of innovation (Dayan et al., 2012). A herbicide with a new target site has not been commercialized in nearly 20 years (Dayan et al., 2012). Lack of innovation has resulted in an Ag-chem industry now facing serious challenges, ranging from herbicide-tolerant weed species to the environmental and ecological impacts of herbicide overuse (Benbrook, 2012). The issue of herbicide-resistant weed species has become especially contentious of late, with the emergence of the glyphosate-resistant weed Palmer amaranth now prevalent in 23 states (Gilbert, 2013). From a non-science perspective, public opinion varies widely on the use of GMO-derived food products (Hug, 2008; Bawa and Anilakumar, 2013; Kamle and Ali, 2013), putting further pressure on the Ag-biotech industry. To continue to thrive, the Ag-chem industry should develop innovative new products that circumvent the need for genetic engineering, and are specific to the unmet needs of modern agriculture.

Innovative chemical solutions for crop protection may be informed from studies that predate the GMO era. Most major crop plants are monocots that contend with dicotyledonous weed species, necessitating herbicides that selectively inhibit dicots. This led to the development of broad leaf herbicides that exploit differences between monocot and dicot seedling development. For example, the broad leaf herbicide mesotrione, which inhibits the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD), is slowly

transported and quickly metabolized by maize (Mitchell et al., 2001). Given that monocots and dicots diverged 140–150 million years ago (Chaw et al., 2004), it is perhaps not surprising that differences in their metabolism can result in a herbicide that is more effective in one class of plants versus another. This divergence does raise questions, however, of whether compounds can be identified that exploit interspecies variation on a smaller scale for agronomic benefit. A first step to addressing these questions is a better understanding of the pharmacogenetic variation across the plant kingdom.

The accessibility of modern genomics now affords unparalleled opportunity to query genetic variation across plant species. Genome sequences can be mined to identify species-specific pathways that could form the basis of targeted herbicide treatments. Exploiting interspecific variation that has evolved in essential pathways can enhance herbicide specificity (Walker et al., 1988; Brown, 1990). For example, Auxinic herbicides are thought to mainly target the auxin hormone receptor (Grossmann, 2010). These compounds show species specific potencies based on differences in uptake and metabolism (Sterling and Hall, 1997). Intriguingly, mutants in *Arabidopsis* have been identified that are resistant to the picolinate auxin picloram but not 2,4-D (**Figure 1**; Walsh et al., 2006). One mechanism for this genotype-specific resistance appears to be mutations in one of the five *Arabidopsis* TIR1 auxin receptors (Walsh et al., 2006). Interestingly, a selective resistance to picloram but not to 2,4-D has been documented in the field (Fuerst et al., 1996; Sabba et al., 2003). In principal, these types of studies demonstrate that natural variation in conserved essential pathways could be exploited to develop compounds that inhibit a weed species yet are ineffective in a favored crop. Pharmacogenetic-based bioinformatics could first identify target alleles in weeds and crops that could form the basis of chemical screens for compounds that exhibit specificity toward the weed protein versus the crop version.

TURNING OVER A NEW LEAF: PROMOTING PRODUCTIVITY, RATHER THAN DEATH

Aside from its role in herbicide discovery, the Ag-biotech industry also has a long history in the development of growth regulators that enhance useful plant attributes (**Figure 1**). From the perspective of plant breeding, genetic manipulation of growth regulators has been central to both horticulture and agriculture. Perhaps the best example involves the impressive yield increases of the green revolution of the 1960s (Davies, 2003), driven by breeding semi-dwarfed varieties for decreased gibberellic acid (GA) biosynthesis in rice and GA signaling in wheat (Hedden, 2003). In parallel to breeding approaches, chemical inhibition of kaurene metabolism, the metabolic precursor to GA, has been used to promote beneficial plant traits in crops (Rademacher, 1991; Gianfagna, 1995). Compounds such as AMO1618 and phosphon D, which inhibit kaurene synthesis, or ancymadol and triazole analogs that inhibit kaurene oxidation, prevent lodging in cereals, increase fruit set in grapes, control size in fruit trees and excessive vegetative growth in cotton (**Figure 1**; Gianfagna, 1995). As an alternative to genetic manipulation and dependence on elite crop varieties, chemical treatments allow farmers the flexibility to adjust crops in response to changing environmental conditions.

These and many other examples of chemical applications for horticultural or agronomic crop improvement were discovered anecdotally by testing known growth regulators on various plant species (**Table A1** in Appendix; Gianfagna, 1995). The chemical genomics era should now allow for a more systematic analysis in this approach. For example, the plant hormone abscisic acid (ABA) has important roles in protecting plants from abiotic stresses such as drought and cold (Ben-Ari, 2012). Rational approaches to this problem have involved attempts to make stable ABA analogs, but have met with limited success (Zaharia et al., 2005). Recently, a chemical screen identified a synthetic naphthalene sulfonamide ABA agonist, Pyrabactin, which preferentially binds an ABA receptor (Park et al., 2009). Although Pyrabactin is mostly active during the germination stage (Park et al., 2009), focused chemical screens built around a sulfonamide substructure identified Quinabactin, which localizes ABA effects to vegetative tissues and results in improved drought tolerance (Okamoto et al., 2013). Thus, stable synthetic compounds that modulates ABA synthesis or signaling may improve the plants response to abiotic stress and promote productivity. It is easy to envision a future in which a farmer that is experiencing drought will apply Quinabactin-like compounds as a treatment to avoid crop losses. By systematically screening for compounds that mimic the activity of a plant hormone, researchers were able to identify chemicals that act through a canonical hormone-signaling pathway. As Pyrabactin bares no structural resemblance to ABA, it is unlikely that modifying the natural ligand to the receptor would have led to the discovery of a compound such as Pyrabactin. This highlights the benefit of screening chemical libraries to identify compounds that can be used as scaffolds in the development of novel agonists or inhibitors of hormone perception.

BACK TO THE FUTURE

Plant hormones such as auxin, GA, and ABA continue to be excellent targets for the Ag-chemical industry, and have a long-standing history of being manipulated in plant biotechnology (Gianfagna, 1995). Given the successes of these biotechnological advances, other hormones that regulate agriculturally important traits could form the basis of future discovery. Functional analogs of the hormone salicylic acid (SA), such as benzothiadiazole (BTH), promote plant resistance to pathogens and have been developed for use in the field (Gorlach et al., 1996; Lawton et al., 1996). The success of these analogs demonstrated the utility of targeting the SA pathway in the development of compounds that promote crop productivity. This idea formed the basis of high-throughput chemical screens that target SA signaling. Screening through compounds using cell suspension cultures treated to pathogenic *Pseudomonas* identified compounds that promote pathogen resistance in *Arabidopsis* by invoking the hypersensitive cell death pathway in response to pathogen attack (Noutoshi et al., 2012b). The inhibition of SA glucosyltransferases promoted pathogen resistance by increasing SA accumulation (Noutoshi et al., 2012b), whereas a set of functional analogs induced SA signaling *in planta* (Noutoshi et al., 2012a). Whether either of these approaches to plant defense signaling will translate to field applicability remains to be seen, however, this approach has led to viable leads in the development of new

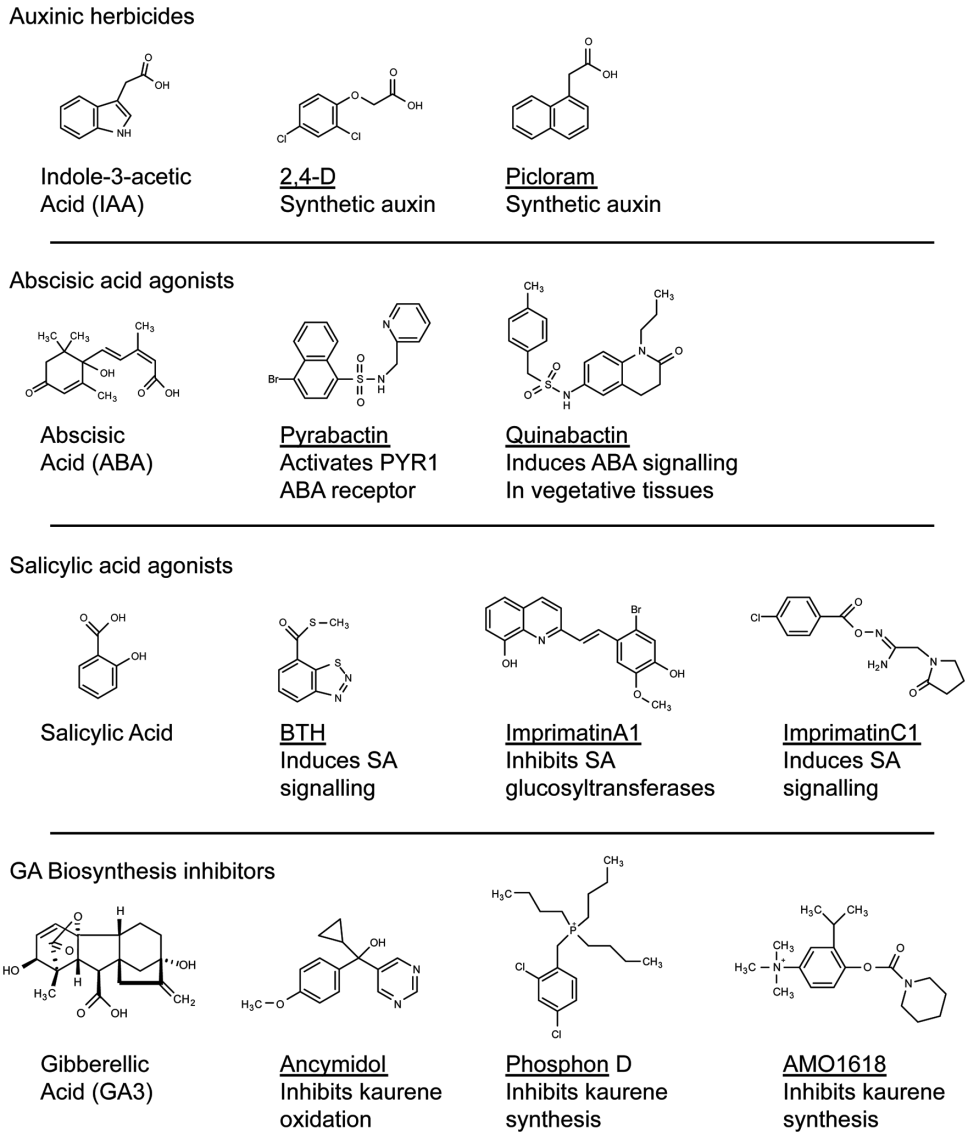

FIGURE 1 | Structural diversity of compounds that modify plant hormone signaling. Compounds that modulate hormone signaling have been identified for major plant hormones. By targeting perception or metabolism, these compounds can induce or inhibit hormone activity. High-throughput screening can identify compounds that act on the hormone receptor yet bare no structural resemblance to the natural ligand (for example, compare Pyrabactin with ABA). These compounds can be used as scaffolds in the development of novel agonists or antagonists of plant hormone perception.

agricultural treatments. At the very least, these chemicals can be used to probe SA signaling pathways in plants (Noutoshi et al., 2012a).

Though hormones present an obvious point to manipulate plant output through chemical biology, metabolites can also serve as signaling molecules that impact plant growth and development (Kim et al., 1999; Stevenson et al., 2000; Hirai et al., 2003; Stokes et al., 2013). By definition, proteins involved in metabolism bind small molecules, and should therefore be druggable (Hopkins and Groom, 2002). This should enable the development of chemicals that antagonize metabolic signaling pathways by acting as competitive inhibitors. Over 1/3 of the *Arabidopsis* genome appears to be involved in metabolism, and close to 200,000 enzymes have been annotated across 17 species for which information is available[2]. The plant metabolome may represent an area of untapped potential through which chemical biology can facilitate the development of novel plant growth regulators.

Once a metabolite has been discovered to influence the development of an important plant trait, chemical screens can uncover modifiers of this response. For example, the presence of glutamate influences root system architecture by restricting primary root elongation and promoting the proliferation of lateral roots (Walch-Liu et al., 2006). Modifying root system architecture can benefit plant growth in response to new

[2]http://www.plantcyc.org

environments and abiotic stresses (Lynch, 1995; Comas et al., 2013). Screening for compounds that antagonized glutamate perception uncovered novel components of a glutamate signaling pathway and facilitated the development of chemical tools that promote root development in response to endogenous cues (Forde et al., 2013). Targeting metabolic pathways by high-throughput chemical screening should glean new insight into the mechanisms through which metabolites influence plant growth and development.

Directing metabolic output through genetic engineering has been a goal of plant scientists for some time. The development of Golden Rice, engineered to synthesize β-carotene in its seeds (Ye et al., 2000; Paine et al., 2005), demonstrated the potential of metabolic engineering to enhance nutritional value of staple crops (Tang et al., 2009). Vitamin A deficiency is a major health concern in many parts of the developing world, and can result in permanent blindness and death (Underwood and Arthur, 1996). Consuming β-carotene, the precursor to Vitamin A, can help combat malnutrition in some of the world's poorest populations (Tang et al., 2009). Unfortunately, efforts to implement this technology in regions that stand to benefit the most from it have been stymied by governments and activists in opposition of genetic modification (Enserink, 2008).

Despite the difficulties bringing Golden Rice to the field, it has demonstrated that metabolic engineering can promote nutritional value in crops and raises questions about the ability to use chemicals analogously to genetic engineering in directing the metabolic output of plant. If enzymes make good drug targets then it should be possible to uncover chemicals that can direct metabolic flux by modulating biosynthetic pathways. Presumably, blocking metabolism at crucial time-points during plant development can promote the accumulation of specific metabolites that could have economic or nutritional value. Methods that enable quick assessment of metabolite abundance would facilitate screens in search of compounds that promote the accumulation of metabolites of interest. Though we are not necessarily advocating for increased application of chemicals to food products, we believe that targeted manipulation of plant metabolism through chemical biology does have the potential to promote nutritional value in crops and to enhance the accumulation of rare or expensive natural products in some species.

Similarity between crop species is beneficial to plant researchers because treatments that are effective in one species are likely to be useful in a related species. In principal, this should facilitate translation from laboratory science to real-world applications. Despite this, there seems to be a paucity of published examples in which leads from high-throughput screens in *Arabidopsis* were then tested across agriculturally important species. Some characteristics of the model plant *Arabidopsis*, including its small size and rapid growth, make it an obvious choice as the subject of phenotype-based high-throughput chemical screening (Robert et al., 2009). As sequenced genomes become readily available and as new tools are developed for other plant species, compounds identified using *Arabidopsis* should be assayed in other plants to assess the utility of these leads in commercial applications. Focus on the development of compounds that modify traits in important species might encourage collaboration between the Ag-biotech sector and academic research groups, a relationship that has stimulated innovation in the pharmaceutical industry (Scudellari, 2011; Loregian and Palu, 2013).

LESSONS FROM BIG PHARMA

In many ways, the Ag-chem industry is facing a similar situation to the pharmaceutical industry, in which exorbitant costs of drug development have become prohibitive. This has resulted in a stagnating supply of innovative new products coming through the research and development pipeline (Bennani, 2011; Pammolli et al., 2011). The increasing market share being lost to generics and some valuable patents expiring over the past few years have put pressure on Big Pharma to restructure their lead development strategy (Cuatrecasas, 2006; Loregian and Palu, 2013). Over the past decade, an increasing number of large pharmaceutical companies have established fruitful collaboration with academic research laboratories, effectively "outsourcing" discovery-based lead generation (Scudellari, 2011; Fishburn, 2013). In support of this, universities across the world have established high-throughput screening facilities that enable drug discovery (Loregian and Palu, 2013). In this model, discovery-based research is handled by the academic institution; commercialization and product development are generally managed by the corporation.

This relationship has allowed the burden of high-risk projects to be taken by the research institute, whose incentives and measures of success may differ from that of the corporate partner (Fishburn, 2013; Loregian and Palu, 2013). An academic group may put greater value in publications and training opportunities (Loregian and Palu, 2013), or may be more interested in pursuing high-risk projects that attempt to drug difficult targets, such as transcription factors and protein–protein interactions (Loregian and Palu, 2013). In this sense, the needs of society benefit from close collaboration between academic labs and Big Pharma. These collaborations can mean more attention paid to rare or neglected diseases, greater propensity to tackle historically difficult targets, and the generation of new molecular entities that can be developed into therapeutic treatments.

A similar strategy would benefit the Ag-biotech industry, in which academic chemical biology labs could make use of the available high-content screening platforms to develop new herbicides and agricultural chemicals. The prevalence of herbicide-resistant weeds, coupled with the increased abiotic stresses crippling agricultural output are putting pressure on the Ag-chem industry to develop innovative methods of crop protection that sidestep the need for genetic modification. Modern genomic analysis should enable researchers to quickly understand the mechanism of resistance, and scientists now have the tools available to develop tailored chemical treatments that target specific classes of weeds and other pests. Taking a lead from Big Pharma, the private sector and academic laboratories should collaborate to establish translational research programs that promote innovation and open new opportunities to sustain agricultural productivity.

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APPENDIX

Table A1 | Plant growth regulators used in agriculture.

Growth regulator	Mode of action	Agronomic benefit	Species
Hormone action			
IBA	Auxin analog	Rooting of stem cuttings	Many plants
2,4-D	Auxin analog	Prevent fruit drop	Apple, pear, citrus
4-CPA	Auxin analog	Stimulate fruit setting	Tomato
NAA	Auxin analog	Fruit thinning	Apples, pear
GA	Plant hormone	Fruit size increase	Grapes
GA	Plant hormone	Delay fruit ripening	Apple, pear, citrus
GA	Plant hormone	Increase yield	Sugarcane
GA	Release enzymes	Malting	Barley
Ethephon	Ethylene release	Rubber production	Hevea
Ethephon	Ethylene release	Promote abscission	Cherry, walnut, olive
Ethephon	Ethylene release	Fruit ripening	Apple, tomato
Ethephon	Ethylene release	Color, fruit acidity	Grapes
Ethephon	Ethylene release	Promoting leaf senescence	Tobacco
Benzyladenine (BA)	Cytokinin analog	Lateral bud formation	White pine
Accel	Cytokinin analog	Increase lateral branching	Carnations
Promalin	Mixture $GA_{4/7}$+BA	Fruit diameter	Apple
Promalin	Mixture $GA_{4/7}$+BA	Increase lateral branching	Apple trees
Growth retardants			
Chloromequat	GA synthesis inhibitor	Stem growth	Poinsettia
Ancymidol	GA synthesis inhibitor	Stem growth	Easter lily
Mepiquat	GA synthesis inhibitor	Reduce excessive growth	Cotton
Chlorflurenol	GA synthesis inhibitor	Reduce growth	Turf grass
Diaminozide	GA synthesis inhibitor	Increase fruit set	Grapes
Diaminozide	GA synthesis inhibitor	Color	Cherry
Diaminozide	GA synthesis inhibitor	Flower bud formation	Apple, pear
Paclobutrazol	GA synthesis inhibitor	Control tree size	Fruit trees
Miscellaneous			
Maleic hydrazide	Unknown	Inhibit sprouting	Onion, potato
Glyphosine	Glyphosate analog	Increases sugar yield	Sugarcane
Dimethipin	Unknown	Defoliant	Cotton

Chemicals that promote a variety of agriculturally important growth traits have been developed for use in the field.

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Unraveling plant hormone signaling through the use of small molecules

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Plants have acquired the capacity to grow continuously and adjust their morphology in response to endogenous and external signals, leading to a high architectural plasticity. The dynamic and differential distribution of phytohormones is an essential factor in these developmental changes. Phytohormone perception is a fast but complex process modulating specific developmental reprogramming. In recent years, chemical genomics or the use of small molecules to modulate target protein function has emerged as a powerful strategy to study complex biological processes in plants such as hormone signaling. Small molecules can be applied in a conditional, dose-dependent and reversible manner, with the advantage of circumventing the limitations of lethality and functional redundancy inherent to traditional mutant screens. High-throughput screening of diverse chemical libraries has led to the identification of bioactive molecules able to induce plant hormone-related phenotypes. Characterization of the cognate targets and pathways of those molecules has allowed the identification of novel regulatory components, providing new insights into the molecular mechanisms of plant hormone signaling. An extensive structure-activity relationship (SAR) analysis of the natural phytohormones, their designed synthetic analogs and newly identified bioactive molecules has led to the determination of the structural requirements essential for their bioactivity. In this review, we will summarize the so far identified small molecules and their structural variants targeting specific phytohormone signaling pathways. We will highlight how the SAR analyses have enabled better interrogation of the molecular mechanisms of phytohormone responses. Finally, we will discuss how labeled/tagged hormone analogs can be exploited, as compelling tools to better understand hormone signaling and transport mechanisms.

Keywords: phytohormones, hormone signaling, structure-activity relationship, labeled molecule, agonists and antagonists

INTRODUCTION

Plants produce a wide variety of endogenous small molecules, allowing them to thrive in the face of internal or external challenges. Among these molecules, phytohormones are growth regulators, which are effective at low concentrations, controlling a vast range of developmental and adaptive processes (Rubio et al., 2009). Our comprehension of plant hormone biology (metabolism, transport, perception, and signaling) has increased tremendously during the last decade. Most of this knowledge has been gained using genetic approaches in the model plant *Arabidopsis thaliana*, however in recent years, chemical genetics has been introduced as a compelling tool in plant science. The application of small molecules allows instantaneous, reversible and conditional alteration of a phenotype and thereby offers circumvention of the limitations of classical genetic approaches, including genetic redundancy, lethality and pleiotropism (Toth and Van Der Hoorn, 2010). Chemical genetics has been extensively employed to study molecular mechanisms of complex and highly dynamic processes such as plant hormone signaling, leading to new possibilities and perspectives in hormone biology. This new knowledge on plant hormone chemistry has not only led to the identification of structurally related compounds for commercial applications, but has also and most importantly provided the basis for the rational design of novel analog molecules as chemical tools probing phytohormone-regulated responses. Determination of the bioactive moieties of many phytohormone molecules in combination with synthetic chemistry has generated an assortment of novel compounds including phytohormone agonists and antagonists and tagged/labeled phytohormone-analogous molecules. Application of those compounds has contributed significantly to our current understanding of the modes of action of phytohormones. Thus, the inter-connection between chemistry and plant biology provides new insights into plant hormone biology. Here, we will review some prominent examples of the use of chemical genomic strategies in plant hormone research. We will focus on abscisic acid (ABA), salicylic acid (SA), auxin, cytokinin (CK), brassinosteroid (BR), and strigolactone (SL) signaling pathways. The review by Chini and co-authors in this issue covers similar topic for jasmonate related-research. This review will highlight how the integration between chemistry and biology improves the potential to dissect hormone signaling.

AGONIST AND ANTAGONIST MOLECULES

ABA AGONISTS AND ANTAGONISTS

ABA (**Figure 1A**) is a sesquiterpenoid plant hormone, which is involved in both biotic/abiotic stress responses and regulation of important aspects of plant growth and development (Cutler et al., 2010). Based on a chemical biology strategy, a variety of small ABA-related bioactive compounds have been identified or designed with the aim to elucidate the mode of action of ABA in plants (Kitahata and Asami, 2011). The most salient example is the selective ABA agonist named pyrabactin (**Figure 1A**), which inhibits seed germination but has no effect on other ABA responses (Zhao et al., 2007; Park et al., 2009). Genetic isolation of mutants insensitive to pyrabactin in a seed germination assay led to the identification of PYRABACTIN RESISTANCE 1 (PYR1) as well as 13 PYR1-like (PYL) members, a new class of START domain proteins, as the long-sought-after intracellular ABA receptors in *Arabidopsis* (Park et al., 2009). Structural biology analyses using ABA/pyrabactin-bound receptors revealed a gate-latch-lock mechanism for ABA perception (Melcher et al., 2009; Miyazono et al., 2009;

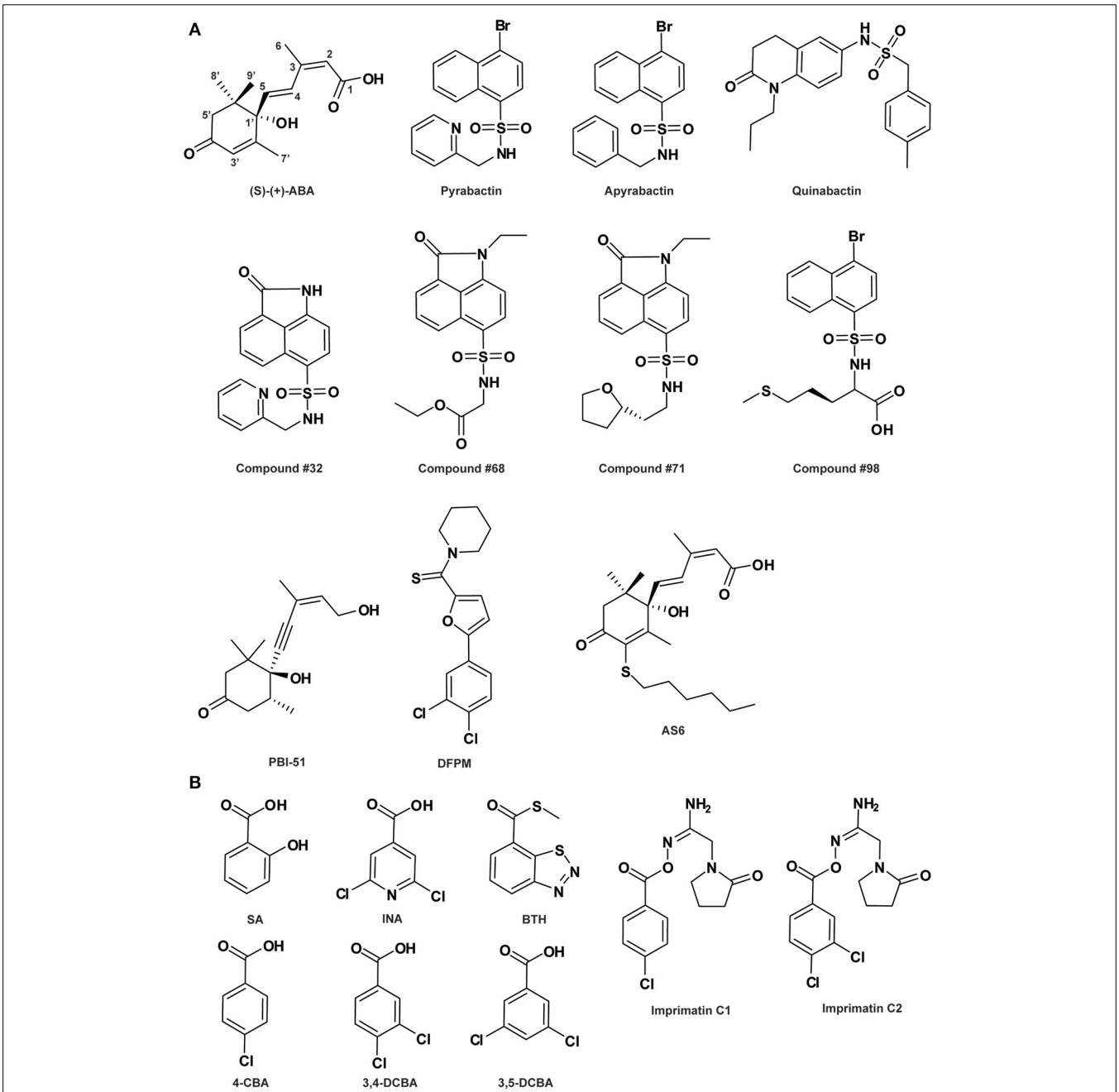

FIGURE 1 | Abscisic acid- (A) and salicylic acid-related compounds (B). See **Table 1** for the full name of each compound.

Table 1 | Names of the phytohormone-related chemical compounds described in the review.

Common name	IUPAC name
Abscisic acid (ABA)	(2Z,4E)-5-((S)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-enyl)-3-methylpenta-2,4-dienoic acid
Pyrabactin	4-bromo-N-(pyridin-2-ylmethyl)naphthalene-1-sulfonamide
Apyrabactin	N-benzyl-4-bromonaphthalene-1-sulfonamide
Quinabactin	N-(2-oxo-1-propyl-1,2,3,4-tetrahydroquinolin-6-yl)-1-p-tolylmethanesulfonamide
Compound #32	2-oxo-N-(pyridin-2-ylmethyl)-1,2-dihydrobenzo[cd]indole-6-sulfonamide
Compound #68	ethyl 2-(1-ethyl-2-oxo-1,2-dihydrobenzo[cd]indole-6-sulfonamido)acetate
Compound #71	(S)-1-ethyl-2-oxo-N-(2-(tetrahydrofuran-2-yl)ethyl)-1,2-dihydrobenzo[cd]indole-6-sulfonamide
Compound #98	2-(4-bromonaphthalene-1-sulfonamido)-5-(methylthio)pentanoic acid
PBI-51	(4S,5R)-4-hydroxy-4-((Z)-5-hydroxy-3-methylpent-3-en-1-ynyl)-3,3,5-trimethylcyclohexanone
DFPM	(5-(3,4-dichlorophenyl)furan-2-yl)(piperidin-1-yl)methanethione
3′-hexylsulfanyl-ABA (AS6)	(2Z,4E)-5-((S)-3-(hexylthio)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-enyl)-3-methylpenta-2,4-dienoic acid
Salicylic acid (SA)	2-hydroxybenzoic acid
INA	2,6-dichloroisonicotinic acid
Benzothiadiazole (BTH)	S-methyl 1,2,3-benzothiadiazole-7-carbothioate
Imprimatin C1	(E)-N′-(4-chlorobenzoyloxy)-2-(2-oxopyrrolidin-1-yl)acetimidamide
Imprimatin C2	(E)-N′-(3,4-dichlorobenzoyloxy)-2-(2-oxopyrrolidin-1-yl)acetimidamide
4-CBA	4-chlorobenzoic acid
3,4-DCBA	3,4-dichlorobenzoic acid
3,5-DCBA	3,5-dichlorobenzoic acid
IAA	indol-3-acetic acid
NAA	1-naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Picloram	4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid
5-F-IAA	5-fluoro-indol-3-acetic acid
BH-IAA	8-(tert-butoxycarbonylamino)-2-(1H-indol-3-yl)octanoic acid
PEO-IAA	2-(1H-indol-3-yl)-4-oxo-4-phenylbutanoic acid
Auxinole	4-(2,4-dimethylphenyl)-2-(1H-indol-3-yl)-4-oxobutanoic acid
FITC-IAA	2-(1-(3′,6′-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthene]-5-ylcarbamothioyl)-1H-indol-3-yl)acetic acid
RITC-IAA	N-(9-(2-carboxy-6-(3-(carboxymethyl)-1H-indole-1-carbothioamido)phenyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-N-ethylethanaminium
Terfestatin A (TrfA)	(2S,3R,4S,5S,6R)-2-(2,4-dihydroxy-3,6-diphenylphenoxy)-6-(hydroxymethyl)oxane-3,4,5-triol
trans-Zeatin (tZ)	(E)-4-(9H-purin-6-ylamino)-2-methylbut-2-en-1-ol
N^6-(2-hydroxy-3-methylbenzylamino) purine (PI-55)	2-((9H-purin-6-ylamino)methyl)-6-methylphenol
N^6-(2,5-dihydroxybenzylamino) purine (LGR-991)	2-((9H-purin-6-ylamino)methyl)benzene-1,4-diol
N^6-(benzyloxymethyl) adenosine (BOMA)	(2R,3R,4S,5R)-2-(6-(benzyloxymethylamino)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol
S-4893	3-(6-chloro-4-phenylquinazolin-2-ylamino)propan-1-ol
N^6-benzyladenine (BA)/6-benzylaminopurine (BAP)	N-benzyl-7H-purin-6-amine
Brassinolide (BL)	(3aS,5S,6R,7aR,9aS,10R)-10-((2S,3S,4S,5R)-3,4-dihydroxy-5,6-dimethylheptan-2-yl)-5,6-dihydroxy-7a,9a-dimethyltetradecahydro-1H-benzo[c]indeno[5,4-e]oxepin-3(12bH)-one
Bikinin (BIK)	4-(5-bromopyridin-2-ylamino)-4-oxobutanoic acid
Brassinopride (BRP)	N-benzyl-N-(1-cyclopropylethyl)-4-fluorobenzamide
Castasterone (CS)	(2R,3S,5S,10R,13S)-17-((2S,3S,4S,5S)-3,4-dihydroxy-5,6-dimethylheptan-2-yl)-2,3-dihydroxy-10,13-dimethyltetradecahydro-1H-cyclopenta[a]phenanthren-6(10H)-one
Alexa Fluor 647-castasterone (AFCS)	2-((1E,3E,5Z)-5-(3-(6-(5-(2-((E)-((2R,3S,10R,13S)-17-((2S,3S,4S,5S)-3,4-dihydroxy-5,6-dimethylheptan-2-yl)-2,3-dihydroxy-10,13-dimethyloctahydro-1H-cyclopenta[a]phenanthren-6(10H,12H,13H,14H,15H,16H,17H)-ylidene)aminooxy)acetamido)pentylamino)-6-oxohexyl)-3-methyl-5-sulfo-1-(3-sulfopropyl)indolin-2-ylidene)penta-1,3-dienyl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)-3H-indolium

(Continued)

Table 1 | Continued

Common name	IUPAC name
(+)-Strigol	(3aR,5S,8bS,E)-5-hydroxy-8,8-dimethyl-3-(((R)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,5,6,7,8,8b-octahydro-2H-indeno[1,2-b]furan-2-one
Karrikin1 (KAR1)	3-methyl-2H-furo[2,3-c]pyran-2-one
GR24	(3aR,8bS,E)-3-(((R)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one
Cyano-isoindole-strigolactone-analog-1 (CISA-1)	(E)-ethyl 2-(1-(but-3-enyl)-3-cyano-2H-isoindol-2-yl)-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)acrylate
4-Br debranone	5-(4-bromophenoxy)-3-methylfuran-2(5H)-one
3′-methyl-GR24	(3aR,8bS,Z)-3-(((R)-3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one
tia-3′-methyl-debranones-like molecule	5-((4-Chlorophenyl)thio)-3,4-dimethylfuran-2(5H)-one
AR36	(2E,4E)-Methyl 5-((3,4-Dimethyl-5-Oxo-2,5-Dihydrofuran-2-Yl)Oxy)-4-Methylpenta-2,4-Dienoate
BOPIDY	4,4-difluoro-4-bora-3α,4α-diaza-s-indacene
HR	BF2 Chelate of (Z)-5-(3,5-dimethyl-1Hpyrrol-2-yl)-N-(4-((E)-1,4-dimethyl-2-((4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3-oxo-1,2,3,4-tetrahydrocyclopenta[b]indol-7-yl)phenyl)-5-(3,5-dimethyl-2H-pyrrol-2-ylidene)pentanamide
EG	BF2 Chelate of (E)-6-((3,5-dimethyl-1H-pyrrol-2-yl)(3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)-2,2-dimethyl-2H-pyran-4(3H)-one

Both the common name and the IUPAC name of each compound are listed. When an abbreviation for the compound is available, it is included in the parenthesis following the corresponding common name.

Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009): ligand binding causes conformational changes in these receptor proteins, which induces closure of the "gate" and "latch" loops surrounding the ligand-binding pocket. Ligand-induced closure of the gate creates an interaction surface required for binding TYPE 2C PROTEIN PHOSPHATASES (PP2Cs), which are negative regulators of ABA signaling (Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Yoshida et al., 2006; Nishimura et al., 2007). With no or low concentration of ABA, PP2Cs like ABA INSENSITIVE 1 (ABI1), ABI2, HOMOLOGY TO ABI1 (HAB1) and PP2CA/ ABA-HYPERSENSITIVE GERMINATION 3 (AHG3), suppress ABA responses by dephosphorylating and inactivating downstream SUCROSE NON-FERMENTING-1 (SNF1)-RELATED PROTEIN KINASE 2 (SnRK2) kinases, the positive regulators in ABA signaling (Gómez-Cadenas et al., 1999; Mustilli et al., 2002; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). An increase in ABA level inhibits the phosphatase activity of PP2C via the formation of an ABA-receptor-PP2C ternary complex, thereby allowing SnRK2s to be activated by phosphorylation (Cutler et al., 2010; Weiner et al., 2010; Miyakawa et al., 2013). Activated SnRK2s in turn phosphorylate and activate downstream effectors mediating various ABA responses (Kobayashi et al., 2005; Furihata et al., 2006; Cutler et al., 2010). The selectivity of pyrabactin for a subset of the PYR/PYL ABA receptors has been exploited to effectively bypass the genetic redundancy in the *pyr*/*pyl* gene family, which was always eluded in classical genetic mutation analyses (Park et al., 2009).

In consistence with being a selective agonist, pyrabactin is an activator for only a subset of PYR/PYL ABA receptors (Park et al., 2009; Melcher et al., 2010). Moreover, it is intriguing that while pyrabactin is an agonist of PYR1 and PYL1, it is an antagonist of PYL2, competitively blocking ABA-dependent PYL2 activation (Melcher et al., 2010). This unique property of pyrabactin was exploited by Melcher et al. (2010) to decipher the mechanism of ABA receptor antagonism at the molecular level by the combinatorial approaches of structural, biochemical and molecular biological studies. They elaborately showed that it is the closed or open conformation adopted by the ligand-bound receptor that determines activation or inhibition of the ABA receptor. This antagonism model is complementary to the perception-activation mechanism of ABA receptors revealed by ABA *per se*, providing a full view of the mechanisms underlying receptor perception and activity regulation. Furthermore, based on this rational model of ABA receptor agonism and antagonism, virtual screening and docking analysis followed by *in vitro* validation has identified at least four pyrabactin-based small molecules as novel ABA-receptor agonists (compounds #32, #68, #71, and #98 in **Figure 1A**; Melcher et al., 2010), highlighting the efficacy of the application of pyrabactin as a chemical tool in ABA biology.

The same small molecule however, named differently as quinabactin or ABA MIMIC 1 (AM1), was identified as a new synthetic selective ABA agonist in two independent chemical library screens where a yeast two-hybrid assay and an *in vitro* protein interaction assay was applied, respectively (**Figure 1A**; Cao et al., 2013; Okamoto et al., 2013). This compound possesses broader receptor spectrum activity and increased bioactivity relative to pyrabactin, although both quinabactin/AM1 and pyrabactin belong to the sulfonamide type of compounds (Cao et al., 2013; Okamoto et al., 2013). On one hand, unlike pyrabactin's unique selectivity on seed germination in *Arabidopsis*, the physiological effects of quinabactin/AM1 are highly similar to those of ABA, triggering substantial ABA-like responses in vegetative tissues and promoting drought tolerance in adult plants

(Cao et al., 2013; Okamoto et al., 2013). Based on their ligand-free oligomeric states, cytosolic ABA receptors can be divided into two major classes: PYR1 and PYL1-PYL3 are homodimers in solution, whereas PYL4-PYL12 are monomers (Miyakawa et al., 2013). Biochemical and genetic analyses showed that quinabactin's ABA-mimic effects in vegetative tissues are primarily mediated by dimeric ABA receptors (Okamoto et al., 2013). Thus, the use of quinabactin/AM1 as a selective agonist for a restricted subset of ABA receptors, i.e., dimeric ABA receptors, facilitates the revelation of the critical role of dimeric receptors in mediating ABA responses in vegetative tissues. On the other hand, although both quinabactin/AM1 and pyrabactin are sulfonamides, their chemical structures differ from one another: the naphthalene double ring and pyridine ring at each end of the sulfonamide linker in pyrabactin are replaced by a dihydro-quinolinone ring and benzyl group, respectively, in quinabactin/AM1 (**Figure 1A**). Comparison between the crystal structures of quinabactin/AM1- and pyrabactin-receptor-PP2C ternary complexes revealed that the binding mode of quinabactin/AM1 with the receptor more closely mimics that of ABA than pyrabactin, which is consistent with their physiological effects. The binding features of similarities to ABA and differences to pyrabactin provide a structural basis for designing the next generation of ABA-selective agonists, which are potential chemical reagents applicable in drought stress management for agricultural crops (Cao et al., 2013). Very recently, a panel of ABA analogs, each with a bulky group substitution on a specific position around the ABA ring, was assembled as agonists with varying efficacy to probe the specific activities of PYR1/PYL receptor-PP2C complex pairs and the resultant physiological effects in *Arabidopsis* based on biochemical and physiological assays (Benson et al., 2014). The findings from this study provide a comprehensive view of ABA structure-activity and ABA receptor-physiological relationships, as well as modification principles for the future design of selective ABA agonists.

ABA antagonists are potential chemical tools not only for studying ABA perception and signal transduction, but also for resolving the roles of ABA in phytohormone cross-talk responses. In an early study, a stereoisomeric acetylenic analog of ABA, (-)-4(Z)-(4*S*,5*R*)-4-hydroxy-4-(5-hydroxy-3-methylpent-3-en-1-ynyl)-3,3,5-trimethylcyclohexanone (PBI-51; **Figure 1A**), was recognized to act as an ABA antagonist inhibiting ABA-regulated gene expression in cress seed germination (Wilen et al., 1993). This compound is useful for studying the relationship between osmotic stress and ABA in the regulation of seed development. In another chemical library screen designed to identify candidate chemicals capable of antagonizing ABA-induced gene expression, a small molecule [5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM; **Figure 1A**) was identified. DFPM was characterized as a selective ABA antagonist for a subset of ABA responses, including ABA-responsive gene expression and ABA-regulated stomatal movement, by disrupting partial ABA signaling network (Kim et al., 2011). Further analyses established that the antagonistic effects of DFPM on ABA signal transduction are mediated through activation of the early plant immune system. These data suggest the existence of a crosstalk between biotic and abiotic stress signaling pathways, where activation of early components in plant innate immune pathways negatively regulates ABA-mediated abiotic stress responses. Therefore, the potent small molecule DFPM can be used as a chemical tool for mechanistic dissection of both plant immunity and ABA signaling interference (Kim et al., 2011). In fact, evidences provided by biochemical and electrophysiological analyses of DFPM inhibitory activity indicated that DFPM disruption of ABA signaling occurs at the level of or downstream of intracellular Ca^{2+} signaling (Kim et al., 2011).

Very recently, based on the well-characterized structural features of ABA receptor system, a new type of ABA analogs, i.e., 3′-alkylsulfanyl-substituted ABAs called AS*n* compounds with *n* representing the alkyl chain length, was created by the structure-guided rational design strategy (Takeuchi et al., 2014). Among them, 3′-hexylsulfanyl-ABA (AS6; **Figure 1A**) was clarified as a potent ABA antagonist. Except for the six-carbon alkyl chain, it is structurally nearly identical to ABA. This chemical characteristic makes AS6 bind to PYL in a highly similar way as ABA with a comparable affinity, while positions its long *S*-hexyl chain protruding out onto PLY's PP2C-interaction surface, preventing ABA-induced PYL-PP2C interaction, consequently blocking plant ABA responses (Takeuchi et al., 2014). In addition to the potential agrichemical value in regulating stress responses and seed germination for crops, AS6 provides a new tool for dissecting ABA's multiple roles, particularly in non-model systems lacking genetic resources.

SA AGONISTS AND ANTAGONISTS

SA (**Figure 1B**) is a phenolic phytohormone known for its primary function as an endogenous signal mediating plant defense responses against pathogens, as well as influencing responses to abiotic stresses and other important aspects of plant growth and development (Vlot et al., 2009; Rivas-San Vicente and Plasencia, 2011). A complex SA-mediated disease resistance signaling network has been identified in recent years, in which NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1), a transcription co-regulator, plays a central role (Vlot et al., 2009; Pajerowska-Mukhtar et al., 2013). Intriguingly, both NPR1 and its paralogs NPR3 and NPR4, two adaptors that bridge between the CULLIN 3 (CUL3) ubiquitin E3 ligase and its substrate, function as the long-sought-for SA receptors (Fu et al., 2012; Wu et al., 2012; for review: Pajerowska-Mukhtar et al., 2013), while NPR1 protein levels are precisely controlled via $CUL3^{NPR3}$- and $CUL3^{NPR4}$-mediated turnover through the proteasome (Spoel et al., 2009). However, the detailed mechanisms of SA perception by distinct receptors under specific physiological conditions and the immediate downstream NPR1 regulation are still elusive.

A number of compounds have been developed as synthetic analogs of SA and employed in disease control for crop protection. Among them, 2,6-dichloroisonicotinic acid (INA; **Figure 1B**) and benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (benzothiadiazole or BTH; **Figure 1B**) are two notable molecules that have also been widely used in studies interrogating components in SA signaling and response (Uknes et al., 1992; Lawton et al., 1996). Meanwhile, selective

agonists have been proven as powerful tools to delineate the function of individual members of functionally redundant receptors. Using a high-throughput chemical screening strategy targeting selective identification of immune-priming compounds, Noutoshi et al. (2012) isolated imprimatinC chemicals, including two structurally similar molecules imprimatin C1 and C2 (**Figure 1B**), as partial agonists of SA. These compounds effectively induce the expression of SA-responsive defense-related genes and increase disease resistance in *Arabidopsis*, while exhibiting no effects on the positive feedback loops in SA signaling and antagonism to jasmonic acid (JA) signaling (Noutoshi et al., 2012). It has been known that elucidation of SA-mediated early defense signaling events is often hampered by various feedback loops and cross-talk with other phytohormones that modulate the SA signal (Vlot et al., 2009). Thus, imprimatinC compounds can potentially assist to better understand the molecular events involved in SA defense signaling. Furthermore, structure-activity relationship (SAR) analyses implicated that the potential downstream metabolites of imprimatinC compounds, including 4-chlorobenzoic acid (4-CBA), 3,4-dichlorobenzoic acid (3,4-DCBA) and their derivative 3,5-DCBA (**Figure 1B**), also act as partial agonists of SA with various potencies (Noutoshi et al., 2012). Therefore, imprimatinC compounds and their potential functional metabolites can serve as valuable tools to address the complexity intrinsic to the activities of SA receptors, providing insights into the mechanisms governing early SA perception and NPR1 regulation and its role in plant immune signaling.

AUXIN AGONISTS AND ANTAGONISTS

Auxin is an important small-molecule phytohormone regulating almost every aspects of plant growth and development (Woodward and Bartel, 2005; Vanneste and Friml, 2009). Indole-3-acetic acid (IAA; **Figure 2A**) is the predominant form of naturally occurring auxin in plants, although indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA) have also been identified endogenously in different plant species (Simon and Petrášek, 2011). Elucidation of the cellular and physiological roles of auxin and its mode of action is historically reliant on the use of diverse bioactive small molecules, ranging from natural metabolites from plants or microbes to synthetic compounds. In recent years, the rapid development of chemical biology has contributed significantly to enhance our understanding of auxin biology, which has been comprehensively summarized in several recent reviews (De Rybel et al., 2009a; Hayashi and Overvoorde, 2013; Ma and Robert, 2014). Here, we intend to concentrate on the employment of auxin agonists and antagonists in interrogating the molecular mechanisms underlying auxin signaling and its regulation.

Auxin transcriptional response starts with the perception of the auxin ligand by the members of the auxin receptor protein family TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOX1 (AFB1) to AFB5, which are F-box subunits of the S-PHASE KINASE-ASSOCIATED PROTEIN1-CULLIN1-F-BOX (SCF) type E3 ubiquitin ligase complex (Dharmasiri et al., 2005a,b; Kepinski and Leyser, 2005). This binding stabilizes the interaction between $SCF^{TIR1/AFB}$ and co-receptors named AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) repressor proteins, which are negative regulators of auxin signaling (Abel et al., 1995; Gray et al., 2001; Tan et al., 2007). The ubiquitylation and subsequent degradation of Aux/IAA repressors via $SCF^{TIR1/AFB}$-mediated 26S proteolysis removes the repression of (derepresses) activities of AUXIN RESPONSE FACTOR (ARF) transcription factors, leading to the transcription of downstream genes (Weijers et al., 2005; Szemenyei et al., 2008; Dos Santos Maraschin et al., 2009; Bargmann and Estelle, 2014). In this model, auxin behaves like molecular glue between the TIR1/AFB binding pocket and the recognition domain (DII) in the Aux/IAA proteins by stabilizing the co-receptor complex (Tan et al., 2007).

Various synthetic compounds capable of eliciting auxin-like responses were identified in the early years of auxin research and used as auxin agonists to examine and manipulate auxin signaling pathways (De Rybel et al., 2009a; Hayashi and Overvoorde, 2013; Ma and Robert, 2014), most notably 1-naphthaleneacetic acid (1-NAA) and the widely used herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) (**Figure 2A**). Genetic analyses of resistance to these compounds or their derivatives assisted in the isolation of a number of key components in auxin signaling, such as AUXIN-RESISTANT1 (AXR1) to AXR3, AXR5, AXR6, AFB4, and AFB5 (Estelle and Somerville, 1987; Woodward and Bartel, 2005). The highly selective resistance of either *afb4* and *afb5* to picolinate-type or *tir1* and *afb5* to benzoic acid-type synthetic auxins indicated that members of the auxin receptor family have different recognition specificities toward diverse auxinic molecules (Walsh et al., 2006; Gleason et al., 2011; Greenham et al., 2011). This was further corroborated by heterologous experiments using a yeast system showing that distinct auxin agonists differentially stabilize the TIR1-Aux/IAA co-receptor complex and AFB5 exhibits higher affinity to the synthetic auxin picloram (Calderón-Villalobos et al., 2012). Furthermore, based on auxin-dependent yeast 2-hybrid assays, biochemical properties of TIR1/AFB-Aux/IAA co-receptor complexes were systematically assessed, indicating that different co-receptor pairs yield a wide range of auxin-binding affinities which seem to be mainly governed by the Aux/IAA (Calderón-Villalobos et al., 2012). In *Arabidopsis*, there are 6 TIR1/AFBs and 29 Aux/IAAs; the cellular context-specific combinations between them may generate many co-receptors with distinct auxin-sensing capacities, resulting in distinct physiological effects (Bargmann and Estelle, 2014). Thus, agonists selectively affecting auxin-related physiological processes of interest represent novel chemical tools for examining specific aspects of auxin signaling.

The molecular structure and mechanism of auxin perception revealed by the crystallographic analysis of the auxin-bound co-receptor complex lay a good foundation for rational structure-based molecular design of auxin antagonists or anti-auxins, specifically blocking $SCF^{TIR1/AFB}$-Aux/IAA-mediated nuclear auxin signaling. Three anti-auxins were generated by this strategy, i.e., *tert*-butoxycarbonylaminohexyl-IAA (BH-IAA), α-(phenylethyl-2-oxo)-IAA (PEO-IAA) and α-(2,4-dimethylphenylethyl-2-oxo)-IAA (auxinole) (**Figure 2A**), listed in order of increasing potency (Hayashi et al., 2008a, 2012b). These molecules bind with auxin receptors the same

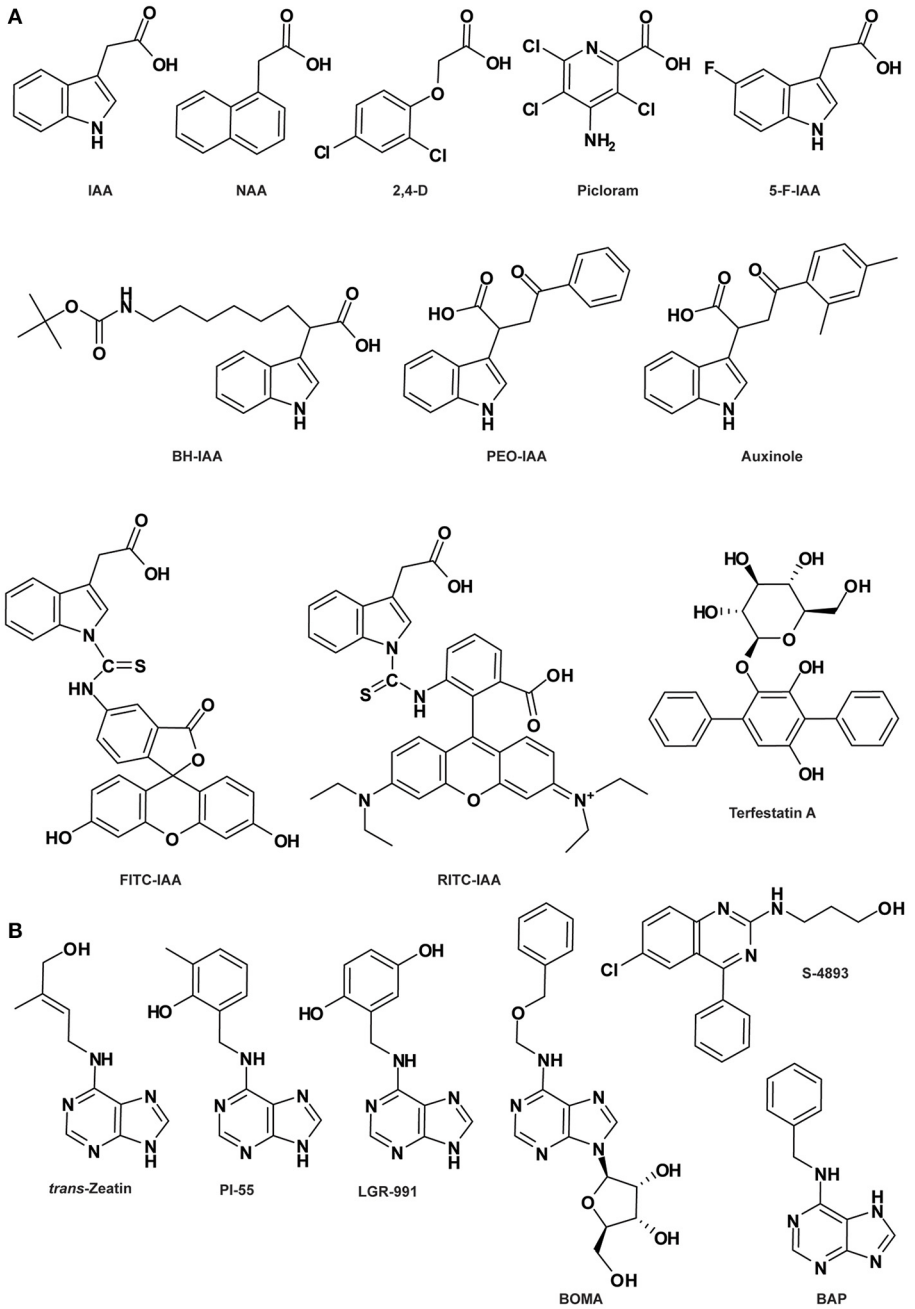

FIGURE 2 | Auxin- (A) and cytokinin-related compounds (B). See **Table 1** for the full name of each compound.

way as IAA, but prevent Aux/IAA docking and the formation of functional co-receptor complexes due to the hindrance caused by the alpha-substituted bulky groups. Thus, the competitive binding between anti-auxin and endogenous IAA inactivates the TIR1/AFB signaling pathway (Hayashi et al., 2008a, 2012b).

In parallel to the nuclear auxin receptors, the extracellular and cell surface-localized AUXIN BINDING PROTEIN1 (ABP1) has been proposed as another important receptor sensing extracellular auxin and mediating rapid non-transcriptional auxin responses centering on the plasma membrane (Sauer and Kleine-Vehn, 2011; Scherer, 2011), including auxin-induced inhibition of clathrin-mediated endocytosis (Robert et al., 2010) and auxin-dependent activation of RHO-RELATED PROTEIN OF PLANTS (ROP) Rho-GTPases governing cell polarity (Xu et al., 2010). It has also been shown that ABP1-mediated auxin signaling negatively regulates the $SCF^{TIR1/AFB}$ pathway (Tromas et al., 2013). ABP1 was first purified from maize coleoptiles by immunoaffinity chromatography nearly 30 years ago (Löbler and Klämbt,

1985) and subsequently proven to bind auxin using photoaffinity labeling method (Jones and Venis, 1989). The crystal structure of ABP1 in complex with auxin was also resolved by Woo et al. (2002). Despite of these significant progresses, the molecular mechanism of ABP1-mediated auxin perception and signal transduction is mostly unresolved. Very recently, a breakthrough has been made on the characterization of the transmembrane kinase (TMK) receptor-like kinases as one group of the long-sought-after ABP1 docking proteins transmitting the extracellular ABP1-perceived auxin signal across the plasma membrane to induce cytoplasmic responses (Xu et al., 2014). Auxin binding to ABP1 prompts its interaction with the extracellular domain of TMK, forming an ABP1-TMK auxin perception complex on the cell surface that activates ROP activity and downstream signaling pathways (Xu et al., 2014). This groundbreaking finding opens a door for addressing many of the mysteries around this longest known but less characterized auxin signaling pathway.

It is reasonable to envision that chemical probes (agonists and antagonists) specifically targeting the ABP1 pathway, similar to those exemplified above for the $SCF^{TIR1/AFB}$-Aux/IAA pathway, could enable identification of novel components in this pathway, shedding more light on ABP1-regulated aspects of auxin biology. In fact, two of such chemical probes have already been identified. *DR5* is a synthetic auxin-responsive element widely used to monitor nuclear TIR1/AFB-mediated auxin signaling (Ulmasov et al., 1997), while inhibition of clathrin-dependent PIN-FORMED (PIN) endocytosis is a hallmark phenomenon for ABP1-mediated auxin signaling. PEO-IAA is a specific antagonist of TIR1/AFB and therefore unable to induce *DR5* expression, but intriguingly inhibits clathrin-dependent PIN endocytosis (Robert et al., 2010), implying that it works as an agonist for ABP1. Conversely, 5-fluoroindole-3-acetic acid (5-F-IAA; **Figure 2A**), a halogenated IAA with auxin activity, is inactive in inhibiting PIN endocytosis while very effective in inducing *DR5* expression (Robert et al., 2010; Simon et al., 2013), functioning as an agonist for TIR1/AFB. Thus, the unique behaviors of these two bioactive molecules structurally analogous to IAA can be utilized in future studies to discriminate between nuclear TIR1/AFB- and extracellular ABP1-dependent auxin signaling pathways. Elucidation of the crystal structure of the auxin-bound ABP1-TMK perception complex will facilitate the development of ABP1-targeted auxin agonists and antagonists, representing novel tools for better understanding of the molecular events controlling this cell surface-cytoplasmic auxin perception and signaling system. Although the transmembrane feature of the TMK protein might impose some difficulties for protein crystallization, the finding that auxin-prompted physical interaction occurs between ABP1 and the extracellular domain of TMK could alleviate this problem to some extent (Xu et al., 2014).

CK AGONISTS AND ANTAGONISTS

CK are classical plant hormones responsible for the regulation of various aspects of plant growth and development such as cell division coordination, cell proliferation, seed germination and root and leaf differentiation (Mok and Mok, 2001; Werner et al., 2001, 2003). Based on the structure of the side-chain, natural CKs are adenine derivatives classified as isoprenoid or aromatic CKs. The isoprenoid CKs, such as *trans*-zeatin (tz; **Figure 2B**), are the ones most frequently found in plants. N^6-benzyladenine (BA; also named 6-benzylaminopurine [BAP]; **Figure 2B**) and its derivatives, such as *meta*- and *ortho*-topolin and the most characterized CK kinetin, belong to the aromatic CKs (Sakakibara, 2006; Bajguz and Piotrowska, 2009; Lomin et al., 2012). Some derivatives of urea also display CK activity, like diphenurea and thidiazuron (Arata et al., 2010). CK signaling occurs through a phosphorylation cascade, which is initiated by the CK receptor HISTIDINE KINASE (HK). In Arabidopsis, three types of CK receptors have been identified: CYTOKININ RESPONSE 1 (CRE1), also called ARABIDOPSIS HISTINE KINASE 4 (AHK4), AHK2 and AHK3.

In the seventies, several synthetic CK derivatives, such as pyrazolo[4,3-d]pyrimidines (Hecht et al., 1971; Skoog et al., 1973), pyrrolo[2,3-d]pyrimidines (Iwamura et al., 1974, 1975) and 7-deaza analogs of 2-methylthioadenine CK (Skoog et al., 1975) were classified as anti-CKs. It was later shown that these compounds do not act as CK antagonists on CK receptors, as was initially suspected, but that at least some of them act as cyclin-dependent kinase inhibitors (Spíchal et al., 2007; Arata et al., 2010). Recently, two BAP derivatives displaying anti-CK activity have been described (Spíchal et al., 2009; Nisler et al., 2010). Among them, N^6-(2-hydroxy-3-methylbenzylamino) purine (PI-55; **Figure 2B**) blocks the binding of the natural tz to the receptor CRE1/AHK4 in a competitive manner. PI-55 is also effective on root growth and branching and stimulates seed germination, supporting the notion that PI-55 inhibits CK perception *in planta*. Moreover, the antagonistic activity of PI-55 was also demonstrated in other species such as tobacco and wheat (Spíchal et al., 2009). Despite its antagonistic effect on CRE1/AHK4, PI-55 at high concentration may weakly induce its interaction with AHK3, leading to AHK3 partial activation (Spíchal et al., 2009). In contrast, another synthetic compound N^6-(2,5-dihydroxybenzylamino) purine (LGR-991; **Figure 2B**), structurally similar to PI-55, acts as an antagonist to the CK receptor CRE1/AHK4 with the same efficiency as PI-55, while competitively antagonizing AHK3 (Nisler et al., 2010). In comparison, LGR-991 presents a lower agonistic effect on the expression of the *ARR5:GUS* reporter gene and consistently induces a phenotype related to a reduction of CK level/signaling. More recently, a synthetic analog of N^6-adenosine, N^6-(benzyloxymethyl) adenosine (BOMA; **Figure 2B**), was described as a novel anti-CK. BOMA is highly specific to CRE1/AHK4 but not AHK3, similarly to PI-55 (Krivosheev et al., 2012).

Interestingly, the phenylquinazoline derivative S-4893 (**Figure 2B**) has been characterized as a novel type of CK antagonist targeting the CK receptor CRE1/AHK4 in a non-competitive way (Arata et al., 2010). S-4893 has been suggested to bind to CRE1/AHK4 differently from the natural CK, and may prevent the conformational modifications of the CK receptor that are required to induce CK-mediated signal transduction. At the physiological level, S-4893 inhibits CK effects on root growth and callus formation in Arabidopsis and other species such as rice (Arata et al., 2010).

Over the past few years, the discovery of synthetic molecules modulating CK signaling has considerably increased our

knowledge of CK perception and provided new opportunities to better understand CK biology.

BR AGONISTS AND ANTAGONISTS

BRs are steroid plant hormones that regulate cell division, elongation and differentiation and are essential for development of organs such as the shoot/hypocotyl, root, leaf and pollen tube. Additionally, BRs are involved in developmental and environmental responses like senescence and biotic and abiotic stress integration (Yang et al., 2011). BRs are perceived by the extracellular domain of the receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1), leading to its dissociation from and association with BRI1 KINASE INHIBITOR 1 (BKI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1; also named SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 [SERK3]), respectively. Phosphorylation of BRI1 is required for the complete activation of the BR signaling pathway (Yang et al., 2011; Wang et al., 2012). Chemical screens based on *Arabidopsis* hypocotyl elongation identified modulators of BR response such as the activator bikinin (BIK; **Figure 3**; De Rybel et al., 2009b) and the inhibitor brassinopride (BRP; **Figure 3**; Gendron et al., 2008). BIK triggers BR signaling by binding to the adenosine triphosphate (ATP) pocket of the major BR-signaling regulator BR-INSENSITIVE2 (BIN2), thus preventing phosphorylation of the downstream transcription factor BRI1-EMS-SUPPRESSOR1 (BES1; De Rybel et al., 2009b). However, BRP's mode of action remains elusive.

Among all the endogenous BRs, brassinolide (BL; **Figure 3**) is the most potent. However, a decrease in its bioactivity can be induced by the engineered modifications of 2-O, 3-O, 22-O or 23-O-methylation (Back et al., 2002; Back and Pharis, 2003). Crystal structure analysis of the BRI1-BL complex revealed that the reduction in the activities of these structural analogs might be due either to their inhibitory effects on the BAK1/SERK3-BRI1 interaction or their lower affinity for BRI1 itself (Hothorn et al., 2011; She et al., 2011; Muto and Todoroki, 2013). To distinguish between these two hypotheses, 2,3-acetonide-BL, 22,23-acetonide-BL and 2,3:22,23-acetonide-BL (diacetonide) were produced, all showing no BL-like activity (**Figure 3**; Muto and Todoroki, 2013). However, 2,3-acetonide-BL and to some extent, 22,23-acetonide-BL, display BL antagonist behavior. The potential activity of diacetonide could not be tested due to its high hydrophobic property preventing it from crossing the cell wall. The weaker antagonist activity of 22,23-acetonide-BL compared to 2,3-acetonide-BL strongly suggests that the 2,3-dihydroxyl group is central for its interaction with the receptor BRI1 (Muto and Todoroki, 2013). Furthermore, it was demonstrated that the interaction between BRI1 and SERK1 is promoted by the presence of BL, which acts as a molecular glue (Santiago et al., 2013). Within SERK1, the residue Phe^{61} and its closest histidine interact with the BL C-ring and the 2α,3α vicinal diol moiety of the hormone, respectively (Santiago et al., 2013). However, whether both hydroxyl groups at C-2 and C-3 or only one of them is required for a potent antagonist effect remains elusive. Taken together, these studies demonstrate the possibility to improve the understanding of BL signaling via chemically modulating BL-BRI1 interaction.

SL AGONISTS AND ANTAGONISTS

The group of SL-related molecules has been described as being involved in general plant development such as root growth, stem secondary development and leaf senescence (Seto et al., 2012). Additionally, SLs act as signals in the rhizosphere for both parasitic and symbiotic interactions (Xie et al., 2010). Karrikins (KARs) and SLs are natural plant signaling molecules involved in common processes such as seed germination and seedling photomorphogenesis (Nelson et al., 2012; Seto et al., 2012; Waters et al., 2014). KARs have been identified in the smoke of burning vegetation and cannot be strictly considered as phytohormones. Both types of molecule contain an enol ether and a substituted methyl butenolide ring, both essential for their stimulatory activity on seed germination (**Figure 4A**). However, KAR structure is simpler than that of SL: the butenolide moiety of KAR is fused to a pyran ring, while it is connected to a tricyclic lactone (ABC-ring) in SL (**Figure 4A**). Although SL and KAR signaling processes are mediated by a common unique F-box protein MORE AXILLARY GROWTH 2 (MAX2), MAX2 is coupled with one of two distinct α/β-hydrolase fold proteins, depending on the phytohormone: DECREASED APICAL DOMINANCE 2 (DAD2)/DWARF14 (D14) for SL or KARRIKIN INSENSITIVE 2 (KAI2) for KAR (Nelson et al., 2011; Hamiaux et al., 2012; Waters et al., 2012). This particular example demonstrates that small structural differences within natural compound enable high specificity for receptor and co-receptor interaction.

As substantial quantities of natural SLs are difficult to obtain, SL synthetic analogs have been engineered. Among them, GR24, in which the A-ring is substituted by an aromatic ring (**Figure 4A**), is the main SL-like compound currently used. As does endogenous SL, GR24 interacts with and is cleaved by the α/β-hydrolase fold protein DAD2/D14 (Hamiaux et al., 2012; Kagiyama et al., 2013; Zhao et al., 2013). Cyanoisoindole-strigolactone-analog-1 (CISA-1) is structurally related to nijmegen-1 (**Figure 4A**; Nefkens et al., 1997) and has also been shown to act through a MAX2-mediated signaling pathway (Rasmussen et al., 2013). Remarkably, CISA-1 is more active and stable than GR24, and possesses interesting fluorescent properties (Rasmussen et al., 2013, see also the "Labeled molecules: compelling tools to understand the action of signaling molecules" section). Moreover, novel SL analogs have been identified as specifically targeting the plant developmental processes via a MAX2-dependent signaling pathway, such as 4-Br debranone (5-[4-bromophenoxy]-3-methylfuran-2[5H]-one) (**Figure 4A**; Fukui et al., 2011, 2013), 3′-methyl-GR24, tia-3′-methyl-debranones-like molecule and AR36 (**Figure 4A**; Boyer et al., 2012, 2014). Their weak potencies on rhizosphere define them as promising SL plant growth regulators (Fukui et al., 2013; Boyer et al., 2014).

FROM STRUCTURE TO ACTIVITY

SAR analyses investigate the relation between a molecule's structure and its bioactivity by testing the potency of multiple natural or synthetic analogs and have been widely used in medical chemistry, pharmacology, cosmetics, toxicology and environmental science (Hasdenteufel et al., 2012). Additionally, determination of the active moieties sheds light on their modes of action. By this

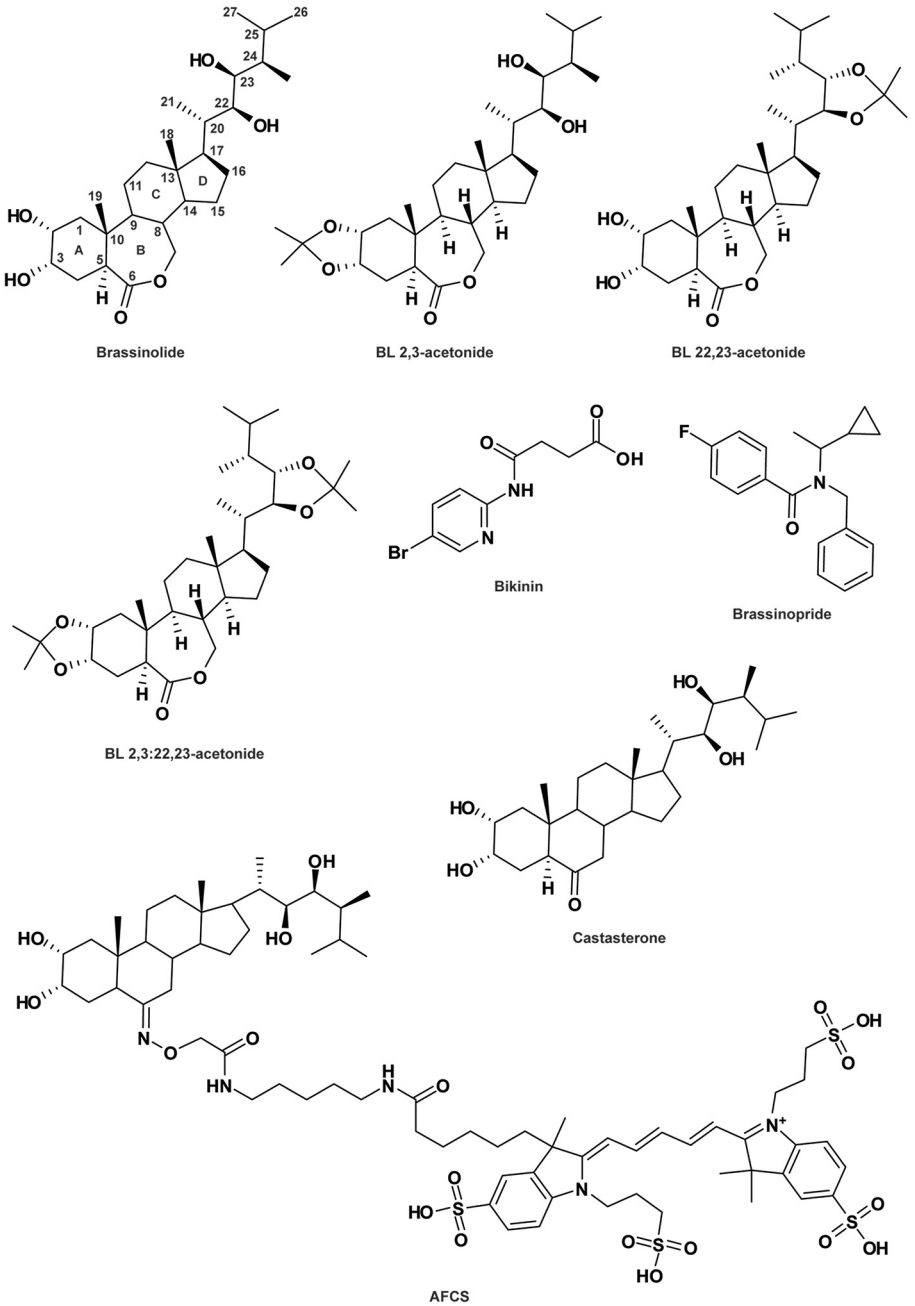

FIGURE 3 | Brassinosteroid-related compounds. See **Table 1** for the full name of each compound.

means, analogs can be identified and purchased in open-access databases or designed and synthesized by combining chemistry.

SAR TO REVEAL THE IMPORTANCE OF THE COMPLEXITY

BRs are plant steroid hormones containing a 5α-cholestane carbon skeleton with a side chain at the C17 position. The first steroidal lactone, named BL (**Figure 3**), was isolated in 1979 from *Brassica napus* pollen (Grove et al., 1979) and since then, more than 50 natural BRs have been characterized throughout the plant kingdom (Fujioka, 1999; Bajguz and Tretyn, 2003). They present some natural variations in the side chain and the substituents in the A and B-rings (**Figure 3**). To better understand BR mode of action, structural requirements for BR bioactivity have been widely studied by the establishment of numerous bioassays including rice leaf lamina inclination and elongation, and curvature and splitting of the bean second internode (Mandava, 1988; Zullo and Adam, 2002; Back and Pharis, 2003). First of all, the trans-A/B-ring conformation, the presence and spatial position of the oxygen atom on the B-ring, and the importance of the 2α, 3α vicinal diol moiety on the A-ring have been shown

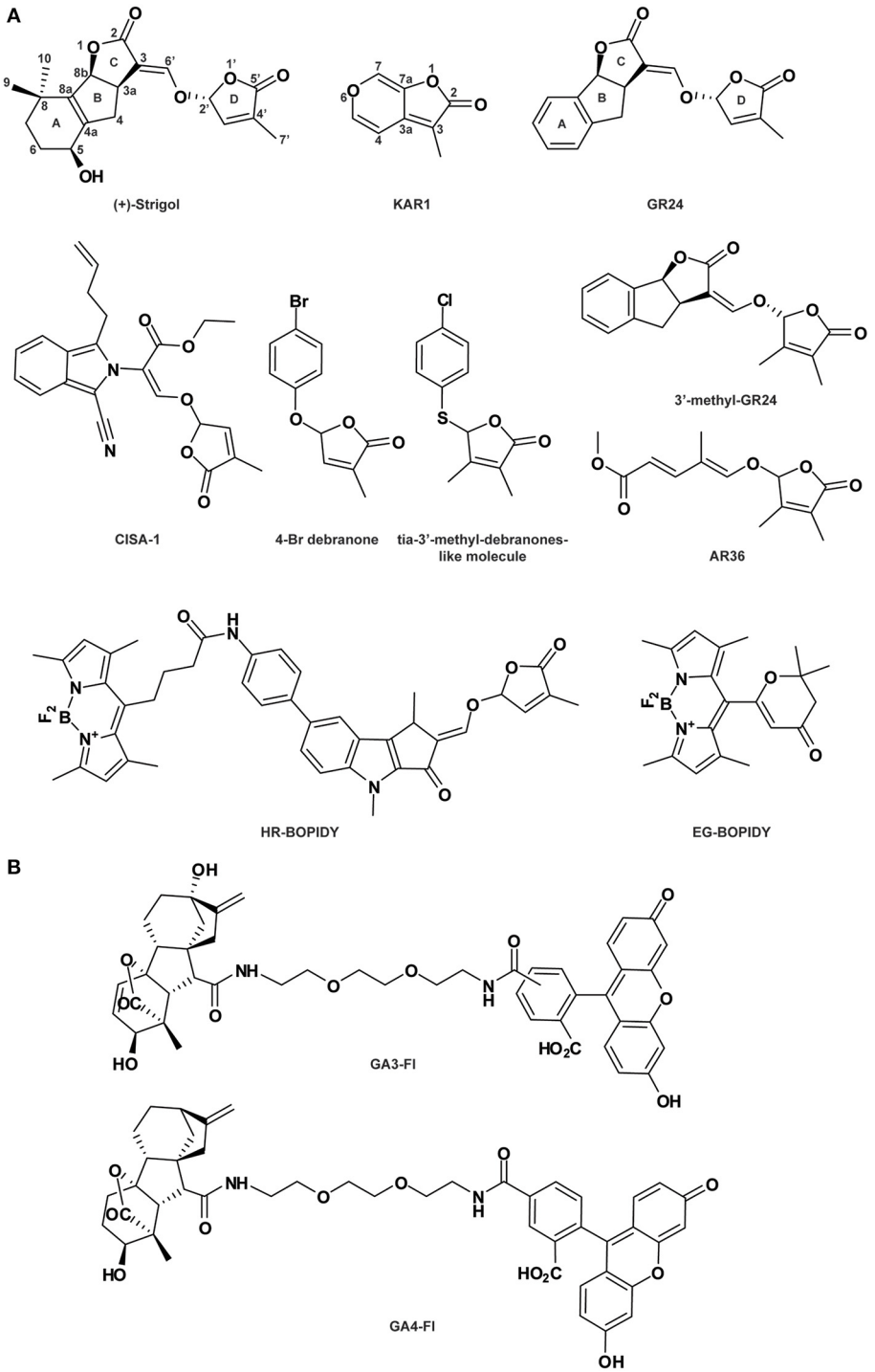

FIGURE 4 | Strigolactone- (A) and gibberellin-related compounds (B). See **Table 1** for the full name of each compound.

to be essential for providing BL-like activity (Mandava, 1988; Baron et al., 1998; Seto et al., 1999; Back and Pharis, 2003; Bajguz, 2011). Regarding the B-ring structure, natural BRs are divided into four types including 7-oxalactone, 6-oxo (6-ketone), 6-deoxo (non-oxidized) and 6-hydroxy. Interestingly, the 6-oxo BRs (such as castasterone [CS]; **Figure 3**; Yokota et al., 1982) display a lower potency than the 7-oxalactone type (for example BL; Bajguz, 2011), suggesting that the seven-membered B-ring lactone is required for optimum activity. The non-oxidized BRs do not show any activity (Bajguz, 2011). Additionally, a substitution of the C-6-keto (in the B-ring) by an α or β hydroxyl group is not favorable for BR potency, indicating that the presence of

an electronegative charge is required to maintain a high level of chemical activity (Ramirez et al., 2005). On the other hand, the side chain of the steroid nucleus is also involved in BR activity determination. Bioactivity is mostly maintained for the BR lacking the methyl group at the C26, C27, or C28, as well as the one presenting a methylidene- or ethylidene- substitution at C24 (Back and Pharis, 2003). Moreover, 2-O, 3-O, 22-O or 23-methylation critically reduces bioactivity (Back et al., 2002; Back and Pharis, 2003). Although the SAR analyses reveal possibilities to modulate BR structure, it appears that each part of the BR chemical structure is a major actor in bioactivity determination. Additionally, new insights into the molecule-receptor binding mechanisms revealed by crystal structure analyses support previous SAR analysis data (Hothorn et al., 2011; She et al., 2011).

SAR TO REVEAL SPECIFIC ACTIVITY

SAR analyses hold great potential for dissecting the functions of endogenous compounds. Several natural SLs have been identified throughout the plant kingdom with a large spectra of activities including the promotion of parasitic weed seed germination, arbuscular mycorrhizal (AM) fungus branching induction and plant growth regulation (Zwanenburg and Pospíšil, 2013). The common structure of endogenous SL includes a tricyclic lactone (ABC-ring) connected via an enol ether bridge to a butenolide group (the D-ring; see Strigol, **Figure 4A**). Importantly, it has been shown that structural requirements to specific activity are divergent.

The active core (or bioactiphore) of SL to stimulate germination of parasitic weeds such as *Orobranche* and *Striga* species has been determined by multiple SAR analyses (Zwanenburg et al., 2009; Janssen and Snowden, 2012; De Saint-Germain et al., 2013). Endogenous SL and the structurally simplified SLs, GR24 (replacement of the A-ring by an aromatic ring), GR7 (lacking the A-ring), GR5 (completed deletion of A and B-rings; Johnson et al., 1981), ABC scaffold and D-ring (2-ethyxybutenolide; Zwanenburg et al., 2009) have been analyzed. This SAR investigation has revealed that the CD but not the ABC part of the molecule is sufficient for seed germination stimulation, suggesting that the SL active core resides in the CD group (Mangnus and Zwanenburg, 1992; Mangnus et al., 1992; Zwanenburg et al., 2009; Zwanenburg and Pospíšil, 2013). The original SL D-ring must be preserved, as the C4 methyl group is essential for SL potency on seed germination (Mangnus and Zwanenburg, 1992; Zwanenburg et al., 1994).

SAR analysis was also conducted on SL to understand SL activity as a plant hormone controlling shoot branching (Fukui et al., 2011; Boyer et al., 2012, 2014). As for root parasitic seed stimulation, the D-ring is essential for shoot branching bioactivity and small changes in the C3 could affect interaction with the receptor. Surprisingly, presence of substitutions on the A and B-rings and a change in the stereochemistry on the C2 do not affect bioactivity (Boyer et al., 2012; Chen et al., 2013). According to Boyer et al. (2012), the SL structure could be replaced by the D-ring only for bud outgrowth inhibition. In agreement, SL analogs (Debranones) in which the D-ring is only linked to an aromatic cycle present the same bioactivity as GR24 for both rice and *Arabidopsis* branching inhibition (Fukui et al., 2011, 2013) and for pea shoot branching inhibition (Boyer et al., 2014). Additionally, the ABC-part could be substituted by an unsaturated acyclic carbon chain without affecting the shoot branching inhibition on pea (Boyer et al., 2014).

Remarkably, the SAR analysis results on AM fungus branching induction are slightly divergent from those mentioned above. As for SL-dependent germination stimulation, the D-ring is required (Akiyama et al., 2010) and the SL stereochemistry is critical (De Saint-Germain et al., 2013). Additionally, the enol-ether bridge connecting the C-D-ring is also critical for SL optimum function (Kondo et al., 2007; Akiyama et al., 2010). However, modifications of the ABC substructure (in particular the A-ring) drastically diminish bioactivity (Besserer et al., 2006; Akiyama et al., 2010; Prandi et al., 2011; Cohen et al., 2013; Boyer et al., 2014). As an example, GR5 stimulates *Orobranche* seed germination but does not induce hyphal branching in AM fungus assays (Johnson et al., 1976; Akiyama et al., 2010). Then, the structure requirement for AM branching is highly specific and small modifications induce a drastic effect on the bioactivity (Boyer et al., 2014).

Overall, the SAR studies performed on multiple endogenous and synthetic SLs reveal that the structural requirements, as an effector of plant development, AM fungal branching or root parasitic seed germination present some noticeable differences. Accordingly, news SL analogs mimicking specific SL activities could be synthetized such as done by Fukui and co-authors (Fukui et al., 2011, 2013). Overall these studies demonstrate that that SL signaling functions through distinct modes of perception in different systems (Boyer et al., 2012, 2014; Chen et al., 2013; Cohen et al., 2013; De Saint-Germain et al., 2013).

SAR TO UNCOUPLE HORMONAL CROSSTALKS

SAR analysis can also disentangle crosstalk between hormone-mediated pathways. As an example, BRP has been characterized as not only a BR signaling inhibitor (see the "Agonist and antagonist molecules" section) but also an inducer of ethylene response (Gendron et al., 2008). Interestingly, one of the BRP derivatives targets essentially the ethylene signaling pathways, highlighting the potential of close structural analogs to separate diverse targeted pathways (Gendron et al., 2008).

The SAR is a powerful approach for dissecting the modes of action of signaling molecules. Indeed, SAR analysis results in the discovery of the required moiety for bioactivity. Interestingly, this approach could also lead to the identification of "dead analogs" (Toth and Van Der Hoorn, 2010). For example, the investigation of several pyrabactin derivatives revealed that its bioactivity requires the pyridyl nitrogen, as the apyrabactin analog (**Figure 1A**) is inactive (Park et al., 2009). Additionally, these totally inactive "dead analogs" could be essential controls in biological assays. In other cases, SAR analysis helps in the design of new antagonist derivatives such as 2,3-acetonide-BL (Muto and Todoroki, 2013) described earlier.

LABELED MOLECULES: COMPELLING TOOLS TO UNDERSTAND THE ACTION OF SIGNALING MOLECULES

The determination of the required structure for a molecule's bioactivity by SAR analysis is central for the successful design

of active tagged/labeled compounds (see as an example in bacteria Chorell et al., 2012). In animal biology, fluorescence-labeled ligand analogs are currently used to study the localization of their receptors as well as the distribution of active endogenous molecules. For example, this strategy was used in research on the dopamine transporter (DAT) involved in dopamine re-uptake. The neurotransmitter DAT is also the principal target for psychostimulants such as cocaine (Chen et al., 2004; Gether et al., 2006; Torres and Amara, 2007). The conception of fluorescent cocaine analogs was essential to permit direct visualization of DAT and to directly follow its cellular trafficking, as no efficient antibody or labeled protein could be generated (Eriksen et al., 2009). The production of fluorescent analogs also creates possibilities to visualize the uptake and *in vivo* distribution of molecules, as illustrated by the use of a fluorescence-tagged glucose probe (Kim et al., 2012).

FLUORESCENT LABELED MOLECULES

The synthesis of fluorescent or tagged compounds has become increasingly attractive for plant researchers over the past few years and has provided new tools to unravel phytohormone signaling and distribution. Several endeavors to generate fluorescent auxin conjugates have been successful. The first attempt was conducted by Muscolo and co-authors, who synthesized fluorescein isothiocynate (FITC) conjugates of IAA and humic substances potentially able to interact with auxin receptors (Muscolo et al., 2007). More recently, new fluorescent auxin conjugates have been produced by coupling with FITC (**Figure 2A**) or rhodamine isothiocynate (RITC; **Figure 2A**; Sokolowska et al., 2014). These two conjugates present an auxin-like activity and are transported via the auxin transport machinery, making them promising tools to study auxin transport and function *in planta*. IAA-FITC and IAA-RITC are both stable at room temperature, however the electrospray ionization tandem mass spectrometry (ESI-MS) analysis conducted on IAA-FITC revealed a degradation of the auxin conjugates. According to the authors, the reason for this may be that the ESI process itself directly reduces the stability of most conjugates. However, the potential IAA-FITC fragmentation *in planta* must be considered.

Since 2009, Bhattacharya and co-authors have generated a new class of bioactive SL analogs named PL series, some of which present luminescent properties under UV radiation at 360 nm (Bhattacharya et al., 2009; Prandi et al., 2011). These compounds are generated by substitution of various functional groups on the A and C-rings of the SL ABC nucleus and provide valuable data for SAR analysis. Although all these analogs show bioactivity as stimulators of germination in *Orobranche aegyptiaca* and hyphal branching in *Gigaspora margarita*, their luminescent properties are not suitable for observation using microscopy-based analysis. However, based on these results, other fluorescently labeled SL analogs have been designed and used successfully *in vivo* in plants and fungi (Prandi et al., 2013). Four new molecules have been produced using different fluorophores inserted on the aromatic ring, which include 5-dimethylaminophtalene-1-sulfyl (dansyl) for (E)-N-(4-(1,4-dimethyl-2-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)-3-oxo-1,2,3,4-tetrahydrocyclopenta[b]indol-7-yl)phenyl)-5-(dimethylamino)naphthalene-1-sulfonamide (AO), the fluorophore fluorescein for (E)-5-(3-(4-(1,4-dimethyl-2-((4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3-oxo-1,2,3,4-tetrahydrocyclopenta[b]indol-7-yl)phenyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid(BL), and 4,4-difluoro-4-bora-3α,4α-diaza-s-indacene (BOPIDY) for the molecules BF2 Chelate of (Z)-5-(3,5-dimethyl-1Hpyrrol-2-yl)-N-(4-((E)-1,4-dimethyl-2-((4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3-oxo-1,2,3,4-tetrahydrocyclopenta[b]indol-7-yl)phenyl)-5-(3,5-dimethyl-2H-pyrrol-2-ylidene)pentanamide (HR) and BF2 Chelate of (E)-6-((3,5-dimethyl-1H-pyrrol-2-yl) (3,5-dimethyl-2H-pyrrol-2-ylidene)methyl)-2,2-dimethyl-2H-pyran-4(3H)-one (EG). The two tagged molecules HR-BOPIDY and EG-BOPIDY (**Figure 4A**) show strong stimulatory effects on *Phelipanche aegyptiaca* seed germination. Additionally, their absorption-emission spectra are suitable for confocal analysis. HR and EG are efficiently taken up by *Medicago truncatula* root hairs and show a cytoplasmic distribution. During the same time period, a new fluorescent SL named CISA-1 has been synthesized by a simple procedure (**Figure 4A**; Rasmussen et al., 2013). A classical genetic approach performed on *Arabidopsis* Columbia wild-type, *max1*/*max4* (SL-deficient mutants) and *max2* (SL-insensitive mutant) confirms its SL-like activity. Similarly to GR24, CISA-1 reduces the number of adventitious roots and inflorescence stems in the SL-deficient mutants, while the SL-insensitive mutant *max2* is not affected. These data suggest that CISA-1 acts downstream of MAX1 and MAX4 through a MAX2-dependent signaling pathway. Furthermore, like GR24, CISA-1 suppresses *MAX4* expression after 24 h of treatment, probably due to feedback regulation from the increased endogenous SL level (Umehara et al., 2008; Mashiguchi et al., 2009). The fluorescent property of CISA-1 has been observed at 10 mM in solution with the excitation and emission spectra between 300-380 nm and 400 nm, respectively, but unfortunately fluorescence detection *in planta* still needs to be improved (Rasmussen et al., 2013).

Two fluorescently labeled bioactive gibberellins (FLBG) have been synthesized with different spacers (1,4-dithiobutylene or 1,3-dithiopropylene chain) between the fluorescein and the gibberellin (GA) molecule (Pulici et al., 1996). Interestingly, the FLBG with the longer chain displayed a stronger GA activity, suggesting that the implementation of a long spacer facilitates the interaction between the active GA moiety and its receptor. Later on, this fluorescence-labeled GA was used tomonitor the potential cell-to-cell movement of GA and its role in releasing chilling-induced dormancy of *Betula pubescens* (Rinne et al., 2001). Very recently, two other bioactive and stable fluorescent GAs were generated (GA_3-Fl and GA_4-Fl; **Figure 4B**) and used to analyze the spatial distribution of GA in *Arabidopsis* roots (Shani et al., 2013). According to studies on the stability of the GA_3 conjugates, the fluorescein has been linked via an amide bond to the GA_3 molecule on the C6 position (Liebisch et al., 1988). The same strategy was also used for GA_4. These two labeled compounds are bioactive due to the existence of an intact GA molecule within their structures, retaining their interaction with the GA receptor. However, they are not suitable substrates for *in vivo* GA metabolism, making them ideal to study GA transport processes. After application, labeled GAs accumulate in the endodermis layer within the elongation zone of the root (Shani

et al., 2013). Pharmacological studies combined with the analysis of mutants defective in endodermal cell layer identity revealed that the GA accumulation is regulated by an active mechanism (Shani et al., 2013). Furthermore, by using fluorescent GAs, it was confirmed that GA distribution is regulated by ethylene, adding another dimension to GA function in plant development (Shani et al., 2013). This study elegantly demonstrates how fluorescently labeled GAs can help to dissect GA localization and real time transport *in planta*.

Recently, a bioactive fluorescently labeled BR analog named Alexa Fluor 647-castasterone (AFCS; **Figure 3**) has been produced to analyze BR signaling processes (Irani et al., 2012). The position of the fluorophore AF467 at the C6 of the B-ring of CS was chosen based on previously generated biotin-tagged photoaffinity CS and is in accordance with the ligand-binding pocket structure of the receptor BRI1 (Kinoshita et al., 2005; Hothorn et al., 2011; She et al., 2011). AFCS was validated as a bioactive BR, although its potency is lower than that of the native BR or CS. AFCS internalization has been shown to be mediated by BRI1, as its uptake is increased in plants overexpressing the BR receptor and reduced in the *bri1* mutant. This fluorescently tagged BR thereby enabled visualization of the ligand-receptor interaction via AFCS-BRI1. In addition, it revealed internalization of the BR-BRI1 complex by live imaging, which is dependent on clathrin-mediated endocytosis and ADP-ribosylation factor-guanine nucleotide exchange factors (ARF-GEFs) (Irani et al., 2012). This study validates the potential of fluorescently labeled compounds not only to dissect hormone transport, but also to visualize ligand-receptor interaction *per se*, as well as trafficking of the ligand-receptor complex.

Labeled molecules are valuable tools to identify direct targets of bioactive endogenous or synthetic compounds. In particular, the application of biotin-tagged compounds facilitates the isolation of compound targets such as receptors by affinity chromatography and could even lead to the determination of the molecule-binding site.

TAGGED MOLECULES

Reizelman et al. (2003) have produced a plethora of tagged SLs with radioactive, photoaffinity, biotin and fluorescent (dansyl) groups to isolate the SL receptor. Germination assays on *Striga hermonthica* seeds revealed that bioactivity of the labeled analogs is retained, demonstrating that the SL binding site tolerates a large substituent on the SL A-ring. Although a 60 kDa membrane-bound protein was isolated by the authors as a SL receptor in *Striga hermonthica* seeds (Zwanenburg et al., 2009; Zwanenburg and Pospíšil, 2013), direct evidence is not yet available and further experiments are required to confirm these results. Nevertheless, the synthesis and use of biotin-tagged photoaffinity CS (BPCS) has helped to demonstrate the direct binding between BRI1 and physiologically active BRs (Kinoshita et al., 2005). BPCS is a bioactive CS analog containing a carbene-generating phenyldiazirine moiety and a biotin tag, which allows its detection by an anti-biotin antibody. Under UV radiation, the phenyldiazirine moiety enables covalent liaison between BPCS and the binding region of the specific receptor. Binding analyses using BPCS, ^{3}H-labeled BL and recombinant BRI1 fragments were performed to characterize the minimum required region for BR perception. These data showed that the minimum region required is composed of 94 amino acids in the extracellular domain of BRI1 constituted by the island domain (70 amino acids located between the 21st and 22nd leucine-rich repeat [LRR] domain of BRI1) and LRR 22. However, structural analysis of the steroid complex demonstrates that the hormone-binding site is larger than this initial prediction (Hothorn et al., 2011).

Interestingly, not only phytohormone analogs but also compounds with antagonist activity such as Terfestatin A (TrfA; **Figure 2A**) can be used to isolate cognate receptors (Yamazoe et al., 2004, 2005). TrfA has been shown to disturb auxin signaling independently from the canonical auxin receptor TIR1 (Yamazoe et al., 2005). Therefore, it can be exploited to identify novel auxin receptors. Determination of the active core of TrfA by SAR analysis could provide the possibility to design a biotin-tagged active TrfA or a solid support-linked TrfA suitable for affinity chromatography of the direct target protein (Hayashi et al., 2008b). Nevertheless, no results using this tagged compound have yet been published.

CAGED MOLECULES

Recently, development of novel technologies based on the creation of caged compounds has created the possibility to control the distribution of active compounds in a temporally and spatially (at the intracellular level) defined way. Caged compounds display an inducible activity as a result of the photo-removable structure, which blocks their functional groups but is easily released by photolysis. This is very useful for modulating the intracellular level of a molecule within a single cell and for investigating the direct consequences of these changes at the cellular level. The design of the cage is a critical step, as the caged compound must be soluble, cell permeable and stable. Diverse bioactive elements have been caged and extensively used, such as messenger ribonucleic acid (mRNA), deoxyribonucleic acid (DNA), nucleotides, peptides, calcium, neurotransmitters and inositol (Ellis-Davies, 2007). Over the past few years, the synthesis of caged auxin, GA, ABA, JA, and SA have been described (Ward and Beale, 1995; Allan et al., 1998). However, detailed biological properties are not provided for all of them. The bioactivity of caged ABA has been successfully validated in stomata guard cells (Allan et al., 1994, 1998). More recently, novel caged auxin (Kusaka et al., 2009) and caged CK (Hayashi et al., 2012a) have been engineered and their bioactivity has been verified by bioassays using specific hormone-responsive marker *Arabidopsis* lines. The caged hormones could be used as a trigger to control hormonal distribution inside the cell, making them potential tools to detail the hormone's cellular response. These caged molecules could thereby help to gain a better comprehension of hormone function, adding new strategies to dissect hormone-mediated signaling.

Taken together, these studies demonstrate that labeled/tagged hormone analogs can be helpful toward a better understanding of hormone biology, in particular with respect to hormone signaling and transport mechanisms. Indeed, fluorescent analogs enable a direct visualization of the tempo-spatial distribution and/or intracellular trafficking of the ligand-receptor complex. However, some fluorescently labeled analogs require further structural

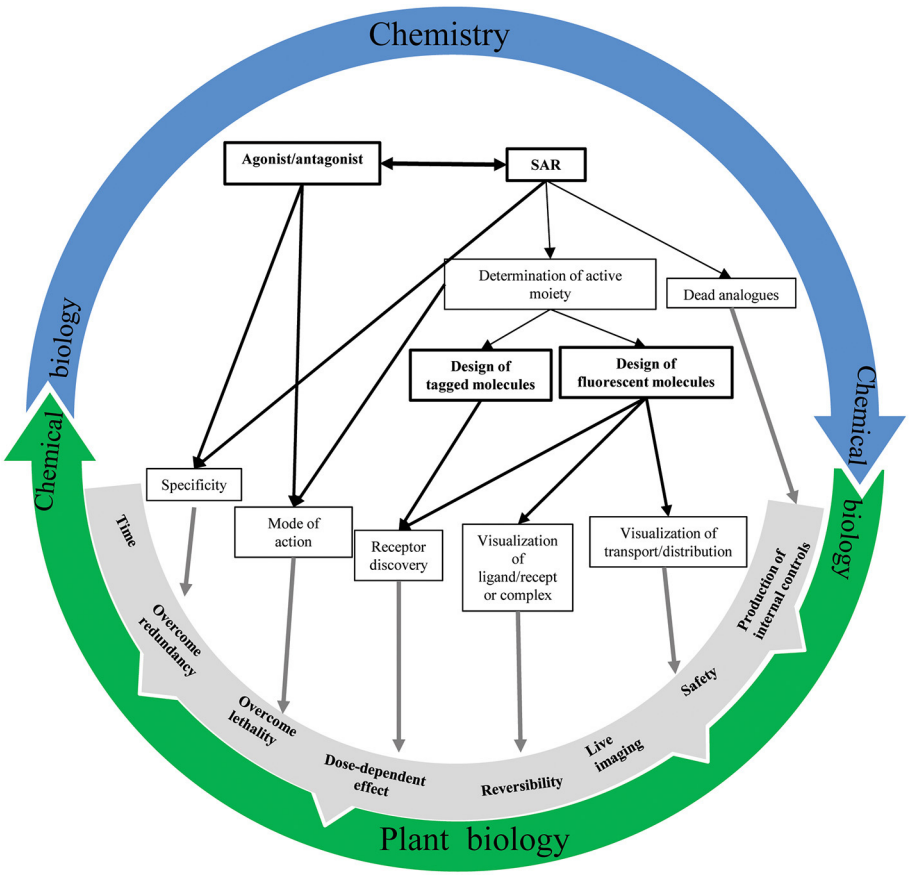

FIGURE 5 | Chemistry-plant biology relationship. An overview of the interconnection possibilities between chemistry and biology to better understand phytohormone signaling mechanisms.

modifications to achieve the spectrometric properties suitable for live imaging studies. The development of new dyes with enhanced characteristics should be explored to generate new conjugates with stronger signal and sensitivity. Modified growth regulators carrying a biotin tag would also be helpful for isolating the direct target protein by affinity chromatography and for determining the binding site of the known receptor. Furthermore, the use of these compounds overcomes several laboratory problems, such as the difficulty to obtain efficient antibodies against the receptor, the long time needed to produce transgenic lines with tagged receptors and the safety issues related to radio-labeled molecules.

CONCLUSIONS

Our understanding of plant hormone signaling has been advanced tremendously by the use of small molecules (**Figure 5**). Increased knowledge of phytohormone structure has provided essential information such as the hormone's chemical properties and its active moiety. Ultimately, these details combined with structural characterization of the target protein facilitate the rational design of new derivatives targeting one specific component of the signaling pathways. Additionally, the engineering of labeled analogs can enable the isolation of hormone receptors and the direct visualization/monitoring of the hormone's tempo-spatial distribution as well as the ligand-receptor complex localization. Remarkably, subtle changes in plant hormone structure count and promote the possibility to precisely dissect the hormone's signaling pathways and the discovery of new endogenous actors. However, it should be noted that structural changes of a molecule could affect tremendously its binding affinity to the receptor, its transport or diffusion rate as well as the way it is uptaken and modified by the metabolic machinery. Along with the expansion of metabolomic technologies and a full coverage of endogenous molecule space, chemical biology will become essential for a better understanding of the molecular mechanisms governing phytohormone regulation. Computerized modeling of potential receptor structure in association with *in silico* molecule docking analysis has opened the door for the systematical investigation of hormone-mediated signaling pathways in plants. In this way, a tight collaboration between chemistry and plant biology is vital toward enhancing our understanding of plant hormone signaling.

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Molecular locks and keys: the role of small molecules in phytohormone research

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Plant adaptation, growth and development rely on the integration of many environmental and endogenous signals that collectively determine the overall plant phenotypic plasticity. Plant signaling molecules, also known as phytohormones, are fundamental to this process. These molecules act at low concentrations and regulate multiple aspects of plant fitness and development via complex signaling networks. By its nature, phytohormone research lies at the interface between chemistry and biology. Classically, the scientific community has always used synthetic phytohormones and analogs to study hormone functions and responses. However, recent advances in synthetic and combinational chemistry, have allowed a new field, plant chemical biology, to emerge and this has provided a powerful tool with which to study phytohormone function. Plant chemical biology is helping to address some of the most enduring questions in phytohormone research such as: Are there still undiscovered plant hormones? How can we identify novel signaling molecules? How can plants activate specific hormone responses in a tissue-specific manner? How can we modulate hormone responses in one developmental context without inducing detrimental effects on other processes? The chemical genomics approaches rely on the identification of small molecules modulating different biological processes and have recently identified active forms of plant hormones and molecules regulating many aspects of hormone synthesis, transport and response. We envision that the field of chemical genomics will continue to provide novel molecules able to elucidate specific aspects of hormone-mediated mechanisms. In addition, compounds blocking specific responses could uncover how complex biological responses are regulated. As we gain information about such compounds we can design small alterations to the chemical structure to further alter specificity, enhance affinity or modulate the activity of these compounds.

Keywords: phytohormones, chemical genomics, hormone perception and signaling, hormone crosstalk, plant chemical biology, jasmonates, agonist and antagonist, small molecules

INTRODUCTION

FROM PHENOTYPES TO MOLECULES: EARLY CHEMICAL GENOMICS APPROACHES

Plant growth, development and adaptation to the environment require the integration of many environmental and endogenous signals that, together with the intrinsic genetic program, determine overall plant responses. In this context, signaling molecules and growth regulators, collectively known as phytohormones, act as central hubs for the integration of complex environmental and cellular signals. Phytohormones such as auxins, cytokinins (CK), gibberellins (GAs), abscisic acid (ABA), ethylene (ET), brassinosteroids (BRs) salicylic acid (SA), jasmonates (JAs), and strigolactones act at low concentrations and, either alone or in combination with other hormones, regulate multiple aspects of plant development, defense and adaptation. The search for both synthetic plant hormones and hormone mimics with increased stability/activity has been central to the development of the agrochemical industry and the "green revolution" in the past century (Brown et al., 2014). Initially, organic chemists used chemically synthesized hormonal derivatives to identify novel compounds mimicking or reversing the phenotypes induced by endogenous phytohormones. For example, the discovery of the structure of the naturally occurring auxin phytohormone indole-3-acetic acid (IAA) allowed chemical synthesis of a wide array of analogs and derivatives, and phenotypic screens. These approaches identified molecules such as 1-naphthaleneacetic acid (1-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2-methyl-4-chlorophenoxyacetic acid (MCPA) that are still widely used today as growth promoters or herbicides (Overbeek and Vélez, 1946; Grossmann, 2010) (Supplemental Table 1). Similarly, phenotypic screens of functional analogs of the endogenous salicylic acid signals identified compounds such as benzothiadiazole (BTH) and 2,6-dichloroisonicotinic acid (INA) that were employed in the field to enhance plant disease resistance (Conrath et al., 1995; Görlach et al., 1996; Lawton et al., 1996) (**Table 1** and Supplemental Table 1).

Table 1 | List of molecules described in this review including molecular targets, biological activity and references.

Common name	Target	Biological activity	References
AUXIN			
Gravacin	PGP19	Strong inhibitor of root and shoot gravitropism	Rojas-Pierce et al., 2007
L-kynurenin	TAA1/TARs	Inhibitor of auxin synthesis and of ethylene responses	He et al., 2011
BUM	ABCB/MBR/PGP efflux carriers	Selective inhibitor of ABCB efflux carriers. Allows discrimination with PIN	Kim et al., 2010
Alcoxy-auxins	Auxin transporters PIN, ABCB and AUX	Selective inhibitors of auxin transport. Not recognized by the receptors	Tsuda et al., 2011
α-Alkyl auxins	TIR1	Rationally designed auxin agonists and antagonists	Hayashi et al., 2008
Auxinole	TIR1/AFBs	Rationally designed auxin antagonist	Hayashi et al., 2012
Picloram	AFB5	Picolinate auxin. Agonist of auxin signaling	Walsh and Chang, 2006; Calderón Villalobos et al., 2012
IAA-Trp, JA-Trp	Unknown	Innhibitors of several auxin mediated responses	Staswick, 2009
GIBBERELIN			
GA3—Fluorescein	GID1 receptor	Florescent GA mimetics recognized by the receptor	Shani et al., 2013
CYTOKININ			
Phe-Ade	CKX and AHK3 and CRE1/AHK4 receptors	Week binding to cytokinin AHK3 and AHK4 receptors and inhibition of Cytokinin Oxidase/dehydrogenase (CKX) on cytokinin degradation	Motte et al., 2013
S-4893	CRE1 receptor	Non-competitive cytokinin antagonist by targeting CRE1 receptor	Arata et al., 2010
SS-6772 and S-4607	CRE1 receptor	CRE1 antagonists	Arata et al., 2010
ABA			
Pyrabactin	PYR1 and PYL1	Affects seed germination by interacting with a sub-set of PYR/PYL/RCAR ABA receptors	Park et al., 2009; Okamoto et al., 2013
Quinabactin	PYR1, PYL1-3,4	Stomatal closure. Interacts with a sub-set of ABA receptors	Okamoto et al., 2013
ASn	PYR/PYL	ABA antagonists. Block the interaction PYR/PYL –PP2C	Takeuchi et al., 2014
JASMONIC ACID			
Coronatine	COI1/JAZs	Produced by Pseudomonas syringae, is a potent agonist of JA. Binds the receptor complex	Xie et al., 1998; Katsir et al., 2008; Fonseca et al., 2009b
Vernolic acid	AOC2	Inhibits AOC2 and limits OPDA production by 50%. Affects JA synthesis	Hofmann et al., 2006
Phenidone	LOX	Animal LOX inhibition. Little effect on JA biosynthesis	Engelberth, 2011
PACOR, PAJAIle	COI1/JAZ1	Biotin-tagged photoaffinity labeled molecules that promote COI1/JAZ interaction	Yan et al., 2009a
JM-8686	AOS	Strong inhibitor of AOS activity	Oh et al., 2006
Jarin-1	JAR1	Inhibits the last step of JA-Ile biosynthesis	Meesters et al., 2104
(+)-7-*iso*-JA-L-Ile	COI1/JAZs	Endogenous jasmonate recognized by the receptor	Fonseca et al., 2009b; Sheard et al., 2010
(+)-JA-L-Ile	COI1/JAZs	Synthetic agonist of the endogenous (+)-7-iso-JA-Ile	Fonseca et al., 2009b
COR-MO	COI1/JAZs	Coronatine rational designed antagonist. Blocks JA and COR perception	Monte et al., 2014
Fluorescent jasmonate	Unknown	Migrates in tomato	Liu et al., 2012; Liu and Sang, 2013
Bestatin	Unknown	Inhibitor of aminopeptidases. Mutants insensitive to bestatin render alleles of *myc2* and *med25*	Schaller et al., 1995; Zheng et al., 2006; Chen et al., 2012
BRASSINOSTEROID			
Brassinazole	Cytochromes P450 DWF4 and CPD	Inhibits BR biosynthesis	Asami et al., 2000, 2001
Fluorescent castasterone	BRI1	Bioactive fluorescent labeled BR, recognized by the receptor BRI1	Irani et al., 2012
Bikinin	GSK3-like kinases, BIN2 included	Induces constitutive BR-related phenotypes by inhibiting GSK3 kinases	De Rybel et al., 2009
Brassinopride	Unknown	Inhibitor of BR action. Acts on BR synthesis and activates ethylene responses	Gendron et al., 2008

(Continued)

Table 1 | Continued

Common name	Target	Biological activity	References
STRIGOLACTONES			
GR24	MAX2/DAD2/D14	A potent synthetic strigolactone analog	Gomez-Roldan et al., 2008; Umehara et al., 2008
Karrikin - KAR2	MAX2/KAI2	Generated in the smoke, structurally similar to strigolactones. Inducers of germination	Nelson et al., 2011; Hamiaux et al., 2012; Waters et al., 2012; Guo et al., 2013
Cotylimides	Unknown	Strigolactones agonist in germination, hypocothyl development and cotyledon bleeching. Revealed a crosstalk between strigolactones and light	Tsuchiya et al., 2010
SALICYLIC ACID			
BTH (benzothiadiazole)	Unknown	Inducer of SA-mediated defense responses, enhancing plant disease resistance in the field	Görlach et al., 1996; Lawton et al., 1996
INA	Unknown	Inducer of SA-mediated defense responses, enhancing plant disease resistance in the field	Conrath et al., 1995
Imprimatins	Two SA glucosyltransferases (SGT)	Activator of endogenous SA accumulation by blocking SA turnover. Enhancers of pathogen activated cell death	Noutoshi et al., 2012

A complementary chemical approach for the identification of bioactive molecules mimicking the activity of endogenous hormones can be based on the analysis of plant-interacting organisms. This approach revolves around organisms that have evolved the capability to produce phytohormones or phytohormone mimics to enhance disease susceptibility and counteract plant defenses. For example, characterization of the fungal pathogen *Gibberella fujikuroi* [responsible for the *bakanae* disease in rice (Kurosawa, 1926)] allowed the identification of gibberellic acid derived phytohormones (Shimada et al., 2008; Robert-Seilaniantz et al., 2011), and analysis of the bacterium *Pseudomonas syringae pv. Tomato* was instrumental for the identification of the phytotoxin coronatine (COR) (Feys et al., 1994). This is a jasmonate functional analog that the bacteria use to hijack the plant defense signaling network (Kloek et al., 2001; Brooks et al., 2004; Gimenez-Ibanez and Solano, 2013; Xin and He, 2013) (**Table 1** and Supplemental Table 1).

Despite the profound contribution of those early chemical approaches in phytohormone research, these methodologies had two important limitations. Firstly, the success of these approaches relies on the serendipity of identifying a structurally amenable product from a relatively small number of natural sources. Secondly, the large collections of hormonal derivatives frequently lack chirality and their structural diversity is limited to variations in attachments within a restricted number of common skeletons (Brown et al., 2014). Therefore, these approaches only cover a small fraction of the structural possibilities present within the chemical space, and therefore reduce their potential versatility.

FROM MOLECULES TO FUNCTION: PLANT CHEMICAL BIOLOGY IN THE GENETIC ERA

Recent decades have seen the development of a whole host of molecular and genetic tools as well as the release of complete genome sequences. Therefore, genetic strategies such as the isolation of mutations that confer altered hormonal responses and the identification of the downstream target genes have substituted the early chemical approaches and quickly became the preferred methods to elucidate the molecular mechanisms underlying phytohormone action. These genetic approaches have significantly enhanced our understanding of the molecular basis of phytohormone action (for review see Browse, 2009). In spite of its success, the use of well-established genetic tools (such as large collections of knockout and activation tagged mutants) for the identification of components in plant hormonal networks has now reached such a stage that it is becoming increasingly challenging to identify the remaining components. This recalcitrant to genetic approaches is largely due to a combination of gene redundancy, where multiple genes regulate the same process and individual knockouts have no discernable phenotype, and gene lethality, which prevents the identification of loss-of-function mutations in essential genes (Robert et al., 2009; Fernández-Calvo et al., 2011; Acosta et al., 2013).

Fortunately, the development of genetic tools has gone hand in hand with advances in combinatorial synthesis. These advances have enabled access to highly diverse chemical libraries containing both wider spectra of molecular shapes and range of biological targets than traditional combinatorial libraries (Schreiber, 2000; Hicks and Raikhel, 2012). These chemical libraries are being used to overcome many of the limitations of purely genetic approaches. They can be used to address genetic redundancy, as small molecules are capable of modulating the active sites of whole classes of protein targets. They can also address gene lethality, as small molecules can enable the temporal and spatial blockage of specific hormonal responses in a reversible manner (McCourt and Desveaux, 2010; Tóth and van der Hoorn, 2010; Hicks and Raikhel, 2012). Hence, in the last two decades agrochemical biased libraries have been widely used in combined genetic and chemical screens aimed at the dissection of multiple physiological processes in plants. These screens have yielded valuable bioactive compounds such as gravacin (Rojas-Pierce et al., 2007), morlin (DeBolt et al., 2007), sortins (Zouhar et al., 2004; Rosado et al., 2011), hypostatin (Zhao et al.,

2007), and endosidins (Robert et al., 2008; Drakakaki et al., 2011) (**Figure 1** and **Table 1**). All these compounds are currently used to modify the activity of individual proteins or protein families in a tuneable, reversible and spatial-temporal controlled manner.

We now know that in some cases the mechanisms for perceiving individual hormones are conserved, and the same recognition systems are able to mediate response to several hormones, while in other cases unique perception strategies have evolved for individual molecules (Tan et al., 2007; Murase et al., 2008; Shimada et al., 2008; Park et al., 2009; Lumba et al., 2010; Sheard et al., 2010; Kumari and van der Hoorn, 2011). A paradigmatic example of a conserved recognition system is the "molecular glue" mechanism, first described for the auxin receptor complex, in which the auxin molecule promotes the formation of its receptor complex (Tan et al., 2007; Mockaitis and Estelle, 2008). The F-box TIR1 (TRANSPORT INHIBITOR RESPONSE 1) or AFBs (AUXIN SIGNALING F-BOX) cannot bind, or bind at very low affinity, auxin without the interaction of the co-receptors Aux/IAA (AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE) and the inositol hexakisphosphate (IP6) cofactor. Only the structural modifications produced by the formation of the tetrameter stabilize the hormone perception. The same mechanism also occurs in jasmonate perception, since the hormone induces the formation of the receptor tetramer complex formed by JA-Ile, the F-box COI1 (CORONATINE INSENSITIVE1), the co-receptor JAZ (JASMONATE ZIM DOMAIN PROTEIN) and the inositol pentakisphosphate (IP5) cofactor (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). Gibberellins are also sensed by a similar perception system: active GAs promote the establishment of the complex formed by GID1 (GIBBERELLIN INSENSITIVE DWARF1) receptor and the F-box SLY1 (SLEEPY1) (Murase et al., 2008; Shimada et al., 2008). In contrast, other phytohormones are perceived by specific protein complex based on different

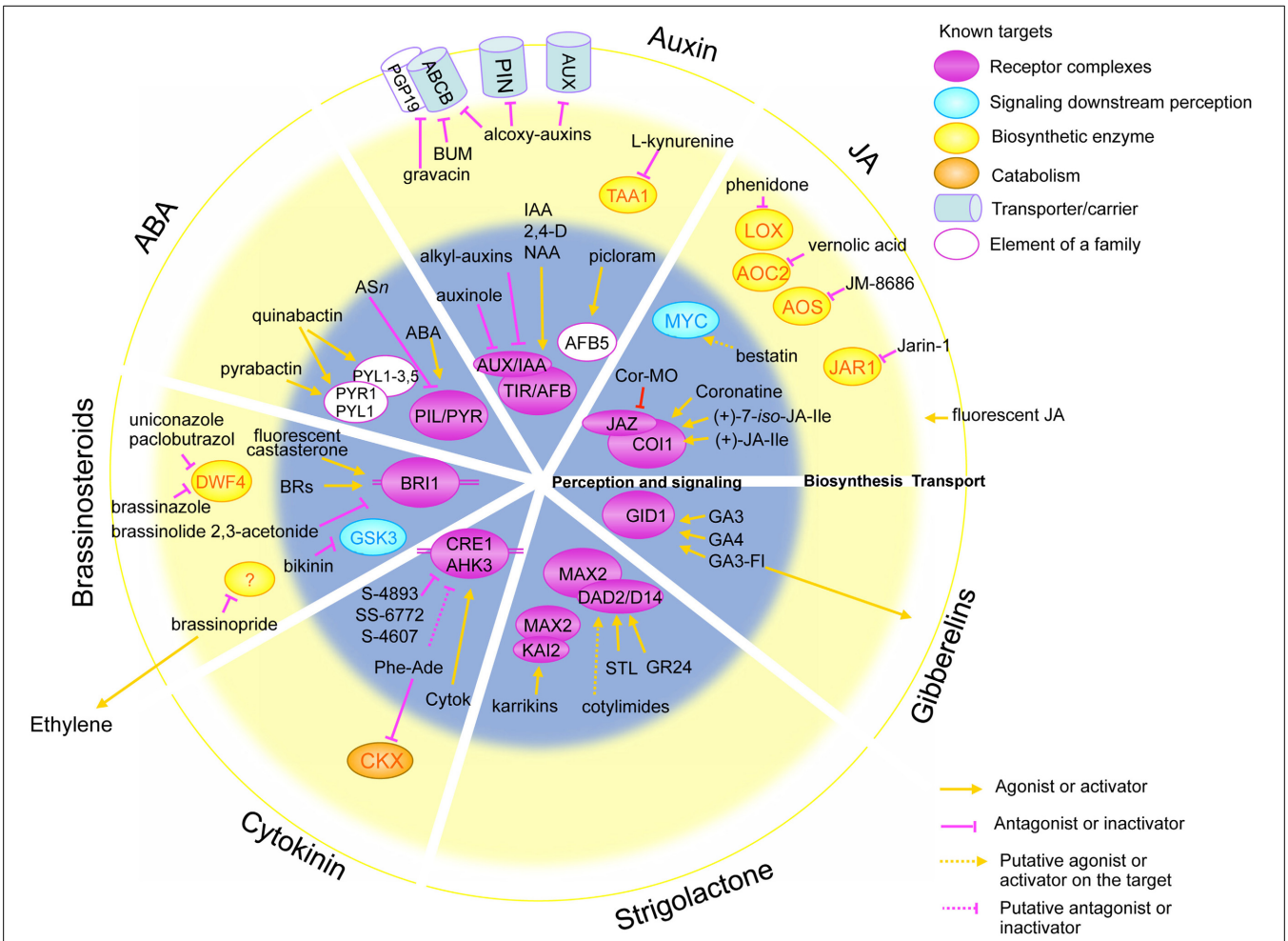

FIGURE 1 | Schematic representation of the molecular targets of small molecules acting in different hormonal pathways. Concentric circles in the background represent the distinct biological processes in hormonal pathways: perception and signaling (gray inner circle), biosynthesis (yellow middle circle) and transport (white outer circle). Circles are divided in quadrants for distinct hormones, from the top clockwise: auxin, jasmonic acid (JA), gibberellins, strigolactones, cytokinins, brassinosteroids and abscisic acid (ABA). Ovals represent the molecular targets: receptor complexes (violet), signaling components (blue), biosynthetic enzyme (yellow) and catabolic enzymes (orange). Cylindrical shapes represent transporters and carriers. Molecules acting as activators are represented with an orange arrow toward their targets, whereas pink blocked arrows highlight antagonists and inhibitor molecules.

recognition systems. For example, the PYR1 (PYRABACTIN RESISTANT 1) and PYL (PYRABACTIN RESISTANT-LIKE) receptors bind ABA directly in cooperation with the co-receptors type 2C protein phosphatases, such as ABI1 (ABA INSENSITIVE 1) and ABI2 (ABA INSENSITIVE 2). The subsequent inactivation of the phosphatases induces the SNF1-type kinase activity, which in turn regulates ABA-dependent gene expression and downstream signaling cascades (Weiner et al., 2010). CK perception and signal transduction pathway occur through a phosphorelay similar to bacterial two-component response systems. Briefly, CK binds directly to the membrane-located HISTIDINE KINASE (AHK) receptors. This initiates a phosphorelay cascade where a phosphoryl group is translocated via the HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEINS (AHPs) and then to the RESPONSE REGULATOR (ARRs) transcription factors. Type-B ARRs regulate the transcription of cytokinin responsive genes and type-A ARRs acting as negative feedback regulators to desensitize plants to excess cytokinin (Kieber and Schaller, 2014).

The discovery of each of these hormonal response mechanisms has enabled the implementation of innovative chemical genomics approaches, and the rational design of chemical tools for phytohormone studies. These will be described in details within this review.

FROM FUNCTION TO TARGETS: SCREENING FOR NOVEL PROTEINS/COMPLEX USING SCREENS AND TAGGED-MOLECULES

Bioactive chemicals identified from forward or reverse chemical screens are very useful for the dissection of complex biological processes. One advantage of this technique is that it can either target specific proteins or multiple members of redundant gene families. However, the identification of the cognate biochemical target/s remains a very complex process that depends on the type and affinity of the chemical-target interaction, as well as the abundance of the target sites (Robert et al., 2009). Throughout the years, researchers have performed diverse genetic screens in *Arabidopsis thaliana* for resistance to specific chemicals. These have allowed the subsequent biochemical and genetic identification of cognate targets. For example, chemical genetic screens for resistance to bikinin (an activator of the brassinosteroids responses) showed that it could bind directly and inhibit a subset of the GSK3 (GLYCOGEN SYNTHASE KINASE 3) kinase family (De Rybel et al., 2009) (**Table 1** and Supplemental Table 1). Similarly, a screen for resistance to gravacin (a strong inhibitor of root and shoot gravitropism) identified the auxin efflux transporter PGP19 (P-GLYCOPROTEIN 19) as its molecular target (Rojas-Pierce et al., 2007) (**Table 1** and Supplemental Table 1).

These genetic-based approaches require further validation of the target since mutations can prevent the drug from reaching the site of action due to either metabolic alterations or uptake/translocation defects. As an alternative, different biochemical tools have been developed for target identification (Kolb and Sharpless, 2003). These include collections of "tagged" chemical libraries that possess reactive moieties permitting the immobilization of active compounds through "click chemistry." Although there are several potential click reactions, the Copper (I) catalyzed synthesis of triazoles from azides and acetylenes has become the standard for the generation of "click libraries" and the chemical species in those libraries possess an amine handle that enables affinity resin synthesis via reaction with activated carboxylic acid affinity resins (Kolb et al., 2001). For example, a library of tagged molecules was used in a high throughput approach to detect active proteins in the whole proteome of *Arabidopsis thaliana* (Van der Hoorn et al., 2011). Additionally these compounds can also contain a fluorophore to enable visualization of hits in living cells or other contexts.

In the following sections of this review we will describe recent landmark chemical genomics approaches and place special emphasis on their roles in the elucidation of the molecular mechanisms underlying hormonal regulation, considering all stages from the biosynthesis to the perception of the signal.

PHYTOHORMONE HOMEOSTASIS

Different endogenous and environmental stimuli regulate the tissue-specific biosynthesis of phytohormones. The synthesis and catabolism of these molecules are tightly regulated as they are very bio-active. For example at least three, partially redundant, biosynthetic pathways have been identified so far for synthesizing auxin (Stepanova et al., 2008; Zhao, 2008). The complete biosynthetic network is not yet fully understood. However, the use of auxin analogs played an important role in identifying many of the mutants impaired in auxin biosynthesis. For example, the *tir2* (*transport inhibitor response 2*) mutant was isolated as an NPA (1-N-naphthylphthalamic acid)-resistant mutant and subsequently shown to encode TAA1 (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1), one of the key enzymes in the indole-3-pyruvic acid (IPA) auxin biosynthetic pathway (Yamada et al., 2009). However, mutants with modestly perturbed levels in auxin show strong pleiotropic effects and this restrict their usefulness in investigating specific aspects of auxin action. In addition, the enzymes that mediate key biosynthetic steps are often redundant and require the generation of higher order combinations of mutants to detect observable phenotypes. Therefore, the identification of compounds enabling the manipulation or blockage of specific biosynthetic pathways is invaluable. An example of such compound is the recently isolated inhibitor of the auxin biosynthesis L-kynurenine (He et al., 2011) (**Figure 1**, **Table 1** and Supplemental Table 1).

L-kynurenine (Kyn) was originally identified as an inhibitor of the ethylene-induced auxin biosynthesis in roots (He et al., 2011) (**Figure 1**). Subsequently, He and colleagues demonstrated that Kyn is an alternate substrate for auxin biosynthetic enzymes TAA1/TAR (TRYPTOPHAN AMINOTRANSFERASE RELATED) and that it competitively inhibits TAA1/TAR activity (Stepanova et al., 2008). Strikingly, Kyn binds to the substrate pocket of TAA1/TAR proteins in a highly effective and selective manner, but does not bind to other aminotransferases. The use of Kyn has overcome some genetic limitations of traditional approaches, such as the sterility and lethality of double and triple mutants in the redundant *TAA1/TAR* gene family, and has enabled the blockage of auxin biosynthesis in specific tissues or plant stages (Stepanova et al., 2008). Kyn has added value to classic genetic studies. For example, by combining Kyn

treatments with mutants impaired in auxin biosynthesis, it was recently shown that root-based auxin biosynthesis is required in addition to polar auxin transport to correctly pattern the root xylem axis (Ursache et al., 2014). Most enzymes within the auxin biosynthetic network are well conserved between plant species, including mosses and lichens (Finet and Jaillais, 2012). Consequently, molecules such as kynurenine that inhibit auxin biosynthesis could easily be used on other species, providing a wide range of possible applications.

Many molecules regulate the complex signaling networks responsible for plant defense, however salicylic acid plays a central role in restricting the activity of biotrophic pathogens. Genetic screens for mutants with enhanced disease resistance have mainly uncovered dwarf mutants with elevated SA levels (Murray et al., 2002; Shirano et al., 2002; Grant et al., 2003; Zhang et al., 2003; Vlot et al., 2009). To avoid the pleiotropic effects of plants with altered SA levels, researchers have long sought-after compounds enabling the manipulation of SA in a tuneable and reversible manner. Recently, a high-throughput chemical genomic screen identified the imprimatin family of molecules as enhancers of pathogen-activated cell death (Noutoshi et al., 2012). Imprimatin treatments induce the accumulation of SA, reduce its inactive metabolite SA-glucoside, and enhance plant disease resistance (**Table 1** and Supplemental Table 1). Further analyses have shown that imprimatins block SA turnover through specific inhibition of two SA GLUCOSYLTRANSFERASES (SAGTs). Double knockout mutants of these SAGTs are semi-dwarf plants that consistently showed the same SA-accumulation and enhanced disease resistance as imprimatin-treated plants (Noutoshi et al., 2012). Imprimatins offer an exciting way in which synthetic compounds that can be applied to different plant species to trigger accumulation of the active endogenous SA and overcome the pleiotropic effects associated with constitutive high levels of SA. Besides the biotechnological applications, these molecules can also be used in phytohormone research to induce the accumulation of endogenous SA transiently at specific plant developmental stages, avoiding the need to use of semi-dwarf mutant lines in the redundant *SAG* genes.

Cytokinins have been long known to regulate cell division, differentiation as well as many aspects of plant development—including root growth, root/shoot branching architecture and vascular development (Werner and Schmülling, 2009; Hwang et al., 2012). Cytokinins are adenine derivatives, and the incorporation of specific side chains at the N6-position triggers their recognition as ligands for specific receptors or substrates for enzymes regulating their homeostasis. One key group of enzymes catalyzing the oxidative removal of the side chain and thereby degrading cytokinins are the CYTOKININ OXIDASE/DEHYDROGENASE (CKX) family. In *Arabidopsis* there are 7 members of the CKX family, and each has subtly different substrate specificity (Kowalska et al., 2010). A recent high-throughput chemical screen based on *in-vitro* cytokinin-dependent shoot regeneration (Motte et al., 2013) identified one novel compound, Phe-Ade (N-phenyl-9H-purin-6-amine) (**Figure 1**, **Table 1** and Supplemental Table 1). Further biochemical studies showed that Phe-Ade induces the accumulation of endogenous cytokinin by acting as a competitive inhibitor of the cytokinin-degrading CKX enzymes and preventing cytokinin catabolism.

Brassinosteroid biosynthesis is regulated by a complex network of three redundant pathways that convert the common precursor campesterol into the active BRs. The BR biosynthetic pathways requires the activity of the cytochrome P450 DWARF 4 (DWF4), a key rate limiting P450 monooxygenase that acts on multiple intermediates in the BR biosynthesis pathways (Asami et al., 2000, 2001; Chung and Choe, 2013) and represents an ideal target to bypass the redundancy of the BR biosynthesis pathways. Uniconazole and paclobutrazol are both inhibitors of P450 monooxygenases that act as weak BR inhibitors and are able to induce accumulation of the precursor campesterol (Asami and Yoshida, 1999) (**Figure 1**). Subsequent analysis into the structure-activity relationship identified brassinazole (BRZ) as strong inhibitor of BR biosynthesis blocking the cytochrome P450 monooxygenase DWF4 and therefore preventing the hydroxylation of BR precursors (Asami and Yoshida, 1999; Asami et al., 2000) (**Table 1** and Supplemental Table 1). BRZ was subsequently used for a genetic screen to isolate BRZ insensitive mutants. *bzr1-1D* (*brassinazole resistant 1*) and *bes1* (*bri1-ems-suppressor 1*) mutants respectively showed insensitivity to BRZ and enhanced constitutive BR responses. The phenotypes of these mutants were later shown to be caused by the stabilization of the transcription factors BZR1 (BRASSINAZOLE RESISTANT 1) and BES1/BZR2 (BRI1-EMS-SUPPRESSOR 1/BRASSINAZOLE RESISTANT 1) (Wang et al., 2002; Yin et al., 2005a). BZR1 and BES1/BZR2 are the fundamental activators of the BR signaling pathway, which regulate the expression of most BR responsive genes (Vert and Chory, 2006). The use of BZR is exemplary of the potential of integrating chemical genomics with classical genetics to identify key regulators of a hormone signaling pathway.

Jasmonic acid-isoleucine (JA-Ile) is an end product of the oxylipin biosynthetic pathway and, together with additional oxylipin molecules, it mediates several developmental processes and stress responses (Fonseca et al., 2009a; Wasternack and Hause, 2013). The oxylipin biosynthetic pathway is well understood and several inhibitors of key steps in this pathway have been reported (Wasternack and Hause, 2013). The JA-Ile biosynthesis is believed to start with the conversion of free α-linolenic acid by 13-lipoxygenases. Therefore, several general inhibitors of animal lipoxygenases (such as phenidone, aspirin, ibuprofen and ursolic acid) were tested in plants; however, they show only with limited inhibitory effects on oxylipin biosynthesis in plants, possibly due to functional redundancy or differences between animal and plant lipoxygenases (Wasternack, 1993; Farmer, 1995; Engelberth, 2011).

The subsequent biosynthetic step is catalyzed by the ALLENE OXYDE SYNTHASE (AOS) and ALLENE OXYDE CYCLASE 2 (AOC2), that mediate a non-redundant, coupled reaction producing the first cyclic oxylipin 12-oxo-phytodienoic acid (OPDA). The complete loss of cyclic oxylipins in *aos1* mutant generates sterile plants and confirmed the essential role of AOS (Park et al., 2002; Wasternack and Hause, 2013). Therefore, AOS and AOC2 represent ideal targets to inhibit the whole cyclic oxylipin pathway. Vernolic acid is a naturally occurring oxylipin first described as competitive inhibitor of the AOC of maize by Hamberg and

Fahlstadius (1990). More recently, the crystal structure of AOC2 determined the direct binding of the competitive JA inhibitor vernolic acid within the AOC2 hydrophobic barrel cavity (Hofmann et al., 2006) (**Table 1** and Supplemental Table 1). Biochemical assays also demonstrated that vernolic acid inhibited approximately 50% of the AOC2-mediated production of OPDA *in vitro*. In addition, the imidazole derivative JM-8686 was designed to inhibit the activity of AOS, most likely by direct binding of the imidazole group of JM-8686 to the heme iron of AOS (Oh et al., 2006). However, the subsequent use of vernolic acid and JM-8686 was very limited because the residual activity of AOS/AOC2 coupled reaction can produce enough cyclic oxylipin to mediate most plant responses.

The final step of the biosynthetic pathway is performed by JASMONOYL-L-ISOLEUCINE SYNTHETASE (JAR1), that synthesizes the bioactive hormone (+)-*7-iso*-JA-Ile by conjugating JA with the amino acid isoleucine (Staswick and Tiryaki, 2004; Fonseca et al., 2009a). Very recently, Meesters et al. reported jarin-1 as the first small molecule inhibitor of jasmonate responses identified in a chemical screen (Meesters et al., 2104). Jarin-1 inhibits many JA-mediated responses *in planta*, but did not affect reactions induced by JA-Ile, suggesting an inhibitory activity on the JA-Ile producing enzyme JAR1. Further biochemical data confirmed that jarin-1 impairs the JA-Ile synthesis and inhibits the activity of JAR1, whereas closely related enzymes are not affected. Molecular modeling suggests a direct jarin-1 binding to the active site of JAR1. Overall, jarin-1 is the first direct, specific inhibitor of JAR1 (Meesters et al., 2104) (**Figure 1**, **Table 1** and Supplemental Table 1).

PHYTOHORMONE TRANSPORT

In plants, most hormones are mobile molecules whose inter or intra-cellular transport is required for function and control of physiological responses. With the textbook exception of auxin polar transport, the molecular mechanisms and components of hormone transport are still relatively unknown. In the case of auxins, genetics and chemistry both played essential roles in identifying and characterizing the three families of auxin transporters, AUX1/LAX (AUXIN RESISTANT 1/LIKE AUXIN RESISTANT), ABCB/MDR/PGP (ATP-BINDING CASSETTE subfamily B/ MULTIDRUG RESISTANCE/ P-GLYCOPROTEIN) and PINs (PIN-FORMED). For example, the *aux1* mutant was isolated through exploring the permeability differences between the membrane-permeable auxin 1-NAA and the membrane-impermeable auxin analog 2,4-D (**Figure 1**, **Table 1** and Supplemental Table 1). The *aux1* mutant was discovered through its agravitropic phenotype that could only be rescued by 1-NAA (Bennett et al., 1996). AUX1 was subsequently characterized as the first IAA influx carrier (Marchant et al., 1999; Swarup et al., 2001; Yang et al., 2006). Some members of the proteins ABCB/MDR/PGP transporters have been identified as proteins with binding affinity to the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) (Noh et al., 2001; Robert and Friml, 2009; Ma and Robert, 2014) (**Figure 1**). The initial identification of the PIN family of auxin efflux carriers occurred through the genetic isolation of the *pin1* mutant, which shows a phenotype resembling that caused by the pharmacological inhibition of polar auxin transport (Okada et al., 1991; Gälweiler, 1998).

Recently, a chemical genomic screen based on phenotyping a suite of morphological traits such as growth rate and flowering time identified a novel and potent inhibitor of ABCB efflux carriers, BUM (2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid). BUM directly binds and inhibits ABCBs, although ABCB1 appears to be the primary target. This binding occurs without directly affecting PIN transporters, and therefore allows the specific analysis discriminating between PIN and ABCB efflux systems (Kim et al., 2010).

Auxin perception allows regulation of the intracellular accumulation of endogenous auxins by modifying the localization of several transporters. As a consequence, it is often difficult to uncouple auxin perception from auxin transport. To overcome this limitation, rationally designed molecules such as alkoxy-IAA derivates (alkoxy-auxins) were developed that specifically target auxin transporters (Tsuda et al., 2011) (**Figure 1**, **Table 1** and Supplemental Table 1). Structural modeling testing the docking of alkoxy-auxins to the TIR1-Aux/IAA receptor suggested that these molecules could not fit into the auxin-binding pocket of the TIR1. It has been shown experimentally that these molecules fail to interfere with auxin perception, Aux/IAA degradation, and the downstream auxin signaling pathway (Tsuda et al., 2011). In contrast, alkoxy-auxins block the auxin transport activity of the PIN, ABCB, and AUX1 transporters in both yeast assays and *in planta*. Therefore, alkoxy-auxins are meant to become important tools to uncouple perception and transport in complex auxin mediated processes (Tsuda et al., 2011; Ma and Robert, 2014).

Long distance transport has also been reported for several hormones, but the molecular mechanisms are just emerging. ABA, cytokinin, strigolactones and jasmonates were detected in phloem or xylem, suggesting that these molecules could either be actively extruded from the cell or simply cross membranes by diffusion into the vascular tissue (Thorpe et al., 2007; Kudo et al., 2010; Kohlen et al., 2011). As in the case of auxins, small molecules provide useful tools to analyse the transport of other hormones. For example, specific ABC transporters inhibitors such as glibenclamide, verapamil and vanadate have been used to confirm role of the proteins AtABCG25 and AtABCG40 as ABA transporters (Kuromori et al., 2010; Kang et al., 2010).

New evidence exists that gibberellins too are actively transported; Shani and colleagues (2013) synthesized fluorescein labeled GA molecules (GA4- and GA3-fluorescein) that could be visualized in root cells and preferentially accumulate in the endodermal cells (**Figure 1**, **Table 1** and Supplemental Table 1). By using mitochondrial ATP synthesis inhibitors such as antimycin A, oligomycin A and myxothiazol, the researchers demonstrated the specific GA accumulation in the endodermis relies on active, energy dependent mechanisms, suggesting an active GA transport (Shani et al., 2013).

The idea of cytokinin-specific transporters is still an open question (Bishopp et al., 2011a). Podlešáková et al. (2012) generated a series of novel analogs of cytokinin and observed that some of these compounds had different transport affinities, hinting at the possibility of identifying immobile CK analogs. The structure-activity analysis of these immobile CK as well as the

identification of their targets might help to define components of the CK transport system.

Wounding triggers systemic responses that depend on the *de novo* synthesis of JA and JA-Ile in distal leaves in *Arabidopsis* (Koo et al., 2009; Wasternack and Hause, 2013), whereas grafting experiments with mutants excluded systemic formation of JA in tomato (Li et al., 2002; Koo and Howe, 2009). In principle this advocates against the transport of JA or JA-Ile. However, using radioactively labeled molecules, Me-JA, JA and JA-Ile were all found in phloem and/or xylem (Baldwin and Zhang, 1997; Thorpe et al., 2007; Matsuura et al., 2012). In addition, a functional fluorescent-labeled jasmonate probe was reported to migrate in the vascular tissues of tomato plants (Liu et al., 2012; Liu and Sang, 2013). We envision that the development of fluorescent-labeled hormones combined with the use of chemicals inhibiting different transport mechanisms will be essential tools with which to address the transport of jasmonates (Rigal et al., 2014). Many hypotheses have been proposed to explain the nature of systemic wound signals, being electric signals a possibility, and recently glutamate receptor-like genes (GLR), similar to those involved in synaptic activity in animals, have been implicated (Mousavi et al., 2013). In addition, three GLR antagonists were identified through a pharmacological screen for molecules inhibiting the growth of tobacco pollen tubes. Furthermore, the analysis of the GLR agonistic amino acids showed that D-serine is the most active agonist promoting pollen tube growth. D-serine is secreted naturally by the pistil to mediate pollen tube guidance (Michard et al., 2011). As D-serine is a modulator of animal neuronal circuits, this finding shows an astonishing analogy between electrochemical signal transduction in plants and animals.

PHYTOHORMONE PERCEPTION

Phytohormones are active at very low concentrations due to their high-affinity recognition systems. Since perception is the first step for the activation of downstream signaling cascades, researchers have prioritized the identification hormone receptors and perception components. Although many components of the hormonal perception system were identified by classical genetic approaches, the use of phytohormone analogs and chemical genomics was important for the detailed dissection of the underlying molecular mechanisms through which they function. For example, NPA was instrumental in identifying several components of the auxin pathway. These include *TIR1*, the founder member of the auxin receptor family TIR1/AFB proteins (Ruegger et al., 1997; Mockaitis and Estelle, 2008).

Coronatine, the bacterial mimic of JA-Ile, was instrumental in the identification of the *coi1* (*coronatine insensitive 1*) mutant. It was subsequently discovered that *coi1* was impaired in the F-box component of the JA-Ile receptor (Xie et al., 1998; Sheard et al., 2010). In addition, a small-scale screen of oxylipins, JA precursors and derivatives identified the synthetic isomer (+)-JA-L-Ile as a strong jasmonate agonist (Fonseca et al., 2009b). The structure of coronatine and the synthetic (+)-JA-L-Ile suggests that the stereochemical orientation of the cyclopentanone-ring side chains greatly affects receptor binding. Purification of the two natural epimers demonstrated that pure (−)-JA-L-Ile is inactive and that the active hormone is (+)-*7-iso*-JA-L-Ile, which is structurally more similar to coronatine (Fonseca et al., 2009b). Besides, the activity of COI1 as the JA-Ile receptor was first demonstrated by using radiolabeled coronatine in competitive binding assays (Katsir et al., 2008). To assess the direct binding of jasmonates to the COI1 receptor, biotin-tagged photoaffinity probes of JAs were designed (Yan et al., 2009a). The coronatine photoaffinity probe (PACOR), which retained weak biological activities, physically binds with the purified COI1 protein, further supporting that COI1 directly binds to COR and serves as a receptor for jasmonate (Yan et al., 2009a). All of these results show clearly the importance of JA-Ile analogs in several of the most important advances in phytohormone research.

THE REDUNDANCY/SPECIFICITY PARADOX OF HORMONE RECEPTORS

Chemical genomic studies can also be used to address the striking receptor redundancy/specificity paradox. Many components of hormone receptor complexes belong to large gene families and are functionally redundant. For example, the *Arabidopsis* genome encodes 14 *PYR/PYL* genes and 12 *JAZ* genes (Chini et al., 2007; Thines et al., 2007; Park et al., 2009). Although members of these families regulate the same hormone-mediated responses, individual members confer some tissue- and process-specificity.

The auxin perception complex shows the greatest redundancy of all the pathways discussed in this review. It is composed of one F-box member (among the 6 possible TIR1/AFB proteins), one co-receptor (among the 29 possible Aux/IAAs) alongside the single IP6 cofactor. The identification of auxin analogs has helped to address both the redundancy and specificity of various components within the auxin perception machinery. For example, mutations in the auxin receptor, AFB5, were identified in a genetic screen for lines resistant to the picolinate auxin (Walsh and Chang, 2006). *afb5* is highly resistant to picolinate auxins (such as picloram or DAS534) but not to other auxin isoforms such as 2,4-D or IAA (**Table 1** and Supplemental Table 1). This suggests that picolinate is a highly specific agonist of the auxin pathway (Walsh and Chang, 2006). Interestingly exogenous application of picloram mimics some aspects of auxin responses that application of 2,4-D or IAA application fails to reproduce, such as hypocotyl elongation. Although TIR1 and AFB5 show an almost identical secondary structure, biochemical analyses show that the TIR1–IAA7 and AFB5–IAA7 co-receptor complexes exhibit different auxin-binding affinities (**Figure 1**). Indeed, a single amino acid substitution has been identified through docking analyses that is responsible for the change in affinities of TIR1 and AFB5 for IAA and picloram (Calderón Villalobos et al., 2012). These data demonstrate that the AFB5-Aux/IAA co-receptor selectively binds picloram, but not IAA, whereas TIR1-Aux/IAA accepts IAA, but not picloram, providing the first mechanistic explanation for specificity in auxin perception.

FROM MOLECULES TO FUNCTIONS: THE POWER OF CHEMICAL GENOMICS

Chemical genomics approaches have also been instrumental in the discovery of the redundant ABA receptors, as different compounds show specificity to certain groups of receptors. Pyrabactin was originally identified as a synthetic inhibitor of only one ABA-mediated response, seed germination (Zhao et al., 2007).

A screen was performed for pyrabactin-resistant mutants aiming to identify redundant components of the ABA pathway (Cutler and McCourt, 2005). Indeed, single pyrabactin resistant mutants (*pyr*) were sensitive to ABA, whereas only multiple mutants in *PYR1*/*PYR1-like* (*PYL*) genes exhibited ABA insensitivity, demonstrating the functional redundancy of family members (Park et al., 2009). Additional studies using small molecules assessed the structural requirements of the binding pocket of the PYR/PYL receptors (Cao et al., 2013; Okamoto et al., 2013). For example, pyrabactin binds and activates two of the PYR/PYL receptors, while quinabactin activates three additional PYR/PYLs (**Table 1** and Supplemental Table 1). Since pyrabactin affects ABA-related processes in seeds and quinabactin regulates ABA-dependent stomatal closure, these chemicals are shedding light on the partially redundant functions of the PYR/PYL ABA receptors (**Figure 1**).

In the case of cytokinin, a chemical genomic approach was employed to identify molecules antagonizing the activity of the cytokinin receptor CRE1 (CYTOKININ RESPONSE 1; Arata et al., 2010). The authors elegantly generated a yeast system based on the *Arabidopsis CRE1* gene conferring cytokinin dependent growth. This system allowed a high-throughput screen looking for growth defects in yeast grown in the presence of cytokinin, and identified two compounds (SS-6772 and S-4607) that inhibited the CRE1-dependent yeast growth (**Table 1** and Supplemental Table 1). These compounds were chemically quite distinct from previous reported cytokinin receptor antagonists and new variations of these compounds were generated introducing minor modification of the quinazoline ring (Spíchal et al., 2009; Arata et al., 2010; Nisler et al., 2010). A new antagonist, S-4893, was confirmed as a strong inhibitor of cytokinin signaling in both yeast system and *in planta*. Further biochemical and genetic studies revealed that S-4893 acts as a non-competitive inhibitor of CRE1 not only in *Arabidopsis* but also in rice, suggesting that this compound operates in a range of plant species to antagonize cytokinin-mediated processes (**Figure 1**).

Perception of BR occurs at the plasma membrane by the receptor BRASSINOSTEROID INSENSITIVE (BRI1). In order to investigate endocytosis of the receptor-ligand complex, researchers developed a bioactive fluorescent labeled BR, called fluorescent castasterone (AFCS) (Irani et al., 2012) (**Figure 1**, **Table 1** and Supplemental Table 1). They used this tool to show that trafficking and endocytosis of the BRI1-AFCS complex is dependent on clathrin, ARF GTPases and the Rab5 GTPase pathway. However, concanamycin A, a specific inhibitor of the trans-Golgi network/early endosome (TGN/EE) blocked the BRI1-AFCS complex at the TGN/EE without affecting the BR signaling. The integration of these chemical and genetic data showed that retention of active BRI1 at the plasma membrane, rather than in endosomes, is an important factor in activation of BR signaling.

The recent identification of many components of several phytohormone receptor complexes opens the opportunity to generate new molecular tools. Most plant co-receptor complexes are able to perceive their targets in heterologous systems such as yeast. For example, yeast two hybrid (Y2H) systems have been used to induce the formation of TIR1-Aux/IAA complex in an auxin-inducible manner (Calderón Villalobos et al., 2012) and in a similar way JA-Ile or COR promotes COI1-JAZ interaction in yeast (Fonseca et al., 2009a; Chini, 2014). As the hormone co-receptors are the only plant proteins expressed within these heterologous systems, they represent unique tools to identify small molecules directly perturbing the hormone perception. Compounds able to induce the formation of the perception complex can subsequently be used to identify novel active forms of the hormone. In contrast, compounds inhibiting the hormone-dependent co-receptor complex might be direct antagonist molecules.

FROM RECEPTOR STRUCTURES TO MOLECULES: RATIONAL DESIGN OF PHYTOHORMONE ANALOGS

In the last decade, the crystal structures of several perception complexes were solved (Tan et al., 2007; Murase et al., 2008; Shimada et al., 2008; Park et al., 2009; Sheard et al., 2010). These structural data open new opportunities for the rational design of antagonist molecules specifically binding to and blocking the active pockets of individual receptors. The methodology of ligand-based rational design has been exploited extensively in medical research, but is just emerging in the agrochemical field (Lamberth et al., 2013). For example, this methodology has permitted the rational design of alfa-alkyl auxin molecules (**Figure 1**, **Table 1** and Supplemental Table 1). These auxin analogs are able to specifically bind and block the formation of the hormone receptor complex was very successful and have allowed systematic structure-activity analysis of the alfa-position of IAA (Hayashi et al., 2008, 2012) (**Figure 1**). An advanced modification of one of these compounds generated the auxinole molecule (alfa-[2,4-dimethylphenylethyl-2-oxo]-IAA). This binds TIR1 strongly to block the formation of the TIR1-IAA-Aux/IAA receptor complex. Molecular docking studies have provided novel insights of the molecular mechanism of auxinole activity, predicting that auxinole strongly interacts with the Phe82 residue. This residue of TIR1 that is crucial for Aux/IAA recognition and blocks TIR1 activity by interacting with this critical amino acid. Hayashi et al. showed that auxinole and alfa-alkyl auxin molecules retain their antagonistic activity in crop plants such as tomato as well as in distant relatives, such as the moss *Physcomitrella patens* (Hayashi et al., 2008, 2012).

The same principle of rational design used around the crystal structure of the COI1/JAZ co-receptor to design a COR-derivative that binds to COI1 but spatially impedes the interaction of the COI-JAZ co-receptors (**Figure 1**). This compound, COR *O*-methyloxime (COR-MO), shows a strong activity inhibiting the formation of the COI1-JAZ perception complex and preventing JAZ degradation (Monte et al., 2014). COR-MO reverses the effects induced by exogenous JA-Ile or COR treatments on several JA-mediated responses efficiently, thereby underpinning its usefulness in dissecting the JA-pathway (**Table 1** and Supplemental Table 1). Moreover, COR-MO enhances plant defense by preventing the effectiveness of the bacterial effector COR during *Pseudomonas syringae* infections. As this compounds works in a variety of different plant species, it further highlights the potential of such compounds in biotechnological and agronomical processes (Monte et al., 2014) (**Figure 1**).

In contrast to JA-Ile and auxins, which act as molecular glues by holding receptor complexes together, ABA binds within a cavity in its receptor where it induces conformational changes that in turn promote the interaction with the active site of group-A PROTEIN PHOSPHATASE 2C (PP2Cs) (Melcher et al., 2010). Following the resolution of the crystal structure of several ABA/PYR/PP2C complexes, Takeuchi and colleagues designed a series of ABA analogs (AS*n*) with long alkyl chains of the ABA 3′ ring CH, that they predicted would spatially block the PYL-PP2C interaction (**Table 1** and Supplemental Table 1). A six-carbon alkyl chain was sufficient to produce a potent ABA antagonist able to block multiple ABA-mediated responses *in vivo* such as germination, the expression of known downstream response genes and PP2A activity (Takeuchi et al., 2014) (**Figure 1**).

Brassinolide (BL) is a potent brassinosteroid that binds the BR receptor BRI1 directly and induces the interaction between BRI1 and SERK1 (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1; Santiago et al., 2013). Based on the crystal structure of the BRI1-BL complex Muto and colleagues generated a alkylated version of BL called brassinolide-2,3-acetonide. This compound was able to bind BRI1 but sterically interferes with the SRRK1 interaction (Muto and Todoroki, 2013) (**Figure 1**, **Table 1**). Indeed, brassinolide-2,3-acetonide showed a clear BL antagonistic effect in rice seedlings and opens the opportunity to develop a set of chemical tools modulating BR perception and further dissect the BR response pathway.

Collectively, these examples of antagonist molecules highlight the usefulness of the structure-based design of hormone analogs specifically binding for and blocking the active pocket of the receptors. This approach provides a novel methodology for generating bioactive hormone analogs.

SPECIFICITY AND REDUNDANCY

Another important contribution of chemical genomic screens is the possibility to assess specificity within signaling pathways or specific developmental processes. Essentially this notion is based on the fact that chemicals can overcome functional redundancy by inhibiting multiple members of a redundant protein family (Cutler and McCourt, 2005). A good example described earlier is pyrabactin, a compound affecting a single ABA-mediated response, germination (Zhao et al., 2007). The analyses of the first pyrabactin resistant (*pyr*) and further *pyr/pyl* mutants revealed nicely the functional redundancy of the 14-member PYR/PYL family for multiple ABA responses (Park et al., 2009).

Bikinin was identified in a screen for molecules inducing constitutive BR-related phenotypes such as hypocotyl elongation, petiole elongation and pale, blade shaped leaves (De Rybel et al., 2009). Strikingly, bikinin induces BR responses in mutants deficient in BR biosynthesis, perception and signaling. Bikinin also stimulates BR responses in *bin2-1*, a gain of function mutation in BIN2 (BRASSINOSTEROID-INSENSITIVE2). BIN2 is a GSK3 (GLYCOGEN SYNTHASE KINASE3) kinase that phosphorylates and inactivates the key transcription factors in the BR pathway, BZR1 and BES1/BZR2 (He et al., 2002). Bikinin acts as a competitive inhibitor of ATP binding and binds BIN2 directly causing the inhibition of seven of the 10 GSK3 kinases (Vert and Chory, 2006; De Rybel et al., 2009; Yan et al., 2009b). One bikinin-inhibited GSK3 kinases, ASK θ, interacts directly with and phosphorylates BEH2 (BES1/BZR1 HOMOLOG 2), a BR responsive transcription factor closely related to BZR1 and BES1/BZR2 (Yin et al., 2005b; Rozhon et al., 2010). Therefore, the discovery of bikinin allowed the identification of new components of the BR pathway and also enabled the conditional blockage of multiple key regulators in BR signaling, providing an essential tool to study the BR regulatory mechanisms.

Bestatin is an inhibitor of aminopeptidase and powerful inducer of JA- and wound-response genes in tomato (Schaller et al., 1995). The root growth inhibitory effect of bestatin depends on the key transcription factor of the JA pathway MYC2 but seems independent of the JA-Ile receptor COI1 (**Figure 1**). Therefore, Zheng et al. (2006) used bestatin to identify new components of the wounding signaling pathway dependent on JA-Ile and MYC2. Several bestatin resistant mutants (*ber*) were isolated, some of which allelic to *jin1/myc2*. In addition, *ber6* carries a mutation in *MED25/PFT1* (*MEDIATOR 25/PHYTOCHROME AND FLOWERING TIME 1*). This gene encodes for a subunit of the eukaryotic transcription mediator system (Zheng et al., 2006). MED25/PFT1 was first described as a positive regulator of shade avoidance and has subsequently been shown to also be required for plant defense (Cerdán and Chory, 2003; Kidd et al., 2009). Recent studies showed that MYC2, MYC3 and MYC4 have redundant roles in plant defense; MED25 directly interacts with MYC2 and it is required for MYC2-dependent pathogen defense (Fernández-Calvo et al., 2011; Çevik et al., 2012; Chen et al., 2012; Schweizer et al., 2013). MYC2, MYC3, and MYC4 are regulated by light quality and are involved in shade avoidance responses (Robson et al., 2010; Chico et al., 2014). Therefore, the use of bestatin to isolate mutants in *MED25/PFT1* suggested the redundant role of the MYC2, MYC3, and MYC4 in defense and shade avoidance responses. The use of bestatin can potentially identify new regulators of the MYCs and help to assess redundancy.

Strigolactones have long been studied because of their importance in stimulating the growth of the parasitic *Striga* and *Orobanche* on several crops. Structure-activity relationship analyses showed that several analogs mimic strigolactone functions (reviewed by Janssen and Snowden, 2012). Different structural requirements regulate strigolactone-mediated processes such as seed germination, hyphal branching of arbuscular mycorrizal fungi and shoot branching inhibition (Kondo et al., 2007; Zwanenburg et al., 2009; Akiyama et al., 2010; Fukui et al., 2011, 2013; Boyer et al., 2012; reviewed by Zwanenburg and Pospísil, 2013). Furthermore, newly synthesized strigolactone competitive analogs suggest that *Arabidopsis*, Orobanche and arbuscular mychorrial fungi possess variations in the sensitivity to strigolactone analogs, providing additional support to the idea that variations in strigolactone receptors among the different species should exist (Cohen et al., 2013; reviewed by Janssen and Snowden, 2012).

Karrikins are compounds structurally similar to strigolactones. They promote germination, but unlike strigolactones, karrikins are not produced in plants, but instead are found in the smoke of burning plant material. Despite this, in many ways they

behave as hormones, as small quantities of the signal is sufficient to trigger a signal transduction pathway. Genetic screenings for karrikin insensitive mutants showed that karrikins perception share a common mechanism with strigolactones. The F-box MAX2 (MORE AXILLARY BRANCHES 2) is required to perceive both kinds of compounds. In both cases an alfa/beta hydrolase fold protein [KAI2 (KARRIKIN INSENSITIVE 2) or DAD2/D14] is part of the receptor complex (Nelson et al., 2011; Hamiaux et al., 2012; Waters et al., 2012; Guo et al., 2013). The strigolactone and karrikin pathways are a good example of how structurally similar molecules rely on similar—or even common—perception mechanisms and confer overlapping physiological responses while maintaining their identity in terms of structure-function (**Figure 1**, **Table 1** and Supplemental Table 1).

PHYTOHORMONE CROSSTALK

It has been well documented that most biological processes are not regulated by a single hormone but rather by complex signaling networks controlled by multiple hormones or other signaling components. For example, auxin and cytokinin act in consort to control the formation of the embryonic root, root meristem size, root branching, vascular pattern and shoot phylotaxy (Dello Ioio et al., 2008; Bishopp et al., 2011b; Besnard et al., 2014). Chemical genomic approaches not only facilitate the dissection of hormonal pathways but could also shed light on the non-linear networks within which they operate as well as identify new downstream biological functions. For example, screening for compounds that affect gravitropism led to the identification of multiple chemicals that affect membrane trafficking in auxin dependent and independent manners (Surpin et al., 2005). Such important and sometimes unexpected results demonstrate the power of screens for small molecules regulating related biological processes.

Recently, a chemical genomic screen for molecules perturbing germination identified cotylimides, a class of compounds structurally similar to strigolactones that recapitulate cotyledon bleaching promoted by GR24 (Tsuchiya et al., 2010). The subsequent screen for mutants insensitive to cotylimides isolated several suppressor lines showing elongated hypocotyls, a phenotype commonly observed in mutants defective in light signaling. The analysis of strigolactones in light responses showed that these compounds mimic light in seedling growth and increase the accumulation of HY5 (ELONGATED HYPOCOTYL5), a protein directly targeted by COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) for degradation. Tsuchiya and colleagues elegantly revealed the molecular mechanism behind this response. Strigolactones mediate the nuclear exclusion of COP1, this stabilizes HY5, which in turn reduces hypocotyl elongation.

As described earlier in this review, L-kynurenine was identified in a chemical genomic screen. This compound was instrumental in showing that auxin induces the nuclear accumulation of the key activator of the ET pathway, EIN3 (ETHYLENE-INSENSITIVE3). Kyn was used to unravel a positive feedback loop between auxin biosynthesis and ET signaling (He et al., 2011), that was not detectable by conventional genetic analysis.

Brassinopride (BRP) was identified in a screen for inhibitors of brassinosteroids, based on the inhibition of BR-mediated hypocotyl growth in the dark and activation of the BR-responsive reporter gene *CPD:GUS* (Gendron et al., 2008) (**Figure 1**). The site of action of BRP has not been defined yet, however, application of brassinolide can reverse BR related effects of BRP. This suggests that BRP could perturb BR biosynthesis. Unexpectedly, BRP also induced exaggerated formation of apical hooks, resembling plant subjected to ET treatments. Additionally, the apical hook phenotype could be blocked by a chemical inhibitor of ethylene perception or by ET insensitive mutants, suggesting that BRP activates ET responses. Phenotypic analysis of ET and BR mutants in combination with analysis of the effects of BRP analogs, revealed a crosstalk between ET and BR in etiolated seedlings. Moreover, variation among BRP analogs suggest that modifying the side groups of BRP can have specific effects on BR or ET functions (Gendron et al., 2008).

Recently, it has also been proposed that hormone derivates can interfere with different signaling pathways (Katsir et al., 2008; Staswick, 2009; Gutierrez et al., 2012). For example, hormone conjugation is a common process in plants to activate, store or deactivate phytohormones, however, some conjugates show unexpected activity. When combined with either auxin or JA, tryptophan (Trp) conjugates with indole-3-acetic acid (IAA-Trp) or with jasmonic acid (JA-Trp). These conjugates act as an inhibitors of auxin responses, preventing several auxin-mediated responses, such as gravitropism, lateral root production and expression of known auxin response genes (Staswick, 2009). The evidence that an endogenous JA derivate inhibits the auxin pathway adds a novel level of regulation between the jasmonate and auxin pathways. It was hypothesized that IAA-Trp and JA-Trp could directly interfere with the auxin receptor TIR1-IAA/Aux as the TIR1 is required for IAA-Trp and JA-Trp inhibition and because these conjugated compounds are structurally similar to the active forms. However, IAA-Trp and JA-Trp do not directly alter the IAA-dependent interaction between TIR1 and Aux/IAA7 and their precise mode of action remains unknown (Staswick, 2009).

FUTURE PROSPECTIVES

Plant chemical biology has enabled significant advances in phytohormone research. In addition to the classical phytohormone analogs widely used by the scientific community, we have recently witnessed the extensive use of chemical genomic approaches. The straightforward availability of large, diverse chemical libraries and the natural chemical resources will surely facilitate a further extension of this methodology and the identification of novel compounds regulating many biological processes of hormone synthesis, transport and response. Complementary, rational design of novel molecules and molecule labeling are emerging but successful strategies.

Therefore, we envision the continued use of plant chemical biology not only to identify novel components or regulation mechanisms of phytohormone pathways but also to better understand their mode of action and molecular networks. The discovery of molecules with certain specificity constrains will certainly contribute toward more rational and sustainable agriculture systems.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00709/abstract

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Target sites for chemical regulation of strigolactone signaling

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Demands for plant growth regulators (PGRs; chemicals that control plant growth) are increasing globally, especially in developing countries. Both positive and negative PGRs are widely used to enhance crop production and to suppress unwanted shoot growth, respectively. Strigolactones (SLs) are multifunctional molecules that function as phytohormones, inhibiting shoot branching and also functioning in the rhizospheric communication with symbiotic fungi and parasitic weeds. Therefore, it is anticipated that chemicals that regulate the functions of SLs will be widely used in agricultural applications. Although the SL biosynthetic pathway is not fully understood, it has been demonstrated that β-carotene isomerases, carotenoid cleavage dioxygenases (CCDs), and a cytochrome P450 monooxygenase are involved in strigolactone biosynthesis. A CCD inhibitor, abamine, which is also an inhibitor of abscisic acid biosynthesis, reduces the levels of SL in several plant species and reduces the germination rate of *Orobanche minor* seeds grown with tobacco. On the basis of the structure of abamine, several chemicals have been designed to specifically inhibit CCDs during SL synthesis. Cytochrome P450 monooxygenase is another target enzyme in the development of SL biosynthesis inhibitors, and the triazole-derived TIS series of chemicals is known to include SL biosynthesis inhibitors, although their target enzyme has not been identified. Recently, DWARF14 (D14) has been shown to be a receptor for SLs, and the D-ring moiety of SL is essential for its recognition by D14. A variety of SL agonists are currently under development and most agonists commonly contain the D-ring or a D-ring-like moiety. Several research groups have also resolved the crystal structure of D14 in the last two years. It is expected that this information on the D14 structure will be invaluable not only for developing SL agonists with novel structures but also in the design of inhibitors of SL receptors.

Keywords: plant growth regulator, strigolactone, biosynthesis inhibitor, agonist, antagonist, 3D structure, *in silico* screening

INTRODUCTION

Chemicals are widely used in agriculture to increase the yields of crops. For example, pesticides, including herbicides, fungicides, insecticides, and/or insect growth regulators, protect crops from the attack of pests that damage them, such as weeds, fungal diseases, and insects. Because pesticides usually protect crops by killing these pests, they are thought of as negative regulators of pests. However, because plant growth regulators (PGRs) are chemicals that control plant growth and benefit crop production by enhancing crop quantities and quality and by improving the postproduction quality of some plants, they are thought of as positive regulators of plants. In developing countries, such as China, the plant growth regulator industry has seen remarkable progress and shows attractive future market potential (http://www.reuters.com/article/2010/05/28/idUS145314+28-May-2010+BW20100528). It is likely that PGRs will be utilized for large numbers of species and cultivars.

The most popular target of PGRs is gibberellin (GA) biosynthesis. In this case, PGRs are considered plant growth retardants and are applied to agronomic and horticultural crops to reduce unwanted longitudinal shoot growth without lowering plant productivity (Rademacher, 2000). Their targets are copalyl-diphosphate synthase and *ent*-kaurene synthase, which are involved in the early steps of GA metabolism; cytochrome P450-dependent monooxygenases, which are involved in the oxidization of *ent*-kaurene to *ent*-kaurenoic acid; and dioxygenases, which catalyze the late steps of GA formation by mimicking 2-oxoglutarate (Rademacher, 2000). Enzymes similar to those involved in GA biosynthesis also play important roles in the formation of strigolactones (SLs), brassinosteroids, abscisic acid, and other plant hormones.

The importance of the chemicals that control plant function has recently been increasing, not only in agriculture but also in the

plant sciences. The primary advantage of using bioactive chemicals to analyze the roles of plant hormones in plants, rather than plant-hormone-deficient mutants, is that they can be applied regardless of the plant species. The phenotypic changes induced by chemical treatments can reveal the physiological functions associated with the target proteins. Furthermore, genetic redundancy does not significantly influence the effects of these inhibitors. Plants treated with an antagonist or an inhibitor of biosynthesis show phenotypes almost identical to those of untreated plants, as is seen in multiple mutants when the target protein is redundant. Finally, chemicals easily regulate the functions of their target proteins only transiently (Kitahata and Asami, 2011). Therefore, the utilization of biosynthesis inhibitors or receptor inhibitors is a useful alternative to mutations for dissecting biological processes (Blackwell and Zhao, 2003).

Recently, the scientific discipline of chemical biology has increased in popularity. The goal of chemical biology is to clarify biological mechanisms using small bioactive organic compounds. In plant hormone research, the increasing use of chemical-biology-based methods has been very effective. For example, with plant hormone biosynthesis inhibitors, researchers can create plant hormone deficiencies in specific plants and under specific conditions. For instance, molecular genetic studies have been conducted using inhibitors of plant hormone biosynthesis to select mutants that are resistant to those inhibitors. This approach has been very successful, especially in brassinosteroid research, in which the brassinosteroid biosynthesis inhibitor Brz was used to isolate the Brz-resistant mutant *bzr1* to identify the novel protein BZR1, which functions in the brassinosteroid signal transduction pathway (Wang et al., 2002).

Strigolactones are terpenoids that contain a lactone ring in their molecules, and are produced in a variety of plant species (compound **1** in **Figure 1**). They are multifunctional molecules, acting as germination stimulants in root parasitic weeds, root-derived signals that induce hyphal branching in arbuscular mycorrhizal fungi, and plant hormones that regulate various phenomena, such as shoot branching, root morphology, secondary growth, and so on (Cook et al., 1966; Akiyama et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008; Seto et al., 2012). Several branching mutants have been identified as mutants of SL biosynthesis and signaling. At present, two carotenoid cleavage dioxygenases (CCDs; AtMAX3 and AtMAX4), one carotenoid isomerase (AtDWARF27 (AtD27), and one cytochrome P450 (AtMAX1) are known to be involved in the biosynthesis of SLs in *Arabidopsis*. AtMAX2 and AtDWARF14 (AtD14), an F-box protein and an α/β hydrolase, respectively, act in SL signaling (Waters et al., 2012a,b). A screen for genetic suppressors of *Atmax2* mutant identified that members of SMXL protein family act downstream of AtMAX2 in SL signaling (Stanga et al., 2013). More recently, DWARF53 (D53), a member of the SMXL protein family in rice, was reported to be a substrate of the SCF^{D3} complex and rapidly degraded in the presence of SL. These data suggest that D53 is a repressor of SL signaling (Jiang et al., 2013; Zhou et al., 2013).

As described above, chemicals that regulate the functions of SLs will be very useful, so several approaches have been used to develop chemically synthesized SL agonists. As a consequence, several chemicals have been reported that are germination stimulants of root parasitic weeds, regulators of shoot branching, and inducers of hyphal branching in arbuscular mycorrhizal fungi (Akiyama et al., 2010; Yoneyama et al., 2010; Zwanenburg and Posplsil, 2013). One of the great successes in this field has been the identification of GR24 (coumpound **2** in **Figure 1**) by Zwanenburg and Posplsil (2013). GR24 (**2**) is now widely used as a standard mimic in SL research and is known to be more stable than natural SLs (Akiyama et al., 2010). However, there has been no report of any antagonists of SLs. Because D14 has been identified as an SL receptor (Jiang et al., 2013; Nakamura et al., 2013; Zhou et al., 2013) and its three-dimensional (3D) structure is available (Hamiaux et al., 2012; Kagiyama et al., 2013; Nakamura et al., 2013; Zhao et al., 2013), the design and development of SL agonists and antagonists will be a fascinating target for chemists.

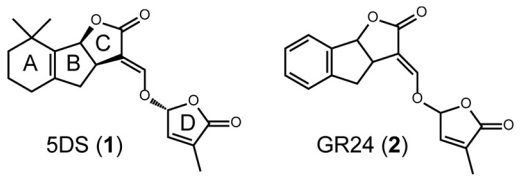

FIGURE 1 | Structures of SLs. Structure of a natural SL, 2′-*epi*-5-deoxystrigol (5DS; **1**), and a synthetic SL, GR24 (**2**).

The SL biosynthetic pathway is not yet fully understood. However, the involvement of Fe-containing β-carotene isomerase (D27 in rice), CCD7, CCD8, and cytochrome P450 monooxygenase (MAX1 in *Arabidopsis*) in SL biosynthesis has been demonstrated (Seto et al., 2012). Alder et al. (2012) demonstrated that conversion of all-*trans*-β-carotene (**3**) to 9-*cis*-β-carotene (**4**) by D27 and cleavage of 9-*cis*-β-carotene (**4**) by CCD7 and CCD8 generates carlactone (**5**), which has a carbon skeleton similar to that of the SLs, including a methylbutenolide ring, a characteristic part of the SL structure (**Figure 2**). Because all of these enzymes include an iron atom in their molecules and nitrogen has a lone pair electrons that can form a coordinated bond with the 3D orbital of the iron atom, chemicals that include a nitrogen atom(s) in their molecules and have binding affinity for the substrate-binding pocket of these enzymes could be inhibitors of SL biosynthesis. For example, abamine (**6**), the first CCD inhibitor, which includes a nitrogen in its molecule, inhibits 9-*cis*-epoxycarotenoid dioxygenase (NCED) in the abscisic acid biosynthetic pathway and reduces abscisic acid levels in abamine (**6**)-treated *Arabidopsis* (**Figures 2** and **3A**; Han et al., 2004a,b). 1H-1,2,4-triazole or 1H-1,3-imidazole derivatives, such as uniconazole-P and paclobutrazol, inhibit a variety of members of the cytochrome P450 enzyme group. The triazole or imidazole moiety is a key component in the action of cytochrome P450 inhibitors because the nitrogen atoms in these groups are essential for binding the heme iron in the cytochrome P450s. In this paper, we review the recent research into the regulators of SL functions, including SL biosynthesis inhibitors and agonists, and the possibility of finding SL antagonists based on the 3D structure of the SL receptor D14.

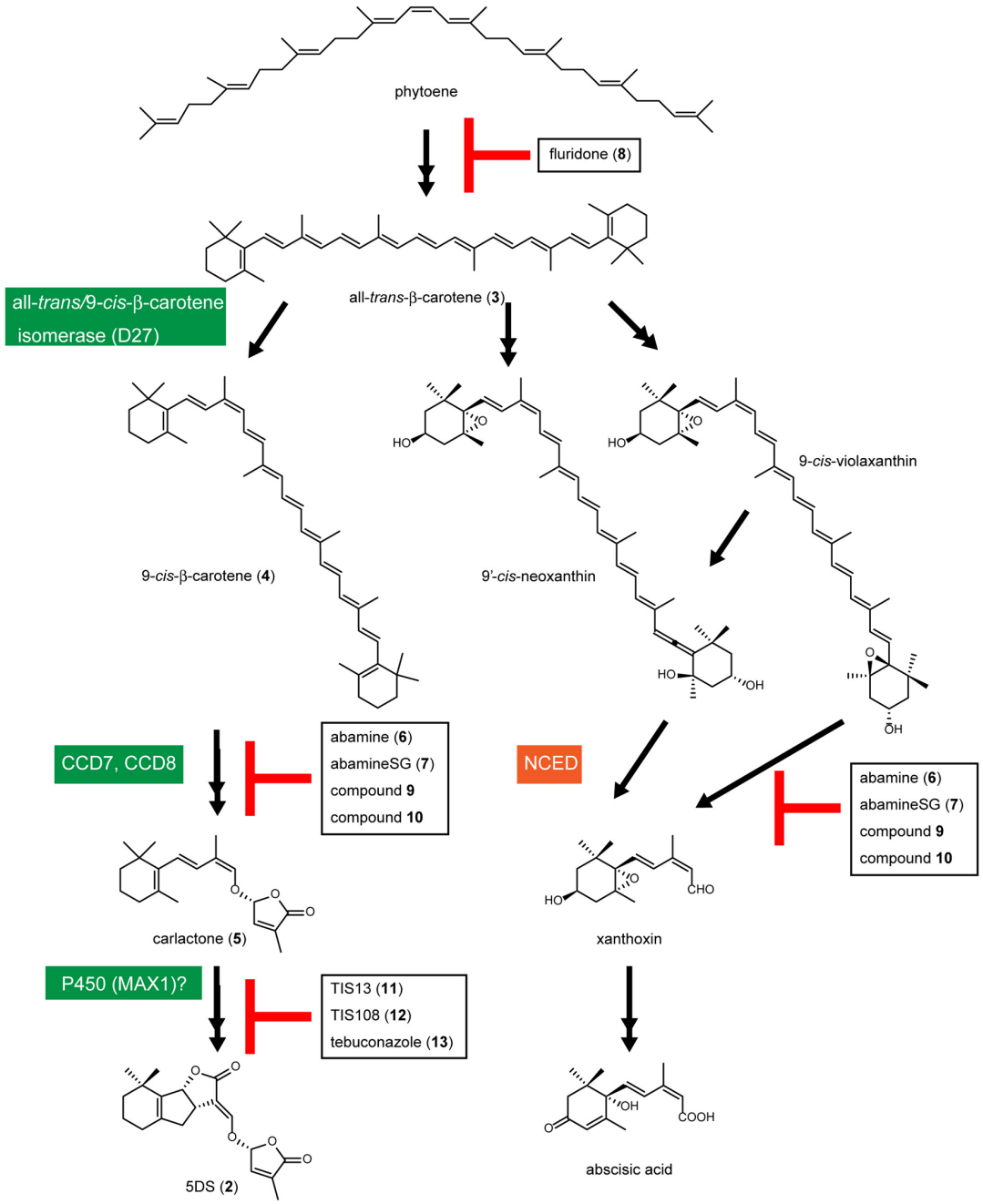

FIGURE 2 | Predicted biosynthetic pathway of SLs and abscisic acid. In the SL biosynthetic pathway, D27 catalyzes the isomerization of all-*trans*-β-carotene (**3**) to 9-*cis*-β-carotene (**4**), and 9-*cis*-β-carotene (**4**) is converted to carlactone (**5**) by CCD7 and CCD8. Carlactone (**5**) would be oxidized by some enzymes including a cytochrome P450 monooxygenase MAX1 (CYP711A) and converted to SLs including 5DS. Abscisic acid is also synthesized from all-*trans*-β-carotene (**3**). In the abscisic acid biosynthetic pathway, 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the cleavage of 9-*cis*-epoxycarotenoid at the 11–12 double bond to produce a precursor of ABA, xanthoxin.

SL BIOSYNTHESIS INHIBITORS

Lignostilbene-alpha,beta-dioxygenase cleaves the olefinic double bond of phenolic stilbenes with a mechanism similar to that of NCED, a key enzyme in abscisic acid biosynthesis (**Figure 2**). Han et al. synthesized several analogs of stilbene and found that several types of lignostilbene analogs that contain nitrogen in the C–C bond are inhibitors of lignostilbene-alpha,beta-dioxygenase (Han et al., 2002, 2003). On the basis of these findings, we started to design and synthesize NCED inhibitors and identified abamine (**6**) as a specific inhibitor of abscisic acid biosynthesis, targeting NCED (**Figures 2** and **3A**; Han et al., 2004a,b). A structure-activity relationship study of abamine (**6**) identified a more

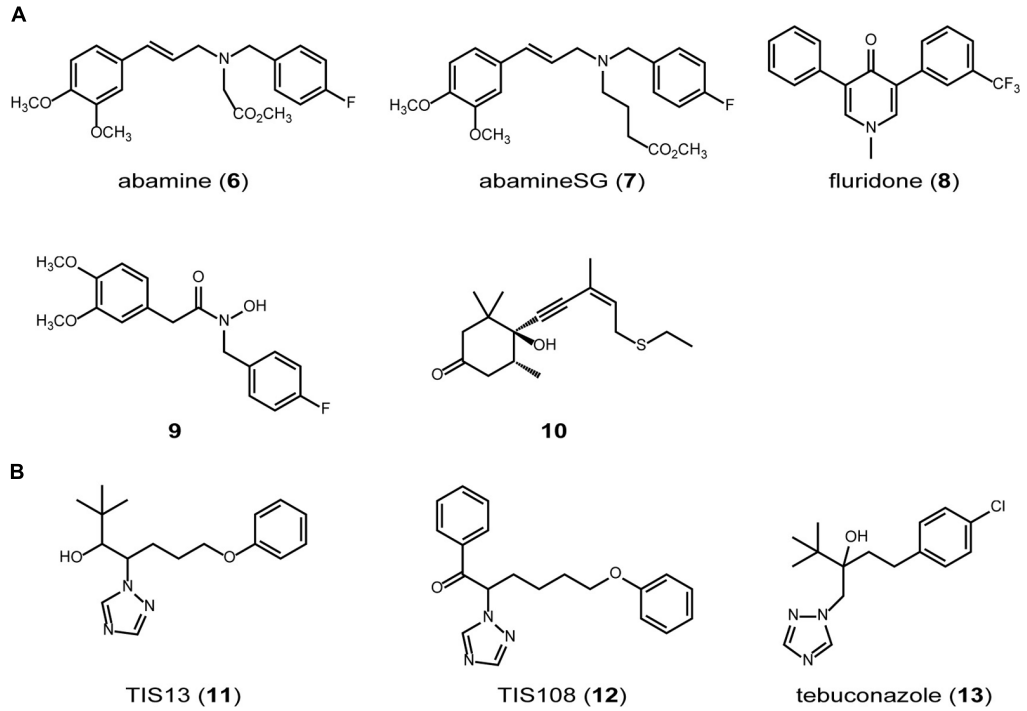

FIGURE 3 | SL biosynthesis inhibitors. (A) carotenoid synthesis/cleavage inhibitors. **(B)** cytochrome P450 inhibitors.

potent and specific NCED inhibitor, abamineSG (**7**; **Figures 2** and **3A**). Abamine (**6**) and abamineSG (**7**) contributed significantly to the recent findings that abscisic acid plays a role in controlling the number of nodules on the roots of leguminous plants (Han et al., 2004a; Suzuki et al., 2004) and in the high-light response of *Arabidopsis* (Galvez-Valdivieso et al., 2009). Fluridone (**8**) inhibits the biosynthesis of all carotenoids and carotenoid-derived metabolites (**Figures 2** and **3A**). Because carotenoid biosynthesis is necessary for the biosynthesis of normal levels of SLs (Matusova et al., 2005; López-Ráez et al., 2008), fluridone (**8**) may also be an inhibitor of SL biosynthesis. However, because fluridone (**8**) causes the photodestruction of chlorophyll and lethal damage to cells, it is not an ideal inhibitor with which to study the biological roles of SLs in plants. We consider that specific SL biosynthesis inhibitors could be useful tools for the biochemical analysis of SL biosynthesis and could extend our understanding of the biological roles of SLs in plants. In this context, we have begun to search for inhibitors of SL biosynthesis (**Figure 3A**).

9-*cis*-epoxycarotenoid dioxygenases belong to the family of CCDs and control the rate-limiting step in the abscisic acid biosynthetic pathway in plants (Iuchi et al., 2001). It has been suggested that abamine (**6**) also targets other CCDs (Kitahata et al., 2006), but the potency of abamine (**6**) in the regulation of SL production is yet to be determined. Because CCD7 and CCD8 are involved in the SL biosynthetic pathway and share similar functions and sequences with all the CCDs, abamine (**6**) might affect SL biosynthesis by inhibiting CCD7, CCD8, or other related enzymes in addition to NCED. It therefore has potential as a new scaffold for the development of regulators of SL biosynthesis. In this context, we evaluated the potency of abamine (**6**) in the regulation of SL production. We found that abamine (**6**) reduces the levels of SLs in several plant species and the germination rate of *Orobanche minor* seeds grown with tobacco (Kitahata et al., 2011). Taken together, these data suggest that abamine (**6**) can be used as a scaffold for the development of specific regulators of SL production. The actual structure of abamine (**6**) offers clues to the design of new CCD inhibitors. Hydroxamic acid analogs (**9**; **Figure 3A**), which were designed based on the structures of abamine (**6**) and abamineSG (**7**), inhibited the activities of many CCDs, including AtCCD7, and increased the number of branches in inhibitor-treated *Arabidopsis* at a concentration of 100 μM (Sergeant et al., 2009). Similarly, sesquiterpene-like inhibitors of NCED (**10**; **Figure 3A**) have been designed based on the structure of abamine (**6**; Boyd et al., 2009). Therefore, abamine (**6**) can be used as a scaffold for designing new inhibitors targeting several types of CCDs, including CCD7 and CCD8, and CCDs may be good targets for designing and developing specific inhibitors of SL biosynthesis.

Another target enzyme class that may be useful in developing SL biosynthesis inhibitors is the cytochrome P450 monooxygenases. At least one cytochrome P450 monooxygenase (CYP711A) is probably involved in the SL biosynthetic pathway in *Arabidopsis* (Booker et al., 2005) and there are five CYP711 family members in rice (Nelson et al., 2004). We screened a chemical library of triazole derivatives, constructed in our laboratory by Min et al. (1999) and Sekimata et al. (2001, 2002), to find new SL biosynthesis inhibitors that induce tiller bud outgrowth in rice seedlings

(**Figure 3B**). We selected TIS13 (**11**) as a candidate inhibitor of SL biosynthesis (Ito et al., 2010). TIS13 (**11**) reduced the levels of SL in both the roots and root exudates, and TIS13-induced second tiller bud outgrowth was suppressed by its coapplication with 1 μM GR24 (**2**). Furthermore, the root exudates of rice treated with 10 μM TIS13 (**11**) had less germination-stimulating activity on the root parasitic weed *Striga hermonthica* than those of the control plants. These results strongly suggest that TIS13 (**11**) inhibits SL biosynthesis in rice. Because we considered TIS13 (**11**) a useful lead compound for developing specific and potent SL biosynthesis inhibitors, we performed a structure–activity relationship study of TIS13 (**11**) using chemical modification, which led to the identification of the more potent SL biosynthesis inhibitor, TIS108 (**12**; Ito et al., 2011, 2013a). Besides the TIS series (**11** and **12**), we found that the fungicide tebuconazole (**13**), which targets cytochrome P450 in fungi, is also a potent SL biosynthesis inhibitor (Ito et al., 2013b). These results strongly suggest that chemicals targeting cytochrome P450 are good scaffolds upon which to design and develop inhibitors of SL biosynthesis.

Because the TIS series (**11** and **12**) and tebuconazole (**13**) are triazole-type inhibitors and have potential affinity for the cytochrome P450s, their target could be CYP711A (**Figure 2**). However, several biosynthetic steps in the SL biosynthetic pathway have still to be clarified and other P450s may be involved in SL biosynthesis. The target site of the TIS series will be identified in future studies.

DESIGN OF SL AGONISTS AND ANTAGONISTS

Precise knowledge of the mechanism of molecular SL recognition will greatly assist chemists in designing and developing SL agonists and antagonists. Numerous studies have revealed that SL is received by D14 class of α/β hydrolase proteins in rice (Arite et al., 2009; Liu et al., 2009; Kagiyama et al., 2013; Nakamura et al., 2013), *Arabidopsis* (Waters et al., 2012b; Zhao et al., 2013; Chevalier et al., 2014) and petunia (Hamiaux et al., 2012). Karrikins are smoke-derived compounds that stimulate seed germination, and karrikin signals, which probably mimic unknown endogenous signals, are closely related and partially overlapped with SL signals (Waters et al., 2014). Karrikin is recognized by KAI2, a close relative of D14, in *Arabidopsis* (Guo et al., 2013). While numerous lines of evidence indicate that D14 and KAI2 are receptor proteins of SLs and karrikins, respectively, Toh et al. (2014) recently showed that GR24 binds KAI2 as well as D14, suggesting that KAI2 may perceive SLs. At present, several groups have resolved the crystal structure of D14 (Hamiaux et al., 2012; Kagiyama et al., 2013; Nakamura et al., 2013; Zhao et al., 2013), and have made it available on the Protein Data Bank (PDB: 4DNP and 4DNQ; 4IH1, 4IH4, 4IH9, and 4IHA; 3W04, 3W05, and 3W06; 3VXK and 3WIO). However, D14 has a unique mechanism of SL recognition, in that its enzymatic activity hydrolyzes its ligand molecule, SL. In a recent paper, we reported that rice D14 hydrolyzes SLs to produce D-OH, and that D14 then forms a complex with D-OH that is important for its interaction with a rice DELLA protein, SLR1 (Nakamura et al., 2013). Since DELLA proteins are key regulators of GA signaling, this SL-dependent D14–SLR1 interaction is presumed to mediate the crosstalk between SL and GA signaling, although the genetic data to support this interaction is absent at present. In the D14–D-OH crystal, D-OH is located at a site far from the catalytic triad, Ser147–His297–Asp268, and is surrounded by Val194, Ser270, and several aromatic residues, including Phe186, Trp205, Tyr209, and Phe245. These aromatic residues allow favorable hydrophobic and/or van der Waals interactions with D-OH. Mutations at Phe186, Trp205, and Phe245 extinguish the D14–SLR1 interaction, supporting the idea that the D14–D-OH interaction is required for the D14–SLR1 interaction. Therefore, we assume that D-OH is a strong candidate for an active form of SL and designate it "branin" (branching inhibitor).

On the basis of the results discussed above, it is assumed that the structural requirements for active SL are as follows: (1) it must be hydrolyzable by D14; and (2) it must produce D-OH after hydrolysis. These requirements are consistent with the hypothesis proposed by Boyer et al. (2012) on the basis of laborious structure–activity relationship analyses, that the presence of a D-ring is essential for the hormonal activity of SL. To confirm this hypothesis, we prepared various SL homologs (**14–17**) that are expected to be hydrolyzed by D14 to induce the D14–SLR1 interaction, and determined their ability to inhibit rice tillering (**Figure 4A**; Nakamura et al., 2013). A strong relationship was observed between their tillering inhibition activity and the induction of the D14–SLR1 interaction, suggesting that the yeast two-hybrid system is a useful tool for screening SL agonists. We could not perform enzymatic assays of all the SL homologs because we encountered several difficulties, e.g., stability of the reaction buffer, detectability of the reaction products, etc. Therefore, further studies are required to test this hypothesis more rigorously.

As described above, the sequential conversion of all-*trans*-β-carotene (**3**) by D27, CCD7 and CCD8 generates carlactone (**5**), which has a carbon skeleton similar to that of the SLs, including a methylbutenolide ring, a characteristic part of the SL structure (**Figure 2**). When applied exogenously, carlactone (**5**) rescued the shoot-branching phenotype of *d27* and *d10*, but not that of *d3*, suggesting that carlactone is a biosynthetic intermediate of the SLs (Alder et al., 2012). Supporting this hypothesis, Seto et al. (2014) demonstrated that carlactone (**5**) is transformed into 2′-*epi*-5-deoxystrigol (**1**) when ^{13}C-labeled carlactone is fed to rice (**Figure 2**). However, it is still possible that carlactone (**5**) itself is recognized and hydrolyzed by D14 to produce D-OH and elicit SL activity, because carlactone (**5**) has a methylbutenolide ring connected to a carbon chain *via* an enolether moiety, which can be hydrolyzed to yield D-OH, as shown above. Recently, avenaol (**18**) was reported to mimic SLs in stimulating the germination of root parasitic plants (**Figure 4B**; Kim et al., 2014). Avenaol (**18**) lacks the B-ring and has an additional carbon atom between the A- and C-rings. However, it contains the C–D moiety of the SLs, the structural feature common to all known SLs. The plant hormonal activity of avenaol (**18**) has not yet been determined, but we assume that it is active because its molecule contains a C–D moiety that is hydrolyzed by D14.

Debranones are phenoxy furanone derivatives that inhibit the outgrowth of tillering buds in rice (Fukui et al., 2011). Like SLs, debranones have a D-ring [3-methylfuranone-2(5H)-one]

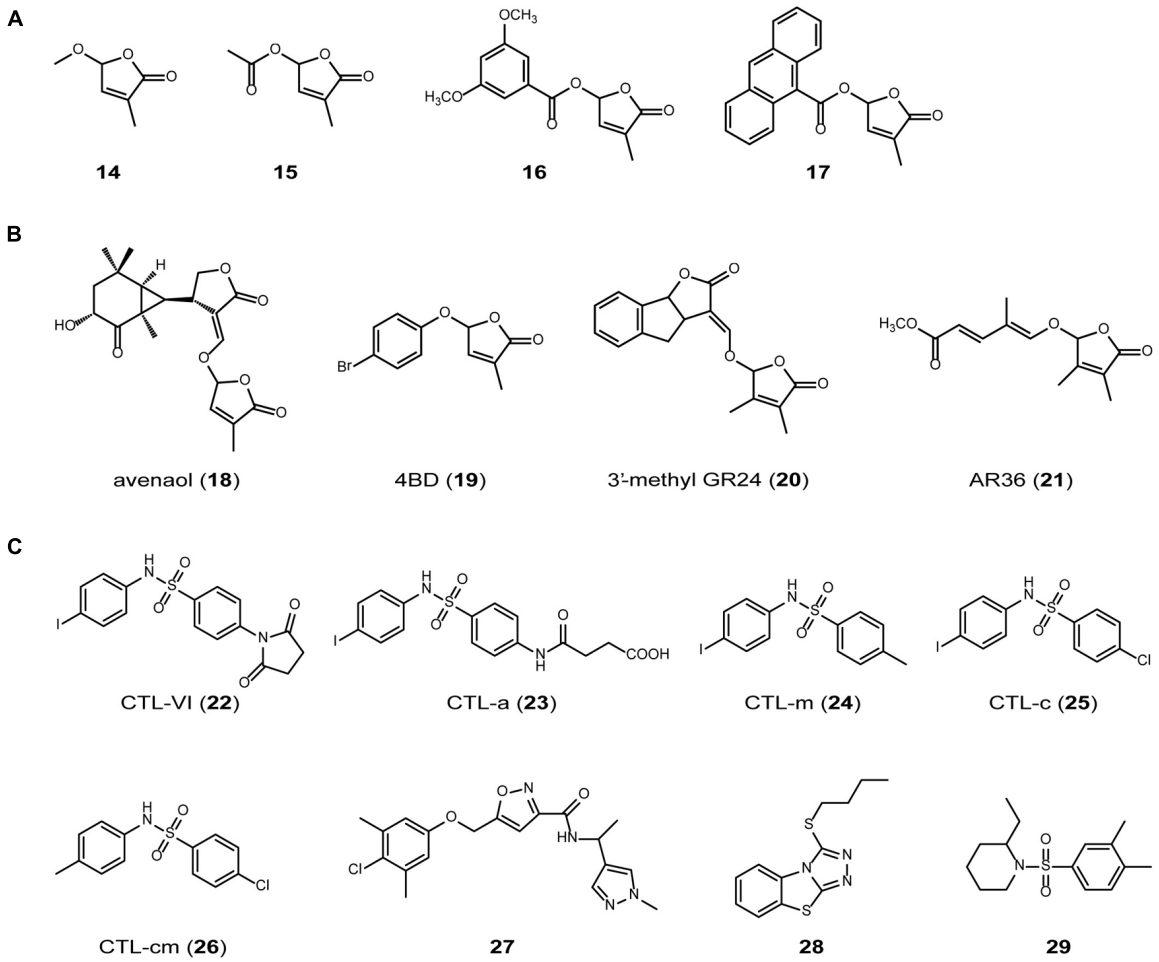

FIGURE 4 | SL agonists. (A) SL homologs that are expected to be hydrolyzed by D14 and have tillering inhibition activity in rice. **(B)** SL mimics reported recently (Fukui et al., 2011, 2013; Boyer et al., 2014; Kim et al., 2014). **(C)** SL agonists without butenolide miety (Toh et al., 2014).

but they lack an enolether moiety. A structure–activity relationship study showed that 5-[4-bromophenoxy]-3-methylfuranone-2(5H)-one (4BD, **19**) had similar activity to that of GR24 (**2**) in many aspects of a biological assay in plants, but is far less active in inducing seed germination in parasitic weeds (Fukui et al., 2013). This suggests that the structural requirements for its hormonal activity in plants and for its activity in the rhizosphere differ, and that 4BD (**19**) could be useful for controlling the plant architecture without inducing the growth of parasitic weeds.

Recently, Boyer et al. (2014) reported another type of SL analog, including 3′-methyl-GR24 (**20**) and AR36 (**21**; **Figure 4B**), which have a dimethylbutenolide motif instead of the D-ring of SL. 3′-methyl-GR24 (**20**) and AR36 (**21**) show hormonal activity in pea, but not in parasitic weed germination or fungal hyphal growth, indicating that the dimethylbutenolide structure is recognized by the SL receptor involved in its branching-inhibition activity, but not by the receptor involved in the action of SL in the rhizosphere (Boyer et al., 2014). This suggests that, as well as 4BD (**19**), dimethylbutenolide-containing compounds are useful for controlling the plant architecture without inducing the growth of parasitic weeds.

Cotylimide (CTL) compound (CTL-VI; **22**; **Figure 4C**) was firstly identified as a molecule that increases SL synthesis and regulates light adapted growth through AtMAX2 in *Arabidopsis* (Tsuchiya et al., 2010). Recently, Toh et al. (2014) demonstrated that CTL-VI (**22**) and its analogs (**23–26**; **Figure 4C**) bind KAI2 and promote interaction between KAI2 and AtMAX2. These CTL compounds inhibits hypocotyl growth of *Arabidopsis*. These results indicate that they are SL agonist. Toh et al. (2014) also screened 4,182 small molecules to identify novel compounds that promote KAI2-AtMAX2 interaction using the yeast two-hybrid system and obtained three lead compounds (**27–29**; **Figure 4C**). These three compounds showed SL activity in *Arabidopsis* hypocotyls and *Striga* seed germination assay. Although the germination stimulation activity of these lead compounds was much weaker than that of GR24, this approach can be useful to identify novel SL agonists.

With the recent increase in computer power and the development of bioinformatics algorithms, computer-assisted drug

design, which utilizes the 3D structures of proteins determined with X-ray crystallography, has become a common method of drug discovery. Because we now have considerable information on the 3D structure of D14, we are planning to use an *in silico* drug design method to screen for novel agonists and antagonists of D14. For this purpose, we are trying the *in silico* screening of ligands of D14 by using the 3D structure of D14 complexed with D-OH (PDB: 3WIO). Using the LigandScout software (Wolber and Langer, 2005; Wolber et al., 2007), we have selected candidate chemicals on the basis of a ligand-based pharmacophore model, with reference to the structural information for the D14–D-OH complex (PDB: 3WIO). Their structures are quite different from those of known SLs, indicating that SL agonists and/or antagonists with novel structures can be obtained with this *in silico* screening method.

CONCLUDING REMARKS

In this review, we have described recent attempts to design inhibitors of SL biosynthesis based on the recently accumulating knowledge of the enzymes involved in SL biosynthesis, and to design SL agonists and SL antagonists based on our recent model of SL recognition in plants. These chemicals have potential utility in both agricultural applications and in basic science, to dissect the mechanisms underlying the wide spectrum of SL functions. The stability of these chemicals is an important feature of their agricultural use. Boyer et al. (2014) has developed stable SL mimics by substituting the D-ring with a dimethylbutenolide moiety, but its structure is not very different from that of the D-ring. For almost all the chemicals that mimic SL activities, a D-ring or its derivative is necessary for their SL-like activities. The single possible exception may be CTL (Tsuchiya et al., 2010; Toh et al., 2014), which has no butenolide moiety in its molecule but shows SL-like activity. Although its potency in binding D14 and inducing the D14–D53 and D14–DELLA interactions is not yet clear, the investigation of these characteristics will be interesting.

In addition to the trials described above, we anticipate that the protein–protein interactions necessary for SL signaling may be alternative and efficient targets for the design of novel inhibitors of SL functions. There are many examples of the pharmacological screening of inhibitors of protein–protein interactions (Wilson, 2009), although inhibitors of such interactions are not common among PGRs.

It has been reported that petunia DAD2, a petunia homolog of D14, interacts with petunia MAX2 (Hamiaux et al., 2012) and that *Arabidopsis* MAX2 interacts with BES1 and BZR1 (Wang et al., 2013), which are major brassinosteroid signaling factors. Therefore, the application of inhibitors of SL receptors might have pleiotropic effects on plant growth. In this context, protein–protein interactions, such as the SL-dependent D14–D53 interaction, will be good targets for the regulation of plant growth by chemicals.

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Validated method for phytohormone quantification in plants

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Phytohormones are long time known as important components of signaling cascades in plant development and plant responses to various abiotic and biotic challenges. Quantifications of phytohormone levels in plants are typically carried out using GC or LC-MS/MS systems, due to their high sensitivity, specificity, and the fact that not much sample preparation is needed. However, mass spectrometer-based analyses are often affected by the particular sample type (different matrices), extraction procedure, and experimental setups, i.e., the chromatographic separation system and/or mass spectrometer analyser (Triple-quadrupole, Iontrap, TOF, Orbitrap). For these reasons, a validated method is required in order to enable comparison of data that are generated in different laboratories, under different experimental set-ups, and in different matrices. So far, many phytohormone quantification studies were done using either QTRAP or Triple-quadrupole mass spectrometers. None of them was performed under the regime of a fully-validated method. Therefore, we developed and established such validated method for quantification of stress-related phytohormones such as jasmonates, abscisic acid, salicylic acid, IAA, in the model plant *Arabidopsis thaliana* and the fruit crop *Citrus sinensis*, using an Iontrap mass spectrometer. All parameters recommended by FDA (US Food and Drug Administration) or EMEA (European Medicines Evaluation Agency) for validation of analytical methods were evaluated: sensitivity, selectivity, repeatability and reproducibility (accuracy and precision).

Keywords: phytohormones, HPLC-MS/MS, quantification, *Arabidopsis thaliana*, *Citrus sinensis*, iontrap

INTRODUCTION

Phytohormones constitute a distinct class of signaling molecules in plants. They can be classified according to their chemical structure—jasmonates [jasmonic acid (JA) and derivatives 12-oxo-phytodienoic acid (OPDA)], auxins (in particular indole-3-acetic acid, IAA), cytokinins, gibberellins, abscisic acid (ABA), salicylic acid (SA), brassinosteroids, ethylene—or according to their biological function—regulator of plant growth, development and reproduction or mediators during biotic and abiotic stresses (Santner and Estelle, 2009).

Frequently these molecules act at low concentrations and play key roles in ecological interactions between plants and other organisms (Pozo et al., 2005; Pieterse et al., 2009; Santner et al., 2009) Therefore, quantification of phytohormones is an essential step to understand their functions in plant metabolism and ecological interactions. While the first highly sensitive methods for quantitative phytohormone analyses relied on immunoassays (Weiler, 1984), in the last 15–20 years many methods have been developed for quantification of these compounds, particularly using hyphenated techniques such as GC-MS (Kowalczyk and Sandberg, 2001; Müller et al., 2002, 2006; Engelberth et al., 2003) and LC-MS (Wilbert et al., 1998; Forcat et al., 2008; Pan et al., 2008, 2010; Müller and Munné-Bosch, 2011; Balcke et al., 2012; Liu et al., 2012).

These techniques provide a powerful analytical tool for quantifying secondary metabolites in plant tissues, especially due to their high sensitivity, specificity and reproducibility. However, different approaches might be adopted depending on the separation method (GC or HPLC) and the spectrometer (triple quadrupole, iontrap, TOF) applied during the quantification studies. Moreover, mass spectrometry analyses are strongly influenced by other compounds present in the plant tissues which can suppress or increase the analyte ionization, a fact that is often not considered. Hence, the matrix effect and several other parameters (like analyte stability and recovery) must be controlled during a quantification study and validation strategies should be employed in order to produce reliable analytical methods for quantification of plant metabolites.

Several papers and reviews covering validation of analytical methods have been published (Shabir, 2003; Bliesner, 2006; Chandran and Singh, 2007). As a rule, these papers describe important parameters such as accuracy, precision (repeatability and intermediate precision), specificity, detection and quantification limits, linearity, range, robustness, etc. All this set of

information should be obtained in the same laboratory as a part of repeatability assays. However, for proceeding with reproducibly assays an inter-laboratory experiment is often necessary. Collaborative trials are used to test the performance (generally the precision) of the analytical method demonstrating that it can be used in more than one laboratory, producing reliable and true results (Hund et al., 2000).

In this present paper we describe the development and inter-laboratory validation of an analytical method for quantification of six phytohormones—the auxin indole-3-acetic acid (IAA), ABA, JA, isoleucine jasmonic acid conjugate (JA-Ile), SA, and 12-oxo phytodienoic acid (OPDA)- in *Arabidopsis thaliana* and *Citrus sinensis* using an iontrap mass spectrometer.

MATERIALS AND METHODS

REAGENTS AND STANDARDS

All solvents used during extraction procedures were analytical grade except for methanol (MeOH). Chromatographic separation was carried out using MeOH HPLC grade purchased from Roth (Carl Roth GmbH, Germany) or J. T. Baker (Xalostoc, Mexico). IAA (purity > 99%), ABA (purity > 99%) and SA (>98%) were purchased from Sigma-Aldrich. 12-oxo phytodienoic acid were purchased from Cayman (Biomol GmbH, Hamburg, Germany). JA was synthesized by saponification of commercially available methyl-JA. Jasmonic acid isoleucine conjugate (JA-Ile) was synthetized according to Kramell et al. (1988). Deuterated standards: [^{2}H5] indole-3-acetic acid (d5-IAA), [^{2}H4] salicylic acid (d4-SA) and [^{2}H6] (+)-cis, trans-abscisic acid (d6-ABA) were purchased from OlChemIm Ltd (Olomouc, Czech Republic) and jasmonic-d5 acid 2,4,4-d3 acetyl-2,2-d2 (d5-JA) was purchased from CDN isotopes (Quebec, QC, Canada).

APPARATUS

HPLC-MS/MS analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) connected to a LTQ Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Chromatographic separation was carried out in a Luna Phenyl-Hexyl column (150 × 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany). Formic acid (0.05%, v/v) and MeOH with 0.05% (v/v) of formic acid were employed as mobile phases A and B, respectively. The elution profile was: 0–10 min, 42–55% B in A; 10–13 min, 55–100% B; 13–15 min 100% B; 15–15.1 min 100–42% B in A; and 15.1–20 min 42% B in A. The mobile phase flow rate was 1.1 mL/min. Injection volume was 25 μL. The LTQ mass spectrometer was equipped with an Electrospray ionization source, operating in the negative and positive ion modes. Negative measurements were carried out using the following ionization parameters: source voltage: 4.4 kV, capillary voltage: −48 V, tube lens −113 V, declustering potential 10 V, turbo gas temperature: 300°C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min. For positive analyses ionization parameters were set at: source voltage: 4.2 kV, capillary voltage: 29 V, tube lens 45 V, declustering potential 10 V, turbo gas temperature: 300°C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min.

Selected reaction monitoring (SRM) experiments were used to monitor specific precursor ion → product ion transitions for each phytohormone and internal standard. Collision energy, precursor ion isolation width and activation Q were optimized for each compound separately.

During the inter-laboratory reproducibility, the analyses were performed in an Acquity HPLC (Waters Co.) coupled with Quattro Premier XE (Micromass Technology) mass spectrometer, using a Luna Phenyl-Hexyl column (150 × 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany) and the same elution conditions mentioned above. The ionization parameters used during these analyses were: In negative mode (capillary: 3.4 kV, extractor 3 V, source temperature 110°C, desolvation temperature 350°C, desolvation gas flow: 800 L/h, cone gas flow: 10 L/h), and in positive mode (capillary: 3.4 kV, extractor 3V, source temperature 110°C, desolvation temperature 350°C, desolvation gas flow: 800 L/h, cone gas flow: 10 L/h). Cone voltage and collision energy were optimized for each compound individually.

PLANT MATERIAL

A. thaliana was cultured for 4 weeks under short day conditions (10 h light/14 h dark photoperiod), 40% humidity and 23°C. After harvesting, plants were immediately frozen in liquid nitrogen and ground in a GenoGrinder (SPEXSample Prep, München, Germany) for 2 × 30 s at 1500 rpm. After homogenization, 100 mg of plants were weighted into 1.5 mL tubes and stored at −80°C until the measurements.

C. sinensis was cultured in a greenhouse (Araraquara, Brazil) under normal light conditions and temperature average of 26°C (day) and 18°C (night). Light green leaves from small trees were collected, immediately frozen in liquid nitrogen and ground in a mortar. After homogenization, 100 mg of frozen plant material were weighted into 1.5 mL tubes and stored at −80°C until the measurements.

PHYTOHORMONES EXTRACTION AND ANALYSIS

Optimization of phytohormones extraction

Two parameters were evaluated during the optimization of phytohormones extraction: composition of extraction solution and type of plant samples (fresh or dry material). Initially tubes containing 100 ± 1 mg of plant material were either kept at −80°C or dried overnight in a freeze drier at −42°C. The extraction was performed adding 1.0 mL of either ethyl acetate, dichloromethane, isopropanol, MeOH or MeOH:water (8:2) into each tube containing dry or fresh plant material. Samples were shaken for 30 min in the Starlab shaker and centrifuged at 16,000 g and 4°C for 5 min. The supernatant was transferred into a new 1,5 micro-centrifuge tube and dried in speed vac. After drying, 100 μL of MeOH were added to each sample, homogenized under vortex and centrifuged at 16,000 g and 4°C for 10 min. The supernatant was analyzed by HPLC-MS/MS.

In a second set of analyses, the influence of both MeOH:water ratio and addition of acid in the extraction mixture was evaluated. The extraction procedure was performed as described above using 3 different MeOH:water ratios (7:3, 6:4, and 1:1) pure, or containing 0.2% of HCl.

Preparation of standards solutions

Stock solutions of each original phytohormone standard were prepared at 1 mg/mL in MeOH. For deuterated

compounds, stock solutions were prepared in acetonitrile at 100 μg/mL.

Working solutions of original phytohormones standards were prepared diluting stock solutions in MeOH:water (7:3), at different concentration for each phytohormone depending on the range of the calibration curve: ABA and IAA (100 μg/mL), JA and SA (200 μg/mL), OPDA (50 μg/mL), and JA-Ile (40 μg/mL).

The internal standard stock solutions (d5-JA, d6-ABA, d4-SA, and d5-IAA) were combined and diluted (final concentration 10 ng/mL for d4-SA and d5-IAA and 20 ng/mL for d5-JA and d6-ABA) with MeOH:water (7:3) yielding the extraction solution.

Final method for phytohormones extraction

Tubes containing 100 mg of fresh and ground plant material were kept at −80°C, and transferred to liquid nitrogen before the extraction. The samples were removed from the liquid nitrogen and 1 mL of extraction solution containing the internal standards (d5-JA, d6-ABA, d5-IAA, and d4-SA), prepared as described in Preparation of Standards Solutions, were directly added. The samples were briefly mix with a vortex, and spiked with phytohormones standards as described in Method Validation to generate the calibration curve and quality control (QC) samples. The spiked samples were shaken for 30 min in the Starlab shaker and centrifuged at 16,000 g and 4°C for 5 min. The supernatant was transferred into a new 1,5 micro-centrifuge tube and dried in speed vac. After drying, 100 μL of MeOH were added to each sample, vortexed and centrifuged at 16,000 g and 4°C for 10 min. The supernatant was analyzed by HPLC-MS/MS.

METHOD VALIDATION

Limit of detection and limit of quantification

The limits of detection (LOD) and quantification (LOQ) for analytical methods based on HPLC analysis can be expressed in response units (signal-to-noise levels). Usually LOD is established using matrix samples spiked with the low amount of standards. However, as none analyte-free matrix was available the LODs were determined in solvent as three times the noise level.

For each matrix, LOQs were defined according to the amount of phytohormones present in 10 independent blank samples, which were extracted as described in Optimization of Phytohormones Extraction. For all the LOQ the signal-to-noise ratios were higher than 10.

Calibration curve and linearity

The calibration curves were prepared in matrix using three different spiking solutions: spiking solution A containing ABA (at 4, 8, 40, 100, 200, 1000, 3000, and 4000 ng/mL), IAA (2, 4, 20, 50, 100, 500, 1000, 2000 ng/mL), and JA-Ile (0.8, 1.6, 8, 20, 40, 200, 400, and 800 ng/mL); spiking solution B containing SA (at 50, 100, 200, 500, 1000, 2000, 4000, and 8000 ng/mL) and JA (at 25, 50, 100, 250, 500, 1000, 2000, and 4000 ng/mL); and spiking solution C containing OPDA (at 500, 1000, 2000, 4000, 6000, 7000, 8000, and 10,000 ng/mL). All spiking solutions were prepared (in MeOH:water, 7:3) by serial dilution of working solutions.

Samples for calibration curve were prepared adding 50 μL of each spiking solution (A, B, and C) into the tubes containing 100 mg of ground fresh plant material and extracted as described in Preparation of Standards Solutions. For a flow sheet see **Scheme 1** (Supporting Material).

Quality controls

QC were used to assess the method's accuracy and precision. QCs were prepared spiking plant material with three different levels of each phytohormone (low, medium and high; **Scheme 1**, Supporting Material).

High quality controls (HQC) were prepared spiking 100 mg of plant material with 50 μL of: high spiking solution A (containing 2800 ng/mL of ABA and IAA and 280 ng/mL of JA-Ile); high spiking solution B (containing 5600 ng/mL of SA and 2800 ng/mL JA) and high spiking solution C (containing 2800 ng/mL of OPDA). Medium quality controls (MQC) were prepared spiking 100 mg of plant material with 50 μL of: medium spiking solution A (containing 700 ng/mL of ABA and IAA and 140 ng/mL of JA-Ile); medium spiking solution B (containing 2800 ng/mL of SA and 1400 ng/mL of JA) and medium spiking solution C (containing 1400 ng/mL of OPDA). And low quality controls (LQC) were prepared spiking 100 mg of plant material with 50 μL of: low spiking solution A (containing 14 ng/mL of ABA and IAA, and 2.8 ng/mL of JA-Ile); low spiking solution B (containing 280 ng/mL of SA and 140 ng/mL of JA) and low spiking solution C (containing 450 ng/mL of OPDA). All QC were prepared in quintuplicates.

Recovery

Recovery was calculated comparing the amount of each phytohormone present in spiked/extracted and extracted/spiked QC. The spiked/extracted QC were prepared as described in Quality Controls. The extracted/spiked samples were spiked with 150 μL of MeOH:water (7:3)—simulating the addition of spiking solutions—and extracted as described in Preparation of Standards Solutions. The dry residues were reconstituted in MeOH containing the final concentration of each phytohormone, which corresponds to half of spiking solution concentration.

Validation in Citrus sinensis

Linearity, reproducibility, recovery, and matrix effects were also evaluated for quantification of phytohormones in leaves of orange, *C. sinensis*. Initially, 10 samples were analyzed to establish the basal level of the six phytohormones in *C. sinensis* tissues. Due to the high content of IAA and ABA and low content of OPDA, the range of calibration curves and QC levels were adjusted to better fit to the new matrix.

The calibration curves were prepared in matrix using three different spiking solutions: spiking solution A contained ABA (at 4, 8, 40, 100, 200, 1000, 3000, and 4000 ng/mL), and JA-Ile (0.8, 1.6, 8, 20, 40, 200, 400, and 800 ng/mL); spiking solution B containing SA (at 50, 100, 200, 500, 1000, 2000, 4000, and 8000 ng/mL), JA (at 25, 50, 100, 250, 500, 1000, 2000, and 4000 ng/mL) and IAA(25, 50, 100, 250, 500, 1000, 2000, and 4000 ng/mL); and spiking solution C contained OPDA (at 60, 120, 240, 480, 640, 800, 1000, and 1200 ng/mL). All spiking solutions were prepared (in MeOH:water, 7:3) by serial dilution of working solutions. Samples for calibration curves were prepared adding 50 μL of each spiking solution (A, B, and C) into the tubes containing 100 mg of ground fresh plant material and extracted as

described in Preparation of Standards Solutions. For a flow sheet see **Scheme 1** (Supporting Material).

HQC were prepared by spiking 100 mg of plant material with 50 μL of: high spiking solution A (containing 2800 ng/mL of ABA and 280 ng/mL of JA-Ile); high spiking solution B (containing 5600 ng/mL of SA and 2800 ng/mL JA and IAA) and high spiking solution C (containing 840 ng/mL of OPDA). MQC were prepared spiking 100 mg of plant material with 50 μL of: medium spiking solution A (containing 700 ng/mL of ABA and 140 ng/mL of JA-Ile); medium spiking solution B (containing 2800 ng/mL of SA and 1400 ng/mL of JA and IAA) and medium spiking solution C (containing 600 ng/mL of OPDA). And LQC were prepared spiking 100 mg of plant material with 50 μL of: low spiking solution A (containing 14 ng/mL of ABA and 2.8 ng/mL of JA-Ile); low spiking solution B (containing 280 ng/mL of SA and 140 ng/mL of JA and IAA) and low spiking solution C (containing 90 ng/mL of OPDA). All QC were prepared in quintuplicates.

Recovery of phytohormones in *C. sinensis* samples was evaluated for the QC samples as described in Recovery.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

Optimization of ion trap parameters for quantification of phytohormones

Due to their high sensitivity, specificity, and the fact that not much sample preparation is necessary, HPLC-MS/MS experiments, especially those involving SRM, are used as reference for quantitative analyses. These also include phytohormone quantifications.

SRM experiments are based on two stages of ion selection. The precursor ion (a protonated or deprotonated target molecule) is selected in the first stage of tandem mass spectrometer, fragmented under a controlled process, thereby generating a specific fragment ion, which is then selected in the second stage of tandem mass spectrometer. Hence, the specificity of SRM experiments relies upon the choice of a specific precursor-fragment ion transition, while the sensitivity depends on the yield and stability of both precursor and fragment ions (Kowalczyk and Sandberg, 2001). Moreover, selection of precursor and fragment ions as well as fragmentation mechanism occurs in different ways for distinct mass spectrometers (triple quadrupole, ion trap, time of flight). Therefore, different approaches and parameters optimization are needed depending on which kind of detector is used in the SRM experiments.

Quantification of phytohormones in plant tissues has been so far carried out using either triple quadrupole or Q-trap instruments (Forcat et al., 2008; Pan et al., 2008, 2010; Balcke et al., 2012; Liu et al., 2012), which are well known for their high performance in SRM experiments (Rousu et al., 2010; Tanaka et al., 2011) Ion trap mass spectrometers, on the other hand, are widely available due to their high versatility, capability of doing MS^n, and for its low cost compared with triple quadrupole, which make it an attractive option for compound identification, screening and qualitative analyses. However, they present specific challenges for quantification experiments, since the scan speed and fragmentation mode do not fit the best with SRM experiments. Therefore, many parameters must be carefully adjusted in order to reach good sensitivity in ion trap mass spectrometers, specially the injection time and activation Q (Evans et al., 2000).

During the present work all parameters for ionization, fragmentation and detection of phytohormones (ABA, IAA, SA, JA, JA-Ile, and OPDA) were optimized, in order to achieve good sensitivity and selectivity in an ion trap mass spectrometer. The values of precursor ion isolation width (ISO), collision energy (CID) and activation Q (Act Q) that presented the best sensitivity and the more stable signals for each phytohormone are shown in **Table 1**. Activation Q must be adjusted before choosing the product ion, since it determines the range of product ions that can be generated. Modification in the default value (0.250 for the equipment used in this work) can provide new fragment ions, which can be interesting for quantification (stable and with high intensity). Injection time for all SRM transitions was 100 ms.

Optimization of phytohormones extraction

The efficiency of phytohormones' extraction was evaluated for both dry and fresh plant material using different organic solvents/mixtures [acetate, dichloromethane, isopropanol, MeOH, MeOH:water (8:2), MeOH:water (7:3), MeOH:water (6:4), MeOH:water (1:1)]. The influence of acidification by hydrogen chloride in the phytohormone extraction was also tested. The results are presented in Figure A (Supplementary Material). When the extraction is performed using non-polar organic solvents (ethyl acetate and dichloromethane) there is a clear difference in the extraction efficiency between fresh and dry material. However, for polar and aqueous mixtures such difference decreased drastically. Mixtures of MeOH and water provided higher extraction efficiency for all phytohormones. Here, the ratio of 7:3 was chosen as extraction solution due to its good performance in extracting the phytohormones and the low content of chlorophyll present in the final sample.

During the evaluation of method repeatability, the concentration of OPDA in the QC samples did not fit with the added amount. The concentration present in the QC was always higher than expected. After more detailed analyses it was observed that

Table 1 | Fragmentation parameters for the phytohormones.

	Precursor ion (m/z)	ISO* (Da)	CID** (V)	Act Q***	Fragments (Da)
ABA	263.0	2.0	30	0.250	152.0–154.0
d6-ABA	269.0	2.0	30	0.250	158.0–160.0
IAA	176.0	2.0	20	0.250	129.0–131.0
d5-IAA	181.0	2.0	20	0.250	134.0–136.0
JA	209.0	1.0	25	0.210	58.0–60.0
d5-JA	214.0	1.0	25	0.210	61.0–63.0
JA Ile	322.0	2.0	30	0.250	129.0–131.0
OPDA	291.0	2.0	18	0.250	164.0–166.0
SA	137.0	1.0	28	0.250	92.0–94.0
d4-SA	141.0	2.0	28	0.250	96.0–98.0

**Precursor ion isolation window; **Collision-induced dissociation energy; ***Activation Q.*

such issue occurred due to the increase of OPDA content in the plant samples during the sample preparation. Actually, all the samples were put on ice, spiked with internal standards and extracted by addition of extraction solution containing the internal standards. As the QC were prepared after the calibration curve samples, the increment in OPDA content in the QC was bigger than in the calibration curve samples. Therefore, the changes in the OPDA content in the plants samples were evaluated while QCs were kept on ice. For this purpose, 18 tubes containing 100 mg of fresh and ground plant tissues were transferred from liquid nitrogen onto ice. The OPDA concentration was evaluated for samples kept on ice for 0, 5, 10, 15, 30, and 45 min. For each point, three tubes containing plant material were removed from ice and added with 1.0 mL of extraction solution. The extraction was carried out as described in Final Method for Phytohormones Extraction. The graphs present in **Figure 1** shows the changes in OPDA content.

These data suggest that OPDA content varies quickly in the wounded/ground plant tissues even when the samples are kept on ice. After 5 min it increased by 50% and the amount doubled within the first 10 min. This might be due to remaining enzyme activities releasing lipid-bound OPDA from plastids localized galactolipids, which are well known for Arabidopsis (Stelmach et al., 2001). These results showed clearly the importance of keeping plant tissues frozen as long as possible, even during weighting and before adding the extraction solvents. Therefore, samples must be maintained at very low temperature (−80°C or liquid nitrogen) before the extraction, and the extraction solvent must be added immediately after removing the samples from such conditions.

These results also highlight the importance of the validation studies for quantification methods, since many parameters involved in the extraction and analysis cannot be proper addressed when statistical figures are not evaluated. In this way, the use of QC as defined in validation protocols can be of great value even during method development. For this reason, validation of each assay or test method should be performed on a case-by-case basis, to ensure that the parameters are appropriate for the method's intended use.

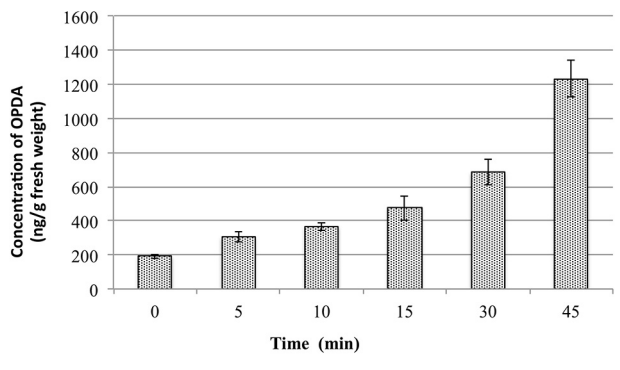

FIGURE 1 | Changes in OPDA content in *Arabidopsis thaliana* leaf samples depending on the time the samples were kept on ice.

VALIDATION OF ANALYTICAL METHOD

The validation studies are conducted in order to demonstrate that the analytical method is applicable for the aimed purpose and to ensure that the obtained values are close to the unknown content of the analyte present in real samples (EMEA, 2006; González and Herrador, 2007; European Commission, 2010). In this work, we evaluated the selectivity/specificity, limits of detection and quantification, linearity, recovery, repeatability and reproducibility of analytical method for quantification of phytohormones in *A. thaliana* and *C. sinensis* tissues.

Selectivity, limit of detection and quantification

Selectivity is defined as the ability of quantification method to discriminate the analyte from the other sample components, giving pure, symmetric and resolved peaks (Green, 1996). For methods which include chromatographic separation, selectivity can be assessed by chromatographic resolution, evaluating whether the peak relative to the analyte is separated from the other peaks present in the matrix. When no blank matrix is available, the selectivity can also be assessed comparing the MS/MS spectrum related to the analyte present in the matrix with the MS/MS spectrum of original standard. If there is no additional peaks MS/MS spectrum for the band correspondent to the analyte in the matrix comparing to MS/MS spectrum of original standards, it suggests that the method is selective.

Therefore, the present method is considered selective/specific for the phytohormones quantification, since the SRM chromatograms present in Figure B (Supplementary Material) contain either only one or well-resolved peaks for all phytohormones. For JA, JA-Ile, and OPDA the peaks are very symmetric and sharp (width less than 30 s). Although for IAA and SA the peaks are broader and not symmetric, the selectivity of the method was also confirmed by the very similar profile of MS/MS spectra related to these bands (**Figure 2**) and the original standards prepared in solvent (Figure B of Supplementary Material).

The limit of detection (LOD) is the lowest analyte concentration, which can be distinguished from the noise in blank samples [it is defined as a concentration with signal/noise (S/N) of 3]. When no analyte-free matrix is available, the detection limit can be calculated in solvent (LOD of the equipment) or by dilution of matrix until reaching an S/N of 3. Since the dilution of the matrix also reduces the matrix effect, thereby not presenting huge advantages compared with the measurements in solvent, in the present work the LODs were evaluated for the HPLC-MS/MS system and the values are shown in **Table 2**.

The limit of quantification (LOQ) is defined as the lowest analyte concentration, which can be quantified precisely and accurately. According to EMA and FDA it corresponds to the concentration of analyte, which yield a peak with S/N of 10. However, as can be seen in **Figure 2**, the amount of every phytohormone in the blank sample yield peaks with S/N of at least 30. Therefore, it is not possible to calculate the LOQ using the conventional definition. For this reason the LOQ for this method was established as the lowest point of the calibration curve (**Table 2**). The SRM chromatogram of this point for every phytohormone is shown in the Figure C (Supplementary Material).

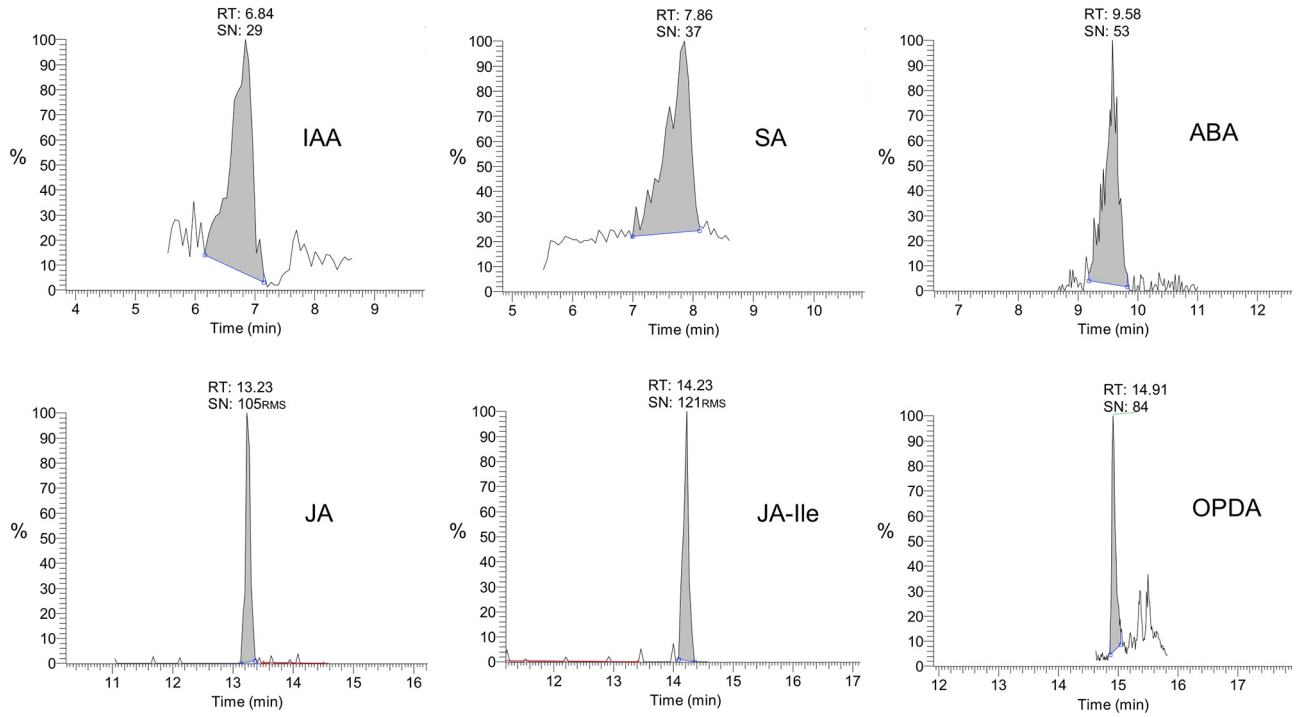

FIGURE 2 | Selected reaction monitoring (SRM) chromatograms for various phytohormones in *Arabidopsis thaliana* leaf extracts.

Table 2 | Parameters of calibration curve for each phytohormone: curve range, regression, weighting, correlation coefficient limit of quantification (LOQ) and amount of each phytohormone present in blank (untreated) *Arabidopsis thaliana* samples.

Analyte	Range (ng/g FW)	Curve*	R^2	LOQ (ng/g)	Amount in blank samples** (ng/g FW)	Matrix effect***
IAA	2–2000	Y = 0.0929916 + 0.0239565*X	0.992	2.0	7.13 ± 1.62	−31%
ABA	2–2000	Y = 0.0726676 + 0.0159863*X	0.998	2.0	5.32 ± 0.88	+11%
JA-Ile	0.4–400	Y = 0.146972 + 0.106572*X	0.993	0.4	1.64 ± 0.23	−25%
JA	12.5–2000	Y = 0.335095 + 0.00835023*X	0.997	12.5	41.32 ± 7.80	+7%
SA	25–4000	Y = 0.80327 + 0.00608683*X	0.989	25.0	123.59 ± 12.89	+46
OPDA	75–2000	Y = 3.03745 + 0.00598094*X	0.998	75.0	447.41 ± 57.21	−87%

**A weighting factor of $1/x^2$ was applied to all curves, except for OPDA, which used a factor of 1/x.*

***Values are average ± standard deviation. Concentrations represent the amount of each phytohormone in plant tissues (ng/g of fresh weight, FW), which is corresponding to the concentration (ng/mL) in the injection solution.*

****Values correspond to $((m_{matrix}/m_{solvent}) - 1)*100\%$.*

The S/N of the first calibration point for all phytohormones is much higher than 10, which is established as the minimum S/N ration for the LOQ. It proves the lowest calibration limit for all phytohormones is above to the LOQ of this method.

Calibration curve and linearity

The range of calibration curves was defined for each compound based on the amount of each compound present in the matrix (**Table 2**) and the changes that might occur during experiments. It is important that the calibration curves include the concentration of the phytohormones present in the blank (untreated control) samples, since it usually corresponds to the control in biological experiments. Hence, the analytical method must be suitable to quantify the amount of each phytohormone in control samples. Here it should be mentioned that the phytohormone concentrations measured in this study are in the same range as published by other groups (e.g., Müller et al., 2002; Pan et al., 2008).

Both correlation coefficient (R^2) and residual plots were used to evaluate the linearity of calibration curve for each phytohormone.

Homoscedasticity tests were performed in order to select the best weighting for the linear regression. In these tests, the residual of each point of calibration curve (difference between the calculated and theoretic values) is plotted against the concentration level. For an adequate regression model (regression and weighting) the residuals are normally distributed along the X-axis

(Almeida et al., 2002). To support the data shown in **Table 2**, **Figure 3** presents the residual plots for the best regression and weighting applied to the calibration curve of each phytohormone. For IAA, ABA, JA-Ile, JA, and SA the weighting factor that fits the best to the linear curve is $1/x^2$. For OPDA, it was 1/x. A linear regression was used in the calibration curve for all phytohormones. Thus, those factors and regression were applied in every analytical curve during the whole validation study.

MATRIX EFFECT

The matrix components can affect the analyte stability, extraction and ionization. As was shown above for OPDA, some enzymes present in wounded *A. thaliana* tissues can modify the basal concentration of OPDA, even when the tissues are kept at low temperature (4°C). In other cases, some enzymes can also degrade the analyte or modify the efficiency of analyte extraction. Moreover, for HPLC-MS/MS methods, some constituents of the matrix affect the efficiency of the analyte ionization, when both have the same retention time. In this case, the matrix interferes can either suppress the analyte ionization (decreasing the response) or enhance it (producing higher responses). The effects of matrix on quantitative methods are not completely understood and varies depending on both analyte and matrix composition.

During the validation, we evaluated the influence of *A. thaliana* constituents on quantification of every phytohormone, analysing the slope (m) of each calibration curve prepared

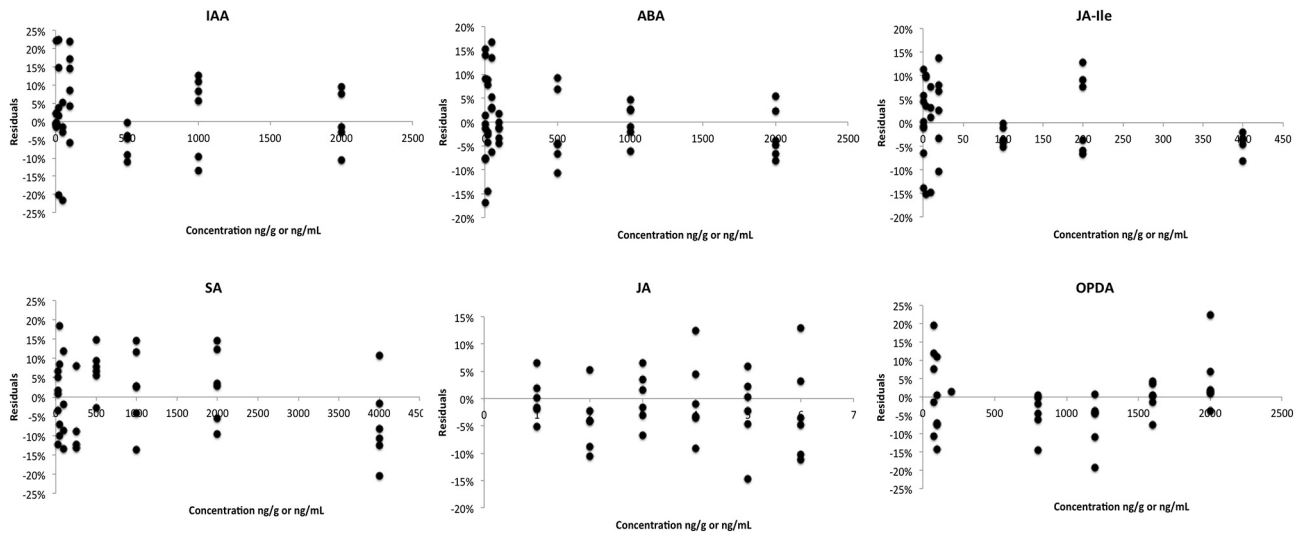

FIGURE 3 | Residual plot associated with the best regression and weighting for calibration curve of each phytohormone.

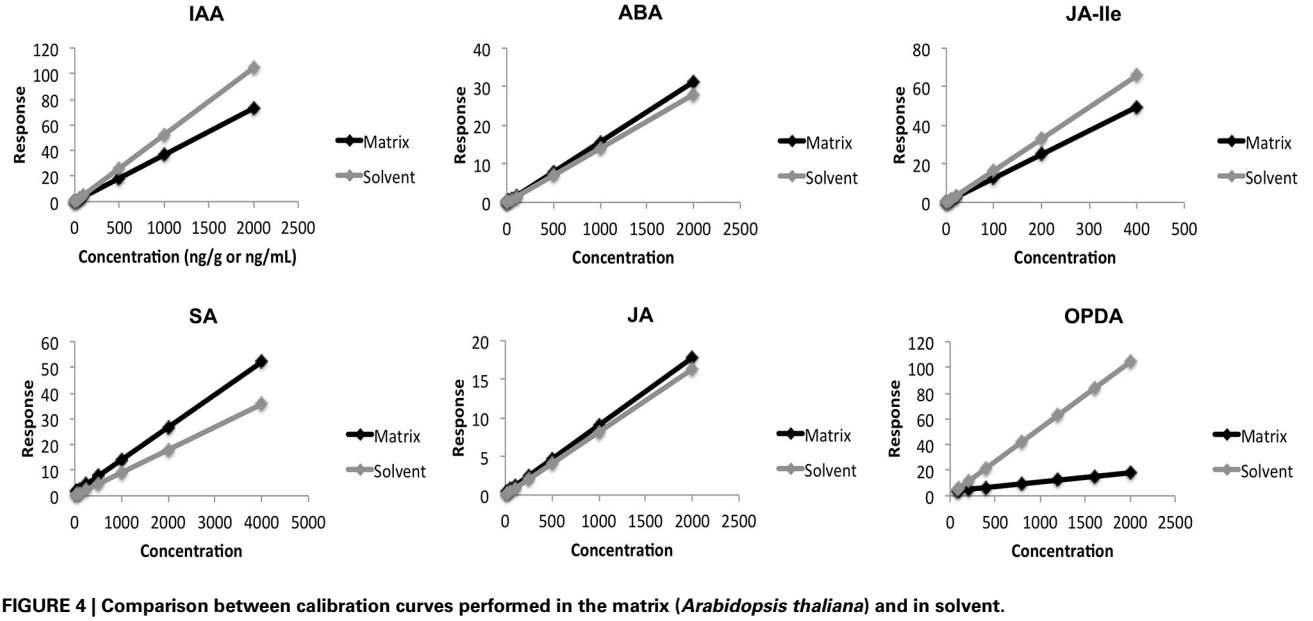

FIGURE 4 | Comparison between calibration curves performed in the matrix (*Arabidopsis thaliana*) and in solvent.

in both solvent and matrix. Comparison between these slopes ($m_{matrix}/m_{solvent}$) showed that the matrix has small influence for quantification of JA and ABA, +7% and +11%, respectively (**Table 2** and **Figure 4**). For the other phytohormones components present in *A. thaliana* affected the measurements in two different ways: increasing the response for SA (+46%) and decreasing it for JA-Ile (−25%), IAA (−31%), and OPDA (−87%). These data proved that the components present in the matrix can indeed influence the response of each analyte in different ways and intensities. Therefore, one of the most reliable ways to evaluate all the matrix effects on quantitative results is using quantification methods (calibration curve and QC) fully developed in the presence of the matrix.

Repeatability, within-laboratory reproducibility, and inter-laboratory reproducibility

Repeatability, within-laboratory reproducibility and inter-laboratory reproducibility (**Scheme 1**) were evaluated in order to define the method's accuracy and precision (EMA and European Commission). They were assessed by overall mean, standard deviation and coefficient of variation for three QC levels (low, medium and high) for independent samples prepared using three spiking solutions.

Repeatability was evaluated by standard deviation and coefficient of variation of three batches (curve and QC) prepared in the same day by the same analyst. While the within-laboratory reproducibility was evaluated comparing the mean, standard deviation (RSD), and coefficient of variation (error) obtained during repeatability measurements and those obtained for samples prepared by a second analyst. The error and standard deviation for practically all QC (low, medium, and high) of all phytohormones were below 15% (**Table 3**). It indicates that this method is precise and accurate for quantification of phytohormone when the measurements are performed in a same laboratory (same equipment, solvents and standards) even when the samples are prepared by different analysts.

Inter-laboratory reproducibility was assessed by collaborative study with Accert Chemistry and Biotechnology Inc., where three new batches of both calibration curve and QC samples were prepared and analyzed using the same extraction procedure as described for repeatability. The error and standard deviation are very low (<10%). It proves that this method is precise and accurate and, hence, it can be used in different laboratories for quantification of phytohormones in *A. thaliana* tissues in order to generate directly comparable data. It is important to highlight that all measurements (calibration curve and QC) must be done in the matrix and for each batch of real samples a calibration curve and the QC (quintuplicate) must be analyzed before samples in order to guarantee the accurateness of the results.

Table 3 | Values of repeatability, within-laboratory reproducibility and inter-laboratory reproducibility obtained during the validation of the method for quantification of various phytohormones (ABA, IAA, JA-Ile, SA, JA, and OPDA) in *Arabidopsis thaliana*.

	Expected conc. (ng/g FW)*	Repeatability *n*=3			Within laboratory reproducibility *n*=6			Inter-laboratory reproducibility *n*=9		
		Mean ± *SD*	RSD (%)	Error (%)	Mean ± *SD*	RSD (%)	Error (%)	Mean ± *SD*	RSD (%)	Error (%)
ABA	7.00	6.67 ± 0.59	8.91	−4.73	6.92 ± 0.56	8.11	−1.15	7.01 ± 0.31	4.37	0.21
	350.00	344.22 ± 11.30	3.28	−1.65	361.58 ± 26.76	7.40	3.31	359.20 ± 22.21	6.18	2.63
	1400.00	1331.25 ± 57.27	4.30	−4.91	1422.62 ± 144.62	10.17	1.58	1401.39 ± 122.85	8.77	0.10
IAA	7.00	7.12 ± 0.71	9.96	1.67	7.29 ± 0.47	6.43	4.09	7.36 ± 0.21	2.86	5.09
	350.00	313.07 ± 8.70	2.78	−10.55	326.16 ± 22.11	6.78	−6.81	332.73 ± 17.28	5.13	−4.94
	1400.00	1306.57 ± 63.12	4.83	−6.67	1378.70 ± 7.32	7.32	−1.52	1353.86 ± 105.62	7.80	−3.30
JA-Ile	1.40	1.57 ± 0.11	6.76	12.34	1.56 ± 0.10	6.36	11.76	1.54 ± 0.03	2.19	10.32
	70.00	70.30 ± 1.85	2.64	0.43	72.24 ± 4.02	5.56	3.20	71.04 ± 3.47	4.88	1.48
	280.00	273.76 ± 3.36	2.36	−2.23	290.30 ± 22.26	7.67	3.68	286.83 ± 22.10	7.71	2.44
SA	140.00	138.46 ± 16.48	11.9	−1.10	138.25 ± 11.59	8.38	−1.25	136.18 ± 3.54	2.60	−2.73
	1400.00	1367.10 ± 43.35	3.17	−2.35	1355.08 ± 84.25	6.22	−3.21	1321.77 ± 56.00	4.24	−5.59
	2800.00	2598.57 ± 112.39	4.32	−7.19	2667.01 ± 145.34	5.45	−4.75	2612.61 ± 139.18	5.33	−6.69
JA	70.00	73.62 ± 3.79	5.15	5.18	70.87 ± 5.63	7.95	−1.25	69.83 ± 3.36	4.82	−0.25
	700.00	691.744 ± 76.12	11.00	−1.18	690.70 ± 63.21	9.15	−1.33	674.38 ± 27.99	4.15	−3.66
	1400.00	1348.90 ± 59.33	4.40	−3.65	1371.20 ± 107.43	7.83	−2.06	1354.27 ± 44.22	3.27	−3.27
OPDA	225.00	220 ± 12.11	5.48	−1.78	233.74 ± 23.54	10.07	3.89	231.11 ± 17.02	7.36	2.71
	700.00	696.42 ± 105.51	15.15	−0.51	711.56 ± 76.76	10.79	1.65	685.69 ± 52.24	7.62	−2.04
	1400.00	1371.79 ± 187.81	13.69	−2.02	1450.72 ± 161.20	11.11	3.62	1411.55 ± 129.21	9.15	0.83

*Corresponds to ng/mL.

Recovery

As described in Recovery, recovery was determined by the ratio between the amount of each phytohormone present in spiked/extracted and extracted/spiked samples. The extracted/spiked samples contained all the matrix interferes and 100% of the phytohormones concentration, since the standards were not subjected to the extraction procedure. On the other hand, in the spiked/extracted samples, the standards were added to the plant samples and the whole extraction procedure was performed afterwards. Thus, the spiked/extracted samples mimicked what happened with the phytohormones during the extraction procedure. The values of recovery for the different QC are shown in the **Table 4**. The overall recovery corresponds to the mean of recovery in different levels. For IAA and ABA the recovery was high, nearly 100%. However, for OPDA the overall recovery was 67.95%. It proved that the matrix affects the recovery distinctly depending on the analyte and on the concentration level. It also shows the significance of performing the calibration curve in the matrix and of validating the analytical method, once the different recoveries were enclosed for the entire range of the calibration curve developed in the matrix.

Quantification of phytohormones in Citrus sinensis

In order to transfer this method to another plant, we choose one of the most the important fruit crops, *C. sinensis*. Thus, statistical parameters such as linearity, repeatability (accuracy and precision), matrix effect and recovery were also evaluated for quantification of phytohormones in leaves of this plant.

The basal level of each phytohormone is shown in **Table 5**. Both range of calibration curve and QC levels had to be modified in order to adjust the quantification method to the content of phytohormones present in citrus. As mentioned above it is important that the calibration curves include the concentration of the phytohormones present in the blank (untreated control) samples, since it usually corresponds to the control in biological experiments.

Linear regression was used for all phytohormones calibration curves with weighting of $1/x^2$ for IAA, ABA, JA-Ile, JA, and SA and 1/x for OPDA. Linearity was assessed by correlation factor (**Table 5**) and matrix effect corresponds to the ratio between the angular coefficient of calibration curve in matrix and in solvent.

This particular matrix (*C. sinensis*) had a small effect in the calibration curve for IAA, ABA, and SA. However, for OPDA, JA-Ile, and JA the matrix had a strong influence in the inclination of the calibration curves (**Table 5** and Figure D, Supplementary Material). The comparison between these results and those presented in **Table 2** (for *A. thaliana*) highlights the importance of performing the calibration curve in the presence of each individual matrix, since they interfere differently in the quantification of each phytohormone.

Table 4 | Percentage of recovery during the extraction of phytohormones in *Arabidopsis thaliana*.

Compound	% of Recovery (Mean + Error)			
	Low*	Medium*	High*	Overall average of recovery
IAA	88.94 ± 12.75	90.98 ± 15.64	97.09 ± 15.15	92.34 ± 4.24
ABA	98.60 ± 11.33	104.50 ± 7.48	105.48 ± 8.52	102.86 ± 3.72
JA-Ile	73.31 ± 13.18	80.99 ± 9.24	77.26 ± 8.50	77.19 ± 3.84
JA	85.35 ± 15.98	75.32 ± 5.71	75.65 ± 12.50	78.77 ± 5.70
SA	86.37 ± 9.31	86.72 ± 9.42	93.71 ± 9.39	88.93 ± 4.14
OPDA	80.05 ± 11.45	63.90 ± 10.97	59.89 ± 5.91	67.95 ± 15.70

**Corresponding to the concentrations given in Quality Controls.*

Table 5 | Parameters of calibration curve for each phytohormone: curve range, regression, weighting, correlation coefficient, limit of quantification (LOQ) and amount of each phytohormone present in blank *Citrus sinensis* samples.

Analyte	Range (ng/g FW)	Curve*	R^2	LOQ (ng/g)	Amount in blank samples** (ng/g FW)	Matrix effect***
IAA	25–4000	Y = 2.09382 + 0.0199062*X	0.989	25	111.47 ± 17.95	−16%
ABA	20–2000	Y = 3.463782 + 0.012662*X	0.994	20	262.07 ± 6.71	+2%
JA-Ile	0.4–400	Y = 0.176041 + 0.0702724*X	0.994	0.4	1.91 ± 0.10	+86%
JA	12.5–2000	Y = 0.74606 + 0.013142*X	0.987	12.5	54.32 ± 9.43	+147%
SA	25–4000	Y = 0.152181 + 0.00719421*X	0.998	25.0	29.60 ± 6.35	−4%
OPDA	30–600	Y = 1.41748 + 0.00743165*X	0.981	30	85.15 ± 1.49	−32%

**A weighting factor of $1/x^2$ was applied to all curves, except for OPDA, which used a factor of 1/x.*

***Values are average ± standard deviation. Concentrations represent the amount of each phytohormone in plant tissues (ng/g of fresh weight, FW), which is corresponding to the concentration (ng/mL) in the injection solution.*

****Values correspond to $((m_{matrix}/m_{solvent}) - 1)*100\%$.*

Table 6 | Values of repeatability (accuracy and precision) obtained during the validation of the method for quantification of various phytohormones (ABA, IAA, JA-Ile, SA, JA, and OPDA) in leaves of *Citrus sinensis*.

	Expected conc.(ng/g FW)*	Repeatability $n = 3$			% Recovery (mean + error)	Overall average of recovery
		Mean ± *SD*	RSD (%)	Error (%)		
ABA	70.00	73.13 ± 3.42	4.68	4.48	84.53 ± 13.04	82.77 ± 8.06
	350.00	369.09 ± 15.06	4.08	5.46	75.63 ± 8.08	
	1400.00	1479.93 ± 73.67	4.98	5.71	88.15 ± 4.83	
IAA	140.00	142.75 ± 9.55	6.69	1.97	55.65 ± 7.97	66.16 ± 8.51
	1400.00	1488.05 ± 23.62	1.59	6.29	70.29 ± 7.42	
	2800.00	3023.73 ± 164.79	5.45	7.99	72.54 ± 5.86	
JA-Ile	1.40	1.43 ± 0.17	11.87	2.38	39.74 ± 8.91	63.23 ± 10.90
	70.00	69.96 ± 2.74	3.92	−0.06	73.36 ± 7.59	
	280.00	300.15 ± 10.17	3.39	7.20	76.59 ± 5.82	
SA	140.00	160.94 ± 2.88	1.79	14.96	61.16 ± 5.56	73.95 ± 5.19
	1400.00	1611.94 ± 106.92	6.63	15.14	81.15 ± 7.58	
	2800.00	3205.07 ± 107.74	3.36	14.47	79.56 ± 3.75	
JA	70.00	76.17 ± 4.97	6.52	8.82	63.61 ± 8.97	75.59 ± 7.40
	700.00	747.05 ± 8.03	1.07	6.72	83.47 ± 7.73	
	1400.00	1496.15 ± 108.73	7.27	6.87	79.68 ± 4.81	
OPDA	90.00	90.67 ± 7.34	8.10	0.74	66.19 ± 10.58	71.00 ± 10.70
	300.00	303.39 ± 34.64	11.42	1.13	74.00 ± 9.77	
	420.00	450.81 ± 60.86	13.50	7.34	72.81 ± 6.57	

**Corresponding to the concentrations given in Validation in Citrus sinensis.*

Statistical parameters accuracy (error) and precision (RSD) were also evaluated for quantification of phytohormones in *C. sinensis* and the results are presented in **Table 6**. Basically, all values are lower than 15%, proving that this method is suitable for quantification of phytohormones in citrus.

Recovery was calculated by comparison between spiked/extracted and extracted/spiking samples as described in Recovery. In the same way as discussed for matrix effects, recovery depends on both matrix and the nature of each compound. Recovery of IAA, for example, is strongly different between *Arabidopsis* and *Citrus*. Therefore, to compare the content of phytohormone in different matrix both calibration curve and recovery must be evaluated in every individual matrix.

GENERAL COMMENTS

In the present work we developed and validated a reliable, precise and accurate method for quantification of six different phytohormones (IAA, ABA, SA, JA, JA-Ile, and OPDA) in tissues of two different plants, the model plant *A. thaliana* and the fruit crop *C. sinensis*. As it was possible to transpose the method to a second, independent laboratory, its applicability and reproducibility in different laboratory environments with different set-ups was successfully demonstrated. Moreover, we showed the significance of the validation of the analytical method for the understanding of analyte stability and the matrix effect in the different levels of the analyte concentrations and for different matrixes. This study shows that it is possible to reach comparable standards for phytohormone measurements, independent where the analyses are performed.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00417/abstract

Scheme 1 | Needs to establish a validated method.

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Peptides and small molecules of the plant-pathogen apoplastic arena

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Plants reside within an environment rich in potential pathogens. Survival in the presence of such threats requires both effective perception of, and appropriate responses to, pathogenic attack. While plants lack an adaptive immune system, they have a highly developed and responsive innate immune system able to detect and inhibit the growth of the vast majority of potential pathogens. Many of the critical interactions that characterize the relationship between plants and pathogens are played out in the intercellular apoplastic space. The initial perception of pathogen invasion is often achieved through specific plant receptor-like kinases that recognize conserved molecular patterns presented by the pathogen or respond to the molecular debris caused by cellular damage. The perception of either microbial or damage signals by these receptors initiates a response that includes the production of peptides and small molecules to enhance cellular integrity and inhibit pathogen growth. In this review, we discuss the roles of apoplastic peptides and small molecules in modulating plant-pathogen interactions.

Keywords: innate immunity, apoplastic immunity, small molecules, host-pathogen interactions, MAMP, PRR

INTRODUCTION

Plants are armed with a sophisticated array of preformed mechanical and chemical barriers to defend themselves against invasion and colonization by pathogens. The first line of plant defense is the physical barrier of the leaf cuticle, which covers the leaf epidermis and prevents invasion of leaf tissue by the viruses, bacteria and filamentous pathogens found on the leaf surface. The plant also protects both the leaf surface and apoplastic space with a host of constitutively produced defensive molecules collectively called phytoanticipins, which act to prevent pathogen colonization and infection (reviewed in González-Lamothe et al., 2009).

While these standing defenses are sufficient to prevent some disease, they are not capable of completely protecting the plant from parasitism. Many pathogens that are capable of bypassing these initial measures take up residence within the apoplastic space, which affords them a potentially protected and beneficial environment in which to reproduce. It is within this space where the fate of many host-pathogen interactions is determined.

The plant cell surface is decorated with a complex array of receptors tightly integrated with dedicated intracellular signaling pathways, all of which are coordinated to quickly perceive and respond to potential apoplastic invaders. This initial detection of invading microorganisms depends in large part on the apoplastic perception of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) expressed by the host plant. This basal response in plants is commonly termed MAMP-triggered immunity (MTI; also referred to as PAMP-triggered immunity and basal immunity; Nicaise et al., 2009). This basal immune response does not rely solely on the perception of MAMPs and therefore may more accurately be referred to as PRR-triggered immunity (PTI) as we will in this article. In addition to the direct perception of MAMPs, plants have also evolved a system through which they can indirectly monitor for pathogens through the perception of products of the pathogenic life-style. This can occur when lytic enzymes expressed by the pathogen or host degrade nearby cells and produce cellular debris. Specific components of these cellular remains can act as danger signals for the plant (Boller and Felix, 2009). Successful pathogens must overcome this basal immunity in order to establish an active infection, and many have evolved mechanisms to inhibit PTI through the translocation of effector proteins into host cells. The plant has in turn evolved nucleotide binding leucine-rich repeat (NLR) resistance proteins, which allow for the direct or indirect detection of the pathogen effectors. This secondary immunity is termed effector-triggered immunity (ETI) and is often accompanied by the hypersensitive response, a localized cell death that limits infection, as well as systemic acquired resistance (SAR), which protects distal tissues from subsequent infections (reviewed in Durrant and Dong, 2004). While ETI is generally a stronger immune response than PTI and is critical for the effective control of many pathogens, the triggering of ETI occurs within the plant cell and thus falls outside of the purview of this review. It is interesting to note that while PTI and ETI have been classified as separate phenomena, recent work has suggested that perhaps the two should be viewed instead as overlapping responses that differ in speed and amplitude (reviewed in Thomma et al., 2011).

Plant responses to pathogen challenge can be broadly divided into two areas; those that result in the direct killing or inhibition of the pathogen, and those that reinforce the immune response locally or act to prime immunity in distal tissues. Apoplastic

immunity has been the subject of a number of excellent reviews (Hoefle and Hückelhoven, 2008; Doehlemann and Hemetsberger, 2013; Stotz et al., 2014). In this review we will highlight studies of the peptides and small molecules produced by both pathogens and plants in the apoplastic space which mediate the relationship between the organisms.

INDUCIBLE CHEMICAL DEFENSES OF THE PLANT

IDENTIFYING THE INTRUDER – PERCEPTION OF EXOGENOUS MOLECULES

Microbe-associated molecular pattern perception is the dominant means by which apoplastic pathogens are recognized and PTI elicited. MAMPs are regions of highly conserved microbe-derived molecules that are recognized by host PRRs, and are therefore broadly analogous to immune epitopes. A wide range of MAMPs have been described from fungal, oomycete, and bacterial pathogens, which span molecular classes including oligosaccharides, lipids, and peptides (**Table 1** and **Figure 1**). Regardless of their source and nature, these molecular signatures provide a signal of potential pathogen attack to the host. Some MAMPs are perceived across large swaths of the plant kingdom, while perception of others is more phylogenetically restricted (Boller and Felix, 2009). Overall, MAMP-induced PTI plays a critical role in the control of pathogen success and has enormous potential to influence crop disease resistance and productivity. Meanwhile, the protection afforded to the plant through these epitopes provides a strong evolutionary pressure on the pathogen to avoid this recognition, resulting in numerous pathogenic strategies to avoid MAMP-perception.

Pattern recognition receptors are responsible for monitoring the apoplastic space for the presence of MAMPs. Upon MAMP detection, PRRs initiate signaling cascades that induce the cellular events associated with PTI. PRRs are cell surface receptors that typically consist of an extracellular MAMP-binding domain, a single transmembrane domain, and an intracellular serine/threonine kinase signaling domain (Zipfel, 2014). While the nature of the binding domain varies according to the chemical nature of the ligand, the peptide specific PRRs contain a series of leucine rich repeats (LRRs). PRRs are members of the receptor-like kinase (RLK) family, while the closely related receptor-like proteins (RLPs) have a similar structure, but lack the intracellular signaling domain (Wang et al., 2008). The *Arabidopsis thaliana* (hereafter *Arabidopsis*) genome contains a total of 216 LRR containing RLKs and 57 RLPs, suggesting a wide diversity of potential binding specificities and illustrating the importance of this system to the plant host (Shiu and Bleecker, 2001; Wang et al., 2008). In addition, many of these proteins are transcriptionally up-regulated upon MAMP treatment, further supporting their importance in governing and potentially amplifying a PTI-primed state (Zipfel et al., 2006). While there have been several recent advances toward the identification of novel MAMPs in various plant systems, it remains challenging to identify their cognate PRRs.

SIGNS OF INVASION – FLAGELLIN

In spite of great effort and interest, there are still relatively few examples of peptide MAMPs and corresponding receptors to be found in the plant literature (reviewed in Albert, 2013). The prototypical example is bacterial flagellin (FliC), which was first shown to elicit a defense response in treated tomato cells (Felix et al., 1999). As this represents the most complete description of a MAMP and its molecular mechanism of action, we will focus on it as a case study to illustrate how MAMPs and their cognate PRRs have been identified. We will also note recent advances in PTI research and highlight the molecular mechanisms of MAMP activity within the apoplast.

To effectively study the elicitors of plant immunity first requires a screening method to observe and quantitate their activity. The accumulation of phytoalexins within plant tissue was one of the first methods adopted to quantitate elicitor activity (Albersheim and Valent, 1978), and allowed novel elicitors to be identified from complex mixtures of pathogen molecules through biochemical means. The activity of FliC was first described in a similar manner, using the alkalinization of tomato cell culture medium to measure the elicitation activity of bacterial cells and lysates (Felix et al., 1999). Once activity was observed, biochemical purification was used to identify the protein responsible. In addition to phytoalexin production and extracellular alkalinization, there are now many well established assays that measure defense activation upon PTI induction. These include assays that measure oxidative burst (Keppler, 1989; Thordal-Christensen et al., 1997; Felix et al., 1999), deposition of callose and lignification to reinforce the plant cell wall (Eschrich and Currier, 1964; Bruce and West, 1989; Chapple et al., 1992), and induced pathogen resistance *in planta* (Zipfel et al., 2004). These techniques complement each other to give insight into the intensity and kinetics of the specific response to individual MAMPs.

The elicitation capacity of the FliC protein has been extensively studied, and the responsible region has been localized to the *N*-terminal 22 amino acids of the protein. This flg22 peptide is active at sub-nanomolar levels and induces alkalinization of the extracellular media and production of reactive oxygen species (ROS) and ethylene (Felix et al., 1999). Further studies have shown that flg22 treatment can also strongly induce callose deposition, up-regulate defense gene expression, and inhibit seedling growth (Gómez-Gómez et al., 1999; Zipfel et al., 2004). Most importantly, treatment of plants with flg22 protects against subsequent pathogen challenge, providing direct evidence that it drives an effective immune response *in planta* (Zipfel et al., 2004).

The identification and characterization of the flg22 epitope represents the pathogen contribution to this communication, with the plant providing the receptor used to decipher its message. The cognate *Arabidopsis* PRR that perceives flg22 in the apoplastic space is FLAGELLIN-SENSING 2 (FLS2), an RLK that binds directly to flg22 and mediates its cellular effects (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006). The search for FLS2 again serves as an excellent primer on the tools used to identify plant PRRs.

The first clue about the identity of the flg22 receptor came from the discovery that Ws-0, a naturally occurring *Arabidopsis* ecotype, is refractory to flg22 treatment. A genetic cross between Ws-0 and Col-0 (a flg22-sensitive ecotype) identified a locus required for flg22 perception (Gómez-Gómez et al., 1999). A forward genetic

Table 1 | Elicitors found in the apoplastic space.

Elicitor	Source	Receptor	Receptor type	Reference
Exogenous				
csp22	Bacterial cold shock protein	Unknown		Felix and Boller (2003)
elf18	Bacterial Elongation Factor Tu (EF-Tu)	EFR	LRR	Kunze et al. (2004), Zipfel et al. (2006)
flg22	Bacterial flagellin	FLS2	LRR	Felix et al. (1999), Gómez-Gómez et al. (1999) Gómez-Gómez and Boller (2000) Chinchilla et al. (2006)
Pep13	Oomycete transglutaminase	Unknown		Brunner et al. (2002)
CBD2synt	Oomycete cellulose-binding elicitor lectin (CBEL)	Unknown		Gaulin et al. (2006)
Peptidoglycan (PGN)	Bacterial cell wall (Gram positive)	Lym1, Lym3	LysM	Gust et al. (2007), Willmann et al. (2011)
Lipopolysaccharide (LPS)	Bacterial cell wall (Gram negative)	Unknown		Newman et al. (1995)
Chitin fragments	Fungal cell wall	CeBip, CERK1, AtCERK1	LysM	Felix et al. (1993), Kaku et al. (2006) Miya et al. (2007) Shimizu et al. (2010)
Beta Glucan (GE)	Oomycete cell wall	Beta Glucan Binding Protein (GBP)	Glycosyl hydrolase family	Albersheim and Valent (1978) Umemoto et al. (1997), Fliegmann et al. (2004)
Xylanase (EIX)	Fungal xylanase	EIX1/2	LRR	Bailey et al. (1990), Ron and Avni (2004) Bar et al. (2010)
Endogenous				
Cutin monomers	Plant cell wall	Unknown		Schweizer et al. (1996) Fauth et al. (1998)
Hydroxyproline-rich glycopeptides (HypSys)	Systemin Cytosolic plant protein	Unknown		Pearce et al. (2001)
Oligogalacturonides (OGs)	Plant cell wall	WAK1	EGF-like	Hahn et al. (1981), Brutus et al. (2010) Nothnagel et al. (1983)
*At*Peps	Cytosolic plant protein	PEPR1/PEPR2	LRR	Huffaker et al. (2006), Yamaguchi et al. (2006) Yamaguchi et al. (2010)
Systemin	Cytosolic plant protein	Unknown		Pearce et al. (1991)

approach was then used to isolate flg22-insensitive mutants from a pool of chemically mutagenized plants, allowing further mapping of the responsible locus (Gómez-Gómez and Boller, 2000). This work made use of the fact that seedlings grown in the presence of flg22 peptide in liquid culture show a characteristic inhibition of development that can be both visually inspected and quantified through the measurement of seedling fresh weight. This high-throughput screening technique provided the requisite power needed to screen the enormous numbers of mutants required to isolate the responsible gene. Only one gene present in the implicated locus resembled a plant resistance protein, and also contained a single mutation in all insensitive mutants (Gómez-Gómez and Boller, 2000). The evidence of direct interaction between radiolabelled flg22 peptides and FLS2 conclusively showed that FLS2 is indeed the receptor for flg22 (Chinchilla et al., 2006). Binding assays remain a key tool in PRR confirmation, but have also been used for the identification of novel PRRs (Zipfel et al., 2006).

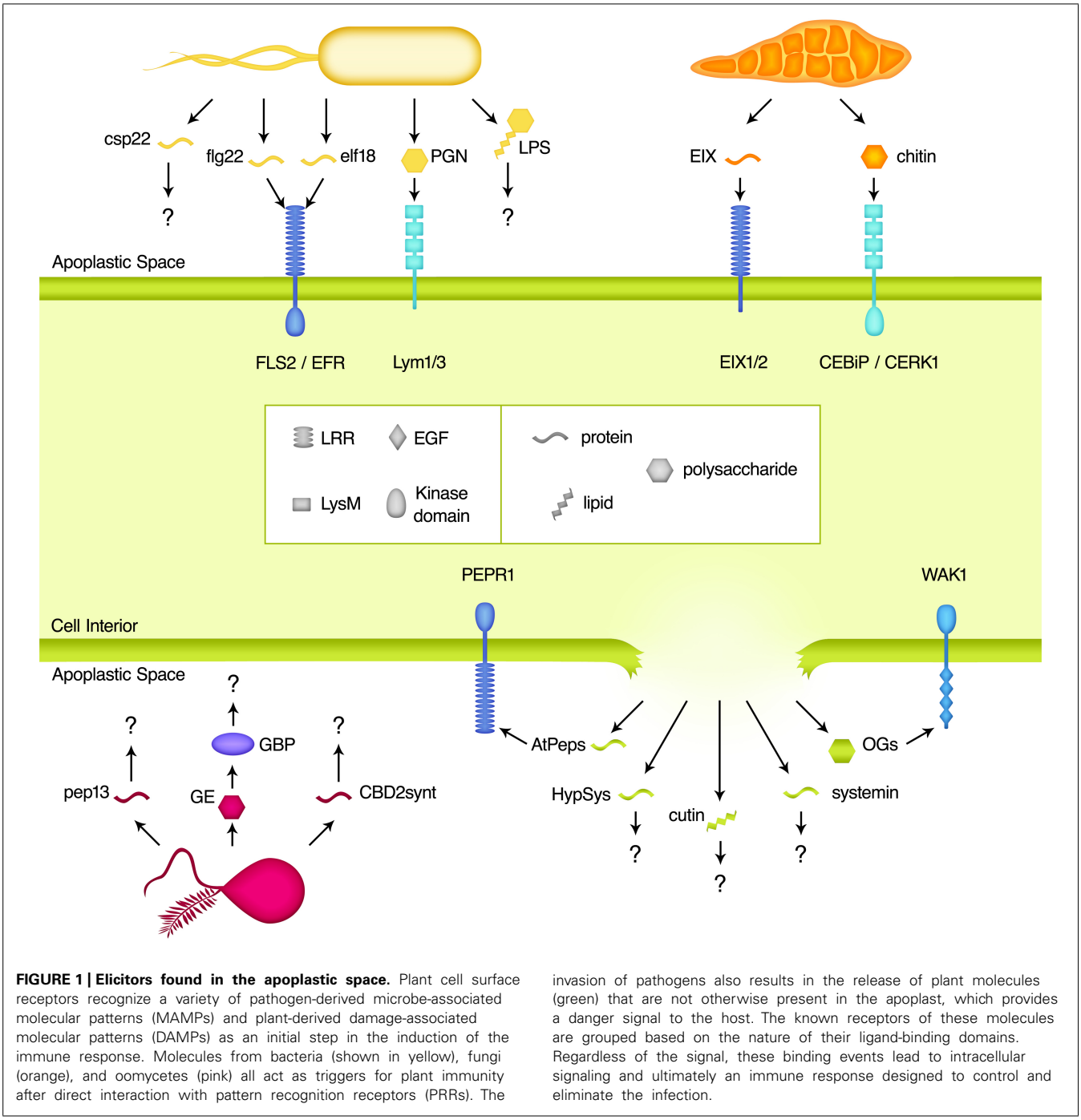

FIGURE 1 | Elicitors found in the apoplastic space. Plant cell surface receptors recognize a variety of pathogen-derived microbe-associated molecular patterns (MAMPs) and plant-derived damage-associated molecular patterns (DAMPs) as an initial step in the induction of the immune response. Molecules from bacteria (shown in yellow), fungi (orange), and oomycetes (pink) all act as triggers for plant immunity after direct interaction with pattern recognition receptors (PRRs). The invasion of pathogens also results in the release of plant molecules (green) that are not otherwise present in the apoplast, which provides a danger signal to the host. The known receptors of these molecules are grouped based on the nature of their ligand-binding domains. Regardless of the signal, these binding events lead to intracellular signaling and ultimately an immune response designed to control and eliminate the infection.

One such application of using a labeled peptide to identify an unknown receptor is found in the case of the *At*Pep1 peptide and its cognate receptor (Yamaguchi et al., 2006). Yamaguchi et al. (2006) used a labeled version of the peptide to identify a plant protein displaying a specific binding activity. Subsequent mass spectrometry analysis identified PEPR1 as the protein responsible for *At*Pep binding. In addition to a direct binding assay, this same research used the ectopic expression of PEPR1 to confirm the receptor identity. In this case, ectopic PEPR1 expression was used to confer sensitivity to *At*Pep1 elicitation in a normally refractory tobacco cell culture, thus confirming the receptor activity (Yamaguchi et al., 2006). One observed limitation to such an approach is the potential lack of conservation in the elicitor-induced signaling pathways across plant species. This can be overcome using a domain-swapping approach in which the extracellular elicitor-binding domain of the candidate receptor is fused to the intracellular signaling domain of a native receptor to induce novel elicitor responsiveness (Brutus et al., 2010).

The identification of MAMP/PRR pairs also allows for a thorough analysis of the binding reaction. Recently the crystal

structure of flg22 bound to FLS2 and the co-receptor BRASSINOSTEROID INSENSITIVE 1-associated kinase 1 (BAK1) has been solved (Sun et al., 2013). Interestingly, the flg22 peptide is bound by both BAK1 and the LRR repeats of FLS2, demonstrating that MAMP-binding is accomplished via interactions with both proteins of the receptor complex. These structural studies have also identified residues that determine binding specificity through both direct bond formation and by exerting steric constraints upon the complex. An example of such a structural requirement is the presence of a glycine residue at position 18 in flg22, where any other amino acid side-chain would create a steric conflict with BAK1 in that region (Sun et al., 2013). The details of the flg22-FLS2/BAK1 interaction also provide context to studies regarding the evolutionary mechanisms by which the pathogen can avoid perception and PTI induction by elucidating the mechanism by which these molecules interact.

The robust immune response that follows MAMP perception produces strong evolutionary pressures on the pathogen to avoid, dampen, or suppress this recognition. Multiple publications have shown that naturally occurring polymorphism within the flg22 epitope results in changes to the extent of PTI elicitation by peptides, suggesting that mutation of the flg22 epitope is an effective strategy to avoid PTI (Sun et al., 2006; Clarke et al., 2013). Interestingly, these examples show little variation of the critical flg22-FLS2/BAK1 interaction residues defined from the crystal structure (Sun et al., 2013). It will be fascinating to determine if variant residues that reduce flg22 perception also influence the flg22-FLS2/BAK1 complex and if so, how this polymorphism influences the MAMP-PRR complex interface. In addition to allelic variation, pathogens can also to suppress MAMP presentation by limiting their availability to the receptor. In the case of flg22, *Pseudomonas syringae* produces an alkaline protease (AprA) that degrades monomeric flagellin, thus denying the plant access to the MAMP and repressing PTI and enhancing pathogenicity (Pel et al., 2014).

Direct signatures of positive and negative selection can also be used to shed light on functionally important residues within MAMPs as well as identify previously unknown MAMPs. Positive selection, or selection for diversity, can be recognized by an excess of substitutions that change the amino acid sequence relative to substitutions that do not (e.g., neutral substitutions), while negative selection, or selective constraints, can be recognized by a deficiency of substitutions that change the amino acid sequence relative to neutral substitutions. While flg22 is under strong positive selection for residues that circumvent perception by FLS2, the flagellin protein as a whole is under strong negative selection to maintain its critical function. It has been shown that this function is required for bacterial viability and is conserved in the known allelic variants of the flg22 peptide (Clarke et al., 2013). McCann et al. (2012) used these opposing selective pressures to develop a computational methodology to identify novel MAMPs. Using comparative genomic data from six strains of *Pseudomonas syringae* and *Xanthomonas* spp., they identified over 50 highly conserved proteins that also showed a small number of individual amino acid residues under strong positive selection. In many of these cases, the positively selected residues were clustered along the protein sequence. Peptides spanning these regions were then synthesized and tested in a number of standard immunity assays, and ultimately shown to elicit PTI in *Arabidopsis*. Confirmation of these peptide elicitors as *bone fide* MAMPs awaits the identification of corresponding PRRs. A bioinformatics approach to MAMP identification overcomes an important limitation of biochemical analyses, namely that weak elicitors will be masked by more potent epitopes (such as flg22) limiting the identification of novel MAMPs. As another approach to overcome this, *Arabidopsis* plants lacking the FLS2 receptor were used to identify the elicitation activity of elongation factor Tu (EF-Tu, elf18; Kunze et al., 2004). However, the use of this genetic strategy becomes limiting with the discovery of each additional MAMP, favoring predictive methods in the future.

Identifying the cognate PRRs of MAMPs remains an important challenge of plant immunity research. Forward genetic screens to identify MAMP-insensitive plants have been a successful approach that will be enhanced in throughput by the advent of next generation mapping technologies. In addition, whole genome sequencing information can be used to predict all possible PRRs within a plant species. In *Arabidopsis*, the coupling of bioinformatic predictions of all candidate PRRs with the availability of insertional mutants allows for reverse genetic screens to rapidly screen a limited number of plant genotypes for loss of MAMP perception.

Another important question that remains unanswered not just for flg22, but for peptide MAMPs in general is the identity of the biologically relevant MAMP molecules within the apoplast. Most PTI research uses elicitor peptides such as flg22 and elf18, but it is unclear for both whether these peptides exist in the apoplast. The EF-Tu protein encoding elf18 is strictly cytoplasmic, while the flg22 peptide is predicted to be buried within the FliC protein (Song and Yoon, 2014). There is no evidence for the mechanism by which the MAMP containing proteins are released from the bacterial cells in which they normally reside, nor for whether they are degraded into peptides at all. It may be that while these peptides are sufficient for PTI induction, it is larger molecules that are responsible for elicitation in the case of a natural infection. The nature of the bioavailable molecule and their apoplastic concentrations may impact their stability and motility within the apoplast as well as their ability to interact with receptors and perhaps other MAMPs to produce more complex signatures of infection. In fact, flagellin monomers induce a non-host hypersensitive response in *Nicotiana benthamiana* whereas the flg22 peptide induces a basal immune response, demonstrating important differences in the immune eliciting potential of an isolated peptide versus an intact protein (Taguchi et al., 2003; Oh and Collmer, 2005; Hann and Rathjen, 2007; Nguyen et al., 2010).

With respect to bioavailability, the oligosaccharide MAMPs have proven to be a more tractable system of study. Several examples provide clear evidence of a role for plant enzymes in the release of this class of MAMPs from the surface of the invading pathogen cell walls (reviewed in van Loon et al., 2006). One of the best studied examples is that of the release of short-chain chitin oligosaccharides that can act as MAMPs and drive host immune reactions (Felix et al., 1993; Shibuya et al., 1993). The chitin MAMPs are liberated by the actions of exochitinases, which

reside within the apoplastic space and actively provide the signal to initiate the plant defense program. It will be of interest to see what roles, if any, plant enzymes play in the release and processing of MAMPs derived from pathogen proteins.

EVIDENCE OF DESTRUCTION – PERCEPTION OF ENDOGENOUS IMMUNE DRIVERS

In addition to the direct recognition of pathogens via the presence of MAMPs, the plant is also able to detect the by-products of pathogen activity in the apoplastic space. Damage associated molecular patterns (DAMPs) are endogenous compounds that are released from larger molecules or structures through the activity of enzymes produced by the pathogen, or by the host in response to the presence of a pathogen (**Table 1**). Like MAMPs, the appearance of DAMPs in the apoplastic space leads to perception by PRRs of the RLK family and the induction of basal immune responses from the plant. Many of the studies identifying DAMPs and their cognate receptors mirror those of MAMPS, so this section will focus on the characteristics that differentiate the DAMPs.

Like MAMPs, DAMPs vary in chemical composition, but also have additional features that are unique from the pathogen derived MAMPs. As their name suggests, DAMPs are a product of degradative processing event, however, they can be divided into two groups based their processing mechanism and the primary purpose of the processed molecule. DAMPs such as cutin monomers and oligogalacturonides (OGs) are similar to the MAMPs in that they are derived from structures that serve crucial functions (i.e., the structure of the plant cell wall) and their recognition induces PTI (reviewed in Ferrari et al., 2013; Serrano et al., 2014). As the studies involving this class of DAMPs mirror those of MAMPs, we will instead focus on DAMPs that are processed from an inactive precursor protein, whose primary function in the plant is pathogen surveillance. The *At*Pep family is one such example, and we will focus on it to illustrate this distinct mechanism by which plants perceive infection.

*At*Peps

The *At*Peps are a widely distributed family of defense-inducing peptides, which were originally identified in *Arabidopsis* based on their ability to promote extracellular alkalinization using the same techniques outlined above for MAMP identification (Huffaker et al., 2006). The novelty of the system became apparent when it was shown that the *At*Peps act to induce basal defenses only after post-transcriptional processing releases the active epitope from the *C*-terminal of the elicitor peptide precursors (PROPEPs), in a manner reminiscent to that of mammalian cytokines (Huffaker et al., 2006). Originally the PROPEP family was described to have seven members in *Arabidopsis*, but a more recent analysis using more sensitive bioinformatic tools identified an eighth family member (Bartels et al., 2013). The presence of PROPEPs has been predicted for many plant species based on sequence homology (Huffaker et al., 2006), and one such homolog (*Zm*Pep1) from maize has been functionally validated suggesting that this family is largely conserved across the plant kingdom (Huffaker et al., 2011).

The presence of multiple family members within a single species raises the question of whether these represent functionally distinct or redundant proteins. Recent work has shown that all eight *At*Peps, when applied exogenously, induce similar defense responses *in planta* (Bartels et al., 2013). While this result demonstrates functional redundancy, the same work describes distinct temporal and spatial expression patterns for the PROPEP family members under normal conditions and in response to various stressors. This use of bioinformatics coupled with *in planta* expression localization shows that only a subset of the PROPEPs are expressed in a manner consistent with a role in pathogen defense, while the expression pattern of others is more consistent with a role in reproduction and development (Bartels et al., 2013). While a more detailed examination of the groups is required, these observations are suggestive of cross-talk between defense signaling and plant development.

The discovery of the receptor for *At*Pep1 also presents some lessons that expand upon our understanding of MAMP/DAMP signaling in the apoplast. As discussed above, PEPR1 was identified by photo-affinity labeling and purification from *Arabidopsis* extracts (Yamaguchi et al., 2006). While PEPR1, a typical LRR kinase, binds to *At*Pep1 and confers *At*Pep1 responsiveness to transgenic tobacco cells expressing PEPR1, AtPep1 induced immune responses were only partially compromised in T-DNA insertional mutants of *pepr1*. Subsequent phylogenetic analysis identified PEPR2 as a likely alternate receptor, and its ability to bind *At*Pep1 was subsequently demonstrated (Yamaguchi et al., 2010). Double mutants of *pepr1* and *pepr2* completely abolished *At*Pep1 immune responses demonstrating that there is functional redundancy at the level of the DAMP receptor. While both receptors are capable of binding to *At*Pep1, it is also interesting to note that the two have differential binding abilities for other family members (Bartels et al., 2013), and further study is required to determine what role those affinities have in defense, development and reproduction.

THE PLANT RESPONSE TO PATHOGEN PERCEPTION – CHEMICAL DEPLOYMENT

Once an apoplastic pathogen has been detected by the immune system, the plant responds with molecules that limit pathogen growth and also prepare distal parts of the plant for future infection. This section will focus on the chemicals and small molecules produced by the plant within the apoplastic space to fight infection and the tools available for their study (**Table 2**).

The majority of these compounds have been shown to have direct effects on the pathogen, though this observation may simply arise from a bias toward research aimed at identifying novel therapeutics. These compounds include the phytoalexins, a heterogeneous group of plant secondary metabolites with antimicrobial activity (reviewed in Denoux et al., 2008). One of the best-studied phytoalexins is camalexin from *A. thaliana*, which is induced upon pathogen challenge and has been associated with growth limitation of pathogens (reviewed in Glawischnig, 2007). Another class of anti-microbial compound is the cyclotides, a group of small proteins from plants that are characterized by head-to-tail cyclic backbone and conserved disulphide knot. While their precise role *in planta* remains unclear, it is interesting to note that they are expressed throughout the plant including in the leaves (Trabi and Craik, 2004), and they show potent anti-microbial properties to many bacteria and fungi (Tam et al., 1999). The plant

also responds to infection by expressing a host of proteins not normally found in healthy tissues called plant pathogenesis-related (PR) proteins, including some which are active peptides (reviewed in Sels et al., 2008). These include protease inhibitor peptides to prevent enzymatic destruction by the pathogen, and several classes of peptides that directly cause pathogen lysis or death (Stec, 2006; Carvalho and Gomes, 2007; De Coninck et al., 2013).

In addition to products that directly impact pathogen survival in the apoplast, there has recently been increasing interest in plant molecules that serve an apoplastic signaling role in response to infection. Of note, several publications have investigated the role of extracellular adenosine triphosphate (eATP) in the plant response to pathogens (Chivasa et al., 2009). The recent discovery of an eATP receptor in plants (Choi et al., 2014) expands on this area of research and suggests a greater role for this molecule than previously appreciated. In general the active compounds in the apoplast are identified either due to their increased production following a pathogen challenge, or following their isolation on the basis of their anti-microbial activity. The significant interest in many of these classes of molecules as therapeutics in plants and other systems has led to increased research in this area, with a significant fraction of those investigations focused on identifying novel compounds to address human health concerns.

This body of research also nicely illustrates one of the central balancing acts in the plant immune response. While many of these compounds directly impact the pathogen, several also play roles in the induction of programed cell death (PCD) in plant cells. While PCD is effective against biotrophic invaders, it increases susceptibility to necrotrophs, requiring that the immune response be appropriately tuned to counter the specific threat that is faced.

In order to explore the plant response to pathogenic insult, and to illustrate many of the central themes discussed above, we will examine the regulation of the oxidative state of the apoplast. The oxidative burst that results from pathogen recognition within the apoplast is one of the best studied plant responses to infection and is therefore where we will focus in this section.

OXIDATIVE BURST

One of the earliest reactions of the plant host upon detection of pathogen invasion is the production of toxic ROS. This production occurs within minutes of MAMP detection and is classically associated with direct microbial killing (Peng and Kuc, 1992). The most common techniques used to study the production of the oxidative burst *in planta* are an assay of luminol chemiluminescence in the presence of hydrogen peroxide (Keppler, 1989; Felix et al., 1999), or staining the locations of hydrogen peroxide production in leaf tissue with 3,3′-diaminobenzidine (DAB; Thordal-Christensen et al., 1997). These techniques have been invaluable in studying ROS production following elicitor treatment or pathogen infection.

In addition to its toxic properties, ROS also serves to limit pathogen ingress by contributing to stomatal closure and reinforcement of the plant cell wall. The stomatal aperture can be observed and measured directly through microscopy and these assays have shown that ROS promote stomatal closure, thus limiting apoplastic access to pathogens (McAinsh et al., 1996). Treatment of plant cells with ROS also results in both callose deposition and changes in the cell wall proteome consistent with an active defense response (Daudi et al., 2012; O'Brien et al., 2012).

Increases in apoplastic ROS concentration also has direct effects on the contents of the apoplastic space. Plant cell culture and chromatography techniques have shown that ROS stimulates phytoalexin production in the apoplast, demonstrating a direct relationship between redox signaling and the presence of defense molecules at the site of pathogen ingress (Apostol et al., 1989; Qiu et al., 2012). Further investigations into the changing chemistry and molecular make-up of the apoplast following pathogen

Table 2 | The plant response to pathogen challenge.

Plant Product	Function	Molecular description	Reference
Reactive oxygen species (ROS)	Oxidative damage to pathogens		O'Brien et al. (2012)
Nitric oxide radical	Signaling molecule		Mur et al. (2013)
Phytoalexins	Anti-microbial	Low MW secondary metabolites	Ahuja et al. (2012)
Polyamines		Basic small molecules	Walters (2003)
Cyclotides	Anti-microbial	Cyclic peptides (∼3 kDa)	Craik (2012)
Extracellular ATP	Signaling molecule	Nucleoside triphosphate	Chivasa et al. (2009)
Proteinase Inhibitor (PR-6)	Enzyme inhibition, interference with replication	Peptides (∼8 kDa)	Sels et al. (2008)
Defensins (PR-12)	Induced pathogen cell death	Basic peptides (∼5 kDa)	De Coninck et al. (2013)
Thionins (PR-13)	Increased pathogen plasma membrane permeability	Cysteine-rich peptides (∼5 kDa)	Stec (2006)
Lipid transfer proteins (LTPs, PR-14)	Increased pathogen plasma membrane permeability	Basic peptides (7 or 10 kDa)	Carvalho and Gomes (2007)

challenge can be accomplished using multiple techniques that allow for the collection of the apoplastic contents (Bernstein, 1971; Hartung et al., 1988; Long and Widders, 1990; Lohaus et al., 2001). These techniques, coupled with continuing advancement in metabolomics and high-throughput proteomic techniques, will no doubt prove to be powerful tools for future research into the changing molecular make-up of this niche.

Perhaps not surprisingly, production of ROS and the subsequent change in the apoplastic redox balance results in wholesale changes to gene expression, including increased expression of several known defense genes (Desikan et al., 1998; O'Brien et al., 2012). In addition to descriptive studies, large-scale gene expression profiling is also used to perform sensitive comparative studies between the effects of different defense-inducing stimuli (Denoux et al., 2008). This approach has shown that while the basal defense response induced by different MAMPs is broadly similar (reviewed in Jones and Dangl, 2006), the response to each specific MAMP also contains unique features including the kinetics and amplitude of the resulting defence response. Denoux et al. (2008) showed in particular that while the transcriptional effects of flg22 and OGs are largely similar, flg22 was a much more potent elicitor as measured by both the scale and scope of its effects. These studies are also being extended to investigate the effects of MAMPs used in combination, which have been shown to have additive, synergistic, or even antagonistic effects on defense induction (Aslam et al., 2009). This is important to note, as most studies to date have focused on single elicitors, while in nature plants would encounter these molecules as complex mixtures of epitopes. In order to gain a true understanding of the biological roles of these molecules more holistic studies will be required in the future.

Other factors beyond ROS influence the oxidative state of the apoplast. For example, the nitric oxide radical (NO) plays a similar role to ROS in its interactions with the pro- and anti-oxidants in the apoplastic space. NO is also induced in response to various stress stimuli *in planta* (Leitner et al., 2009; Mur et al., 2013) and, via interactions with ROS, plays a role in both pathogen defense and hypersensitive cell death (Delledonne et al., 2001; Hong et al., 2008; Mur et al., 2013). This suggests that the overall oxidative state of the apoplastic space plays an important role in determining how a plant responds to a broad range of pathogen challenges. While the interactions between these networks are becoming clarified, there still remains much more to learn about the relationships between them.

THE INVADER FIGHTS BACK – VIRULENCE FACTORS IN THE APOPLASTIC SPACE

Our focus thus far has been on how the plant prepares itself to fight invasion and responds upon detecting an attack, but of course at the same time pathogens work to evade detection and manipulate the plant to its benefit. There are numerous examples of such subversion from the filamentous pathogens and these will be the focus of this section.

The relationship between plant hosts and invasive fungi and oomycetes can be broadly divided into necrotrophic or biotrophic, and determines the method by which the pathogen derives nutrition from the host. The phytotoxins produced by filamentous pathogens have a large range of targets, whether they are employed by necrotrophs to induce cell death, or by biotrophs to satisfy their nutritional needs in living tissue (reviewed in Howlett, 2006). Recent advances in genome sequencing and interrogation have given new insights into the mechanisms by which these pathogen virulence factors result in successful infection. Phytotoxins can cause direct damage to cell membranes, alter gene expression, inhibit plant protein function, mimic plant hormones, and induce cell death through the production of ROSs (reviewed in Möbius and Hertweck, 2009). While much progress has been made in our understanding of these molecules, the nature of many phytotoxins remains to be resolved, and as such our understanding in this area may rapidly change in the future.

KILLING TO EAT – NECROTROPHIC VIRULENCE FACTORS

There are numerous examples of phytotoxins that act in the apoplast to induce plant cell death, which plays a central role in providing a source of nutrition for necrotrophic pathogens. The identification and characterization of these molecules mirrors the methods used in MAMP studies (i.e., isolation of an active molecule from pathogen cultures and subsequent genetic confirmation). As such we will not focus on these techniques, but rather give an example to illustrate the current state of understanding of these small molecules in the apoplast. One of the few phytotoxins from this class that is a peptide and thus falls within the scope of this review on small molecules is the PtrToxB peptide from *Pyrenophora tritici-repentis*. The toxin has a predicted molecular weight of 6.5 kDa and causes a characteristic chlorosis in susceptible wheat cultivars (Martinez et al., 2001). The chlorosis results from the degradation of chlorophyll, the process of which is light-dependent and likely requires ROS production (Strelkov et al., 1998). While PtrToxB has no known protein domains, it is hypothesized to be localized to the apoplast based on its protease resistance (Ciuffetti et al., 2010). While the description of PtrToxB and other apoplastic phytotoxins demonstrate that fungal invaders are actively modifying this niche to favor their survival, many further studies, including detailed structural analyses, should provide more insight into the range of these molecules and their specific activities. It is also important to note that not all virulence factors are protein derived, and the production of oxalic acid by necrotrophic fungi provides an excellent example of a chemical that plays an important role in pathogenicity (Cessna et al., 2000).

BIOTROPHS BENDING THE PLANT TO THEIR WILL

In contrast to the goals of the necrotrophic pathogens, biotrophs derive nutrition from the host while maintaining plant survival. The fungal pathogen *Cladosporium fulvum*, which infects tomato, represents a unique system for the study of pathogen nutrition, as its *in planta* growth is limited to the apoplast (Lazarovits and Higgins, 1976; De Wit, 1977). By directly measuring the nitrogen content of the infected apoplast, it was shown that infection of tomato with *C. fulvum* results in increased levels of many amino acids and a particular increase in the concentration of the non-protein amino acid γ-aminobutyric acid (GABA). Given its high levels it was hypothesized that GABA would provide a ready nitrogen source for the fungi (Solomon

and Oliver, 2001) and a subsequent study showed that *C. fulvum* expresses a GABA transaminase, further suggesting that GABA is used as a nitrogen source by the fungus (Solomon and Oliver, 2002). More recently it has been shown that the wheat fungal pathogen *Stagonospora nodorum* requires GABA metabolism for full pathogenicity, suggesting that this may be a common source of nitrogen within the apoplast for pathogens (Mead et al., 2013). While the mechanism by which fungal infection results in increased GABA concentration in the apoplast remains to be deciphered, the presence of the pathogen within this space suggests that the process involves manipulation of the plant cell at the apoplastic interface.

CONCLUDING REMARKS

Plant science has long sought to increase disease resistance in plants and thus improve crop yields. Originally the goal was pursued through selective plant breeding, but modern science has allowed for a more rational approach by elucidating the molecular determinants of plant disease and immunity. From identification of pathogen components that mimic disease symptoms and plant extracts that are toxic to microbes, to the recent use of bioinformatic tools to predict novel elicitors of plant immunity, we have exponentially increased our understanding of the communication between host and pathogen at a molecular level.

Many of the peptides and small molecules that are directly responsible for causing disease and inducing the plant immune reaction are now known, and their molecular mechanisms are being rapidly elucidated. However, we still remain far from a complete and clear vision of the interplay between these individual players that determines the ultimate result of an infection. Advances on that front will require a more holistic approach to plant immunity research, which will allow us to better assess the interface between pathogen and host as it occurs in nature. Early forays in these directions have shown sometimes surprising results, and do not reflect a simple additive relationship between these effects.

It will also be important to transition these investigations into a wider variety of plant species. Plant species show great variation in their response to even the most potent elicitors of the immune response, suggesting that there may need to be much work done in order to translate the lessons learned in one system to plant immunity more broadly. At the same time the study of pathogen variability on immune elicitation will surely lend new insights into our understanding of the determinants of pathogenicity.

While the techniques used to study the apoplastic space have changed, the ultimate goal remains unchanged. The study of the changing environment in which these pathogens exist still holds the key to improving plant health, and thereby improving human health. This area of research holds the promise of advancing our basic understanding of plant biology, while simultaneously opening up novel targets for therapeutic intervention.

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Synthetic plant defense elicitors

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To defend themselves against invading pathogens plants utilize a complex regulatory network that coordinates extensive transcriptional and metabolic reprogramming. Although many of the key players of this immunity-associated network are known, the details of its topology and dynamics are still poorly understood. As an alternative to forward and reverse genetic studies, chemical genetics-related approaches based on bioactive small molecules have gained substantial popularity in the analysis of biological pathways and networks. Use of such molecular probes can allow researchers to access biological space that was previously inaccessible to genetic analyses due to gene redundancy or lethality of mutations. Synthetic elicitors are small drug-like molecules that induce plant defense responses, but are distinct from known natural elicitors of plant immunity. While the discovery of some synthetic elicitors had already been reported in the 1970s, recent breakthroughs in combinatorial chemical synthesis now allow for inexpensive high-throughput screens for bioactive plant defense-inducing compounds. Along with powerful reverse genetics tools and resources available for model plants and crop systems, comprehensive collections of new synthetic elicitors will likely allow plant scientists to study the intricacies of plant defense signaling pathways and networks in an unparalleled fashion. As synthetic elicitors can protect crops from diseases, without the need to be directly toxic for pathogenic organisms, they may also serve as promising alternatives to conventional biocidal pesticides, which often are harmful for the environment, farmers and consumers. Here we are discussing various types of synthetic elicitors that have been used for studies on the plant immune system, their modes-of-action as well as their application in crop protection.

Keywords: plant activators, systemic acquired resistance, plant innate immunity, pesticide, crop protection, salicylic acid, chemical genetics, plant defense

INTRODUCTION

THE PLANT IMMUNE SYSTEM

Plants serve as a source of nutrients for a wide variety of heterotrophic microorganisms that can cause diseases in their hosts. Physical barriers, such as a waxy cuticular layer and rigid cell walls, as well as preformed antimicrobial chemicals can provide some protection against attacking phytopathogens (Nürnberger and Lipka, 2005). In addition, plants have evolved an inducible immune system that is based on the specific recognition of pathogen-derived molecules (Chisholm et al., 2006; Jones and Dangl, 2006). Two classes of plant immune receptors are critical for defense activation (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Pattern recognition receptors (PRRs) directly interact with highly conserved microbe associated molecular patterns (MAMPs) activating pattern-triggered immunity (PTI; Gómez-Gómez and Boller, 2002; Zipfel et al., 2004; Segonzac and Zipfel, 2011). PTI can be attenuated or blocked by effector molecules that are secreted into plant cells by microbial pathogens that are well-adapted to their hosts (Abramovitch and Martin, 2004). The remaining weakened host immunity operating during such compatible plant/pathogen interactions [a state also referred to as effector-triggered susceptibility (ETS)] is called basal defense (Glazebrook et al., 2003; Chisholm et al., 2006; Jones and Dangl, 2006). While basal defense can limit the spread of virulent pathogens in their hosts, it is typically insufficient to prevent disease.

A second class of plant immune receptors, encoded by disease *resistance* (*R*)-genes, recognize the presence or activity of effectors and induce effector-triggered immunity (ETI), a manifestation of the well-described phenomenon of gene-for-gene resistance or race-specific resistance which leads to incompatible interactions (Flor, 1971; Nimchuk et al., 2003; Jones and Dangl, 2006; Elmore et al., 2011). ETI is a strong immune response that efficiently protects plants from avirulent pathogens and is often associated with the hypersensitive reaction (HR), a form of programmed death of plant cells at infection sites. Purified molecules or crude biochemical preparations from pathogens triggering PTI have also been referred to as general elicitors, while those triggering ETI, or race-specific resistance, have been termed race-specific elicitors (Wevelsiep et al., 1991).

Numerous studies have shown that ETI, basal defense and PTI utilize a common set of signaling components including multiple regulatory proteins, reactive oxygen intermediates (ROIs) as well as the phytohormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA; Nimchuk et al., 2003; Glazebrook, 2005; Spanu, 2012). Levels of ROI, SA, ET, or JA often increase in plant tissues after pathogen infections. While basal defense seems mainly to be

a weakened form of PTI, ETI has been proposed to result from boosted basal defense- or PTI-associated responses (Tao et al., 2003; Jones and Dangl, 2006; Shen et al., 2007).

Inducible immune responses are tightly associated with extensive transcriptional- and metabolic–reprogramming controlled by a complex regulatory network (Glazebrook et al., 2003; Tsuda et al., 2009; Sato et al., 2010). While historically 10 classes of pathogenesis-related (*PR*) genes had been recognized, which exhibit transcriptional up-regulation in defense-related biological situations (Kombrink and Somssich, 1997), more recent genome-wide transcript profiling studies have revealed that 100–1000s of genes typically respond to defense induction by transiently altered transcript levels. Numerous signal transducers and transcription factors have been implicated in the plant defense network (Katagiri, 2004; Eulgem, 2005; Jones and Dangl, 2006). This network can be subdivided into various defined sectors that can interact with each other (Tsuda et al., 2009; Sato et al., 2010). For example, distinct defense signaling sectors dependent on early MAMP-activated MAP kinases (MAPKs) or the hormones SA or JA, have been described. Interestingly, some of these sectors were found to largely interact in an additive or synergistic fashion during PTI, while they are partially antagonistic to each other during ETI (Tsuda et al., 2009). The latter phenomenon seems to allow for compensatory effects if a defined sector is disabled due to interferences with pathogen effectors.

The complexity of this network is likely the result of two separate co-directional evolutionary pressures. Firstly, the asymmetrical arms race between plants and pathogens/pests manifested in continuous co-evolution of effectors and their host targets may have resulted in an ever-increasing diversity of plant defense regulators and regulatory circuits. Secondly, the need to fine-tune defense outputs appropriate for the respective attacker(s), which may exhibit biotrophic, hemibiotrophic, or nectrotrophic lifestyles, requires a complex regulatory system that allows for extensive crosstalk and compensatory interactions (Tsuda et al., 2009). An additional level of complexity likely arose from the need to link effector recognition mechanisms, which appear to be of recent evolutionary origin to more ancient regulatory processes mediating PTI (Chisholm et al., 2006; Holub, 2008).

While PTI, basal defense and ETI are transient local responses limited to pathogen infected tissues, plants can also activate long-lasting systemic immunity. Such systemic immunity can be initiated by local compatible or incompatible interactions resulting in systemic acquired resistance (SAR) or triggered by certain strains of non-pathogenic plant growth-promoting rhizobacteria (PGPR) leading to induced systemic resistance (ISR; Pieterse et al., 1998; van Wees et al., 2000). SAR mediates long-lasting broad-spectrum resistance to a wide range of pathogens in uninfected tissues and organs (Ward et al., 1991; Fu and Dong, 2013). In addition to local pathogen infections, exogenous application of SA or SA analogs (see below) can induce SAR-like responses (White, 1979; Metraux et al., 1991; Ward et al., 1991). SAR and related systemic immune responses have been demonstrated in several plant systems, such as cucumber, watermelon, tobacco, and *Arabidopsis thaliana* (*Arabidopsis*; White, 1979; Kuc, 1982; Metraux et al., 1991; Ward et al., 1991). Typically SAR is associated with a local and systemic increase of SA levels that conditions enhanced expression of several classical *PR* genes (Rasmussen et al., 1991; Ward et al., 1991; Vernooij et al., 1994; Wildermuth et al., 2001; Durrant and Dong, 2004). Some of these *PR* genes, such as *PR1*, *PR2,* and *PR5* serve as robust markers for this systemic immune response (Kombrink and Somssich, 1997).

While local and systemic accumulation of SA is critical for SAR induction, this hormone seems not to serve as a mobile signal mediating immunity in uninfected distal tissues. Several other small molecules have been proposed to fulfill such a role, such as methyl-salicylic acid (MeSA), azelaic acid, glycerol-3-phosphate, the abietane diterpenoid dehydroabietinal, JA, and the amino acid-derivative pipecolic acid (Park et al., 2007; Fu and Dong, 2013). A central regulator of SAR is the transcriptional co-factor NON-EXPRESSOR OF PR GENES1 (NPR1; Dong, 2004). By interacting with TGA bZIP transcription factors, NPR1 seems to mediate up-regulation of the vast majority of SAR-associated genes (Fu and Dong, 2013). NPR1 activity has been proposed to be controlled by the SA-binding proteins NPR3 and NPR4, which can physically bind to NPR1 in a SA-concentration-dependent manner (Fu et al., 2012).

In contrast to SAR, induction of ISR is not associated with the accumulation of SA and *PR* transcripts (Sticher et al., 1997; van Wees et al., 2000). ISR has been shown to be triggered by the *Pseudomonas fluorescens* strain WCS417r (WCS417r) and other non-pathogenic rhizobacteria in several plant species including *Arabidopsis* (Wei et al., 1996; Sticher et al., 1997; Pieterse et al., 1998; Yan et al., 2002; Vallad and Goodman, 2004). In *Arabidopsis*, WCS417r-induced ISR acts against *P. syringae* pv. tomato, is dependent on JA and ET signaling, but does not require SA. Intriguingly, ISR is blocked in the *Arabidopsis npr1* mutant. Thus, *NPR1* also plays an important role in the ISR signaling pathway (Pieterse et al., 1998; Glazebrook, 2001).

Upon perception of several exogenous defense-related stimuli, plants can establish an enhanced capacity to activate immune responses. This sensitization process, which is called priming, can be triggered by treatment of plants with necrotizing pathogens, beneficial microorganisms, wounding or with various natural and synthetic compounds (Conrath et al., 2002, 2006; Conrath, 2006; Beckers and Conrath, 2007; Goellner and Conrath, 2008). Once a pathogen infects primed plants, defense responses are activated faster and more robustly (Conrath et al., 2006; Goellner and Conrath, 2008). Although this phenomenon has been known for years, its molecular basis is still only partly understood (Conrath, 2006, 2011; Conrath et al., 2006). Chromatin modifications, accumulation of dormant mitogen-activated protein kinases and alterations of primary metabolism have been shown to be associated with this process (Conrath et al., 2002, 2006; Beckers et al., 2009; Conrath, 2011; Jaskiewicz et al., 2011).

A BRIEF HISTORY OF SYNTHETIC ELICITORS

Synthetic elicitors are small molecules that can induce plant immune responses and are structurally distinct from natural plant defense inducers, such as general or race-specific elicitors or endogenous plant defense signaling molecules. Synthetic elicitors may trigger defense reactions by mimicking interactions of natural elicitors or defense signaling molecules with

their respective cognate plant receptors or by interfering with other defense signaling components. Often the term "plant activators" is used for molecules that can protect plants from diseases by inducing immune responses. However, this term does not discriminate between synthetic and natural elicitors. One of the first synthetic elicitors was identified by Gianinazzi and Kassanis (1974), who found Polyacrylic acid derivatives of 3500 Da or lower molecular weights to mediate resistance of tobacco (*Nicotiana tabacum*) against tobacco mosaic virus (TMV) or tobacco necrosis virus (TNV) and to activate *PR1* gene expressions in tobacco (Gianinazzi and Kassanis, 1974; Kassanis and White, 1975). At the same time, 2,2-dichloro-3,3-dimethylcyclopropane-carboxylic acid (WL28325) was described as a compound suitable for controlling rice blast in rice. WL28325 affects the phenol metabolism of rice plants by enhancing peroxidase activities (Langcake and Wickins, 1975a,b). Two years later, 3-allyloxy-1,2-benzisothiazole-1,1-dioxide, widely called Probenazole (PBZ), was described. It activates defense-related enzymes and triggers dramatic increases of tolerance against rice blast in rice. It has effectively been used in agriculture for over three decades against rice blast (Watanabe et al., 1977; Schreiber and Desveaux, 2008).

Exogenous application of SA and other benzoic acid derivatives, such as acetylsalicylic acid (Aspirin), was reported to induce resistance of tobacco against TMV and to cause the accumulation of PR-proteins (White, 1979). This discovery was a major breakthrough and paved the way for the identification of more potent related compounds by the Switzerland-based pharmaceutical corporation Ciba-Geigy (now Syngenta). Ciba-Geigy researchers reported 2,6-dichloro-isonicotinic acid (INA) and its ester derivative CGA 41397 as potent SAR-inducers in 1987. They also identified benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), which has similar effects as INA, but was later found to be more suitable for applications in crop protection (Metraux et al., 1990; Ward et al., 1991; Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996; Uknes et al., 1996). As INA and BTH mimic the defense-associated effects of SA, but are less phytotoxic and more efficient than this natural plant defense hormone, they have been abundantly used as defense triggers in basic and applied studies on plant immunity. As outlined in detail below, these two compounds have been among the most frequently used synthetic elicitors in research for the past 15–20 years. However, recent improvements in combinatorial chemistry (Blackwell and Zhao, 2003; Stockwell, 2004; Dean, 2005; Raikhel and Pirrung, 2005) have enabled scientists outside the private sector to perform systematic screens for synthetic elicitors. Thus, a plethora of new compounds with defense-inducing properties distinct from INA and BTH or other established synthetic elicitors is currently emerging (**Table 1**). Such second-generation synthetic elicitors will equip researchers with an extensive repertoire of new chemical tools to dissect the plant defense network in an unprecedented fashion and to explore their use as active ingredients of novel types of pesticide alternatives and other agrochemicals.

FUNCTIONAL ANALOGS OF SALICYLIC ACID

The natural plant defense hormone SA (2-hydroxybenzoic acid) serves as an endogenous signal to activate certain immune responses and to establish disease resistance. Various defense-related stimuli have been shown to trigger enhanced SA levels in local and systemic plant tissues. Exogenous application of SA can induce ROI production, *PR* gene expression and immunity against various pathogens with biotrophic or hemibiotrophic lifestyles (Glazebrook, 2005; Vlot et al., 2009).

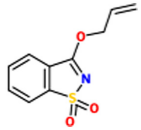
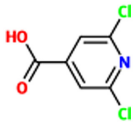
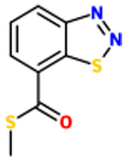
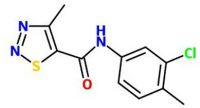
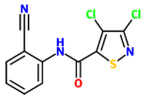
In plants, SA can be synthesized from the shikimate pathway-derived primary metabolite chorismate either via phenylpropanoid derivatives in the cytoplasm or via isochorismic acid in chloroplasts (Pieterse et al., 2012; An and Mou, 2014). Although both metabolic pathways are not fully understood, several of their enzymes have been identified. The production of SA and its levels are normally tightly regulated (Wildermuth, 2006). Critical for the production of the majority of defense-associated SA in *Arabidopsis* is isochorismate synthase 1 (ICS1), which is transcriptionally induced by defense-related stimuli (Wildermuth et al., 2001). Two distinct forms of SA glucosyltransferase (SAGT) enzymes convert most of the produced SA to either salicyloyl glucose ester (SGE) or SA-O-β-glucoside (SAG), which is stored in the vacuole. Additional SA derivatives are known in plants, such as MeSA. SAG, SGE, and MeSA are likely biologically inactive (Vlot et al., 2009; Fu and Dong, 2013).

Salicylic acid plays a pivotal role in defense signaling and several proteins have been proposed to bind to SA and to potentially serve as SA receptors. The first putative SA-binding protein reported in the literature was SABP1 from tobacco, a potential catalase (Chen et al., 1993). It was proposed that SA inhibits its ability to convert H_2O_2 to O_2 and H_2O (Conrath et al., 1995; Du and Klessig, 1997; Vlot et al., 2009). However, this claim is controversial, as much higher SA-concentrations seem to be needed for catalase inhibition than observed in defense-activated plants (Chamnongpol et al., 1996; Tenhaken and Rubel, 1997). Similarly, it was shown that SA can also bind to ascorbate peroxidase (APX) and inhibit its activity upon application of high concentrations of exogenous SA (Durner and Klessig, 1995; Vlot et al., 2009). An additional tobacco SA-binding protein, SABP2, functions as a MeSA esterase. SABP2 shows a high binding affinity for SA, which inhibits its esterase activity (Kumar and Klessig, 2003; Forouhar et al., 2005). SABP2 seems to play an important role in the activation of SAR in tobacco by catalyzing the release of SA from the transport metabolite MeSA in systemic tissues (Park et al., 2007). Another SA-binding protein, SABP3, a tobacco chloroplastic carbonic anhydrase, is involved in HR and has antioxidant function (Slaymaker et al., 2002; Vlot et al., 2009). However, it remains to be determined whether this function can affect plant defense.

In *Arabidopsis*, NPR1 plays a critical role in the interpretation of the SA signal. NPR1 is responsible for activating a large set of defense genes in response to SA-related signals (Dong, 2004; Fu and Dong, 2013). Moreover, the NPR1 paralogues NPR3 and NPR4 function as SA receptors, and their interactions with NPR1 are directly regulated by binding to SA (Fu et al., 2012). In addition, NPR1 itself has also been shown to be capable of binding SA independently of NPR3 and NPR4 and to respond to interactions with this ligand by conformational changes (Wu et al., 2012).

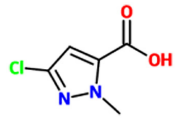
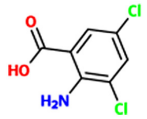
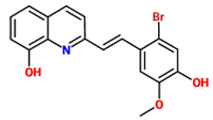
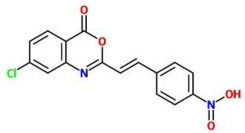
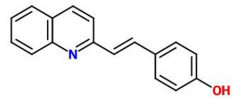
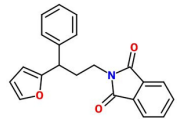
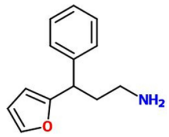
With several proteins capable of binding to SA, defense mechanisms controlled by this phytohormone feature a set of "drug-able" targets potentially interfering with SA-related synthetic molecules.

Table 1 | Synthetic elicitors discussed in the main text.

Chemical names	Chemical structures	Biotic interactions*	Application methods	Concentrations**	Reference
3-allyloxy-1,2-benzisothiazole-1,1-dioxide (Probenazole, PBZ)		*Oryza sativa* – *Magnaporthe grisea*	Root drench	896 uM (200 ppm)	Watanabe et al. (1977)
2,6-dichloro-isonicotinic acid (INA)		Cucumber (*Cucumis sativus*) – *Colletotrichum lagenarium*	Foliar spray	104 uM (20 ppm)	Metraux et al. (1991), Ward et al. (1991), Uknes et al. (1992)
		Nicotiana tabacum -*Tobacco mosaic virus* (TMV)	injection into leaves	1000 uM	
		Arabidopsis thaliana (Ler) – *Hyaloperonospora arabidopsidis*	Soil drench	52 uM	
		A. thaliana (Col-0) – *Pseudomonas syringae* pv 'tomato' DC3000	Foliar spray	650 uM	
benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH)		*A. thaliana* (Col-0) – *P. syringae* pv 'tomato' DC3000	Foliar spray	300 uM	Friedrich et al. (1996), Görlach et al. (1996), Lawton et al. (1996)
		A. thaliana (Col-0) – *H. arabidopsidis*	Foliar spray	300 uM	
		A. thaliana (Col-0) – *Turnip crinkle virus*	Foliar spray	300 uM	
		N. tabacum – *Cercospora nicotianae*	Foliar spray	1200 uM	
		N. tabacum – *Erwinia carotovora*	Foliar spray	1200 uM	
		N. tabacum – *Phytophthora parasitica*	Foliar spray	1200 uM	
		N. tabacum – *P. syringae* pv. Tabaci	Foliar spray	1200 uM	
		N. tabacum –TMV	Foliar spray	1200 uM	
N-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (Tiadinil, TDL)		*N. tabacum* –TMV	Root drench	1 mg/pot	Yasuda et al. (2004)
Isotianil		*O. sativa* – *M. grisea*	Foliar spray	840 uM (250 ppm)	Ogava et al. (2011)
N-cyanomethyl-2-chloroisonicotinamide (NCI)		*O. sativa* – *Pyricularia oryzae*	Root drench	240 g a.i (active ingredient)/10a (are)	Yoshida et al. (1990a)

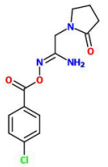
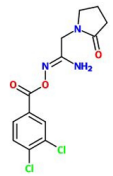
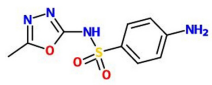
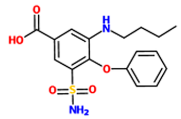
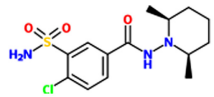
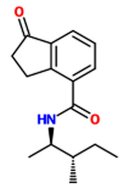
(Continued)

Table 1 | Continued

Chemical names	Chemical structures	Biotic interactions*	Application methods	Concentrations**	Reference
3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA)		*O. sativa – P. oryzae*	Root drench	0.05 mg/pot	Nishioka et al. (2003, 2005)
3,5-dichloroanthranilic acid (DCA)		*A. thaliana – H. arabidopsidis*	Foliar spray	100 uM	Knoth et al. (2009)
2-[(E)-2-(2-bromo-4-hydroxy-5-methoxyphenyl)ethenyl] quinolin-8-ol) (Imprimatin A1)		*A. thaliana–P. syringae pv tomato DC3000 avrRpm1* or *A. thaliana–P. syringae pv. tomato DC3000*	Root drench	100 uM	Noutoshi et al. (2012d,e,f)
7-chloro-2-[(E)-2-(4-nitrophenyl)ethenyl]-4H-3,1-benzoxazin-4-one) (Imprimatin A2)		*A. thaliana – P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana – P. syringae* pv. tomato DC3000	Root drench	100 uM	Noutoshi et al. (2012d,e,f)
4-[(E)-2-(quinolin-2-yl)ethenyl]phenol) (Imprimatin A3)		*A. thaliana – P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana – P. syringae* pv. tomato DC3000	Root drench	100 uM	Noutoshi et al. (2012d,e,f)
2-(3-(2-furyl)-3-phenylpropyl) benzo[c]azoline-1,3-dione) (Imprimatin B1)		*A. thaliana – P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana – P. syringae* pv. tomato DC3000	Root drench	100 uM	Noutoshi et al. (2012d,e,f)
3-(2-furyl)-3-phenylpropylamine) (Imprimatin B2)		*A. thaliana – P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana – P. syringae* pv. tomato DC3000	Root drench	100 uM	Noutoshi et al. (2012d,e,f)

(Continued)

Table 1 | Continued

Chemical names	Chemical structures	Biotic interactions*	Application methods	Concentrations**	Reference
[(E)-[1-amino-2-(2-oxopyrrolidin-1-yl)ethylidene]amino] 4-chlorobenzoate) (Imprimatin C1)		*A. thaliana* – *P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana* – *P. syringae* pv. Tomato DC3000	Root drench	100 uM	Noutoshi et al. (2012c)
[(E)-[1-amino-2-(2-oxopyrrolidin-1-yl)ethylidene]amino]3,4-dichlorobenzoate) (Imprimatin C2)		*A. thaliana* – *P. syringae* pv tomato DC3000 avrRpm1 or *A. thaliana* – *P. syringae* pv. tomato DC3000	Root drench	100 uM	Noutoshi et al. (2012c)
Sulfamethoxazole (Smex)		*A. thaliana* – *P. syringae* pv. tomato DC3000	Foliar spray	100 uM	Schreiber et al. (2008)
3-(butylamino)-4-phenoxy-5-sulfamoylbenzoic acid (Bumetanide)		*A. thaliana* – *P. syringae* pv. tomato DC3000 (Pst) avrRpm1	Root drench	100 uM	Noutoshi et al. (2012b)
3-benzyl-1,1-dioxo-6-(trifluoromethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide (Bendroflumethiazide)		*A. thaliana* – *P. syringae* pv. tomato DC3000 (Pst) avrRpm1	Root drench	100 uM	Noutoshi et al. (2012b)
4-chloro-*N*-(2,6-dimethyl-1-piperidyl)-3-sulfamoyl-benzamide (Clopamide)		*A. thaliana* – *P. syringae* pv. tomato DC3000 (Pst) avrRpm1	Root drench	100 uM	Noutoshi et al. (2012b)
1-oxo-indanoyl-L-isoleucine methyl ester		*Pennisetum glaucum* – *Sclerospora graminicola*	Seeds are soaked with chemical	75 uM	Deepak et al. (2007)

** Biotic interactions tested and affected by compound; ** Converted to molarity if information on concentration available.*

Consequently, some synthetic elicitors have been found to mimic a subset of known SA functions; likely by directly interfering with known or unknown receptors of this defense hormone. Besides such SA agonists, which molecularly mimic SA, other synthetic elicitors may trigger transcriptional and physiological responses related to those induced by SA without directly interfering with SA targets. For this review we consider both types of SA mimics as functional SA analogs. Synthetic elicitors of this type are described in the section, below.

PROBENAZOLE (PBZ)

Several biologically active 1,2-benzisothiazole derivatives have been found to exhibit a broad spectrum of pharmacological activities and to serve as antibacterials, fungicides and anti-inflammatory agents (De, 1981; Trapani et al., 1985; Zani et al., 1996; Vicini et al., 2002). Some of them also show auxin-like activity and have been used as herbicides (Giannella et al., 1971; Branca et al., 1975). Inspired by the potency of some of these compounds, researchers of Meiji Seika Kaisha Ltd. in Japan performed systematic tests with representatives of this class of molecules (Watanabe et al., 1977). They found 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (now widely known as PBZ), to efficiently control rice blast (*Magnaporthe oryzae*; anamorph: *Pyricularia oryzae*) infections in rice (*Oryza sativa*; Watanabe et al., 1977; Schreiber and Desveaux, 2008). This compound showed remarkable effects in suppressing rice blast at a dose of 896 uM (200 ppm) when applied by drenching roots (Watanabe et al., 1977) and has been commercially used under the name Oryzemate® for more than 30 years in the field protecting rice from rice blast fungus and bacterial leaf blight as well as corn from southern corn leaf blight (Iwata, 2001; Oostendorp et al., 2001). PBZ does not influence the growth of various tested crops, such as tomato, cucumber, Chinese cabbage, kidney bean, or rice, when sprayed at a concentration of 2240 uM (500 ppm), but at 4480 uM (1000 ppm) some abnormalities in plant development can be observed (Watanabe et al., 1977).

Probenazole affects various stages of the blast fungus infection cycle and inhibits hyphal penetration into the host tissue, lesion expansion and sporulation (Watanabe et al., 1977). From PBZ-treated rice plants anticonidial germination substances were isolated and characterized as toxic against fungi. These antifungal plant metabolites included a mixture of fatty acids, such as octadecatrienoic acid, palmitic acid, linoleic acid, and linolenic acid (Sekizawa et al., 1981; Shimura et al., 1983). Moreover, activities of defense-related enzymes, such as peroxidase, polyphenoloxidase, PAL, tyrosine ammonia-lyase and catechol-O-methyltransferase, increased dramatically in rice upon treatment with PBZ, as they do in response to infection with rice blast fungus (Midoh and Iwata, 1996; Iwata, 2001).

A PBZ-induced cDNA termed *PBZ-responsive gene* (*PBZ1*) has been cloned from rice. PBZ1 transcript accumulation was found to serve as a robust marker for responses to this synthetic elicitor. PBZ-induced PBZ1 mRNA accumulates in a dose-dependent manner. *PBZ1* expression is also induced by rice blast fungus, but not wounding. *PBZ1* belongs to the *PR-10* family of classical *PR* genes. One of the metabolites of PBZ, 1,2-benzisothiazole-3(2H)-one-1,1-dioxide (BIT) was found to be as potent in inhibiting rice blast as PBZ, but does not induce the accumulation of the PBZ1 transcripts (Midoh and Iwata, 1996; Nakashita et al., 2001, 2002b; Yoshioka et al., 2001). Thus, induced *PBZ1* expression seems not to be needed for rice blast resistance.

Microarray and RT-PCR analysis revealed up-regulation of UDP-glucose:SA glucosyltransferase (*Os*SGT1) transcripts in response to PBZ treatment in rice (Umemura et al., 2009). RNAi-mediated *Os*SGT1 knockdown in transgenic rice plants resulted in reduced PBZ-mediated resistance against blast. Although mechanistic details of its role in defense induction are unclear, *Os*SGT1 appears to be critical for PBZ-mediated defense induction (Umemura et al., 2009).

In *Arabidopsis*, both PBZ and its metabolite BIT stimulate expression of *PR* genes and induce SA accumulation and SAR. PBZ and BIT do not activate plant immunity in *npr1* mutants or *nahG* plants. Thus, SA and NPR1 seem to be required for PBZ- and BIT-mediated defense responses and both compounds mimic effects of SA (Yoshioka et al., 2001; Nakashita et al., 2002b). However, in contrast to INA, BTH, and DCA, which are likely authentic SA agonists (see below), PBZ and BIT appear to interfere with defense signaling steps upstream from SA accumulation and not to interact with downstream targets of SA.

2,6-DICHLORO-ISONICOTINIC ACID (INA)

Kunz et al. (1988) of Ciba-Geigy reported screening of a large number of compounds for activation of resistance in cucumber (*Cucumis sativus*) against the fungal pathogen *Colletotrichum lagenarium* and identified 2,6-dichloro-isonicotinic acid (INA) and its ester derivative CGA41397 (Kunz et al., 1988; Metraux et al., 1991). High levels of protection of cucumber against *C. lagenarium*, were achieved by foliar-spray application of 104 uM (20 ppm) INA or CGA41397 as well as root drench application of 10-fold lower concentrations of each compound. In these chemically-treated plants, responses were similar to those observed in systemic tissues of plants whose lower leaves were inoculated with TNV *or C. lagenarium* that induce SAR in upper leaves. Under field conditions, INA provided pathogen resistance in pear, pepper and rice (Kuc, 1982; Metraux et al., 1991). INA was also shown to induce SAR in tobacco and *Arabidopsis (*Ward et al., 1991; Uknes et al., 1992*)* and provide significant protection of tobacco against TMV, *Cercospora nicotianae, Peronospora tabacina, Phytophthora parasitica* var nicotianae, and *P. syringae* pv. tabaci (Ward et al., 1991).

In *Arabidopsis* INA can trigger long-lasting *PR* gene expression and disease resistance. In this species it can reduce susceptibility to virulent strains of the oomycete *Hyaloperonospora arabidopsidis (Hpa*) or *P. syringae* pv. tomato DC3000 without directly affecting viability of these pathogens (Uknes et al., 1992; Knoth et al., 2009). As injection of 1 mM INA into tobacco leaves induces transcript accumulation of the same characteristic set of *PR* genes as SA application, it is considered a functional SA analog. Although INA partially mimics defense-associated effects of SA, it does not trigger any changes of SA levels and, unlike SA or PBZ, induces SAR in *nahG* transgenic tobacco and *Arabidopsis* plants (Delaney et al., 1994; Vernooij et al., 1995). Thus, INA must be interfering with targets that operate downstream from SA accumulation

and are likely involved in the interpretation of SA levels. Consistent with this assumption, INA has been reported to mimic several proposed biochemical and physiological effects of SA, such as inhibition of catalase and APX activity or the induction of cellular H_2O_2 accumulation (Chen and Klessig, 1991; Chen et al., 1993, 1995; Conrath et al., 1995; Durner and Klessig, 1995). The modulation of ROI levels seems to be a critical aspect of INA activity, since antioxidants can block the INA-dependent induction of *PR* gene expression (Chen et al., 1995; Durner and Klessig, 1995).

Through mutant screens to identify genes required for SAR in *Arabidopsis*, the *npr1/nim1* (*non-expresser of PR genes 1, no immunity 1*) mutants that are insensitive to SA and INA were discovered (Cao et al., 1994; Delaney et al., 1995). Both biologically- and INA-induced SAR as well as basal defense were found to be compromised in either one of these mutants. The *npr1* and *nim1* mutants are in different *Arabidopsis* accessions, but were found to be allelic and to have defects in the same gene (Cao et al., 1994, 1997; Ryals et al., 1997). A large body of literature has reported on molecular roles of NPR1 as a transcriptional cofactor, since its identification as a major regulator of SAR. These studies have been summarized in several excellent reviews (Dong, 2004; Durrant and Dong, 2004; Fu and Dong, 2013). Most importantly, NPR1, together with NPR3 or NPR4, have been found to serve as SA receptors (Fu et al., 2012; Fu and Dong, 2013). NPR3 can bind to NPR1 in a SA dose-dependent manner, while NPR4-NPR1 interactions are constitutive and inhibited by SA. In yeast two-hybrid assays, in addition to SA, INA can promote NPR1–NPR3 interactions. INA can also reduce the binding affinity of SA to NPR3 and NPR4 by competing with this defense hormone (Fu et al., 2012). Thus, INA appears to be a true SA agonist.

In addition to *npr1* mutants, triple or quadruple mutants of closely related TGA-bZIP transcription factors, which are known to physically interact with NPR1, are also blocked in INA-induced *PR* gene expression and pathogen resistance (Zhang et al., 2003; Wang et al., 2006). Thus, INA seems to mediate its defense-related effects upon interactions with NPR1-related proteins, which control several TGA transcription factors. Interactions with other SA-binding proteins, such as SABP1 and SABP2 may also to contribute to the activity of this SA analog. So far, INA has been applied to many plant species and was found to induce resistance against a wide variety of pathogens (Hijwegen and Verhaar, 1993; Conrath et al., 1995; Van Kan et al., 1995; Han et al., 2000; Lee et al., 2009). However, because INA and its derivatives have phytotoxic side effects in crops, none of these compounds has been commercialized as agrochemicals (Oostendorp et al., 2001). Still, INA is being continually used as an efficient chemical tool to study SAR.

BENZOTHIADIAZOLE (BTH)

Another SAR-inducer screening by Ciba-Geigy with a large number of benzo[1,2,3]thiadiazole-7-carboxylic acid derivatives resulted in the identification of benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl-ester [benzothiadiazole (BTH); acibenzolar-S- methyl (ASM), CGA245704] as a potent inducer of plant immune responses (Schurter et al., 1993; Kunz et al., 1997; Oostendorp et al., 2001). BTH was subsequently shown to trigger in various plant species resistance against a wide variety of pathogens, such as TMV, *Cercospora nicotianae, Erwinia carotovora, Phytophthora parasitica* and *P. syringae* pv. tabaci (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996; Kunz et al., 1997). As BTH did not show any direct effect on a number of plant pathogens *in vitro*, BTH is not antimicrobial (Friedrich et al., 1996). In *Arabidopsis*, BTH triggers *NPR1*-dependent SAR (Lawton et al., 1996).

At the molecular level, BTH induces the same characteristic set of SAR-related responses that are induced by pathogens or SA, including up-regulation of *PR* genes. Thus, like INA, BTH appears to be a functional analog of SA (Friedrich et al., 1996; Wendehenne et al., 1998). INA and BTH share several characteristic functional features. Both compounds do not induce accumulation of SA in plants (Vernooij et al., 1995; Friedrich et al., 1996) and share the ability to induce SAR and *PR* gene expression in transgenic *nahG* lines (Vernooij et al., 1995; Lawton et al., 1996). Thus, both INA and BTH seem to activate SA-response mechanisms by interfering as SA agonists with targets operating downstream from SA accumulation. Like SA and INA, BTH was also proposed to inhibit both APX and catalase functions (Du and Klessig, 1997; Wendehenne et al., 1998). However, BTH is a much more effective inhibitor of catalase than SA and the catalase inhibition mechanisms of BTH and SA are different. While SA seems to inhibit catalase function in an H_2O_2- and time-dependent manner, BTH inhibits this activity independently from time and H_2O_2. INA was not included in these experiments. For APX inhibition, however, BTH and SA exhibit similar dose-response curves (Wendehenne et al., 1998).

Recent data suggested that BTH is converted into acibenzolar by SABP2 and this product is critical for SAR induction. When BTH was sprayed on SABP2-silenced tobacco plants, it failed to induce PR1 protein expression and SAR. On the contrary, when the same transgenic plants were treated with acibenzolar, SAR was fully induced (Tripathi et al., 2010).

In rice, it was shown that the *Os*WRKY45 transcription factor plays a pivotal role in BTH-induced defense responses against rice blast disease. This BTH-triggered defense mechanism seems independent of NH1, a rice ortholog of *A. thaliana* NPR1 (Shimono et al., 2007). WRKY45 knockdown lines exhibited strongly reduced levels of BTH-induced resistance to the fungal pathogen *M. oryzae* and the bacterial pathogen *Xanthomonas oryzae* pv. oryzae (Xoo; Shimono et al., 2007). Interestingly, *Os*WRKY45 is an ortholog of *At*WRKY70, which also can act in an NPR1-independent manner in SA signaling in *Arabidopsis* (Li et al., 2004; Knoth et al., 2007, 2009). In addition to BTH, PBZ and Tiadinil (TDL; see below) partly induced blast resistance in rice through a WRKY45-dependent pathway (Shimono et al., 2012). Recently, WRKY45-regulated BTH-responsive genes were identified by microarrays (Nakayama et al., 2013).

BTH can also prime plant defense reactions. Low doses of BTH that are insufficient to trigger detectable levels of defense responses, can prime parsley cells and increase their sensitivity for MAMP-triggered coumarin phytoalexin secretion. This effect is associated with potentiated activation of genes encoding phenylalanine ammonia-lyase (PAL), which is critical for coumarin biosynthesis. In addition to BTH, also SA and INA can prime parsley cells for the activation of coumarin secretion by low MAMP doses (Kauss et al., 1992; Katz et al., 1998;

Thulke and Conrath, 1998; Conrath et al., 2002). BTH can also prime *Arabidopsis* plants for enhanced pathogen-responsiveness of *PAL* gene expression. BTH-mediated defense priming in *Arabidopsis* is dependent on NPR1 (Kohler et al., 2002; Goellner and Conrath, 2008). An interesting mechanism involving two known defense-associated MAPKs, MPK3, and MPK6, seems to contribute to this priming phenomenon in *Arabidopsis*. BTH induces the accumulation of mRNA and inactive protein forms of both MAPKs. Subsequent stress treatment results in phosphorylation and activation of MPK3 and MPK6 (Beckers et al., 2009). In addition, epigenetic chromatin marks appear to be involved in defense-priming processes. The *AtWRKY29*, *AtWRKY6,* and *AtWRKY53* genes showed a typical priming response and were strongly transcribed after stress application following pre-treatment with BTH. BTH pre-treatment also triggered in these experiments various histone modifications that are typically found at actively transcribed genes, such as H3K4me3, H3K4me2, H3ac, or H4ac at *AtWRKY29* and H3K4me3 or H3K4me2 at *AtWRKY6* and *AtWRKY53*. BTH-induced trimethylation of H3K4 is reduced in the priming-deficient *npr1* mutant. On the contrary, the constitutively primed *cpr1* and *sni1* mutants exhibit high levels of H3K4me3 in the absence of BTH treatment. Thus, elevated H3K4me3 levels are closely associated with BTH-induced defense gene priming (Jaskiewicz et al., 2011).

In contrast to INA, BTH was found to be suitable for agricultural crop protection. It became a commercial product under the trade name of BION® (in Europe) in 1989 and Actigard® (in the US) in 1990 (Schurter et al., 1993; Kunz et al., 1997; Oostendorp et al., 2001). BTH activates very wide spectrum of resistances of various plant species against fungal, bacterial, or viral pathogens and several insects and nematodes.

N-(3-CHLORO-4-METHYLPHENYL)-4-METHYL-1,2,3-THIADIAZOLE-5-CARBOXAMIDE (TIADINIL, TDL)

Thiadiazoles are known to have many pharmacological activities (Camoutsis et al., 2010; Chaudhary et al., 2010; Kharb et al., 2011; Singh et al., 2011). Tests of various 1,2,3-thiadiazole derivatives for their ability to control rice blast disease by Nihon Nohyaku Co., Ltd. (Japan) resulted in the discovery of *N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (Tiadinil, TDL), which provided protection against this disease without exhibiting any antimicrobial activity (Tsubata et al., 2006). Since 2003, this compound has been commercially available under the trade name V-GET®in Japan. Its metabolite 4-methyl-1,2,3-thiadiazole-5-carboxylic acid (SV-03), exhibited similar levels of anti-rice blast activity as TDL (Tsubata et al., 2006; Toquin et al., 2012). In addition to rice blast, TDL is also used to control the pathogenic fungi *Colletotrichum theaesinensis* and *Pestalotiopsis longiseta* on tea leaves (Yoshida et al., 2010).

In tobacco, TDL and SV-03 induce SAR and increased local resistance to TMV, the virulent bacterial pathogen *P. syringae* pv. tabaci and powdery mildew (*Oidium lycopersici*) without affecting these pathogens directly. Both compounds also induce *PR1*, *PR2* and *PR5* gene expression in *Arabidopsis* and enhance basal resistance of this species to *P. syringae* pv. tomato DC3000 (Yasuda et al., 2004, 2006; Yasuda, 2007). TDL or SV-03 treatment does not induce accumulation of SA in tobacco. Moreover, TDL or SV-03-treated *nahG* transgenic tobacco plants exhibit enhanced resistance to TMV and *P. syringae* pv. tabaci and induced *PR* gene expression. However, TDL- or SV-03-triggered defense responses are blocked in *Arabidopsis npr1* mutants. Taken together, these results suggest that, similar to BTH and INA, TDL and SV-03 trigger disease resistance by interfering with signaling steps downstream of SA (Yasuda et al., 2006; Yasuda, 2007).

The thiadiazole derivative, 1,3,4-oxadiazole, has also been shown to exhibit antifungal and antibacterial activities (Kharb et al., 2011; Singh et al., 2011). By combining different heterocyclic thiadiazole-related moieties, including oxadiazoles, new compounds were designed and evaluated regarding their performance in crop disease protection. Although only three out of the 23 tested compounds elicited SAR more efficiently than TDL, combining thiazole- and oxadiazole moieties may be a promising approach in designing new crop protectants (Fan et al., 2009).

ISOTIANIL

As a result of a comprehensive search for isothiazole-based compounds, Isotianil was discovered by Bayer AG (now Bayer CropScience AG) in Germany in 1997 and developed jointly with the Japanese company Sumitomo Chemical Co., Ltd. as a crop protectant against rice blast and bacterial leaf blight in rice. It also activates defense responses against a wide range of additional pathogens in various plants. Moreover, Isotianil does not show any direct antimicrobial activity against bacteria and fungi (Ogava et al., 2011; Toquin et al., 2012). In 2010, it was registered under the name Stout® in Japan and China, where it substantially increased rice production (Ogava et al., 2011; Brozek et al., 2012; Yoshida et al., 2013). Its efficiency against rice blast seems unusually high, as lower dosages of Isotianil are needed than of any other existing plant defense activator, such as PBZ and TDL (Ogava et al., 2011).

At the molecular level, Isotianil treatment triggers accumulation of defense-related enzymes such as lipoxygenase or PAL in rice. Affymetrix whole genome microarray analysis revealed that Isotianil treatment induces some defense-related genes, including *OsWRKY45,* that are involved in SA signaling (Ogava et al., 2011; Toquin et al., 2012). Further microarray analyses showed that Isotianil likely primes rice for more intense defense activation in response to pathogen infections. At this point no published information on its mode-of-action is available.

N-CYANOMETHYL-2-CHLOROISONICOTINAMIDE (NCI)

A screen of 2-chloroisonicotinamide derivatives for effective rice blast control agents were performed by Nihon Nohyaku Co., Ltd. (Japan), resulted in the identification of *N*-cyanomethyl-2-chloroisonicotinamide (NCI) as a potent defense inducer (Yoshida et al., 1989, 1990a,b). NCI showed one of the highest anti-blast activities compared to other *N*-alkyl-2-chloroisonicotinamides and its efficacy was equal to that of PBZ. It does not show antifungal activity against rice blast *in vitro* at concentrations as high as 1100 uM (500 ppm). Its activity is long-lasting, as it was found to be still effective against rice blast 30 days after a single application.

NCI treatment inhibits mycelial development of *P. oryzae* at inner epidermal cells and increases the number of small brownish lesions that are correlated with active immunity of rice. These results suggest that NCI efficiently induces plant defense mechanisms (Yoshida et al., 1990a).

In tobacco, NCI can induce SAR and mediate local resistance to TMV, *Oidium lycopersici* and *P. syringae* pv. tabaci. It also induces expression of *PR1*, *PR2* and *PR5* and is active in transgenic *nahG* tobacco plants. Thus, it does not require SA for activation of defense (Nakashita et al., 2002a). In *Arabidopsis*, NCI reduces growth of virulent *P. syringae* and induces resistance independently from SA accumulation, ET and JA, but requires NPR1. Thus, like INA and BTH, NCI seems to interfere with defense signaling steps operating between SA and NPR1 (Yasuda et al., 2003a; Yasuda, 2007).

3-CHLORO-1-METHYL-1H-PYRAZOLE-5-CARBOXYLIC ACID (CMPA)

A screen by Nishioka et al. (2003) targeting new chemicals to control blast disease in rice resulted in the discovery of pyrazolecarboxylic acid derivatives as potent inducers of systemic immunity. The most efficient anti-blast compound identified in this screen was 3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA). CMPA does not directly affect pathogen viability up to a concentration of 623 uM (100 ppm), while it can significantly induce rice blast resistance at 10-fold lower concentrations. Thus, its anti-blast activity is not dependent on antimicrobial activity and this compound seems to activate systemic plant defense mechanisms (Nishioka et al., 2003). Although, CMPA, BTH, and PBZ trigger rice blast resistance with similar efficacies, CMPA induces PBZ1 transcript accumulation in rice at levels lower than PBZ or BTH (Nishioka et al., 2005).

In tobacco, CMPA enhances resistance to *P. syringae* pv. tabaci and *Oidium sp.*. CMPA also induces expression of *PR1*, *PR2,* and *PR5* in wild-type as well as *nahG* transgenic tobacco. Therefore, CMPA seems not to require SA to induce SAR-like disease resistance and may interfere with defense signaling downstream from SA. Consistent with this assumption, CMPA was found to act through NPR1 in *Arabidopsis* (Yasuda et al., 2003b; Yasuda, 2007).

3,5-DICHLOROANTHRANILIC ACID (DCA)

The compound 3,5-dichloroanthranilic acid (DCA) is one of 114 synthetic elicitor candidates that were identified by a comprehensive screening of 60,000 diverse compounds for inducers of the pathogen-responsive *CaBP22::GUS* reporter gene in *Arabidopsis* (Knoth et al., 2009; Knoth and Eulgem, 2014). DCA efficiently triggers resistance of *Arabidopsis* against virulent strains of the oomycete *Hpa* and *P. syringae* DC3000. It up-regulates transcript levels of various known SA-responsive defense-related genes, such as *PR1*, *WRKY70,* and *CaBP22*. Like INA and BTH, its activity does not require accumulation of SA. However, unlike these well-characterized SA analogs, DCA-mediated immunity is not fully blocked in *npr1 Arabidopsis* mutants. DCA-triggered immune responses are to a large extent independent from NPR1, but partially blocked in *wrky70* mutants. Thus DCA partially targets a WRKY70-dependent branch of the defense signaling network that does not require NPR1 (Knoth et al., 2009).

Microarray analyses revealed that DCA, INA, and BTH trigger partially overlapping transcriptional responses in *Arabidopsis* (Wang et al., 2006; Knoth et al., 2009; Bhattarai et al., 2010). For example, transcripts of a set of 202 genes were found to be commonly up-regulated by each one of these three synthetic elicitors. However, DCA, INA, and BTH also induce unique transcriptional changes. Taken together, these and other observations suggest that each of these SA analogs interferes with targets in the SA response pathway in a unique manner.

ADDITIONAL FUNCTIONAL ANALOGS OF SA

Besides the functional analogs of SA that are discussed above, additional derivatives of this defense hormone were tested (Conrath et al., 1995; Knoth et al., 2009). This includes 3,5-dichlorosalicylic acid, 4-chlorosalicylic acid, and 5-chlorosalicylic acid, which mimic SA, induce *PR1* gene expression and enhance disease resistance to TMV infection in tobacco (Conrath et al., 1995). Furthermore, 3-chlorobenzoic acid and 3,5-dichlorobenzoic acid induce basal defense against *Hpa* as well as *CaBP22::GUS* expression in *Arabidopsis* (Knoth et al., 2009). In contrast, the SA-related compounds benzoic acid, 2-aminobenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and 4-amino-SA did not show any defense-inducing activity (Chen and Klessig, 1991; Conrath et al., 1995; Durner and Klessig, 1995).

Furthermore, several agonists of the peroxisome proliferator-activated receptor were found to mimic effects of SA in local HR responses, but not *PR* gene expression or SAR, in soybean. The latter finding suggested that the roles of SA in local and systemic defense induction are distinct (Tenhaken et al., 2001).

IMPRIMATINS

A screen of 10,000 small molecules to identify plant immune priming compounds by Noutoshi et al. (2012d) and coworkers resulted in the identification of three distinct classes of compounds that can prime *Arabidopsis* cells to exhibit enhanced immunity against virulent and avirulent *P. syringae*. These immune-priming compounds were termed Imprimatins. Based on structural similarities they were classified as Imprimatin A, -B or -C, representatives, respectively (**Table 2**; Noutoshi et al., 2012c,d,e,f).

A common feature of Imprimatin A and Imprimatin B compounds is that they only prime plants for enhanced defense reactions and cannot directly induce immune responses (Noutoshi et al., 2012e,f). Application of each of these compounds increases levels of endogenous SA and decreases levels of the inactive SA metabolite SAG suggesting they inhibit SAGTs (Noutoshi et al., 2012e,f). Supporting this view, single and double knockout mutants of the *Arabidopsis* SAGT genes *UGT74F1* and *UGT76B1* showed increased disease resistance and free SA levels and resemble in this respect wild-type *Arabidopsis* plants treated with Imprimatins A_1, A_2, A_3, B_1, or B_2 (Noutoshi et al., 2012e). The enzymatic activities of UGT74F1 and UGT76B1 were also blocked *in vitro* by each of these Imprimatins at concentrations effective for immune priming. These results suggest that Imprimatin A and -B representatives have a unique mode-of-action in defense priming and specifically inhibit SAGTs (Noutoshi et al., 2012e,f).

Table 2 | Imprimatins.

Main type	Common name	Systematic name
Imprimatin A	Imprimatin A_1	2-[(E)-2-(2-bromo-4-hydroxy-5-methoxyphenyl)ethenyl] quinolin-8-ol)
	Imprimatin A_2	7-chloro-2-[(E)-2- (4-nitrophenyl)ethenyl]-4H-3,1-benzoxazin-4-one)
	Imprimatin A_3	4-[(E)-2-(quinolin-2-yl)ethenyl]phenol)
Imprimatin B	Imprimatin B_1	2-(3-(2-furyl)-3-phenylpropyl) benzo[c]azoline-1,3-dione)
	Imprimatin B_2	3-(2-furyl)-3-phenylpropylamine)
Imprimatin C	Imprimatin C_1	[(E)-[1-amino-2-(2-oxopyrrolidin-1-yl)ethylidene]amino] 4-chlorobenzoate)
	Imprimatin C_2	[(E)-[1-amino-2-(2-oxopyrrolidin-1-yl)ethylidene]amino]3,4-dichlorobenzoate)

Two members of class C of Imprimatins, C_1 and C_2, were found to be SA analogs, as they activate downstream SA signaling steps and induce expression of known SA-responsive genes. However, their defense-inducing activity is weaker than that of SA. Further structure-function analyses suggested that these compounds may be converted in *Arabidopsis* to 4-chlorobenzoic acid and 3,4-chlorobenzoic acid, which can mimic the defense-related effects of Imprimatins C1 and C2 (Noutoshi et al., 2012c).

SULFONAMIDES

SULFANILAMIDES

In order to identify small molecules that reduce susceptibility of *Arabidopsis* to virulent *P. syringae*, a small collection of 200 molecules from the LATCA library (Library of Active Compounds in *Arabidopsis*; Zhao et al., 2007) was screened for candidates that reduce cotyledon bleaching in liquid grown seedlings. *P. syringae* induced bleaching of *Arabidopsis* cotyledons is a robust disease symptom that develops within 4–5 days post-inoculation with this pathogen (Schreiber et al., 2008). Among other candidates, the sulfanilamide compounds, sulfamethoxazole (Smex), sulfadiazine (Sdiz), and sulfapyridine (Spyr) were found to reduce this bleaching phenotype. Although, sulfanilamides have been widely used as antibiotics, the authors showed that these three candidates did not directly reduce bacterial viability and growth at concentrations that suppress their virulence. Thus, these compounds seem to act by inducing plant immune responses (Schreiber et al., 2008).

Sulfamethoxazole was found to be the most potent one of the three identified sulfanilamides. Smex can prevent cotyledon bleaching at a concentration of 100 uM. Interestingly, Smex does not induce *PR1* expression and is active in *npr1* mutants. Thus, Smex is likely to induce defense mechanisms unrelated to the canonical SA defense pathway. Smex-mediated disease protection is also independent from JA, ET, and ABA signaling and does not require an oxidative burst (Schreiber and Desveaux, 2008; Schreiber et al., 2008).

Sulfanilamides are structural analogues of *p*-aminobenzoic acid (PABA), which can inhibit dihydropteroate synthase, an enzyme that catalyzes an important step in the folate biosynthetic pathway. Smex-mediated inhibition of folate biosynthesis may induce plant defense mechanism independently from *PR1* expression (Schreiber et al., 2008, 2012). A screen performed by the same lab to identify compounds that protect *Arabidopsis* against the fungal pathogen *Fusarium graminearum* resulted, besides Smex, in the identification of the indole alkaloid gramine as a plant defense inducer. Both gramine and Smex reduced severity of *F. graminearum* infection in wheat as well (Schreiber et al., 2011).

OTHER SULFONAMIDES

Noutoshi et al. (2012a), additional sulfonamide compounds were also reported to induce disease resistance in plants. By using the same chemical screening strategy that was used for Imprimatins, chemical libraries representing 2677 bioactive molecules and small natural compounds were screened to identify immune-priming molecules. Four different sulfonamide compounds, sulfameter (SFM), sulfamethoxypyridazine (SMP), sulfabenzamide (SBA), and sulfachloropyridazine (SCP) were identified in this screening and further characterized. They increased the occurrence of cell death of *Arabidopsis* suspension cell cultures infected by an avirulent *P. syringae* strain and were classified as immune-priming compounds. However, unlike Smex, these compounds can induce *PR1* gene expression and, unlike Imprimatin A or B representatives, they do not inhibit SAGTs (Noutoshi et al., 2012a).

DIURETICS

Diuretics are pharmaceutical drugs that are widely used in clinical medicine, especially to treat hypertensive and oedematous states (Plant, 2003). Three diuretics, 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoic acid (Bumetanide), 3-benzyl-1,1-dioxo-6-(trifluoromethyl)-3,4dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide (Bendroflumethiazide) and 4-chloro-*N*-(2,6-dimethyl-1-piperidyl)-3-sulfamoyl-benzamide (Clopamide; McNeil et al., 1987; Breyer and Jacobson, 1990; Pacifici, 2012) were identified as plant immune-priming compounds through the screening of a chemical library of 2000 known bioactive compounds (Noutoshi et al., 2012b). They stimulate pathogen-induced cell death in *Arabidopsis* in a concentration-dependent manner. In *Arabidopsis* they can enhance disease resistance to both avirulent and virulent *P. syringae* strains. Effects of 100 uM diuretic on defense induction are comparable to those triggered by 50 uM SA and they do not directly inhibit bacterial growth up to concentration of 200 uM. Application of these diuretics significantly decreases the growth of avirulent bacteria compared to mock treatment and mediates enhanced *PR1* gene expression after infection with *P. syringae*. These compounds potentiate disease resistance by enhancing plant defense responses, but, unlike SA and its analogs, do not induce *PR1* expression in the absence of pathogen infection (Noutoshi et al., 2012b).

Diuretics exhibit pharmacological effects in humans by acting on proteins of the SLC12A family, which are sodium-coupled chloride co-transporters that are located along the renal tubule of

the kidney nephron. Diuretics inhibit these co-transporters by binding to their Cl^- binding site (Breyer and Jacobson, 1990; Gamba, 2005). The *Arabidopsis* genome encodes only a single protein closely related to SLC12A, *At*1g30450 (*At*CCC1). Thus, diuretics-triggered defense priming may be mediated via *At*CCC1. However, no results regarding this possible role of *At*CCC1 have been reported.

Interestingly, diuretics contain a sulfonamide moiety similar to those identified in the defense-inducing sulfanilamide compounds sulfamethoxazole, sulfadiazine, and sulfapyridine (Schreiber et al., 2008). Both diuretics and sulfanilamides can decrease bacterial growth *in planta*. The presence of sulfonamide moieties seems to be essential for their ability to induce defense reactions, as diuretics without sulfonamide groups do not exhibit this activity (Schreiber et al., 2008; Noutoshi et al., 2012b). Further studies with diuretics and sulfanilamides are needed to uncover their modes-of-action.

ADIPIC ACID DERIVATIVES

In order to identify chemical mixtures that can delay senescence and induce immunity in plants, various mixtures of adipic acid monoethyl ester derivatives were tested. Application of a mixture of furfurylamine and 1,2,3,4-tetra-O-acetyl-β-D-glucopy-ranose (FGA) increased chlorophyll content, cell wall sugar content and delayed the chlorophyll degrading rate along with senescence in tomato and pepper (Flors et al., 2001). FGA also increased PAL activity as well as the concentration of flavonoids and phenolic compounds and strengthened plant immunity against various different pathogens such as *Phytophthora citrophthora* and *Altemaria solani* in tomato (*Solanum lycopersicum L.*) as well as *Alternaria solani* in pepper (*Capsicum annuum* L.; Flors et al., 2001). Individual application of three novel amides of adipic acid, 5-carbamoil ethyl pentanoate (N1), 5-(2-furfurylmethylcarbamoil) ethyl pentanoate (N2) and 5-(3-aminopropylcarbamoil) ethyl pentanoate (N3) was shown to strongly induce resistance against *Alternaria solani* in pepper. However, many other adipic acid derivatives were most effective when used as a mixture (Flors et al., 2003a,b). Although these chemicals reduced pathogen growth in their hosts, many of them did not show any direct antimicrobial effect to pathogens and, therefore, likely induce plant immune responses (Flors et al., 2001, 2003a,b, 2004). However, the mode-of-action underlying this function remains unresolved.

JASMONIC ACID ANALOGS

Jasmonic acid and its methylester, methyl-jasmonate (MeJA), are important members of the family of jasmonates which are biologically active fatty-derived cyclopentanones, that are broadly present in the plant kingdom. They are synthesized rapidly by the octadecanoid (and possibly hexadecanoid) biosynthesis pathways upon pathogen or insect attack and activate defense responses (Howe, 2010; Wasternack and Hause, 2013). Jasmonates are known to control stress responses against nectrotrophic pathogens, herbivores and wounding, but are also known to perform various important roles in plant development related to leaf senescence, growth inhibition and floral development (He et al., 2002; Balbi and Devoto, 2008; Zhang and Turner, 2008; Oh et al., 2013; Santino et al., 2013). Upon synthesis, JA can either be metabolized to MeJA or conjugated to L-isoleucine leading to jasmonoyl-isoleucine (JA-Ile), which is an active form of JA (Svoboda and Boland, 2010; Pieterse et al., 2012).

Together with Jasmonate ZIM-domain (JAZ)-type transcriptional repressors, the F-box protein Coronatine Insensitive1 (COI1) functions as JA-Ile receptors. Recruitment of JAZ proteins into COI1-containing SKP1-Cullin-F-box (SCF^{COI1}) complexes results in proteasome-mediated degradation of these transcriptional repressors. Consequently expression of a large number of JA-responsive genes is de-repressed and defense responses are activated (Browse, 2009; Pieterse et al., 2012; Monte et al., 2014). Jasmonates typically promote defense responses against necrotrophic microbial pathogens. For example, exogenous application of JA or MeJA was shown to protect barley against *Erysiphe graminis* f.sp. hordei (Schweizer et al., 1993). In *Arabidopsis*, MeJA up-regulates transcript levels of the *PDF1.2* gene family along with 100s of additional genes (Schenk et al., 2000; Jung et al., 2007; Scranton et al., 2013) and enhances resistance to various necrotrophic pathogens, such as the fungi *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998; Seo et al., 2001; Rowe et al., 2010).

Systematic structural modifications of JA revealed the minimal structural requirements required for its bioactivity allowing for the synthesis of JA-mimics (Svoboda and Boland, 2010). The synthetic JA mimic coronalon (2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester) mediated induction of stress responses in various plant species (Schüler et al., 2004). In addition, coronalon and its unsubstituted form (1-oxo-indanoyl-L-isoleucine methyl ester) increased levels of nicotine and trypsin proteinase inhibitors which are known MeJA-activated defense products in *N. attenuata*. They also triggered transcriptional up-regulation of the majority of genes that are known to be responsive to MeJA (Pluskota et al., 2007). The compound 1-oxo-indanoyl-L-isoleucine methyl ester was also shown to enhance activity of defense-related enzymes such as PAL or peroxidases and to induce resistance against downy mildew (Deepak et al., 2007). Additional synthetic JA mimics were shown to induce jasmonate signaling and immune responses in various plant species (Krumm et al., 1995; Fliegmann et al., 2003; Pluskota et al., 2007), However, none of these compounds were studied at the molecular level and nothing is known about their modes-of-action.

CONCLUSIONS AND PERSPECTIVES

In this review article we have provided an overview of the discovery and functional characteristics of synthetic elicitors as well as their potential for basic research and crop protection. In our opinion, three major observations stand out.

(1) The vast majority of known synthetic elicitors belongs to the large group of functional SA analogs and mimics roles of this messenger molecule in defense induction. Many of these compounds are structurally related to SA. This strong trend may be partially due to a bias in the used compound screening strategies, most of which were based on the use of known SA-triggered immune responses as an indicator of defense induction. However, the dominance of functional SA analogs

among known synthetic elicitors may also reflect that the SA-response pathway is particularly enriched for drug-able targets (which often have natural ligand binding pockets) and may involve more than just one type of SA receptor. This is consistent with the fact that responses triggered by different SA analogs do often not fully overlap and are partly unique. Thus, many functional SA analogs may constitute selective SA agonists, each of which interferes in a distinct manner with natural SA targets.

(2) Synthetic elicitors can be successfully applied in crop protection. Several examples illustrate the utility of plant immune-stimulants or -inducers in agriculture. Most likely more examples will follow, providing attractive alternatives to conventional biocidal agrochemicals.

(3) Synthetic elicitors can also serve as potent tools in basic research approaches expanding our knowledge of plant immunity. A particularly prominent example highlighting their potency in this respect is the role of INA in the discovery of NPR1 as a central regulator of SA-dependent immune responses.

While additional screens for synthetic elicitors that are more potent and possibly distinct from those that are known are desirable, a rich arsenal of interesting plant defense-inducing compounds is already at hand. What is missing at this point, is a comprehensive systematic comparison of their functional characteristics in a single plant system, such as *Arabidopsis*. We anticipate specific interactions of many of these compounds with the plant immune system to define distinct "points of reference," that can be probed and further examined with each compound. A next critical step will be the identification of direct synthetic elicitor targets and their roles in plant defense. This may lead to the discovery of so far unknown components of the plant immune system and reveal novel regulatory interactions controlling plant defense reactions. Furthermore, innovative screening designs are needed to complement the set of available compounds. A greater diversity of synthetic elicitors will not only be beneficial for basic research, but may also be necessary for the design of innovative multifunctional crop protectants that stimulate multiple aspects of the plant defense system and can provide resistance against a broader spectrum of plant pathogens.

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