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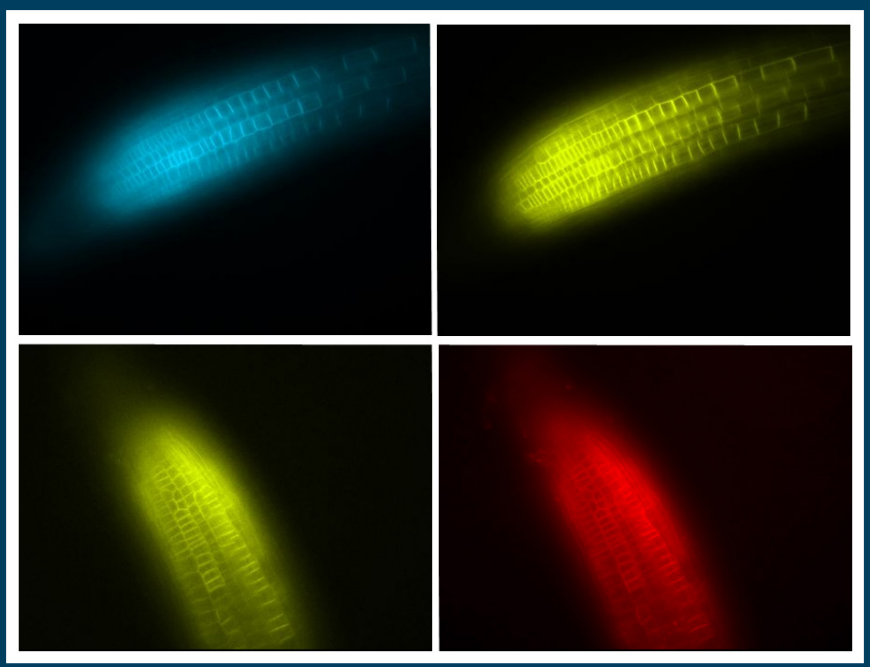
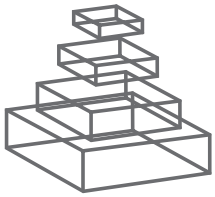


ROOT SYSTEMS BIOLOGY

Topic Editor
Wolfgang Schmidt

frontiers in PLANT SCIENCE



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ISSN 1664-8714
ISBN 978-2-88919-275-5
DOI 10.3389/978-2-88919-275-5

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ROOT SYSTEMS BIOLOGY

Topic Editor:
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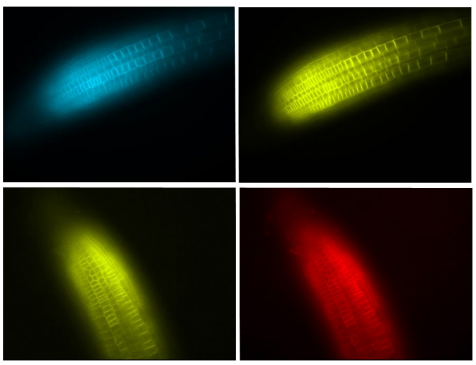

Expression of VSFP2s from the RPS5 promoter in root tips.

Figure taken from: Matzke AJM and Matzke M (2013) Membrane "potential-omics": toward voltage imaging at the cell population level in roots of living plants. *Front. Plant Sci.* 4:311. doi: 10.3389/fpls.2013.00311

The understanding of biological complexity has been greatly facilitated by cross-disciplinary, holistic approaches that allow insights into the function and regulation of biological processes that cannot be captured by dissecting them into their individual components. In addition, the development of novel tools has dramatically increased our ability to interrogate information at the nucleic acid, protein and metabolite level. The integration and interpretation of disparate data sets, however, still remain a major challenge in systems biology.

Roots provide an excellent model for studying physiological, developmental, and metabolic processes. The availability of genetic resources, along with sequenced genomes has allowed important discoveries in root biochemistry, development and function. Roots are transparent, allowing optical investigation of gene activity in individual cells and experimental manipulation. In addition, the predictable fate of cells emerging from the root meristem and the continuous development of roots throughout the life of the plant, which permits simultaneous observation of different developmental stages, provide ideal premises for the analysis of growth and differentiation. Moreover, a genetically fixed cellular organization allows for studying the utilization of positional information and other non-cell-autonomous phenomena, which are of utmost importance in plant development. Although their ontogeny is largely invariant under standardized experimental conditions, roots possess an extraordinary capacity to respond to a plethora of environmental signals, resulting in distinct phenotypic readouts. This high phenotypic plasticity allows research into acclimative and adaptive strategies, the understanding of which is crucial for germplasm enhancement and crop improvement.

With the aim of providing a current snapshot on the function and development of roots at the systems level, this Research Topic collated original research articles, methods articles, reviews, mini reviews and perspective, opinion and hypotheses articles that communicate breakthroughs in root biology, as well as recent advances in research technologies and data analysis.

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frontiers in
PLANT SCIENCE

EDITORIAL
published: 19 May 2014
doi: 10.3389/fpls.2014.00215

Root systems biology

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Keywords: root architecture, root hairs, systems biology, synthetic biology, auxin, regulatory peptides, nutrient acquisition, gene co-expression analysis

Plant roots, which are essential for providing anchorage to the soil, acquiring mineral nutrients and water, and for synthesizing a plethora of metabolites, provide an excellent model for studying physiological, developmental, and metabolic processes at a systems level. The challenge to understand such processes has been compared with deciphering the principle of a radio by a reductionist approach, i.e., by randomly removing parts from a series of identical radios and observing the "phenotypes" resulting from this procedure (Lazebnik, 2002). Undoubtably, understanding (root) biology as a whole represents a much bigger challenge, but the constant development of novel tools and algorithms as well as technical progress on omics technologies facilitate rapid progress toward a more integrative, holistic picture of root biology. The 13 articles in this ebook highlight the latest results, approaches, and resources in root systems biology.

One challenge when studying roots is their multicellular complexity. Qiao and Libault (2013) describe a method in which an ultrasound aeroponic system is employed to generate a large quantity of root hair cells, allowing for an uniform and long-term treatment of a single cell type with various biotic and abiotic stimuli for downstream functional genomics applications. Root hair development is affected by soil environmental factors that maximize the absorption capacity and, ultimately, the fitness of the plant. Lee and Cho (2013) summarize the role of auxin as a key player and organizing node for environmental/hormonal modulation of root hair growth. Auxin plays also a key role in the formation of lateral roots which, post-embryonically initiated from the primary root in response to developmental and environmental stimuli, provide a high level of plasticity to the root system architecture. New generation imaging techniques and high-throughput approaches, often used in combination with computational modeling, have triggered a revival of root development research. In their review article, Cuesta et al. (2013) describe traditional and novel tools, and evaluate their potential to address longstanding questions on lateral root organogenesis at a qualitatively new level.

Root architecture is closely interconnected with and shaped by the availability of nutrients, in particular nitrate and phosphate. Strategies for enhanced resource acquisition in crops are of increasing importance to secure sustainable food production. Such strategies have recently focused on root traits with the aim of a more efficient utilization of soil resources that would facilitate the transition from high-input monoculture-based agriculture to productive, sustainable agro-ecosystems with low inputs. Tian and Doerner (2013) evaluate the importance of root resource foraging and the possibility of exploiting natural variants in landraces or wild relatives of crops for breeding programs with the aim of producing crops with root traits that allow for a more resilient performance when experiencing environmental stresses such as phosphate deficiency. Nitrogen, mainly taken up as nitrate, is another essential nutrient that strongly affects root architecture and is critical for plant productivity. The modulation of root development by N availability has great agricultural importance and its understanding provides the basis for the generation of germplasms with improved root architecture. Mohd-Radzman et al. (2013) provide an update of the current knowledge of the signaling components involved in N-mediated root architecture, giving special emphasis on the legume root system. Deficiency of nitrate results in the expression of approximately 2000 genes from which only a minority has yet been functionally characterized. By integrating publicly available microarray data from 27 independent nitrate-related experimental datasets, Canales et al. (2014) generated several highly co-expressed gene clusters with robust functions in nitrate transport, signaling, and metabolism in *Arabidopsis* roots. In addition to prioritizing potentially important genes for further functional characterization, the meta-analysis uncovered several putative key regulatory factors that control these gene network modules and highlight novel nitrate-controlled developmental processes such as root hair formation.

The transition zone of the root connects the highly sensitive root apex with the elongation zone in which responses to environmental stimuli are accomplished, resulting in changes in cell fate and alterations in root architecture. Baluška and Mancuso (2013) discuss the specific features of the transition zone and hypothesize that it acts as a command zone that integrates environmental information received from the apex to regulate responses of cells in the elongation zone. Abiotic stress such as drought, salinity, flooding, and cold adversely affect plant growth and decline crop productivity. Stressor-specific protein signatures that dictate adaptive mechanisms are described from a proteomics perspective by Ghosh and Xu (2014). Advances in mass spectrometry and peptide fragmentation dramatically improve the coverage of proteomic profiles and opens up new perspectives for the dissection of molecular mechanisms underlying adaptive responses to abiotic stresses.

Intrinsically disordered proteins do not adopt a folded structure in their functional form, but perform functions of critical importance in signaling cascades and transcription factor networks. Owing to their intrinsic conformational flexibility,

disordered proteins can bind multiple partners with high specificity and low affinity, thereby adding complexity to the interactomes. Far from being rare or anecdotal, disordered proteins are among the most important proteins in a given proteome, apparently contradicting the classical structure-function relationship. Pazos et al. (2013) postulate that protein disorder is particularly important for the sessile lifestyle of plants, providing them with a fast mechanism to obtain intricate, interconnected, and versatile molecular networks for interacting with the environment.

More than 7000 small, unannotated open reading frames, many of which may encode regulatory peptides, exist in the *Arabidopsis* genome (Hanada et al., 2013). Small signaling peptides are a growing class of regulatory molecules which are part of the myriad of signaling networks that control the development of plant roots. Delay et al. (2013) review the involvement of regulatory peptides in several aspects of plant root development, including but not limited to meristem maintenance, the gravitropic response, lateral root development and vascular formation, highlighting the recent leap in our understanding of their role in the regulation of developmental programs.

Gene regulatory networks (GRNs) are an excellent tool for the integration and analysis of complex biomolecular systems at the structural and dynamic level. However, most GRN models are incomplete because they likely lack components or interactions due to sketchy experimental data and computational limitations. Azpeitia et al. (2013) propose a set of procedures for detecting and predicting missing interactions in Boolean networks and evaluate their applicability to predict putative missing interactions using a previously published *Arabidopsis* root stem cell nice network as an example (Azpeitia and Alvarez-Buylla, 2012).

Research into root biology has greatly profited from engineering plants to express multi-component DNA constructs such as promoter/reporter gene fusions. Emami et al. (2013) introduce an optimized protocol for the rapid and inexpensive generation of multi-component transgenes based on the Golden Gate cloning strategy. Simultaneous monitoring of membrane potential changes in populations of cells would provide a quantifiable characteristic to evaluate together with global changes in gene activity and metabolite levels in systems biology research. Matzke and Matzke (2013) describe the production of transgenic plants engineered to express different versions of genetically encoded voltage-sensitive fluorescent proteins that are targeted to the plasma membrane and internal membranes of plant cells. Their Hypothesis and Theory article describes progress toward adapting a technology originally used on animal nerve cells to record electrical patterns that transcend single cell boundaries and single membrane systems in response to various stimuli in living plants.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 April 2014; accepted: 30 April 2014; published online: 19 May 2014.
Citation: Schmidt W (2014) Root systems biology. Front. Plant Sci. 5:215. doi: 10.3389/fpls.2014.00215
This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

METHODS ARTICLE
published: 26 November 2013
doi: 10.3389/fpls.2013.00484

Unleashing the potential of the root hair cell as a single plant cell type model in root systems biology

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Plant root is an organ composed of multiple cell types with different functions. This multicellular complexity limits our understanding of root biology because -omics studies performed at the level of the entire root reflect the average responses of all cells composing the organ. To overcome this difficulty and allow a more comprehensive understanding of root cell biology, an approach is needed that would focus on one single cell type in the plant root. Because of its biological functions (i.e., uptake of water and various nutrients; primary site of infection by nitrogen-fixing bacteria in legumes), the root hair cell is an attractive single cell model to study root cell response to various stresses and treatments. To fully study their biology, we have recently optimized procedures in obtaining root hair cell samples. We culture the plants using an ultrasound aeroponic system maximizing root hair cell density on the entire root systems and allowing the homogeneous treatment of the root system. We then isolate the root hair cells in liquid nitrogen. Isolated root hair yields could be up to 800 to 1000 mg of plant cells from 60 root systems. Using soybean as a model, the purity of the root hair was assessed by comparing the expression level of genes previously identified as soybean root hair specific between preparations of isolated root hair cells and stripped roots, roots devoid in root hairs. Enlarging our tests to include other plant species, our results support the isolation of large quantities of highly purified root hair cells which is compatible with a systems biology approach.

Keywords: stress response, root hair cell, single plant cell type, systems biology, ultrasound aeroponic system

EXPERIMENTAL OBJECTIVES

Our understanding of root biology (i.e., root development, root cell differentiation and elongation, response to biotic and abiotic stresses) is based on -omic studies performed at the level of the entire root system or specific regions of the root as well as from the identification of mutants showing defects in root development. These mutants were characterized from the model plant *Arabidopsis thaliana* (Benfey et al., 1993; Rogg et al., 2001; Mishra et al., 2009) as well as other plants where genetic tools are well developed [e.g., *Medicago truncatula* (Tadege et al., 2008), *Oryza sativa* (Kurata and Yamazaki, 2006), *Lotus japonicus* (Schauser et al., 1998; Perry et al., 2003)]. These valuable studies led to the identification of important genes and even gene networks controlling plant development and adaptation to stresses (Schiefelbein et al., 2009; Bruex et al., 2012).

To enhance our current understanding of root biology, a systems biology approach is needed to take advantage of the recent improvements in technologies such as mass spectrometry and high-throughput sequencing. One challenge when studying root biology is the multicellular complexity of plant roots. For example, -omic analysis at the level of a complex organ such as the root represents an average of the responses of the different cells composing the sample. Consequently, cell specific transcripts, proteins and metabolites as well as cell-specific epigenomic changes will not be revealed resulting in a partial understanding of the specific response of a cell or cell type to a stress and difficulties to fully integrate the various -omic data sets.

To demonstrate that a single cell type model represents an attractive alternative to overcome plant multicellular complexity and to better understand gene networks, we compared the transcriptomes of the soybean root hair to that of the whole root (Libault et al., 2010b). Of the 5671 transcription factor (TF) genes known in soybean (Schmutz et al., 2010; Wang et al., 2010), we were able to detect transcripts for 3960 TF genes mining the whole root transcriptome. Out of the 1711 TFs undetected in the whole root transcriptome, 425 (25%) were only detected in the root hair cell transcriptome. This result is surprising since root hair cells were clearly one of the cell type represented in the root samples used for transcriptomic analysis. We are assuming that the low proportion of root hair cells in the root sample led to a dilution of root hair specific transcripts challenging their detection. This analysis strongly supports the need to work on a single cell type such as the root hair cell rather than an entire tissue to enable a more sensitive and accurate depiction of transcript abundance and, as a consequence, plant cellular responses to environmental perturbation. In addition, working at the single cell level will provide data more amenable to the development of computational models and the mapping of gene networks. Using a single cell type system as a model, the information obtained will be clearly unambiguous and would lead to a better characterization of gene networks.

The understanding of root hair cell biology requires the application of the full repertoire of functional genomic tools. However, major challenges in characterizing the biology of a single differentiated root cell type are the limited access to the root system and

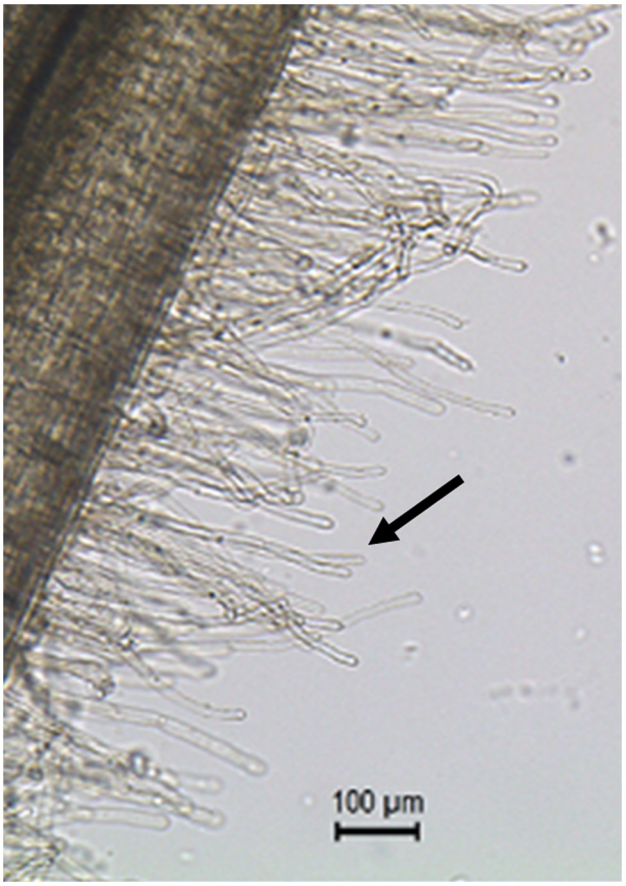

FIGURE 1 | Root hair cells (black arrow pointing at one of the root hair cells) are single tubular root cells. Their distinctive lateral elongation increases the surface of exchange between the plant's root system and the soil. The main function of root hairs is the uptake of water and nutrients from the rhizosphere.

the isolation of the root cells of interest. In this manuscript, we describe a method to: (1) homogeneously treat the plant's root hair cells; (2) easily access the root system and, *a fortiori*, the root hair cell; (3) isolate large quantities of this single cell type.

LIMITATIONS OF CURRENT TECHNIQUES

The isolation of single differentiated root cell types is limited by: (1) the accessibility to the root system; (2) the cell wall which confers the rigidity of the plant and its overall structure. Laser capture microdissection is a popular technique to isolate specific cells types but it is labor-intensive and cell yields are very limited. Nevertheless, it has been successfully applied to study root biology (Klink et al., 2005; Ithal et al., 2007; Santi and Schmidt, 2008; Takehisa et al., 2012). A second method based on the labeling of cell type by the GFP has been recently established to measure *Arabidopsis thaliana* single plant cell type transcriptomes and their regulation in response to environmental stresses (Zhang et al., 2005; Petersson et al., 2009). Using a collection of transgenic plants expressing the GFP in different root cell lines, *Arabidopsis thaliana* root cell types were isolated after digestion of the cell wall and isolation of the resulting GFP positive protoplasts using cell sorting technology. This strategy allowed the identification of root cell type-specific genes validating the concept of root cell-specific transcriptomes. However, as reported by the authors of these studies, the digestion of the cell wall also led to a few changes of the plant transcriptome independently of the cell line or treatment. In addition, several studies highlighted a massive restructuration of the chromatin and epigenetic marks in leaf protoplasts in comparison to differentiated leaves cells (Zhao et al., 2001; Tessadori et al., 2007; Ondřej et al., 2009; Chupeau et al., 2013). A third method, the INTACT method, was applied on *Arabidopsis thaliana* to isolate hair and non-hair cells and analyze their transcriptome and epigenome (Deal and Henikoff, 2010, 2011). This method is based on the expression of biotinylated nuclear envelope protein under the control of a cell type-specific promoter sequence and the isolation of labeled nuclei using streptavidin-coated magnetic beads. The characterization of a cell-specific promoter is a pre-requisite to the INTACT method. While RNA and chromatin structure can be accessed using the INTACT method, other aspects of the biology of the plant cell such as its entire proteome and metabolome cannot be reached with this method.

Another strategy to study plant single-cell biology is to massively isolate easily accessible cell types. Such method has been successfully applied on aerial parts of the plant. For example, cotton fiber and pollen cells were isolated to investigate plant cell elongation mechanisms (Franklin-Tong, 1999; Ruan et al., 2001; Arpat et al., 2004; Padmalatha et al., 2012). More recently, the soybean root hair (**Figure 1**) has emerged as a new single cell type model (Libault et al., 2010a). Various studies validate the use of the root hair cell as a model in systems biology through the analysis of the infection of soybean root hair cells by mutualistic symbiotic bacteria [i.e., the soybean root hair cell is the first site of infection by *Bradyrhizobium japonicum*, the nitrogen-fixing symbiotic bacterium involved in soybean nodulation (Gage, 2004; Kathryn et al., 2007)]. In these studies, soybean seedlings were germinated on agar plate preliminary to the inoculation of the plants with *B. japonicum* followed with the isolation of the root hair cells. Various -omics approaches were successfully used to decipher root hair cell biology, including transcriptomic (Libault et al., 2010b), proteomic (Wan et al., 2005; Brechenmacher et al., 2009, 2012), phosphoproteomic (Nguyen et al., 2012) and metabolomic (Brechenmacher et al., 2010) methods. In addition to being a model to investigate plant microbe interactions, the root hair cell is also an excellent model to decipher plant cell regulatory networks in response to abiotic stresses. This is based on their primary role in water and nutrient uptake.

To utilize full potential of this attractive single cell type as a model in root systems biology, root hairs must be evenly treated preliminary to their isolation from the rest of the root system in quantities compatible with any -omic analysis, and a fortiori, transgenic root hair cells must be isolated to perform functional genomic studies at the level of a single cell type. To reach these two goals, we developed the method described below combining the use of an ultrasound aeroponic system to generate and evenly treat a large population of root hair cells and the purification of frozen root hair cells using a highly selective filtration system. This method overcomes the limitations related to the

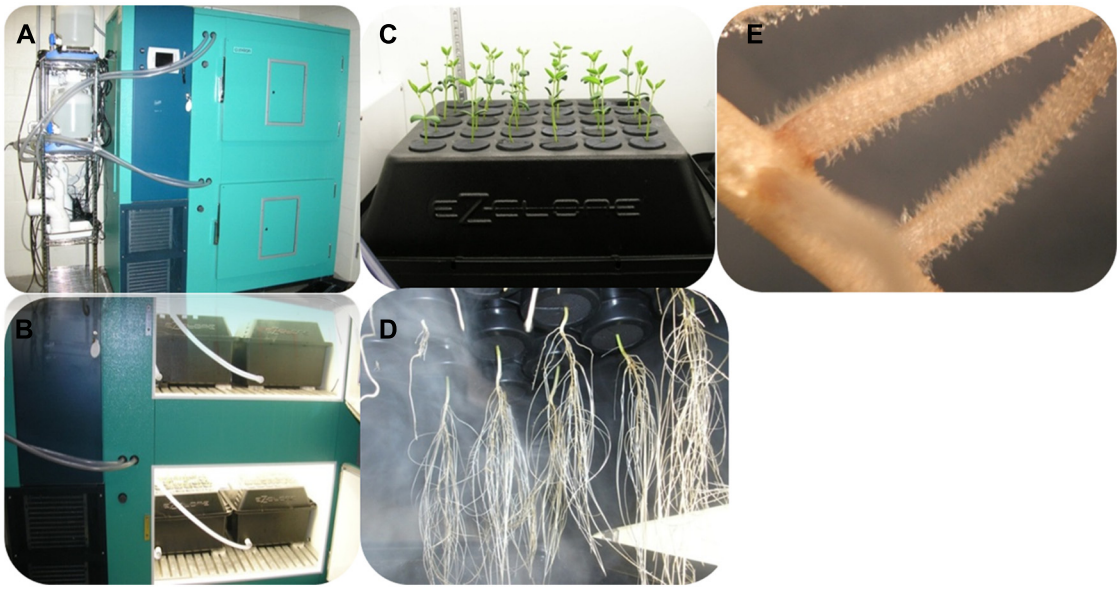

FIGURE 2 | Soybean seedlings grown in the ultrasound aeroponic system; (A,B) the whole system for plant culturing; (C,D) the plants in the EZ-cloner; (E) soybean root showing a high density in root hair cells.

use of the agar media to germinate seedlings such as the heterogeneity of the root hair cell population produced (i.e., root hair cells interact with the agar or are expanding in the atmosphere impacting their physiology) and open new avenues to investigate root hair cell biology because enabling functional genomic studies (see below). To date, we focused on the isolation of soybean root hair cells but the method described below has been validated using other plant models such as maize, sorghum, and rice.

DETAILED PROTOCOL OF THE OPTIMIZED METHOD

USE OF AN ULTRASOUND AEROPONIC SYSTEM TO ENHANCE ROOT HAIR DENSITY AND TREATMENT

The study of root hair cell response to stresses presupposes:

1. The even treatment of the root system under control and stressed conditions to minimize biological variations;
2. The optimization of the growth conditions of the root system and the enhancement of the differentiation of root hair cells on the root system;
3. An easy access to the root hair cell compatible with their observation and isolation;
4. The development of methods to efficiently isolate them.

We recently developed a method which fulfills these different requirements. Five days-old soybean seedlings germinated on a mixture of vermiculite and perlite (3:1) were transferred to the ultrasound aeroponic system under controlled conditions (long day conditions, 25–27°C, 80% humidity; **Figure 2**). This system is composed of two units: the fogger system and the cloner unit (EZ-CLONE Enterprises Inc.). The fogger system relays on the production of a 5 micrometres (μm) droplets of nutritive solution by ultrasound misters (OCEAN MIST®, DK24) which atomize nutrient solution into a nutrient-rich mist by vibrating at an ultrasonic frequency [in the case of soybean, we are using the B&D nutritive solution (Broughton and Dilworth, 1971)]. An air flow pushes the cool mist into the cloner unit where plants are growing. The quantity of mist produced by the fogger system is controlled by the number of mist makers used per fogger system as well as by a timer controlling the frequency and duration of the production of mist. Using a thin mist to feed the plant maximized the oxygenation of the root system, an important factor contributing to a higher density in root hair cells of the root system [(Shiao and Doran, 2000); **Figures 2C,D**]. Altogether, this unique system optimizes root growth, enhances root hair cell density and offers an easy access to the root hair cell compatible with their observation and isolation (**Figure 2E**).

ROOT HAIR ISOLATION PROCEDURE

Root hair cell isolation has been repetitively applied on soybean (Wan et al., 2005; Brechenmacher et al., 2010; Libault et al., 2010b; Nguyen et al., 2012). Concomitantly to the development of the aeroponic system, the method used to isolate soybean root hair cell was updated to reach two objectives: (1) maintain or enhance the level of purity of the root hair cell preparation from the rest of the root system; (2) maximize root hair yields. Several methods exist to isolate root hairs including gentle brushing of the frozen root system into liquid nitrogen (Bisseling and Ramos Escribano, 2003) or stirring of the roots immersed in the liquid nitrogen with glass rod preliminary to their isolation (Roehm and Werner, 1987; Bucher et al., 1997). The first method maximizes root hair purification but root hair yields are low and the method is labor intensive. The second method provides large quantities of plant material but the root hair cell preparation could be easily contaminated

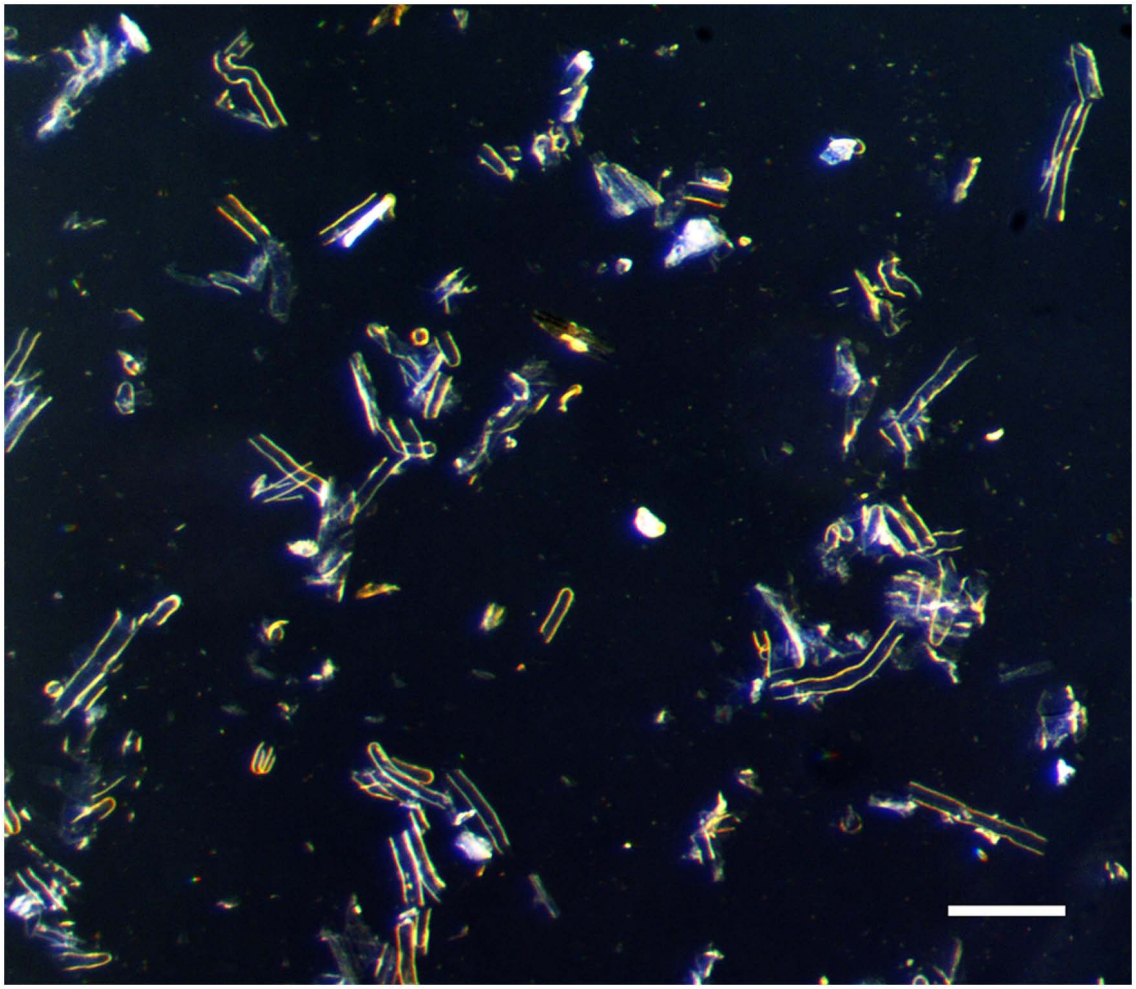

FIGURE 3 | Isolated root hairs in light microscope. Bar = 100 μm.

by non-root hair cells such as root fragments resulting from the stirring.

We optimized the latter method as described below. Briefly, the root systems of 3 weeks-old soybean plants are isolated, rapidly wiped off to remove extra moisture then immediately immersed into liquid nitrogen. This rapid freezing prevents undesirable stress of the root and root hair cells due to their manipulation. All subsequent steps are performed in liquid nitrogen. Frozen roots are gently stirred into liquid nitrogen by a glass rod for 10 min. The flow of liquid nitrogen is sufficient to break root and root hairs. The liquid nitrogen containing the root hairs is filtered through 90 μm sieve into a beaker. Based on stereomicroscopic observations, this mesh offers the best compromise to maximize the level of purification of the root hair cells without compromising the yield (**Figure 3**). The stripped roots are rinsed 5–7 times to collect the remaining root hair cells and increase the yield (i.e., as much as 1000 mg of isolated root hair cells were isolated from 63-week old soybean plants). The plant material harvested is usable the most up-to-date molecular approaches.

MOLECULAR QUANTIFICATION OF THE LEVEL OF PURITY OF THE ROOT HAIR CELL PREPARATIONS

To evaluate the purity of the root hair cell preparations, we quantified the expression of several "root hair-specific" genes in both isolated root hair and stripped root samples. These genes were selected from the soybean transcriptome atlas (Libault et al., 2010c) based on their high or specific expression in root hair cells compared to stripped roots (**Figure 4A**). We are assuming that the low transcript abundance of these "root hair-specific" genes in stripped roots is the consequence of the presence of remaining root hair cells or root hair cell nuclei in the stripped root samples (i.e., the nucleus of mature root hairs are located in the base of the cell).

The fold change of gene expression level in root hair cell *versus* stripped root ranged from 11.9 (Glyma09g05340) to 44.1 (Glyma15g02380) based on RNA-seq data (**Figure 4A**). Applying qRT-PCR methods, we analyzed the quality of the plant material collected using our optimized method compared to a previous root hair cell isolation method (Wan et al., 2005; **Figure 4B**). Independently of the root hair isolation method

A

Soybean gene	Normalized Illumina reads numbers		Putative function of the protein
	Root hair cell	Stripped Root	
Glyma02g01970	700.2	21.6	Pollen proteins Ole e I family
Glyma03g34560	1289.4	84.6	Pollen proteins Ole e I family
Glyma04g04800	8.1	0	Helix-loop-helix DNA-binding domain
Glyma08g12130	9.1	0	AP2 domain
Glyma09g05340	1132	95.4	Peroxidase
Glyma11g35560	602.3	15.8	Pollen proteins Ole e I family
Glyma15g02380	256.3	5.8	Profilin
Glyma17g14230	1199.6	45.6	Rare lipoprotein A (RlpA)-like double
Glyma18g02870	752.6	21.6	Pollen proteins Ole e I family

B

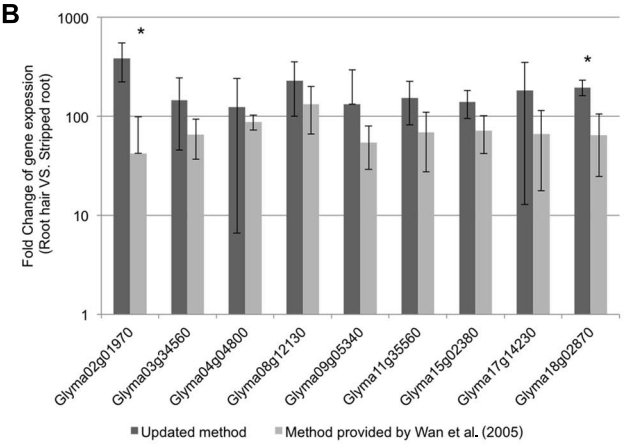

FIGURE 4 | Expression analyses of soybean root hair specific genes. (**A**) Relative expression levels of nine soybean genes in root hair cells and stripped roots from the Illumina read data (Libault et al., 2010c); (**B**) The fold-change of the expression of nine root hair specific genes was quantified between isolated root hairs and stripped roots. The plant material was generated using our optimized protocol (dark bars) and the method provided by Wan et al. (2005) (light gray bars). For each experiment, a minimum of three biological replicates were performed and analyzed. The student *t*-test was applied to highlight significant differences between these two methods. The asterisk indicates significantly difference ($*p < 0.05$).

used, we observed a higher abundance of transcripts encoded by the nine candidate genes in isolated root hairs compared to stripped roots supporting high levels of purification of the root hair cells. Using root hair cells and stripped roots collected using the method described by Wan et al. (2005), the fold changes of expression of root hair specific genes between root hairs and stripped roots ranged between 42.4 ± 56.6 (Glyma02g01970) and 133.0 ± 67.1 (Glyma08g12130). Our optimized root hair cell method repetitively led to fold changes of expression of root hair specific genes ranging between 124.4 ± 117.8 (Glyma04g04800) and 385.8 ± 164.0 (Glyma02g01970). This result supports a higher enrichment in root hair cells in root hair cell preparation using the updated method compared to Wan et al. (2005) method.

APPLICATIONS OF THE ULTRASOUND AEROPONIC SYSTEM TO INVESTIGATE BIOLOGICAL QUESTIONS

The flexibility of the use of the ultrasound aeroponic system is fully compatible with the homogeneous treatment of the root system to analyze root hair response to biotic and abiotic stresses. The nature of the abiotic stresses allowed by the aeroponic system is diverse including: (1) changes of the chemical composition of the nutritive solution to analyze root hair response to nutrient deprivation, low and high pHs, salinity or heavy metal contaminations, etc.; (2) changes of the environmental conditions such as temperature, water potential, etc.; (3) inoculation of plants with pathogenic and symbiotic microorganisms. The latter was validated by inoculating 2 weeks-old root systems of the hypernodulation soybean mutants [i.e., NOD1-3, NOD2-4, and NOD3-7; Ito et al., 2007] with a bacterial suspension of *B. japonicum*, the soybean nitrogen-fixing symbiont. As soon as 10 days after inoculation, nodules emerged. Thirty days after inoculation, a large number of nodules were developing on the hypernodulating soybean roots [NOD1-3 (106.8 ± 27.7 nodules per plant), NOD2-4 (159.7 ± 42.7 nodules per plant), NOD3-7 (99.7 ± 29 nodules per plant)]. Compared to these mutants, 4.4- to 7-fold fewer nodules were counted on wild type root system (22.8 ± 9.25 nodules per plant). Although, we found that the number of nodules per root system is lower in the aeroponic system grown plants compared to vermiculite grown plants (i.e., wild type, NOD1-3, NOD2-4, and NOD3-7 mutants showed 67, 441, 344, and 143 nodules per plant, 17–18 days after inoculation, respectively; Ito et al., 2007), the NOD hypernodulating phenotype is observed in the aeroponic system. These data support that this technology is fully compatible with the analysis of the early and late stages of legume nodulation. We assume that additional experiments and tests using the aeroponic system would maximize the number of nodules per plant.

Another potential attractive application of the aeroponic system is the generation of composite plants (i.e., plants carrying a mixture of transgenic and non-transgenic roots growing from a wild type shoot) and, *a fortiori*, the easy access to a large mass of transgenic roots compatible with their observation and various molecular analyses. To test this potential utilization of the aeroponic system, we inoculated soybean shoots with *Agrobacterium rhizogenes* carrying our transgene of interest (in this case, a fusion between the cassava vein mosaic virus promoter and the *UidA* gene which encodes the beta-glucuronidase). Ten days after bacteria inoculation, a callus was formed and roots started to emerge (**Figure 5A**). Four weeks after inoculation, the emerged root system was stained using X-Gluc to reveal the β-glucuronisase activity (Libault et al., 2010d; **Figure 5B**). In average, we observed seven transgenic roots emerging from each composite plant. Stereomicroscopic observations revealed that these roots carry an impressive number of transgenic root hair cells (**Figure 5C**).

CONCLUSION

In this manuscript, we combined the use of an ultrasound aeroponic system with updated method to isolate root hair cells to maximize the potential of plant root hair cell as a single cell type model for systems biology. This updated method has the following advantages: (1) enhance root hair cell density on the root system; (2) even and long-term treatment of the entire population of root hair cells to access the molecular response of the root hairs to various biotic and abiotic stresses.; (3) compatibility with the microscopic observation of the root hair cells; (4) leading to high yields of isolated root hair cells compatible with any -omic analyses.

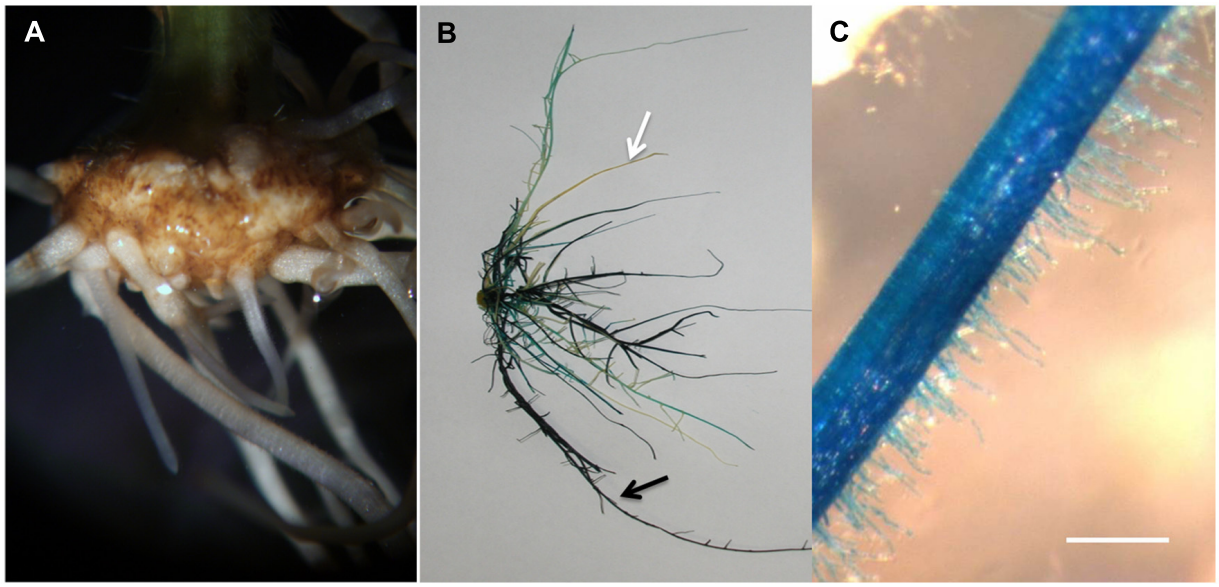

FIGURE 5 | Soybean transgenic roots and root hairs generated in the ultrasound areoponic system; (A) transgenic roots emerging from the callus 10 days after *Agrobacterium rhizogenes* inoculation; (B) *GUS*-stained soybean root system, the black arrows point at the transgenic root and the white arrows point at the non-transgenic root; (C) *GUS*-stained transgenic root hair cells. Bar = 200 μm.

In addition to be well-suited to perform –omics analyses at the level of one single cell type, the ultrasound aeroponic system has been validated to study plant-bacteria interactions and to produce large quantities of easy accessible plant material allowing functional genomic studies. Undoubtly, our updated method of generating large amount of pure root hair cells will promote the progress of deciphering the regulatory mechanism of plant cell biology including plant cell response to environmental stresses.

EXPERIMENT PROCEDURE

PLANT GROWTH

Soybean seeds (*Glycine max* [L.]) were surface-sterilized by three sequential treatments with 1.65% sodium hypochlorite (10 min each), rinsed three times with deionized water before a 10 min treatment with 10 mM hydroxychloride. Seed were finally washed three times with sterile water before sowing on sterilized mixture of vermiculite and perlite (3:1 ratio). Seeds were germinated under permanent light conditions at 25°C. One week later, the seedlings were transferred into fogger system and supplement with the mist of B&D plus 10 mM KNO_3. Cultured for another 2 weeks in areoponic system, the seedlings were collected into liquid nitrogen for root hair isolation.

RNA EXTRACTION AND cDNA SYNTHESIS

Total RNAs were extracted using Trizol Reagent (Invitrogen). Six to ten μg of total RNA were extracted from one preparation of isolated root hairs. Total RNA samples were treated with the TURBO DNase (Ambion) according to the protocol provided by the manufacturer before to reverse transcribe 1 μg of DNA-free RNA using oligodT and the Moloney murine leukemia virus reverse transcriptase as previously described (Libault et al., 2010b).

Table 1 | qRT-PCR primers.

Soybean gene ID	Forward primer	Reverse primer
Glyma02g01970	TGGCTGCAAAGTGAAAATGA	TCAATTCTTCGTGCCAATGA
Glyma03g34560	ATGAGTTGGGGCAGTACGAC	TAGTTGAGCTTGACGCCAGA
Glyma04g04800	CCAACGGAACAAAGGTTGAT	TATCGGAGCGTACATCCACA
Glyma08g12130	GCCCAACAAAGGATTAACGA	TATCCTCCACATGGCACTCA
Glyma09g05340	GGCATGACAAGGGCTCATAC	GCCTGTTCCGTTGTTGT
Glyma11g35560	TGCTACGTGAAGCCTGTT	AGTGGAGCACCATTGAGA
Glyma15g02380	CAAGGTGAACCTGGAGCTGT	TCTCCCAACCTCTCAACGAT
Glyma17g14230	CGTGATGAATGTTGGAGGTG	GTTGCAAATGCCTGGTATGA
Glyma18g02870	GACCCTTAGCTTTCCGTCCT	TCTCAATGCATGGTCAAAGG

QUANTITATIVE REAL-TIME PCR AND DATA ANALYSIS

Quantitative real-time PCR (qPCR) primers were designed using Primer3 software[1] **Table 1**.

Quantitative real-time PCR reactions were performed as described by Libault et al. (2008) including an initial denaturation step of 3 min at 95 °C followed by 39 cycles of 10 s at 95 °C and 30 s at 55°C. Dissociation curves were obtained using a thermal melting profile performed after the last PCR cycle: a constant increase in the temperature between 65 and 95°C.

Cycle threshold (Ct) values were obtained based on amplicon fluorescence thresholds. According to Vandesompele et al. (2002), delta Ct were generated using the geometric mean of the cycle threshold of three reference genes [*Cons6*, *Cons7*, and *Cons15* genes Libault et al., 2008]. PCR efficiency (P_{eff}) for each sample was calculated using LinRegPCR (Ramakers et al., 2003), and the expression level (E) were calculated using the equation $E = P_{\text{eff}}{}^{(-\Delta Ct)}$. The fold change of the gene expression levels between root hair *versus* stripped root was calculated for each root hair specific gene. Three independent biological replicates were generated for each condition and Student t-tests with two tails and two samples equal variance were applied to display the significant differences of gene expression between root hair and stripped root samples. P value < 0.05 was regarded significant.

CLONING AND SOYBEAN HAIRY ROOT TRANSFORMATION

As described by Libault et al. (2010c), cloning of the cassava vein mosaic virus promoter upstream of the *UidA* gene was performed using the Gateway® system (Invitrogen[2]). The cassava vein mosaic virus promoter fragment was introduced first into the pDONR-Zeo vector (Invitrogen) using the Gateway® system BP Clonase® II enzyme mix, then into pYXT1 destination vectors carrying the *UidA* genes, using the Gateway® LR Clonase® II enzyme mix.

Two weeks-old soybean plants grown on pro-mix were used to generate composite plants. K599 *Agrobacterium rhizogenes* bacterial strain carrying the transgene of interest, a transcriptional fusion between the cassava vein mosaic virus promoter and the *UidA* gene, was grown at 30°C in LB medium supplemented with kanamycin. The bacteria were pelleted by centrifugation, and re-suspended in B&D medium supplemented with 10 mM potassium nitrate and acetosyringone (20 μM) to an optical density at 600 nm = 0.35.

Soybean shoots were cut between the first true leaves and the first trifoliate leaf and placed into rock-wall cubes (Fibrgro). Each shoot was inoculated with 4 mL of *Agrobacterium rhizogenes* suspension and then allowed to dry for approximately 3 days (23°C, 50% humidity, long-day conditions) before watering with deionized water. After 1 week, instead to transfer the composite plants into vermiculite-perlite as described by Libault et al. (2009), the transformed soybean shoot were transferred into the ultrasound aeroponic system supplemented with B&D medium plus 10 mM potassium nitrate. After 2 weeks, the β-glucuronisase activity of the soybean root system was revealed as described by Libault et al. (2010d).

[1]http://biotools.umassmed.edu/bioapps/primer3_www.cgi

[2]http://www.invitrogen.com/

ACKNOWLEDGMENT

This work was funded by a grant from the National Science Foundation (Plant Genome Research Program, #IOS-1339194).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 August 2013; accepted: 07 November 2013; published online: 26 November 2013.

Citation: Qiao Z and Libault M (2013) Unleashing the potential of the root hair cell as a single plant cell type model in root systems biology. Front. Plant Sci. ***4****:484. doi: 10.3389/fpls.2013.00484*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 12 November 2013
doi: 10.3389/fpls.2013.00448

Auxin, the organizer of the hormonal/environmental signals for root hair growth

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The root hair development is controlled by diverse factors such as fate-determining developmental cues, auxin-related environmental factors, and hormones. In particular, the soil environmental factors are important as they maximize their absorption by modulating root hair development. These environmental factors affect the root hair developmental process by making use of diverse hormones. These hormonal factors interact with each other to modulate root hair development in which auxin appears to form the most intensive networks with the pathways from environmental factors and hormones. Moreover, auxin action for root hair development is genetically located immediately upstream of the root hair-morphogenetic genes. These observations suggest that auxin plays as an organizing node for environmental/hormonal pathways to modulate root hair growth.

Keywords: auxin, boron deficiency, ethylene, jasmonate, phosphate deficiency, root hair, root hair-specific genes, strigolactone

INTRODUCTION

The root hair develops as a tubular structure from the root hair-forming root epidermal cell. The root hair development on the root epidermal cell consists of two major steps: the fate determination step, which produce hair or non-hair cells, and the root hair differentiation (or morphogenesis) step, where the root hair initiates and elongates from the root hair cell (Grierson and Schiefelbein, 2002, 2009). The fate determination step in *Arabidopsis* has been genetically well characterized. In the non-hair cell position, a complex of WEREWOLF (WER, a MYB transcription factor), GLABRA3/ENHANCER OF GLABRA3 [GL3/EGL3, basic helix-loop-helix (bHLH) transcription factors], and TRANSPARENT TEST GLABRA (TTG, a WD40 protein) positively modulates the expression of GLABRA2 (GL2, a homeodomain transcription factor); GL2, then, works as a negative regulator against root hair differentiation by inhibiting the expression of genes for root hair morphogenesis (Grierson and Schiefelbein, 2009). On the other hand, in the hair cell position, a Leu-rich repeat receptor-like protein kinase (LRR-RLK) called SCRAMBLED (SCM) is likely to receive external signals from the inner tissues and suppress the expression of WER, and thus of GL2, so as to release the inhibition against root hair morphogenesis of the root hair cell (Grierson and Schiefelbein, 2009). The lack of GL2 in the hair cell seems to lead to the activation of a bHLH transcription factor, ROOT HAIR DEFECTIVE 6 (RHD6), which is necessary for root hair initiation (Masucci and Schiefelbein, 1996; Menand et al., 2007). RHD6 then positively controls other downstream bHLH transcription factors (Yi et al., 2010) and root hair-specific (RHSs) morphogenetic genes, *RHS* (Won et al., 2009).

Partially, independent of the developmental genetic pathway, auxin-related environmental factors and phytohormones affect the hair morphogenetic process (Masucci and Schiefelbein, 1994, 1996; Okada and Shimura, 1994; Katsumi et al., 2000; Lee and Cho, 2008). Root hair-modulating phytohormones include auxin, ethylene, jasmonic acid (JA), brassinosteroid (BR), and strigolactone (SL). Amongst these phytohormones, auxin has been most intensively studied regarding its role in root hair growth. Auxin shows an obvious positive effect on root hair elongation without affecting the fate determination step (Masucci and Schiefelbein, 1994, 1996; Pitts et al., 1998; Cho and Cosgrove, 2002). Auxin genetically works downstream of RHD6 as exogenous auxin restores root hairs in the root hair-defective *rhd6* mutant (Masucci and Schiefelbein, 1996). Recent studies have added diverse hormonal and environmental factors affecting root hair development and shown that majority of these factors work together with auxin to control root hair development. In this review, we divide the upstream pathway of root hair development into fate-determining pathway and environmental/hormonal pathway and locate auxin at the organizing node where diverse environmental and hormonal signals for root hair growth converge.

AUXIN SIGNALING AND HOMEOSTASIS OPERATE CELL-AUTONOMOUSLY FOR ROOT HAIR GROWTH

Among three major nuclear auxin signaling components, auxin receptors [TRANSPORT INHIBITOR RESPONSE1 [TIR1]/AUXIN SIGNALING F-BOX PROTEINs (AFBs)] and their substrates or auxin-signaling repressors [AUXIN/INDOLE-3-ACETIC ACIDs (Aux/IAAs)] have been well defined to affect root hair growth. The *tir1* mutant, along with the mutants of its paralogs *afb1, afb2,* and *afb3*, showed a decreased root hair growth (Dharmasiri et al., 2005), whereas RHS over-expression of TIR1 considerably enhanced root hair growth (Ganguly et al., 2010). These results are in accordance with the auxin receptors' nature in that they cause degradation of repressors (Aux/IAAs) for auxin responses. Conversely, the genetic data have demonstrated diverged roles of these repressors during root hair development. Degradation-resistant mutants of *AUXIN*

RESISTANT2 (*AXR2*)/*IAA7* (Wilson et al., 1990; Masucci and Schiefelbein, 1996), *AXR3*/*IAA17* (Leyser et al., 1996), *SOLITARY ROOT* (*SLR*)/*IAA14* (Fukaki et al., 2002), and *IAA28* (Rogg et al., 2001) showed inhibition of root hair growth, indicating their negative function in auxin-mediated root hair growth, whereas the similar gain-of-function mutant of *SHY2/IAA3* (Knox et al., 2003) showed enhanced root hair growth, suggesting its positive role in root hair growth. In contrast, the role of another major auxin signaling component, AUXIN RESPONSE FACTORs (ARFs), has scarcely been characterized in root hair growth.

Cell type-specific gene manipulation experiments have demonstrated that auxin signaling and homeostasis for root hair growth are operational in a hair-cell autonomous way, where changes of auxin levels and auxin signaling components in the root hair cell directly affect root hair growth (Cho et al., 2007a; Lee and Cho, 2008). When genes were root hair-specifically expressed using a RHS promoter (Cho and Cosgrove, 2002; Kim et al., 2006a), the dominant *axr2-1* mutant gene specifically suppressed root hair growth (Won et al., 2009) while, as mentioned above, TIR1 greatly enhanced hair growth (Ganguly et al., 2010). In a complementary manner, when the dominant *axr3-1* mutant gene was expressed specifically in the non-hair cells to cause defects in auxin signaling exclusively within the non-hair cells, it did not show any effect on root hair growth in the hair cell (Jones et al., 2008). These studies together suggest that the auxin signaling for root hair growth is operational in the root hair cell.

Although the auxin signaling for root hair growth is hair cell autonomous, auxin concentration seems to be higher in the non-hair cell than in the hair cell. The expression of AUX1 (an auxin influx carrier) was shown to localize specifically to the non-hair cell, whereas PIN2 (PIN-FORMED2, an auxin efflux carrier) was evenly expressed in both hair and non-hair cells, which would cause more auxin accumulation in the non-hair cell than in the hair cell (Jones et al., 2008). Another study showed a similar result where exogenous auxin (1-naphthalene acetic acid, NAA) induced a much higher response of DR5::GUS, the auxin responsive reporter, in the non-hair cell than in the hair cell (De Rybel et al., 2012). Because defects of auxin signaling in the non-hair cell did not influence hair growth in the hair cell, the high accumulation of auxin in the non-hair cell is thought to provide sustainable auxin concentrations for the root hair cells where the non-hair cell file works as an auxin pipeline to supply auxin from the root tip auxin maximum to the root hair differentiation zone (Jones et al., 2008). The short-haired *pin2* mutant phenotype also supports this hypothesis (Cho et al., 2007b). PIN2 is mainly expressed and asymmetrically localized at the upper side (toward the shoot) in the epidermis of root meristem and elongation zones (Luschnig et al., 1998; Müller et al., 1998). Therefore, the loss of PIN2 would cause defects in supplying auxin from the root tip to the hair-differentiation zone, which in turn results in suppression of root hair growth.

Studies with various auxin transporters have demonstrated that the auxin homeostasis of the root hair cell is critical for root hair growth. RHS expression of auxin efflux carriers such as PINs (PIN1-4, PIN7, and PIN8) and P-GlycoProteins (PGPs)/ATP-Binding Cassette transporter Bs (ABCBs), dramatically suppressed *Arabidopsis* root hair growth, and this root hair inhibition was suppressed by the auxin efflux carrier inhibitor, suggesting that auxin efflux carrier-mediated root hair inhibition occurs due to depletion of auxin in the root hair cell (Lee and Cho, 2006; Cho et al., 2007a,b; Ganguly et al., 2010). Indole-3-butyric acid (IBA), an auxin precursor, provides another point-of-view on the relationship between auxin concentration and root hair growth. IBA goes through peroxisomal modification to be converted into IAA in the root cap. Interestingly, the loss-of-function mutant of an IBA-transporter, PLEIOTROPIC DRUG RESISTANCE8 (PDR8) /PENETRATION3/ABCG36, an IBA-specific efflux carrier protein, was shown to increase root hair length: under the *pdr8* mutant, IBA accumulation was increased, leading to an increased concentration of IAA (Strader and Bartel, 2009). As IBA serves an auxin reservoir, proper IBA transport from the root tip region to the hair-differentiation zone can be important for root hair growth.

Impairment of auxin transport caused by metabolite also supports the idea that changes in auxin concentration affect root hair development. D'orenon, a C_{18}-ketone (5*E*,7*E*)-6-methyl-8-(2,6,6-trimethylcyclohex-1-enyl)octa-5,7-dien-2-one, an early cleavage product of β-carotene, was shown to affect auxin homeostasis by increasing abundance of PIN2 in the epidermal cells, leading to a decrease in auxin levels in the root hair cell similarly to RHS PIN overexpression aforementioned (Schlicht et al., 2008).

CROSS-TALK BETWEEN AUXIN AND OTHER HORMONES FOR ROOT HAIR GROWTH

Many phytohormones have been shown to control root hair development by cross-talking with auxin. To date, the influence of auxin and ethylene on root hair development has been most heavily studied. However, the influence of other phytohormones, such as SL, BR, and JA has been steadily inching their way into root hair development.

Although the functional relationship between ethylene and auxin for plant development can be both positive and counteractive depending on the tissue type, both hormones are in a positive relationship in regard to root hair growth (Cary et al., 1995; Tanimoto et al., 1995; Masucci and Schiefelbein, 1996; Muday et al., 2012). For example, the root hair defect of *rhd6* mutant was rescued both by auxin and by ACC (1-aminocyclopropane-1-carboxylic acid, the ethylene precursor; Masucci and Schiefelbein, 1994), the long-haired phenotype of the ethylene overproducing *eto1* mutant was suppressed by the *aux1* mutation (Strader et al., 2010), and the *aux1 ein2* double mutant showed an additive root hair defect (Rahman et al., 2002). These results consistently demonstrate a positive relationship between auxin and ethylene. A transcriptome analysis further demonstrates that auxin and ethylene act on the common pathway for root hair development since almost 90% of the genes were commonly up-regulated by both auxin and ethylene (Bruex et al., 2012). However, the pathways that these two hormones take for root hair growth seem to be complicated.

According to the experimental data so far reported, auxin appears to work both upstream and downstream of ethylene.

Auxin was able to restore root hair growth in the ethylene-insensitive mutant *ein2-1* (Rahman et al., 2002). Similarly, auxin-resistant mutants, *ibr5*, *tir1*, *axr1*, and *aux1*, were shown to suppress the long root hair phenotype of the ethylene-overproducing mutant *eto1* (Strader et al., 2010). In addition, ethylene enhances auxin biosynthesis in the root tip and stimulates basipetal auxin transport toward the root elongation zone (Stepanova et al., 2005; Růžička et al., 2007; Swarup et al., 2007). These results suggest that auxin may work downstream of ethylene for root hair growth. On the other hand, a competitive inhibitor of ethylene, 1-methylcyclopropene (1-MCP), inhibited auxin-induced restoration of root hair growth in *rhd6* (Cho and Cosgrove, 2002), and ethylene was shown to initiate the auxin-induced microtubule randomization which is necessary for root hair elongation (Takahashi et al., 2003). The root hair growth of the auxin-signaling defective *arf7 arf9* double mutant did not respond to auxin, but ACC greatly enhanced root hair growth in this mutant (Kapulnik et al., 2011b). These latter cases suggest that ethylene may be working downstream of auxin for root hair growth.

Strigolactone positively affects root hair development via ethylene and auxin. The treatment of synthetic SL, GR24, under the *max2* (defective in MORE AXILLARY GROWTH2, the SL signaling component) mutant did not enhance root hair growth, whereas such growth was evident under the *max3* or *max4* mutant (SL biosynthetic mutants), signifying that SL affects root hair growth via the MAX2-mediated SL signaling pathway (Kapulnik et al., 2011a). SL seems to work via ethylene to stimulate root hair growth: *max2* is sensitive to ACC, but *ein2* and *etr1* are insensitive to GR24 in root hair growth (Kapulnik et al., 2011b). SL directly influences ethylene production by increasing the transcription level of ACS2 (ACC Synthase 2; Kapulnik et al., 2011b), an enzyme necessary for ethylene biosynthesis.

Unlike the SL-ethylene case, SL and auxin interact with each other in multiple levels for root hair growth. First, sub-effective concentrations of auxin and SL together enhanced root hair growth more greatly than when they were applied individually, indicating their synergistic effect on root hair growth (Kapulnik et al., 2011b). Second, while auxin failed to enhance root hair growth in the *arf7 arf9* double mutant, root hair growth in this mutant was normally stimulated by SL, suggesting that SL works independently or downstream of auxin for root hair growth (Kapulnik et al., 2011b). As ethylene was shown to enhance root hair growth of the *arf7 arf9* double mutant and SL was shown to work through ethylene, the effect of SL on the *arf7 arf9* double mutant could take place through ethylene. Third, auxin works downstream of SL for root hair growth as exogenous auxin could restore the defective root hair growth of *max2* and *max4* mutants almost to the wild-type level (Mayzlish-Gati et al., 2012). In addition, although they are not specified to the root hair, the results that SL modulates auxin transport and auxin signaling by regulating the expression of PINs (Bennett et al., 2006) and TIR1 (Mayzlish-Gati et al., 2012) suggest that SL superimposes the auxin action.

Brassinosteroid has been shown to inhibit root hair growth. Application of epi-brassinolide (epiBL, a synthetic BR) significantly inhibited root hair growth of the *Arabidopsis* seedling root, and this was phenocopied by Aux/IAA overexpression (Kim et al., 2006b). The expression of root hair-related Aux/IAAs, such as AXR2/IAA7, AXR3/IAA17, and SLR/IAA14, was increased by epiBL and suppressed in the BR-insensitive *bri1* mutant, suggesting a possibility that BR may inhibit root hair growth by suppressing auxin signaling in the root hair (Kim et al., 2006b). This observation and interpretation is interesting. Although auxin also induces expression of Aux/IAAs, it also simultaneously causes the degradation of these repressors. However, while BR stimulates the expression of Aux/IAAs, it would not cause their degradation, resulting in accumulation of Aux/IAA repressors and suppression of auxin signaling. This can be a rare case of auxin-BR interactions, probably specific to root hair growth, because auxin and BR generally show synergistic effects in diverse developmental processes (Hardtke et al., 2007).

Jasmonic acid positively affects root hair growth where exogenous JA enhanced root hair growth in a dosage-dependent manner (Zhu et al., 2006). JA also affects root hair morphogenesis as it increases branched root hairs (Zhu et al., 2006). Although the JA signaling to the root hair development has not been well characterized, it can be cross-connected with auxin and ethylene signaling. The interconnectivity between auxin and JA signaling is shown as the auxin signaling mutants *axr1* was resistant to exogenous JA in the primary root inhibition assay (Tiryaki and Staswick, 2002), which was exemplified when the JA response mutant, *jar1-1*, was found to be an allele of the *AXR1* gene (Tiryaki and Staswick, 2002). In addition, JA was shown to promote auxin biosynthesis by up-regulating *YUCCA8* and *YUCCA9* (Hentrich et al., 2013). However, it has not been directly shown whether JA affects root hair development via auxin. On the other hand, the crosstalk between JA and ethylene for root hair growth has been shown. JA-induced root hair growth was blocked by AVG or Ag^{+}, the inhibitors of ethylene biosynthesis and signaling, respectively, and in the ethylene-insensitive *etr1-3* mutant (Zhu et al., 2006), suggesting that ethylene signaling is required for JA-mediated root hair growth. Conversely, the treatment of JA biosynthesis inhibitors, ibuprofen and SHAM, suppressed ethylene-mediated root hair growth, implying that JA and ethylene mutually require each other for root hair growth (Zhu et al., 2006). The likely converging point of JA and ethylene signaling is EIN3/EIL1 (ETHYLENE INSENSITIVE3/EIN3-LIKE1). JAZ (JA ZIM-DOMAIN, a transcriptional repressor), which is degraded by JA, represses EIN3/EIL1 by physically interacting with them, and JA treatment relieves JAZ from EIN3/EIL1 leading to the expression of ethylene-responsive genes and the increase of root hair growth (Zhu et al., 2011).

THE INTERACTION BETWEEN ENVIRONMENTAL FACTORS AND AUXIN FOR ROOT HAIR GROWTH

Root hair growth is also affected by environmental factors including phosphate (Pi), boron, and glucose. Among these, the implication of Pi in root hair development has been most intensively studied. Due to the immobile nature of Pi ion in the soil, plant roots frequently experience Pi deficiency, which stimulates root hair formation and elongation (Schmidt and Schikora, 2001). Pi deficiency at least partly modulates root hair development by affecting auxin signaling and transport. The *APSR1* (*ALTERED PHOSPHATE STARVATION RESPONSE1* encoding a potential

transcription factor) gene plays a negative role in root hair elongation during normal Pi conditions and is down-regulated under low Pi states leading to an enhanced root hair growth (González-Mendoza et al., 2013). The loss of APSR1 caused a clear decrease in PIN7 protein levels. Although the decrease of PIN7 expression in the root hair cell can restore auxin levels and thus growth of the root hair (Ganguly et al., 2010), whether ASPR1 directly modulates root hair growth via PIN7 remains unknown. However, this study supports the idea that auxin mediates Pi deficiency-induced root hair growth. In contrast, a different study indicates that Pi deficiency-induced root hair growth and formation may work downstream or independently of auxin signaling. Auxin insensitive signaling and transport mutants such as *axr1*, *axr2*, and *aux1,* show shorter and fewer root hair phenotypes, and Pi deficiency restored both growth and number of root hairs (Schmidt and Schikora, 2001).

Phosphate was also shown to affect root hair development via the SL pathway. The response to Pi starvation was reduced under the defects in SL biosynthesis and signaling. *max2* and *max4* mutants showed a decrease in expression of Pi starvation-induced (PSI) genes while GR24 was able to rescue the reduced Pi response under the *max4-1* mutant (Mayzlish-Gati et al., 2012). Since SL was known to work upstream of ethylene (Kapulnik et al., 2011b), Pi may affect the ethylene signaling via SL, creating a linear signaling pathway from an external influence, in this case Pi, to the root hair development via phytohormones (Mayzlish-Gati et al., 2012). Complementing the ideas above, the *hsp2* (*HYPERSENSITIVE TO PHOSPHATE STARVATION 2*, an allele of *CTR1* or *CONSTITUTIVE TRIPLE RESPONSE 1*) mutant showed a hypersensitivity to Pi starvation, indicating that ethylene signaling is involved in Pi-mediated root hair development (Lei et al., 2011). In a similar fashion, the *etr1*-1 and *ein2-5*, ethylene signaling mutants, reduced the expression of *PT2* (a high-affinity phosphate transporter gene) while the ethylene over-producing *eto1-1* mutant increased *PT2* expression, further exemplifying the relationship between Pi and ethylene to guarantee better acquisition of Pi for the plant (Lei et al., 2011). However, because Pi deficiency could restore root hair growth and formation in the ethylene signaling mutants, *etr1* and *ein2*, Pi deficiency may also take an ethylene-bypassing pathway for root hair development (Schmidt and Schikora, 2001).

In addition to its effect on hair growth, Pi deficiency can affect the fate determination step in root hair development. Under Pi-deficient conditions, root hairs not only grow longer in the hair cells but also are formed ectopically in the non-hair cell position (Schmidt and Schikora, 2001; Müller and Schmidt, 2004). Pi deficiency greatly enhanced the root hair number in the non-hair cell position, and this increase occurred partly even in the *wer, gl2,* and *ttg* fate determination mutants (Müller and Schmidt, 2004). Recent finding of bHLH32, a negative regulator of PSI genes, provides a link between Pi deficiency and the fate determination pathway, where bHLH32 was shown to interact with TTG and GL3 and high Pi conditions did not inhibit root hair development under the *bhlh32* mutant background (Chen et al., 2007). However, it has to be elucidated how bHLH32, interacting with TTG and GL3, affects hair/non-hair cell fate determination.

Boron also is implicated in root hair development. Boron deficiency causes enhanced root hair growth and formation where at least ethylene signaling has been shown to be implicated. Low boron-mediated increase of root hair growth was shown to be blocked in the *ein2-1* mutant, and the ethylene responsiveness was considerably enhanced by low boron in the elongation and differentiation zone of the root (Martín-Rejano et al., 2011). Although it has not been directly shown whether boron deficiency-enhanced root hair development requires auxin, the possibility exist as low boron increased auxin-sensitive DR5:GUS reporter gene expression in the root and low boron-mediated inhibition of the primary root was suppressed in the *aux1* mutant, suggesting that low boron signaling may use auxin signaling (Martín-Rejano et al., 2011).

Besides environmental factors, artificial high glucose conditions affect root hair development, and this seems to be linked with the expression of auxin-related genes (Mishra et al., 2009). As auxin and glucose cause numerous common responses, Mishra et al. (2009) compared auxin- and glucose-responsive transcriptomes and analyzed the relationship between auxin and glucose on root hair development. High glucose up-regulated *YUCCA2*, *PIN1*, *PIN2*, *ARF*, and *ABP1* genes while down-regulated *TIR1* and several *SAUR*, *Aux/IAA,* and *GH3* genes in the whole seedling level. Oddly, glucose suppressed auxin-induced DR5:GUS reporter expression in the root. Although glucose effects on auxin biosynthesis and signaling are complicated in the whole seedling level, glucose seems to require auxin signaling for root hair growth because mutants such as *tir1, slr1, axr3,* and *axr2* showed defects in glucose-induced root hair growth (Mishra et al., 2009).

THE POSITION OF AUXIN IN THE OVERALL SIGNALING FOR ROOT HAIR DEVELOPMENT

As mentioned earlier, auxin works downstream of RHD6 for root hair growth. Recently, a bHLH transcription factor, called RHD6-LIKE4 (RSL4), was found to be a direct downstream target of RHD6 (Yi et al., 2010). RSL4 is expressed in the hair cell file of the elongation and differentiation zone of the root, and its loss of function mutant *rsl4-1* grew much shorter and fewer root hairs than wild type, indicating that RSL4 is indeed needed for root hair growth and initiation. Consistently, the RSL4 overexpression (under the CaMV 35S promoter) lines kept growing root hairs more than four times longer than those of wild type. Auxin was able to increase transcription of *RSL4* not only in wild type but also in the *rhd6* mutant background, suggesting that *RSL4* is the target of auxin in the downstream of RHD6. Furthermore, auxin failed to restore root hair growth in the *rsl4-1* mutant background, which is contrasted to the auxin effect in the *rhd6* mutant. These data collectively showed that the auxin pathway and the fate determination pathway via RHD6 converge on to RSL4 to modulate root hair growth.

Root hair growth and morphogenesis should require root hair-specifically functioning genes, as well as essential house-keeping morphogenetic genes, in which the RHS genes should specify all the hair cell-specific events for root hair morphogenesis. Diverse *RHS* genes have been functionally identified and these *RHS* genes commonly carry the characteristic root hair-specific *cis*-element (RHE) on their promoters (Kim et al., 2006a; Won et al., 2009). The function of RHE has been conserved at least in the angiosperm lineage since RHE was shown to be cross-functional between monocots and dicots, suggesting that the RHE-binding

or *RHS*-modulating transcription factor also has been conserved in angiosperms (Kim et al., 2006a). The expression of *EXPANSIN A7*, a *RHS* gene, has been demonstrated to be regulated by RHD6 (Cho and Cosgrove, 2002; Won et al., 2009) but in an indirect way (Yi et al., 2010). This leads us to a hypothesis that *RHS* genes are located downstream of RSL4. The transcriptome analysis with wild type, *rsl4-1* mutant, and *RSL4* overexpressor revealed that RSL4 indeed up-regulated many *RHS* genes (Yi et al., 2010). Moreover, Pi deficiency was able to restore root hair from *rhd6* but failed to do that in *rsl4-1*, suggesting that the Pi deficiency signaling via auxin also requires RSL4 to promote root hair growth (Yi et al., 2010). The comparison between three independent transcriptome analyses further indicates that auxin and RSL4 commonly act on *RHS* genes. Auxin up-regulated 97 genes in the *rhd6* mutant background (Bruex et al., 2012), and RSL4 overexpression up-regulated 83 genes (Yi et al., 2010). Won et al. (2009) found that 24 *RHS* genes were down-regulated in the *rhd6* mutant background, among which 16 *RHS* genes were found in both transcriptome collections from Bruex et al. (2012) and Yi et al. (2010). These results further suggest that auxin operates upstream of RSL4 to stimulate *RHS* gene expression.

Recently, a membrane-anchored MYB (maMYB), an R2R3-type MYB transcription factor, has been implicated in root hair growth (Slabaugh et al., 2011). The silencing of maMYB via RNAi shortened root hair length without affecting hair initiation, indicating that maMYB is specifically involved in hair elongation. The interesting point is that exogenous auxin rescued the short root hair phenotype of the maMYB–RNAi line and promoted the transcription of maMYB of the wild type plant. These results suggest that maMYB works upstream of RSL4 for root hair growth. The same study showed that maMYB affects the expression of a *RHS* gene (*RHS14*) but in a negative way. It is unlikely that maMYB directly binds RHE to suppress *RHS* genes because RHE works in a positive manner (Won et al., 2009). maMYB might modulate *RHS* genes by binding *cis*-elements other than RHE or by interacting some upstream factors to negatively regulate *RHS* genes. Not all *RHS* gene products seems to positively work for root hair growth. Some of them, such as RHS1 and RHS10, negatively regulate root hair growth (Won et al., 2009), suggesting that the overall root growth process is in a balance of both positive and negative modulators. maMYB may provide another level of regulatory tool to fine-tune root hair growth between RSL4 and auxin.

CONCLUDING REMARKS

Root hair-controlling factors listed in this review can be classified largely into fate-determining developmental factors, hormonal factors, auxin-related environmental factors, and finally root hair morphogenetic genes (**Figure 1**). These factors show diverse interactions; not only linear but also networking and mutual. Environmental factors generally take advantage of hormonal signaling to modulate root hair growth, in which sometimes multiple hormones are implicated to mediate the environmental factor (e.g., Pi deficiency). Environmental factors maximize their effects on root hair development also by affecting the fate-determining developmental steps, resulting in increased root hair number. It is noticeable that most root hair-affecting hormones intensively interact with auxin in various levels; biosynthesis, transport, and signaling of auxin. In the overall signaling pathway for root hair growth, auxin funnels the upstream environmental pathways, and other hormonal signaling right onto the master regulator (RSL4) for root hair growth and morphogenesis. Considering these aspects, auxin is likely to play a role as an organizing center for environmental/hormonal signaling for root hair growth.

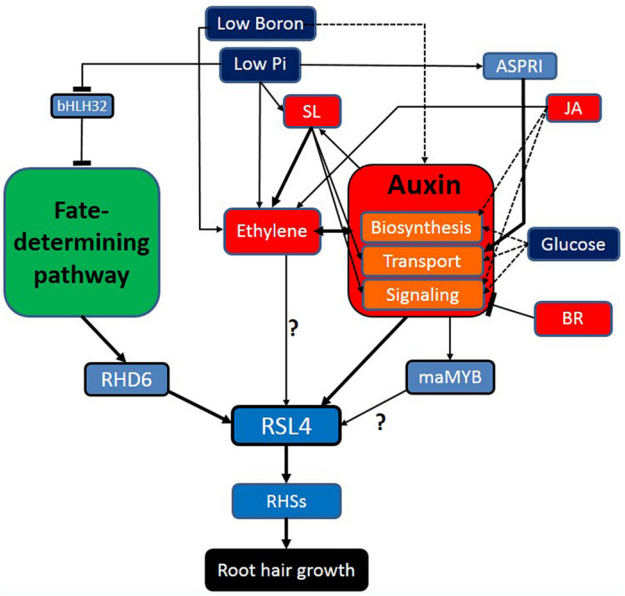

FIGURE 1 | Auxin plays an organizing center for environmental/hormonal pathways for root hair growth. Navy blue, red, light blue boxes indicate environmental factors, hormones, and genetic factors, respectively. Broken-lined arrows represent the cases where the direct effect on the root hair by the factor has not been shown. The blunt bar end indicates an inhibitory effect.

ACKNOWLEDGMENTS

This work was supported by grants from the Mid-career Researcher Program (2011-0017242, NRF, MEST) and the Next-Generation BioGreen 21 programs (TAGC PJ00820701 and SSAC PJ00951404) of Rural Development Administration.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 August 2013; accepted: 20 October 2013; published online: 12 November 2013.

Citation: Lee RD-W and Cho H-T (2013) Auxin, the organizer of the hormonal/environmental signals for root hair growth. Front. Plant Sci. ***4****:448. doi: 10.3389/fpls.2013.00448*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 26 December 2013
doi: 10.3389/fpls.2013.00537

Systems approaches to study root architecture dynamics

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Reviewed by:
Masanori Arita, University of Tokyo, Japan
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The plant root system is essential for providing anchorage to the soil, supplying minerals and water, and synthesizing metabolites. It is a dynamic organ modulated by external cues such as environmental signals, water and nutrients availability, salinity and others. Lateral roots (LRs) are initiated from the primary root post-embryonically, after which they progress through discrete developmental stages which can be independently controlled, providing a high level of plasticity during root system formation. Within this review, main contributions are presented, from the classical forward genetic screens to the more recent high-throughput approaches, combined with computer model predictions, dissecting how LRs and thereby root system architecture is established and developed.

Keywords: root system, lateral root, genetic screening, transcript profiling, genomics studies, systems approach

The root as an underground organ is of vital importance for plant life. It provides anchorage to the soil, supplies minerals and water, synthesizes metabolites, and interacts with symbiotic organisms (Den Herder et al., 2010). The root system architecture is shaped by the environmental signals and other external cues, which modulate root growth direction and kinetics (Luschnig et al., 1998; Lavenus et al., 2013), as well as its surface by affecting root hair growth (Lan et al., 2013) and frequency of branching (Sanz et al., 2011). In particular, formation of lateral roots (LRs) is one of the key determinants of the root architecture with an eminent impact on the efficiency of soil exploitation. For example, nitrate, phosphate, or sulfate availability modulate both primary root growth as well as LR formation and outgrowth (Linkohr et al., 2002; Hubberten et al., 2012), demonstrating close interconnection between nutrient availability and root architecture. LR organogenesis (**Figure 1**) is a well-defined process with discrete developmental steps including (i) priming, (ii) initiation, (iii) LR primordia (LRPs) organogenesis, (iv) LR emergence, and (v) activation of the LR apical meristem (Laskowski et al., 1995; Malamy and Benfey, 1997). These distinct developmental phases are under specific control mechanisms, providing a high level of plasticity during root system formation. To shape the root architecture in response to various external cues, plant hormones play an important role of rapid endogenous signal mediators (López-Bucio et al., 2003; Malamy, 2005). The core of this hormonal regulatory network comprises two antagonistically acting molecules: auxin and cytokinin. Auxin as a key stimulatory factor triggers and coordinates LR organogenesis, while cytokinin interferes with both initiation and LRP organogenesis (Benková et al., 2003; Laplaze et al., 2007; Fukaki and Tasaka, 2009; Bielach et al., 2012a).

The exploration of the development of the root system has been confronted with various obstacles, such as the interplay between primary root growth and LR organogenesis, and technical challenges, such as limitations on isolating specific tissue layers of LRP which are hidden within tissues of the primary root. Therefore, studying root growth has required the development and implementation of specific tools and approaches. On other hand, some experimental advantages are obvious, e.g., there is a defined spatio-temporal frame or developmental window when cells are competent to initiate LRs (Dubrovsky et al., 2006, 2011), a capacity of ectopic induction and synchronization of LR initiation along the primary root by hormone application (Himanen et al., 2002), or easy-to-perform imaging of the LRPs.

Recently, root development research experienced great revival, especially because of an implementation of new generation imaging techniques and high-throughput approaches. The aim of this review is to acknowledge those techniques that have contributed to the deeper understanding of the development of the root system, as well as to present the most novel tools and their application potential. To present a broad picture of the formation and development of the root system we have discussed some of most prominent approaches for studying LR development that range from live-based methodologies to high-throughput technologies, combined with predictions from computer models of LR morphogenesis.

LATERAL ROOT FORMATION AND DEVELOPMENT IN LIGHT OF REAL-TIME IMAGING

Implementation of the modern imaging techniques enabled to address longstanding questions on the LR organogenesis at qualitatively new level. In particular real-time imaging with high tissue resolution have provided a close spatio-temporal view on the early phases of lateral root initiation (LRI) and brought new insights into the dynamics of LR organogenesis.

In *Arabidopsis thaliana*, within the developmental window the position of the newly initiated LR is defined, i.e., the root zone where the cells exhibit the highest competence to initiate LRs (Dubrovsky et al., 2006). Early attempts to monitor LRI dynamics revealed that early events resulting in LRI correlate

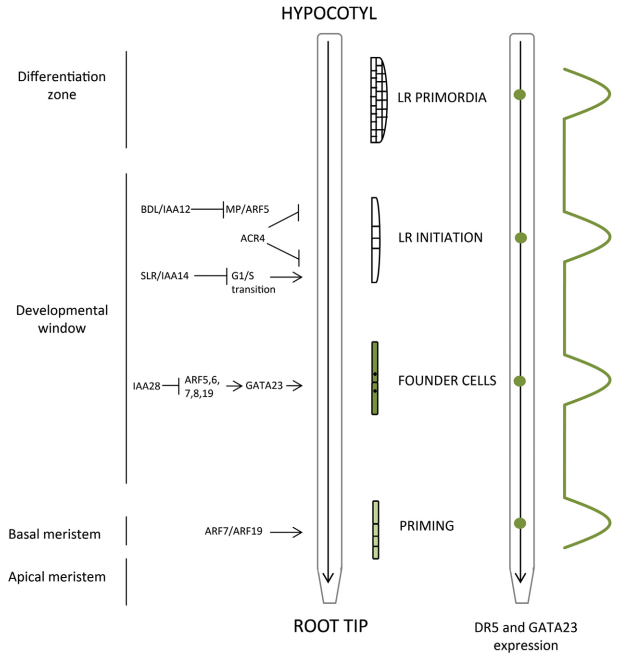

FIGURE 1 | Spatiotemporal control of lateral root organogenesis in primary root. Priming of LRP precedes LR initiation and occurs in the basal meristem. Developmental window defines the occurrence of the FC establishment and most distal LRI, and LRPs develop and emerge through adjacent tissues in the differentiated part of the root. Correlation between the root zones (apical and basal meristem, developmental window, differentiation zone) with phases of LR development (priming, founder cells, LR initiation, LR primordia) along with the main molecular events are presented. The oscillatory mechanism that determines LR positioning as reported by DR5::LUC (auxin response marker) and GATA23 is depicted (green dots).

with regular fluctuations of an auxin sensitive reporter *DR5*::β-*glucuronidase* (*GUS*) in the protoxylem cells in the basal root meristem (De Smet et al., 2007). This recurrent enhancement of auxin activity, named priming, occurs with regular intervals of approximately 15 h, corresponding with the frequency of LRI during root growth. Later by the implementation of a real-time monitoring system using the *DR5::LUCIFERASE* reporter, more detailed studies of LRI dynamics could be performed (Moreno-Risueno et al., 2010). Luciferase imaging confirmed the oscillatory nature of the auxin response in the root, and correlated pulses of luciferase activity with prebranch sites that precede the establishment of static points where LRs are formed (Moreno-Risueno et al., 2010). Application of high-throughput transcriptome profiling synchronized with the oscillatory auxin response led to the discovery that besides auxin, a complex network of oscillating transcription factors contributes in setting up a prebranch site.

Another approach that combined the high end real-time imaging with computational modeling pointed out that the change in a shape of root cells as a consequence of gravitropic or mechanical bending redirects auxin flow toward pericycle cells, thereby resulting in an auxin accumulation in these cells that triggers LRI (Ditengou et al., 2008; Laskowski et al., 2008).

The next step in LR formation after priming and founder cell (FC) specification is the initiation of primordia organogenesis by series of anticlinal divisions. The identification of mutants in which despite FC establishment no LRI takes place (Celenza et al., 1995; DiDonato et al., 2004; Dubrovsky et al., 2008; De Rybel et al., 2010) indicates that the specification of FCs does not lead by default to LRI and that these two events might be uncoupled. Real-time imaging of LRI has shown that auxin continues to accumulate in the FCs, until it reaches a maximum just prior the actual initiation event (De Rybel et al., 2010). Later, an auxin reflux pathway, which is transiently established during the early phases of LRI, was found. This reflux via the PIN-formed (PIN3) auxin efflux carrier reinforces the auxin flow from the endodermal cells to the FCs, thereby enabling to reach the auxin threshold required to transit from founder to LRI phases (Marhavý et al., 2013).

LRP organogenesis continues by a series of cell divisions and differentiation coordinated by auxin. An auxin gradient with a maximum at the primordia tip is instructive for proper organ formation, and modulation of auxin distribution interferes with the progress of LR organogenesis (Benková et al., 2003). High resolution imaging of LRPs in real time turned out to be a powerful approach to bring further insights into the mechanisms controlling LRP organogenesis. 3D/4D image analysis (Lucas et al., 2013) revealed that early stage LRPs exhibit tangential divisions that create a ring of cells enclosing a population of rapidly dividing cells. The division patterns in the latter cell population during LRP morphogenesis are not stereotypical, although the shape of new LRPs is highly conserved. Interestingly, manipulating the properties of overlaying tissues disrupted LRP morphogenesis, indicating that the interaction with overlaying tissues might be more important for LRP morphogenesis than the precise division pattern.

Recently, monitoring LRP development when cytokinin is present revealed a rapid change in the PIN1 auxin efflux carriers expression caused by this hormone. Based on these observations cytokinin was proposed to interfere with LR organogenesis through regulation of the constitutive cycling of PIN1 by its alternative sorting to lytic vacuoles and subsequent degradation (Marhavý et al., 2011).

Similarly, understanding LR emergence and their interplay with surrounding tissues rapidly advanced with improved imaging techniques. In light of these observations it became clear that LR emergence is a tightly coordinated process during which auxin acts as local inductive signal to control cell separation in overlaying tissues (Swarup et al., 2008; Péret et al., 2012; Kumpf et al., 2013; Lucas et al., 2013). Moreover, applying real-time imaging improved our view on the acquisition of LR gravity sensing properties when emerging out of the parental root. The regulation of the gravitropic response of LRs, defining the gravitropic setpoint angle, is crucial for the radial expansion of the root system (plagiotropism). Real-time analysis demonstrated that acquiring a gravity sensitive stage strongly correlates with the modulation of asymmetric auxin transport rates in LR columella, the differentiation of statoliths and the establishment of a connection to the primary root vasculature (Guyomarc'h et al., 2012; Rosquete et al., 2013).

GENETIC STUDIES

A traditional strategy to identify the molecular components and mechanisms involved in a developmental process is forward genetics. Chemical agents (e.g., ethyl methanesulfonate) or radiation are used to induce mutations causing a certain phenotype, and using a mapping strategy the responsible gene can be identified (Lukowitz et al., 2000).

This approach has been successfully applied to reveal key components of LR formation (**Table 1**). Early screens based on the LR phenotype by Celenza et al. (1995) identified the *aberrant lateral root formation* (*alf*) mutants. Three different *alf* mutants were characterized: (i) *alf1-1*, with an increased number of LRs caused by IAA overproduction, turned out to be an allele of *SUPERROOT1* (*SUR1*) and *ROOTY1* (*RTY1*) [Boerjan et al. (1995) and King et al. (1995), respectively]; (ii) *alf3-1*, with arrested LRPs, that can be rescued by exogenous application of auxin; and (iii) *alf4-1*, unable to form LRs. Further work was done on the latter mutant by DiDonato et al. (2004), showing that ALF4 is required to maintain the developmental plasticity of pericycle cells and their meristem-like properties. Hence, *alf4-1* can perceive the LR induction signal but initiation cannot proceed because the xylem-adjacent pericycle cells cannot divide, since a mitotically active state is not maintained in the mutant. Another screen based on LR abundance has identified additional components specifically involved in the early phases of LRI, such as *Reduces Lateral root Formation* (*RLF*) (Ikeyama et al., 2010). The *RLF* gene codes for a cytosolic protein containing a cytochrome b5-like heme/steroid binding domain, and it seems to be involved in the activation of pericycle cell divisions at LRI sites downstream of auxin signaling.

Besides root oriented screens, investigation of other plant phenotypes (e.g., auxin defects, shoot appearance) has revealed remarkable components of LR formation and development. A screen for mutants defective in the shoot gravitropic response led to the identification of the *solitary root-1* (*slr-1*) mutant that completely lacks LRs (Fukaki et al., 2002). The mutation in *IAA14* belonging to the *Aux/IAA* auxin signaling repressor gene family stabilizes the IAA14 protein and as a consequence the auxin dependent initiation of LRs is disrupted. In efforts to reveal additional components of this SLR-mediated pathway, a suppressor screening was conducted on *slr-1*, identifying mutants such as *slr-1R1* (Fukaki et al., 2002) or *SSL2* (Fukaki et al., 2006). In the case of *slr-1R1*, an intragenic suppressor of *slr-1*, root hair formation is restored, but LR formation gets poorly recovered, indicating that LR and root hair formation require different mechanisms involving SLR/IAA14. The second mutant identified (*pickel/ssl2*) is an extragenic suppressor of *slr-1*. *PICKEL/SSL2* is a homolog of the animal chromatin-remodeling factor CHD3/Mi-2, implicating a role for chromatin remodeling mediated by PKL/SSL2 in the negative regulation of auxin-mediated LR formation in *Arabidopsis*.

Also screens targeting auxin signaling pathway resulted in the identification of mutants defective in LR organogenesis, highlighting the importance of auxin signaling in LR formation. That is the case for the auxin receptors TIR1 and related F box proteins AFB1, 2 and 3 (Dharmasiri et al., 2005). The loss of these genes resulted in a progressive decrease in auxin response during

Table 1 | Summary of the genes involved in LR initiation and development, including those summarized in Casimiro et al. (2003), De Smet et al. (2006) and Péret et al. (2009).

Gene	Mutant	LR phenotype	Publication
ABA1	*aba1*	Inhibition of LR	Vartanian, 1996; Signora et al., 2001
ABI3	*abi3-6*	Required for correct auxin response in LR	Brady et al., 2003
ABI4	*abi4*	Inhibition of LR	Signora et al., 2001
ABI5	*abi5*	Inhibition of LR	Signora et al., 2001
ACR4*	*acr4*	Increased LR	De Smet et al., 2008
AFB1	*afb1*	Decrease auxin response on LR	Dharmasiri et al., 2005
AFB2	*afb2*		
AFB3	*afb3*		
AGB1	*agb1-1, agb1-2*	Increased LR	Ullah et al., 2003
ALF1/SUR1/ RTY1*	*alf1-1/sur1/rty1*	Increased LR	Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995
ALF3*	*alf3-1*	Arrested LR	Celenza et al., 1995
ALF4*	*alf4-1*	Lack of LR	Celenza et al., 1995; DiDonato et al., 2004
ARABIDILLO	*arabidillo1/2*	Reduced LR	Coates et al., 2006
ARF7/ARF19*	*arf7xarf19*	Lack of LR	Okushima et al., 2005
ARF8	*arf8-1*	Increased LR	Tian et al., 2004
	Overexpression 35s::ARF8	Reduced LR	
ARF 10/16/17	*Overexpression ARF10/16/17*	Reduced LR	Mallory et al., 2005; Wang et al., 2005
ARR3	*arr3*	Reduced LR	To et al., 2004
ARR4	*arr4*		
ARR5	*arr5*		
ARR6	*arr6*		
ARR8	*arr8*		
ARR9	*arr9*		
ATHB-2	*35s::ATHB-2*	Reduced LR	Steindler et al., 1999
	reverse ATHB-2 sequence	Increased LR	
	35s::αATHB-2		
ATHB-8	*Overexpression*	Reduced LR	Baima et al., 2001
AUX1*	*aux1* alleles	Reduced LR	Marchant et al., 2002
AXR1	*axr1-3, axr1-12*	Reduced LR	Lincoln et al., 1990
AXR2*	*axr2-1/iaa7*	Increased LR	Nagpal et al., 2000
AXR4	*axr4-1, axr4-2, axr4-1 axr1-3*	Reduced LR	Hobbie and Estelle, 1995
AXR6	*axr6-1, axr6-2*	Reduced LR	Hobbie et al., 2000

(Continued)

Table 1 | Continued

Gene	Mutant	LR phenotype	Publication
BRX2	*brx-2*	Increased LR (on cytokinin)	Li et al., 2009
CEG	*ceg*	Increased LR	Dong et al., 2006
CKX1	*Overexpression 35s::CKX1*	Increased LR	Werner et al., 2003
CKX3	*Overexpression 35s::CKX1*	Increased LR	Werner et al., 2003
DFL1	*dfl1-D, sense 35s::DFL1*	Reduced LR	Nakazawa et al., 2001
	antisense 35s::DFL1as	Increased LR	
E2Fa*	*E2Fa*	Reduced LR	Berckmans et al., 2011
ERA1	*era1-2*	Increased LR	Brady et al., 2003
ETA3	*eta3*	Reduced LR	Gray et al., 2003
GNOM	*gnom* alleles	Reduced LR	Geldner et al., 2004
GPA1	*gpa1-1, gpa1-2*	Reduced LR	Ullah et al., 2003
HAT2	*Overexpression*	Reduced LR elongation	Sawa et al., 2002
HOBBIT	*hbt* allele	LR meristem defect	Willemsen et al., 1998
HY5	*hy5-1, hy5-Ks50*	Increased LR	Oyama et al., 1997
IAA1	*axr5-1*	Reduced LR (on auxin)	Yang et al., 2004
IAA3*	*shy2-2*	Reduced LR	Tian and Reed, 1999
	shy2-22, shy2-24	Increased LR	
IAA14*	*slr-1*	Lack of LR	Fukaki et al., 2002
	slr-1R1	Poorly restored LR	
	PICKLE/SSL2	Partial restored LR	Fukaki et al., 2006
IAA18	*iaa18/crane*	Reduced LR	Uehara et al., 2008
IAA19	*msg2-1*	Reduced LR	Tatematsu et al., 2004
IAA28*	*iaa28-1*	Reduced LR	Rogg et al., 2001
IAR3 ILL2 ILR1	*ilr1 iar3 ill2*	Reduced LR	Rampey et al., 2004
ILR2	*ilr2-1*	Reduced LR	Magidin et al., 2003
KNAT6	*35s::RNAi*	Increased LR	Dean et al., 2004
KRP2	*35s::KRP2*	Reduced LR	Himanen et al., 2002
LAX3*	*lax3*	Reduced LR	Swarup et al., 2008

(Continued)

Table 1 | Continued

Gene	Mutant	LR phenotype	Publication
LIN1	*lin1*	No LR repression	Malamy and Ryan, 2001
MRP5*	*mrp5-1*	Increased LR	Gaedeke et al., 2001
NAC1	*Antisense 35s::NAC1*	Reduced LR	Xie et al., 2002
	Overexpression 35s::NAC1	Increased LR	
PAS1	*pas1*	Reduced LR	Faure et al., 1998; Vittorioso et al., 1998
PAS2	*pas2*	Increased LR	Faure et al., 1998; Bellec et al., 2003
PAS3	*pas3*	Reduced LR	Faure et al., 1998
PGP4	*pgp4*	Increased LR	Santelia et al., 2005
PIN1*	*Overexpression 35s::PIN1*	Delay LR development	Benková et al., 2003
PIN3*	*pin1 pin,3 pin3 pin7*	Reduced LR	
PIN4	*pin4 pin7, pin1 pin4 pin7, pin1 pin3 pin4*		
PIN7*	*pin1 pin3 pin7*		
PINOID	*Overexpression 35s::PID*	Reduced LR	Christensen et al., 2000; Benjamins et al., 2001
PLT1 PLT2	*plt1 plt2*	Increased LR	Aida et al., 2004
PXA1	*pxa1*	Reduced LR	Zolman et al., 2001
RanBP1c	*Antisense AtRanBP1c*	Reduced LR	Kim et al., 2001
RAV1	*Overexpression*	Delay LR development	Hu et al., 2004
RCN1	*rcn1*	LR growth less NPA sensitive	Rashotte et al., 2001
RIB1	*rib1*	Increased LR	Poupart and Waddell, 2000
RLF*	*rlf-1*	Reduced LR	Ikeyama et al., 2010
RML1	*rml1*	Arrested LR	Cheng et al., 1995
RML2	*rml2*	Lack of LR	Cheng et al., 1995
ROP2	*CA-rop2*	Increased LR	Li et al., 2001
	DN-rop2	Reduced LR	
SBR	*sbr*	Reduced LR	Subramanian et al., 2002

(Continued)

Table 1 | Continued

Gene	Mutant	LR phenotype	Publication
SEU	*seu-3*	Reduced LR	Pfluger and Zambryski, 2004
SINAT5	*Overexpression 35s::SINAT5*	Reduced LR	Xie et al., 2002
	Dominant negative 35s::SINAT5 (C49S)	Increased LR	
SUR1	*sur1*	Increased LR	Seo et al., 1998
SUR2	*sur2/rnt1*	Increased LR	Delarue et al., 1998; Barlier et al., 2000; Bak et al., 2001
TIR1	*tir1-1*	Reduced LR	Ruegger et al., 1998; Dharmasiri et al., 2005
TIR3 (BIG)	*tir3-1, asa1/umb1*	Reduced LR	Ruegger et al., 1997; Gil et al., 2001; Kanyuka et al., 2003
WAK4	*DEX-induced WAK4 antisense*	Inhibition LR development	Lally et al., 2001
XBAT32	*xbat32-1*	Reduced LR	Nodzon et al., 2004
XPL1	*xipotl*	Increased LR	Cruz-Ramírez et al., 2004
YDK1	*ydk1-D, 35s::YDK1*	Reduced LR	Takase et al., 2004

*Genes discussed within this review.

LR formation. Several Aux/IAA gain-of-function mutants, like *shy2/iaa3* (Tian and Reed, 1999) or *iaa28-1* (Rogg et al., 2001) exhibited dramatically reduced number of LRs, or like *axr2-1/iaa7* (Nagpal et al., 2000) showed an increased number of LRs. Similarly, the forward genetics approach has been employed to seek for new molecular components mediating the interaction between the auxin-cytokinin pathways during LR formation (Bielach et al., 2012b). Several *primordia on auxin and cytokinin* (*pac*) mutants in which the basal LRI process was not affected, but a cytokinin resistance phenotype appeared in the presence of auxin, might reveal new players balancing the auxin-cytokinin developmental output.

An alternative strategy is the reverse genetics approach, which is the analysis of mutants in genes selected based on prior knowledge about their role in specific pathways connected with LR formation. That is the case of the *Auxin Response Factor* (*ARF*) gene family, encoding transcriptional regulators that are core components of the auxin signaling pathway (Ulmasov et al., 1999). A PCR-based screening approach was conducted, identifying T-DNA insertions affecting the *ARF* genes (Okushima et al., 2005). By mutant phenotype characterization of several members of this family (specifically *ARF7* and *ARF19*), their role in LR formation was discovered. Similarly, by detailed mutant analyses the function of AUX1 and LAX3 auxin influx and PIN1, PIN3, PIN7, PGP1 and PGP19 efflux transporters in different phases of LR organogenesis has been recognized (Gaedeke et al., 2001; Marchant et al., 2002; Benková et al., 2003; Mravec et al., 2008; Swarup et al., 2008).

PROTEIN INTERACTION STUDIES

The lasting challenge in elucidating how LR formation is controlled is a complete dissection of the regulatory pathway components. DNA-protein or protein-protein interaction studies, such as yeast one-hybrid or yeast two-hybrid, are powerful approaches to uncover more new molecular players. By implementing this approach in the study of LR formation and development, a direct molecular link between auxin signaling, cell cycle machinery and LRI has been shown (Berckmans et al., 2011). The *E2Fa* transcription factor (regulator of cell cycle initiation) has been identified as a direct target of the *LATERAL ORGAN BOUNDARY DOMAIN18/ASYMMETRIC LEAVES2-LIKE20* (*LBD18/ASL20*) transcription factor downstream of auxin signaling and its role in triggering the first asymmetric division during LRI has been demonstrated (Berckmans et al., 2011). Additionally, by tandem affinity purification (protein-protein interaction) other proteins involved were identified, including LBD33. The data suggest that a LBD18/LBD33 dimer is necessary for *E2Fa* expression.

TRANSCRIPT PROFILING STUDIES

Genome-wide transcript profiling is a high-throughput technology which enables the efficient evaluation of the complete transcript regulation in a certain process (Hennig et al., 2003). Besides particular genes, the identification and analysis of clusters of co-expressed genes might provide important insights on the physical or functional connection between gene products during the regulation of certain developmental process.

The true challenge in identifying regulatory genes involved in LR organogenesis by genome-wide profiling arises from the fact that LRI is restricted in time and space to a small number of pericycle cells hidden within surrounding primary root tissues. To circumvent this obstacle, a lateral root-inducible system (LRIS) was implemented to boost the frequency of LRI in a largely synchronized manner (Himanen et al., 2002). By combining LRIS with transcript profiling, Himanen et al. (2004) identified genes linked with early phases of LR initiation. Besides expected targets, such as components of the auxin signaling pathway and the cell cycle, clusters of regulatory genes co-regulated in course of the early phases of LRI were recognized. Later, using the LRIS set up, transcript profiles of the control and the lateral rootless *solitary root/iaa14* (*slr-1*) mutant were compared to extract genes linked with LRI (Vanneste et al., 2005). Within the genes identified, cell division-related genes were found (*APC8/CDC23*, *PCNA1*), directly linking auxin signaling and cell cycle activation during LRI both at the S-phase and the G2-to-M transition. Similarly, by extracting the auxin-regulated genes whose expression is strongly suppressed in the *arf7* and *arf19* mutants defective in LR organogenesis *LATERAL ROOT PRIMORDIUM1 (LRP1)*, *AUXIN-REGULATED GENE INVOLVED IN ORGAN*

SIZE (*ARGOS*) or *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*), family genes were uncovered for their role in LR organogenesis (Okushima et al., 2005).

Although the LRIS significantly increased the frequency of the LRIs, the limitation of tissue specificity was not overcome, since the material used included the whole root segment and contaminating tissues. Considering that a few cells within the pericycle layer are involved in a process such as LR formation, it is very likely that some important regulators could be missed. An elegant solution for this appeared to be an implementation of the Fluorescent Activated Cell Sorting (FACS) technique in combination with transcriptome profiling. To monitor the transcriptome of the xylem pole pericycle cells exclusively, the Gal4-GFP enhancer trap line J0121 with pericycle-specific expression was used (Laplaze et al., 2005). This improved strategy led to the identification of the membrane-localized receptor-like kinase *ARABIDOPSIS CRINKLY4* (*ACR4*), specifically transcribed in the small daughter cell after the first asymmetric pericycle cell division. The ACR4 is a key factor in promoting formative cell divisions in the pericycle, as well as in constraining the number of these divisions once organogenesis has been started (De Smet et al., 2008).

Later phases of LR development have also been targeted by transcript profiling studies (i.e., high-throughput quantitative RT-PCR). Namely, impact of the environmental signals, such as salt stress on primordia development has been examined (Duan et al., 2013). It was disclosed that the water stress-associated hormone abscisic acid (ABA) mediates suppression of LR emergence, acting primarily at the endodermis by tissue-specific ABA signaling pathways.

With the extensive increase of data generated by genome-wide profiling interesting targets might be easily overlooked. Hence, specialized algorithms and computational pipelines are developed to refine data mining and evaluation. A recently released spatio-temporal transcriptional map of the *Arabidopsis* root (the RootMap) (Brady et al., 2007) became an outstanding tool for evaluating expression patterns and gene correlations in root tissues.

A new tool named Visual Lateral Root Transcriptome Compendium (Visual RTLC) was developed by Parizot et al. (2010), in order to combine and compare the different datasets focused on LR organogenesis. These new appearing methods for data mining provide great opportunities to scale up the identification of novel regulators of LR organogenesis.

CHEMICAL GENOMICS

Using the above mentioned approaches, crucial components of the LR regulation have been identified, and functional connections between key regulatory pathways (auxin, cytokinin, cell cycle-related), underlying root system architecture control, have been recognized.

Lately, the chemical biology opened new ways to study biological systems. The ability of chemical compounds to enhance, mimic, interfere or block a specific developmental process rises as a powerful tool to discover new regulatory components. The chemical approach is based on the ability of small synthetic molecules to modify the activity of proteins or pathways, resulting in the understanding of the protein function at a level that would be difficult to achieve through gene-based perturbation (Robert et al., 2009). Additionally, a tight temporal control can be accomplished, allowing for instance to overcome limitations related to mutational approaches (e.g., the long-term effect of disrupting the process can lead to lethality). Even more, its combination with other approaches, such as genomic studies, provides an additional power, i.e., the compound effect on a mutant that exhibits a certain phenotype.

The high-throughput screening of the chemical library must first be optimized in order to identify compounds that interfere with a specific developmental process. Aimed at LR organogenesis, an efficient screening method based on the LRIS was established (De Rybel et al., 2012; Audenaert et al., 2013). Using this platform, a naxillin, the non-auxin-like synthetic molecule that induces LR formation, was found as an activator of LRI, being more effective than known synthetic or natural auxins (De Rybel et al., 2012). A chemical approach combined with transcriptome profiling showed that 2581 vs. 401 genes are de-regulated by either auxin or naxilin, respectively, indicating a narrower mechanism of naxillin action when compared to natural auxin (De Rybel et al., 2012). Interestingly, genes involved in the early events of LR development such as *GATA23*, *LBD33,* and *LBD29*, were found to dominate in the naxillin induction profile. Forward genetics resulted in the identification of the *naxillin resistant 1* (*nar1*) mutation in the *IBR3* gene, linking naxillin activity with the regulation of the peroxisomal IBA-to-IAA conversion to promote the development of LRs.

COMPUTER MODELING APPROACHES

Computer models of plant development typically integrate experimentally identified interactions between genes and proteins (regulatory networks) to predict the dynamics of such regulatory networks in the developmental context (Prusinkiewicz and Runions, 2012). Numerous computer models of LR development have been developed to predict putative mechanisms underlying LR morphogenesis. These computer models often integrate experimental observations to identify a minimal mechanistic framework for LRI (Laskowski et al., 2008; Lucas et al., 2008). For example, mechanical deformation of cells was found to occur in the curved region of the primary root and to dramatically affect the size and shape of cells (Ditengou et al., 2008). Laskowski et al. (2008) demonstrated that a subtle change in the cell shape can be instructive for the auxin accumulation in pericycle cells and thus LRI. They also proposed that a feedback between auxin and expression of auxin influx carriers in pericycle cells further builds up this auxin maximum and thus promotes the LRI (Swarup et al., 2005; Laskowski et al., 2008; Péret et al., 2013). Hence, the combination of mechanical tension and auxin feedback on its transport can guide LR development in a self-organizing manner. On the other hand, a model developed by Lucas et al. (2008) suggests that root branching could be controlled by lateral inhibition—a different mechanism that depends on competition or distance between initiation sites and already emerged LR primordia.

Yet another approach attempted to approximate the complexity of sub-cellular regulatory networks that involve crosstalk

between auxin and cytokinin that could influence both size and location of division and differentiation regions within the primary root as well as the putative periodicity of LR branching (Muraro et al., 2011, 2013). This type of modeling approach serves as a very useful tool to explore how dynamic response of auxin-cytokinin interaction network might change with respect to various mutant-like perturbations.

Finally computer modeling approaches have been applied to understand the physics and mechanics of LR development (Szymanowska-Pulka et al., 2012). Szymanowska-Pulka and colleagues reconstructed LRP morphogenesis based on anatomical observations and proposed a dynamic model of LRP growth that integrates acquisition of cell patterning that determines the final shape of the organ. Similar to that model the combination of live biological imaging, 3D/4D microscopic image reconstruction and dynamic computer model, have also revealed the relevance of coordinated patterning processes occurring in the proximity of the developing LRP that are central to the proper emergence of LRs (Lucas et al., 2013).

Taken together, a synergy of modeling and experimental efforts presented herein is likely to further generate new insights in LR patterning processes and ultimately broaden our understanding of the complex root system architectures.

NOVEL TOOLS AND FUTURE PERSPECTIVES

GENETIC STUDIES: SEMI-AUTOMATED PHENOTYPE ANALYSIS

Developing methods based on acquiring and analyzing developmental processes in real-time are continuously improving. Among others, the implementation of automated systems on root phenotype analyses combined with accurate images is a desirable feature. Fast and high-throughput phenotyping methods were developed to monitor the dynamic of root growth. For instance, the GiA Roots semi-automated software tool for high-throughput analysis of root system architecture (Galkovskyi et al., 2012), the semiautomated 3D *in vivo* imaging and digital phenotyping pipeline that enables high-throughput and accurate measurements of root system architecture through time (Topp et al., 2013), or the RootNav image analysis tool that allows the semiautomated quantification of complex root system architectures (Pound et al., 2013) were established.

Monitoring gene expression by live microscopy on a large number of specimens growing under controlled conditions to assess their spatio-temporal expression turns out to be another challenge. For this purpose, a microfluidic device (RootArray) where the roots are repeatedly imaged by confocal microscopy, coupled with an image analysis platform that includes automated real-time detection and tracking of samples, has been developed (Busch et al., 2012). This platform provides the ability to compare the reporter gene expression in *Arabidopsis* roots at tissue level in different developmental zones.

GENETIC STUDIES: FAST-FORWARD GENETICS

A classical forward genetics approach implies the generation of a large mapping population, a high density of genetic markers for achieving a high resolution mapping, and the screening for recombinants in order to define the genetic interval where the mutation is placed (Lukowitz et al., 2000). Taking the advantage of Next Generation Sequencing, fast-forward genetics (SHOREmap pipeline) has been introduced, where the mapping is directly performed by sequencing (Schneeberger and Weigel, 2011). The SHOREmap pipeline covers from mapping to *de novo* marker identification during the sequencing process, and final annotation of candidate mutations (Schneeberger et al., 2009), hence profoundly increasing the efficiency of mutant identification.

TRANSCRIPT PROFILING STUDIES: RNA SEQUENCING

An improved high-throughput transcript profiling technology—RNA sequencing (RNA-seq), has appeared in the last years (Wang et al., 2009). This innovative technique allows the evaluation of the entire transcriptome. It can be used to determine the structure of genes, their splicing patterns and other post-transcriptional modifications, to detect rare and novel transcripts, and to quantify the changing expression levels of each transcript. When compared to microarrays, RNA-seq can detect all expressed genes without the generation of an array of probes, with reduced background noise and large dynamic range. This turned out to be particularly important in species such as tomato, where publicly available microarrays cover only one-third of the complete genome. The RNA-seq approach was used to analyze the transcriptome of tomato roots with the main focus on the spatial patterning and regulation of genes in the root by the hormones cytokinin and auxin. This transcriptome analysis of hormone regulation in tomato root revealed novel genes regulated by each of these hormones and can further be utilized as a reference to conduct future research on tomato roots (Gupta et al., 2013).

CONCLUDING REMARKS

Root system development is central for the plant to reach optimal growth. Hence, understanding the mechanisms that determine root architecture is of great agronomic importance, since they provide a basis for targeted engineering of plant architecture, e.g., for regulating root growth and branching to exploit less nutritious and arid soils. The availability of genome information has made it possible to study the gene expression on a genome scale, observing the behavior of many genes at a time, and obtaining a comprehensive, dynamic molecular picture. In the systems biology century, the only way to get insight in a developmental process is by combining synergistically the different available techniques, which include the most novel tools and advances. From this perspective, new available approaches are ready to be undertaken to obtain deeper insight in LR formation and development.

ACKNOWLEDGMENTS

We thank Annick Bleys for help in preparing the manuscript. This work was supported by the European Research Council with a Starting Independent Research grant (ERC-2007-Stg-207362-HCPO) and the Czech Science Foundation (GA13-39982S) to Eva Benková. We apologize that, because of space restrictions, the scientific contributions of only a limited number of original articles could be cited and discussed.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 September 2013; accepted: 11 December 2013; published online: 26 December 2013.

*Citation: Cuesta C, Wabnik K and Benková E (2013) Systems approaches to study root architecture dynamics. Front. Plant Sci. **4**:537. doi: 10.3389/fpls.2013.00537*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 12 August 2013
doi: 10.3389/fpls.2013.00303

Root resource foraging: does it matter?

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Edited by:
Wolfgang Schmidt, Academia Sinica, Taiwan

Root traits have recently come out of the dark to take center stage in efforts to increase food production and transition toward sustainability, with calls for a "Second Green Revolution" focused on roots (Lynch, 2007; Den Herder et al., 2010). We will highlight some of the challenges *en route* to developing fruitful strategies for enhanced resource acquisition in crops. The article will focus on resource foraging, the process by which root system architecture (RSA, the ensemble of the root system in space) changes over time to acquire resources. Most studies on foraging in plants have focused on resource competition between wild species, using plants subjected to natural selection, not to agricultural selection (Cahill and McNickle, 2011). These studies have been extremely useful to begin to identify the mechanisms and selective forces that shape adaptation and survival in natural environments (Kembel et al., 2008; McNickle et al., 2009; de Kroon et al., 2012; Craine and Dybzinski, 2013; Padilla et al., 2013). A striking, albeit not surprising, conclusion from these studies is the diversity of root behavior, including foraging, observed in different species, even in different accessions of the same species, revealing its adaptive nature.

WHAT IS RESOURCE FORAGING?

The Oxford Dictionary defines foraging as: "a wide search over an area in order to obtain something, especially food or provisions." For many animals, foraging is a motile behavior essential for survival. To survive, sessile plants must acquire resources that are differentially and non-uniformly distributed in space and time: Nitrate is relatively mobile in most soils—hence will percolate into deeper layers with water, and be more evenly distributed; by contrast, inorganic phosphate is relatively immobile, will distribute heterogeneously, in fertile agricultural soils mostly in the surface layer (Marschner, 1995). Thus, when plants explore their environment there is no single, optimal strategy equally suited for all nutrients. Root behavior can be defined as the tropisms and growth activities that vary quantitatively over time, such as changes to growth rate (of each meristem), direction (angle, tortuosity), and root density (rate of lateral root primordia formation and emergence, and demography of lateral root meristems per unit cell number of lower order root). While each of these parameters can impact foraging capacity, those that affect the behavior of lateral organs stand out, because they define the capacity to exploit locally enriched resources.

HOW DO PLANTS FORAGE FOR RESOURCES?

Foraging in animals is defined as "searching" for resources—do plant roots *prospect* for resources, that is grow without prior perception of resources; or does the *perception* of resources promote growth behaviors that facilitate their exploitation? This distinction is not semantic, because targeted strategies to improve resource acquisition would focus on different processes.

Recent work in Arabidopsis has shown that lateral root primordia (LRP) are formed in a strictly acropetal sequence, with no new LRP formed between already developed lateral roots (LR) or LRP (Dubrovsky et al., 2000, 2001, 2006; Moreno-Risueno et al., 2010). However, not every LRP develops into an LR (Zhang et al., 1999; Dubrovsky et al., 2006), indicating the presence of a facultative checkpoint, which may be responsive to developmental, environmental or physiologic cues. Note that these experiments were performed in artificial, axenic conditions: synthetic media with uniform nutrient distribution at the onset of the experiment and the absence of any root-microbe interactions, or of obstacles, whose mechanical impedance affects growth behavior (Fang et al., 2013). Therefore, their significance for root systems in more natural environments remains to be confirmed. However, precisely these conditions permitted a very rigorous, detailed analysis of the initiation and early development of LRP, which is challenging in a soil environment. Despite these caveats, the findings of these and other studies suggest that resource perception stimulates outgrowth, of at least a fraction of LRP in response to environmental cues. Resource perception likely occurs through processes localized in the root cap, which is involved in the perception and orchestration of responses to several environmental cues (Eapen et al., 2005; Svistoonoff et al., 2007; Baldwin et al., 2013), and which is consistent with a requirement for pre-existing LRP to be activated in the vicinity of resource patches, rather than *de novo* production of primordia.

Root growth behavior is very variable ("plasticity"), irrespective of the growth substrate. Several studies have shown that for example, growth rates of individual lateral roots, even in "homogeneous" environments, vary greatly (Pages, 1995; Freixes et al., 2002; Forde, 2009). Thus, the magnitude of growth activities is not simply proportional to resources in the environment, which seems to contradict the earlier conclusion that perception of resources promotes growth behaviors that facilitate their exploitation. Such growth variability has been termed "developmental noise" to highlight its stochastic nature, and it has been argued that the contribution of developmental noise to the overall elaboration of the root system exceeds that of resource-responsive modulation of LR growth (Forde, 2009).

In a classical set of experiments, Drew and collaborators showed that barley root system growth responds differently to similar environmental

nutrient concentrations, dependent on resource distribution. When nutrients were uniformly distributed throughout the root's environment, LR growth was not greatly stimulated, whereas when heterogeneously distributed, root growth in the vicinity of high-nutrient patches was significantly enhanced (Drew, 1975; Drew and Saker, 1975). These distinct responses appear adaptively advantageous, when one considers that plants need to invest previously assimilated limiting resources, such as photosynthate and amino acids, to grow roots. Earlier work has shown a strong correlation between the hexose concentration in individual LR meristems and their growth rate (Freixes et al., 2002); strong demand for and consumption of hexoses in active meristems reinforces local phloem unloading, which ensures elevated assimilate provision to active meristems. Moreover, it was recently shown that soluble carbohydrates (sucrose, glucose) also stimulate auxin biosynthesis in a dose-dependent manner (Sairanen et al., 2012). Together, these observations imply the existence of positive feedback mechanisms that reinforce the outcome of competition between individual LRP and LR meristems for systemic resources within the plant. Such mechanisms to limit the number of active LR meristems may largely underpin the phenomenon of "developmental noise."

In addition to the factors controlling root growth rate and density, other aspects also play important roles in a root system's capacity to acquire resources: growth angle and tortuosity, as well as root hairs. The former parameters are particularly important in domesticated plants in an agricultural context, because managed soil environments generally have higher nutrient levels in the surface layer, partly caused by fertilizer provision. Thus, unsurprisingly, modern barley cultivars not only have different RSA than their wild progenitors or landraces, furthermore, the growth angles of their nodal roots (which grow close to the surface) are shallower (Grando and Ceccarelli, 1995; de Dorlodot et al., 2007). Tortuosity, the ratio between the actual length of the root and the shortest distance between its origin and tip (Tracy et al., 2012), which is a surrogate measure for the extent to which a soil volume is sampled for nutrients, may not only be the outcome of obstacle avoidance, but could be part of a foraging strategy for immobile nutrients. Recently, it was shown that some root growth-regulatory peptides enhance tortuosity by interfering with auxin-dependent control of root growth direction (Whitford et al., 2012; Fernandez et al., 2013), which may be exploited in the future to enhance nutrient capture. Root hairs are important to extend the reach of roots radially; their length responds to the availability of immobile resources such as iron or phosphate (Schmidt and Schikora, 2001; Müller and Schmidt, 2004).

RESOURCE CAPTURE IN NON-DOMESTICATED PLANTS

Studies in wild plants have revealed a remarkable level of RSA variability, shaped by inter- and intra-species competition in plant communities for limiting resources (Cahill and McNickle, 2011). These studies showed that many plants sense, and can distinguish, the presence of kin and non-kin plants, and can adjust RSA responses correspondingly. Competition and survival under limiting and non-uniformly distributed resource availability have selected for not necessarily the most energy-efficient strategies (maximizing resource acquisition with minimal energy input), but rather aim at resource pre-emption (super-optimal resource acquisition to negate their availability to potential competitors) (Craine et al., 2005; McNickle et al., 2009; McNickle and Cahill, 2009; Cahill et al., 2010; Cahill and McNickle, 2011; Nord et al., 2011). It is not yet clear, whether that strategy is universal; if it were and applicable to domesticated crops, this would point to a future bottleneck in crop improvement efforts, for which optimizing the plant's energy expenditure in relation to resource acquisition would be important.

IS INCREASED FORAGING CAPACITY IN CROPS DESIRABLE?

Although agricultural practice in rich countries is characterized by regular inputs, particularly in the form of high-level fertilization and irrigation, this practice is not sustainable (Foley et al., 2011; Mueller et al., 2012). While increasing foraging capacity and efficiency in crops would not bypass the requirement to supplement resources, it will allow crops to utilize soil resources more extensively and efficiently, reduce resource losses due to environmental run-off, and, most importantly, facilitate the transition to productive agro-ecosystems with low inputs. The largest impact of this would be felt where it matters most - in those countries where farmers cannot afford high inputs. Improving foraging capacity would also aim to increase resource acquisition efficiency (RAE), although it is not certain that it would simultaneously increase resource utilization efficiency (RUE), which is the efficiency with which a unit of resource is converted into (harvestable) biomass (Rose and Wissuwa, 2012).

CONCLUSIONS

Despite many recent advances in our understanding of the genetic and mechanistic control of root growth, we are still very far from an operational understanding of the genetic mechanisms that underpin root system behavior. Recent technological advances, such as X-ray tomography, will without question facilitate progress by enabling a much more detailed understanding of the spatial and temporal aspects of root system growth (Mairhofer et al., 2012; Tracy et al., 2012). Further technological breakthroughs are needed, particularly in the analysis of RSA when multiple plants, kin or non-kin, are growing together to better understand the conditional interaction of homo- and heterologous root systems in different soil environments, as this will be important for the development of future cropping systems and the suitability of specific cultivars.

Resource acquisition in roots is a complex trait, governing root growth behavior and acquisition by transporters and assimilation into metabolic pathways. Therefore it is dependent on allelic-specific interactions of many genes, although some cases of single, large effect quantitative trait loci (QTL) underpinning improved performance are known (Gamuyao et al., 2012). Targeted approaches based on single-gene based transgenic approaches are necessary, but unlikely to be sufficient or deliver results in time to provide a doubling of food production by 2050 (The Royal Society, 2009). Therefore, it is an urgent priority to exploit the natural variants selected for over centuries in landraces or

wild relatives of crops that perform well in soils with poor resources (Lynch, 2007), to provide material for breeding programs that combine yield traits from elite varieties with resource traits from landraces or wild accessions. Such breeding programs have the potential to rapidly deliver results, particularly where it matters most: in poor countries, to markedly enhance yields in low-input agricultural systems. This should be a priority, as investments here will yield the greatest initial returns, together with the highest likelihood of rapid adoption.

To secure sustainable food production, it is imperative to pursue both fundamental research into root growth mechanisms to inform transgenic approaches *and* breeding programs based on existing germplasm. Since sustainable agricultural practices aim to transform current high-input monoculture-based agroecosystems into ecosystems more similar to natural environments, with lower inputs and multiple or mixed cropping systems, research into root foraging should not be restricted to crop plants and their interactions. We have much to learn from wild plants how inter-species competition and different niches have shaped root foraging strategies in evolution in the quest for more resilient performance for crops experiencing environmental stresses.

ACKNOWLEDGMENTS

Work in the Doerner lab was supported by ANR and the Darwin Trust.

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Received: 06 July 2013; accepted: 22 July 2013; published online: 12 August 2013.

Citation: Tian X and Doerner P (2013) Root resource foraging: does it matter? Front. Plant Sci. ***4****:303. doi: 10.3389/fpls.2013.00303*

This article was submitted to Frontiers in Plant Systems Biology, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 01 October 2013
doi: 10.3389/fpls.2013.00385

Nitrogen modulation of legume root architecture signaling pathways involves phytohormones and small regulatory molecules

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Nitrogen, particularly nitrate is an important yield determinant for crops. However, current agricultural practice with excessive fertilizer usage has detrimental effects on the environment. Therefore, legumes have been suggested as a sustainable alternative for replenishing soil nitrogen. Legumes can uniquely form nitrogen-fixing nodules through symbiotic interaction with specialized soil bacteria. Legumes possess a highly plastic root system which modulates its architecture according to the nitrogen availability in the soil. Understanding how legumes regulate root development in response to nitrogen availability is an important step to improving root architecture. The nitrogen-mediated root development pathway starts with sensing soil nitrogen level followed by subsequent signal transduction pathways involving phytohormones, microRNAs and regulatory peptides that collectively modulate the growth and shape of the root system. This review focuses on the current understanding of nitrogen-mediated legume root architecture including local and systemic regulations by different N-sources and the modulations by phytohormones and small regulatory molecules.

Keywords: nitrogen regulation, legumes, root development, lateral root, nodulation, phytohormone, microRNA, small regulatory peptides

INTRODUCTION

Understanding how plants grow and develop under diverse environmental conditions is crucial for improving crop productivity. As plants are sessile, they are highly sensitive to the environment and respond accordingly for growth and survival. Of particular importance is nitrogen (N) which provides the building blocks for protein production in plants and dictates crop yield and productivity. The root system adapts to soil N-levels by modifying its architecture (Hodge, 2006). In legumes, during N-limitation, specialized root organs called nodules, can form through symbiotic interaction with rhizobia which are specialized nitrogen-fixing bacteria. Rhizobia convert atmospheric N_2 to ammonium to provide legumes with N for growth. Some of this fixed N is recycled back into the soil to sustain subsequent plant growth. Due to this ability, legumes are used as rotational or cover crops to replenish soil N (Collette et al., 2011). As legume root architecture is strongly regulated by N, understanding N-regulation of root development has great agricultural importance. The recent discovery of small regulatory molecules such as microRNAs and regulatory peptides provide additional facets to the classic phytohormone mediated pathways of root development. Therefore this review aims to give a brief perspective on the current knowledge of the signaling components involved in N-mediated root architecture with emphasis on the legume root system.

IMPROVING PLANT ROOT ARCHITECTURE FOR BETTER N USE EFFICIENCY

Nitrogen levels strongly influence root architecture and crop yields (Hodge, 2006; Garnett et al., 2009). The enhanced crop production during the Green Revolution was mostly attributed to N fertilizer use to alleviate soil N-limitation (Tilman, 1998; Xu et al., 2012). However, there is also an internal control in plants for N use – N use efficiency (NUE) which determines the efficiency of a plant to transport, assimilate and uptake N from the environment (Xu et al., 2012). Poor NUE often translates into utilization of only 30–40% of externally supplied N and this wastage is exacerbated by the energy intensiveness of the Haber-Bosch process which consumes 1–2% of the world energy supply (Cocking, 2009; Hao et al., 2011). In addition, current food crop production has reached a NUE plateau, limiting further yield increases. Poor NUE has also led to excessive N fertilizer usage, generating adverse environmental effects including nitrate-derived water pollution, the production of reactive N, algal blooms and water and soil acidification (Tilman, 1998; Galloway et al., 2008; Rockstrom et al., 2009). Therefore, suboptimal NUE poses a major challenge. On one hand, crop production must increase to sustain world population growth (Collette et al., 2011). On the other, the further environmental damage that will ensue if NUE is not improved will undermine these efforts. Disturbances in the global N-cycle are already negatively impacted global biosphere health and reactive N gases contribute to global warming (Rockstrom et al., 2009).

A more sustainable alternative involves utilizing the biological N fixation ability of legumes to replenish soil N. Therefore, a better understanding of legume development with respect to N-mediated root growth is required for agricultural sustainability.

IMPORTANCE OF LEGUMES TO PROVIDE AN ALTERNATIVE N-SOURCE IN SUSTAINABLE AGRICULTURE

Nitrogen-fixing root nodules form from the legume-*Rhizobium* symbiosis. A "zone of maximum susceptibility" occurs in the elongation zone near the root tip (Bhuvaneswari et al., 1980; Sargent et al., 1987). *Rhizobium*-derived nodulation (Nod) factors are required to induce root hair curling, infection and nodule primordium formation. Rhizobia colonize mature nodules and fix N. The legume-*Rhizobium* symbiosis contributes between 14 and 140 kg of N/acre/year and 33% of human protein globally (Graham and Vance, 2003). A 15 year study involving the co-cultivation of maize with soybean compared to growing maize grown alone showed a significant reduction of carbon and N loss to the environment (Drinkwater et al., 1998). The Food and Agriculture Organization (FAO) promotes sustainable agriculture by increasing legume usage in crop-rotations and as cover crops to enrich soil N levels (FAO, 2009; Collette et al., 2011). Although N-limitation has been long known as a prerequisite for nodulation to occur, the mechanism behind legume root susceptibility for nodulation is still unknown. Since lateral root and nodule development and overall root architecture are strongly influenced by N-availability, a comprehensive understanding of these processes is required to optimize legume utilisation for sustainable agriculture.

N REGULATION OF ROOT ARCHITECTURE IS MEDIATED THROUGH SYSTEMIC AND LOCAL SIGNALING PATHWAYS

Local and systemic controls influence N-mediated root architecture regulation (**Figure 1**). In addition, homogenous and heterogeneous N-regimes impart differential responses in dicots and monocots. Local control is exemplified by the stimulation of lateral root elongation by high N-patches in the soil (Robinson et al., 1999). In the systemic pathway, root architecture is dictated by the plant's overall N-status (Zhang and Forde, 1998; Robinson et al., 1999). Homogeneous high nitrate (e.g. $\geq$10 mM) imparts systemic inhibition of lateral and primary root growth (**Figure 1**) whereas homogeneous low nitrate (e.g. $\leq$1 mM) promotes both (Robinson et al., 1999; Zhang et al., 1999; Walch-Liu et al., 2006; Ruffel et al., 2011). Coordinated systemic and local regulations are observed in split-root experiments where the root system is split into two, with each side exposed to different treatments. Split-root exposed to low and high N-level on each side respectively shows more lateral roots form on the side exposed to high nitrate (Ruffel et al., 2011). The root foraging mechanism exploits the high N-patches and minimal investment is made by the plant to the N-limited roots (Robinson et al., 1999). Compared to these N-regulations of lateral roots, less is known about nodule regulation by local and systemic N pathways.

Local and systemic pathways also regulate nodule numbers. The earliest formed nodules stimulate systemic autoregulation which suppresses further nodulation in younger root regions (**Figure 1**). Autoregulation can also be observed in split-root experiments: nodules forming on one split-root will inhibit nodulation on

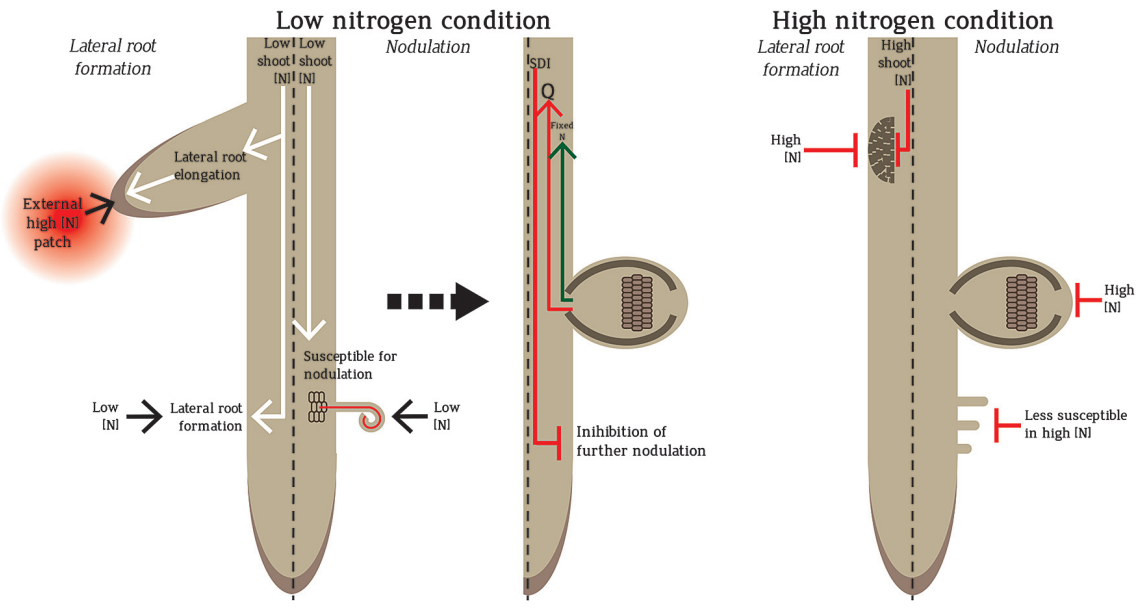

FIGURE 1 | Regulation of nodule and lateral root formation in low and high N. Low N promotes the formation of lateral roots and nodules. Lateral root formation increases in low N to promote foraging. However, if the root senses a patch of high N during N-limitation, lateral root elongation is promoted towards the high N-patch to exploit the available N-source for the plant use. In low N, legume roots are susceptible to rhizobial infection and form nodules in which N is fixed by rhizobia and transported into the plant. The formation of nodules also sends a root-derived signal "Q" to the shoot, which triggers the production of a shoot-derived inhibitor (SDI) that travels back to the root to inhibit further nodulation. In homogenous high N conditions, both lateral roots and nodules are inhibited. In high N, the root is less susceptible for nodulation and infection, nodule number development and N fixation capacity are reduced. High shoot N also leads to less lateral root formation.

the second split-root (Kosslak and Bohlool, 1984; Sargent et al., 1987). Nodules formed by the first inoculation produce root-derived signal (Q) which travels to the shoot via the xylem to be ultimately perceived by a leucine-rich repeat receptor-like kinase (LRR-RLK). Functional orthologues of this LRR-RLK have been identified in *Lotus japonicus* (HAR1; hypernodulation aberrant root 1), *Medicago truncatula* (SUNN; super numeric nodules), soybean (NARK; nodule autoregulation receptor kinase) and pea (SYM29; Krusell et al., 2002; Searle et al., 2003; Schnabel et al., 2005). After Q perception, the shoots produce a shoot-derived inhibitor (SDI) that suppresses nodulation in other parts of the roots (**Figure 1**; Caetano-Anolles and Gresshoff, 1991). Systemic control is also observed through selective discrimination of rhizobia based on N-fixation and nodulation efficiency (Sargent et al., 1987; Laguerre et al., 2012). Local high nitrate strongly inhibits nodulation locally and initiates a systemic response. The systemic response can be measured using split-roots (Ruffel et al., 2008; Jeudy et al., 2010). As plants need to invest a lot of energy to maintain nodules, the coordinate regulation of local and systemic pathways ensures that plants have sufficient N with the least energy investment. Therefore, plants will opt for nodulation only when they have a high requirement for N but utilize an external N source whenever available.

INORGANIC AND ORGANIC N INFLUENCE ON PLANT ROOT DEVELOPMENT

Plants take up N from the soil in either inorganic (e.g., nitrate or ammonium) or organic (e.g., amino acids) forms. Most plants prefer nitrate to ammonium because excess nitrate can be stored in vacuoles and high ammonium can be toxic (Glass et al., 2002). In legumes, high nitrate and ammonium (>3 mM) inhibit nodulation but lower concentrations (0.5–2 mM) can stimulate nodulation (Bollman and Vessey, 2006; Barbulova et al., 2007). Different N-sources regulate roots differentially (Bollman and Vessey, 2006; Ruffel et al., 2008). Split-root experiments comparing the effects of growth by nitrate, ammonium or N-fixation revealed that nitrate is the only N-source that compensates growth during N-limitation (Ruffel et al., 2008; Jeudy et al., 2010). When one side of the split-root is in sufficient nitrate and the other is N-limited, the root will compensate the systemic N-limitation by increasing nitrate uptake from the sufficient side (Jeudy et al., 2010). Both ammonium and N-fixation do not seem to have compensatory regulation for growth during N-limitation. However, long-term N-limitation leads to nodule growth stimulation in the sufficient N-side of the split-root and inhibits nodulation in the N-limited side (Salon et al., 2009; Jeudy et al., 2010). These differential root responses to nitrate, ammonium and N-fixation demonstrate the ability of legumes to distinguish between the N-regimes. This is likely to be mediated by different sensory components regulating the plant responses to the respective N-forms.

Root growth in ammonium is partly regulated by ammonium transporters (AMTs) which are involved in maintaining optimal ammonium levels *in planta* and modulating root responses to prevent ammonium toxicity. The AMT1 and AMT2 families were identified in *Arabidopsis* (Yuan et al., 2007). The AMT1 family controls ammonium transport and acquisition while the AMT2 family is involved in regulatory processes (Sohlenkamp et al., 2002; Yuan et al., 2007). In *Lotus*, three AMT1s and two AMT2s have been characterized (Salvemini et al., 2001; Simon-Rosin et al., 2003; D'Apuzzo et al., 2004; Rogato et al., 2010a). LjAMT1;1 and LjAMT1;2 are up-regulated during N-limitation. LjAMT1;3 is up-regulated by high ammonium (D'Apuzzo et al., 2004) and is a putative ammonium transceptor that mediates root responses to toxic ammonium levels (Rogato et al., 2010a,b). LjAMT2;1 is postulated to recover ammonium lost from cellular efflux in nodules and other organs (Simon-Rosin et al., 2003) whereas LjAMT2;2 is required for N-acquisition during mycorrhizal associations (Guether et al., 2009). Ammonium negatively impacts nodulation by inhibiting root hair curling and repressing the expression of *NIN* (*NODULE INCEPTION*), an essential gene for nodule formation (Barbulova et al., 2007). These results indicate that ammonium inhibition is upstream of the Nod factor pathway and that ammonium perception needs to be relayed quickly for rapid nodule inhibition to occur.

Nitrate, the predominant form of soil inorganic N, strongly affects lateral root and nodule formation. In contrast to ammonium, nitrate inhibition occurs downstream of the nodulation pathway just before cortical cell division (Barbulova et al., 2007). The *Lotus* autoregulation mutant, *har1,* is nitrate-insensitive but retains sensitivity to ammonium. Autoregulation mutants from other species are also nitrate insensitive suggesting that nitrate is involved in the autoregulation pathway (Schnabel et al., 2010). Two nitrate transporter families, NRT1 and NRT2, mediate nitrate-dependent responses. NRT1s are mostly low affinity transporters (LATs) and NRT2 are mostly high affinity transporters (HATs; Tsay et al., 2007). In *Medicago*, two NRT1 transporters were identified: NIP/LATD (numerous infections and polyphenolics/lateral root-organ defective), which is involved in root architecture regulation (Bagchi et al., 2012), and NRT1.3, which regulates nitrate uptake in N-deficient conditions (Morère-Le Paven et al., 2011). NIP/LATD acts as a HAT under low nitrate conditions and NIP/LATD mutants have severe defects during nodule and lateral root formation, which can only be partially rescued by an *Arabidopsis* NRT1 homologue (Bagchi et al., 2012). This suggests additional functions for NIP/LATD apart from transporting nitrate (Yendrek et al., 2010; Bagchi et al., 2012). MtNRT1.3, which encodes a dual-affinity nitrate transporter similar to AtNRT1.1, is postulated to regulate nitrate uptake during N-limitation (Morère-Le Paven et al., 2011). Since AtNRT1.1 is known to be a transceptor, the legume NRT homologs could be involved in nitrate-dependent nodulation signaling pathways. Nitrate inhibits nodulation not because of its nutritional effect but more likely as an important signaling cue to regulate nodulation (Carroll and Mathews, 1990).

Apart from inorganic N, free amino acids, particularly glutamine, also affect root architecture. High glutamine inhibits root growth by acting as an internal N-status signal for mediating root development (Zhang et al., 1999). In legumes, the glutamine, asparagine and ureides produced by nodules may also regulate nodulation. A high level of fixed-N in the phloem lowers nitrogenase activity in nodules (Parsons et al., 1993; Imsande and Touraine, 1994; Sulieman et al., 2010). These reductions in nitrogenase activity through feedback regulation might contribute to nodule modulation (Parsons et al., 1993; Serraj and Sinclair, 2003; Sulieman and Tran, 2013).

N-mediated regulation of root development ensures that sufficient N acquisition occurs to support the formation of the photosynthetic apparatus in the shoot. The shoot then invests carbon to promote root development to explore the soil for more N or initiate symbiosis to fix N (Garnett et al., 2009; Nunes-Nesi et al., 2010). Therefore, the communication between local and systemic pathways is tightly regulated by phytohormones and other regulatory molecules including small regulatory molecules.

PHYTOHORMONES: WELL-KNOWN MEDIATORS OF LONG-RANGE SIGNALING IN N-REGULATION

Auxin is the major hormone implicated in root development and N-mediated control of root architecture. High nitrate is thought to reduce local auxin accumulation suggesting that auxin may be a shoot-to-root signal of "N-status" (Bao et al., 2007; Okushima et al., 2011). In *Arabidopsis*, high shoot N-levels are speculated to reduce shoot-to-root auxin transport resulting in reduced lateral root formation (Reed et al., 1998; Forde, 2002). However studies with *Medicago* reveals that high shoot N-levels increase shoot-to-root auxin transport (Jin et al., 2012). The correlation between shoot-to-root auxin transport and lateral root formation suggests that systemic N-regulation via auxin acts through modulating auxin levels for the formation of lateral root founder cells (Dubrovsky et al., 2011). This response is demonstrated by using *Medicago sunn-1* mutant, which has insensitive shoot-to-root auxin transport regardless of the N-level. In *sunn-1*, the correlation between N-dependent auxin transport and lateral root regulation is lost (Jin et al., 2012). However, SUNN-dependent shoot-to-root auxin transport seems to only apply to nitrate-mediated lateral root regulation but not nodule regulation suggesting that auxin-dependent N-regulation of nodulation acts locally in the root (Jin et al., 2012). Auxin regulation of root development involves crosstalk with other phytohormones such as ethylene.

The gaseous hormone, ethylene, is directly regulated by soil nitrate levels and is involved in local nitrate-dependent root regulation. In several species, high nitrate increases root ethylene evolution (Ligero et al., 1986, 1987; Caba et al., 1998; Tian et al., 2009). High ethylene levels inhibit nodules and lateral roots formation while low ethylene increases lateral roots and promotes nodulation (Peters and Crist-Estes, 1989; Lee and Larue, 1992; Nukui et al., 2000; Oldroyd et al., 2001). Several rhizobia generate better nodulation responses by inhibiting localized ethylene by synthesizing an ethylene precursor mimic or by producing the aminocyclopropane-deaminase enzyme which degrades aminocyclopropane, the ethylene precursor (Ma et al., 2002). Ethylene also imparts positional control of nodulation as increased ethylene levels opposite the phloem poles favors nodule formation opposite the xylem poles (Heidstra et al., 1997). Ethylene regulation of lateral roots and nodules likely occurs through the control of cell cycle pathways (Dan et al., 2003; Spadafora et al., 2012) however little detail is known how this occurs. Cell cycle regulation by ethylene occurs partly through crosstalk with cytokinin (Spadafora et al., 2012) which is also involved in nitrate-regulated development.

Cytokinin directly regulates the cell cycle and is a mediator for communicating N-status between the shoot and root via the phosphorelay pathway (Sakakibara et al., 2000). In this pathway, nitrate replenishment of an N-starved root system increases cytokinin synthesis which is then transported to the shoot, signaling the root's N status. As shoot N-supply is depleted, cytokinin is transported back to the root to signal the shoot's low N-status (Sakakibara et al., 2000; Ruffel et al., 2011). Cytokinin also regulates the cell cycle during lateral root development by acting directly on lateral root founder cells to inhibit root initiation (Li et al., 2006; Laplaze et al., 2007). However, once differentiation occurs, high cytokinin promotes lateral root elongation (Li et al., 2006). As lateral root elongation is also stimulated by high nitrate patches, it would be interesting to examine the cytokinin-nitrate interaction during this process. In legumes, cytokinin is an upstream component of the nodulation pathway and exogenous cytokinin application induces the expression of several nodulation genes (Fang and Hirsch, 1998; Gonzalez-Rizzo et al., 2006). Cytokinin receptor mutants of *Lotus* (Murray et al., 2007) and *Medicago* (Gonzalez-Rizzo et al., 2006) show reduced nodulation while a cytokinin receptor gain-of-function mutants leads to spontaneous nodulation (Murray et al., 2007; Tirichine et al., 2007). Although N-mediated root development involves cytokinin, auxin and ethylene, small regulatory molecules fine-tune these pathways.

SMALL REGULATORY MOLECULES FOR FINE-TUNING PLANT DEVELOPMENTAL RESPONSES

Regulatory microRNAs and peptides act as fine-tuners of local cellular development. These small regulatory molecules are likely to act as cellular cues in response to environmental conditions including N-availability. The signaling cascades then activate the phytohormone pathways to modulate the root system. For example, the auxin receptor, Auxin signaling F-box protein 3 (AFB3), is a target of microRNA, miR393 (Vidal et al., 2010). Since AFB3 and miR393 are both nitrate-induced, this leads to a transient up-regulation of AFB3 in a feed-forward loop prior to the subsequent induction of miR393 which down-regulates AFB3 (Vidal et al., 2010). The rapid down-regulation of AFB3 by miR393 provides a fine-tuned mechanism of the root system to dynamically respond to N in real time. This interaction is an excellent example of a small regulatory molecule integrating nitrate availability with auxin signaling. Recently, soybean miR160 has also been shown to modulate auxin during nodulation. miR160 is down-regulated by *Rhizobium* inoculation while its *Arabidopsis* homologue is upregulated by N-starvation (Subramanian et al., 2008; Liang et al., 2012). Over-expressing miR160 in soybean leads to auxin hypersensitivity which reduces nodule formation demonstrating the importance of auxin regulation by miR160 during nodulation (Turner et al., 2013). miR169 is also involved in N-regulation of root development. Overexpressing miR169 in *Arabidopsis* reduces nuclear factor Y-A (NF-YA) transcript levels leading to a low N-stress phenotype coupled with low N acquisition (Zhao et al., 2011). In *Medicago*, miR169 targets HAP2-1, a NF-YA involved in nodulation (Combier et al., 2006). This *Medicago* NF-YA is closely regulated with *NIN* (Soyano et al., 2013). *NIN* and its *Arabidopsis* homolog, *NLP7* are up-regulated by low N (Wang et al., 2009). As *nlp7* displays N-stressed root phenotypes even in N-sufficient conditions (Castaings et al., 2009), it would be interesting to see if miR169 or other regulatory molecules are involved in NIN/NLP7 dependent pathway during N-limitation in roots.

Other regulatory molecules in plants include small signaling peptides. The most well-studied regulatory peptide families in plants are the CLE peptide family. Several members of this family are involved in root development including CLE40, which involves in the maintenance of the root apical meristem (RAM) by regulating cellular differentiation (Stahl et al., 2009). In legumes, nodule-specific CLE peptides also regulate nodulation (Saur et al., 2011; Mortier et al., 2012). Several CLEs are nitrate-regulated like GmNIC1 in soybean which is involved in the autoregulation of nodulation (Reid et al., 2011, 2013). During autoregulation, GmNIC1 interacts with the NARK receptor in the root (Reid et al., 2011, 2013). Recently, the LjCLE-RS2 peptide in *Lotus* was demonstrated to be the "Q" signal (**Figure 1**) which interacts with HAR1 in the shoot (Okamoto et al., 2013). LjCLE-RS2 is also up-regulated by nitrate and is hypothesized to integrate nitrate inhibition of nodulation via the HAR1-dependent autoregulation pathway (Okamoto et al., 2009). Nodule inhibition by these nitrate-regulated CLEs suggests possible crosstalks between autoregulation and nitrate-regulation of nodulation. These CLEs are likely to be involved in signal transduction pathways which further regulate the cytokinin-mediated nodulation pathway (Saur et al., 2011). Therefore, these small regulatory peptides provide a fine-tuning mechanism for nitrate-mediated control of root architecture.

CONCLUSION

Nitrogen is an essential nutrient for plant productivity and its environmental availability strongly regulates root architecture. To optimize N acquisition, nitrate and ammonium transporters/transceptors provide the sensory components of N-mediated root development in legumes. The signal for N-availability is translated into an array of phytohormone pathways which regulates root development. Small regulatory molecules such as microRNAs and peptides provide further fine-tuning of these phytohormone signals to produce highly dynamic and plastic root responses to N-levels. Hence these regulatory pathways, which integrate environmental sensory signals with the modulation of phytohormones and small regulatory molecules could be exploited to improve legume root architecture for better NUE.

ACKNOWLEDGMENTS

Nadiatul A. Mohd-Radzman was supported by ANU International PhD Scholarship. This work was supported by an Australian Research Council grant to Michael A. Djordjevic and Nijat Imin (DP140103714).

AUTHOR CONTRIBUTIONS

Nadiatul A. Mohd-Radzman wrote the paper. Nijat Imin and Michael A. Djordjevic edited the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 July 2013; accepted: 11 September 2013; published online: 01 October 2013.

*Citation: Mohd-Radzman NA, Djordjevic MA and Imin N (2013) Nitrogen modulation of legume root architecture signaling pathways involves phytohormones and small regulatory molecules. Front. Plant Sci. **4**:385. doi: 10.3389/fpls.2013.00385*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 07 February 2014
doi: 10.3389/fpls.2014.00022

Systems analysis of transcriptome data provides new hypotheses about *Arabidopsis* root response to nitrate treatments

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Nitrogen (N) is an essential macronutrient for plant growth and development. Plants adapt to changes in N availability partly by changes in global gene expression. We integrated publicly available root microarray data under contrasting nitrate conditions to identify new genes and functions important for adaptive nitrate responses in *Arabidopsis thaliana* roots. Overall, more than 2000 genes exhibited changes in expression in response to nitrate treatments in *Arabidopsis thaliana* root organs. Global regulation of gene expression by nitrate depends largely on the experimental context. However, despite significant differences from experiment to experiment in the identity of regulated genes, there is a robust nitrate response of specific biological functions. Integrative gene network analysis uncovered relationships between nitrate-responsive genes and 11 highly co-expressed gene clusters (modules). Four of these gene network modules have robust nitrate responsive functions such as transport, signaling, and metabolism. Network analysis hypothesized G2-like transcription factors are key regulatory factors controlling transport and signaling functions. Our meta-analysis highlights the role of biological processes not studied before in the context of the nitrate response such as root hair development and provides testable hypothesis to advance our understanding of nitrate responses in plants.

Keywords: meta-analysis, root hairs, nitrate, systems biology, gene co-expression analysis, transcription factors, Gene Ontology (GO)

INTRODUCTION

Nitrogen (N) is a constituent of nucleic acids, amino acids, chlorophyll, and phytohormones among many other biomolecules. N is quantitatively the most abundant element in plant tissues after C, H, and O, representing up to 5% of dry weight (Miller and Cramer, 2005). However, biologically available N is often in short supply in natural as well as agricultural systems, limiting plant growth and development. Understanding the molecular mechanisms underlying plant N responses is an important issue for plant biology as well as agriculture. Transcriptomics approaches have been used extensively to investigate these mechanisms. Transcriptomic studies have evaluated different aspects of the plant nitrogen response, such as the effect of N source (e.g., Wang et al., 2007; Patterson et al., 2010), time after N treatments (e.g., Krouk et al., 2010a), N concentration (e.g., Wang et al., 2007), tissue type (e.g., Wang et al., 2003), and cell type (e.g., Gifford et al., 2008). These studies have identified a myriad of genes and functions modulated by N nutrient/metabolites such as uptake, transport and metabolism (reviewed in Vidal and Gutiérrez, 2008; Alvarez et al., 2012).

The nitrate signaling pathway is still poorly understood with only a handful regulatory factors modulating gene expression at different levels identified. Plants perceive changes in the concentration of nitrate by specific receptors. It has been established that the dual-affinity nitrate transporter NRT1.1 is able to function as nitrate sensor in *Arabidopsis thaliana* (Ho et al., 2009). Additional regulatory factors include protein kinases from the CIPK family. For example, loss of function of *CIPK8* gene reduces by 40–65% the expression of several primary response genes such as *NRT1.1*, *NIA1*, or *GLN2* (Hu et al., 2009). Another level of control of gene expression by nitrate is carried out by transcription factors. Only a few transcription factors directly implicated in regulating nitrate responses have been characterized to date (Gutiérrez, 2012). Some of these transcription factors are important for the control of nitrate assimilation, while others have been involved in modulation of root system architecture. The first transcription factor identified in the N response was a MADS box transcription factor called *ANR1* (Zhang et al., 1999). *ANR1* is involved in the control of lateral root growth in response to localized nitrate supply. Recent studies indicate NLPs are also important transcription factors in the nitrate response because they regulate many known nitrate signaling and assimilation genes (Konishi and Yanagisawa, 2013; Marchive et al., 2013). Additional transcription factors known to regulate N responses include members of the LBD family (Rubin et al., 2009); *HY5*, which is related to phytochrome-mediated effects on enzymes involved in nitrogen assimilation (Lillo, 2008); and a nitrate-induced *NIGT1* member of the GARP family, which has been suggested to be involved in the control of nitrate utilization (Sawaki et al., 2013). Recently, *NAC4* was found to be a key regulatory element controlling a nitrate-responsive network and lateral root development in *Arabidopsis* (Vidal et al., 2013). However, nitrate regulation of other important features of

root system architecture such as primary root growth and root hair development have not yet been explored in depth (Forde and Walch-Liu, 2009). Several studies reported root hairs are important for nutrient uptake, including nitrogen uptake (Libault et al., 2010). Wang et al. (2002) observed root hair growth in response to nitrate treatment in rice. In the case of other macronutrients, such as phosphate, many genes involved in root morphological response to nutrient availability (Niu et al., 2013), including root hair responses (Lin et al., 2011a,b), have been identified. However, the role of root hairs and root hair development in nitrate responses has not been addressed.

The development of high-throughput technologies enabled identification of more than 2000 genes with changes in gene expression in response to various N treatments (Krouk et al., 2010b). However, functional characterization of these genes is long and laborious and lags far behind global gene expression studies. In order to aid prioritizing, we integrated publicly available root nitrate transcriptome data obtained under various experimental conditions. The analysis presented here (i) identifies the most consistent genes and biological functions regulated in response to nitrate treatments across multiple experiments (ii) highlights important biological process associated with nitrate root responses that have not been previously addressed and (iii) proposes key regulatory factors controlling major gene network modules. Our integrated systems analysis provides concrete testable hypothesis to advance our understanding of plant root nitrate responses.

METHODS

MICROARRAY DATA COLLECTION AND PREPROCESSING

Microarray data used in this work was obtained from experiments published in: (Wang et al., 2003, 2004, 2007; Gutiérrez et al., 2007a; Gifford et al., 2008; Hu et al., 2009; Krouk et al., 2010a; Patterson et al., 2010; Ruffel et al., 2011; Vidal et al., 2013 and Álvarez et al., submitted.) CEL files of these experiments are available in the public microarrays databases GEO, EBI, or NASC. Accession numbers for each data set are indicated in **Table 1**. Background correction and normalization of the raw data sets was performed using Robust MultiChip Analysis (RMA) implemented in "affy" R package (Gautier et al., 2004).

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES

The non-parametric RankProduct method (Breitling et al., 2004) was used to identify differentially expressed genes between treatment and control conditions (potassium nitrate vs. potassium chloride), as this method has been shown to be robust in analyzing microarray data that comprise small numbers of replicates (Hong and Breitling, 2008). A gene was considered statistically significant if its false discovery rate (FDR) adjusted *p*-value was equal or smaller than 0.05.

NETWORK CONSTRUCTION AND COEXPRESSION MODULE DETECTION

A weighted gene coexpression network was constructed using the WGCNA R package version 1.27.1 (Langfelder and Horvath, 2008) with differentially expressed genes. First, the Pearson correlation matrix was weighted by raising it to a power (β). To choose the appropriate power, the network topology for various soft-thresholding powers was evaluated using pickSoftThreshold function and $\beta = 7$ was chosen because this ensured an approximate scale-free topology of the resulting network as previously described (Zhang and Horvath, 2005). Next, the pairwise measure of gene coexpression of the resulting weighted network was transformed into a topological overlap (TO) similarity measure, which is a robust measure of pairwise interconnectedness (Yip and Horvath, 2007). A TO similarity measure between two genes (ij) is defined as: $\mathrm{TO}_{ij} = \frac{\sum_u a_{iu}a_{uj} + a_{ij}}{\min(k_i,\ k_j) + 1 - a_{ij}}$ where $k_i = \sum_u a_{iu}$ was the node connectivity and a is the network adjacency. Finally, TO similarity measure coupled with average linkage hierarchical clustering was performed for module detection using the Dynamic Tree Cut algorithm (Langfelder et al., 2008). The coexpression network was visualized using Cytoscape v 3.0 (**File S1**) and analyzed using the NetworkAnalyser plugin (Doncheva et al., 2012). In order to simplify the display of the network and to focus on relevant relationships, only edges in this network of the corresponding TO similarity measure above a threshold of 0.10 are shown in **Figure 3**. Further, NetworkAnalyser plugin was used to assess which genes in the network form hubs. VirtualPlant platform (Katari et al., 2010) was used to generate a subnetwork of transcription factors and putative targets taking into account the TO similarity measure and over-represented transcription factors binding sites.

FUNCTIONAL ENRICHMENT ANALYSIS

Fisher's exact test was performed for declaring a GO (Gene Ontology) category as significantly over-represented (Benjamini–Yekutieli method for controlling FDR, adjusted p-value < 0.05) using PlantGSEA toolkit (Yi et al., 2013). GO level was defined as the number of edges in the shortest path connecting a node to the root node, this information was calculated using SQL queries on the GO database. To determine protein sequence similarity for genes associated to GO terms, we performed pair-wise BLASTP sequence comparisons. We only analyzed alignments with an *E*-value smaller or equal than 10^{-10}. To compare the results we used protein sequence similarity scores normalized by query length.

RESULTS AND DISCUSSION

MEMBERS OF G2-LIKE AND LBD TRANSCRIPTION FACTOR FAMILIES ARE THE MOST CONSISTENTLY REGULATED GENES IN RESPONSE TO NITRATE TREATMENTS

Meta-analysis of microarray data has been used to define robust sets of regulated genes (e.g., Gutiérrez et al., 2007b; Bhargava et al., 2013). To gain new insights into the plant N response we carried out a meta-analysis of transcriptome data based on the following criteria: First, we focused on experiments performed with nitrate as the only experimental factor. Nitrate is one of most important nitrogen sources in agricultural soils (Glass, 2009) and it can act as a signal to regulate global gene expression as well as different aspects of plant growth and development (Wang et al., 2004). Second, we focused on transcriptome studies carried out in root organs because it is the first organ encountering nitrate and initiates plant responses (Wang et al., 2003) and it is the organ for which more data sets are available. Finally, we focused on studies with wild-type genetic background and Affymetrix microarray platform to avoid cross-platform variation as well as unbalanced data sets. Using these criteria, 27 experimental datasets corresponding to 131 ATH1 Affymetrix genechip hybridizations were

Table 1 | Summary of microarray datasets and experimental parameters.

References	Accession number	Database	Number of arrays selected	Name in this paper	Nitrate concentration (mM)	Time treatment (min)	Tissue	Growth stage	Growth condition
Wang et al., 2003	NASCARRAYS-479	NASC Arrays	4	1	0.25	20	Whole roots	Seedling	Hydroponic
Wang et al., 2004	NASCARRAYS-480	NASC Arrays	4	2	5	120	Whole roots	Seedling	Hydroponic
Gutiérrez et al., 2007a	E-MEXP-828	Array Express	8	3	5	480	Whole roots	Adult	Hydroponic
				4	10	480	Whole roots	Adult	Hydroponic
				5	15	480	Whole roots	Adult	Hydroponic
Gifford et al., 2008	GSE7631	GEO	42	6	5	120	Lateral root cap	Seedling	Hydroponic
				7	5	120	Epidermis + Cortex	Seedling	Hydroponic
				8	5	120	Endodermis + Pericycle	Seedling	Hydroponic
				9	5	120	Pericycle	Seedling	Hydroponic
				10	5	120	Stele	Seedling	Hydroponic
				11	5	120	Whole roots	Seedling	Hydroponic
				12	5	210	Whole roots	Seedling	Hydroponic
Krouk et al., 2010a	GSE20044	GEO	24	13	1	3	Whole roots	Seedling	Hydroponic
				14	1	6	Whole roots	Seedling	Hydroponic
				15	1	9	Whole roots	Seedling	Hydroponic
				16	1	12	Whole roots	Seedling	Hydroponic
				17	1	15	Whole roots	Seedling	Hydroponic
				18	1	20	Whole roots	Seedling	Hydroponic
Hu et al., 2009	GSE9148	GEO	6	19	25	30	Whole roots	Seedling	Hydroponic
Ruffel et al., 2011	GSE22966	GEO	18	20	5	120	Whole roots	Seedling	Agar plates
				21	5	480	Whole roots	Seedling	Agar plates
				22	5	2880	Whole roots	Seedling	Agar plates
Patterson et al., 2010	GSE29589	GEO	9	23	1	90	Whole roots	Seedling	Hydroponic
				24	1	480	Whole roots	Seedling	Hydroponic
Vidal et al., 2013	GSE35544	GEO	6	25	5	120	Whole roots	Seedling	Hydroponic
Álvarez et al., submitted	GSE43011	GEO	6	26	5	120	Whole roots	Seedling	Hydroponic
Wang et al., 2007	NASCARRAYS-481	NASCArrays	4	27	0.25	20	Whole roots	Seedling	Hydroponic

selected for further analysis (**Table 1**). To focus on relevant N-responsive genes, RMA normalization (Irizarry et al., 2003) was performed and Rank Products (Breitling et al., 2004) was used to identify genes that exhibited expression differences between nitrate treatment and control (KCl) samples in each experiment. A total of 2286 genes were identified as differentially expressed in at least one experiment, with a maximum FDR of 5% (**Table S1**). Next, we ranked differentially expressed genes by the number of experiments in which they were regulated. The top 50 genes according to this criterion are shown in **Table 2**. No gene was differentially expressed in response to nitrate treatments in all experiments. The expression of all genes in **Table 2** was induced by nitrate treatments in at least 40% of the experiments analyzed, with a maximum of 75%. The majority of these core nitrate-response genes were upregulated. Starvation pre-treatment causes a decrease in expression levels of many genes related to nitrate assimilation, signaling, and transport. An increase in the expression of many genes is therefore expected after the nitrate treatment. Interestingly, the top 5 most consistent genes found in our analysis code for transcription factors. *HRS1* (AT1G13300), a G2-like transcription factor, was statistically induced in 20 out of 27 experiments (**Table 2**). *HRS1* is expressed in root hairs and is induced under low phosphate concentration (Liu et al., 2009). *HRS1* may be involved in modulation of primary root and root hair growth in response to phosphate starvation (Liu et al., 2009). Our results suggest that in addition to phosphate, *HRS1* is a regulator of root growth in response to nitrogen availability. Three other members of this transcription factor family respond to nitrate in multiple experiments: AT3G25790, AT1G68670, and AT1G25550. AT1G68670 is a direct target of *SHORT ROOT* (*SHR*) (Cui et al., 2011), a key regulator of root growth and development in *Arabidopsis*. Moreover, *SHR* regulates cytokinin homeostasis by directly controlling transcription of *CYTOKININ OXIDASE 3* gene (Cui et al., 2011). Several studies indicate nitrate induces the expression of cytokinin biosynthesis genes resulting in cytokinin accumulation in response to nitrate (Kiba et al., 2011). These observations suggest G2-like transcription factor (AT1G68670) is part of the *SHR* regulatory network modulating root development and cytokinin levels. The second G2-like family gene AT1G25550, was found to bind the *E2Fa* promoter. *E2Fa* is an essential transcription factor in *A. thaliana* that regulates asymmetric cell division marking lateral root initiation (Berckmans et al., 2011). These findings suggest that G2-like transcription factors may be important regulators of root morphology in response to nitrate availability. Other transcription factors that respond to nitrate in a large number of experiments (70%) are *LBD37* and *LBD39*. Transcription factors of the LBD family have been shown to have important roles in regulation of anthocyanin synthesis and nitrogen metabolism by repressing transcripts that are critical for nitrate transport and assimilation (Rubin et al., 2009). Additional transcription factors involved in *Arabidopsis* nitrate root responses include other previously characterized NAC and NLP transcription factors as well as a list of new candidates. **Figure 1** shows the most represented transcription factor families in decreasing order. The most represented families are ERF/AP2 (31) and WRKY (15) transcription factors, with an overrepresentation of nitrate responsive genes as expected from their gene family size. These two transcription factor families are involved in plant development and responses to biotic and abiotic stress (Rushton et al., 2010; Mizoi et al., 2012). However, their role in nitrate responses is not clear yet. Another family of transcription factors with many members are MYB-type transcription factors (22). *MYB75* is known for its role in response to nitrogen limitation (Lea et al., 2007). In other plant species, MYB proteins are known to control expression of genes coding for important nitrogen assimilation enzymes, such as glutamine synthetase (Gómez-Maldonado et al., 2004) and asparagine synthetase (Canales et al., 2012). These results point toward specific transcription factors as candidate regulators controlling hallmark responses to nitrate treatments such as metabolism and root system architecture.

NITRATE REGULATES A CORE SET OF BIOLOGICAL FUNCTIONS REGARDLESS OF THE EXPERIMENTAL CONTEXT

As shown in **Figure 2A**, ∼60% of differentially expressed genes were regulated by nitrate in only one experiment, consistent with the idea that nitrate regulation of gene expression depends largely on the experimental context (Gutiérrez et al., 2007b; Krouk et al., 2010b). However, analysis of regulated biological functions showed that responses at the functional level are more robust from experiment to experiment as compared to genes (**Figure 2B**). The average number of genes shared between any two experiments is 6.7%, while 19.5% of overrepresented GO terms (FDR < 0.05) are shared in the same two experiments (**Figure 2B**). This difference in the percentage of shared genes vs. GO terms increases with the number of experiments compared. For example, the number of GO terms shared between any five experiments is 10 times higher than the number of shared genes. To evaluate whether this difference between intersection of genes and GO terms was biological or an artifact due to the nature of the gene to GO association data we carried out three control experiments: (1) Conservation of GO terms could be explained by genes annotated to very general GO term categories increasing the chance of intersection at the GO term level. To address this potential issue, we compared the distribution of total and shared over-represented GO terms between any combination of two experiments. As shown in **Figure S1A**, the distribution of levels is similar for total and shared GO terms indicating differentially expressed genes are not biased toward general GO term categories. (2) Nitrate-responsive genes identified here may have many GO annotations, thus, explaining increased overlap in GO terms. To rule out this possibility, we analyzed the number of GO terms associated with the list of 2286 nitrate responsive genes used in this study and compared with the GO annotations found in 1000 lists of 2286 randomly selected genes represented in the ATH1 Affymetrix Gene Chip. As shown in **Figure S1B**, the average number of GO terms and their distribution are very similar in both cases. This result indicates nitrate responsive genes have no more annotations than a random set of genes of the same size. (3) If the genes are shared when comparing experiments the GO terms will be shared as well. However, GO terms shared between any two experiments contained on average only 22.4% of the same genes (**Figure S2**). This result indicates most of the genes contributing to over-represented GO terms are different in each experiment.

Table 2 | Ranking of the top 50 most consistent genes in response to nitrate.

AGI locus	Description	Total	Upregulated	Downregulated
At1g13300	G2-like transcription factor family protein (*HRS1*)	20	20	0
At5g67420	LOB domain-containing protein 37 (*LBD37*)	19	19	0
At4g37540	LOB domain-containing protein 39 (*LBD39*)	19	19	0
At3g25790	G2-like transcription factor family protein	19	19	0
At1g49500	G2-like transcription factor family protein	18	18	0
At1g24280	Glucose-6-phosphate dehydrogenase 3 (*G6PD3*)	17	17	0
At5g01740	Nuclear transport factor 2 family protein (*NTF2*)	16	16	0
At3g48360	BTB and TAZ domain protein 2 (*BT2*)	16	16	0
At1g25550	G2-like transcription factor family protein	16	16	0
At1g80380	P-loop containing nucleoside triphosphate hydrolases	16	16	0
At5g40850	Urophorphyrin methylase 1 (*UPM1*)	15	15	0
At5g10210	Unknown	15	15	0
At1g77760	Nitrate reductase 1 (*NIA1*)	15	15	0
At1g78050	Phosphoglycerate/bisphosphoglycerate mutase (*PGM*)	15	15	0
At2g15620	Nitrite reductase 1 (*NIR*)	15	15	0
At2g26980	CBL-interacting protein kinase 3 (*CIPK3*)	15	15	0
At4g02380	Senescence-associated gene 21 (*SAG21*)	14	14	0
At3g07350	Unknown	14	14	0
At5g19970	Unknown	13	13	0
At5g62720	Integral membrane HPP family protein	13	13	0
At5g09800	ARM repeat superfamily protein	13	13	0
At3g49940	LOB domain-containing protein 38 (*LBD38*)	13	13	0
At3g16560	Protein phosphatase 2C family protein	13	13	0
At1g30510	Root FNR 2 (*RFNR2*)	13	13	0
At2g16060	Hemoglobin 1 (*AHB1*)	13	12	1
At5g41670	6-phosphogluconate dehydrogenase family protein	12	12	0
At5g19120	Eukaryotic aspartyl protease family protein	12	12	0
At4g25835	P-loop containing nucleoside triphosphate hydrolases	12	12	0
At4g05390	Root FNR 1 (*RFNR1*)	12	12	0
At1g49860	Glutathione S-transferase 14 (*GSTF14*)	12	12	0
At1g68880	Basic leucine-zipper 8 (*bZIP*)	12	11	1
At1g16170	Unknown	12	12	0
At2g48080	2OG-Fe(II) oxygenase family protein	12	12	0
At2g30040	Mitogen-activated protein kinase kinase kinase 14 (*MAPKKK14*)	12	12	0
At5g13110	Glucose-6-phosphate dehydrogenase 2 (*G6PD2*)	11	11	0
At5g45340	Cytochrome P450 (C*YP707A3*)	11	11	0
At3g62930	Thioredoxin superfamily protein	11	11	0
At3g47980	Integral membrane HPP family protein	11	11	0
At4g37610	BTB and TAZ domain protein 5 (*BT5*)	11	11	0
At4g32950	Protein phosphatase 2C family protein	11	9	2
At4g18340	Glycosyl hydrolase superfamily protein	11	11	0
At1g78090	Trehalose-6-phosphate phosphatase (*TPPB*)	11	11	0
At1g32920	Unknown	11	11	0
At1g68670	G2-like transcription factor family protein	11	11	0
At5g15830	Basic leucine-zipper 3 (*bZIP3*)	10	10	0
At5g14760	L-aspartate oxidase (*AO*)	10	9	1
At5g04950	Nicotianamine synthase 1 (*NAS1*)	10	10	0
At3g60750	Transketolase	10	10	0
At3g02850	STELAR K+ outward rectifier (*SKOR*)	10	10	0
At1g08090	Nitrate transporter 2.1 (*NRT2.1*)	10	10	0

Total number of experiments in which each gene was regulated as well as if this regulation was up or downregulated are indicated.

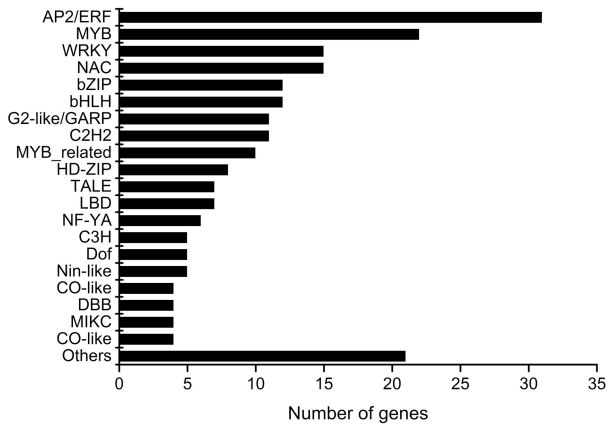

FIGURE 1 | Distribution of the 219 nitrate-responsive transcription factors according to family. Transcription factors were classify following PlantTFDB2.0 database annotation (Zhang et al., 2011).

A prediction of our hypothesis is that different members of the same gene family should be found contributing to shared GO terms between different experiments. To systematically test this hypothesis, we performed pair-wise comparisons of protein sequences for all genes annotated to shared GO terms between any two experiments. We then compared the distribution of protein sequence similarities for all pair-wise comparisons between genes contributing to a shared GO term in our data set vs. all genes annotated to that GO term. We found shared GO terms (74.5%) have more pairs of similar protein sequences coming from different experiments than expected by chance ($\alpha < 0.05$). For example, *NRT2.2* gene was found differentially expressed in experiment 21 and *NRT2.5* was found differentially expressed in experiment 23. Both *NRT2.2* and *NRT2.5* are annotated to the shared GO term "Transport (GO:0006810)." Similarly, the shared GO term "Response to Carbohydrate (GO:0009743)" contains *GLN1;2* and *GLN1;1*, each regulated in different experiments.

The easiest interpretation of these results is that nitrate responses at the biological function level are more robust to experimental context than genes. This phenomenon could be explained by functional redundancy of different genetic components, a feature that is common to biological networks and has been proposed as a mechanism for robustness toward stochastic fluctuations (Whitacre, 2012). A similar idea is the degeneracy concept proposed by Edelman and Gally (2001), which defines the property whereby structurally different elements may perform the same or similar functions. This feature has been attributed not only to gene networks but also to neural networks and evolution (Edelman and Gally, 2001). This phenomenon may be particularly relevant in plants, where increased gene family sizes may provide higher adaptive capacity to environmental perturbations.

ROBUST NITRATE RESPONSIVE BIOLOGICAL PROCESSES HIGHLIGHT NEW NITRATE CONTROLLED DEVELOPMENTAL PROCESSES

Which biological functions are most relevant for nitrate responses in roots? To answer this question, we ranked GO terms by the number of experiments in which they were present based on regulated genes with the corresponding annotation. In order to focus on specific functions, we only considered GO terms at level 7 and 8 and removed redundant terms using the REVIGO tool (Supek et al., 2011). **Table 3** shows the list of the most consistent biological functions. In contrast to genes, the most consistent GO terms appear regulated in ∼90% of the experiments analyzed. The most consistent biological functions are those related to nitrate transport and carbon metabolism. We also found categories associated with root morphogenesis that have not been studied in the context of nitrate responses such as trichoblast differentiation (GO:0010054, 64 genes). Trichoblasts are a subset of specialized epidermal cells from which root hairs emerge. These specialized cells play an important role in the uptake of water and nutrients by increasing root absorption surface (Gilroy and Jones, 2001). Phytohormones are important regulators of root hair growth and development. It has been reported that auxin and ethylene promote root hair elongation and growth (Muday et al., 2012). Interestingly, biological processes that are statistically enriched in response to nitrate (**Table S2**) include auxin biosynthesis (GO:0009851, 32 genes, p-value $= 1.25 \times 10^{-7}$). Genes associated with this function include several genes from the tryptophan-dependent auxin biosynthetic pathway (Mano and Nemoto, 2012) such as *TAR2* (Stepanova et al., 2008), *CYP79B2*, and *CYP79B3* (Zhao et al., 2002). Nitrate can also regulate the expression of several auxin-signaling genes including the *AFB3* auxin receptor (Vidal et al., 2010, 2013). It has been recently reported that nitrogen and small GTPase proteins act synergistically to regulate root hair growth in *Arabidopsis* (Bloch et al., 2011). In addition, it is known that auxin signaling pathway controls root hair growth (Lee and Cho, 2013). Based on these observations, we hypothesize that nitrate modulates root hair development by modulating auxin biosynthesis and signaling.

WEIGHTED CORRELATION GENE NETWORKS PREDICT FUNCTIONAL MODULES IN RESPONSE TO NITRATE

To analyze functional relationships among the 2286 nitrate responsive genes identified above we inferred a network as described previously (Langfelder and Horvath, 2008). In our analysis, 11 coexpression modules were identified and functional analysis indicated 9 out of the 11 modules had overrepresented biological functions. Interestingly, the gene network modules identified include robust functions of the nitrate response such as ion transport, carbon metabolism, response to chemical stimulus and trichoblast differentiation (**Figure 3**). The most consistent biological functions found associated to the nitrate response (**Table 3**) metal ion transport, monocarboxylic acid metabolic process, nitrate transport, glucose catabolic process or regulation of transcription are also overrepresented in these modules. Below we describe in more detail gene network modules 1, 6, 7, 8, and 9 containing these functions.

Module 1 is the largest module in the nitrate-responsive gene coexpression network, in terms of number of genes and number of connections. The top categories in this module are anion transport (GO:0006820, p-value $= 2.60 \times 10^{-18}$) and response to nitrate (GO:0010167, p-value $= 7.75 \times 10^{-17}$). Other biological

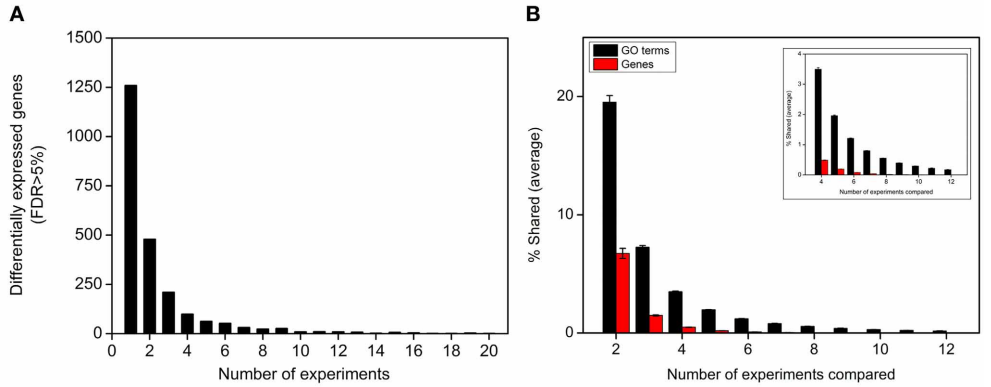

FIGURE 2 | Biological functions are more robust than gene identities in the nitrate response. (A) Histogram of 2286 significantly nitrate responsive genes (rankproduct, FDR < 5%) vs. the number of experiments in which were regulated. **(B)** Histogram of the average percentage of elements (over-represented GO terms or genes) shared between different number of experiments. Inset shows the same graph but starting from 4 experiments.

functions involved in the nitrate response were also overrepresented, such as signaling (GO:0023052, p-value $= 1.42 \times 10^{-7}$) and regulation of transcription (GO:0006355, p-value $= 8.54 \times 10^{-6}$). Within the latter function, we found several families of transcription factors that were enriched, such as the G2-like or LBD discussed above.

Because hub genes play key functional roles in gene networks (Zotenko et al., 2008; Vidal et al., 2013), we identified those genes with the highest number of connections (degree) within each network module. The gene with highest degree in module 1 is an unknown HPP family protein (AT3G47980, pfam04982). HPP proteins are integral membrane proteins with four transmembrane helices. The identified protein has a predicted size of 27 kDa and a high pI (10.7) and based on his signal peptide sequence it is likely localized in the plastidic membrane. Another HPP family member (AT5G62720) has been identified in chloroplastidic membrane fractions by mass spectrometry (Ferro et al., 2010) and is also induced in response to nitrate. Using the eFP Browser (Winter et al., 2007), we found that the *HPP* genes are expressed in a tissue-specific manner (**Figure S3**). AT5G62720 expression is most abundant in photosynthetic tissues, while AT3G47980 is preferentially expressed in the roots suggesting the physiological roles of these two proteins may be different. Further research will be required to elucidate the role of HPP proteins in plants and their putative role in nitrate responses. However, because module 1 is enriched in transport functions, it is likely that highly connected genes such as the HPP genes identified may play a role in intracellular transport in the context of the nitrate response.

Enriched biological functions in module 7 are ion transport (GO:0006820, p-value $= 1.73 \times 10^{-3}$) and response to nitrate (GO:0010167, p-value $= 2.46 \times 10^{-3}$). These biological functions are also among the most enriched in gene network module 8. Genes of the CIPK family are among the most connected genes in both modules. CIPKs are Ser/Thr protein kinases that interact with calcineurin B-like calcium sensors and are involved environmental stress responses and nutrient sensing (Luan et al., 2009). Specifically, *CIPK3* is the second most connected gene in module 8 and *CIPK8* is the third most connected gene in module 7. *CIPK8* plays a role in regulation of gene expression of primary nitrate response genes (Hu et al., 2009). On the other hand, *CIPK3* has been widely analyzed in several experimental contexts, demonstrating its importance in plant development and adaptation to stress (Kim et al., 2003). However, its role in nitrate response has not yet been addressed. Interestingly, several protein phosphatases are also present in these modules, including PP2C (AT5G27930, AT5G26010, AT5G26010, AT3G16560, AT1G67820), dual phosphatase *ATPFA-DSP1* (AT1G05000), *AtMTM2* (AT5G04540), and *PP2A* (AT5G03470). With respect to the PP2A family, Heidari et al. (2011) showed that PP2A is required for the activation of nitrate reductase (NR). These results suggest the new kinases and protein phosphatases identified here may be important in phosphoproteome homeostasis for signaling and control of nitrogen responses in *Arabidopsis* roots.

Module 9 contains many overrepresented biological functions related to metabolism such as glycolysis (GO:0006096, p-value $= 6.21 \times 10^{-9}$), carboxylic acid metabolic process (GO:0019752, p-value $= 1.12 \times 10^{-4}$), hexose biosynthetic process (GO:0019319, p-value $= 2.62 \times 10^{-3}$), pyruvate metabolic process (GO:0006090, p-value $= 5.85 \times 10^{-3}$) and cellular nitrogen compound metabolic process (GO:0034641, p-value $= 1.23 \times 10^{-3}$). In addition, among the most connected genes we find several genes related to ammonium assimilation, such as glutamine synthetase (AT5G35630), glutamate synthase (AT5G53460), and isocitrate dehydrogenase (AT4G35260).

Finally, module 6 is a particularly interesting module in the context of nitrate responses because it is enriched in genes associated with trichoblast differentiation. Several genes within this module are essential for root hair development. One example is *Arabidopsis EXPANSIN 7* (*AtEXPA7)* gene, which is expressed specifically in root hairs (Lin et al., 2011a,b; Lan et al., 2013). The reduction of mRNA levels of *AtEXPA7* significantly affects root hair length (Lin et al., 2011a,b). Furthermore, it has been

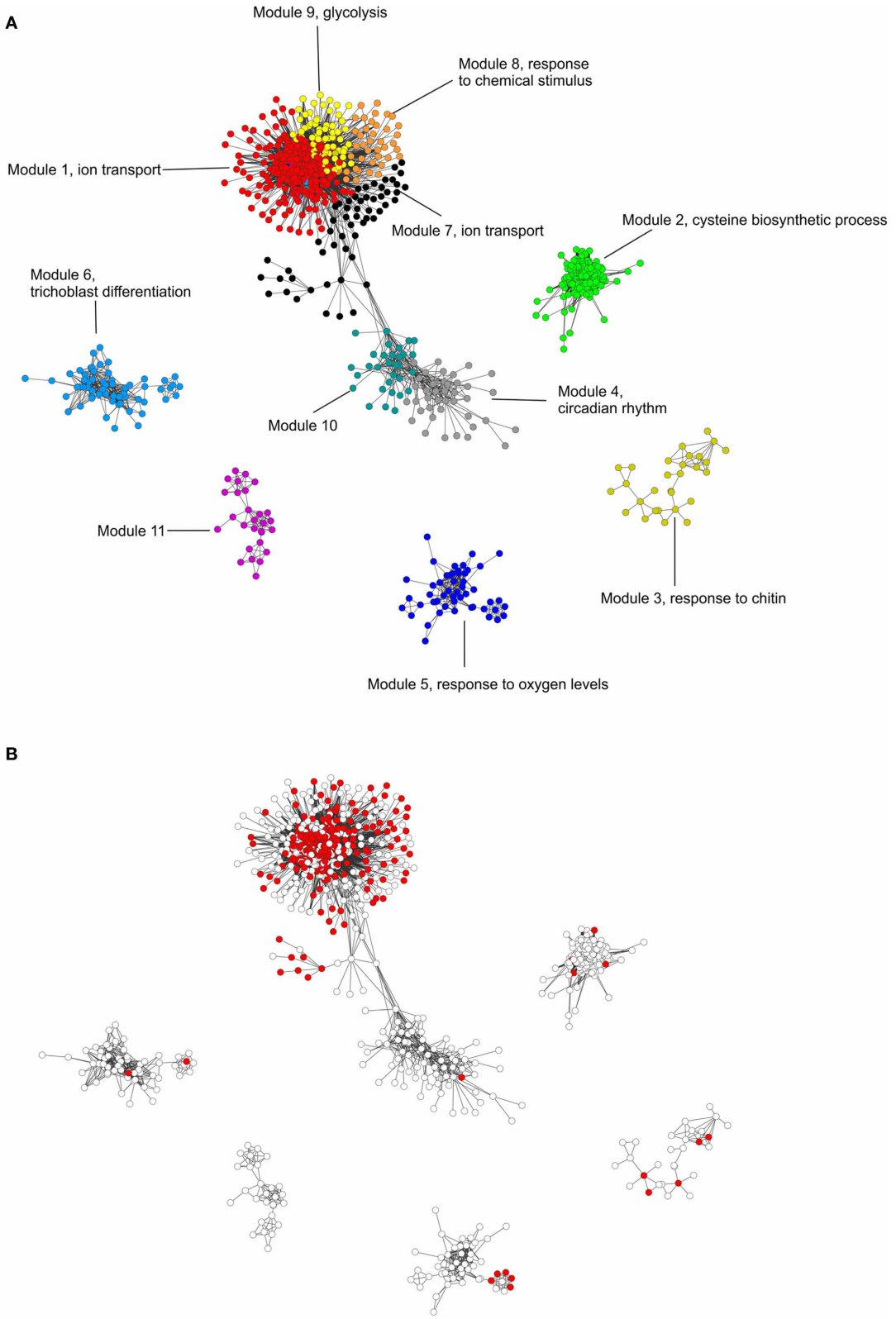

FIGURE 3 | Nitrate-responsive gene coexpression network. (A) Colors are used to distinguish each gene network module. The most over-represented and consistent biological process GO terms (appear in at least 14 different experiments) are indicated in each module (**File S2**). **(B)** Red nodes indicate genes that respond similarly to nitrate treatments in NR-null mutants and wild-type plants indicating they respond directly to a nitrate signal (Wang et al., 2004). X and Y axes does not represent any particular scale (**Table S3**).

Table 3 | Ranking of the most consistent biological functions in the nitrate response.

GO	Description	Number of experiments
GO:0000041	Transition metal ion transport	24
GO:0032787	Monocarboxylic acid metabolic process	23
GO:0015706	Nitrate transport	22
GO:0006007	Glucose catabolic process	21
GO:0006355	Regulation of transcription, DNA-dependent	21
GO:0019375	Galactolipid biosynthetic process	20
GO:0006569	Tryptophan catabolic process	19
GO:0006612	Protein targeting to membrane	19
GO:0006739	NADP metabolic process	19
GO:0009744	Response to sucrose stimulus	18
GO:0019344	Cysteine biosynthetic process	18
GO:0000165	MAPK cascade	17
GO:0010054	Trichoblast differentiation	16
GO:0016567	Protein ubiquitination	16
GO:0045893	Positive regulation of transcription, DNA-dependent	16
GO:0051973	Positive regulation of telomerase activity	16
GO:0006090	Pyruvate metabolic process	15
GO:0006499	N-terminal protein myristoylation	15
GO:0009694	Jasmonic acid metabolic process	15
GO:0043288	Apocarotenoid metabolic process	15
GO:0045892	Negative regulation of transcription, DNA-dependent	15
GO:0009687	Abscisic acid metabolic process	14
GO:0043623	Cellular protein complex assembly	14
GO:0051761	Sesquiterpene metabolic process	14
GO:0055080	Cation homeostasis	14

GO terms with high semantic value (levels 7 and 8) and represented among genes regulated in more than half of the experiments analyzed are shown.

shown that this gene is able to complement a mutation in the rice *OsEXPA17* gene, suggesting functional conservation of root hair expansins in monocots and dicots (ZhiMing et al., 2011). Module 6 also includes the *AtXET14* gene, which encodes a xyloglucan endotransglucosylase enzyme implicated in root hair development (Maris et al., 2009). Adding purified recombinant *AtXET14* protein to MS medium for 2 days decreases growth of initiated root hairs and reduced the root elongation zone. In addition, *MRH6* and *COBL9* genes were also found in this module. These two genes were identified in a screening for root hair morphology mutants (Jones et al., 2006). Finally, peroxidase and extensin genes were over-represented in this module. Hydrogen peroxide is involved in root hair development (Dunand et al., 2007) and peroxidases are proposed candidate genes involved in this developmental process (Kwasniewski et al., 2013).

Recently, an R2R3-MYB transcription factor was found to control development of root hairs in *Arabidopsis* (Slabaugh et al., 2011). We found five members of R2R3-MYB family (AT1G48000, AT5G14340, AT3G06490, AT5G60890, AT1G73410) within module 6. These genes belong to the only transcription factor family over-represented in the trichoblast differentiation network module, representing attractive candidate regulatory factors for root hair development in response to nitrate treatments in *Arabidopsis*. **Figure 4** integrates these findings and proposes a simplified model for how nitrate may modulate root hair development. Nitrate is able to regulate expression of auxin biosynthesis genes. Auxin promotes initiation and growth of root hairs (Muday et al., 2012). It has been demonstrated that a significant amount of auxin is synthesized in the roots (Ljung et al., 2005), specifically in the apical region and *CYP79B2* and *CYP79B3* genes are involved in this localized auxin synthesis. Consistent with this model, we found nitrate induces expression of *CYP79B3* in 4 independent experiments (Gifford et al., 2008; Ruffel et al., 2011; Vidal et al., 2013 Álvarez et al., submitted) and *CYP79B2* in two different microarray experiments (Gifford et al., 2008; Vidal et al., 2013). Based on coexpression network analysis, we propose that these morphogenic changes would be mediated by cell wall proteins as extensins, expansins and peroxidases, which could be regulated by R2R3-MYB transcription factors (**Figure 4B**).

NITRATE SIGNALS AND NOT DOWNSTREAM PRODUCTS OF NITRATE REDUCTION REGULATE GENES FOUND MAINLY IN MODULES 1, 7, 8, AND 9

Analysis of a NR-null mutant has shown that nitrate serves as a signal to control the expression of many genes in *Arabidopsis* (Wang et al., 2004). In order to distinguish nitrate-regulated modules vs. modules controlled by other N forms produced by nitrate reduction and assimilation, we integrated NR-null mutant transcriptome data (Wang et al., 2004) with our network analysis. As shown in **Figure 3B**, most of the nitrate-regulated genes are concentrated in central modules 1, 7, 8, and 9. Based on these results, transport, metabolism, and signaling biological functions represented in these network modules are robustly controlled by a nitrate signal. These results also suggest that biological functions such as circadian rhythms (module 4), response to oxygen levels (module 5), and trichoblast differentiation (module 6) are regulated by products of nitrate reduction. These results are consistent with previous findings such as the case of the master clock gene *CCA1* previously found to be regulated by organic nitrogen signals (Gutiérrez et al., 2008).

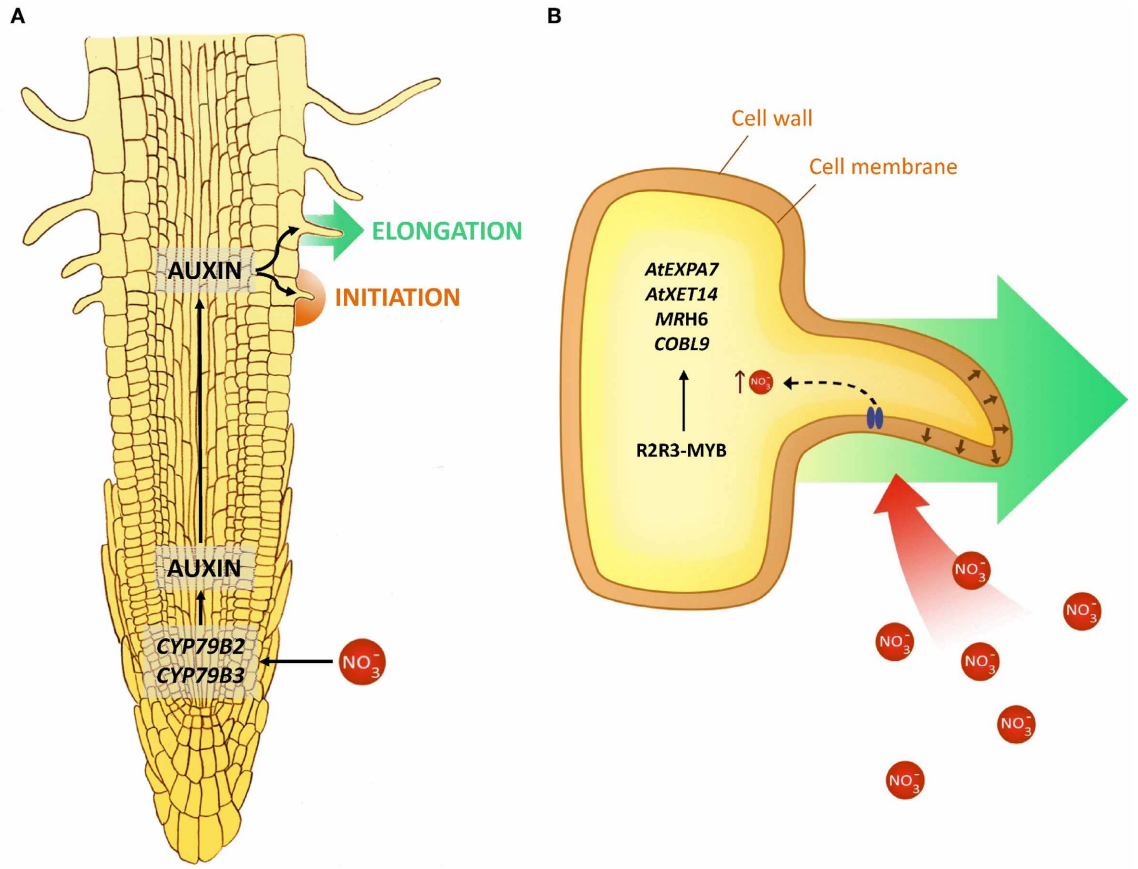

FIGURE 4 | Proposed model of how nitrate modulates root hair development in *Arabidopsis*. (A) Nitrate treatments induce auxin biosynthesis and this phytohormone promotes root hair initiation and elongation. **(B)** Schematic detail of a root hair and genes involved in their growth mediated by nitrate.

NETWORK ANALYSIS PREDICTS CENTRAL NITRATE RESPONSE MODULES ARE CONTROLLED BY bZIP AND Myb TRANSCRIPTION FACTORS

To identify transcription factors that control essential and robust functions in the root nitrate response such as nitrate transport and assimilation, we focused in transcription factors from modules 1, 7, 8, and 9 and their possible targets. **Figure 5** shows the subnetwork with edges between transcription factors and their putative targets taking into account the over-represented binding sites for the transcription factor in the promoter region of corresponding target genes using VirtualPlant software (Katari et al., 2010). In this network, MYB-related (AT5G58900) and bZIP (AT5G65210) genes showed the highest degree. Three different G2-like transcription factors (AT1G68670, AT1G25550, AT1G13300) were also found in top positions of the ranking of transcription factors with higher degree. As shown in **Figure 5B**, MYB-related gene coexpressed with nitrite reductase, 2-oxoglutarate/malate chloroplast transporter and a 6-phosphogluconate dehydrogenase gene from the oxidative pentose phosphate pathway. These results suggest this MYB-related factor controls basic aspects of nitrate metabolism, such as nitrate reduction, GS/GOGAT cycle and the generation of reducing equivalents.

A bZIP transcription factor identified as potential network driver (*TGA1*) belong to the subfamily TGA and another member of this family (*TGA4*) occupied the fifth position in the ranking of transcription factors with higher degree. These transcription factors have been implicated in bacterial defense responses. *tga1/tga4* double mutant plants show a greater susceptibility to infection by *Pseudomonas syringae* (Shearer et al., 2012). However, our analysis suggests that these transcription factors (*TGA1*, *TGA4*) could be important in the nitrate response of *Arabidopsis* roots. *TGA1* is the second gene of this subnetwork in terms of number of connections and it is differentially expressed in 9 different microarray experiments. *TGA1* is highly coexpressed with metabolic genes such as urophorphyrin methylase 1 and phosphoglucose isomerase 1. In fact, the biological function overrepresented with a lower *p*-value among the possible targets of *TGA1* is primary metabolic process (GO:0044238, p-value $= 0.002$). We have recently validated this hypothesis, demonstrating *TGA1* and *TGA4* transcription factors are important regulatory factors of the root response to nitrate treatments in *Arabidopsis thaliana* (Álvarez et al., submitted). Another transcription factor of the bZIP family implicated in N-responses is *bZIP11*. bZIP11 has been shown to regulate asparagine synthetase 1 and proline dehydrogenase 2 in *Arabidopsis* (Hanson et al., 2011).

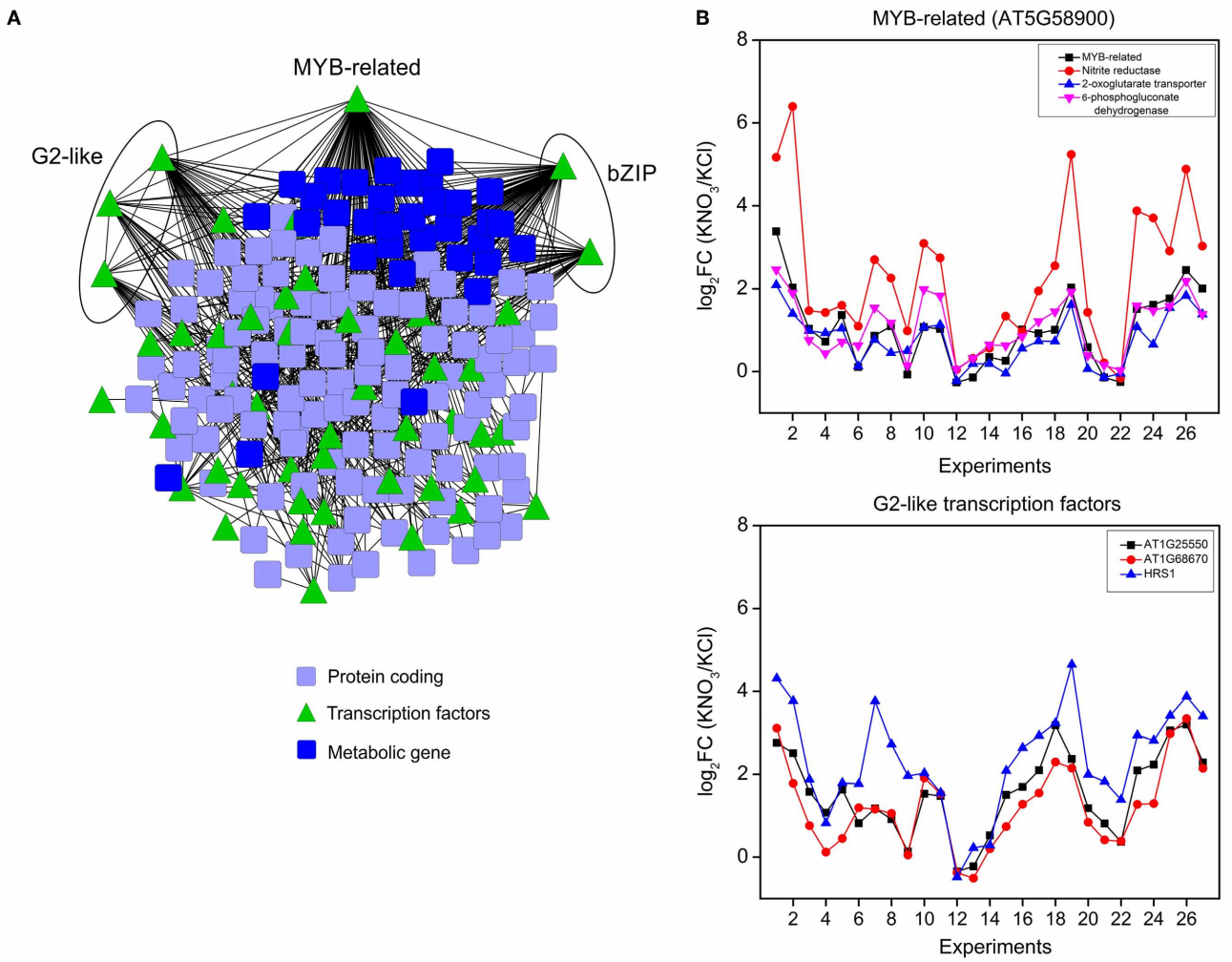

FIGURE 5 | Transcriptional control of the central modules in the nitrate gene network. (A) A subnetwork of transcription factors and their potential targets derived from modules 1, 7, 8, and 9. Edges represent predicted regulatory interactions between transcription factors and target genes based on overrepresented transcription factors binding sites on the promoter of the targets. The most connected transcription factors are highlighted. **(B)** Expression patters of *MYB*-related transcription factor together with their three most correlated targets. Expression profiles of the three G2-like transcription factors are shown below.

G2-like transcription factors are members of the GARP superfamily and are characterized by a conserved domain (GARP) that is a single Myb-related DNA-binding domain (Sawaki et al., 2013). It is interesting to note that there are several connections between the three members of the G2-like transcription factors family, suggesting they respond to nitrate treatments in a coordinated fashion (**Figure 5A**). AT1G68670 is the G2-like transcription factor with the higher degree in this subnetwork and is coexpressed with other transcription factors such as another G2-like (**Figure 5B**), *ZINC FINGER PROTEIN 4* (AT1G66140) and MYB-related (AT5G58900). Moreover, because several protein kinases such as *MAPKKK16*, *WNK7*, and *CIPK1* are also present in this regulatory network, we hypothesize G2-like transcription factors are key regulatory genes involved in nitrate signaling leading to metabolic and developmental responses in *Arabidopsis* roots.

CONCLUSIONS

Integrated network analysis of transcriptome data provided novel hypothesis about functions and regulatory mechanisms by which *Arabidopsis* plants respond to nitrate. Our meta-analysis better assessed the nitrate functional space than any single or integrated transcriptome study previously published. We estimated the mean functional coverage of any single experiment at about 31%. This result highlights the need for integrated data analysis to better map the functional space for any given perturbation. Moreover it underscores the need for using experiments carried out under non-redundant environmental conditions.

Our Systems approach identified nitrate regulation of root hairs as an important component of the plant developmental response to changes in N nutrition, a yet unexplored research area at the intersection of N nutrition and root biology. We provided concrete hypothesis for genes and connections among genes

related to root hair differentiation in response to nitrate that have not been previously highlighted nor addressed experimentally. Our results also highlight the role of bZIP and G2-like transcription factors for regulation of important functions related to nitrate transport and signaling. G2-like transcription factors have not been characterized in the context of nitrate responses. Functional studies of these new candidate genes should help better understand regulatory mechanisms underlying root nitrate responses in *Arabidopsis* and other plants.

ACKNOWLEDGMENTS

This work was funded by Fondo Nacional de Desarrollo Científico y Tecnológico (1100698), the International Early Career Scientist program from the Howard Hughes Medical Institute (Award 55007421), Fondo de Desarrollo de Areas Prioritarias Center for Genome Regulation (Grant 1509007), Millennium Nucleus Center for Plant Functional Genomics (Grant P10-062-F). Javier Canales is supported by Fondo de Desarrollo Científico y Tecnológico postdoctoral fellowship (Grant 3130315). We would like to thank Dr. Elena Vidal for constructive discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00022/abstract

Figure S1 | Controls performed to evaluate GO term robustness. (A) Distribution of total and shared over-represented GO term levels between any combination of two experiments. To generate this graph we analyzed all possible combinations of two experiments. Level 1 is the most general GO term category and level 11 the most specific. The x-axis corresponds to the depth of a concept while the y-axis shows average number of GO terms for a given level. **(B)** Number of GO associated with each 2286 nitrate responsive genes or with 2286 randomly selected genes (average of 1000 iterations).

Figure S2 | Histogram of the percentage of genes associated with the shared GO terms between any combination of two experiments.

Figure S3 | Gene expression patterns of HPP genes (AT3G47980 and AT5G62720) according to the *Arabidopsis* eFP browser.

File S1 | Cytoscape file of the nitrate-responsive gene coexpression network.

File S2 | Cytoscape file of the subnetwork present in Figure 5A.

Table S1 | List of 2286 nitrate-responsive genes.

Table S2 | Biological processes that are statistically enriched in response to nitrate.

Table S3 | List of genes that were commonly expressed in nitrate reductase-null mutant and wild type.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 November 2013; accepted: 20 January 2014; published online: 07 February 2014.

*Citation: Canales J, Moyano TC, Villarroel E and Gutiérrez RA (2014) Systems analysis of transcriptome data provides new hypotheses about Arabidopsis root response to nitrate treatments. Front. Plant Sci. **5**:22. doi: 10.3389/fpls.2014.00022*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 02 October 2013
doi: 10.3389/fpls.2013.00354

Root apex transition zone as oscillatory zone

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Root apex of higher plants shows very high sensitivity to environmental stimuli. The root cap acts as the most prominent plant sensory organ; sensing diverse physical parameters such as gravity, light, humidity, oxygen, and critical inorganic nutrients. However, the motoric responses to these stimuli are accomplished in the elongation region. This spatial discrepancy was solved when we have discovered and characterized the transition zone which is interpolated between the apical meristem and the subapical elongation zone. Cells of this zone are very active in the cytoskeletal rearrangements, endocytosis and endocytic vesicle recycling, as well as in electric activities. Here we discuss the oscillatory nature of the transition zone which, together with several other features of this zone, suggest that it acts as some kind of command center. In accordance with the early proposal of Charles and Francis Darwin, cells of this root zone receive sensory information from the root cap and instruct the motoric responses of cells in the elongation zone.

Keywords: plant roots, plant sensory biology, plant electrophysiology, plant polarity, plant morphogenesis, plant cytoskeleton, plant communication, auxin, neurotransmitter agents

TRANSITION ZONE AS INITIATOR OF ROOT TROPISMS, CELL POLARITY, AND CELL FATE SWITCHES

Since pioneering experiments of Charles and Francis Darwin, published in their book more than 150 years ago, it is known that growing root apices are actively behaving, exploring their environment and searching for water and nutrients. Their root caps are acting as sensory organs transmitting sensory information into motoric subapical root zones (Darwin, 1880). However, as root caps are relatively far away from the elongation region, which drives the differential cell/root growth for the root tropisms, it was not obvious how the sensory root cap effectively controls root growth directions. In 1990, we revealed that a large zone, comparable in size to the meristem, is located between the apical meristem and basal elongation region (Baluška et al., 1990). Later, we reported that microtubules and F-actin filaments accomplish complex and extensive re-arrangements in cells of the transition zone (Baluška et al., 1992, 1997). In addition, it turned out that the basal border of the transition zone is very flexible and it can be regulated very sensitively by gravity. In particular, cells at the upper part of gravistimulated maize roots, especially the outer cortex cells which accomplish the basipetal (shootward) transport of auxin (Kollmeier et al., 2000), enter the rapid elongation zone sooner (closer to the root tip) as they would do if not placed into the horizontal position (Baluška et al., 1993a,b, 1996a). This results in immediate speeding-up of the root surface extension rate at the upper root part, causing roots to bend downward (along the gravity vector). This finding has twofold impacts. Firstly, it indicates that root gravitropism is not initiated, as widely believed, only by differential elongation of cells but also by differential release of transition zone cells into the region of rapid cell elongation (Baluška et al., 1993a,b, 1996a,b). Secondly, the transition zone bending starts almost immediately after the gravistimulation, suggesting that it performs sensory processes and effective sensory-motoric integration (Mancuso et al., 2006; Baluška et al., 2010a; Baluška and Volkmann, 2011). Roots exposed to exogenous auxin, which stops growth completely, are still able to initiate and develop gravitropic curvature (Ishikawa and Evans, 1993; Ottenschläger et al., 2003; Yamamoto, 2003). As the basal (rootward) border of the transition zone fluctuates between diverse root sides or cell files (Baluška et al., 1996b; Ivanov and Dubrovsky, 2013), it emerges that the transition zone cells have flexible fates. At its both the apical and basal transition zone borders, either entering it or leaving it, cells are under tight control of diverse endogenous or exogenous factors, rendering the transition zone developmental buffering capacity (Baluška et al., 2010a; Ivanov and Dubrovsky, 2013). Actually, as we are discussing it below, cellular polarities and fates can switch dramatically in this developmentally flexible root apex zone.

TRANSITION ZONE AS INITIATOR OF ROOT HAIRS AND ORGAN MORPHOGENESIS

Epidermis and pericycle cells represent morphogenetic root tissues as they can, under proper induction, accomplish either root hair formation or lateral root primordial formation. Both root hairs and lateral roots are specified at the basal (shootward) border of the transition zone. The competence for either the root hair initiation or lateral root primordium initiation is present in within this narrow developmental window of root cell fate history. Both, developmental and ecological contexts determine how many roots hairs or lateral roots are generated in particular primary root (Osmont et al., 2007; Péret et al., 2011; Jones and Ljung, 2012). *Arabidopsis* roots, similarly as roots of other Brassicaceae species, are different with respect of root hairs because they are generated irrespective of this ecological context, strictly in the trichoblast cell files. Unique aspect of lateral root primordial formation is that new organs are initiated endogenously, deeply within the root tissues

(Sablowski, 2004). All other important morphogenesis processes in plants are initiated and accomplished in the epidermis layer. However, roots of *Arabidopsis*, and other Brassicaceae, also do not enter into symbiosis with mycorrhizal fungi. Interestingly, symbiotic interfaces between fungal cells and root cells are developed in the inner cortex cells, close to the pericycle of the transition zone. Endodermis as the peripheral tissue of the stele protects against symbiotic fungi and bacteria, but not against haustorial roots of parasitic plants (Tomilov et al., 2005). Roots of some plants enjoy symbiosis with nitrogen-fixing Rhizobia bacteria when bacteria-harboring nodules resemble lateral root primordia, and initiate their development close to this root apex zone. Finally, also parasitic roots target the transition zone of host roots for their invasion (Tomilov et al., 2005).

Our study of root hair initiation in maize root epidermis revealed, that the very early step, known as bulge formation, is accomplished by local modification of cell wall, especially recruitment and activation of expansions (Baluška et al., 2000a,b). This local weakening of the cell periphery complex results in bulging out of the outer cell epidermis portion, switching the cell polarity locally, and subsequent assembly of the F-actin-based tip growth machinery, which drives the root hair formation. Similarly as with the differential root surface extension of gravistimulated roots, also root hair formation can be accomplished even when microtubules are depolymerized (Baluška et al., 1996a, 2000a). Activated tip growth machinery recruits secretory vesicles which, in addition to the Golgi-derived vesicles, rely heavily on endosomes and endocytic recycling vesicles motility of which is under F-actin control (Voigt et al., 2005a). In this respect, the root hair tip zone resembles closely the synaptic recycling domains (Baluška et al., 2005a), with the only difference being that endocytosis balances exocytosis at the root synapses, so that there is no growth or any obvious cell periphery expansion. Nevertheless, it cannot be excluded that the synaptic domains are rhythmically expanding and shrinking, as exocytosis and endocytosis processes are not tightly coordinated. Interestingly in this respect, the tip-growth domains perform inherent oscillations in root hairs and pollen tubes (Feijó et al., 2001; Monshausen et al., 2007; Rounds and Bezanilla, 2013). Furthermore, endocytic vesicle recycling is important for repair of the *stressed* plasma membranes (Baluška and Wan, 2012), as well as for the evolutionary origin of action potentials (Goldsworthy, 1982) which have a potential to protect the integrity of stressed plasma membrane (Baluška and Wan, 2012). Intriguingly, peaks of proton influxes are associated with alkalinization at the clear zone of tip-growing pollen tubes (Feijó et al., 1999), as well as with the unique status of the root apex transition zone (Collings et al., 1992; Baluška and Mancuso, 2013a). Could it be that the evolutionary origin of the transition zone is related to the stress biology of plant roots and their very symbiotic nature? Indeed, land plant evolution has been driven by symbiotic interactions of the earliest land plants with fungi and bacteria (Jorgensen, 1993; Simon et al., 1993; Wang et al., 2010; Sanders et al., 2011; Baluška and Mancuso, 2013b). Evolving plants, and especially their roots, invented very high complexity of proteins of the PIN, PILS, and ABC transporter families (Cho and Cho, 2012; Feraru et al., 2012; Viaene et al., 2013). This allowed evolution of root apex sensory-motoric circuits driving complex architecture and behavior of root systems (Baluška et al., 2009a,b; Baluška, 2012a; Burbach et al., 2012; Wan et al., 2012) exploring effectively heterogenous soil patches (Hodge, 2009; Monshausen and Gilroy, 2009; Trewavas, 2009). Especially the shootward PIN2 transporter and non-genomic auxin receptor ABP1 emerge to be essential for the evolution of plant synapses and root apex transition zone (Baluška et al., 2009a, 2010a; Baluška, 2012a; Baluška and Mancuso, 2013b).

TRANSITION ZONE AS OSCILLATORY ZONE: FROM TRANSPORT OSCILLATIONS AT MEMBRANES, VIA DYNAMIC AND INTEGRATED CYTOSKELETON, TO OSCILLATING GENE EXPRESSION IN NUCLEUS

Recent addition to the unique properties of cells in the transition zone is very prominent ion transport activities. Particularly striking is the fact that ion fluxes shows not only peaks at the transition zone, but also that these show highly oscillating nature specifically in this root zone, feature which is missing in all other root zones (Shabala et al., 1997, 2006; Shabala and Knowles, 2002; Shabala, 2003; McLamore et al., 2010a,b). Moreover, besides ion fluxes, also other transport processes show peaks of their activities and oscillate specifically in the transition zone. Here we can mention oxygen influx and polar auxin fluxes (Mancuso and Boselli, 2002; Mancuso et al., 2005, 2007; McLamore et al., 2010a,b), and nitric oxide (NO) emissions (Illéš et al., 2006; Mugnai et al., 2012). As almost all plasma membrane processes investigated in the transition zone show oscillations, it can be proposed that the primary plasma membrane generated oscillations (**Figure 1**) are transferred, via dynamic cytoskeleton, up to the nucleus which is still centrally localized in cells of the transition zone (**Figures 2** and **3**). This central position of the nucleus is actively maintained via cytoskeletal

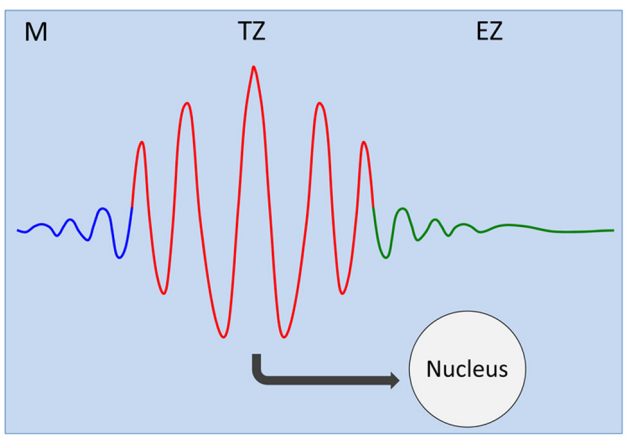

FIGURE 1 | Transition zone as oscillatory zone. Oscillations of the plasma membrane electrical potentials generate, via multiple feedback loops with membrane associated cytoskeleton, self-sustaining and self-regulating cellular oscillator (Shabala et al., 1997, 2006; Mancuso and Boselli, 2002; Shabala, 2003; Mancuso et al., 2005, 2007; McLamore et al., 2010a,b; Mugnai et al., 2012). Oscillatory patterns of ion and auxin fluxes at the plasma membrane feed into the oscillations of gene expression in the nucleus (Moreno-Risueno et al., 2010, 2012; Traas and Vernoux, 2010; Moreno-Risueno and Benfey, 2011). M, meristem; TZ, transition zone; EZ, elongation zone.

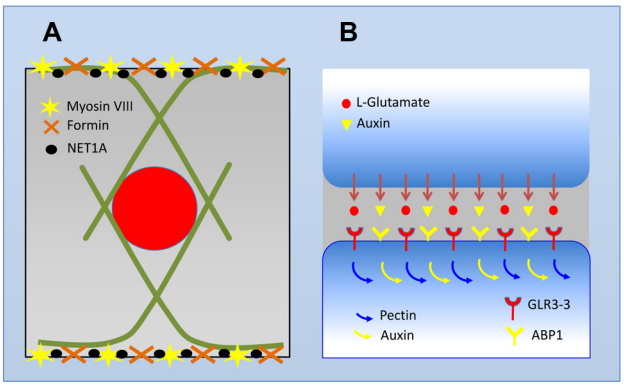

FIGURE 2 | Schematic view of the transition zone cell and its auxin-secreting synapse. (A) Cells in the transition zone maintain their nuclei in the central position. Instead of fine F-actin networks typical for meristematic cells, these cells develop prominent bundles of F-actin which enclose the nucleus in a cage-like structures. Inhibition of endocytic vesicle recycling with brefeldin A results in a disintegration of this F-actin cage and nuclei are shifted out of their central position (Baluška and Hlavacka, 2005). At the plasma membrane, F-actin bundles are anchored at F-actin rich end-poles which are active in endocytosis/endocytic vesicle reycling. Anchoring of F-actin bundles at the plasma membrane and support of dense F-actin meshorks at these domains is accomplished via myosin VIII, group Ie formins, and NET1A actin-binding protein (Baluška et al., 1997, 2000c, 2002, 2009a; Deeks et al., 2005, 2012; Baluška, 2012a). **(B)** Abundant myosin VIII, dense meshworks of F-actin, and very active endocytosis of cell wall pectins crosslinked with boron and calcium allow tight synaptic cell–cell adhesion. At these adhesive and polar cell domains, cells secrete auxin out cells via the endocytic vesicle recycling of pectins and numerous plasma membrane proteins including putative auxin transporters of the PIN family (Baluška et al., 2002, 2008; Mancuso et al., 2005, 2007). Auxin is peceived at the adjacent cells via its plasma membrane/cell periphery receptor ABP1. Glutamate is proposed also to be secreted out of plant cells and glutamate receptor-like protein GLR3.3 is enriched at these synaptic domains (see Figure 1C in Vincill et al., 2013).

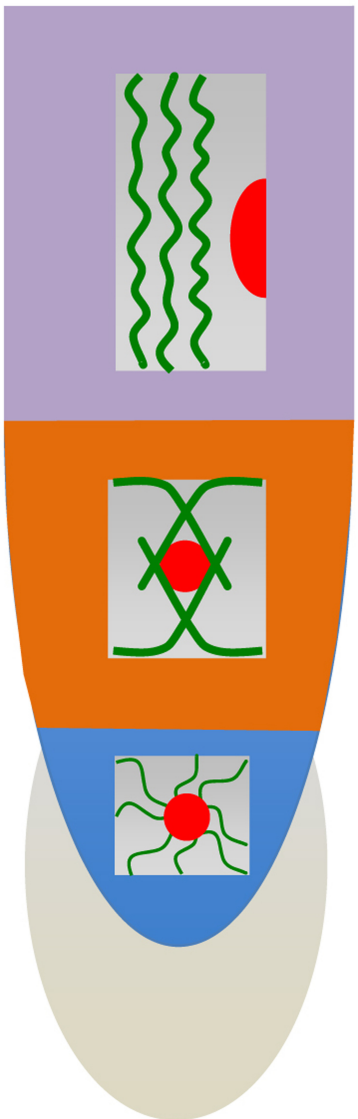

FIGURE 3 | Schematic views of cellular architecture in meristem, transition zone and elongation region. Cells in the meristem are characterized with centrally positioned nuclei suspended in networks of F-actin and radial arrays of perinuclear microtubules (Baluška et al., 1992, 1997, 2000c; Voigt et al., 2005b). In the transition zone, nuclei still keep their central position, but fine F-actin networks are replaced by bundles of F-actin organized via the nuclear surface and the end-poles enriched with myosin VIII (Baluška et al., 1997, 2000c; Reichelt et al., 1999). In the elongation region, cells start to elongate very rapidly and develop their central vacuole which is pushing their nuclei toward the side walls. F-actin bundles obtain longitudinal and wrinkled/loosened appearances (Baluška et al., 1997, 2000c; Voigt et al., 2005b).

connections of the nuclear surface to the plasma membrane, and is important for its information processing roles (Baluška et al., 1998, 2000b, 2004a,b, 2006a). Synchronizations are primarily generated at the plasma membrane (**Figure 1**), and these are then perlocated, via dynamic cytoskeletal elements, up to the centrally positioned nuclei (**Figures 2** and **3**). Importantly, oscillating gene expression waves define the prebranch sites of future lateral root primordia (Moreno-Risueno et al., 2010, 2012; Traas and Vernoux, 2010; Moreno-Risueno and Benfey, 2011). In this respect, the transition zone resembles the segmentation clock of vertebrate embryos. Not only that new root primordia are formed deeply within root tissues, in a stark contrast to other examples of plant organogenesis, but they also show other striking parallels to developing animals (Traas and Vernoux, 2010). Importantly in this respect, whereas the shoot organogenesis is hard-wired and accomplished at the shoot periphery in a spiral-like patterns, the root organogenesis is soft-wired (sensitive to sensory information) and accomplished endogenously in internal tissues. Root primordia are initiated in the transition zone (De Rybel et al., 2010) as probabilistic events whose frequency is based on auxin transport (Laskowski, 2013). As we are discussing it later, the oscillating gene expression pattern in nuclei of the transition zone cells is emerging to be closely linked with the oscillations in synaptic and electric activities (**Figures 3** and **4**) of the plasma membrane and associated recycling vesicles.

With respect of the unique endogenous origin of lateral root primordia, it is very interesting that root apex stem cells are also localized deeper within root apex tissues. Moreover, genes maintaining root stem cells specify also the root identity in the early embryo (Sablowski, 2004), and there are two asymmetric

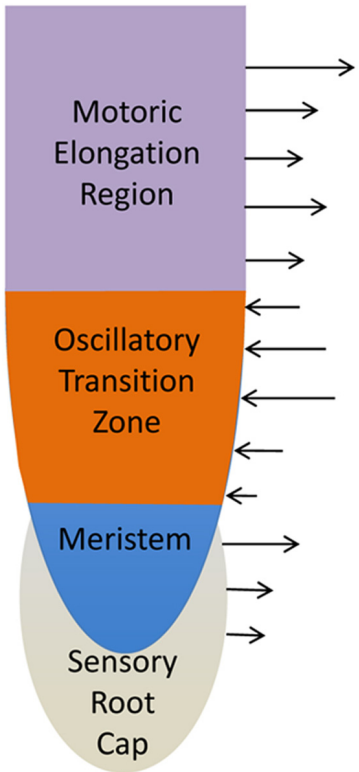

FIGURE 4 | Schematic views of cellular root apex zones and their electric fields. Sensory root cap is enclosing the meristem with dividing cells. These two most apical zones are characterized by the outward electric current (Collings et al., 1992; Baluška and Mancuso, 2013a). Adjacent transition zone is characterized with two inversions of the electric current pattern at the root apex. The apical border of the transition zone accomplishes the outward–inward switch whereas the basal border the inward–outward switch (Collings et al., 1992; Baluška and Mancuso, 2013a). The elongation region is characterized again by the outward electric current. High root synaptic activities at the transition zone are linked with the prominent inward electric currents.

formative cell divisions which underly generation of root body from its stem cells (Scheres, 2007). Intriguingly, transcription factor SHORT-ROOT (SHR) is crucial in this respect as it moves, in association with endosomes and microtubules, from the stele cells into the endodermis and root tip of *Arabidopsis*, where it specifies the endodermal cell identity and stem cell function (Koizumi et al., 2011; Wu and Gallagher, 2012, 2013). Interestingly, root-specific genes and processes underly also regeneration in plants (Sugimoto et al., 2010). Endodermis in the transition zone has unique properties, especially with respect to polar auxin transport (PAT) via PIN3 (Marhavý et al., 2013) and gibberellin signaling (Ubeda-Tomás et al., 2008, 2009; Band et al., 2012; Shani et al., 2013), making this "inner skin-like" tissue (Alassimone et al., 2012; Baluška, 2012b) crucial with respect of the root growth control and the priming of the lateral root primordia initiation (De Smet, 2012; Marhavý et al., 2013). Besides well characterized auxins and cytokinins (Benková and Hejátko, 2009; Vanstraelen and Benková, 2012), especially crucial for the transition zone prove to be gibberellins which control cell growth processes, root apex zonation (Ubeda-Tomás et al., 2008, 2009; Band et al., 2012; Shani et al., 2013), PIN2 stability and the shootward auxin transport (Löfke et al., 2013), as well as the cellular polarity and axiality via transverse organization of cortical microtubules (Baluška et al., 1993a,b; Locascio et al., 2013). Despite the obvious differences in the shoot and root organogenesis, the same PLETHORA transcription factor clade determines patterning of both shoot and root primordia in *Arabidopsis* (Hofhuis et al., 2013). Recently, all cells of the transition zone have been reported to form symplastic domain (Benitez-Alfonso et al., 2013). This high symplastic connectivity is crucial for lateral root patterning, and might be relevant also for the synchronous oscillations characteristic for this root apex zone (**Figure 1**).

OSCILLATING PLANT SYNAPSES: ACTIN–AUXIN OSCILLATOR MEETS AUXIN–PECTIN OSCILLATOR

All root apex cells are polarized along the apical–basal root axis which is inherently linked to the polar cell–cell transport streams of auxin (Baluška et al., 2003b, 2010a). Cortical microtubules determine tubular shape of elongation root cells and F-actin enriched at the non-growing end-poles (Baluška et al., 1997, 2001a), together with endocytic recycling networks, define the identity of apical (rootward) and basal (shootward) recycling domains (Baluška et al., 2003a,b,c, 2010a). F-actin meshworks at the end-poles (**Figure 2A**) are very abundant especially in cells of the transition zone (Baluška et al., 1997, 2009a; Baluška and Hlavacka, 2005) and their abundance correlates closely with the amounts of auxin transported across, and enriched at, these cell–cell adhesion domains (Mancuso et al., 2005, 2007; Schlicht et al., 2006). F-actin is not essential for cell expansion in the transition zone (Baluška et al., 2001b), but it is critical for both endocytosis and endocytic vesicle recycling, which is inherent part of polar cell–cell transport of auxin (Baluška et al., 2003c, 2005a, 2008; Mancuso et al., 2007). Besides abundant F-actin, also plant-specific myosin VIII (Reichelt et al., 1999) is an inherent part of this plant-specific synapses (Baluška et al., 2000c, 2001c) specialized for the sensory-mediated auxin secretion (**Figure 2B**). Plant-specific myosin VIII, enriched at plant synapses, is important for the plant endocytosis (Volkmann et al., 2003; Golomb et al., 2008; Sattarzadeh et al., 2008), being the main driver of the plant synaptic activity. Besides myosin VIII, also formins and actin-binding protein NET1A organize F-actin at the plant-specific root synapses (**Figure 2A**) of the transition zone (Baluška and Hlavacka, 2005; Deeks et al., 2005, 2012).

Importantly, F-actin and auxin transport are connected via feedback interactions which are at the core of actin–auxin oscillations (Nick et al., 2009; Nick, 2010; Qiao et al., 2010; Durst et al., 2013). It was a big surprise when we discovered more than 10 years ago that the major cargo of endocytic recycling vesicles are cell wall pectins cross-linked with boron and calcium (Baluška et al., 2002; Yu et al., 2002). The actin–auxin oscillations characterized in tobacco BY-2 cells, organized into single cell files (Nick et al., 2009; Nick, 2010; Qiao et al., 2010; Durst et al., 2013), are apparently present also in the root apices which are composed of numerous independent but integrated cell files which are interconnected, both symplastically and electrically, via numerous cell–cell plasmodesmata (Baluška et al., 2001c, 2003a; Barlow et al., 2004;

Wojtaszek et al., 2004; Šamaj et al., 2006). Similarly as the plant synapses, also these plasmodesmata, and especially their groupings known as pit-fields, are enriched with abundant F-actin/myosin VIII meshworks and active in endocytosis/endocytic vesicle recycling (Baluška et al., 2001c, 2003a, 2004c; Volkmann et al., 2003; Šamaj et al., 2006; Golomb et al., 2008; Sattarzadeh et al., 2008). Auxin is not only enriched at the active synapses (Schlicht et al., 2006), but it is also "trapped" within the recycling vesicles together with pectins and recycling PIN transporters (Šamaj et al., 2004; Schlicht et al., 2006). As inhibition of endocytosis and endocytic vesicle recycling blocks PAT in the root apex transition zone, here the auxin is acting also as plant-specific neurotransmitter (Baluška et al., 2003c, 2005a, 2008; Mancuso et al., 2007). It is secreted via synaptic-like recycling apparatus and elicits very rapid electric responses on the adjacent (postsynaptic) cells (Felle et al., 1991; Rück et al., 1993). Similarly as in neurons, endocytosis at the plant-specific synapses is controlled via the mechanical status of the plasma membrane (Baluška and Wan, 2012). Auxin represents small multifunctional signaling molecule which not only induces electric and chemical signaling cascades but also regulates endocytosis and vesicle trafficking in the post-synaptic cell (Robert et al., 2010; Baluška, 2012a; Chen et al., 2012). Obviously, auxin acts as plant-specific transmitter for effective cell–cell communication and coordination via continuous streams of the PAT conveying context-relevant sensory information (Baluška et al., 2009a,b, 2010a,b; **Figure 5**). Interestingly, similar mechano-chemical feedback regulatory loops between cell wall mechanics, controlled again via cross-linked cell wall pectins and the PAT, determine also organ formation and phyllotactic patterning in shoots (Peaucelle et al., 2011; Braybrook et al., 2012; Palin and Geitmann, 2012; Wolf et al., 2012; Braybrook and Peaucelle, 2013).

SYNAPTIC RECYCLING OF CELL WALL PECTINS AT SYNAPTIC END-POLES: WHEN "OUTSIDE IS INSIDE"

Unique aspect of cell walls in cells of the transition zone is endocytosis and endocytic recycling of calcium/boron cross-linked pectins (Baluška et al., 2002, 2005b; Yu et al., 2002; Dhonukshe et al., 2006). Importantly, this cell wall pectin endocytosis is effective especially at microtubules depleted and F-actin and myosin VIII-enriched synaptic end-poles (Baluška et al., 1997, Baluška et al., 2001a, 2003a,b; Reichelt et al., 1999; Barlow and Baluška, 2000; Volkmann et al., 2003). These drive cell–cell transport of auxin via endocytic vesicle recycling (Baluška et al., 2003a,b,c, 2005a; Mancuso et al., 2005, 2007; Schlicht et al., 2006), resembling closely the synaptic processes of neurons in animals and humans (Baluška et al., 2003a,b,c, 2005a, 2009a,b). Interestingly in this respect, the polar cell–cell auxin transport is directly linked with sensing of environment, as well as for translating these sensory perceptions into sensory-motor circuits with the transition zone (Baluška et al., 2009a,b; Baluška and Volkmann, 2011; Baluška, 2012a; Baluška and Wan, 2012; Baluška and Mancuso, 2013a). In roots, both development and behavior are closely linked with sensory perceptions and sensory-motoric circuits.

It is crucial in this respect to keep in mind that the inside of endocytic vesicles corresponds topologically to the cellular outside (Baluška and Wan, 2012), so the efflux of auxin out of cells at the plasma membrane corresponds to the influx of auxin into endocytic vesicles. As auxin has one of its receptors (ABP1) exposed to the external plasma membrane leaflet (Napier, 1997; Dahlke et al., 2010), and its distribution and activities are sensitive to exogenous auxin (Diekmann et al., 1995; Tromas et al., 2009; Robert et al., 2010; Sauer and Kleine-Vehn, 2011; Shi and Yang, 2011; Xu et al., 2011; Baluška, 2012a; Chen et al., 2012; Lin et al., 2012; Wang et al., 2013), the vesicular secretion of auxin in cells of the transition zone fits well to the neurotransmitter-like concept of auxin (Baluška, 2012a). In strong support of the neurotransmitter and synaptic views of auxin in plants (Baluška et al., 2003c, 2008; Mancuso et al., 2005, 2007), auxin exerts electric responses when added exogenously to plant cells (Felle et al., 1991; Rück et al., 1993) and these electric responses are ABP1-dependent (Rück et al., 1993). Recent advances in our understanding of ABP1 suggest that this ancient protein (Tromas et al., 2009) regulates, besides the electric responses of plant cells to auxin, endocytosis, and endocytic vesicle recycling (in other words, synaptic activities), also Rho GTPase and receptor-like kinases (RLKs) signaling cascades at the plasma membrane of plant cells (Tromas et al., 2009; Robert et al., 2010; Sauer and Kleine-Vehn, 2011; Shi and Yang, 2011; Xu et al., 2011; Baluška, 2012a; Chen et al., 2012; Lin et al., 2012; Wang et al., 2013). This synaptic view of endocytic recycling and auxin cell–cell transport is relevant for both sensory and motor actions of the root apex. In this sensory-motor integration, electric activities emerge to play a central role (Masi et al., 2009).

TRANSITION ZONE AS CENTER OF SUPRACELLULAR SYNAPTIC AND BIOELECTRIC OSCILLATING ACTIVITIES. RELEVANCES FOR THE DARWIN'S "ROOT-BRAIN" HYPOTHESIS

Our recent discoveries revealed that the transition zone, although negligible with respect of cell growth, is the most active zone in the whole root apex with respect of oscillating electric spike activities (Masi et al., 2009), endocytosis-driven vesicle recycling (Mancuso et al., 2005, 2007; Schlicht et al., 2006), and oxygen demands (Mancuso et al., 2000; Mugnai et al., 2012). Electric activity peaks at the transition zone (Collings et al., 1992; Masi et al., 2009; Baluška and Mancuso, 2013a; **Figure 4**), and perhaps this makes this root apex zone also for an attractive target of pathogenic and symbiotic organisms (Miller et al., 1986). Until now, these synaptic and electric activities represent a mystery, but these would be rather expected on the basis of the Darwin "root-brain" hypothesis (Baluška et al., 2009b; Kutschera and Niklas, 2009; Sahi et al., 2012) first postulated by Charles and Francis Darwin more than 150 year ago (Darwin, 1880; Barlow, 2006). Francis and Charles Darwin, despite having rather simple "country-house" experimental conditions (Kutschera and Briggs, 2009) accomplished relevant experiments which clearly documented that root apex behaves as *brain-like* organ, resembling brains of lower animals (Darwin, 1880; Barlow, 2006; Baluška et al., 2009b; Kutschera and Niklas, 2009). But the leading botanists of that time, Julius Sachs (Baluška et al., 2009b; Kutschera and Niklas, 2009) and Wiesner (1881) argued that experiments performed by Francis and Charles Darwin were flawed. Emil Detlefsen, assistant of Julius Sachs, as well as Julius Wiesner with the assistance from Hans Molisch claimed to demolish the fault results of Francis and Charles Darwin that

the root apex acts as sensory organ controlling root tropisms accomplished in remote growth regions (Wiesner, 1881; Detlefsen, 1882). But it turned out that Francis and Charles Darwin were correct and that, in fact, the experiments of Wiesner, Molisch, Detlefsen, and Sachs were flawed, and obviously performed just to demolish the sensory plant science of Francis and Charles Darwin. Interestingly, Francis Darwin succeeded in turning-down this criticism in 1882, few days after his father death (Darwin, 1882). In fact, later studies documented clearly that the decapped maize roots grow even faster as the intact roots (Juniper et al., 1966; Pilet, 1971, 1972); also because these roots stop to crawl but grow straightly (Baluška et al., 2009a; Burbach et al., 2012). Unfortunately, this important response paper by Francis Darwin from 1882 went almost unnoticed by the botanical mainstream. If it would be taken into account, it might be that the modern plant sciences would develop in rather different directions, including sensory plant biology and root tip-based sensory control of plant root movements.

FROM ALZHEIMER, VIA PARKINSON TO SCHIZOPHRENIA: LESSONS FROM THE TRANSITION ZONE?

The toxicity of aluminum in both plant and animal cell biology is well established, although poorly understood. Aluminum toxicity is one of the most important limiting factor for crop production in acid soils worldwide (Panda et al., 2009). Aluminum is highly toxic to root apices, with the transition zone representing the target of aluminum toxicity (Sivaguru and Horst, 1998; Sivaguru et al., 1999). Surprisingly, aluminum is less toxic to root cells which entered the elongation regions (Sivaguru and Horst, 1998). Importantly, aluminum inhibits basipetal auxin transport (Kollmeier et al., 2000), as well as PIN2 endocytosis (Illéš et al., 2006; Shen et al., 2008), endocytic vesicle recycling of PIN2 (Shen et al., 2008; Amenós et al., 2009), as well as polarity and cell patterning in the root apex (Doncheva et al., 2005), and root behavior (Poschenrieder et al., 2009).

We have reported that aluminum is internalized into the most sensitive cells of the distal portion of the transition zone in *Arabidopsis* root apices (Illéš et al., 2006; see also Babourina and Rengel, 2009), while aluminum also inhibits endocytosis in these cells (Illéš et al., 2006; Shen et al., 2008; Amenós et al., 2009). Intriguingly in this respect, elongating root cells are not sensitive to aluminum (Sivaguru and Horst, 1998; Sivaguru et al., 1999) and there is no internalization of aluminum into elongating root cells (Illéš et al., 2006; Babourina and Rengel, 2009). In support of the endocytosis/endocytic vesicle recycling being the primary processes affected in root cells, endocytosis of aluminum and its toxicity is lowered in the *Arabidopsis* mutant over-expressing of a DnaJ domain protein auxillin which regulates the clathrin-based endocytosis (Ezaki et al., 2006). Moreover, aluminum toxicity affected also NO production which is highest in cells of the distal portion of the transition zone (Illéš et al., 2006; Mugnai et al., 2012). Plant synapses being very active in endocytosis and transporting auxin show highest activities in the transition zone and it might turn out that the active plant synapses represent the aluminum target in the root apices (Panda et al., 2009; Poschenrieder et al., 2009; Baluška, 2010). This scenario is strongly supported by our finding that aluminum causes strongest depolarization of the plasma membrane potential exactly in cells of the root apex transition zone (Illéš et al., 2006). This effect is known to be mediated by glutamate and glutamate receptors (Sivaguru et al., 2003). Importantly in this respect, similarly as auxin, also L-glutamate is amino acids derived transmitter molecule which is released from synapse in form of well-defined quanta (defined by the size of synaptic vesicles). This quantal nature of cell–cell communication (Edwards, 2007) seems to be central for the very high effectivity of synapses in the cell–cell communication. This principle could also represent the basis of the still elusive flux sensor of the PAT (Merks et al., 2007; Stoma et al., 2008; Baluška, 2012a; Walker et al., 2013) which is essential for the canalization concept of PAT of Tsvi Sachs (Sachs, 1969; Sablowski, 2013).

Interestingly, in animals and humans, neuronal cells are extremely sensitive toward aluminum which is also internalized specifically in these cells (Guy et al., 1990). Aluminum was found to be enriched in lysosomes, similarly like the Alzheimer's amyloid β-peptide plaque depositions (Schuurmans Stekhoven et al., 1990). These are also internalized from cell surface and aluminum was reported to inhibit their degradation (Sakamoto et al., 2006). Therefore, in both root cells and brain neurons, endocytosis of aluminum emerges to be relevant to its high cytotoxicity (Illéš et al., 2006; Babourina and Rengel, 2009; Kawahara and Kato-Negishi, 2011). Recent advances in plant cell biology revealed similarities between root cells sensitive to aluminum and neurons (Baluška, 2010). Further studies on these cells might give us crucial clues not just for the plant sciences but also for our understanding of the Alzheimer disease (Kawahara, 2005; Kawahara and Kato-Negishi, 2011). Plants might turn out to be useful also for our better understanding of the Parkinson disease, of the most common neurodegenerative diseases. Protein DJ-1 which is important for the onset of Parkinson disease (Lev et al., 2006), but its role is still not well understood (Aleyasin et al., 2007; Kahle et al., 2009), is present also in plants and, similarly as in neurons, regulates the reactive oxygen species (ROS) homeostasis (Xu and Møller, 2010; Xu et al., 2010). Besides plant-specific roles in chloroplast development (Lin et al., 2011), DJ-1 is expressed also in *Arabidopsis* root apex cells and its expression increased when roots are exposed to light and initiate ROS burst and light-escape tropism (Yokawa et al., 2011, 2013; Burbach et al., 2012). Finally, our understanding of schizophrenia might profit from *Arabidopsis* because the BLOS-1 protein, homologous to human and mice BLOC-1 complex BLOS proteins which are relevant for the schizophrenia (Mullin et al., 2011), controls endocytic vesicular recycling of PIN2 and PIN1 in the transition zone of *Arabidopsis* roots, and modulates their growth (Cui et al., 2010).

TRANSITION ZONE AS EXPENSIVE, PRIVILEGED, AND PROTECTED ROOT APEX ZONE

Brains are the most expensive tissue of animals and humans (Mink et al., 1981; Navarrete et al., 2011; Howarth et al., 2012). For instance in humans, brain represent only about 2% of human body but consumes about 20% of all oxygen demand (Mink et al., 1981). Very high energy budged, due to energetically costly processes such ion channels activities, endocytosis and endocytic vesicle recycling, and dynamic actin cytoskeleton, results in very high demands for oxygen and ATP (Attwell and

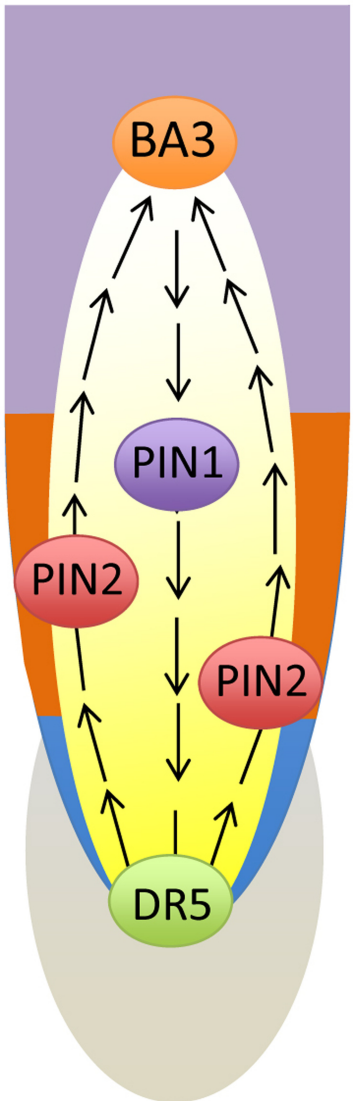

FIGURE 5 | Schematic views of two loops of the polar auxin transport streams at the root apex. In the central stelar tissue, polar auxin transport stream is polarized toward the root cap. Here it is redistributed laterally in a fountain-like manner toward the root periphery at which the stream gets opposite (shootward) polarity and continues up to the basal border of the transition zoner, where it is looping back via an inversed fountain-like manner back to the central stelar tissues. This complex pattern of polar cell–cell transport of auxin is tightly linked with sensory events at the root cap and instruct the motoric events at the apical portion of the elongation region (Baluška et al., 2009a,b, 2010a; Baluška and Mancuso, 2013a).

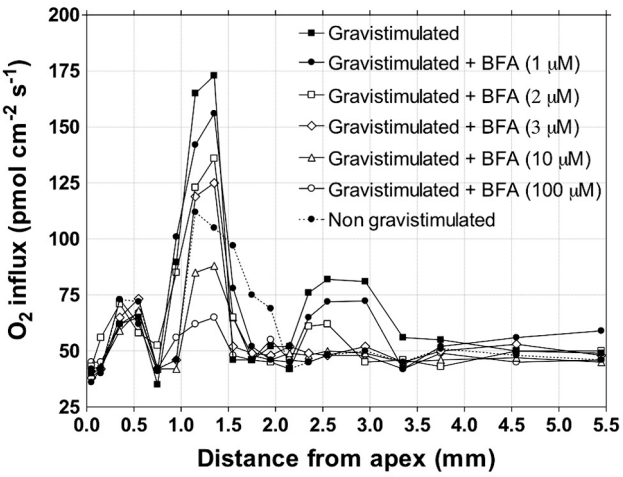

FIGURE 6 | BFA inhibits oxygen influx peak into the transition zone. Effect of different concentrations of BFA on the profiles of the oxygen influx for the upper side in maize roots growing vertically for 5 min after gravistimulation (elicited by rotating the chamber 90° until the root was horizontal. Values are means, $n = 15$. Oxygen fluxes were measured with a vibrating oxygen-selective microelectrode following the method described in Mancuso et al. (2000).

Laughlin, 2001; Laughlin, 2001; Harris et al., 2012). Updated energy budgets for the neural computation in mammalian neocortex and cerebellum neurons report 50% of signaling energy is used on glutamate receptors, 21% on action potentials, 20% on plasma membrane resting potentials, 5% on neurotransmitter secretory release, and 4% on vesicle recycling (Howarth et al., 2012). Surprisingly, often overlooked is also the very high ATP demand for F-actin dynamics and rearrangements (Bernstein and Bamburg, 2003).

Similarly in plants, the highest demand for oxygen has been scored in the root apex transition zone, although these cells do not divide or grow significantly (Mancuso and Boselli, 2002; McLamore et al., 2010a; Mugnai et al., 2012). Moreover, importantly, inhibition of the endocytic vesicle recycling using brefeldin A lowers oxygen demand of the transition zone of maize root apices dramatically (**Figure 6**). This finding suggests that the major sink of the extra-need of these cells is tightly linked to high rates of endocytosis and of endocytic vesicle recycling at plant synapses of the transition zone (Baluška et al., 2003c, 2005a; Mancuso et al., 2005, 2007). Currently favored view is that the transcellular PAT is driven by the plasma membrane-based efflux and influx transported of the PINs and AUXs protein families. However, as a matter of fact, cells of transition zone are secreting auxin via the endocytic vesicle recycling (Mancuso et al., 2005, 2007). Inhibition of endocytic vesicle recycling via brefeldin A resembles effects of classical PAT inhibitors such as NPA and TIBA. Moreover, the PAT inhibition is accomplished sooner as removal of PINs from the plasma membrane (Baluška et al., 2003c, 2005a, 2008; Mancuso et al., 2007). Moreover, also genetic evidences support the conclusion that the PAT peak in the transition zone is based on vesicular synaptic recycling (Mancuso et al., 2007). BFA-mediated inhibition of synaptic-like vesicle recycling lowers high oxygen demand in this zone (**Figure 5**). In other words, in cells of the transition zone, the PAT does not depend on the mere presence of PINs at the plasma membrane, but rather on the high rate of endocytic recycling of vesicles loaded with auxin, its transporters, cell wall pectins, and other recycling molecules (Baluška et al., 2003c, 2005a, 2008; Šamaj et al., 2004; Mancuso et al., 2007). All this fits well with the synaptic concept of PAT (Baluška et al., 2003a, 2005a; 2008; Mancuso et al., 2007). The most active plant synapses evolved in the transition zone, which is strategically well-placed at

the sites of phloem unloading. The cells of the transition zone are flooded with extracellular sucrose (Baluška et al., 2001c) inducing the fluid-phase endocytosis (Baluška et al., 2001c, 2006c; Etxeberria et al., 2005; Baroja-Fernandez et al., 2006). In cells of the maize root transition zone close to the unloading phloem, sucrose is internalized via the F-actin–myosin VIII driven fluid-phase endocytosis is active here (Baluška et al., 2001c, 2004c). Intriguingly in this respect, action potentials control both phloem long-distance transport and unloading of sucrose (Fromm, 1991; Fromm and Bauer, 1994; Fromm and Lautner, 2006); and plant glutamate receptors (GLRs)-like proteins GLR3.2, GLR3.3, and GLR3.4 are strongly expressed in *Arabidopsis* root phloem, especially near their unloading sites at the transition zone (Vincill et al., 2013). It emerges that the phloem long-distance transport and phloem unloading of sucrose are controlled electrically, perhaps via electric signals emerging from the brain-like transition zone (Masi et al., 2009). All 20 GLRs of *Arabidopsis* are expressed in their roots (Chiu et al., 2002; Roy et al., 2008) and it can be expected that they control plant-specific synaptic plasticity in the root apex transition zone, similarly as in animal brains (Lau and Zukin, 2007; Paoletti et al., 2013). Recently, GLR3.3 has been reported to be critical for the plant defense and immunity signaling (Li et al., 2013; Manzoor et al., 2013; Mousavi et al., 2013). Importantly, the GLR3.3 is not only localized at root synapses of the transition zone (see Figure 1C in Vincill et al., 2013), but it is also essential for electrical signaling that rapidly induce defense responses at remote leaves after local herbivore attack (Christmann and Grill, 2013; Mousavi et al., 2013).

Similarly like brain, also the transition zone is not only privileged but also highly protected niche. Under diverse stress situations, stress-induced abscisic acid (ABA) and NO protect cells of the transition zone. NO protects transition zone against aluminum or cadmium toxicity (Wang and Yang, 2005; Tian et al., 2007; Xiong et al., 2009; He et al., 2012) and oxygen deficit (Mugnai et al., 2012), whereas ABA protects the transition zone against water stress (Saab et al., 1990; Sharp et al., 1994; Ober and Sharp, 2003).

AUXIN AS PLANT-SPECIFIC TRANSMITTER: SIMILARITIES TO L-GLUTAMATE AND OTHER AMINO ACIDS-DERIVED NEUROTRANSMITTERS AND NEUROMODULATORS

Auxin is chemically very close to others monoamine transmitters, especially to serotonin and melatonin. Moreover, although auxin is a very small molecule below the size limit of the plasmodesmata transport, it is not transported across these plant-specific cell–cell channels. Obviously, there are selective processes active around plasmodesmata orifices, which prevent auxin to pass freely these cytoplasmic channels between plant cells (Baluška et al., 2003c, 2006a, 2008; Wojtaszek et al., 2004). All this suggests that auxin acts as plant-specific transmitter in plants. Interestingly, monoamine transmitters control adaptive behavior in lower animals such as nematodes (Donnelly et al., 2013), resembling behavior of plant roots. In lower and higher animals, monoamine transmitters and neuromodulators orchestrate chemical codes for behavioral patterns (Bicker and Menzel, 1993; Roeder, 2005; Pirri et al., 2009; Harris-Warrick, 2011; Donnelly et al., 2013). Ability of plants to rapidly modify their behavior in accordance with the environmental challenges is essential for their survival. This principle is very obvious in plant roots, as these are movable despite of the sessile nature of higher plants. Interestingly in this respect, stressed roots accomplish effective escape tropisms if faced with unfavorable environment (Yokawa et al., 2011, 2013; Burbach et al., 2012; Wan et al., 2012). This resembles the escape behavior of nematodes (Pirri et al., 2009; Donnelly et al., 2013). Auxin is chemically very similar to monoamine transmitters such as serotonin, melatonin, dopamine, tyramine, noradrenaline, and other; which control behavior and emotions in animals and humans (Pirri et al., 2009; Lövheim, 2012; Donnelly et al., 2013). Plants synthesize all these monoamines. Although the role of these is still not understood, at least serotonin and melatonin have physiological and perhaps also neuronal-like roles in plants (Ramakrishna et al., 2011; Pelagio-Flores et al., 2011, 2012; Park and Back, 2012). Similarly as auxin, both serotonin and melatonin regulate root system architecture (Pelagio-Flores et al., 2011, 2012; Park and Back, 2012). This resembles also the effects of another transmitter L-glutamate which acts also via neuronal GLRs of plants. It can be expected that the complex behavior of plant roots is controlled not only by auxin but also by other monoamine transmitters and neuromodulators. Recent paper reports end-pole/synapse localization of the GLR3.3 in roots of *Arabidopsis*, whereas GLR3.2 and GLR3.4 are enriched especially in root apex phloem elements, especially and at their sieve plates (Vincill et al., 2013). Interestingly, all these GLRs are also involved in the control of lateral root primordia initiation as revealed by increased amounts of primordia, which are aberrantly placed (Vincill et al., 2013). In addition, the GLR3.3 not only localize to the auxin secreting synapses of the transition zone (Baluška et al., 2005a, 2008; Mancuso et al., 2007), but is also important for root gravitropism (Miller et al., 2010). Rice GLR3.1, which is belonging to the same GLR clade, was found to be essential for root growth via control of the transition zone of rice root apex, which was highly reduced in mutant roots (Li et al., 2006). Finally, L-glutamate and GLRs control also root system architecture via impacts not only on the lateral roots but also via control of the primary root growth (Walch-Liu et al., 2006; Forde and Walch-Liu, 2009). One possibility is that L-glutamate and GLRs control root growth and root system architecture via phloem transport and unloading. As already mentioned, GLRs are enriched in phloem, especially at the sieve plates (Vincill et al., 2013), close to the root apex where phloem is unloaded (Baluška et al., 2001c). Importantly in this respect, both the phloem transport and the phloem unloading are controlled electrically via action potentials (Fromm, 1991; Fromm and Bauer, 1994; Fromm and Lautner, 2006).

PERSPECTIVES AND OUTLOOK

In contrast to the shoot apex, the plant root apex is highly regularly organized with clearly defined zones (**Figures 2** and **3**) and regular cell files (Ishikawa and Evans, 1995; Baluška et al., 1996b, 2006b, 2010a; Verbelen et al., 2006; Ivanov and Dubrovsky, 2013). It is very interesting that this anatomical zonation is associated with clear patterns of electric activities when root cap and elongation region show outward electric currents (**Figure 4**), whereas meristem and especially the transition zone

generates prominent inward electric currents (Collings et al., 1992; **Figure 4**). Importantly, these electric activities not only peak but also oscillate in the transition zone (Masi et al., 2009), where also auxin fluxes oscillate (McLamore et al., 2010a,b), and the root growth rates are tightly linked with the electric currents oscillations (Souda et al., 1990; Hecks et al., 1992). Anoxia or ether exposures block the currents oscillations (Yoshida et al., 1988) and root growth (Hecks et al., 1992), whereas recovery of the root growth is linked with the re-appearance of electric currents oscillations (Hecks et al., 1992). Furthermore, oscillatory patterns of ion transport activities and electric currents at the transition zone of barley root apices are closely linked with the rhythmic patterns of nutrient acquisition (Shabala and Knowles, 2002). In future studies, it will be important to find out further details of this oscillatory strategy for root nutrient acquisition. On the other hand, these root apex-specific electric currents can feed back not only into growth processes but also into morphogenesis of root apex as cell division patterns in maize root apices are sensitive to the extracellular electric fields (Wawrecki and Zagórska-Marek, 2007). In plants, these studies are very few and our knowledge extremely limited. But in animals, it is getting obvious that the emerging bioelectric code is at least as important for the development, embryogenesis and regeneration as the genetic code (Tseng and Levin, 2013). Any living cells, and even their organelles, generate their own electric fields which feedback into the biochemical and molecular processes underlying the cell/tissue polarities (McCaig et al., 2009; Yao et al., 2009; Zhao et al., 2012; Tseng and Levin, 2013). Endocytosis emerges to be sensitive to electric fields. Similarly as in neurons, endosomes of plant cells also process the sensory information for motoric outcomes (behavior) of their organs such as roots (Baluška and Volkmann, 2011; Baluška and Wan, 2012; Wan et al., 2012).

Electric fields generated by the oscillatory transition zone resemble encephalograms scored around brains of animals and humans. These prominent electric fields represent summation of synchronous electric activities of transition zone cells, and suggest two important features of this unique root apex zone. Firstly, the transition zone cells have the highest electric activities from all cells of the whole root (Masi et al., 2009) and; secondly, this activity is highly synchronous (**Figure 1**). Only this synchronicity allows vibrating probes to detect prominent electric currents which form the characteristic pattern of the smaller outward current peak at the meristematic zone and the prominent inward current peak at the transition zone (Baluška and Mancuso, 2013a). It is still mystery why the polarity of electric current suddenly switches from outward to inward at the meristem–transition zone border (**Figure 4**). Interestingly, the inward current switches again, back to the outward current at the transition zone–elongation region border (Collings et al., 1992; Baluška and Mancuso, 2013a). This pattern of electric field was scored in roots of all species tested so far, suggesting that there is something special about the inward current of the transition zone (Baluška and Mancuso, 2013a). As it correlates with high activities of root synapses, it might be possible that sensory stimuli/experiences will modify patterns of root electric fields. In support of this attractive possibility, root apex electric fields change during gravistimulation of maize roots (Collings et al., 1992). In addition, the PAT (**Figure 5**) appears to act as both the sensor and instructor for the adaptive root behavior (Baluška et al., 2009a,b, 2010a,b). The transition zone electric field is affected by inhibitors of PAT (Collings et al., 1992). In future, it will be important to analyze the possible connections between the synaptic activities and the patterns of the electric fields around the oscillatory transition zone (**Figures 1** and **4**). Particularly, how do the sensory inputs modify these electric field patterns, and if modified electric field patterns are related to the motoric output of driving the root behavior. Sensory perceptions and active behaviors are linked to neurons and neuronal systems in both lower and higher animals. Currently, the mainstream of plant sciences considers plants for passive automata-like organisms, lacking any sensory-motoric circuits. But this position, which is based on ancient Aristotelian worldviews, depriving plants of cognition-relevant sensitivities and activities are not tenable anymore (Brenner et al., 2006, 2007; Trewavas, 2007, 2009; Barlow, 2008; Baluška and Mancuso, 2009; Calvo and Keijzer, 2010; Karpiński and Szechyńska-Hebda, 2010; Szechyńska-Hebda et al., 2010; Trewavas and Baluška, 2011; Marder, 2012, 2013; Debono, 2013). Especially root behavior shows cognitive features which are not possible to explain with currently dominated views of passive, automata-like organisms (Baluška et al., 2010b; Barlow, 2010a,b; Gagliano et al., 2012).

In humans and animal brains, neuronal oscillations are important for processing of sensory information (Schroeder and Lakatos, 2008; Uhlhaas et al., 2009, 2010; Hipp et al., 2011; Arnal and Giraud, 2012). Synchrony and oscillatory patterning of anatomically grouped neurons drive sensorimotor networks in animals. Similarly in the root apex transition zone, all cells show synchronous oscillations (**Figure 1**) which are related to root behavior based on plant-specific sensorimotor networks. In future, it will be important to understand possible links between the oscillations at the plasma membrane and gene expression oscillations, as well as the relevance of these oscillations for the *brain-like* command center status of the transition zone and the whole root apex (Darwin, 1880; Barlow, 2006; Baluška et al., 2009b). It is emerging that besides the plant-specific molecules such as auxin, also many of the classical neuronal molecules including glutamate, GABA, serotonin, melatonin, acetylcholine, will be relevant in this respect.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 June 2013; accepted: 22 August 2013; published online: 02 October 2013.

*Citation: Baluška F and Mancuso S (2013) Root apex transition zone as oscillatory zone. Front. Plant Sci. **4**:354. doi: 10.3389/fpls.2013.00354*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 24 January 2014
doi: 10.3389/fpls.2014.00006

Abiotic stress responses in plant roots: a proteomics perspective

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Abiotic stress conditions adversely affect plant growth, resulting in significant decline in crop productivity. To mitigate and recover from the damaging effects of such adverse environmental conditions, plants have evolved various adaptive strategies at cellular and metabolic levels. Most of these strategies involve dynamic changes in protein abundance that can be best explored through proteomics. This review summarizes comparative proteomic studies conducted with roots of various plant species subjected to different abiotic stresses especially drought, salinity, flood, and cold. The main purpose of this article is to highlight and classify the protein level changes in abiotic stress response pathways specifically in plant roots. Shared as well as stressor-specific proteome signatures and adaptive mechanism(s) are simultaneously described. Such a comprehensive account will facilitate the design of genetic engineering strategies that enable the development of broad-spectrum abiotic stress-tolerant crops.

Keywords: proteomics, abiotic stress, root, adaptive response

INTRODUCTION

Abiotic stresses such as drought, salinity, flood, and cold vastly affect plant growth and metabolism that ultimately disturbs plant life (Bray et al., 2000; Ahmad and Prasad, 2012a,b). This has a negative impact on global crop production since majority of world's arable lands are exposed to these abiotic stress conditions (Rockström and Falkenmark, 2000). Upto 50–70% decline in major crop productivities have been attributed to abiotic stresses on several occasions (Mittler, 2006). For their survival under these stress conditions, plants respond by modifying several aspects in their metabolic cascade (Dos Reis et al., 2012). These response mechanisms help plants to survive during the stress period as well as to recover following cessation of the stress.

Abiotic stress responses in plants occur at various organ levels among which the root specific processes are of particular importance. Under normal growth condition, root absorbs water and nutrients from the soil and supplies them throughout the plant body, thereby playing pivotal roles in maintaining cellular homeostasis. However, this balanced system is altered during the stress period when roots are forced to adopt several structural and functional modifications. Examples of these modifications include molecular, cellular, and phenotypic changes such as alteration of metabolism and membrane characteristics, hardening of cell wall and reduction of root length (Gowda et al., 2011; Atkinson and Urwin, 2012). These changes are often caused by single or combined effects of several abiotic stress responsive pathways that can be best explored at the global level using high-throughput approaches such as proteomics (Petricka et al., 2012).

Proteomics allow global investigation of structural, functional, abundance, and interactions of proteins at a given time point. As a technique proteomics is advantaged over other "omics" tools since proteins are the key players in majority of cellular events. In addition to its capability of complementing transcriptome level changes, proteomics can also detect translational and post-translational regulations, thereby providing new insights into complex biological phenomena such as abiotic stress responses in plant roots (Gygi et al., 1999b; Salekdeh et al., 2002). In this review, proteomics studies on root responses against drought, salinity, flood, and cold are discussed with an aim to highlight shared as well as stressor specific protein classes altered due to stress conditions.

TOOLS AND TECHNIQUES FOR PLANT PROTEOMIC ANALYSES

Advances in high-throughput proteomics helped to address complex biological questions in various species. However, plant proteomics still have to deal with several technical challenges. For instance good sample quality is one of the critical factors for successful proteomic experiments and is challenging to obtain from plant tissues. An enriched level of proteases and oxidative enzymes in plant tissues make it extremely difficult to extract stable protein mixtures. Moreover, secondary metabolites produced in plant cells often interfere with subsequent protein fractionation and downstream analyses. Hence it is notoriously difficult to extract complete and representative protein population from plant tissues. Additional hindrance comes from the cell wall that is difficult to fragment. Use of TCA (Trichloroacetic acid)—acetone precipitation and phenol extraction method helped to overcome the above challenges to certain extent (Isaacson et al., 2006). However, optimizations to certain experimental conditions are still essential considering the heterogeneity between species. In addition, low protein content in plant cells has been

another major limitation to effective extraction of proteins from plants.

Protein extraction is usually followed by protein separation and identification that can be achieved with the use of two-dimensional electrophoresis (2-DE) (Wittmann-Liebold et al., 2006) or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Fournier et al., 2007). Although the merits of gel based separation techniques have been debated (Gygi et al., 2000) when compared to the LC-based shotgun approach, both separation strategies are being widely used with their own advantages and disadvantages. Gel based approaches are widely used for their simplicity, reproducibility, wide molecular weight coverage, and detection of post-translational modifications. However, careful manual editing is essential to obtain high precision especially for comparative proteomics. Additionally narrow pI range coverage and inability to detect low abundant proteins limited the use of this technique for broad protein mapping (Gygi et al., 2000). Protein spots obtained upon separation on a 2D gel are then trypsin digested into peptides for further protein identifications. On the contrary, LC-based separation strategy uses digestion before separation in most of the cases. This separation system covers broad molecular weight range along with identification of low abundant proteins (Fournier et al., 2007).

Protein identification followed by separation has mainly outstretched with the advances in mass spectrometry (MS). Firstly, breakthroughs in soft ionization methods such as matrix assisted laser desorption ionization (MALDI) (Tanaka et al., 1988) or electrospray ionization (ESI) (Yamashita and Fenn, 1984) and secondly peptide fragmentation by collision-induced dissociation (CID) in tandem MS (Stephenson and McLuckey, 1998) helped to achieve excellent coverage. Peptides identified through MS and MS/MS is finally searched against particular protein database to obtain a list of proteins. Recent advances in identification of qualitative changes like post-translational modifications allow differentiating between identical peptide mass and its modified variants which are important from biological perspective. Along with the qualitative changes, spatiotemporal variations in quantitative biomolecule ratios within a cell are also of high significance for better explanation of molecular events. Introduction of LC-MS based tagging approaches such as isotope-coded affinity tags (ICAT) (Gygi et al., 1999a), stable isotope labeling by amino acids in cell culture (SILAC) (Martinović et al., 2002; Ong et al., 2002, 2003; Ibarrola et al., 2004), isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004; Choe et al., 2007; Ghosh et al., 2011, 2013) helped to explore this field by relatively quantifying proteins or peptides at a global level. Emergence of statistically robust label free quantitative approach is also helping quantitative proteomics research to analyze large number of clinical samples (Chelius et al., 2003; Liu et al., 2004; Wu et al., 2006). Hence, with the existing as well as ongoing advances in the MS field, proteomics are expected to provide improved ways to reveal biological information. **Figure 1** describes a typical workflow of proteomic studies on plant tissues.

Since genome sequences of many plant species especially crops are still lacking, protein databases available for the model plants such as Arabidopsis and rice are currently used as reference databases for analyzing data obtained from other plant species. Therefore, up to now proteomics data analysis in plants is not well-optimized. However, further improvements are expected with increasing number of genome sequences made available for various plant species.

PROTEOMICS OVERVIEW ON ABIOTIC STRESS RESPONSES IN PLANT ROOTS

Abiotic stress biology research in plants has been enriched with a broad range of transcriptomic and proteomic studies that provide comprehensive information on alteration of gene expression and proteome profile during and following stress conditions (Hakeem et al., 2012; Mizoi et al., 2012). At the transcript level, abiotic stress responses were mainly studied from 30 min to 1 day after stress induction (Kilian et al., 2007). Whereas investigations carried out with comparative proteomic approaches were often performed on plants exposed to a particular stressor for at least 1 day. The time lapse between transcriptomic and proteomic studies was probably determined based on the time required for translation process in eukaryotes (Berthelot et al., 1973). Approximately 50% of the genes responsive to flood, salinity and extreme temperatures were found to encode transcriptional regulators (Kilian et al., 2007; Mizoi et al., 2012). Thus, transcription factors were immensely highlighted as regulators of abiotic stress responses at RNA level studies (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Seki et al., 2001; Kilian et al., 2007; Mizoi et al., 2012). However, identification of low abundant proteins such as transcription factors were limited by the use of gel based separation techniques in proteomic studies. Nevertheless, proteomic studies have led to the identification of various abiotic stress responsive proteins, some of which might be downstream effectors of the transcription factors identified at the transcriptional level. Moreover, MS-based proteomics allow isoform specific protein identification and hence are able to differentiate specific and shared functions within a protein family. This level of detection is often not possible in transcriptomic studies. Thus, proteome-wide identification and functional analysis of proteins provide additional insights into the findings obtained at the transcriptional level and thereby allow a better understanding of abiotic stress response pathways in plants. **Table 1** summarizes various proteomic studies performed with roots of different plant species grown under drought, high salinity, flooding or cold condition. The proteome wide alterations during root responses to each stressor are discussed in detail in the following sub-sections.

DROUGHT STRESS

Prolonged water deficit in the soil causes drought, one of the prevalent abiotic stresses that vastly affect the metabolic and physiological functions of a growing plant. Being the primary organ that detects changes in soil condition, root plays an important role in the drought response. Over the last decades, mechanisms involved in the drought response in roots have been extensively studies at the protein level using comparative proteomic approaches. These proteomic studies have linked various functional protein classes to drought responses.

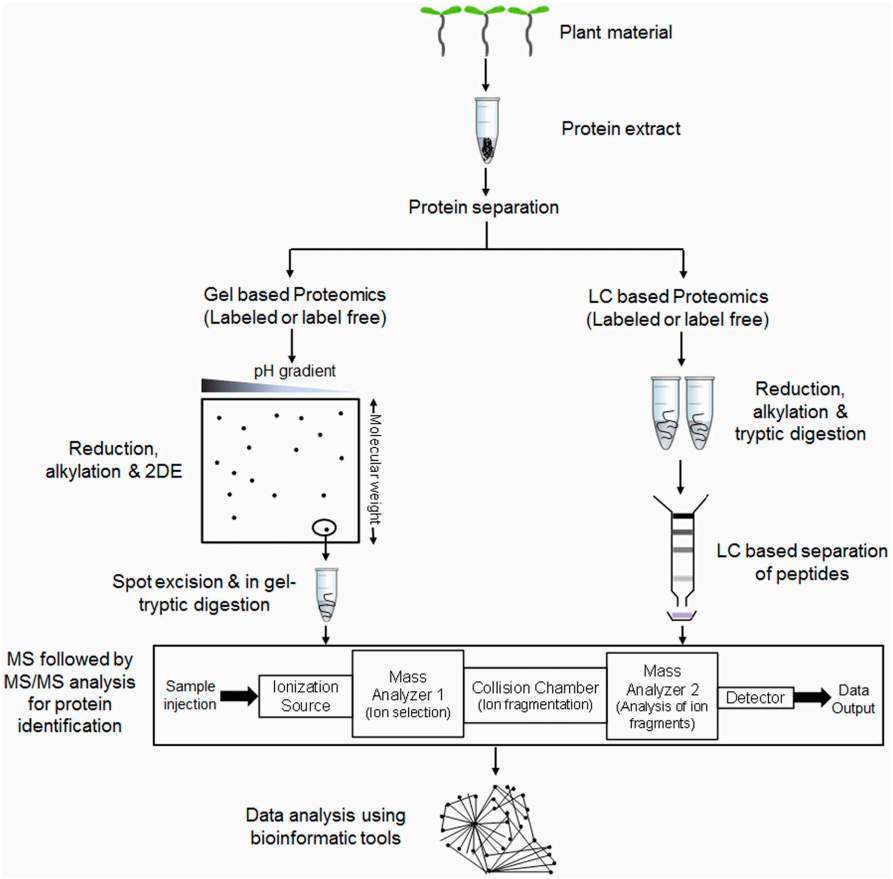

FIGURE 1 | A general workflow of comparative proteomic experiments in plants. Proteins are extracted and subjected to separation via gel (e.g., 2DE or 2 Dimensional gel electrophoresis) or non-gel (e.g., LC or liquid chromatography) based approaches. Reduction, alkylation and digestion are performed before or after the separation step as per the requirement to convert protein mixtures into peptides. Separated peptides are analyzed through mass spectrometer (MS) followed by tandem MS (MS/MS) for determining protein identity. The detected protein list is then used for data analysis using various bioinformatic tools.

Carbon/nitrogen metabolism related proteins such as triosphosphate isomerase, malate dehydrogenase, α-mannosidase, UDP-sugar pyrophosphorylase, NADP-malic enzyme, phosphoglucomutase, and UDP-glucose-6-phosphate dehydrogenase, were reported to be more abundant in roots of soybean (Toorchi et al., 2009; Alam et al., 2010; Mohammadi et al., 2012a), wild watermelon (Yoshimura et al., 2008), and rapeseed (Mohammadi et al., 2012b) 1 day after drought treatment. This reflected an increased energy demand as well as enhanced cellular activities in the root tissues at this stage of the stress. Simultaneously, a relative increase in root growth rate was observed, which was further supported by the abundance of root growth related small G-protein family members such as Ran GTPases (Yoshimura et al., 2008). Such root elongation could be the indication of an effort by the root to absorb water from deep soil layers. To replenish water deficit within the systems, roots also developed other mechanisms such as enhanced pumping of protons into vacuoles (Mohammadi et al., 2012b). As a signature of this, osmolytes and trans-membrane water-channel proteins such as vacuolar-type H^+-ATPases and plasma-membrane associated cation-binding protein 1 were found to be synthesized and stored at high levels in drought-induced plant species (Ishitani et al., 1995).

During drought stress, photosynthetic electron transport chain was markedly suppressed and as a consequence the excess excitation energy was driven towards the production of reactive oxygen species or ROS. To counteract the harmful effects of these ROS, several ROS scavengers were induced during drought stress. These include dehydrins, dehydroascorbate reductase, quinine reductase, γ-glutamyl cysteine synthetase, and glutathione S-transferases as observed from proteomic studies pursued on soybean (Toorchi et al., 2009; Alam et al., 2010; Mohammadi et al., 2012a), wild watermelon (Yoshimura et al., 2008), tomatoes (Shalata et al., 2001; Mittova et al., 2004), sunflower (Di Baccio et al., 2004), and other plant species (Mishra and Das, 2003). Additionally, increased levels of molecular chaperones, such as heat shock proteins, were detected in roots of sugar beet (Hajheidari et al., 2005), wheat (Demirevska et al., 2008), wild watermelon (Yoshimura et al., 2000), and sugarcane (Jangpromma et al., 2010) under drought treatment. These

Table 1 | A summary of comparative proteomic analyses performed with roots treated with different abiotic stresses. Plant species, stress treatment conditions, proteomic approaches, and protein classes identified in these studies are described.

Species	Treatment		Proteomics approach	References	Protein classes identified
	Condition	Duration			
DROUGHT					
Soybean	10% PEG 6000	4 days	2-DE	Mohammadi et al., 2012a	• Metabolic enzymes • Lignin biosynthesis related enzymes • Small G-protein family members • Osmolytes and trans-membrane H_2O-channels • ROS scavengers • Molecular chaperones • Proteosomal factors • Protease inhibitors • Proteolytic enzymes • Translation factors
	Stop watering	5 days	2-DE	Alam et al., 2010	
	PEG 6000	4 days	2-DE	Toorchi et al., 2009	
Wild watermelon	Stop watering	–	2-DE	Yoshimura et al., 2008	
	Stop watering	–	2-DE	Yoshimura et al., 2000	
Rapeseed	Stop watering	1–7 days	2-DE	Mohammadi et al., 2012b	
Wheat	18% PEG 6000	–	2-DE	Demirevska et al., 2008	
Sugarcane	Stop watering	3 weeks	1-DE, 2-DE	Jangpromma et al., 2010	
SALINITY					
Rice	150 mM NaCl	–	2-DE	Cheng et al., 2009	• Plasma membrane receptors • Ca^{++} signaling protein • Kinases • Ethylene receptors • ROS scavengers • Ion channel proteins • Membrane proteins • Metabolic enzymes • Enzymes involved in ETC and ATP synthesis
	200 mM NaCl	1, 3, and 6 h	2-DE	Zhang et al., 2009	
	150 mM NaCl	10 and 24 h	2-DE	Chitteti and Peng, 2007	
	5 μM ABA	48 h	2-DE	Li et al., 2010	
	100 mM NaCl	2 weeks	2-DE	Malakshah et al., 2007	
	150 mM NaCl	5 days	2-DE	Nam et al., 2012	
Wheat	200 mM NaCl	24 h	2-DE	Peng et al., 2009	
	201 mM NaCl	24 h	2-DE	Wang et al., 2008	
Arabidopsis	150 mM NaCl	6 and 48 h	2-DE	Jiang et al., 2007	
Maize	25 mM NaCl	1 h	2-DE	Zörb et al., 2010	
	100 mM NaCl	9 days	2-DE	Zörb et al., 2004	
Wild tomato	200 mM NaCl	–	2-DE	Zhou et al., 2011	
Pea	150 mM NaCl	6 weeks	2-DE	Kav et al., 2004	
Creeping bentgrass	Nacl	28 days	2D DIGE	Xu et al., 2010	
Sugar beet	900 mM Nacl	–	2D DIGE	Yang et al., 2012	
Cucumber	50 mM Nacl	3 days	2-DE	Du et al., 2010	
Barley	250 mM Nacl	13 days	2-DE	Witzel et al., 2009	
	200 mM NaCl	5 days	2-DE (gradient)	Sugimoto and Takeda, 2009	
FLOOD					
Wheat	Submerged in water	2 days	2-DE	Kong et al., 2010	• Disease/defense-related proteins • Metabolic enzymes • Molecular chperones • Cytoskeleton proteins • Cell wall biosynthesis related proteins • Signaling molecules • Proteins related to *de novo* protein synthesis
Soybean	Submerged in water	12–48 h	2D DIGE	Nanjo et al., 2010	
	Submerged in water	1–3 days	2-DE	Salavati et al., 2012	
	Submerged in water	12 h	2-DE	Komatsu et al., 2013	
COLD					
Rice	10°C	24 and 72 h	2-DE	Lee et al., 2009	• Primary metabolism associated enzymes • Antioxidants • Molecular chaperones • Proteins involved in cellulose biosynthesis • Membrane proteins • Signal transduction molecules • Defense-related proteins
	5°C	48 h	2-DE	Hashimoto and Komatsu, 2007	
	15°C, 10°C, and 5°C	24 h	2-DE	Hashimoto et al., 2009	
Chicory	<5°C	–	2-DE	Degand et al., 2009	
Maize	10°C	7 days	2-DE	Kollipara et al., 2002	
Poplar	4°C	4, 7, and 14 days	2-DE	Renaut et al., 2004	
Pea	6–8°C	11 days	2-DE	Dumont et al., 2011	

proteins may play a cytoprotective role in roots by preventing aggregation and assisting refolding of non-active proteins (Hartl, 1996).

Evidence from several proteomic studies showed that roots respond to drought stress using mechanisms similar to those occurring in damaging cells. For instance, enhanced levels of proteosomal factors were detected in drought-tolerant varieties of rapeseed seedlings (Mohammadi et al., 2012b). Similarly, in watermelon roots several proteolytic enzymes known to facilitate degradation of irreversibly damaged proteins were found to be induced by drought (Yoshimura et al., 2008). These include leucine aminopeptidases, ubiquitin family proteases, cysteine proteases, and multicatalytic endopeptidase (Yoshimura et al., 2008). Moreover, defense-related proteins and protease inhibitors, such as phloem serpin-1, Knotted 1 (kn1), pentatrico peptide repeat (PPR) protein (Yoo et al., 2000; Alam et al., 2010), NB-LPR or Nucleotide binding domain, leucine-rich repeat protein (Yu et al., 1998), and jasmonate-inducible proteins (Mohammadi et al., 2012b), were also found in abundance in roots of *Cucurbita maxima*, soybean, wild, and cultivated tomatoes, respectively. Together, these findings strongly suggest that defense-related proteins and proteins reported to regulate programmed cell death (PCD) are also involved in the response of roots to drought stress.

As soon as drought conditions are withdrawn, plants enter the recovery phase that can be characterized by certain proteome signature. For example, enhanced levels of actin isoform B was observed in the leaf, hypocotyl, and root of drought-affected soybean seedlings (Mohammadi et al., 2012a), indicating that actin is involved in repairing injured membranes following drought stress. Moreover, structural components of the cell wall were also altered in the root during the drought recovery phase. Examples of these include proteins related to lignin biosynthesis, such as caffeoyl-CoA 3-O-methyl-transferases and class III plant peroxidases that were found to be induced by drought in wild watermelon (Yoshimura et al., 2008) and maize (Degenhardt and Gimmler, 2000) roots and thereby suggesting enhanced lignin production. Increased amount of lignin builds the mechanical strength of cell wall and thereby protects roots against the dry soil (Yoshimura et al., 2008). In addition, cell wall modification is also used to minimize water loss and cell dehydration, thus helping plants to resist and recover from drought upon availability of water.

SALT STRESS

Salt stress is developed from excessive concentrations of salt, especially sodium chloride (NaCl) in soil. Root is the primary organ of exposure and hence responds rapidly. Several proteomic-based investigations have provided new insight into root responses and adaptation against high salinity.

Proteins involved in signal perception were found to be higher in abundance at the early stage of salt stress (Zhao et al., 2013). These include: (1) receptors in the plasma membrane (PM) or in the cytoplasm, (2) G protein, (3) Ca^{++} signaling protein or Ca^{++} binding protein, (4) phosphoproteins involving activation of kinase cascade, and (5) ethylene receptors. Receptor protein kinases (RPKs) in rice roots (Cheng et al., 2009; Zhang et al., 2009), transforming growth factor β receptor-interacting protein in wheat roots (Peng et al., 2009) and small GTP binding proteins in wheat (Wang et al., 2008), Arabidopsis (Jiang et al., 2007), and rice (Chitteti and Peng, 2007; Zhang et al., 2009) roots were found to be rapidly induced by high salinity. Ca^{++} signaling related proteins such as calmodulin (CaM) and calreticulin (CRT) (Jiang et al., 2007; Cheng et al., 2009; Li et al., 2010; Zörb et al., 2010) were also found in higher levels during salt stress. In addition, salt responsive protein kinase cascade was activated in roots of rice (Chitteti and Peng, 2007), wheat (Peng et al., 2009), maize (Zörb et al., 2004), wild tomato (Zhou et al., 2011), pea (Kav et al., 2004), and creeping bentgrass (Xu et al., 2010). Moreover, 14-3-3 family proteins, such as GF14a and GF14b in rice (Malakshah et al., 2007), 14-3-3 proteins in sugar beet (Yang et al., 2012), and 14-3-3 like protein A in wheat (Wang et al., 2008) were more abundant in roots exposed to high salinity. 14-3-3 proteins are positive regulators of H^+-ATPase activity, which is known to initiate the stress responses (Malakshah et al., 2007) by modulating the electro-chemical gradient across the PM (Finnie et al., 1999).

As adaptive responses to salt stress, root triggers several cellular and molecular events such as (1) alteration in carbohydrate and energy metabolism, (2) changes in ion homeostasis and membrane trafficking, (3) ROS scavenging, and (4) dynamic reorganization of cytoskeleton and redistribution of cell wall components.

Alteration in carbohydrates and energy metabolism under salinity can be addressed by the high abundance of enzymes involved in glycolysis, TCA cycle, electron transport chain (ETC), and ATP synthesis (Kav et al., 2004; Zörb et al., 2004; Chitteti and Peng, 2007; Peng et al., 2009; Du et al., 2010; Manaa et al., 2011). For example, NADH (reduced form of nicotinamide adenine dinucleotide) dehydrogenases, CCOs (cytochrome c oxidases), and ATP synthase subunits were found to be induced by salt stress in the root of many plant species (Chitteti and Peng, 2007; Jiang et al., 2007; Wang et al., 2008; Peng et al., 2009; Yang et al., 2012). Moreover, glycolytic enzymes such as fructose bisphosphate aldolase (FBPA), triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase, enolase, pyruvate decarboxylase, and alcohol dehydrogenase (ADH) were found to be more abundant in roots of rice (Chitteti and Peng, 2007; Cheng et al., 2009; Li et al., 2010), wheat (Wang et al., 2008; Peng et al., 2009), maize (Zörb et al., 2004, 2010), tomato (Manaa et al., 2011), pea (Kav et al., 2004), and cucumber (Du et al., 2010) under salt treatment. A similar trend was observed for TCA cycle enzymes, including pyruvate dehydrogenase, dihydrolipoamide dehydrogenase, aconitate hydratase, isocitrate dehydrogenase, succinyl-CoA ligase, and malate dehydrogenase as reported in proteomic studies with Arabidopsis (Jiang et al., 2007), rice (Chitteti and Peng, 2007; Li et al., 2010; Nam et al., 2012), wheat (Wang et al., 2008; Peng et al., 2009), wild tomato (Zhou et al., 2011), pea (Kav et al., 2004), and cucumber (Du et al., 2010). Enhanced levels of these primary metabolism related enzymes thus indicates that adequate energy is a prerequisite for roots to deal with high salinity.

Salt stress is known to increase Na^+/K^+ ratio in the root that leads to cell dehydration and ion imbalance (Tester and Davenport, 2003; Cavalcanti et al., 2007; Munns and Tester,

2008). To block or reduce cell dehydration and maintain ion homeostasis, plant roots have developed several strategies to enhance Na^+ exclusion and decreases Na^+ entry (Tester and Davenport, 2003). These strategies were implemented by modulating the activity of ion channels, V-ATPases and several salt responsive transporters. Among the ion channel proteins, enhanced levels of voltage-gated potassium channel (VGPC) in wheat roots (Peng et al., 2009), voltage-dependent anion channel protein (VDAC) in roots of maize and wild tomatoes (Zörb et al., 2010; Zhou et al., 2011), and lowered level of cyclic nucleotide-gated channel (CNGC) in wheat roots (Wang et al., 2008) were detected, in agreement with their roles in balancing Na^+/K^+ ratio. Most subunits of V-ATPases were induced in the root proteomic studies performed under salinity stress. These include two isoforms of V-ATPase subunit A in maize (Zörb et al., 2004) and cucumber (Du et al., 2010), five isoforms of subunit E in rice (Cheng et al., 2009), wheat (Wang et al., 2008), and pea (Kav et al., 2004), as well as a subunit in sugar beet (Yang et al., 2012). ABC transporters in wheat roots were found to be more abundant during salt stress (Wang et al., 2008; Peng et al., 2009), thus demonstrating their significant roles in salinity response. Alongside, several membrane associated proteins, such as annexin, remorins, PM polypeptides, and membrane steroid binding proteins, were also identified to be responsible for balancing the ion gradient throughout the membrane during salt stress (Lee et al., 2004; Peng et al., 2009).

As a result of water deficit in roots during salt stress, excess amount of reactive oxygen species (ROS) was produced (Miller et al., 2010). Consequently, various ROS scavengers were found to appear at high levels to decrease the excess ROS levels. Proteomic studies on several salt responsive species such as Arabidopsis (Jiang et al., 2007), wheat (Wang et al., 2008; Peng et al., 2009), wild tomato (Zhou et al., 2011), pea (Kav et al., 2004), cucumber (Du et al., 2010), salt cress (Zhou et al., 2010), and creeping bentgrass (Xu et al., 2010) showed abundance of superoxide dismutase (SOD), indicating its role as a key ROS scavenger during this stress condition. Catalase pathway was found to be deactivated in roots of barley (Witzel et al., 2009) and cucumber (Du et al., 2010) after 7 days of salt stress. By contrast, peroxiredoxin and thioredoxin levels were found to be induced by salt stress in roots of maize (Zörb et al., 2010), cucumber (Du et al., 2010), salt cress (Zhou et al., 2010), rice (Zhang et al., 2009; Nam et al., 2012), and wild tomato (Zhou et al., 2011) respectively. These findings together indicate that the above-mentioned enzymes play key roles in protecting root cells from salt-induced oxidative damage. Moreover, proteomic studies with different tissues/organelles from different species revealed dynamic changes in isoforms of various ascorbate-glutathione (AsA-GSH) cycle-related enzymes. For example, in roots of Arabidopsis (Jiang et al., 2007), rice (Chitteti and Peng, 2007; Nam et al., 2012), wheat (Wang et al., 2008; Peng et al., 2009), barley (Sugimoto and Takeda, 2009), and salt cress (Zhou et al., 2010), most glutathione S-transferase (GST) isoforms were found to be salt-inducible, with the exception of GST11 (Jiang et al., 2007), suggesting that the glutathione peroxidase (GPX/GST) pathway was activated to combat salinity. Furthermore, in Arabidopsis root, salt responsive peroxidase (POD) isoforms showed an initial decrease in abundance under salinity stress followed by an increase (Jiang et al., 2007).

While recovering from salt stress, cytoskeleton organization and cell wall components are commonly altered to maintain cell turgor by adjusting cell size (Ndimba et al., 2005; Li et al., 2011). Basic cytoskeleton components such as actin (Chitteti and Peng, 2007; Jiang et al., 2007; Xu et al., 2010), tubulin (Ndimba et al., 2005; Jiang et al., 2007; Katz et al., 2007; Peng et al., 2009; Pang et al., 2010), and other cytoskeleton-related proteins such as some actin-binding proteins (ABPs) (Yan et al., 2005), kinesin motor (Chitteti and Peng, 2007; Sobhanian et al., 2010), myosin (Cheng et al., 2009; Peng et al., 2009), and xyloglucan endotransglycosylase (XET) hydrolases (Zörb et al., 2010) were found to have altered abundance during recovery from salinity stress. In addition, changes in cytoskeleton organization and cell wall components were reported to be associated with other physiological responses occurred during salinity stress. One of the examples of these physiological changes include the control of cell expansion and morphology by co-migration of tubulin and P-type ATPases (Campetelli et al., 2005) to connect with the PM (Dryková et al., 2003). Another example includes XETs that enabled wall loosening required for cell expansion by nicking and re-ligating the inter-microfibrillar xyloglucan chains (Fry et al., 1992).

FLOODING STRESS

Heavy or continuous rainfall in areas with poorly drained soil causes flood, one of the most severe environmental stresses affecting plants, in particular at their early growth stages. Soybean, wheat, barley, and maize are categorized as flood-sensitive whereas rice is an example of flood-tolerant species (Komatsu et al., 2012). The hypoxic environment formed due to submerged state during flooding stress, affects aerobic respiration (Bailey-Serres and Voesenek, 2008), which leads to a boosted production of ATP and regeneration of NAD^+ through anaerobic respiration (Gibbs and Greenway, 2003). Under this oxygen deprived condition, protein synthesis is hampered as well, since translation is a tremendously energy-intensive process (Nanjo et al., 2010). In order to cope with this stress, plants need to adopt several changes in their gene expression profiles as well as at cellular protein levels.

Proteomic studies with plants grown under flooding stress conditions identified many differentially regulated proteins, which provide insights into flood-induced response mechanisms. One of the early root responses against flood stress could be attributed to the altered abundance of proteins involved in primary metabolism, energy production and secondary metabolism. For example, several proteins involved in the primary metabolism of sugars and polysaccharides (UDP-glucose dehydrogenase, UGP, β-glucosidase G4 and rhamnose synthase), amino acids (aspartate aminotransferase), and lipids (lipoxygenase) were induced as early flood responsive proteins (Jackson and Ram, 2003; Nanjo et al., 2010). On the contrary, phenolics synthesis pathway enzymes such as isoflavonoid synthesis enzyme dihydroflavonol reductase, phenylalanine ammonia-lyase, 6′-deoxychalcone synthase were found to be decreased in abundance. Secondary metabolism related proteins such as S-adenosylmethionine synthetase, caffeic acid 3-O-methyltransferase, and dihydroflavonolreductases were also

declined in level under flood stress as observed in a study with flooded soybean seedlings (Nanjo et al., 2010). These enzymes are members of the phenlypropanoid pathway. A decreased level of these enzymes thus justifies the reason behind inhibited pigmentation during flooding as a way of energy conservation.

Another change induced by flooding was the decrease of cell wall synthesis. For example, rhamnose synthase, a key component of plant cell wall was decreased in abundance as observed in a study with flooded soybean seedling (Nanjo et al., 2010). A proteomic study on cell wall specific proteins in wheat roots also revealed lower levels of methionine synthase, β-1, 3-glucanases, and β-glucosidase suggesting that roots of wheat seedlings respond to flood stress by coordinating methionine assimilation and cell wall hydrolysis, thus restricting cell growth. Together these data suggest that, during flood condition, cell wall synthesis is inhibited to reduce energy consumption. On the other hand, restriction of cell wall polysaccharide hydrolysis helps to preserve carbohydrate resources in the cell wall, which can support plant survival under flooding conditions (Kong et al., 2010).

ROS scavengers such as peroxidase, ascorbic peroxidase, and superoxide dismutase were found to be lower in abundance during flood (Shi et al., 2008; Komatsu et al., 2010, 2012), since generation of ROS is limited during the hypoxic condition. Proteins involved in proteolysis, protein folding, and storage was found to be high in abundance that indicates their probable involvement in excluding damage induced non-active proteins. One of the examples of these categories include heat shock proteins that function as a molecular chaperone in variety of cellular processes such as prevention of protein aggregation, translocation of nascent chains across membranes, assembly, or disassembly of multimeric protein complexes, and targeting proteins for lysosomal or proteasomal degradation (Komatsu et al., 2010, 2012). Moreover, disease/defense-related proteins such as glycosylated polypeptides, α-amylase/subtilisin inhibitors and chitinases were enhanced in abundance in wheat roots during flood condition (Kong et al., 2010). Among these, α-amylase/subtilisin inhibitors function in defense against micro-organisms (Yamasaki et al., 2006) and chitinase is involved in a defense mechanism against pathogens as well as abiotic stresses (Shibuya and Minami, 2001). Higher abundance of these proteins in the root thus indicated that molecular processes such as protein folding and degradation are involved in plant adaptive responses toward unfavorable environmental condition. This kind of adaptation gradually leads to damage and exclusion of root tip cells that were injured due to the flooding stress (Zhang and Glaser, 2002; Yanagawa and Komatsu, 2012).

Simultaneously, roots develop ways for flood recovery that can be characterized by certain proteome-wide changes (Dubey et al., 2003; Komatsu et al., 2012; Salavati et al., 2012). Cytoskeleton associated proteins such as actin isoform B, were enhanced in abundance as observed in post-flooded roots, suggesting cell wall expansion for root elongation (Wasteneys and Galway, 2003). Alongside, cell wall biosynthesis takes place during the recovery period and needs the production of deoxy-sugars through the dTDP-glucose 4-6 dehydratase reaction (Seifert, 2004; Chen et al., 2011). Proteins related to secondary metabolism such as S-adenosylmethionine synthase were found to be high abundant in the post flooding recovery period although they were lowered in abundance during flooding. This indicates its requirement for metabolites formation in order to overcome the flooding effect (Hesse et al., 2004). Protein levels for signaling molecules such as phosphatidyl ethanolamine binding proteins or PEBPs was enhanced during the flood recovery as well. This class of proteins being the regulator of various signaling pathways, control growth and differentiation (Salavati et al., 2012). Abundance levels for this group of proteins were found to be gradually decreasing with progression of recovery time which leads to the indication that these proteins were used up during the recovery process (Karlgren et al., 2011; Salavati et al., 2012). Moreover during post-flooding recovery, *de novo* protein synthesis was activated with an increased demand for synthesis of immunophilins, possessing peptidyl-prolylcis-trans isomerase activity (Romano et al., 2005; Salavati et al., 2012). Together, these findings suggested that re-organization of cytoskeleton, alteration of cell wall structure, synthesis of S-adenosylmethionine associated secondary metabolites and *de novo* protein synthesis are the key cellular processes responsible for the recovery of root from flooding stress (Salavati et al., 2012; Komatsu et al., 2013).

COLD STRESS

Cold or low-temperature stress is one of the major abiotic stresses that severely affect plant growth and survival. Chilling (<20°C) or freezing (<0°C) temperatures can induce ice formation in plant tissues, leading to cellular dehydration (Chinnusamy et al., 2007). To cope with this adverse condition, plants adopt several strategies such as producing more energy by activation of primary metabolisms, raising the level of antioxidants and chaperones, and maintaining osmotic balance by altering membrane structure (Uemura et al., 1995; Prasad, 1996; Sharma et al., 2005).

Comparative proteomics performed on cold tolerant and sensitive plants helped to understand the overall response as well as recovery mechanism against cold stress. For instance, activation of metabolic processes was observed in rice roots upon 24–72 h of chilling stress as indicated by the enhanced levels of several metabolism-associated proteins (Lee et al., 2009). These include a group of carbohydrate metabolism enzymes, such as phosphogluconate dehydrogenase, NADP-specific isocitrate dehydrogenase, fructokinase, and cytoplasmic malate dehydrogenase. In addition, higher abundance of pyruvate orthophosphate dikinase precursors (PPDK), aconitate hydratase, glycine dehydrogenase, and enolase were also identified in chilling stress-related studies (Lee et al., 2009). Among them PPDk is responsible for the production of phosphoenolpyruvate (PEP), the primary acceptor of CO_2(Moons et al., 1998); whereas aconitate hydratase, glycine dehydrogenase, and enolase are involved in the tricarboxylic acid cycle, photorespiration, and glycolysis (Hojoung et al., 2002; Cui et al., 2005), respectively. Similarly, higher abundance of adenylate kinase protein under chilling stress is an indication of enhanced ATP synthesis and energy metabolism. Additionally, peptidylprolyl isomerase Cyp2 and cysteine proteinase was preferentially accumulated in rice roots upon chilling stress (Pradet and Raymond, 1983; Hashimoto and Komatsu, 2007). Taken

together, these results indicated that during cold stress plants require high energy production that comes from activation of metabolic pathways.

Quantitative analysis of PM proteome of rice roots grown under cold stress condition revealed that proteins related to membrane permeability and signal transduction through the membrane were enhanced in level (Hashimoto et al., 2009). Examples of this category include members of the annexin and hypersensitive-induced response (HIR) protein families. Annexins are Ca^{2+}- dependent membrane binding proteins that play vital roles in membrane trafficking and organization, and are known to regulate ion channel activity and phospholipids metabolism (Gerke and Moss, 2002). Thus, elevated levels of annexins led to protection against the osmotic imbalance caused by cold stress. The HIR family of proteins belongs to a structurally-related superfamily, which includes prohibitins, stomatins, and other membrane proteins. These proteins control the ion channel activity and thereby regulate diverse cellular processes like cell division, osmotic homeostasis and cell death (Nadimpalli et al., 2000). Higher abundance of this family of proteins can also be explained as a response to osmotic stress induced by cold treatment. Other proteins that were found to be involved in the cold stress response include dehydrins, 25 KDa dehydrin-like protein, ERD14, and cold acclimation-specific protein 15 (CAS15) as identified in chicory roots under chilling stress (Degand et al., 2009). The first three proteins belong to the dehydrin family and are believed to form complexes with other macromolecules to protect the cells from freeze-induced desiccation (Kosová et al., 2007). CAS15 protein contains characteristic dehydrin K and S segments and thus contributes similarly to cold tolerance like dehydrins (Pennycooke et al., 2008).

On the other hand, cold stress causes oxidative damage to the cells by generating ROS or their precursors. To protect against this damaging effect, several anti-oxidants are produced in the root. For example, oxalyl-CoA decarboxylase, the second enzyme of oxalate catabolism pathway, was enriched in rice roots under chilling stress (Lee et al., 2009). This enzyme causes decarboxylation of activated oxalate molecule that generates ROS through fenton reaction. Unless decarboxylated, it causes generation of hydroxyl or carbonate radicals through its interaction with hydrogen peroxide (Urzúa et al., 1998). Gradual increase of glyoxalase I protein level throughout the cold stress period indicated detoxification of methylglyoxal produced during the stress condition (Espartero et al., 1995; Lee et al., 2009), thus providing another example of antioxidant generation. In addition, ROS scavengers induced commonly in all abiotic stresses, such as superoxide dismutase, catalase, and ascorbate peroxidase, were found in abundance in a study with chicory roots under chilling stress (Lee et al., 2009).

Moreover, heat shock proteins (HSPs) were found to be higher in abundance as a chilling stress response in roots of rice (Cui et al., 2005; Yan et al., 2006), chicory (Degand et al., 2009), maize (Kollipara et al., 2002), and poplar (Renaut et al., 2004), with HSP70 family being the most abundant. These proteins act as molecular chaperones and thus prevent aggregation of the denatured proteins as well as facilitate protein refolding (Lee et al., 2009). In addition, a putative calreticulin precursor with chaperone activity was also detected in a study with rice seedlings under chilling stress (Hashimoto and Komatsu, 2007). Defense-related proteins such as protein disulfide isomerase and disease resistance response protein were also detected in relatively high abundance in pea roots under chilling stress (Dumont et al., 2011) indicating that defense-related pathways are activated in the root when combating cold stress.

Several other proteome signatures provided insights on plants' preparation for recovery once the stress is being released. For example proteins involved in cellulose synthesis, such as UDP-glucose pyrophosphorylase were highly abundant in rice roots upon 48 h chilling stress (Amor et al., 1995; Hashimoto and Komatsu, 2007). This suggested new cell wall synthesis under chilling stress to confer enhanced protection of cells against low temperature.

CONCLUSION

Proteomic analyses on plant roots under various abiotic stress conditions revealed important information on proteins involved in the abiotic stress response. This leads to the identification of molecular and cellular mechanisms that are specific to certain abiotic stress or shared between two or more abiotic stress conditions (**Figure 2**). For instance, during drought, high salinity and cold stress conditions, trans-membrane water, and/or ion channel proteins were found to be higher in abundance, indicating changes in ion and/or osmotic balance. This phenomenon was however not observed in flooded conditions when the roots were submerged in water. In addition, higher abundance of ROS scavengers was detected in roots under drought, high salinity and cold stress and can be looked upon as a preventive measure against oxidative damage caused due to high ROS levels. By contrast, the abundance of ROS scavengers during flood condition was low, which can be explained as a fact that roots are maintained in a hypoxic state during flood. On the other hand, molecular chaperones involved in protein folding, disease-, and defense-related proteins, such as proteolytic enzymes and proteosomal factors, were found to have higher levels during drought, flood, and cold stress conditions, indicating refolding of denatured proteins and proteolytic elimination of damaged proteins. Moreover, all the abiotic stress conditions discussed in this review were shown to induce the protein levels involved in primary metabolism in the root, indicating an enhanced energy demand during the stress condition. However, secondary metabolism associated proteins were found to be low in abundance in roots during flood condition suggesting a mode of energy conservation.

At the recovery phase, increased lignin biosynthesis was found following cold and drought stress. This molecular mechanism results in enhanced mechanical strength of roots by hardening cell wall at the root tip. Changes in abundance for cytoskeleton associated proteins were also observed, that can be looked upon as compensation against reduced cell size as well as repairing injuries caused by drought, salinity, and flood stress. Moreover, the levels of proteins related to *de novo* protein synthesis, growth-related signaling, and secondary metabolism were enhanced during recovery from flood stress as a replenishment

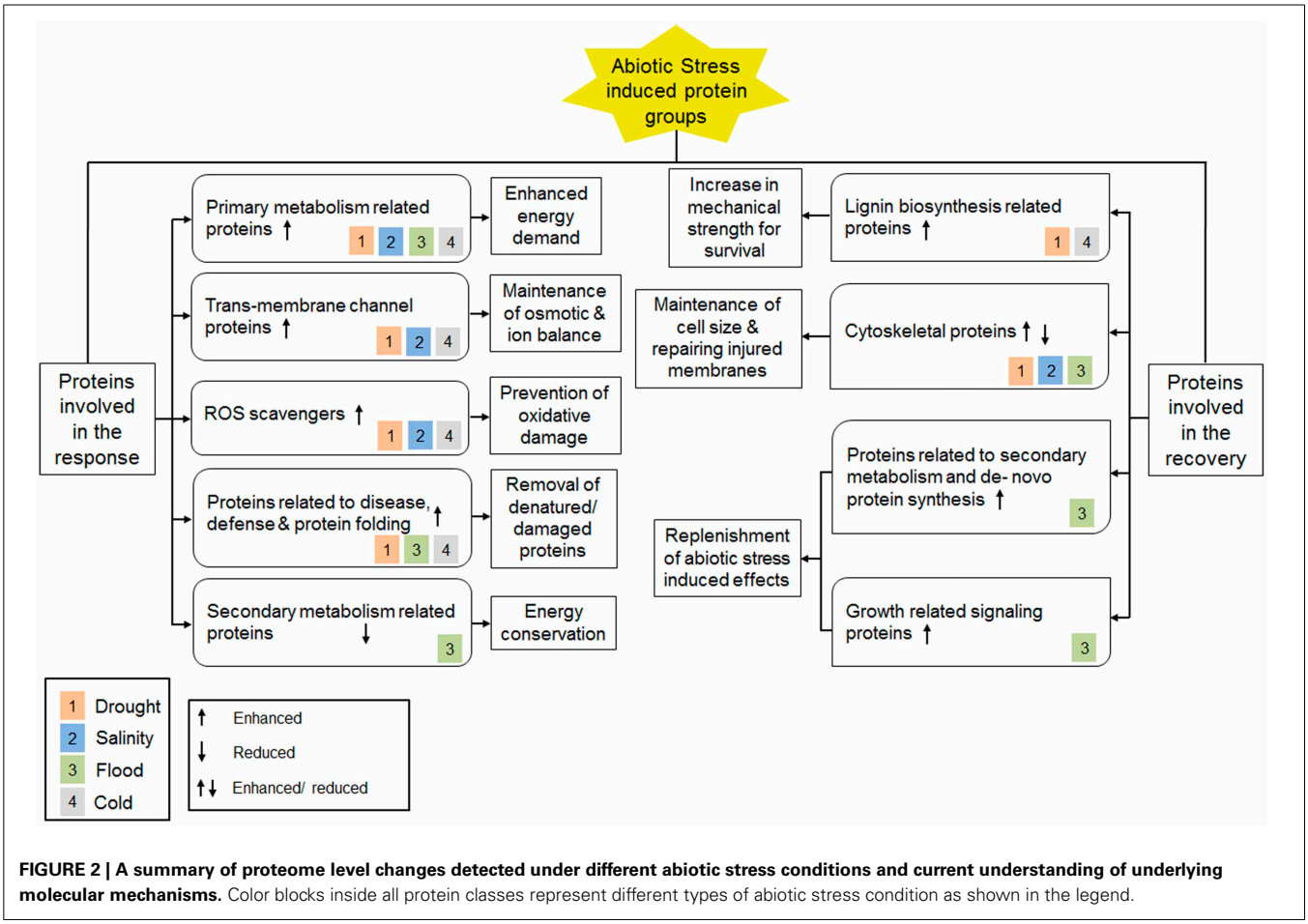

FIGURE 2 | A summary of proteome level changes detected under different abiotic stress conditions and current understanding of underlying molecular mechanisms. Color blocks inside all protein classes represent different types of abiotic stress condition as shown in the legend.

of the stress-induced effects. Together, these changes suggested a compensatory mechanism by which the stress-induced effects could significantly be recovered.

FUTURE PERSPECTIVES

As mentioned in this review, majority of literature reported proteomic analyses of the root response at least after 24 h of exposure to cold, drought, high salinity, or flooding stress. Therefore, early proteomic changes associated with individual abiotic stress remain to be elucidated, which will allow the identification of distinct sets of effectors for stress signaling. The function of these early effectors may be masked by secondary processes at a later stage. Technically, most of these proteomic studies have utilized the gel based separation approaches (**Table 1**) that resulted in identification of mostly high abundant proteins. Use of advanced LC based separation techniques may significantly improve detectability of low abundant proteins such as transcription factors, kinases, and transport proteins. Additionally, post-translational changes such as phosphorylation, glycosylation, and oxidation, which are likely induced by the stressors, are still to be captured with the use of gel based proteomic approaches. Other than these aspects, changes in plant hormone-mediated metabolic programs, as well as alterations in protein-protein interactions and subcellular translocation of proteins, remain to be explored and correlated with different stressors. Future investigations on these areas are expected to improve our understanding of plant root responses to abiotic stress. These researches could be augmented with the use of comparatively new proteomic strategies such as hydrogen–deuterium exchange (H/D exchange), surface plasmon resonance (SPR)-MS together with integrated cell biology approaches such as immuno-precipitation and live imaging analysis. Since proteins are dynamic in nature and most published proteomic studies only focused on a single time point, carefully designed time-course experiments will thus be required in future to advance our knowledge on the time-dependent response or recovery mechanisms (Graves and Haystead, 2002).

ACKNOWLEDGMENTS

We apologize to all colleagues whose original works could not be cited for space constraints. This work is supported by National Research Foundation Singapore under its Competitive Research Programme (CRP Award No. NRF2010NRF-CRP002-018), National University of Singapore Young Investigator Award, Ministry of Education of Singapore Academic Research Fund (Tier 2; MOE2012-T2-1-157) and the Lee Hiok Kwee donation fund.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 August 2013; accepted: 06 January 2014; published online: 24 January 2014.

*Citation: Ghosh D and Xu J (2014) Abiotic stress responses in plant roots: a proteomics perspective. Front. Plant Sci. **5**:6. doi: 10.3389/fpls.2014.00006*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 12 September 2013
doi: 10.3389/fpls.2013.00363

Protein intrinsic disorder in plants

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To some extent contradicting the classical paradigm of the relationship between protein 3D structure and function, now it is clear that large portions of the proteomes, especially in higher organisms, lack a fixed structure and still perform very important functions. Proteins completely or partially unstructured in their native (functional) form are involved in key cellular processes underlain by complex networks of protein interactions. The intrinsic conformational flexibility of these disordered proteins allows them to bind multiple partners in transient interactions of high specificity and low affinity. In concordance, in plants this type of proteins has been found in processes requiring these complex and versatile interaction networks. These include transcription factor networks, where disordered proteins act as integrators of different signals or link different transcription factor subnetworks due to their ability to interact (in many cases simultaneously) with different partners. Similarly, they also serve as signal integrators in signaling cascades, such as those related to response to external stimuli. Disordered proteins have also been found in plants in many stress-response processes, acting as protein chaperones or protecting other cellular components and structures. In plants, it is especially important to have complex and versatile networks able to quickly and efficiently respond to changing environmental conditions since these organisms cannot escape and have no other choice than adapting to them. Consequently, protein disorder can play an especially important role in plants, providing them with a fast mechanism to obtain complex, interconnected and versatile molecular networks.

Keywords: protein function, protein structure, protein interactions, protein intrinsic disorder, biological networks, plant environmental responses

PROTEIN INTRINSIC DISORDER

It is now recognized that a large fraction of the proteome, especially in eukaryotic organisms, lacks a fixed 3D structure in its native form. In these proteins, either the complete chain ("intrinsically disordered/unstructured protein," IDP/IUP) or part of it ("intrinsically disordered/unstructured region," IDR/IUR) do not adopt a folded structure in its functional form, but exist as a flexible mobile polypeptide (Dunker and Obradovic, 2001; Tompa, 2002; Uversky, 2013).

Intrinsically disordered proteins/intrinsically disordered region were detected from diverse experimental evidences of lack of fixed structure: e.g., missing segments in X-Ray-derived structures or lack of constraints to define a unique structure in NMR. At the sequence level, IDRs are characterized by long stretches of charged and polar residues almost lacking hydrophobic residues, which consequently do not allow the formation of hydrophobic cores to initiate folding (Romero et al., 2001; Dyson and Wright, 2005; Tompa, 2005). This particular (highly biased) amino-acid composition was the basis of the first approaches for detecting IDPs/IDRs from primary sequences. Later, as more examples of experimentally determined IDRs accumulated, specific predictors were trained with them, such as PONDR (Romero et al., 1997) or DISOPRED (Ward et al., 2004). These, together with the latest methodologies based on physical principles, e.g., FoldIndex (Prilusky et al., 2005) and IUPRED (Dosztanyi et al., 2005), constitute the current toolbox for predicting disorder from primary sequences.

Research in this type of proteins was delayed in part by the fact that they apparently contradicted the classic "structure-function relationship" paradigm, which states that a protein has to be folded in a fixed 3D conformation in order to perform its function. In IDPs, it is actually their lack of structure what is instrumental to perform their particular functions. This is because in most cases the molecular function of these polypeptides is related to transient binding to multiple (different) partners. Such a particular way of interacting could not be achieved by "fixed" surfaces, but only by those able to adapt to different conformations. Indeed, in many cases IDRs become structured upon binding to a partner, and in some cases the same IDR can adopt different bound structures depending on the partner (Tompa, 2005). This entropy reduction due to the structural gain associated to the binding is in part responsible for the special characteristics of the disorder-mediated interactions. Besides binding, IDRs also act as flexible linkers and "springs" within the cell (Dunker et al., 2002; Tompa, 2002; Cozzetto and Jones, 2013).

Disordered proteins/regions are associated with key cellular processes such as signaling cascades, transcription regulation, cell cycle control and chaperone activity (Iakoucheva et al., 2002; Uversky et al., 2005; Tompa et al., 2006; Xie et al., 2007). These processes require reversible transient interactions of high specificity

and low affinity, eventually with different partners, exactly the type of interactions mediated by these unstructured polypeptides. Consequently, far from being "rare" or anecdotic, disordered proteins are among the most important proteins in a given proteome, and their mutation is, in many cases, either lethal or leads to diseases (Iakoucheva et al., 2002; Midic et al., 2009). Indeed, the possibility of interacting with multiple partners makes IDPs being "hubs" (highly connected nodes) in protein interaction networks (Haynes et al., 2006) which are themselves related to lethality (Jeong et al., 2001). For example, the highly studied human transcription factor (TF) p53 is disordered in half of its length and indeed uses these IDRs to interact with its more than hundred different known partners (Oldfield et al., 2008). Similarly, signaling networks are branched and interconnected, and they require transient interactions of high specificity with different partners, making unstructured proteins excellent candidates for them. Another prototypical example are the molecular chaperones, for which a growing body of evidence points to the involvement of disorder in the activity of many of them (Kovacs and Tompa, 2012). Many chaperones contain IDRs (which are involved in the regulation of the chaperone or in the interaction with the substrate itself) or are fully disordered (IDPs). Interacting through disordered segments allows these chaperones to help in the folding of a much broader range of substrates.

Within these long disordered segments, particular stretches of amino-acids, generally with increased evolutionary conservation, have been found to be important for determining the interaction specificity. They can be seen as a sort of "functional sites" within disordered segments. These include "molecular recognition features" (MoRFs), which have a tendency to form certain secondary structures (α-MoRFs, β-MoRFs, . . .) realized when they bind to a partner (Fuxreiter et al., 2004; Mohan et al., 2006), "eukaryotic linear motifs" (ELMs; Gould et al., 2010), and "short linear motifs" (SLiMs; Diella et al., 2008).

These proteins are not only involved in central cellular processes but they are also more abundant than previously anticipated. The development of specific predictors able to detect IDPs/IDRs from primary sequences, and their massive application to complete proteomes rendered surprising results. Almost 1/3 of eukaryotic proteins are mostly disordered and half of them contains at least one long IDR (>30 residues). This rises to 70% for proteins involved in signaling (Iakoucheva et al., 2002; Vucetic et al., 2003; Ward et al., 2004).

Moreover, there is a relationship between disorder content and what one intuitively regards as "organism complexity." Even if this is controversial mainly due to the imprecise definition and quantification of "organismal complexity," at least there is a clear difference between the relatively low disorder content of prokaryotic organisms and the high disorder found in eukarya (Ward et al., 2004; Schad et al., 2011). This can be related to the involvement of disorder in cellular processes that are apparently more complex and interconnected in higher and multicellular organisms (cell cycle control, signaling cascades, etc.).

Taking together all these observations point toward the involvement of disorder in the generation of the highly-connected and intricate molecular interaction networks which underlie the complex biological processes characteristic of higher organisms. Indeed, protein interactions mediated by IDRs are recognized as a way of introducing plasticity in protein interaction networks (Tompa et al., 2005; Uversky et al., 2005). Along the same line, it has also been shown that in many cases alternative splicing isoforms are characterized by the addition/deletion of IDRs so as to add/remove interacting regions and consequently tune the "wiring" of the networks these isoforms are involved in (Romero et al., 2006; Buljan et al., 2013).

PROTEIN DISORDER IN PLANTS

LARGE-SCALE QUANTIFICATIONS OF DISORDER

In principle, protein disorder in plant proteomes follows the same trends reported for other species. A number of studies focused on plant model organisms showed that disorder is present in the typical processes involving transient interactions with multiple partners. For example, a genome-wide analysis of protein disorder in *Arabidopsis thaliana* (Pietrosemoli et al., 2013) showed that the biological processes more enriched in disordered proteins were related to cell cycle, signaling, DNA metabolism, RNA splicing, etc. In this study, disorder predictions were generated for all proteins in this model organism. These data, together with a functional classification of the proteins in biological processes, allowed to evaluate the degree of disorder of the different biological processes. Carrying out the same process for the Human proteome allows to perform comparative studies on the usage of disorder in both organisms. The proteome of *A. thaliana* follows the expected trend regarding whole disorder content: as an eukaryotic organism, it has much more disorder than bacterial proteomes and, leaving apart discussions on the definition of "organism complexity" and its quantification, *Arabidopsis* is globally less disordered than *Human*, an organism intuitively regarded as of higher complexity (Schad et al., 2011; Pietrosemoli et al., 2013).

In spite of this lower overall disorder content, there are some biological processes that are more enriched in disorder in *A. thaliana* than in *Human*. Many of these processes are related to the detection and response to external (environmental) stimuli (Pietrosemoli et al., 2013). These include processes related to the perception of light, response to abiotic stress, protein folding (chaperones) and secondary metabolism (mediating plant response to stress). A hypothesis to explain that these processes related to the perception and response to stimuli are more disordered in plants than in organisms of higher complexity involves that plants might have evolved very complex, versatile and intricate systems for interacting with the environment since, being sessile organisms, they cannot escape from environmental hazards and changes, as animals do, and have no other option than responding to them (Pietrosemoli et al., 2013). Protein disorder is a possible way for increasing the "wiring" (connectivity) of the molecular networks underlying a given biological system. As a consequence, such system becomes more intricate and complex. This relationship between disorder in plants and their increased ability to respond to changing conditions has also been noted by other authors (Sun et al., 2013).

EXAMPLES OF INVOLVEMENT OF DISORDER IN PLANTS

The involvement of protein intrinsic disorder in a number of plant molecular systems has been studied in detail. Again, in all the cases

protein disorder allows the proteins in these systems to interact transiently with multiple partners with high specificity and low affinity. Moreover, in general these systems follow the trend commented above regarding the relationship between disorder in plants and versatility/complexity in the response to stimuli and changing conditions.

Maybe the most studied example of disordered plant systems are the dehydrins (Mouillon et al., 2006; Kovacs et al., 2008; Sun et al., 2013). This large and diverse family of proteins, involved in the response to drought and other environmental stresses, is almost completely disordered: their content of "standard" secondary structure elements (α-helix and β-strand) is low, and they present a significant content of poly-Pro helices (Mouillon et al., 2006). This family includes protein chaperones such as ERD10 and ERD14 (Kovacs et al., 2008; Tompa and Kovacs, 2010) as well as proteins involved in the binding of metal ions, protection of membranes, and global protection of the cell during the highly compact dry state characteristic of plant seeds (Sun et al., 2013). It looks like dehydrins have evolved for maintaining this disorder state and avoid forming compact folded structures (Mouillon et al., 2006). The conformational flexibility associated to the disordered state allows them to sequester water, ions, proteins (as chaperones), and perform all the other molecular functions associated to their roles in responding to water-related stresses.

Another plant-specific family of proteins heavily relying on disorder for functioning is the GRAS family (Sun et al., 2011; Sun et al., 2012). These proteins play an important role in plant development and are involved in signal transduction cascades, such as those related to hormone response. Within these cascades, they act as integrators of signals (i.e., from different hormones or environmental inputs). It is indeed their disordered region (present in the N-terminal) that allows them to interact with multiple partners through different binding sites (MoRFs, see Introduction) and consequently integrate the signals they represent. The most conserved C-terminal domain of this family (which is actually what characterizes it) is structurally ordered and contains also motifs involved in protein interaction. Among them, there are Leucine-rich regions probably involved in interactions with TFs so as to transduce the integrated signals downstream.

In some cases, this integration of signals occurs at the level of the TF itself, due to the presence of disordered domains (besides the ordered DNA binding domain), which allows the TF to be influenced by multiple partners. For example the NAC family of plant TF is involved in a variety of processes such as plant defense, stress response or development. These proteins present a conserved (structured) N-terminal DNA-binding domain and a more variable intrinsically disordered C-terminal region (Jensen et al., 2010; Sun et al., 2013; **Figure 1**). This region acquires local structure (α-helix) when binding to the multiple partners of these proteins. This mechanism by which a TF is influenced by multiple partners through disordered regions, which also happens with other plant TFs such as the basic leucine zipper domain (bZIP) family (Yoon et al., 2006), is similar to that of the human p53 commented.

It was also proposed that many chloroplast proteins whose genes were originally encoded by the chloroplast genome acquire disordered regions as they are transferred to the nucleus (Yruela and Contreras-Moreira, 2012). In concordance with its prokaryotic origin, proteins coded in the chloroplast genome almost lack disordered regions. Nevertheless, it looks like the "eukaryotic machinery" of the nucleus adds disorder to them once they become coded there. This reinforces the idea of the relationship between disorder and the emergence of the complex molecular machineries associated to eukaryotic organisms.

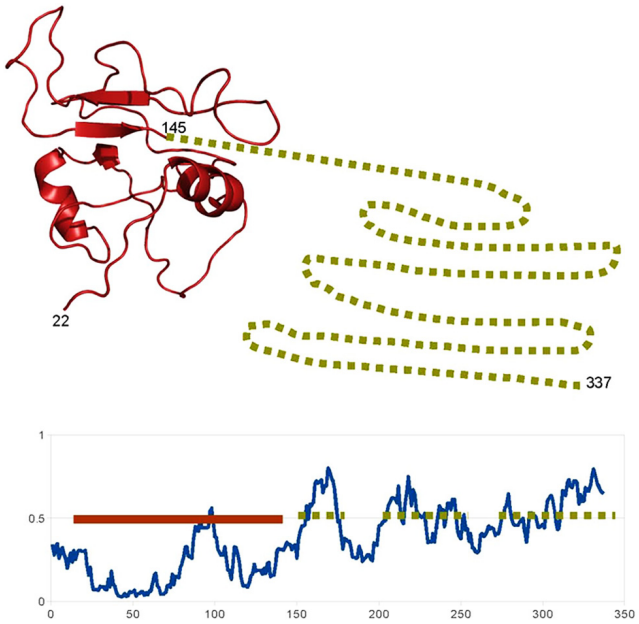

FIGURE 1 | Example of a highly disordered protein in *A. thaliana*. Schematic representation of the structural features of the "putative NAC domain-containing protein 94" (Uniprot: NAC94_ARATH). The disorder prediction of IUPRED (Dosztanyi et al., 2005) show that, according with the standard 0.5 threshold, most of the C-terminal part of the protein (from 150 to 337) is probably unstructured (dotted lines). The N-terminal DNA binding domain is probably structured and, indeed, a structural model can be generated based on the structure of a homolog (PDB:4dul_B, 62% sequence identity; solid line). So probably this protein "looks like" the representation above: a short structured DNA-binding domain followed by a long flexible disordered region, involved in the binding of different partners.

CONCLUSIONS

In summary, recent research is showing that, in contrast to the classical dogma, intrinsic disorder is an important feature for many proteins to function. In general, protein disorder allows interaction versatility and adds complexity to the interactomes. It is likely a way in which evolution can increase the complexity of biological networks without increasing excessively the size of the genomes. In plants, the predominance of intrinsic disorder in proteins involved in responses to environmental conditions could be explained as a requirement of these processes to be more complex due to the special characteristics of these sessile organisms.

ACKNOWLEDGMENTS

We thank the members of the Computational System Biology Group (CNB-CSIC) for interesting discussions. This work was partially supported by project BIO2010-22109 from the Spanish Ministry of Science and Innovation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 June 2013; accepted: 27 August 2013; published online: 12 September 2013.

Citation: Pazos F, Pietrosemoli N, García-Martín JA and Solano R (2013) Protein

intrinsic disorder in plants. Front. Plant Sci. ***4****:363. doi: 10.3389/fpls.2013.00363*
This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 06 September 2013
doi: 10.3389/fpls.2013.00352

Regulation of *Arabidopsis* root development by small signaling peptides

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Plant root systems arise *de novo* from a single embryonic root. Complex and highly coordinated developmental networks are required to ensure the formation of lateral organs maximizes plant fitness. The *Arabidopsis* root is well-suited to dissection of regulatory and developmental networks due to its highly ordered, predictable structure. A myriad of regulatory signaling networks control the development of plant roots, from the classical hormones such as auxin and cytokinin to short-range positional signaling molecules that relay information between neighboring cells. Small signaling peptides are a growing class of regulatory molecules involved in many aspects of root development including meristem maintenance, the gravitropic response, lateral root development, and vascular formation. Here, recent findings on the roles of regulatory peptides in these aspects of root development are discussed.

Keywords: small signaling peptides, small post-translationally modified peptides, regulatory peptides, root development, CLE, RGF/CLEL/GLV, IDA, CEP

INTRODUCTION

The entire plant root system is formed post-embryonically from a single primary root. Growth and development of this system requires coordinated regulation of hardwired developmental programs together with input from environmental signals (Casimiro et al., 2003; Malamy, 2005). While the classical phytohormones are key players in many aspects of root development, cell-to-cell communication is a vital component of most developmental processes. Regulatory peptides are one class of small signaling molecules that mediate intercellular communication. Roles for small signaling peptides in shoot development have been elucidated and are relatively well-characterized (Fukuda and Higashiyama, 2011). Recently, there has been a leap in our understanding of the roles of regulatory peptides in root development.

Small signaling peptides arise from genes that typically encode an N-terminal signal peptide region, one or more conserved peptide domains and variable regions that flank one or both sides of the discrete peptide domains. There are two structural classes of signaling peptides: small (5–20 amino acids) post-translationally modified peptides, which are the focus of this review; and larger cysteine-rich peptides (approximately 50 amino acids) that undergo disulfide bond formation as part of the maturation process. Common post-translational modifications to the smaller peptides class include hydroxylation, sulfation, and arabinosylation. These modifications may increase peptide stability, assist with receptor interactions and provide a further degree of regulation. The precursor proteins undergo processing to form the mature peptide product. While the maturation process is poorly understood, it was recently shown that four residues upstream of the peptide domain are required for CLE (CLAVATA3/ESR-related) peptide endoproteolytic processing (Ni et al., 2011). Most peptides are thought to act as extracellular signaling molecules that are ligands for membrane bound receptors although few ligand/receptor interactions have been validated (Matsubayashi et al., 2002; Hirakawa et al., 2008; Ogawa et al., 2008).

Several families of regulatory peptides, defined by homology of the peptide domain, have been implicated in various developmental processes in the roots. The CLE peptide family has a conserved 12–14 amino acid CLE motif at or near the C-terminus (Cock and McCormick, 2001). The RGF (ROOT GROWTH FACTOR) family of peptides, also known as GLV (GOLVEN) and CLEL (CLE-Like), has a conserved 14 amino acid peptide domain containing the tyrosine sulfation motif Asp-Tyr (Matsuzaki et al., 2010; Meng et al., 2012b; Whitford et al., 2012). The IDA (INFLORESCENCE DEFICIENT IN ABCISSION) peptide family has conserved, functionally active 20 amino acid motif (EPIP; Butenko et al., 2003; Stenvik et al., 2008). The CEP (C-TERMINALLY ENCODED PEPTIDE) family has a conserved 15 amino acid peptide domain with two proline residues that may be hydroxylated (Ohyama et al., 2008).

Recent work implicates regulatory peptides in many aspects of root growth and development, including meristem maintenance, gravitopism, lateral root (LR) development, and protoxylem differentiation (**Figures 1A–G**). This review outlines the recently discovered roles of these peptide families in root development.

ROOT MERISTEM MAINTENANCE

For continual growth and development, roots require a source of new cells that are able to differentiate into various tissue types. The root meristem contains a population of stem cells maintained by the quiescent center (QC). An extraordinary amount of precise regulation is required to ensure that the identity of the QC cells, the surrounding stem cells and their daughters is maintained. Several transcription factors are essential for this. *WOX5* (*WUSCHEL*-related homeobox 5) is expressed in the QC to maintain stem cell identity (Haecker et al., 2004). *SCR* (*SCARECROW*)

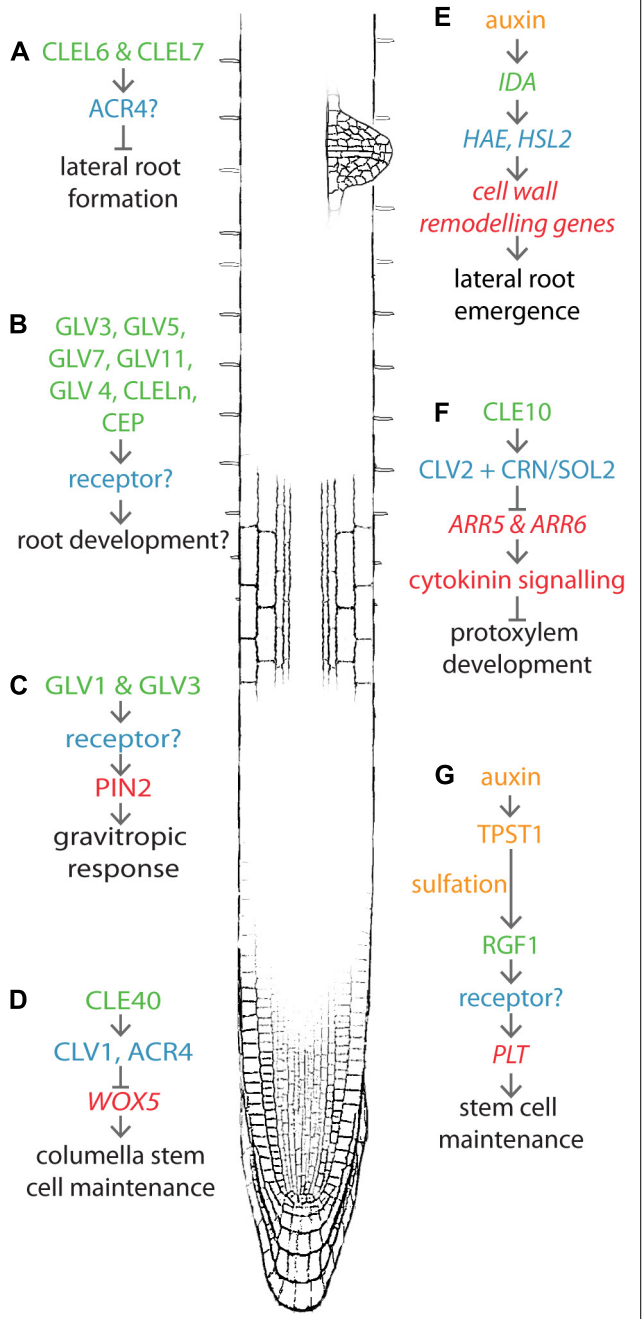

FIGURE 1 | Recent advances in peptide-mediated regulation of root development. CLE, RGF/GLV/CLEL, IDA, and CEP peptides are involved in several aspects of root development including lateral root formation **(A), (B), (D), (E)**, protoxylem development **(F)**, stem cell maintenance **(G)**, and gravitropic response **(C)**. Known pathways discussed in this review are shown near their approximate location in the root. Peptides are indicated in green text, receptors in blue. Upstream processes are yellow and downstream processes are red. Developmental output is indicated by black text.

and *SHR* (*SHORTROOT*) are expressed along the radial axis to provide positional information and also maintain QC identity (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003). *PLT1* (*PLETHORA1*) and *PLT2* (*PLETHORA2*) are auxin responsive genes that form a gradient distribution to maintain stem cell identity when expressed at high levels and promote differentiation when present at low levels (Aida et al., 2004; Galinha et al., 2007). An auxin maximum is also required in the stem cell niche (Sabatini et al., 1999; Friml et al., 2003).

A recent report implicated the CLE40 peptide in maintaining QC and columella stem cell identity (**Figure 1D**; Stahl et al., 2009). *CLE40* is expressed in the stele and in differentiating columella cells. *cle40* loss of function mutants had short roots with irregular tips caused by delayed differentiation of columella stem cells. Excessive CLE40 led to columella stem cell differentiation. The expression of *WOX5* was perturbed when CLE40 was deregulated, extending beyond the QC in the *cle40* mutant and being reduced in the QC and shifted toward the proximal meristem upon CLE40 peptide addition to WT plants. CLE40, together with ACR4 (*Arabidopsis* CRINKLY 4), a receptor-like kinase, regulated the spatial expression of *WOX5* to maintain columella stem cell identity. Additionally, the CLV2 (CLAVATA 2) leucine-rich repeat receptor was found to be necessary for CLE40-dependent root growth suppression, but not for differentiation of the daughters of columella stem cells (Stahl et al., 2009), indicating that the same peptide may interact with more than one receptor to regulate different developmental processes. Indeed, further investigation showed that subcellular localization, environment, and concentration affect the formation of receptor complexes (Stahl et al., 2013). The leucine-rich repeat receptor kinase CLV1 was shown to be involved in the signaling pathway of CLE40 and ACR4 (**Figure 1D**). *ACR4* and *CLV1* have similar expression patterns in the root meristem. CLE40 regulates *ACR4* expression but not *CLV1* expression. Using Förster resonance energy transfer and multiparameter fluorescence image spectroscopy, the transmembrane domains of ACR4 and CLV1 were shown to interact and form homomeric and heteromeric complexes depending on localization (plasma membrane or plasmodesmata). Both the *clv1* and *acr4* single mutants were impaired in aspects of meristem maintenance and responded differently to CLE40 peptide application. This indicated that the homo- and heteromeric complexes differentially regulate root meristem maintenance.

In another recent report, the sulfated RGF1 peptide was found to regulate meristem maintenance by providing a link between *PLT* expression and auxin through sulfation by TPST1 (TYROSYLPROTEIN SULFOTRANSFERASE 1; **Figure 1G**; Matsuzaki et al., 2010; Zhou et al., 2010). The *tpst1* mutant displayed pleiotropic effects including decreased meristem size and cell division activity, QC misregulation and starch granule accumulation. This mutant lacked the only copy of tyrosylprotein sulfotransferase found in the *Arabidopsis* genome (Matsuzaki et al., 2010; Zhou et al., 2010). *TPST1* was induced by auxin and helped to maintain auxin distribution by regulating expression of *PIN-FORMED (PIN)* and auxin biosynthetic genes (Zhou et al., 2010).

The RGF peptide family was found by a bioinformatic search for putatively sulfated peptide-encoding genes (Matsuzaki et al., 2010). Applying synthetic, sulfated RGF1 peptide to *tpst1* roots ameliorated the stem cell misregulation phenotype and increased cell division activity, indicating that sulfated RGF1 was required for these processes. Further investigation showed the sulfated peptides PSK (PHYTOSULFOKINE) and PSY1 (PLANT PEPTIDE

CONTAINING SULFATED TYROSINE 1) were also required for full restoration of cell division activity. Eight of the nine sulfated RGF peptides complemented the *tpst1* mutant in this way, however, non-sulfated peptides were unable to do so. It was found that RGF1 acts by regulating spatial expression and expression levels of *PLT* transcription factors. This work provided a link between auxin levels, the RGF peptide and *PLT* expression through TPST1. These studies also exemplified the extra layer of regulation that can be provided by post-translational modification of signaling peptides.

The *CEP* family has also been shown to affect root meristem development (Ohyama et al., 2008). Several forms of cleaved and modified peptides were confirmed using mass spectrometry on *CEP1* overexpression lines. Synthetic 15 amino acid CEP1 peptide application or *CEP1* overexpression arrested root growth. Meristematic cell division and expansion was repressed by CEP1, although QC specification was not affected. A more detailed analysis of the CEP family and the mode of action of individual CEPs is required.

VASCULAR DEVELOPMENT

Once cells leave the meristematic zone of the root, they go through phases of elongation and differentiation into specific tissue types. Procambial and cambial cells differentiate into xylem and phloem, the vascular tissues required for long-distance transport of water and nutrients though the plant. Several regulatory peptides are known to play important roles in vascular development, including the CLE-family member TDIF (TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR; Ito et al., 2006). More recently, another member of this family, CLE10, was found to inhibit protoxylem formation through interaction with cytokinin signaling (**Figure 1F**; Kondo et al., 2011).

A screen for *CLE* genes up-regulated during vascular development together with synthetic peptide assays implicated *CLE10* in early xylem differentiation (Kondo et al., 2011). CLE10 peptide application rapidly down-regulated the transcription of *ARR5* and *ARR6*, two type A *Arabidopsis RESPONSE REGULATORs* that negatively regulate cytokinin signaling. This indicated that cytokinin signaling was increased by CLE10, leading to decreased protoxylem development, one of the documented roles of cytokinin. The leucine-rich repeat receptor-like protein CLV2 and protein kinase CRN (CORYN)/SOL2 (SUPRESSOR OF LLP2) were both required for CLE10 suppression of *ARR5* and *ARR6* transcription, indicating they may be the receptor module for the CLE10 peptide.

GRAVITROPIC RESPONSE

Response to gravity stimulus is an essential component of root development. This process relies on gravity sensing in the root tip together with manipulation of auxin gradients, mediated by the localization of the *AUX1/LIKE-AUX1* family of auxin influx carriers and the *PIN* family of auxin efflux carriers (Vieten et al., 2007). The GLV/RGF peptide family has been implicated in this process (**Figure 1C**). Using a reverse genetic screen, Whitford et al. (2012) found that *GLV1/RGF6*, *GLV2/RGF9*, and *GLV3/RGF4* overexpression (or sulfated peptide addition) caused an altered gravitropic response in roots. GLV1/RGF6 and GLV3/RGF4 post-transcriptionally regulated PIN2 within minutes of peptide application, presenting evidence that GLV/RGF signaling controls the trafficking and stability of PIN2. A perturbation in the PIN2 pool would abolish the finely tuned auxin gradient necessary for normal gravitropic response. A loss of function *glv3/rgf4* mutant displayed gravitropic defects (Fernandez et al., 2013) implying GLV3/RGF4 is required for correct gravitropic response.

In a separate study, *CLEL/RGF* genes were found by homology with *CLE18*, a peptide precursor gene with a CLE motif in the variable region and a CLEL/RGF peptide domain at the C-terminus (Meng et al., 2012a). Unmodified CLEL6/RGF6 and CLEL8/RGF1 peptide application and *CLEL6/RGF6* and *CLEL7/RGF5* overexpression induced long roots on WT plants as well as an additional "wavy" root phenotype. This wavy root phenotype was reported to be independent of thigmotropism and phototropism. It was also reported to be independent of gravitopism as the phenotype persisted when unmodified CLEL6/RGF6 peptide was applied to *eir1-1* and *aux1-7* mutants, which are impaired in the gravitropic response. This is in disagreement with the aforementioned study which implicates CLEL6/GLV1/RGF6 in gravitropic response.

Interestingly, the peptide-encoding gene at the center of this debate, *CLEL6/GLV1/RGF6*, is reportedly not expressed in any part of the root (Whitford et al., 2012; Fernandez et al., 2013). Furthermore, a *GLV1/RGF6* knockdown line did not show impaired gravitropic response (Fernandez et al., 2013). This indicates regulation of peptide gene expression is paramount to its *in planta* function. Excessive peptide treatment by overexpression or peptide application should be interpreted in a biologically relevant context and should be supported by data from loss of function mutants.

LATERAL ROOT DEVELOPMENT

The formation of LRs allows the plant to exploit water and nutrients in the surrounding soil. LRs arise from a repetitive process which begins in the root meristem region. The earliest described event in LR development is priming or pre-branch site formation. This process requires auxin (De Smet et al., 2007) and the oscillation of over 3,000 genes (Moreno-Risueno et al., 2010). As the cells move into the differentiation zone, a site of relatively low auxin levels, LR founder cells are specified from xylem pole pericycle cells (Dubrovsky et al., 2008, 2011). The founder cells are initiated in an auxin-dependent manner (Dubrovsky et al., 2011) and begin to undergo a series of divisions. Eight stages of LR development have been defined (Malamy and Benfey, 1997), from the first asymmetric division (stage I) through many subsequent rounds of cell division (stages II–VII), to LR emergence (stage VIII). In order for the nascent LR to pass through the cortical, endodermal, and epidermal cell layers before emerging, auxin-dependent degradation and remodeling of cell walls is required (Swarup et al., 2008).

Peptides from the RGF family were reported to regulate LR development in an auxin-independent manner (**Figure 1A**; Meng et al., 2012a). Overexpression of *CLEL6 /RGF6* or *CLEL7/RGF5* resulted in a significant reduction in LR number, due to abnormal cell divisions at stage I of LR development. The authors speculated that CLEL6/RGF6 and CLEL7/RGF5 may interact with the

receptor kinase ACR4, as it too plays an important role in the early stages on LR development. Other RGF genes were found to be specifically expressed during different stages of LR development (Fernandez et al., 2013). Promoter reporter constructs showed that *GLV6/RGF8* was active from stage I of LR development, *GLV5/RGF2* and *GLV10/RGF5* at stage II, *GLV7/RGF3* and *GLV11/RGF1* at stage IV, *GLV3/RGF4* at stage V, and *GLV9* and *GLV2/RGF9* after emergence. Overexpression of these genes resulted in decreased LR number as well as root waving and enlarged root meristems. These data indicate that RGF peptides may act at different stages of LR development, however, specific mechanisms remain to be elucidated.

The IDA peptide and its receptors, HAE (HAESA) and HSL2 (HAESA-Like 2) were recently shown to play a role in LR emergence (**Figure 1E**; Kumpf et al., 2013) in addition to their known roles in floral abscission (Butenko et al., 2003; Stenvik et al., 2008). The *ida*, *hae*, *hsl2* single mutants, and *hae hsl2* double mutant showed a significant reduction in the number of LRs (Kumpf et al., 2013). LR primordia in these mutants encountered difficulties in penetrating the cortical, endodermis, and epidermal cell layers and often displayed irregular flattened shapes. *IDA* was strongly induced by auxin, whereas the two receptors were only transiently induced by auxin, indicating the receptors are used to limit IDA function. Two stages for IDA-mediated cell wall remodeling (CWR) were identified. During early primordia development (stage I and II), auxin from the LR primordium induced *IDA* expression in the endodermal cells, where HAE and HSL2 were already present. IDA signaling led to the expression of CWR enzymes, which allowed the nascent LR to pass through the endodermal cell layer. At a later stage in primordia development (stage V), auxin was derived from the auxin influx carrier LIKE AUX1-3 (LAX3), expressed in the neighboring cortical and epidermal cells. This induced the degradation of SOLITARY ROOT1 which in turn released the transcription factors AUXIN RESPONSE FACTOR (ARF) 7 and 19. ARF7 was required for the subsequent induction of *IDA* that triggered the expression of CWR genes through HAE/HSL2 signaling to allow the primordia to emerge from the parent root.

OTHER ROLES OF SMALL SIGNALING PEPTIDES IN ROOT DEVELOPMENT

A recent report described *CLELn* (*CLE*-Like protein in the nucleus), a RGF family gene that lacked the archetypal N-terminal signal sequence (Meng et al., 2012b). A green fluorescent protein (GFP)-fusion assay suggested this peptide localizes to the nucleus and western blots indicated it is specifically processed from the precursor. Overexpression of this gene gave a long root phenotype similar to *GLV2/RGF9* overexpression, however, synthetic peptide application elicited the long and wavy phenotype seen when GLV1/RGF6 and GLV11/RGF1 synthetic peptides were assayed. These data raise the possibility that regulatory peptides do not act solely as intercellular signals and may play roles in nuclear signaling or are secreted by non-conventional routes.

Regulatory peptides also play important roles in nodule organogenesis in legumes. Nodules form in response to infection by symbiotic bacteria called rhizobia. This process requires systemic regulation (autoregulation of nodulation; Caetano-Anolles and Gresshoff, 1991), a process that has parallels with shoot meristem regulation in *Arabidopsis* by *CLV3* and *CLV1* and their downstream processes (Mayer et al., 1998; Brand et al., 2000; Schoof et al., 2000; Leibfried et al., 2005; Osipova et al., 2012). Two root-specific pathways that involve Nod factors and cytokinin signaling are also required for nodulation. *Medicago truncatula CLE12* and *CLE13* are up-regulated in nodulating roots (Mortier et al., 2010). Upon overexpression, wild type plants do not form nodules. Suppression of nodulation by *Mt*CLE12 and *Mt*CLE13 is dependent upon *SUNN* (*SUPER NUMERIC NODULES*, orthologous to *CLAVATA1* in *Arabidopsis*) and induces type A response regulators, leading to cytokinin signaling (Mortier et al., 2010; Saur et al., 2011). Similar results were found in *Glycine max* (Reid et al., 2011) and *Lotus japonicus* (Okamoto et al., 2009), indicating at least two CLE peptides play an essential role in the autoregulation of nodulation. Recently a nodule-specific CLE in *L. japonicas* (CLE-RS2) was shown to be a root produced arabinosylated peptide (Okamoto et al., 2013). It interacted with HAR1, which shares functional similarity with SUNN, in an arabinose chain and sequence-dependent manner. CLE-RS2 was found in shoot-collected xylem sap, indicating that the CLE-RS glycopeptide is a long distance, root-to-shoot signal that controls autoregulation. These exciting results add a new dimension to how regulatory peptides control root development.

FUTURE PERSPECTIVES

Although a number of peptides from the CLE and RGF families have been implicated in different aspects of root development, the specific function and mechanistic action of other family members remains to be elucidated (**Figure 1B**). In a step toward this, the expression patterns and effects of misregulation of all RGF and CLE peptide family members have been assessed (Jun et al., 2010; Fernandez et al., 2013). Further work is required to elucidate the functions of the CEP family in root development.

While genetic redundancy has hindered elucidation of peptide function by loss of function mutants, a recently reported technology may assist in overcoming this. It was shown that by substituting Gly6 in the *CLV3* peptide domain for Ala or Thr, dominant-negative *clv3* phenotypes were obtained (Song et al., 2013). When applied in combination with the unsubstituted CLV3 peptide, it was shown that the antagonistic effect was a result of competition between the two peptides. It was hypothesized that $CLV3_{Thr6}$ was able to bind the CLV3 receptor without eliciting a response. This concept was also used to make antagonists for CLE8 and CLE22 peptides. This technology may assist in elucidating the function of specific regulatory peptides.

As highlighted in this review, small signaling peptides play important roles in root development. There are several layers of regulation which serve to add specificity to the roles of individual peptides. These include post-translational modification, tissue-specific expression, regulation of receptor expression, the subcellular localization of the receptor and the potential for long-distance movement. Recent bioinformatic approaches have indicated that over 7,000 small, unannotated open reading frames exist in the *A. thaliana* genome (Hanada et al., 2013). As proportion of these are likely to be regulatory peptide-encoding genes,

there is still a long way to go in fully exploring the extent of peptide-mediated developmental regulation.

ACKNOWLEDGMENTS

Christina Delay was supported by an Australia Postgraduate Award and GRDC Grains Industry Research Scholarship (GRS10329). This work was supported by an Australian Research Council grant to Michael A. Djordjeric and Nijat Imin (DP140103714).

CONTRIBUTION

Christina Delay wrote the paper. Nijat Imin and Michael A. Djordjevic critically read and edited the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 July 2013; accepted: 21 August 2013; published online: 06 September 2013.

*Citation: Delay C, Imin N and Djordjevic MA (2013) Regulation of Arabidopsis root development by small signaling peptides. Front. Plant Sci. **4**:352. doi: 10.3389/fpls.2013.00352*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 30 April 2013
doi: 10.3389/fpls.2013.00110

Finding missing interactions of the *Arabidopsis thaliana* root stem cell niche gene regulatory network

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Over the last few decades, the *Arabidopsis thaliana* root stem cell niche (RSCN) has become a model system for the study of plant development and stem cell niche dynamics. Currently, many of the molecular mechanisms involved in RSCN maintenance and development have been described. A few years ago, we published a gene regulatory network (GRN) model integrating this information. This model suggested that there were missing components or interactions. Upon updating the model, the observed stable gene configurations of the RSCN could not be recovered, indicating that there are additional missing components or interactions in the model. In fact, due to the lack of experimental data, GRNs inferred from published data are usually incomplete. However, predicting the location and nature of the missing data is a not trivial task. Here, we propose a set of procedures for detecting and predicting missing interactions in Boolean networks. We used these procedures to predict putative missing interactions in the *A. thaliana* RSCN network model. Using our approach, we identified three necessary interactions to recover the reported gene activation configurations that have been experimentally uncovered for the different cell types within the RSCN: (1) a regulation of *PHABULOSA* to restrict its expression domain to the vascular cells, (2) a self-regulation of *WOX5*, possibly by an indirect mechanism through the auxin signaling pathway, and (3) a positive regulation of *JACKDAW* by MAGPIE. The procedures proposed here greatly reduce the number of possible Boolean functions that are biologically meaningful and experimentally testable and that do not contradict previous data. We believe that these procedures can be used on any Boolean network. However, because the procedures were designed for the specific case of the RSCN, formal demonstrations of the procedures should be shown in future efforts.

Keywords: gene regulatory networks, Boolean models and functions, root stem cell niche, incomplete networks, predictive modeling, *Arabidopsis thaliana*

INTRODUCTION

The *Arabidopsis thaliana* root stem cell niche (RSCN) consists of a group of cells that rarely divide, known as the quiescent center, surrounded by four different types of stem cells (**Figure 1**; Dolan et al., 1993). The root stem cells produce all cell types necessary for the development of the primary root. Due to its architectural simplicity and its accessibility for experimental research at the genetic and molecular levels, the *A. thaliana* RSCN has become an important experimental model for molecular genetic studies in the last few decades. During this time, many important molecular mechanisms involved in the maintenance and development of the RSCN have been described (Sablowski, 2011; Azpeitia and Alvarez-Buylla, 2012). At least three molecular mechanisms have been uncovered as being fundamental for RSCN maintenance and development. The first mechanism involves auxin signaling and the PLETHORA (PLT) transcription factors that regulate auxin signaling (Galinha et al., 2007; Ding and Friml, 2010). The second mechanism involves the transcription factors SHORTROOT (SHR), SCARECROW (SCR), and some of their target genes (TGEN), as well as proteins that interact with them (Sabatini et al., 2003; Welch et al., 2007). The third mechanism includes CLAVATA-like 40 (CLE40) and WUSCHEL-RELATED HOMEOBOX 5 (WOX5; Stahl et al., 2009). Importantly, these three mechanisms are interconnected and present complex non-linear behaviors (reviewed in Azpeitia and Alvarez-Buylla, 2012).

Network models are an excellent tool for the integration and analysis of complex biomolecular systems, such as RSCN molecular mechanisms, at the structural and dynamic levels (de Jong, 2002; Alvarez-Buylla et al., 2007). In such models, the network nodes represent genes, proteins, RNA, or other molecular factors, while the edges correspond to positive or negative regulatory interactions among the nodes. Each node attains different values that

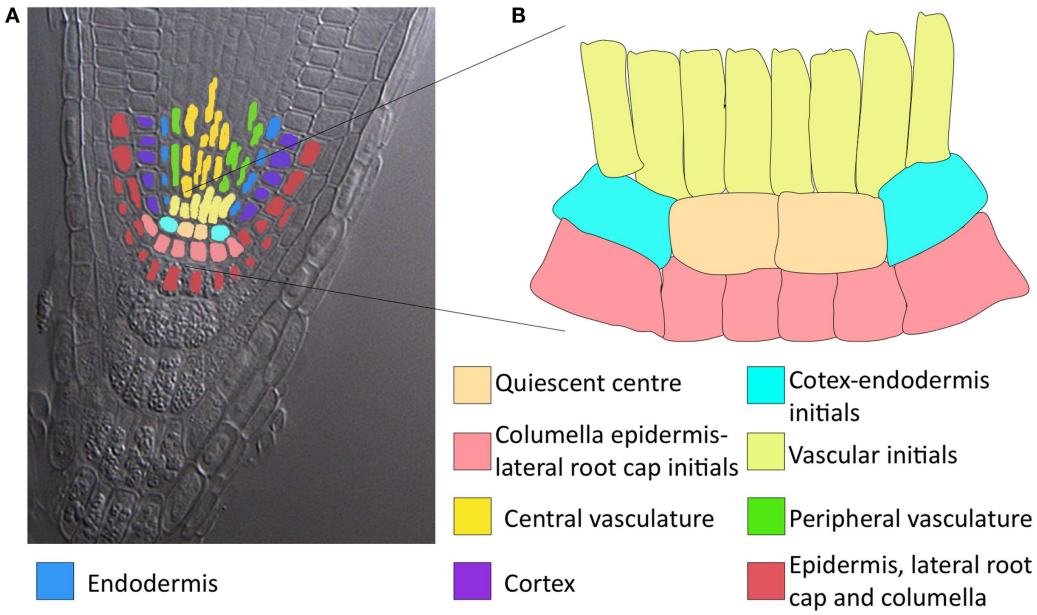


FIGURE 1 | Colour tracing of a confocal longitudinal section of an Arabidopsis root tip and a magnification of the RSCN. **(A)** Cleared root tip of Arabidopsis thaliana. The expected stable gene configurations that characterise each cell type are distinguished with different colours. As observed, some of the expected attractors represent more than one cellular type. QC, quiescent centre; END, endodermis; VI, vascular initials; CEI, cortex-endodermis initials; COR, cortex; PVC, peripheral vascular cells; CLEI, collumela-epidermis-lateral-root-cap initials; LCC, collumela and lateral root cap; and CVC, central vascular cells. **(B)** Amplification of the RSCN.

correspond to its expression or activity level, and the node's state changes in time depending on the state of the regulating nodes. The regulatory functions can be specified by different mathematical formalisms, but in all cases, these rules allow to follow the system's collective dynamics over time and find relevant dynamic properties of the entire regulatory system. Among these properties, self-sustained network states, referred to as attractors, have been found to be particularly relevant. Attractors may be either cyclic or fixed-point.

Dynamic network models allow analyses of the sufficiency of reported data to explain the observed behaviors and properties of a particular system (de Jong, 2002). For example, Kauffman (1969) proposed that the attractors of a given gene regulatory network (GRN) could represent the experimentally observed gene expression profiles or configurations that characterize different cell types in biological systems. If the experimental data are sufficient, the GRN model attractors should coincide with the gene configurations experimentally documented for the different cell types. This hypothesis has been explored and validated with networks based on biological data (e.g., Mendoza and Alvarez-Buylla, 1998; Albert and Othmer, 2003; Espinosa-Soto et al., 2004). In fact, we published a GRN model of the RSCN a few years ago (Azpeitia et al., 2010).

Over the past few years, experimental reports have improved our knowledge about the RSCN GRN (reviewed in Azpeitia and Alvarez-Buylla, 2012). Interestingly, when we incorporated new experimental data, the set of attractors recovered by the model drastically changed. The new GRN model was not able to recover the observed attractors and generated many attractors that had not been observed experimentally. In this case, some key components or interactions are presumed to be missing. In principle, with the inclusion of putative missing components or interactions it should be possible to recover the expected attractors. However, the identification of the missing data in general is a non-trivial task.

In continuous systems, the inference of missing data is complicated partly because once the new information is introduced, new parameters must be estimated or incorporated into the postulated kinetic functions, and this procedure can cause the reformulation of such functions. In contrast, discrete networks usually do not need to deal with complicated parameter estimation or adjustment, and the redesign of the interaction functions is usually simpler. Boolean networks (BNs) are arguably the simplest discrete modeling approach for dynamic networks. In BNs, nodes may attain only one of two values or states: *0* if the node is OFF, and *1* if the node is ON. The level of expression for a given node may be represented by a discrete variable x, and its value at a particular time $(t+\tau)$ depends on the state of other components in the network $(x_1, x_2, \ldots, x_n)$ at a previous time. The state of every node x therefore changes according to the following equation:

$$x_n\ (t+\tau) = F_n\left(x_{n_1}(t),\ x_{n_2}(t),\ \ldots,\ x_{n_k}\right) \qquad (1)$$

In this equation, $(x_{n_1}(t), x_{n_2}(t), \ldots, x_{n_k}(t))$ are the regulators of gene x_n, and F_n is a discrete function known as a Boolean function (BF). Such functions can be highly non-linear. Despite their simplicity, BN models have rich behaviors that yield meaningful information about the properties of the network under study. Because of this characteristic, BNs have been successfully used for the analysis of diverse GRNs (e.g., Albert and Othmer, 2003;

Espinosa-Soto et al., 2004). The main constraint for the detection of putative missing interactions in BNs is that the number of possible BFs for a node increases as a double exponential function, namely 2^{2^i}, where i represents the number of inputs regulating a target node. For example, a node with five regulatory inputs has 2^{32} ($\approx 4 \times 10^9$) possible BFs determining its dynamic response (**Figure 2A**). Similarly, the number of possible network topologies in a network is 2^{n^2}, where n represents the number of nodes. Hence, in a BN with five nodes, a total of 2^{25} ($\approx 3.35 \times 10^7$) possible topologies determine the GRN connectivity (**Figure 2B**). Most GRN topologies can be described by different sets of BFs. Thus, if we consider a BN with five nodes where all nodes interact with each other in every possible manner, $(2^{2^i})^5$ ($\approx 1.46 \times 10^{48}$) sets of BFs describe this topology. As observed, modeling the number of possibilities caused by additional components or links quickly becomes computationally intractable, even for such small networks using a simple Boolean formalism. Nevertheless, the dynamics of BNs with tens of nodes can be exhaustively analyzed in a relatively short amount of time, compared with other types of networks (e.g., Arellano et al., 2011). Thus, a methodology that allows for systematic integration and prediction of missing interactions in BNs would provide an instrumental tool in the proposal of a more complete RSCN GRN model and likely any other GRN.

Pal et al. (2005) studied how to produce a BN with a predefined set of expected attractors. Later, Zou (2010) studied how to obtain a set of expected attractors if the network topology exists and the BFs are partially known. Other researchers have investigated how to construct a BN from knowledge of the state-transition dynamics (e.g., Jarrah et al., 2007). Finally, Raeymaekers (2002) proposed that not all BFs are biologically meaningful and postulated a set of meaningful functions. The work of Raeymaekers is tightly linked to the so-called "canalizing BFs," which produce stable and more biologically realistic BNs (Kauffman et al., 2004). Because the RSCN GRN model already relies on published experimental data, the methodology should be able to not only produce meaningful BFs, maintain the topology and recover the set of expected attractors but also agree with previous data regarding the reported molecular interactions. Moreover, taking into account reported molecular experimental data may greatly reduce the number of possible BFs to test. For example, SHR and SCR are known to directly and positively regulate *MAGPIE* (*MGP*) expression (Levesque et al., 2006; Cui et al., 2007); therefore, the BFs where SHR or SCR do not promote *MGP* expression directly do not need to be tested.

In this paper, we updated the RSCN GRN model using experimental data that were reported after we published our last model. Interestingly, when we incorporated the new experimental data, the set of recovered attractors did not correspond with the experimentally observed gene configuration states in the RSCN. Thus, we designed a set of procedures to add all possible missing interactions one-by-one to the model without contradicting experimental data and to greatly reduce the number of possible BFs when trying to predict missing interactions for a particular node. Using these procedures, we explored the effect of adding putative missing interactions in the set of attractors. We considered that the addition of a putative missing interaction improved our model if the interaction reduced the number of non-expected attractors or increased the number of expected attractors recovered by the model. The interaction that most improved the model, by removing non-expected attractors or adding expected attractors, was incorporated into the model. If more than one interaction equally improved the model, one interaction was randomly selected and added to the BN model. After the inclusion of an interaction, we repeated the process until the inclusion of three consecutive interactions did not improve the model, or we exclusively obtained the set of expected attractors. Based on our results, we proposed three putative missing interactions that were biologically meaningful, could be tested experimentally and in conjunction were sufficient to recover the set of observed attractors of the RSCN GRN; however, these interactions were not sufficient to eliminate the non-meaningful attractors in the model. Interestingly, these three interactions were always the first to appear as putative missing interactions. After adding the three interactions, the procedures produced more putative missing interactions that reduced the number of meaningless attractors. However, this second set of putative missing interactions was more variable, and we were never able to exclusively recover the set of expected attractors, strongly suggesting that additional components are yet to be discovered. Nevertheless, we provide three concise and testable predictions that are in agreement with the data that have been reported on RSCN patterning.

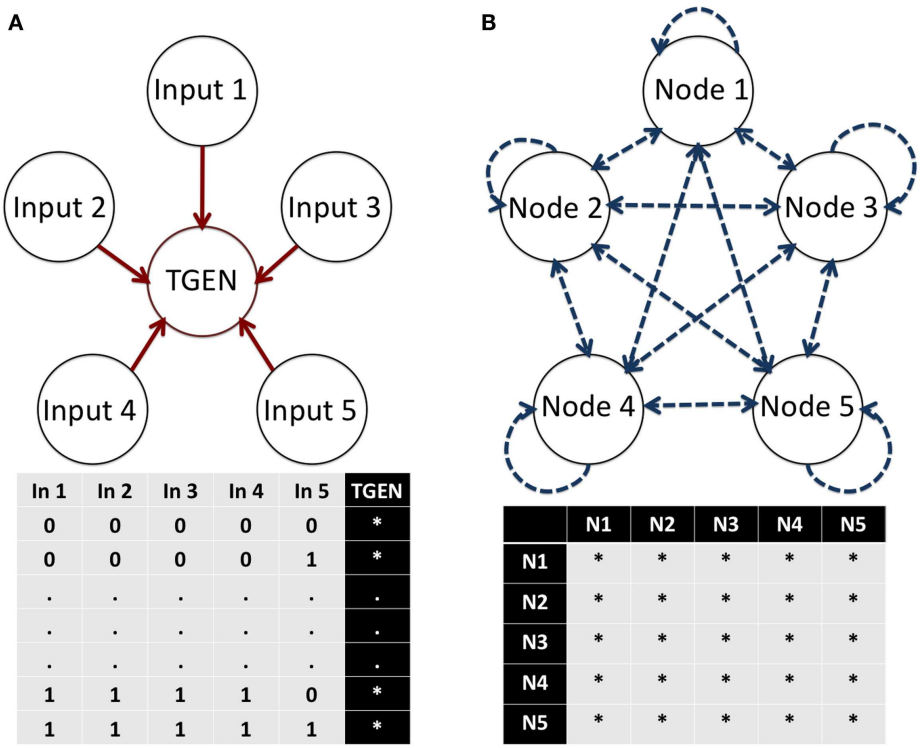
We believe that these procedures are useful for detecting missing interactions and possible incorrect gene regulatory or topological inferences due to incomplete data in any other GRN. However, because the procedures were generated *ad hoc* for the RSCN molecular interactions, generalization, and mathematical demonstrations of the procedures should be performed in the future to formally analyze the implications of using these procedures for any other GRN. Nevertheless, in the context of this study, we believe that these procedures may lead to novel research questions concerning general issues, such as the constraints that a given network topology imposes on the set of attractors.

METHODS

In this section, we describe the update to the RSCN GRN and the procedures used to reduce the number of possible BFs generated when trying to predict missing interactions and maintain previous experimental data. Then, we describe an evolutionary algorithm used to test the procedures in the GRN of the RSCN.

RSCN GRN UPDATE

Three main regulatory mechanisms have been involved in the development and maintenance of the RSCN. The first mechanism involves the transcription factor SHR of the GRAS gene family (Sena et al., 2004). SHR is transcribed in the stele, but its protein moves to the adjacent cell layer (i.e., QC, cortex/endodermis initials, and endodermis) (Nakajima et al., 2001). In the QC, cortex/endodermis initials and endodermis, SHR promotes the expression of SCR, another GRAS transcription factor (Cui et al., 2007). SHR and SCR form a complex and together they regulate the expression of many genes, including other transcription factors and miRNAs. Their targets include the transcription factors



In 1	In 2	In 3	In 4	In 5	TGEN
0	0	0	0	0	*
0	0	0	0	1	*
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
1	1	1	1	0	*
1	1	1	1	1	*

	N1	N2	N3	N4	N5
N1	*	*	*	*	*
N2	*	*	*	*	*
N3	*	*	*	*	*
N4	*	*	*	*	*
N5	*	*	*	*	*

FIGURE 2 | Number of possible BFs in a node and the topologies of a network. **(A)** The number of possible BFs for a particular node depends on the number of inputs or regulators of the node. In each possible state of its inputs, the node can assume a 0 or 1 expression value. Thus, 2^{2^i} possible BFs are available to describe the regulation of a node with i inputs. **(B)** The number of possible topologies of a network depends on the number of nodes. In a network, each node can interact with itself and any other node. Thus, n^2 possible interactions exist. Because each interaction can either exist or not exist, 2^{n^2} possible topologies describe a network with N nodes. E, Exist, and D, Do not exist.

JKD and *MGP*, as well as miRNA165/6 (Sozzani et al., 2010). JKD and MGP physically interact with SHR and SCR and are important for the regulation of *SCR* expression (Welch et al., 2007). The miRNA165/6 moves from its transcription domain and negatively regulates the expression of HD-ZIP III genes in the stele (Carlsbecker et al., 2010). The second mechanism is comprised of the auxin signaling pathway and their TGENs, such as the PLT transcription factors (Galinha et al., 2007). In the auxin signaling pathway, the transcription factors AUXIN RESPONSE FACTORS (ARF) form dimers with proteins of the Aux/IAA family (Guilfoyle and Hagen, 2007). In an Aux/IAA-ARF dimer, the ARFs cannot promote the expression of their TGENs. However, auxin promotes Aux/IAA degradation (Calderón Villalobos et al., 2012). Thus, as auxin concentration increases, the ARFs are released from the Aux/IAA negative regulation and promote the expression of their TGENs. The third mechanism involves the transcription factor WOX5, the mobile protein CLE40 (a negative regulator of WOX5) and their receptor ACR4 (Stahl et al., 2009, 2013). Importantly, these mechanisms interact with each other (Azpeitia et al., 2010).

To update our previous GRN, we first omitted the interactions predicted by our previous work that had not yet been confirmed experimentally and that were rather hypothetical (Azpeitia et al., 2010). The reason for this omission is that the objective of this work was to detect and predict missing interactions using a systematic approach that could be applied to any system. The only prediction in our previous model that we conserved is the negative regulation of WOX5 by CLE40 because this result was experimentally documented while the model was under review (Stahl et al., 2009). Then, we removed *PLT* genes from the model because even though these genes are essential for RSCN maintenance (Galinha et al., 2007), the *PLT* genes do not regulate any other node in the model under analysis and can therefore be collapsed (**Figures 3A,B**). We also corrected or completed data about the interactions among SCR, MGP, and JKD according to the results of Ogasawara et al. (2011). Thus, in this model, MGP does not act as a negative regulator of *SCR*; JKD is a positive regulator of *MGP* and itself; and MGP negatively self regulates (Ogasawara et al., 2011). Stahl et al. (2009) reported that the receptor ACR4 is necessary for CLE40 negative regulation of *WOX5* and is positively regulated by CLE40. Apparently, SHR and SCR regulation of *WOX5* is not direct (Sozzani et al., 2010). Moreover, SHR and SCR promote *miRNA165/6* expression, while miRNA165/6 represses PHB mRNA translation (Carlsbecker et al., 2010). According to Grigg et al. (2009), PHB overexpression prevents *WOX5* expression. Hence, we decided to delete the positive, direct regulation of *WOX5* by SHR and SCR because the regulation is not direct, and incorporate this positive regulation indirectly by the repression of PHB. Recently, PHB was reported to be a negative regulator of *JKD* (Miyashima et al., 2011). Because our model does not incorporate

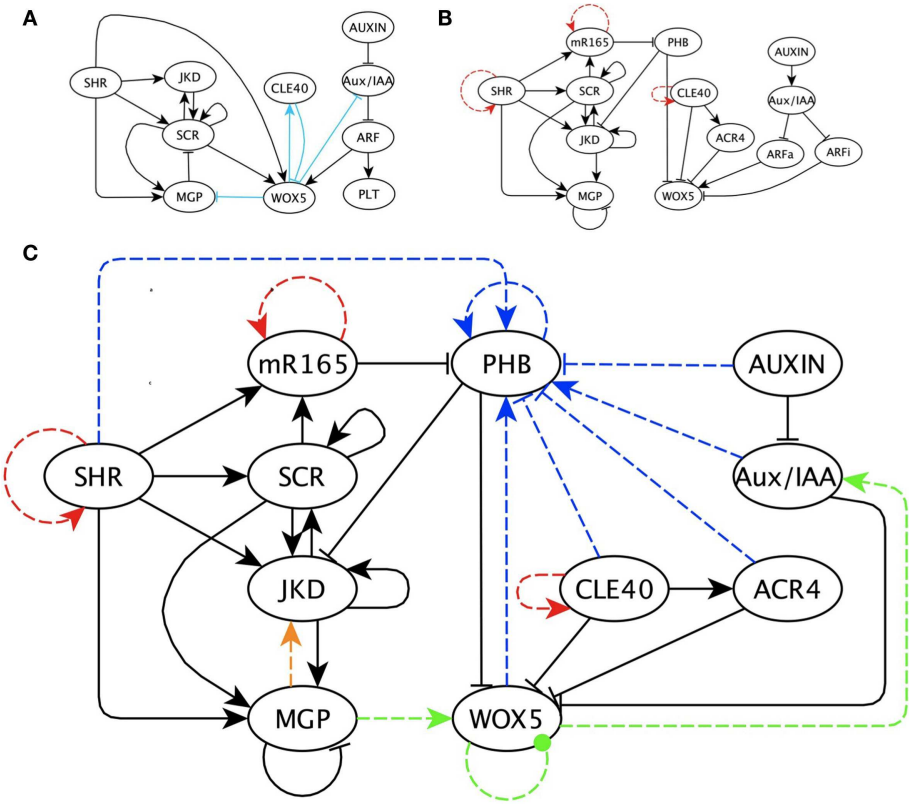


FIGURE 3 | The previous and updated RSCN GRN with predicted missing interactions. **(A)** Previously published RSCN GRN (Azpeitia et al., 2010). The light blue edges indicate previous predicted missing interactions. **(B)** Updated RSCN GRN as explained in the main text. The red edges are the self-regulations introduced to represent protein movement. **(C)** RSCN GRN with predicted missing interactions. For simplicity and clarity, intermediary nodes were not included in this GRN; however, these nodes are available in Supplementary Material. Yellow, green and blue edges are the three predicted interactions required to recover the expected attractors and are grouped according to the node's functions. The blue edges always indicate regulation of PHB. The yellow edge is a positive regulation of JKD by MGP. The green edges correspond to regulation of WOX5. The dotted green edge indicates a negative or positive regulation of WOX5 by itself.

space explicitly, we replaced molecular diffusion and movement by including a positive self-regulatory edge in nodes that move among cells (i.e., SHR, CLE, and miRNA165/6) to allow expression of these nodes where they move and no node positively regulates them. Finally, we reduced the auxin signaling pathway to the auxin and Aux/IAA nodes because the pathway is composed of linear path-like interactions that can be collapsed. In this way, we reduced the number of nodes in our network, and this change reduced the number of possible topologies and BFs describing the network once we incorporated putative missing interactions. In Section "Appendix 1 in the Appendix" we present the data and results of the analysis performed to reduce the auxin signaling pathway. We incorporated this information in the updated regulatory network model proposed here (**Figure 3B**). The main experimental data about gene interactions are presented in **Table 1**.

Importantly, the inclusion of a putative missing interaction in a node with four inputs was excessively demanding. To allow the addition of putative missing interactions in nodes with four inputs, we created intermediary nodes that integrate the influence of two regulators over any gene with four regulators (see Supplementary Material).

INTEGRATING AND FORMALIZING EXPERIMENTAL DATA INTO BN MODELS

As mentioned above, experimental data are formalized as BFs in BNs. BFs follow the equation:

$$x_n(t+\tau) = F_n(x_{n_1}(t), x_{n_2}(t), \ldots, x_{n_k}(t))$$

where $x_n(t+\tau)$ represents the state of node n at time $t+\tau$ (τ representing a positive integer), and $(x_{n_1}(t), x_{n_2}(t), ..., x_{n_k}(t))$ represents the state of the regulators of node x_n at time t. BFs can be described either as logical statements or as truth tables. Logical statements use the logical operators AND, OR, and NOT, while the state of node n at time $t+\tau$ is given for all possible combinations of its k regulator states of activation at time t in truth tables. Using the BFs of all nodes, we can follow the dynamics of the GRN until it reaches a stationary network configuration or state (attractor). A network configuration is the vector comprised of a set of values, where each value corresponds to the state of a specific node of the network. Single-state, stationary configurations are known as fixed-point attractors, while a set of network states that orderly repeat among each other cyclically correspond

Table 1 | Main experimental information used in the RSCN GRN.

INTERACTIONS	EXPERIMENTAL EVIDENCE	REFERENCE
SHR → *SCR*	The expression of *SCR* is reduced in *shr* mutants background. ChIP-QRTPCR experiments show that *SHR* interacts *in vivo* with the predicted regulatory sequences of *SCR* and positively regulate it	Helariutta et al. (2000), Levesque et al. (2006), Cui et al. (2007)
SCR → *SCR*	In *scr* mutant background promoter activity of *SCR* is absent in the QC and CEI. A ChIP-PCR assay confirmed that *SCR* binds to its own promoter and promotes its own expression	Sabatini et al. (2003), Cui et al. (2007)
JKD → SCR	The SCR promoter expression in QC and CEI is not detected in JKD mutants from early heart stage onward. JKD was able to activate luciferase gene expression driven by a 1.5 kb SCR promoter region	Welch et al. (2007), Ogasawara et al. (2011)
JKD → JKD	JKD was able to activate luciferase gene expression driven by a 3.5 kb JKD promoter region	Ogasawara et al. (2011)
JKD → MGP	JKD was able to activate luciferase gene expression driven by a 3.5 kb MGP promoter region	Ogasawara et al. (2011)
MGP −| MGP	MGP addition was able to inhibit the SHR, SCR, and JKD induced luciferase gene expression driven by a 3.5 kb MGP promoter region	Ogasawara et al. (2011)
SHR → KD	The post-embryonic expression of JKD is reduced in shr roots. A CHIP-chip analysis detected JKD as a direct target gene of SHR	Welch et al. (2007), Cui et al. (2011)
SCR → JKD	The post-embryionic expression of JKD is reduced in scr roots	Welch et al. (2007)
SCR → *WOX5*	WOX5 expression is reduced in shr mutants	Sarkar et al. (2007)
SHR → *WOX5*	WOX5 expression is undetectable in scr mutants	Sarkar et al. (2007)
Auxin signalin pathway → WOX5	In mp or bdl mutants background WOX5 expression is rarely detected	Sarkar et al. (2007)
Auxin signalin pathway −| *WOX5*	In *iaa17* mutants background *WOX5* expression is decreased	Ding and Friml (2010)
SCR → *miRNA165/6*	In *scr* single mutants, miRNA165/6 expression is greatly reduced. A ChIP-PCR assay confirmed that *SCR* binds to miRNA165/6 promoter	Carlsbecker et al. (2010), Miyashima et al. (2011)
SHR → *miRNA165/6*	In *shr* single mutants, miRNA165/6 expression is greatly reduced. A ChIP-PCR assay confirmed that *SHR* binds to miRNA165/6 promoter	Carlsbecker et al. (2010), Miyashima et al. (2011)
miRNA165/6 −| *PHB*	Over expression of *miRNA165/6* causes a decrease in the transcript levels of *PHB*. The allele *phb-1d* that expresses miRNA165/6-resistant PHB transcripts has ectopic *PHB* transcripts expression	Zhou et al. (2007), Miyashima et al. (2011)
PHB −| WOX5	In se mutants, which fail to repress PHB expression, embryonic WOX5 expression is absent	Grigg et al. (2009)
PHB → JKD	jkd transcripts levels are reduced in the phb-1d miRNA-resistant PHB allele	Miyashima et al. (2011)
CLE40 → *ACR*	CLE40p treatment strongly increased *ACR* expression	Stahl et al. (2009)
CLE40 −| *WOX5*	In *cle40* mutants the WOX5 expression domain is expanded, and CLE40p treatment reduced WOX5 expression in the QC	Stahl et al. (2009)
SHR → MGP	The expression of MGP is severely reduced in the shr background. Experimental data using various approaches have suggested that MGP is a direct target of SHR. This result was later confirmed by ChIP-PCR	Levesque et al. (2006), Cui et al. (2007, 2011), Welch et al. (2007)
SCR → *MGP*	SCR directly binds to the MGP promoter, and MGP expression is reduced in the scr mutant background	Levesque et al. (2006), Welch et al. (2007)

to cyclic attractors. Importantly, fixed-point attractors usually correspond to the arrays of gene activation states that characterize different cell types. Once we recover the set of attractors in the GRN, we can compare the attractors with the expected attractors, which are the experimentally observed stable gene expression configurations. The expected set of attractors are defined from gene expression patterns obtained from the literature that clearly define the spatio-temporal gene configuration of the system. Different data types, such as that obtained from transcriptional and translational reporter assays and microarrays, can be used to define the expected attractors. If the experimental information is correct, but the recovered and the expected attractors are not the same, then the GRN is likely missing information. One possibility is that there are missing interactions within the network.

To add putative missing interactions, two important issues must be considered.

(1) One needs to understand how the experimental data are contained in the BFs. In general, more than one logical statement exists for most BFs. Importantly, such equivalent logical statements can use different logical operators. For example, the logical statement "RGEN1 AND RGEN2" that uses the AND operator is equivalent to the logical statement "NOT (NOT RGEN1 OR NOT RGEN2)" which uses the OR and NOT logical operators. In contrast, a unique truth table represents each BF, indicating that the truth table is not arbitrarily selected. Indeed, each logical statement has an equivalent representation in a truth table, while each truth table can have many equivalent logical statements. Thus, in this paper, we use truth tables to analyze how the experimental information is formalized and contained in the BFs.
(2) One needs to realize that the same BF can formalize regulatory interactions documented with various types of experimental data. For example, we can infer that TGEN is regulated by Regulatory Gene 1 (RGEN1) through a loss-of-function mutant analysis or with a chromatin immunoprecipitation assay. Consequently, we may need to preserve the information gathered from different experiments and then formalize the same BF. Thus, the procedure through which we add putative missing interactions while maintaining congruence with the available experimental data depends on the specific set of experimental data available. In this work, we generated four different procedures by analyzing how the experimental information of the RSCN GRN is contained in the truth tables. The procedures were designed as follows.

Procedure 1

Add a putative missing interaction generated by gain and loss-of-function mutants (**Table 2**). When this procedure is used, each row of the truth table represents an experiment, and we can only state that under certain conditions the TGEN responds differentially to changes in the expression levels of other genes.

Procedure 2

Add a putative missing interaction to a truth table while maintaining the sign of the regulation of previously reported regulators (**Table 2**). Some experimental data clearly determine whether a gene is a positive or a negative regulator. When this case is true, we want to maintain that regulation with the same sign. Using this procedure, when we add a putative missing interaction, we exclusively generate BFs without changing the sign of the regulation of the RGENs that we want to maintain as positive or negative regulators.

Procedure 3

Add a putative missing interaction to a truth table while maintaining documented protein–protein interactions (**Table 2**). The experimental data can indicate that a pair or a group of genes act as complexes. However, this fact does not mean that all the proteins in a complex only function in the context of the complex. The proteins could act as individual units or form complexes with different proteins. This procedure allows putative missing interactions to be added while maintaining the functionality of the documented complexes. However, in the new BFs, the proteins in the complex can have new functionalities by themselves or with the putative missing regulator; the proteins can be substituted in or deleted from the complex under certain conditions; and new regulators can become part of the complex. For example, imagine a complex formed by proteins A and B. Once protein C is added as a putative missing regulator, the original protein A-B complex will continue to be a protein A-B complex, but now proteins A and B could also function in a protein A-C, B-C, or A-B-C complex.

Procedure 4

Add a putative missing interaction to a truth table where one or more of the nodes can act exclusively as part of a protein complex (**Table 2**). Contrary to the last procedure, the experimental data can indicate that a pair or a group of proteins are only functional when they work together. Using this procedure, we can maintain proteins as functional only when they form a complex, once a putative missing interaction is added. Importantly, proteins cannot be substituted or deleted from the complex under any condition. In contrast to procedure 3, the A-B complex cannot become an A-C or B-C complex. However, protein C could be included in the complex and function in an A-B-C complex.

We also designed two procedures that stem from the limits of the Boolean formalism, and we propose these procedures to simplify the interpretation of the predicted missing interactions. These procedures were designed as follows.

Procedure 5

Add a putative missing interaction while avoiding the generation of BFs where one or more nodes do not influence the activity of the target node (Raeymaekers, 2002) (**Table 2**). Notably, certain types of regulatory interactions cannot be expressed with a Boolean formalism (e.g., the modulation of protein activity by another protein). Thus, a TGEN may be regulated by a given RGEN even if this regulation is not explicitly reflected in the BFs. Given this uncertainty, we avoided generating these BFs.

Procedure 6

Add a putative missing interaction while avoiding the generation of BFs where one or more nodes act as positive and negative regulators in the same truth table (Raeymaekers, 2002) (**Table 2**). This

Table 2 | Summary of the procedures proposed to infer putative missing interactions in data-based network models.

Procedure	Application to inferring putative interactions
PROCEDURE 1	
Adding missing links in congruence with available experimental data that can be represented in single rows of truth tables	This is probably the most simple procedure. It allows modifying the network adding missing putative interactions, and at the same time the regulatory effects of the nodes whose role is based on experimental that is represented by single rows of the true tables is preserved. Examples of experiments represented by single rows are loss and gain-of-function mutants
PROCEDURE 2	
Adding missing links while maintaining the sign of the regulation	Prevents changes in the regulatory sign of genes when we introduce putative missing interactions
PROCEDURE 3	
Adding missing links while maintaining documented protein–protein interactions	This procedure guarantee that the joint action of proteins acting as complexes is respected in the new rows that result from the addition of new interactions. However, it allows new complexes to be formed, replacing, deleting or including one or more components in the complex
PROCEDURE 4	
Adding missing links while maintaining necessary protein–protein interactions	Procedure 4 is similar to procedure 3, since it also guarantee the joint action of proteins acting as complexes. However, this procedure do not allows the substitution or deletion of any of the components of the complexes. Importantly, it does allow the incorporation of other components in the complex
PROCEDURE 5	
Adding missing links without independent TGEN activity	Procedure 5 prevents the generation of BFs where one or more regulator has no effect on its target gene
PROCEDURE 6	
Adding missing links without ambiguous regulators	Procedure 6 prevents the emergence of nodes that act as global positive and negative (ambiguous) regulators at the same time

assumption is a simplification because these types of regulatory interactions have been reported experimentally. However, these interactions appear to be infrequent, and exclusion of these interactions allowed us to greatly reduce the number of BFs when we added a putative missing interaction.

A more detailed description of the procedures and their design is available in Section "Appendix 2 in the Appendix."

APPLICATION OF THE PROCEDURES TO POSTULATE A SET OF POSSIBLE NEW BFS GIVEN PUTATIVE MISSING INTERACTIONS IN THE *A. THALIANA* RSCN GRN

To detect and predict missing interactions, we applied an evolutionary algorithm using the following steps.

(1) Generate all putative single missing interactions. The putative missing interactions were those that were not already present in the model and were not contradicted by any available experimental evidence.
(2) Generate all possible BFs of the putative missing interactions maintaining consistency with available biological data using the above procedures.
(3) Test one-by-one all BFs generated and obtain the set of attractors generated with the added interaction.
(4) Select and incorporate into the model the BFs that most improved the model. The criteria to assess if the addition of a regulatory interaction conferred an improvement in the model were, in order of relevance: (a) if the BF increased the number of expected attractors recovered and (b) if the BF reduced the number of non-expected attractors. Here, we defined our expected attractors as the stable gene expression patterns observed experimentally in the RSCN of *A. thaliana* using transcriptional or translational reporter genes. Many genes have oscillatory expression behavior in the root (Moreno-Risueno et al., 2010). However, to our knowledge, none of the genes considered in the updated version of the RSCN GRN have this type of oscillatory expression behavior. Thus, for this particular case, reducing the number of non-expected attractors included eliminating the cyclic attractors.
(5) If more than one BF equally improved the fitness, one BF was randomly selected and added to the model.
(6) After the inclusion of a putative missing interaction, we returned to step 1 unless the model recovered only the expected attractors, or the inclusion of three consecutive BFs did not further improve the model fitness (**Figure 4**). In **Figure 5**, we present a pseudocode of the algorithm.

Using the above procedures, which greatly reduced the number of BFs to test (see below), the generation of all the predictions for each model implied testing approximately 100,000 different networks, which is a highly demanding computational process. Thus, we performed the algorithm 10 times, resulting in 10 different models that predicted different putative interactions. We were able to generate 10 different models because more than one BF equally improved the model each time, allowing us to randomly select different BFs. We applied our procedures using the algorithm to the Boolean GRN of the *A. thaliana* RSCN.

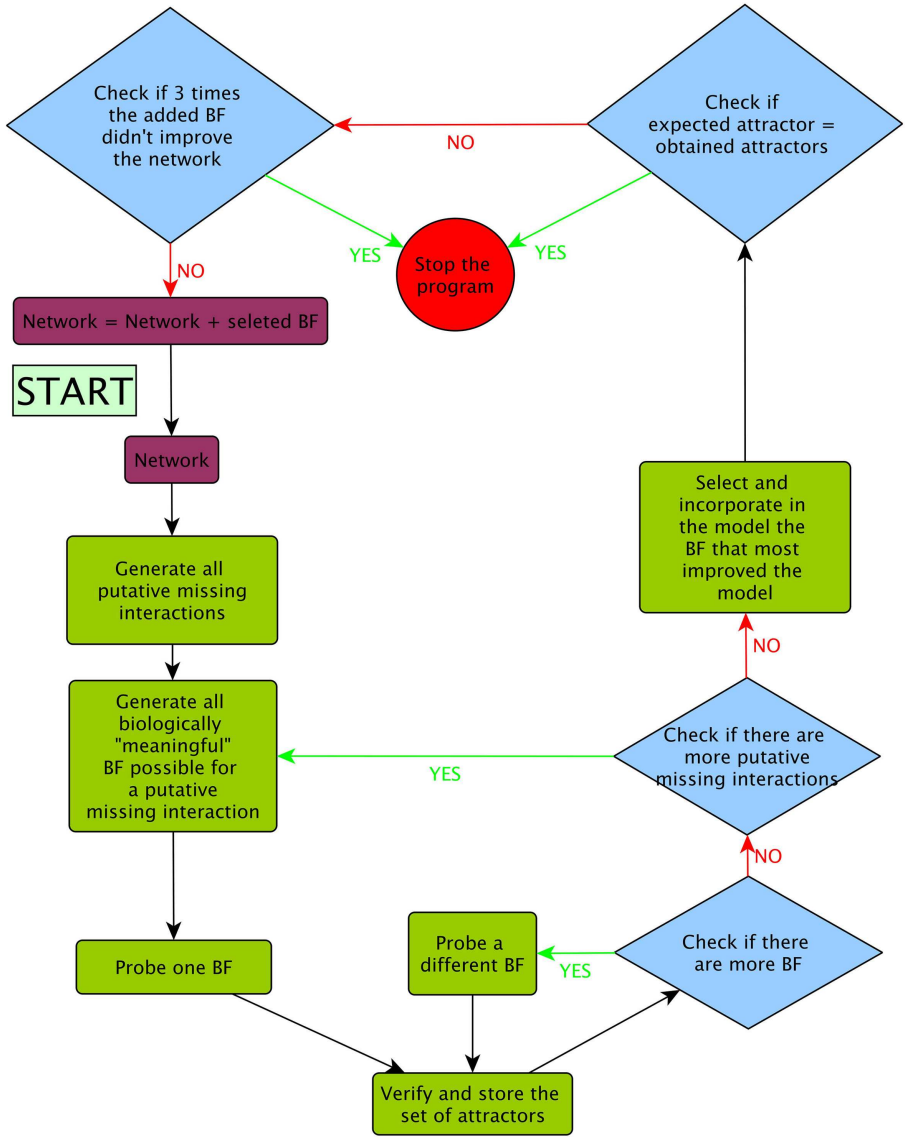


FIGURE 4 | Flux diagram of the evolutionary algorithm followed during the prediction of putative missing interactions using our procedures.

HYPOTHESIS

RSCN GRN updated model behavior

Based on available experimental data, we defined nine expected fixed-point attractors (**Table 3**) for each cell type in the RSCN and some root meristem cell types. Some attractors represented more than one cell type due to lack of experimental data in the model to distinguish among cell types (**Figure 1**). With the updated RSCN GRN model, we obtained 7 of the 9 expected attractors, 21 attractors without biological meaning in the RSCN context, and 4 cyclic attractors. This result suggests that missing data are yet to be incorporated into the RSCN GRN. Hence, we employed our set of procedures as described above to predict possible missing interactions in the network. The procedures used in each node depended on the type of available data for each gene. In **Table 4**, we present the procedures used to propose putative missing interactions for each gene, and the experimental information used in each case is provided in **Table 1**. The self-regulatory loops of nodes with movement must be positive, and hence, we applied procedure 2 in these nodes.

Predicted putative missing interactions in the RSCN GRN

The combined addition of three new regulatory interactions was sufficient to recover the expected attractors in the different cell types in the RSCN (**Figure 3C**). Interestingly, these three interactions were also the first ones to appear. No matter which order we included these three interactions, our methodology never proposed any other putative missing interactions until the three were included in the model. This result suggests that these three interactions are fundamental to recover the observed attractors in the RSCN. However, adding these three interactions was not sufficient

```
def improve_model(network_model, max_tries, expected_atractors):
  fitness = network_model.get_fitness(expected_atractors)
  possible_interactions = []
  new_networks = []
  if max_tries > 0:
    # Make a list of the other possible interactions
    possible_interactions = get_possible_interactions(network_model)
    # Try each additional interaction
    for interaction in possible_interactions:
      # Make a list of the rules for the additional interaction
      possible_rules = get_possible_rules(network_model, interaction)
      # Try each additional rule
      for rule in possible_rules:
        # Make a copy of the model
        new_network_model = NetworkModel(network_model.nodes,
                                         network_model.interactions,
                                         network_model.actualization_rules,
                                         network_model.atractors,
                                         network_model.fitness)
        # Add the new interaction
        new_network_model.add_interaction(interaction)
        # Add the new rule
        new_network_model.change_rule(interaction.target, rule)
        # Calculate the fitness of the new model
        new_fitness = new_network_model.get_fitness(expected_atractors)
        # Store_the_network at the correct place according to its fitness
        add_network_in_place(new_networks, new_network_model)
        if (new_fitness == 1):
          return new_networks
    # Find the fitness of the best new model
  new_fitness = new_networks[len(new_network) - 1].get_fitness(expected_atractors)
    # Find the index of one of the best models
    best_index = find_best(new_networks, expected_atractors)
  if new_fitnes > fitness:
        # If the best new model is better then the original one, continue improving it
    improve_model(new_networks[best_index], 3, expected_atractors)
  else:
        # If the best new model is not better then the original one, try to improve the result again
    improve_model(new_networks[len(new_network) - 1], max_tries - 1, expected_atractors)
```

FIGURE 5 | Pseudocode of the methodology used to incorporate putative missing interactions.

to eliminate cyclic attractors or several biologically meaningless attractors. In fact, the inclusion of these three interactions always increased the number of cyclic or unexpected attractors. We tried to avoid the increase of cyclic attractors by selecting only networks that simultaneously reduced the number of cyclic attractors and recovered biologically significant attractors. However, this procedure was unsuccessful (data not shown).

Interestingly, the three interactions mentioned above were functionally similar in the 10 replicas of the search process (**Figure 3C**). The first interaction is a regulation of PHB that restricts its expression domain to the vascular cells. The regulation of PHB was either positive regulation by those nodes with a similar expression domain (e.g., SHR and Aux/IAA) or negative regulation by those genes with a complementary expression pattern (e.g., CLE and ACR4). We postulate that the likely regulator of PHB is a member of the KANADI (KAN) gene family. *KAN* genes were not included in this GRN model because no connections with any node of the RSCN GRN in the root have been documented yet; however, KAN genes have antagonistic roles with PHB in the shoot and have a complementary expression pattern to *PHB* in the root (**Figure 3C**; Hawker and Bowman, 2004; Izhaki and Bowman, 2007).

The second interaction is a regulation over WOX5. Almost all the networks predicted that this regulation should be a feedback loop. The WOX5 loop could be direct or indirect, as well as positive or negative (**Figure 3C**). Interestingly, some experimental and theoretical evidence supports the existence of such a loop through the auxin signaling pathway (Gonzali et al., 2005; Azpeitia et al., 2010), and our results suggests that this feedback look could exist. However, contradictory experimental evidence has been reported on this issue. Positive regulation of *WOX5* by auxin has been reported (Gonzali et al., 2005), while other data suggest that auxin negatively regulates *WOX5* (Ding and Friml, 2010). Based on the interactome analysis, our model proposes that ARF activators are positive regulators, while ARF inhibitors are negative regulators of WOX5; therefore, this model includes both

Table 3 | Expected attractors.

CT/G	SHR	miR	JKD	MGP	PHB	SCR	IAA	A/I	WOX	CLE	ACR
CVC	1	0	0	0	1	0	0	1	0	0	0
PVC	1	1	0	0	0	0	0	1	0	0	0
End	1	1	1	1	0	1	0	1	0	0	0
Cor	0	1	1	0	0	0	0	1	0	0	0
LCC	0	0	0	0	0	0	1	0	0	1	1
VI	1	1	0	0	0	0	1	0	0	0	0
CEI	1	1	1	1	0	1	1	0	0	0	0
CLEI	0	1	0	0	0	0	1	0	0	1	1
QC	1	1	1	0	0	1	1	0	1	0	0

CT, Cell type; G, Gene; CVC, Central Vascular cells; PVC, Periferal vascular cells; End, Endodermis; Cor, Cortex; LCC, Lateral root-cap and columella cells; VI, Vascular initials; CEI, Cortex-endodermis initials; CLEI, Columella and lateral root-cap-epidermis initials; QC, Quiescent center; miR, miRNA165/6; IAA, Auxin; A/I, Aux/IAA; WOX, WOX5; CLE, CLE40; and ACR, ACR4.

Table 4 | Procedures used when adding putative missing interactions in each node.

	Procedure 1	Procedure 2	Procedure 3	Procedure 4	Procedure 5	Procedure 6
SHR	NO	YES	NO	NO	YES	YES
miR	YES	YES	YES	YES	YES	YES
JKD	YES	YES	YES	YES	YES	YES
MGP	YES	YES	YES	YES	YES	YES
PHB	YES	YES	NO	NO	YES	YES
SCR	YES	YES	YES	YES	YES	YES
Auxin	NO	YES	NO	NO	YES	YES
Aux/IAA	YES	YES	NO	NO	YES	YES
WOX5	YES	YES	YES	NO	YES	YES
CLE40	NO	YES	NO	NO	YES	YES
ACR	YES	YES	NO	NO	YES	YES

possibilities. With this model, we predict that *WOX5* should negatively regulate the auxin signaling pathway. Our model assumed that ARFa was always capable of promoting *WOX5* expression, as proposed by Vernoux et al. (2011). However, if the results that the negative regulation of *WOX5* by the auxin signaling pathway is stronger than the positive regulation, as Ding and Friml (2010) proposed, then the regulation of the auxin signaling pathway by WOX5 should be positive. The third interaction is a positive regulation of *JKD* by MGP (**Figure 3C**). The interplay between JKD, MGP, SCR, and SHR is complex (Welch et al., 2007; Ogasawara et al., 2011), and our simulations suggest that additional possible regulatory mechanisms should be considered, highlighting the ability of our procedures to detect probable missing data.

After the inclusion of 11–15 interactions, the performance of the resulting GRN models no longer improved. After this point, almost all models reduced both the number of cyclic and biologically meaningless attractors to three. Interestingly, some interactions were present in several of the 10 final models. Specifically, the most common interactions were: (1) inhibition of *SHR*, (2) activation of *SHR* by PHB, (3) negative regulation of auxin by PHB, and (4) negative regulation of *CLE40* by Aux/IAA or SHR. The BFs in the original model and the 10 models with putative missing interactions are available in Supplementary Material, and all putative missing interactions predicted by the whole set of simulations are available in Supplementary Material. Importantly, all putative missing interactions that were proposed using our procedures were biological meaningful, did not contradict previous experimental data, and are experimentally testable. Our results indicate key gaps in the data concerning the regulation of nodes in the RSCN GRN. Unraveling how these genes are regulated will be fundamental to our understanding of how the RSCN is maintained. However, our work already suggest possible nodes and missing interactions needed to obtain a sufficient model of RSCN patterning.

Efficiency of the procedures

The reduction of possible BFs obtained with our procedures is astonishing. For example, using procedures 4, 5, and 6 together on all regulatory genes, no matter the number of regulators, always resulted in 4 possible BFs. Using these procedures on all nodes is equivalent to reducing all nodes to 1, which represents a dimer or protein complex. This result was important for the RSCN GRN because SHR and SCR form a dimer that is only functional if both proteins are present (Cui et al., 2007). Thus, the TGENs of the dimer used procedure 4. Using this procedure, we only needed to test tens of BFs from the over four billion possible BFs of JKD

and MGP. Because of this reduction, we only tested ≈3,000 of $\approx 8 \times 10^9$ BFs to generate the first set of possible BFs in the model.

The efficiency of the use of procedures 1, 2, and 3 and combinations of the procedures needs to be formally analyzed in future studies. However, previous work demonstrated that using procedures 5 and 6 reduces the number of BFs from 16 to 8, 256 to 72, and 65,536 to 1882 for a node with 2, 3, and 4 RGENs (Raeymaekers, 2002), respectively. This previous study suggests that using combinations of our procedures should be able to reduce the number of BFs further, making the combinations useful in the prediction of putative missing interactions. The total reduction is completely dependent on the quantity and quality of the available experimental data, which will determine the procedures to use.

Usefulness of the procedures

In addition to the utility of the procedures for predicting putative missing interactions, we detected other important uses of the procedures. The first important use is evident when the experimental data are only sufficient to use procedure 1, or procedure 1 combined with procedures 5 or 6. In this case, positive regulators can be negative regulators, and *vice versa*. Thus, when we apply procedure 1 to predict a putative missing interaction, regulatory genes can change their sign of regulation. This result is important because it demonstrates that some experiments commonly used to infer gene regulatory interactions are not sufficient to assure the sign of regulation (see Appendix 2 in the Appendix). We can use procedure 1 to detect, and later test experimentally, if a positive regulator was identified as a negative regulator, and *vice versa*. We detected a second use when applying any single procedure or combination of procedures, except procedure 4. In this case, single proteins within protein complexes can act as independent units. The proteins are not necessarily required to act as independent units; however, this result helps us detect cases where proteins can, or need to, be substituted in a protein complex or when the proteins can regulate the activity of a TGEN as independent units or as units of different protein complexes. These predictions can be experimentally validated (see Appendix 2 in the Appendix).

DISCUSSION AND CONCLUSION

All, or most, GRN models are incomplete because they likely lack components or interactions due to incomplete experimental data and computational limitations. However, even for small BNs, the detection of such missing data is difficult because the number of possible BFs and topologies describing the interactions rapidly becomes overwhelming as the number of nodes and interactions being considered increases. We have proposed here a set of procedures that greatly reduce the number of possible interactions and enable the detection and prediction of biologically meaningful, putative missing interactions, while maintaining congruence with available and already incorporated experimental data. Our procedures were designed to maintain congruence with different types of experimental data and greatly reduce the number of possible BFs to be tested (≈3,000 out of over $\approx 8 \times 10^9$ in the example of the RSCN GRN). Importantly, we tested our procedures with smaller network motifs to assure that our procedures worked as expected before testing the procedures on the RSCN GRN.

The magnitude of the reduction in the putative BFs greatly depends on the quality of the available data and the nature of the interactions. Depending on the quality of the data, different BFs are generated. Importantly, our procedures demonstrate that some experiments that are usually used to determine the sign of a regulatory interaction are not reliable or are not adequate to uncover the actual interaction in diverse contexts (Lewontin, 2000). Similarly, some experiments that indicate the necessity of a protein complex for the expression of a TGEN are also not reliable. Furthermore, these situations are frequently not intuitive, and the procedures put forward here enable the detection of the circumstances under which such mistaken inferences can occur. Once the circumstance involved is known, we can easily design experiments to dismiss such situations. However, if we have enough experimental data to confirm the sign of the regulation or the presence of a complex, then we can use the proposed procedures to maintain these experimental data contained in the BF without change.

Using these procedures, we have designed an evolutionary algorithm to systematically predict possible missing interactions, and we have applied this approach to the *A. thaliana* RSCN GRN. Our work provides concise predictions concerning additional interactions and a novel RSCN GRN architecture that could be tested experimentally. Importantly, our work has identified three additional key interactions that could be studied: (i) regulation of *PHB* to maintain its expression pattern in the vascular cylinder, (ii) a feedback loop regulating *WOX5*, and (iii) positive regulation of *JKD* by MGP. However, we were not able to recover a network that attained only the experimentally observed gene configurations without the presence of unobserved attractors. Additional missing nodes, such as SCZ (ten Hove et al., 2010) or *root growth factors* (Matsuzaki et al., 2010), may be required to recover only the observed set of configurations. Because we were interested in finding missing interactions within already connected RSCN genes, we decided to dismiss genes that were unconnected from those included in this work, such as SCZ. Another possible explanation for why we never obtained only the expected attractors is that we only included putative missing interactions one-by-one. Including two or more putative missing interactions each time could change the results due to combinatorial effects. As explained previously, the computational demand for including one interaction can be very large. Hence, the computational demand of adding interactions simultaneously rapidly explodes. However, we believe that our approach provides a formal, systematic framework to postulate novel hypotheses concerning the way genes interact. For small networks, testing the effect of adding multiple interactions is possible.

There still are several improvements that could be done to the procedures. The inclusion of a genetic algorithm would allow a search for missing interactions not only one-by-one but also by sets of putative missing interactions at one time. Optimizing with Binary Decision Diagrams (BDDs) or more efficient algorithms could also allow for testing of more than one interaction. A way to simplify the use of our procedures is to incorporate them into an existing dynamic network analyzer (e.g., Arellano et al., 2011). Procedures that use information of the GRN topology or about the effect of how genes in the networks indirectly affect other genes should further reduce the number of BFs generated when we add

putative missing interactions. For example, we could already know that in the RGEN1 loss-of-function mutant, TGEN = 0, while RGEN3 = 1, but that RGEN3 is not a TGEN of RGEN1. In this case, if we add RGEN3 as a putative missing regulator of TGEN, we will know that in the new rows of the TGEN's truth table where RGEN1 = 0, TGEN expression value will be 0 when RGEN3 = 1 and TGEN's expression value will be unknown when RGEN3 = 0. The use of this type of data for the generation of more procedures was not explored in this work, but should be addressed in future research.

The fact that we used BNs in this work implies both strength and weakness. BNs allowed us to exhaustively test all the possible GRNs generated by adding putative missing interactions; however, BFs are unable to represent certain types of regulatory interactions, such as those implying fine-tuning modulations of regulatory activity. An improvement to our procedure would involve extending the procedures to consider multivalued discrete networks that can better evaluate more circumstances, although this method would also increase the computational demand.

Finally, given that the methodology used in this work can be applied to any BN, we believe that this type of exploration could help guide experimental research not only of biomolecular GRNs but also of any biological, physical, or theoretical system that can be formalized as a Boolean interaction network. For example, this methodology can be used to study the constraints that a given network topology imposes on attractor evolvability. However, formal mathematical demonstrations should be performed first.

ACKNOWLEDGMENTS

This paper constitutes a partial fulfillment of the Doctorado en Ciencias Biomédicas of the Universidad Nacional Autónoma de México (UNAM). Eugenio Azpeitia and Nathan Weinstein acknowledges the PhD scholarship and financial support provided by the Consejo Nacional de Ciencia y Teconología (CONACyT), and UNAM. This work is supported by CONACyT (180098; 180380; 167705; 152649; 105678) and DGAPA, UNAM (IN204011-3; IN203113-3; IN226510-3; IB201212-2) grants. Elena R. Alvarez-Buylla is currently sponsored by the Miller Institute for Basic Research in Science, University of California, Berkeley, USA. We thank Rigoberto V. Perez-Ruiz and Diana Romo for technical and logistical assistance. We thank M. S. Ursula Abad, Lynna Kiere, and Emilio Mora for their detailed revision of the manuscript as well as all of their comments. We also thank Dr. David Rosenblueth and Pedro Góngora for all the clarifying discussions about the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Systems_Biology/10.3389/fpls.2013.00110/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 February 2013; paper pending published: 12 March 2013; accepted: 10 April 2013; published online: 30 April 2013.

Citation: Azpeitia E, Weinstein N, Benítez M, Mendoza L and Alvarez-Buylla ER (2013) Finding missing interactions of the Arabidopsis thaliana root stem cell niche gene regulatory network. Front. Plant Sci. ***4****:110. doi: 10.3389/fpls.2013.00110*

This article was submitted to Frontiers in Plant Systems Biology, a specialty of Frontiers in Plant Science.

APPENDIX 1

AUXIN SIGNALING PATHWAY REDUCTION

In this section, we first briefly describe the auxin signaling pathway and then explain the analysis of the ARF-Aux/IAA interactome using MixNet, a publicly available software program designed for structural network analysis. For the interested reader a more detailed explanation of MixNet can be found in this reference (Picard et al., 2009).

In the auxin signaling pathway, the Aux/IAA genes repress the transcriptional activity of the ARFs by forming heterodimers. ARFs can be classified based on their transcriptional activity; ARFs 5, 6, 7, 8, and 19 are transcriptional activators (ARFa), while all other ARFs are putative transcriptional inhibitors (ARFi; Guilfoyle and Hagen, 2007). ARFa and ARFi compete for the same TGENs (Ulmasov et al., 1999). The SCF^{TIR1} ubiquitin ligase complex promotes Aux/IAA degradation in the presence of auxin, releasing ARFs from Aux/IAA inhibition. Once ARFs are released from Aux/IAA inhibition, ARFs are able to perform their transcriptional activity.

Recently, Vernoux et al. (2011) published an ARF and Aux/IAA interactome and analyzed how these proteins interact in the shoot and whole seedling using MixNet (Picard et al., 2009). MixNet uses a probabilistic clustering method that allows for the identification of structural connectivity patterns. Because MixNet relies on an algorithm that does not make any *a priori* assumptions about network structural properties, MixNet allows a blind search of highly or poorly interconnected groups of nodes. MixNet considers that nodes can be divided into Q connectivity classes, with Q being unknown. As a result, MixNet returns to the user a value α, which is the proportion of each group, and π, the connectivity of the groups. Finally, if $Z_{iq} = 1$, then node i belongs to class q. Hence, MixNet describes the network topology using connectivity probabilities among nodes, such that $\pi_q p$ represents the probability for a node from group q to be connected to a node from group p (Picard et al., 2009). Model selection in MixNet can be performed based on the ICL and incomplete data likelihood criteria.

Rademacher et al. (2011) reported the expression patterns of ARFs in the root, while Brady et al. (2007) created a high-resolution expression map of the root that included the Aux/IAA gene family. We defined the Aux/IAA and ARF genes that are expressed in the root based on these previous studies and analyzed their interactome reported previously (Vernoux et al., 2011). Based on these considerations, we considered the following ARF and Aux/IAA genes:

ARFs:
ARF1, ARF2, ARF5, ARF6, ARF7, ARF8, ARF9, ARF10, ARF16, and ARF19.

Aux/IAAs:
IAA1, IAA2, IAA3, IAA5, IAA6, IAA7, IAA8, IAA9, IAA10, IAA11, IAA12, IAA13, IAA14, IAA16, IAA17, IAA19, IAA20, IAA27, IAA28, IAA29, IAA30, IAA32, IAA21, and IAA33.

We applied the MixNet algorithm for $Q = 1$–15 clusters and used the ICL criterion for model selection. As Vernoux et al. (2011), reported, based on the ICL criterion, the MixNet analysis favors four clusters. However, this solution is only valid for a large N; therefore, we used the three cluster ($Q = 3$) solution as reported in Vernoux et al., 2011. This solution implies that the Aux/IAA and ARF proteins are divided into three different groups. The first group was comprised mostly of Aux/IAA proteins, which interact among themselves, and ARFa. The second group was mostly comprised of ARFa, which interacts only with Aux/IAA. The third group was mostly comprised of ARFi, which does not interact with any other group. This model of the auxin signaling pathway is very general; however, as more information becomes available, a more detailed auxin signaling pathway will be possible.

The probability matrix π, the nodes comprising each cluster and the interactions among the Aux/IAA and ARF proteins extracted from the work of Vernoux et al. (2011) are given below.

π Matrix:
0.110916, 0.0848193, 0.275044.
0.0848193, 0.745456, 0.856257.
0.275044, 0.856257, 0.240615.

Cluster 1:
ARF1, ARF2, ARF10, ARF16, ARF18, IAA6, IAA11, IAA29, IAA31, IAA32.

Cluster 2:
IAA1, IAA2, IAA3, IAA7, IAA8, IAA10, IAA12, IAA13, IAA14, IAA16, IAA17, IAA19, IAA20, IAA27, IAA28, IAA30, IAA33.

Cluster 3:
ARF5, ARF6, ARF7, ARF8, ARF9, ARF19, IAA5, IAA9.

The only nodes that do not behave as expected were IAA5, IAA11, IAA29, IAA31, and IAA32, which belong to cluster 1, and IAA5 and IAA9, which belong to cluster 3. We expected these nodes belonged to cluster 2.

The topology of the auxin signaling pathway according to this result eliminated the Aux/IAA-ARFi interaction. In this model, ARFi modulates the ARFa response once ARFa proteins are released from Aux/IAA inhibition. However, in the presence of high auxin concentration, ARFa always activates its TGENs (Vernoux et al., 2011). Boolean models cannot represent the degree of the response due to the ARFa/ARFi ratio. Consequently, we eliminated ARFi from the GRN, resulting in a linear pathway where ARFa activity is only regulated by Aux/IAA in the GRN. Moreover, ARFa proteins are constitutively expressed in all cells of the root meristem, including the RSCN. Hence, ARFa does not need to be included in the GRN because its activity is equally represented by the auxin response that is triggered when the auxin concentration promotes Aux/IAA degradation. Consequently, we reduced the auxin signaling pathway to the auxin and Aux/IAA nodes.

APPENDIX 2

DETAILED DESCRIPTION OF THE PROCEDURES

In this section, we describe how we designed the 6 procedures used to infer putative missing interactions in the RSCN GRN. As

explained in the main text, we used truth tables to analyze how the experimental data are contained in the BFs. However, we should be able to write a negative regulator with a NOT operator and a protein–protein interaction with an AND operator. For this reason, we will continue to use logical statements whenever useful in this section.

Procedure 1: adding missing links while maintaining congruence with available experimental data that can be represented by a single row in the truth table

Let us assume a TGEN is expressed when RGEN1 and RGEN2 are both expressed, which is represented by the last row of the truth table in **Figure A1A**. In addition, we will assume that TGEN is not expressed in the single loss-of-function RGEN1 and RGEN2 mutants, represented by the second and third rows of the truth table, respectively. Finally, we will suppose that TGEN is not expressed when both RGEN1 and RGEN2 are not present, and this scenario is represented in the first row of the truth table. In this example, each experiment is formalized as a single row of the truth table. Now, how can a third RGEN3 be added to the TGEN's BF without contradicting the previously incorporated experimental data? Knowing that each row of the truth table represents one experimental result can help us. As observed, in the first row of the truth table without the addition of RGEN3, the TGEN expression state is *0*. To maintain consistency with these data in the truth table once RGEN3 is added, the TGEN expression state must remain at *0* in at least one of the truth table's rows where RGEN1 and RGEN2 are not expressed. The possible rows that fit these criteria are shown in **Figure A1B**. To maintain consistency with the experimental data contained in the other rows, we perform the same analysis of all other rows in the truth table. Thus, in this example, we must maintain at least one *0* for the expression value of TGEN

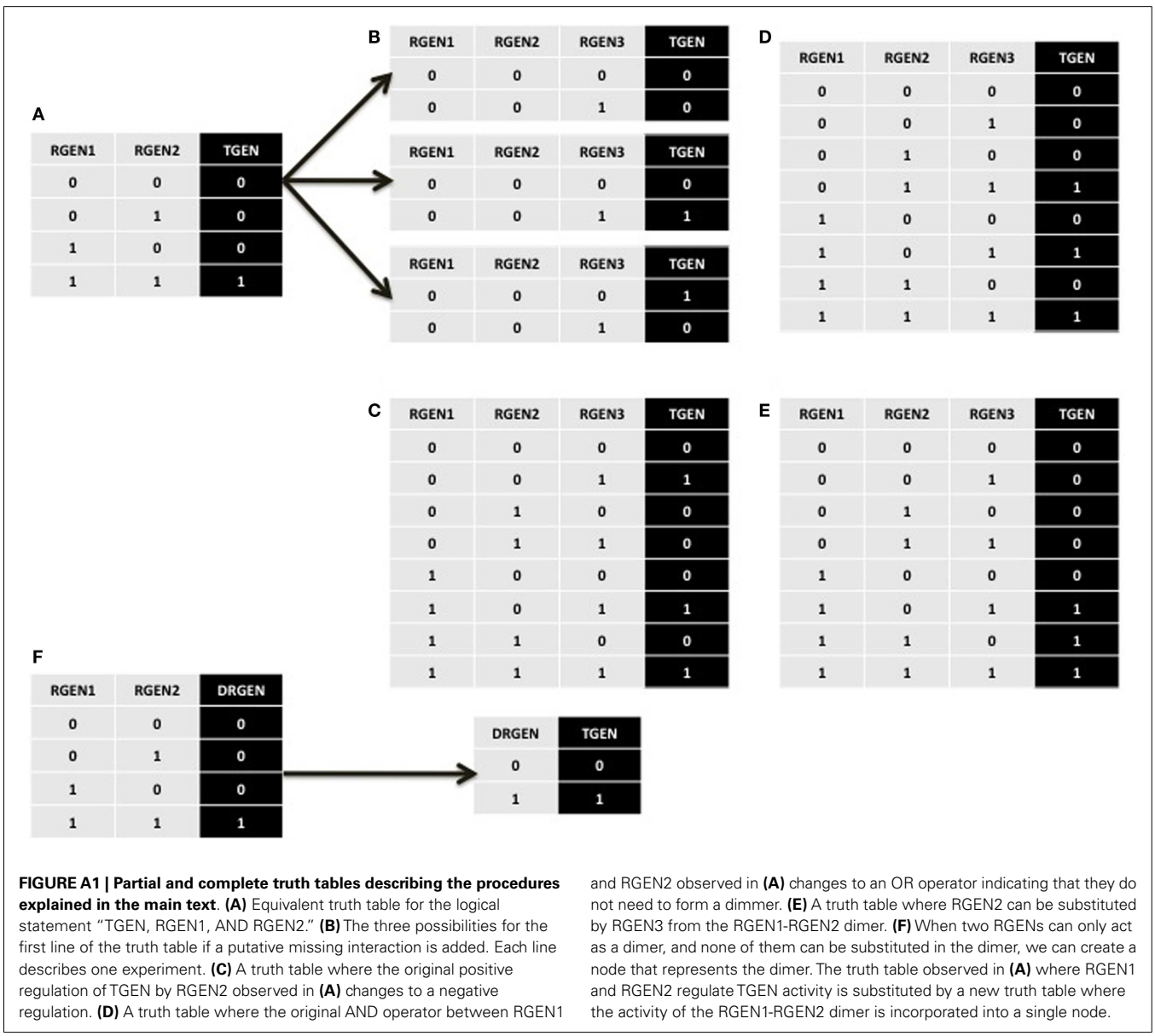

FIGURE A1 | Partial and complete truth tables describing the procedures explained in the main text. **(A)** Equivalent truth table for the logical statement "TGEN, RGEN1, AND RGEN2." **(B)** The three possibilities for the first line of the truth table if a putative missing interaction is added. Each line describes one experiment. **(C)** A truth table where the original positive regulation of TGEN by RGEN2 observed in **(A)** changes to a negative regulation. **(D)** A truth table where the original AND operator between RGEN1 and RGEN2 observed in **(A)** changes to an OR operator indicating that they do not need to form a dimmer. **(E)** A truth table where RGEN2 can be substituted by RGEN3 from the RGEN1-RGEN2 dimer. **(F)** When two RGENs can only act as a dimer, and none of them can be substituted in the dimer, we can create a node that represents the dimer. The truth table observed in **(A)** where RGEN1 and RGEN2 regulate TGEN activity is substituted by a new truth table where the activity of the RGEN1-RGEN2 dimer is incorporated into a single node.

whenever RGEN1 or RGEN2 are not expressed and a value of *1* whenever RGEN1 and RGEN2 are both expressed when RGEN3 is added. Hence, when each experiment is represented by a single row in a truth table, we need to maintain at least one *0* as the TGEN expression value under the conditions where no expression of TGEN was experimentally observed, and at least one *1* under the conditions where TGEN expression was experimentally observed; this process maintains consistency with the previously incorporated experimental data when putative missing interactions are added to the truth table. This procedure generates many possible BFs once we add a putative missing interaction. Nevertheless, the procedures is useful when an experiment is contained in a single row of a truth table. A common use of this procedure occurs when the only available experimental data are single and multiple gain- and loss-of-function mutants.

Procedure 2: adding missing links while maintaining the sign of the regulation

In **Figure A1C**, we present a truth table that we can be generated using procedure 1. One logical statement that can represent this function is "TGEN = RGEN3 AND (RGEN1 OR NOT RGEN2)." In this logical statement, RGEN2 changed from being a positive to a negative regulator of TGEN. However, we need to define positive and negative regulation in the BF context to assure that this change occurred.

If a RGEN positively regulates the TGEN, then we should observe in the truth table that when RGEN is ON, TGEN should also be ON, at least under one condition. Here, we defined a *condition* as the set of states where all RGENs of a TGEN have a fixed expression value, except the RGEN for which we are analyzing the sign of its regulation. Hence, we defined RGEN as a *local positive regulator* of TGEN, when TGEN and RGEN expression states are the same under identical conditions (**Figure A2A**). Conversely, we defined RGEN as a *local negative regulator* when TGEN and RGEN expression values are different under the same conditions (**Figure A2B**). Finally, we defined RGEN as a *local neutral regulator* of TGEN if the latter does not change its expression value irrespective of its regulator state (**Figure A2C**).

An absolute positive regulator of a TGEN should never be able to act as a local negative regulator of the target. However, this rule does not mean that a positive RGEN must activates the TGEN under all conditions. Thus, we defined a global positive regulator as a RGEN that acts as a local positive regulator or as a local positive and local neutral regulator. A global negative regulator acts as a local negative regulator or as a local negative and local neutral regulator. However, a global neutral regulator only acts as a local neutral regulator. Finally, we defined ambiguous global regulators as those RGENs that act as local positive and local negative regulators or as local positive, local negative, and local neutral regulators (**Figure A2D**).

A node labeled as a negative regulator according to our global regulator definitions can always be expressed with a NOT logical operator in the logical statement; however a negative regulator cannot be represented as a neutral regulator and may not be represented as a positive or ambiguous regulator as observed in **Figure A3A**. The same definitions apply to positive nodes. An ambiguous node according to our global regulator definitions can always be expressed as an ambiguous regulator, but not as

A

RGEN1	RGEN2	TGEN
0	0	0
0	1	1

C

RGEN1	RGEN2	TGEN
0	0	0
0	1	0

B

RGEN1	RGEN2	TGEN
0	0	1
0	1	0

RGEN1	RGEN2	TGEN
0	0	1
0	1	1

D

Type of global regulator	Positive local regulation	Negative local regulation	Neutral local regulation
Positive	Necessary	Forbidden	Allowed
Negative	Forbidden	Necessary	Allowed
Neutral	Forbidden	Forbidden	Necessary
Ambiguous	Necessary	Necessary	Allowed

FIGURE A2 | Partial truth tables representing local regulations and definitions of global regulators. A portion of the truth tables where RGEN2 acts as a local positive **(A)**, negative **(B)**, and neutral regulator **(C)**. **(D)** The definitions of global regulators based on local regulator definitions. The table shows which types of local regulations are necessary, allowed, or forbidden for each type of global regulation.

a positive, negative, or neutral regulator (**Figure A3B**). Finally, a truth table with a neutral global regulator can always be written as a logical statement without the inclusion of the regulator, even when the regulator can be included in another equivalent logical statement (**Figure A3C**). Thus, our global regulator definition assures that a regulator labeled as negative, positive, neutral, or ambiguous can be expressed in a logical statement with the correct logical operator.

Consequently, to verify the sign of the regulation, we use our global regulator definitions. Applying the global definitions, we can analyze the truth table in **Figure A1C** and observe that RGEN2 is a local negative and local neutral regulator of TGEN. Therefore, RGEN2 is a global negative regulator. In contrast, in the original truth table without RGEN3 added (**Figure A1A**), RGEN2 acted only as a local positive regulator; hence, RGEN2 was a global positive regulator. Thus, when we use procedure 1 to generate BFs, RGENs can change their sign of regulation. This result indicates that some experiments, such as the ones used in our example and which are commonly used to infer gene regulatory interactions, are not sufficient to assure that a RGEN is a positive or negative regulator. Importantly, the use of procedure 1 can identify the circumstances under which a positive regulator can be falsely identified as a negative regulator, and *vice versa*.

In some occasions, a RGEN is known to be either a positive or a negative regulator of its TGEN. For example, high quality experimental data, such as chromatin immunoprecipitation, might indicate that RGEN1 and RGEN2 are direct positive regulators of TGEN. These data will not change the truth table in **Figure A1A**. We can use our definitions to include putative missing interactions without changing the experimentally observed sign of the regulatory interaction. If RGEN is a known negative regulator, we use procedure 2 to exclusively generate all the BFs where RGEN acts as a negative local regulator or as a negative and neutral local regulator.

Using our global regulator definitions in procedure 2, the RGEN regulation sign can be expressed in the desired manner in a logical statement (e.g., with a NOT if we want a negative regulator). Thus, using our procedure, the RGEN regulatory sign can be expressed in a way that maintains consistency with the sign of regulation reported experimentally.

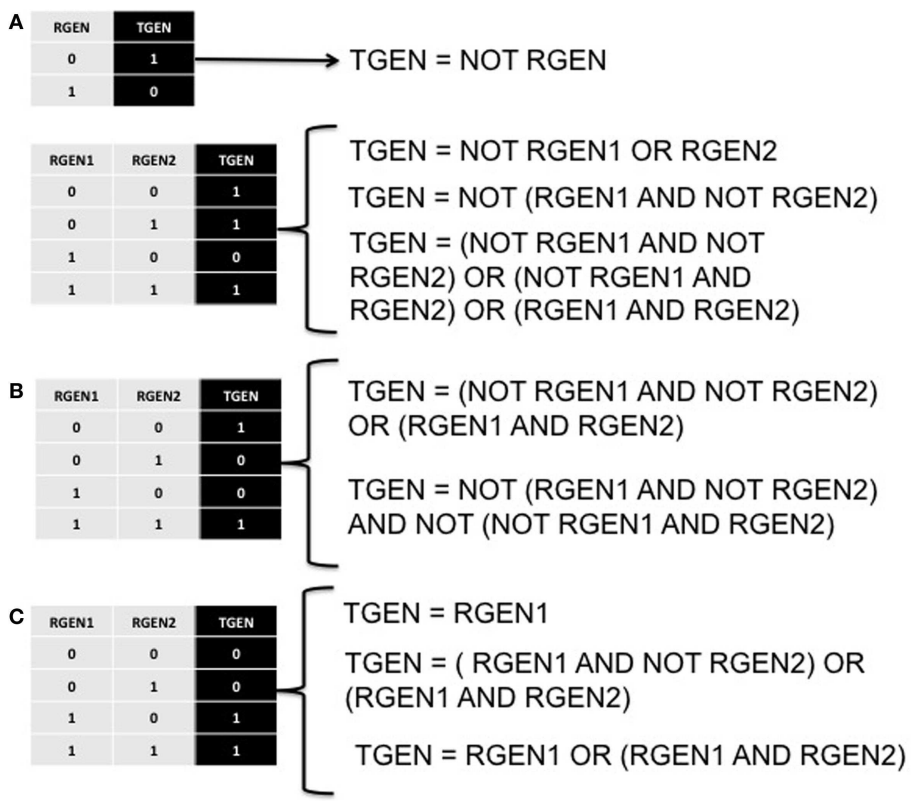

FIGURE A3 | Truth tables and equivalent logical statements for global negative, ambiguous, and neutral regulator definitions. **(A)** Two examples of a negative global regulator. As observed in the first example, the regulator can only be expressed with a NOT logical operator in the logical statement. In the example below, equivalent logical statements where RGEN1 acts as a negative and as an ambiguous regulator can be used. However, we can observe that if RGEN1 is labeled as a negative global regulator, we can always express RGEN1 in a logical statement with a NOT logical operator. **(B)** An example of ambiguous global regulators. In this example, the regulators can only be expressed as ambiguous regulators using a NOT logical operator in one part of the sentence and omitting the NOT operator in the other part of the sentence. **(C)** A neutral global regulator. The logical statement can be written without including the neutral global regulator (RGEN2). However, we can use an equivalent logical statement to include RGEN2 as a positive, negative, or neutral regulator. Neutral global regulators can always be removed from the logical statement.

Procedures 3 and 4: adding missing links while maintaining documented protein–protein interactions

In another scenario, a yeast two hybrid assay, or other method, confirmed that RGEN1 and RGEN 2 are not only positive regulators of TGEN, but RGEN1 and RGEN2 also interact at the protein level and form a dimer. In our example, when we add RGEN3, procedures 1 and 2 can generate the truth table observed in **Figure A1D**. One logical statement that can represent this function is "TGEN = RGEN3 AND (RGEN1 OR RGEN2)." Using this logical statement, RGEN1 and RGEN2 do not need to act as a dimer. However, we need to define the expression of a dimer in a BF before assuring the last statement.

Some transcriptional regulators act as dimers or more complex multimers. A TGEN activity is independent, locally, and globally, of a global neutral RGEN. However, if two RGENs function as a dimer, neither RGEN1 nor RGEN2 can act as local neutral regulators in the dimer. Anyhow, in the truth table in **Figure A1D**, RGEN1 and RGEN2 are local neutral regulators in both conditions where RGEN1 and RGEN2 could form a dimer, which is what we do not want that happens if we want to maintain the dimer functionality. Using the sign definitions defined previously, we can generate a procedure to generate BFs that maintain the dimer functionality, namely procedure 3. In this procedure, to maintain the dimer functionality, we need to verify that at least one local non-neutral regulation is specified for each RGEN in the same row, and in this row they must be capable to act as a dimmer (i.e., have a *1* expression value). Variations to this procedure can be used to maintain different types of interactions among regulators.

Finally, using procedure 3, we can generate the truth table observed in **Figure A1E**. One logical statement that can represent this function is "TGEN = RGEN1 AND (RGEN2 OR RGEN3)." This statement indicates that RGEN3 can substitute for RGEN2 in the dimer. However, the presence of a RGEN in a dimer can sometimes be necessary to regulate the expression of a TGEN. In this situation, the dimer RGEN1-RGEN2 is only functional when both proteins are together, and none of them can be substituted. The simplest way to maintain these data is by creating a new node, namely DRGEN (to indicate a dimer of RGENs) that represents the complex formed by RGEN1 and RGEN2. Subsequently, the TGEN truth table can be redefined in terms of DRGEN (**Figure A1F**). Using this method, none of the RGENs that form a complex can be substituted.

Is important to note that these first four procedures only use the available information about how RGENs regulate their TGENs. This implies that we do not include information about how RGENs affect each other or about the network topology nor any kind of partial information regarding possible indirect regulation of TGEN by RGENs. Thus, the procedures could be improved if we include data about the effect of RGENs on another RGENs of the GRN that are not their TGENs or if we use information about the network topology. The use of this data could reduce even more the number of BFs generated when we add a putative missing interaction, but will complicate the algorithm design and greatly increase the number of procedures. Because we did not include this kind of data to design the procedures, the number of possible BFs could be overestimated. However, the algorithms and procedures design is simpler.

Procedures 5 and 6: adding missing links without ambiguous regulators and incorporating independent TGEN activity

While the procedures described above were dependent on a set of experimental data that are available when reconstructing a truth

A

RGEN1	TGEN
0	0
1	0

RGEN1	TGEN
0	1
1	1

RGEN1	RGEN2	TGEN
0	0	0
0	1	0
1	0	1
1	1	1

B

RGEN1	RGEN2	TGEN
0	0	1
0	1	0
1	0	0
1	1	1

RGEN1	RGEN2	TGEN
0	0	0
0	1	1
1	0	1
1	1	0

FIGURE A4 | Truth tables with global neutral and ambiguous regulations of TGEN. **(A)** Truth tables with global neutral regulations of TGEN by RGEN1 or RGEN2. In the top two truth tables, we observe that the expression of TGEN does not change independently of the expression value of RGEN1. In the truth table below, we observe that the change in TGEN's expression value depends only on the value of RGEN1 but not on the expression value of RGEN2. **(B)** Truth tables with global ambiguous regulation of TGEN by RGEN1 and RGEN2. In both truth tables, TGEN's expression value is positively and negatively regulated by RGEN1 and RGEN2 indicating that both factors are ambiguous regulators of TGEN.

table with added interactions and/or nodes, these two additional procedures stem from the limits of the Boolean formalism, and we propose these procedures to simplify the interpretation of the predicted missing interactions and reduce the number of BFs generated when we add a putative missing interaction.

The activity of a TGEN may be independent of the activity of one or more of its RGENs. For example, in the truth tables in **Figure A4A**, which are represented with the logical statements "TGEN = 0," "TGEN = 1" and "TGEN = RGEN1," TGEN activity is not affected by RGEN1 in the first two and is independent of RGEN2 in the third one. Some of these cases appear because certain gene regulations cannot be represented as BFs due to missing data or the nature of the interactions. For example, the role of some proteins whose function is to modulate the activity of other proteins cannot be represented as a BF. Consequently, BFs where one or more of the RGENs were global neutral regulators were not considered because these BFs indicate that the TGEN activation state is independent of one or more RGEN or the RGENs regulatory effect cannot be represented with a Boolean formalism. We refer to this procedure as procedure 5.

Finally, we decided not to consider BFs where one or more RGENs were ambiguous global regulators (**Figure A2D**; see example of an ambiguous regulator in **Figure A4B**). This is a simplifying assumption, because some genes are indeed ambiguous regulators. However, some authors propose that a dual regulatory role is not common (Davidson, 2001), and a biological interpretation is difficult to provide in cases where the number of ambiguous RGENs increases in the BF. Constraining the BF to only those with unambiguous global regulators greatly reduces the number of BFs (e.g., only 1882 of 65,536 for four regulators; La Rota et al., 2011). We refer to this procedure as procedure 6. Importantly, the use of these last two procedures has been discussed and analyzed previously, demonstrating its utility and biological importance (Raeymaekers, 2002).

Using our set of procedures, we can incorporate putative missing interactions that are congruent with the available experimental data and imply novel predictions without contradicting previously available experimental data. The methodology is explained above.

frontiers in
PLANT SCIENCE

METHODS ARTICLE
published: 02 September 2013
doi: 10.3389/fpls.2013.00339

A robust family of Golden Gate *Agrobacterium* vectors for plant synthetic biology

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Tools that allow for rapid, accurate and inexpensive assembly of multi-component combinatorial libraries of DNA for transformation into plants will accelerate the progress of synthetic biology research. Recent innovations in molecular cloning methods has vastly expanded the repertoire with which plant biologists can engineer a transgene. Here we describe a new set of binary vectors for use in *Agrobacterium*-mediated plant transformation that utilizes the Golden-Gate Cloning approach. Our optimized protocol facilitates the rapid and inexpensive generation of multi-component transgenes for later introduction into plants.

Keywords: molecular cloning, *Agrobacterium* binary vectors, synthetic biology, combinatorial libraries, Golden Gate Cloning, plant transformation

INTRODUCTION

Assembly and transformation of a multi-component DNA construct such as a promoter and a reporter gene fusion is a common task in everyday plant research. Therefore optimization of this process is likely to yield major productivity gains. Traditionally, restriction endonuclease-mediated cleavage in combination with T4 DNA ligase-mediated joining has been used to create the desired DNA construct. However this method is time consuming, sequence dependent and is not well suited for high-throughput assembly of a large number of constructs.

The advent of recombination-based cloning utilizing λ phage Integrase (Hartley et al., 2000), which was later commercialized by Invitrogen as *Gateway® Recombination Cloning* reduced the time needed to assemble a construct. The later version of this strategy (*MultiSite Gateway®*) allowed for simultaneous cloning of up to three DNA fragments into a vector by utilizing mutant versions of the λ phage Integrase recognition sequences that are only functional in specific combinations (Petersen and Stowers, 2011). However, Gateway cloning has significant drawbacks, which include the high cost of cloning kits, the need to use specific bacterial strains to propagate plasmids carrying the *ccdB* gene and the presence of recombination scars in the final product that can have effects on gene expression or protein function.

Other non-Integrase based cloning techniques have been used in order to replace the traditional restriction endonuclease/ligase methods. Many of these techniques use the homology between different fragments of partially single-stranded DNA to anneal the fragments together and build the desired construct. They differ mainly in how the partially single-stranded DNA is initially generated. For example in Uracil Specific Excision Reagent (USER) cloning (Nour-Eldin et al., 2010), uracil bases are first incorporated into each DNA fragment using primers containing uracil instead of thymine. Uracil DNA Glycosylase (UDG) enzyme is then used to excise the uracil bases. Subsequent treatment of the excision site with DNA glycosylase-lyase Endonuclease VIII enzyme will result in a partially single-stranded DNA fragment.

Sequence and Ligase Independent Cloning (SLIC; Li and Elledge, 2012) uses the 3′ exonuclease activity of the T4 DNA polymerase, which is heavily favored in the absence of dNTPs in order to create partially single-stranded DNA fragments. Addition of the dCTPs will force the T4 DNA polymerase to switch from exonuclease to polymerase activity, and the absence of dATPs, dGTPs, and dTTPs results in a paused polymerase. Once different partially single-stranded fragments have been mixed and annealed to their targets, they will be transformed into *E. coli*, where the nicks are repaired.

Gibson cloning (Gibson, 2011) uses T5 exonuclease, which removes nucleotides in the 5′–3′ direction to create a DNA fragment with a 3′ single-stranded overhang. As in USER and SLIC, fragments are annealed together based on sequence homology at these single-stranded ends. Phusion DNA polymerase is used to fill in the gaps between the annealed DNA fragments and ligase seals the nicks. In general, homologous regions are recommended to be ∼25 base pairs (bps) in length although in many cases ≥15 bps overlap can work. In our experience, 70–80% of the colonies generated via Gibson cloning of two DNA fragments, have the desired construct (data not shown).

USER, SLIC and Gibson cloning methods enable the joining of DNA fragments without intervening unwanted DNA sequences. Gibson has been demonstrated to work well for ligating relatively large DNA fragments and was used to assemble the genome of *Mycoplasma mycoides* (Gibson et al., 2010). Both SLIC and Gibson cloning also have a number of weaknesses: (1) repeats in the homologous regions used to anneal DNA fragments can result in undesired side products (2) single-stranded DNA that

has a stable secondary structure such as a hair-pin, will not base-pair with its target (3) fragments smaller than 250 bps could be completely digested by the exonuclease before annealing, so optimization may be needed when working with fragments of this size class.

In Circular Polymerase Extension Cloning (CPEC; Quan and Tian, 2011) linear insert(s) and destination vector are first heat denatured, creating single-stranded DNA fragments that can anneal to their targets using their overlapping sequences. Subsequent DNA polymerase extension allows the previously single-stranded DNA fragments to act as primers to regenerate the desired DNA sequences as insert(s) in the destination vector. The overlapping sequences between inserts and the destination vector need to be carefully designed to be unique and have very similar (±2°C) high melting temperatures (T_m ~ 60–70°C) to minimize nonspecific-hybridization. Due to the dependence of this technique on DNA polymerase extension there is likely to be an upper bound in terms of the size of the final assembly, although an 8.4 kb plasmid assembled from four fragments has been previously reported (Quan and Tian, 2009).

While in theory it is possible to generate combinatorial libraries of constructs, using any of the methods mentioned above, in practice the demonstrated examples have been few and far between. However high-throughput combinatorial libraries of synthetic constructs have been implemented with ease using the Golden Gate Cloning strategy (Cermak et al., 2011; Engler and Marillonnet, 2011).

Golden Gate Cloning (Engler et al., 2008, 2009) uses Type IIS restriction enzymes. These enzymes cut at a single site outside of their recognition-binding site sequence. For example *BsaI* recognizes the sequence 5′-GGTCTC-3′, cleaves DNA one bp 3′ of the recognition site, and creates a 5′ overhang that is four bases in length. The 5′ overhang sequences can be designed to allow for annealing different fragments together. In a typical Golden Gate Cloning reaction, each fragment to be assembled into the destination vector is flanked by *BsaI* sites that, when cleaved, generate unique overhang sequences on either side of the insert. The destination vector will typically have *BsaI* sites that linearize the plasmid when digested and generate overhang sequences for ligation of the insert(s) (**Figures 1**, **A1**). The fragments are concatenated together using DNA ligase to generate the desired product. The digestion and ligation reactions take place in the same tube using alternating temperatures to drive the ligation and digestion reactions. Assembly of the correct vector is processive since ligation of the insert with the vector does not recreate the *BsaI* recognition sequence.

Here we describe a set of binary vectors for *Agrobacterium*-mediated plant transformation based on the Golden Gate Cloning strategy that can be utilized to generate transgenes of variable size and composition. We have also developed a binary vector that uses *SapI*, a Type IIS enzyme that uses a 7-bp recognition sequence and allows for additional flexibility when performing Golden Gate Cloning.

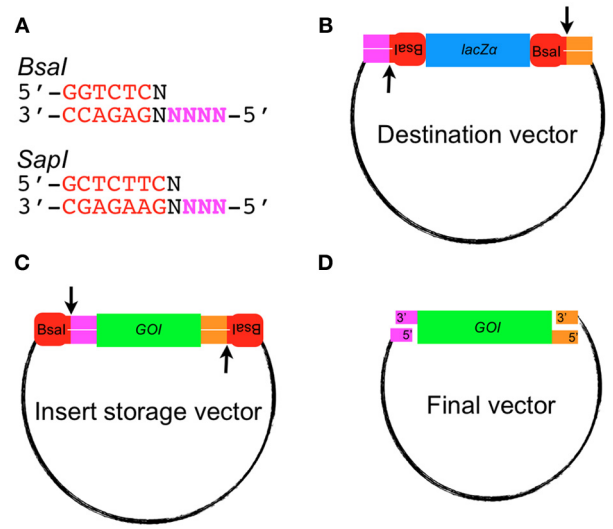

FIGURE 1 | Golden Gate assembly of an insert into the destination vector. (A) Recognition sequences for the Type IIS restriction endonucleases *BsaI* and *SapI*. 5′ overhang sequences used for annealing fragments are shown in bold magenta lettering. **(B)** Example of a Golden Gate-compatible destination vector. Note the orientation of the *BsaI* sites cause excision of the *lacZα* gene. **(C)** Example of a Golden Gate compatible vector containing a gene of interest (GOI) that will be released after digestion with *BsaI*. **(D)** A typical Golden Gate Cloning reaction would involve mixing the destination vector and insert storage vector together into one tube at equal molar ratio with *BsaI* and T4 DNA ligase. The final vector produced would lack *BsaI* recognition sequences and be resistant to digestion.

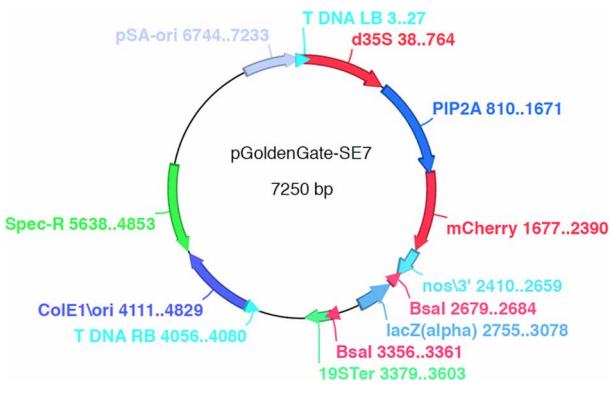

FIGURE 2 | pGoldenGate-SE7 map.

RESULTS

Three *Agrobacterium* binary destination vectors (pGoldenGate-SE7, pGoldenGate-SE9, pGoldenGate-MCY2) (**Figures 2**, **A2**, **A3**) have been constructed. All three plasmids use Spectinomycin resistance as a selectable marker in *E. coli* and *Agrobacterium*.

pGoldenGate-SE7 and pGoldenGate-MCY2 both use the *Pro35S:PM-mCherry* transgene as the selectable marker in plants. *Pro35S:PM-mCherry* drives expression of a plasma-membrane-localized mCherry constitutively in the plant including in the mature seeds (**Figure 3**). This selectable marker allows for the selection of T_1 transgenic plants by screening for red fluorescence in the dry seeds. Thus, seedlings are not exposed to antibiotics

or herbicides that may have a negative effect on normal growth and development, and T_1 plants can be used for analysis. The *Pro35S:PM-mCherry* reporter is also useful at later stages of transgenic line characterization for scoring the segregation ratio of the transgene. Use of this selection method is compatible with high-throughput selection approaches such as automated seed sorting (http://www.unionbio.com/copas). We have also generated the pGoldenGate-SE9, which uses Kanamycin resistance as an alternative selection marker in plants.

Our family of vectors include the *lacZ*α gene cloned between two *BsaI* or *SapI* sites, which allows for selection of recombinant clones based on blue-white colony color. The *lacZ*α gene is flanked by *BsaI* sites on both sides of the selection marker, and is excised from the vector upon digestion with *BsaI*. The excision of *lacZ*α will result in two four-base overhangs on opposite strands shown here in lowercase letters (3′-GAGCTC*tcat*-5′) and (5′-*tgga*AAGCTT-3′) adjacent to the underlined *XhoI* and *HindIII* sites on the destination vectors (pGoldenGate-SE7, pGoldenGate-SE9).

pGoldenGate-MCY2 uses *SapI* instead of *BsaI* as the Type IIS enzyme. *SapI* has a seven bp recognition sequence (5′-GCTCTTC-3′), and thus occurs less frequently in genomes than *BsaI*. *SapI* cleaves DNA one bp 3′ of the recognition site, and creates a 5′ overhang that is three bases in length. The three base overhangs allows for scar-less assembly of coding DNA sequences, since each codon is also three bps long. Digestion of pGoldenGate-MCY2 with *SapI* will result in two three-base overhangs on opposite strands shown here in lowercase letters (3′-GAGCTC*tca*-5′) and (5′-*tgg*AAGCTT-3′) adjacent to underlined *XhoI* and *HindIII* sites in the destination vector.

To introduce a transgene into our Golden-Gate vectors, individual components such as promoter and reporter genes are first amplified with PCR primers that incorporate *BsaI* or *SapI* sites with the desired overhangs at each end (**Figure A1**). These PCR products are cloned for sequence confirmation before use, for example, using pCR™-BluntII-TOPO® (Life Technologies) or other similar PCR-product cloning kits. The pCR-BluntII-TOPO vector itself contains a *BsaI* site, so it's important to avoid using its overhang sequence (5′-*gtta*-3′) when designing *BsaI* Golden Gate overhangs. The pCR-BluntII-TOPO vector also contains two *SapI* sites with overhangs (5′-*tcc*-3′) and (5′-*gca*-3′) which should be avoided when designing *SapI* Golden Gate overhangs.

To maximize success, all plasmids are first quantitated (e.g., by Qubit, Life Technologies) and then combined in an equal molar ratio in the reaction as shown in **Table 1**. We have been able to easily assemble five fragments in a single reaction using the pGoldenGate-SE7 vector. A detailed schematic of the two-component assembly using pGoldenGate-SE7 as the destination vector is shown in **Figure A1**. The largest size of a given insert fragment tested thus far was 3500 bps, and the smallest fragment was only three bps between the two single-stranded overhangs. On average we obtain about three blue colonies for every 1000 white colonies for the Golden Gate reactions. Remarkably of the 46 cloning reactions performed with pGoldenGate-SE7, the success rate of obtaining the correct multi-component plasmid was 100% even when only one white colony was screened per construct. We have successfully assembled constructs using inserts that were either first cloned into plasmids, generated by annealing pairs of short-oligonucleotides or as gel-purified PCR products.

We have used the Golden Gate assembly protocol from J5 (http://j5.jbei.org/j5manual/pages/81.html), except that supplementary ATP is added to optimize ligase activity. Single use aliquots of 10X Ligase buffer and 10 mM ATP are made to reduce freeze-thaw cycles of those buffers. In our experience the addition of more than 1 μl of T4 DNA ligase or 0.75 μl of high concentrated T4 DNA ligase can actually reduce the efficiency of the Golden Gate Cloning reaction. For *SapI*, it is important to use the

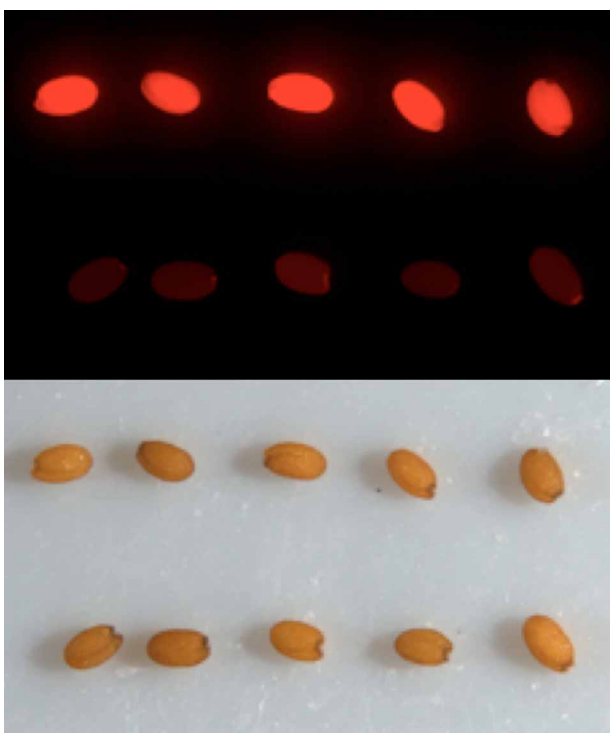

FIGURE 3 | *Pro35S:PM-mCherry* cassette (pGoldenGate-SE7, pGoldenGate-MCY2) enables the selection of transgenic (top row) vs. non-transgenic (bottom row) seeds. Fluorescence (top image) and bright field (bottom) images are shown.

Table 1 | Assembly of Golden Gate reaction.

Components	Amount
Destination vector (e.g. pGoldenGate-SE7)	100 ng
Each additional assembly piece	In equal molar concentration to destination vector
10X NEB T4 DNA ligase buffer	1.5 μl
10 mM ATP	1.5 μl
BsaI or high concentrated *SapI*	1.0 μl
NEB T4 DNA ligase (2 million cohesive end units/ml)	1.0 μl
dH_2O	To bring the total volume to 15 μl

high concentration stock (10,000 units/ml) of *SapI* enzyme. The PCR cycling parameters are shown in **Tables 2**, **3**. In our experience 25 repetitions of the first cycle is more that adequate to assemble two fragments of DNA (**Table 2**). We have used up to 50 repetitions of the first cycle in order to successfully assemble up to five fragments of DNA. 80°C heat treatment is used to inactivate both the *BsaI* and DNA ligase. Before transformation into *E. coli*, 1 μl of fresh *BsaI* is added to the reaction mix (for pGoldenGate-SE7 and pGoldenGate-SE9 plasmids only). The addition of *BsaI* after the inactivation of DNA ligase reduces the number of blue colonies to virtually zero. The total reaction volume before the addition of fresh *BsaI* is 15 μl. For *SapI*, no additional digestion is needed after the 25 cycles of digestion and ligation.

Additional *BsaI* site(s) within one or more of the DNA fragments being used can complicate cloning, since digestion at such sites (1) might generate overhang sequences that interfere with ligation of the intended construct, and (2) will cause cleavage of the vector prior to transformation into *E. coli*. To circumvent this problem, we first treat the destination vector with *BsaI*, and gel-purify the non-*lacZ*α fragment of the digestion. The non-*lacZ*α fragment will be used in place of the destination vector in the Golden Gate Cloning reaction with the PCR cycling parameter as shown in **Table 3**. In order to eliminate the possibility of nonspecific-hybridization to any partially single-stranded DNA other than its intended target, we design each overhang so that it differs in sequence from its non-target overhangs by at least two bps.

Table 2 | PCR cycling parameters for Golden Gate Cloning reaction where there are no internal *BsaI/SapI* sites within the insert fragment(s).

37°C 2 min.	25–50X
16°C 5 min.	
80°C 10 min.	
‡37°C 30 min.	

‡Before the start of this step, 1 μl of fresh BsaI needs to be added to the reaction mix.

Table 3 | PCR cycling parameters for Golden Gate Cloning reaction where *BsaI* site(s) exist within the insert fragment(s).

37°C 2 min.	25–50X
16°C 5 min.	
80°C 10 min.	
*16°C 2 h	

**Before the start of this step, 1 μl of fresh DNA ligase needs to be added to the reaction mix.*

Destination vector (pGoldenGate-SE7 or pGoldenGate-SE9) is first digested with BsaI, and the non-lacZα fragment of the digestion is gel-purify. The non-lacZα fragment is used in place of the destination vector in the Golden Gate Cloning reaction with the PCR cycling parameter as shown.

CONCLUSION

Until fairly recently, assembling a multi-component DNA construct was considered a project bottleneck. Thanks to what seems to be an ever-expanding arsenal of molecular tools and techniques, rapid, accurate and inexpensive assembly of multi-component constructs is now possible. The decreasing cost of gene synthesis allows for the construction of combinatorial multi-component DNA libraries where one or more parts may have variable sequences. Golden Gate Cloning allows for these multi-component libraries to be easily assembled and the use of fluorescence-based selection markers enable high-throughput methods for selection of transgenic plants.

MATERIALS AND METHODS

Invitrogen's One Shot® TOP10 chemically competent *E. coli* cell strain, which is *lacZ*Δ*M15*, was used to allow for blue/white color screening of colonies. Cells were grown on LB/Agar plates with 40 μg/ml each of Spectinomycin and X-gal. *BsaI (R0535L)*, *SapI* (R0569M, 10,000 units/ml), T4 DNA ligase (M0202M) were obtained from New England Biolabs. DNA quantitation was

Table 4 | Sequences to add *BsaI* or *SapI* sites to the PCR products prior to Golden Gate Cloning for a two-part (promoter plus reporter) scar-less assembly into the destination vector.

Primer	Recognition/Overhang	Sequence to add
BsaI forward promoter	5′-GGTCTCN**AGTA**-	Starting from the first bp of your promoter
BsaI reverse promoter	5′-GGTCTCN**TCAT**- [1]	Reverse complement of your promoter starting from the last bp
BsaI forward reporter	5′-GGTCTCN**ATGA**- [1]	Starting from the 5th bp of your reporter
BsaI reverse reporter	5′-GGTCTCN**TCCA**-	Reverse complement of your reporter starting from the last bp
SapI forward promoter	5′-GCTCTTCN**AGT**-	Starting from the first bp of your promoter
SapI reverse promoter	5′-GCTCTTCN**CAT**- [2]	Reverse complement of your promoter starting from the last bp
SapI forward reporter	5′-GCTCTTCN**ATG**- [2]	Starting from the 4th bp of your reporter
SapI reverse reporter	5′-GCTCTTCN**CCA**-	Reverse complement of your reporter starting from the last bp

[1] *Assumes the first 4 bps of your reporter is 5′-ATGA-3′.*

[2] *Assumes the first 3 bps of your reporter is 5′-ATG-3′.*

BsaI primers apply to the pGoldenGate-SE7 and pGoldenGate-SE9 plasmids, whereas SapI primers apply to the pGoldenGate-MCY2 plasmid. Overhangs are shown in bold.

done using the Qubit dsDNA HS Assay kit (Life Technologies Q32854). Gel purification of plasmid DNA was done on agarose gels stained with Crystal Violet (C3886 Sigma) 10 ug/ml. Gels were visualized in room light to avoid damage from UV-trans-illuminators. Excised gel fragments were purified using Qiagen's Qiaquick Gel Extraction Kit. **Table 1** shows the reaction mix used in a typical Golden Gate assembly and **Tables 2**, **3** shows the PCR cycling parameters. **Table 4** shows the sequences needed to add the *BsaI* or *SapI* sites to the PCR products prior to Golden Gate Cloning for a two-part (promoter plus reporter) scar-less assembly into the destination vector. Plasmid sequences and ordering information can be obtained through Addgene.org using the following identification numbers: (pGoldenGate-SE7: 47676), (pGoldenGate-SE9: 47677), (pGoldenGate-MCY2: 47679).

PLASMID CONSTRUCTION

pGoldenGate-SE7

The pCherry-pickerT plasmid (Duan et al., 2013) was used as a starting point for construction of our Golden Gate vectors. An existing *BsaI* site located outside of the *ccdB* gene was first mutated using the QuickChange® Site-Directed Mutagenesis kit (Stratagene catalogue # 200519). A *lacZ*α gene fragment isolated from pUC19 was PCR amplified to include *BsaI* recognition sites and cloned between the *HindIII* and *XhoI* sites of pCherry-pickerT to replace the *ccdB* gene.

pGoldenGate-SE9

A Kanamycin resistance gene was PCR amplified (∼1400 bps) from the dpGreenKanT plasmid and used to replace the *Pro35S:PM-mCherry* gene in pGoldenGate-SE7 between the *XhoI* and *AscI* restriction enzyme sites to create the pGoldenGate-SE9 plasmid.

pGoldenGate-MCY2

The *BsaI* cloning sites flanking the *lacZ*α gene in pGoldenGate-SE7 were replaced with *SapI* sites. The resulting PCR product was digested with *XhoI* and *HindIII* and ligated into pGoldenGate-SE7, resulting in pMCY1. A pre-existing *SapI* site and a second copy of the *lacZ* promoter that was downstream of the 19S terminator and the *HindIII* site were next removed. Gibson assembly was used to create the final pGoldenGate-MCY2 plasmid.

ACKNOWLEDGMENTS

We would like to acknowledge Luke Mackinder for advice on using Gibson cloning. Research in the Dinneny lab on gene regulation using synthetic biology approaches is funded by a National Science Foundation grant (1157895) awarded to José R. Dinneny.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 July 2013; paper pending published: 28 July 2013; accepted: 13 August 2013; published online: 02 September 2013.

*Citation: Emami S, Yee M and Dinneny JR (2013) A robust family of Golden Gate Agrobacterium vectors for plant synthetic biology. Front. Plant Sci. **4**:339. doi: 10.3389/fpls.2013.00339*

This article was submitted to Plant Systems Biology, a section of the journal Frontiers in Plant Science.

APPENDIX

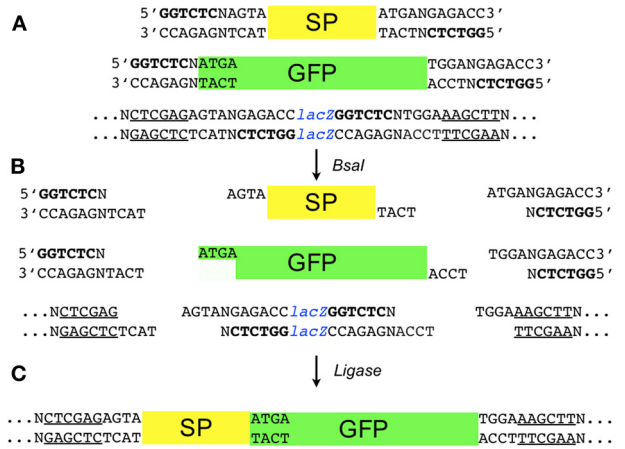

FIGURE A1 | Detail schematic of the two-component assembly using pGoldenGate-SE7 as the destination vector. (A) Synthetic promoter (SP), reporter (GFP), and the pGoldenGate-SE7 destination plasmid containing the *lacZ*α gene are put in the same reaction tube along with *BsaI* and the T4 DNA ligase. The recognition site for *BsaI* (5′-GGTCTC-3′) is shown in bold. The underlined segments are *XhoI* and *HindIII* sites flanking the *lacZ*α gene in the destination vector. **(B)** Shows the partially single-stranded DNA fragment generated after the digestion with *BsaI* **(C)** Ligase is used to assemble the desired product. Note that 5′-ATGA-3′ is the first four bps of the GFP coding DNA sequence and therefore this assembly is scar-less.

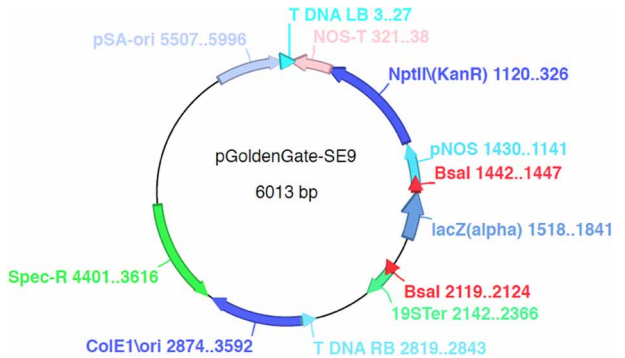

FIGURE A2 | pGoldenGate-SE9 map.

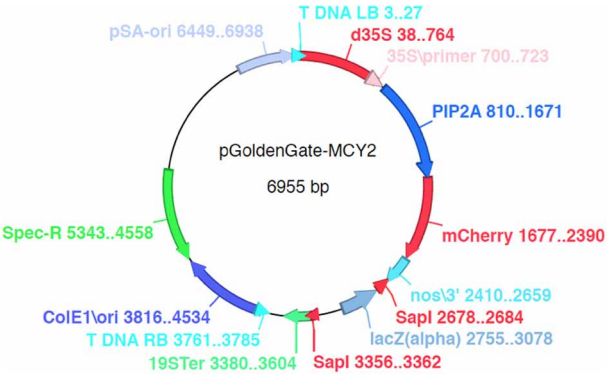

FIGURE A3 | pGoldenGate-MCY2 map.

frontiers in
PLANT SCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 06 August 2013
doi: 10.3389/fpls.2013.00311

Membrane "potential-omics": toward voltage imaging at the cell population level in roots of living plants

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Genetically encoded voltage-sensitive fluorescent proteins (VSFPs) are being used in neurobiology as non-invasive tools to study synchronous electrical activities in specific groups of nerve cells. Here we discuss our efforts to adapt this "light-based electrophysiology" for use in plant systems. We describe the production of transgenic plants engineered to express different versions of VSFPs that are targeted to the plasma membrane and internal membranes of root cells. The aim is to optically record concurrent changes in plasma membrane potential in populations of cells and at multiple membrane systems within single cells in response to various stimuli in living plants. Such coordinated electrical changes may globally orchestrate cell behavior to elicit successful reactions of the root as a whole to varying and unpredictable environments. Findings from membrane "potential-omics" can eventually be fused with data sets from other "omics" approaches to forge the integrated and comprehensive understanding that underpins the concept of systems biology.

Keywords: *Ciona intestinalis* voltage sensor-containing phosphatase, nuclear electrophysiology, nuclear membranes, root cell, systems biology, roots, transmembrane potential, voltage-sensitive fluorescent protein

INTRODUCTION

Systems biology aims to integrate multiple, large-scale "omics" data sets to create a holistic understanding of the functional principles and dynamics of complex biological systems (Zhang et al., 2010). Plant scientists are using many "omics" platforms, such as transcriptomics, proteomics, and metabolomics, to formulate systems-level descriptions of living plants at different developmental stages and under a variety of environmental conditions (Fukushima et al., 2009; Heyndrickx and Vandepoele, 2012; Keurentjes et al., 2013; Kleessen et al., 2013). Although not yet considered among "omics" approaches, the transmembrane electrical potential is an essential and universal feature of living cells and hence must be considered in any multi-scale representation of the living state (Noble, 2013). Simultaneous monitoring of membrane potential changes in populations of cells would provide a quantifiable characteristic to evaluate together with global changes in gene expression, protein abundances, and metabolite levels in systems biology research. Measurement of membrane potential has not traditionally been amenable to high-throughput analysis but recent technical advances are bringing this possibility closer to reality.

In this article, we describe progress toward adapting a technology, used originally on animal nerve cells, to record simultaneous changes in membrane potential in populations of root cells in living *Arabidopsis thaliana* (*Arabidopsis*) seedlings. The method relies on genome-encoded voltage-sensitive fluorescent proteins (VSFPs), which are able to undergo voltage-induced changes in fluorescence. In principle, VSFPs allow optical imaging of changes in membrane potential in single cells, layers of cells, and whole organisms (Peterka et al., 2011; Perron et al., 2012), thus bringing electrophysiological monitoring into the realm of systems biology. We describe the production of transgenic plants expressing various types of VSFPs targeted to different membranes in cells of roots, which are non-photosynthetic and have a low background fluorescence compared to most other plant organs. We discuss the possibilities and challenges of using genetically encoded voltage indicators in plants to study coordinated changes of the plasma membrane potential in cell populations and at multiple membrane systems within single cells.

VOLTAGE-SENSITIVE FLUORESCENT PROTEINS

Classical methods for recording membrane potentials have generally relied on invasive tools such as microelectrodes. Motivated by the desire to carry out functional analyses of selected groups of nerve cells in the brain, neurophysiologists have been developing over the last decade experimental methods for non-invasive and synchronous monitoring of electrical activity from populations of neurons. Foremost among the tools arising from these efforts are VSFPs, which can be stably expressed in specific cell types determined by the promoter used to drive transcription and localized to distinct subcellular compartments by using appropriate targeting signals (Mutoh and Knoepfel, 2013). The technology is steadily improving and the potential for VSFPs to offer fast and sensitive optical monitoring of electrical activity in cells of living organisms led to their being declared a "Method to Watch" by Nature Methods in January 2012 (Pastrana, 2012).

The basic idea behind VSFPs is to fuse a voltage-sensing domain of a membrane protein to a single fluorescent protein or a tandem pair of fluorescent proteins and use, respectively, either changes in fluorescence intensity or FRET (Förster resonance energy transfer) to report shifts in membrane potential (Perron et al., 2009a; Mutoh and Knoepfel, 2013). The voltage-sensing

domain used in the latest versions of VSFPs is derived from the voltage sensor-containing phosphatase of the sea squirt *Ciona intestinalis* (Ci-VSP; Murata et al., 2005). The transmembrane motifs S1 to S4 of Ci-VSP are thought to coordinately operate as the voltage-sensing domain. A history of the development of VSFPs is available in recent reviews (Perron et al., 2012; Mutoh and Knoepfel, 2013).

CONSTRUCTS FOR EXPRESSING VSFPs IN PLANTS

We have assembled constructs for expression of three different VSFPs for use in plants (**Figure 1**). Two of these are from the second generation of VSFPs (VSFP2) and monitor changes in membrane potential through a FRET-based mechanism using a pair of fluorescent proteins. The third is derived from third generation VSFPs (VSFP3) and is based on voltage-dependent alterations in fluorescence intensity of a single fluorescent protein (**Figure 2**; Perron et al., 2009a, 2012; Mutoh and Knoepfel, 2013). The VSFP constructs were obtained from their developer, Thomas Knoepfel, and as described below, introduced into *Arabidopsis* plants under the control of various plant promoters and different subcellular localization signals. Careful selection of transcriptional regulatory signals is important because expression must be sufficiently strong to detect fluorescent signals but not so high as to promote aggregation of the fluorescent fusion proteins or interfere with membrane localization (Mutoh et al., 2011; Perron et al., 2012).

FRET-BASED VSFPs

The FRET-based mechanism of VSFP2 proteins depends on voltage-dependent alterations in protein conformation such that energy transfer between the two chromophores is reversibly modulated by changes of membrane voltage (**Figure 2**). The two VSFP2 probes we have adapted for plants each contain a different pair of donor and acceptor fluorescent proteins fused with the Ci-VSP (Akemann et al., 2010). VSFP2.3 contains cyan-emitting fluorescent protein (monomeric) mCerulean and the yellow-emitting fluorescent protein citrine as donor and acceptor, respectively. VSFP2.4 contains (monomeric) mCitrine as a donor and mKate2, a monomeric far-red-emitting fluorescent protein (Shcherbo et al., 2009), as acceptor (**Figure 2**). Experiments with neurons have demonstrated the usefulness of testing different pairs of fluorescent protein because the species of fluorescent protein can influence the efficiency of membrane deposition and signal amplitude (Perron et al., 2009b; Mutoh and Knoepfel, 2013). The choice of which VSFP variant to use for a given purpose thus needs to be determined empirically and will depend on the cell type, degree of background fluorescence, and membrane to be targeted (Mutoh et al., 2011).

We have placed the respective constructs encoding VSFP2.3 and VSFP2.4 under the control of two distinct promoters (**Figure 1**) that display in our hands somewhat different patterns of expression in the root. The RPS5 promoter (Weijers et al., 2001) drives expression primarily in meristem region and the elongation zone (**Figure 3**). The ubiquitin-10 promoter (Grefen et al., 2010) drives lower expression in the meristem region but higher expression in the elongation and maturation zones including root hairs (**Figure 4**). Transgenic *Arabidopsis* lines homozygous for each construct have been generated and screened for the desired expression properties. In root cells, which also exhibit the aforementioned low background fluorescence, these lines display approximately equal and uniform fluorescent signals from the tandem FRET pair when imaged with the respective excitation and emission wavelengths of the individual chromophores (**Figure 3**). In mammalian cells, VSFPs based on the Ci-VSP

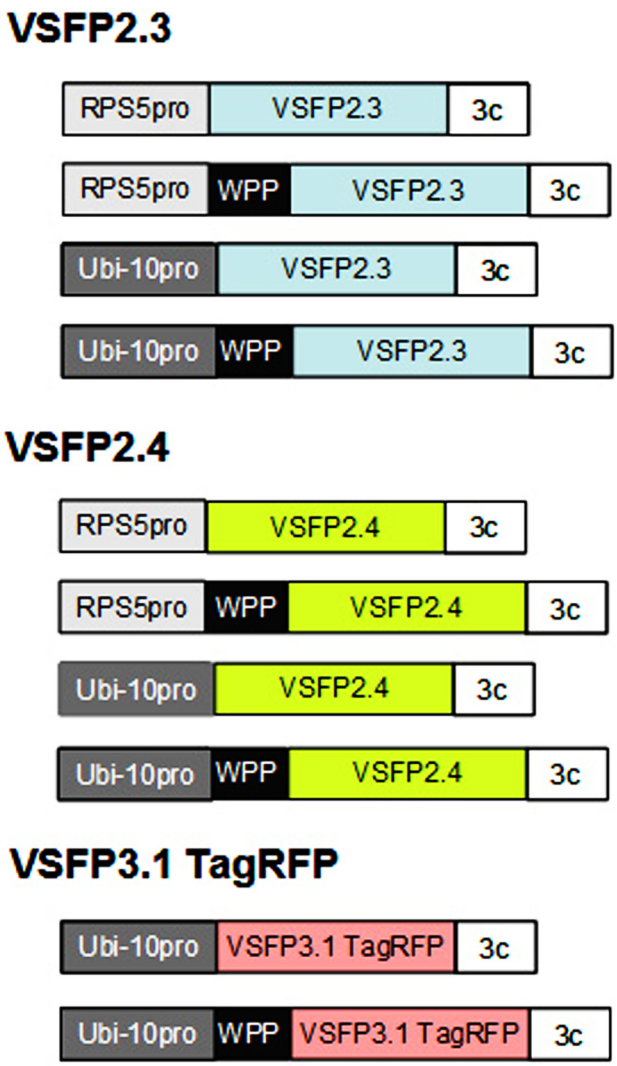

FIGURE 1 | Constructs for expressing VSFPs in plants. Genes encoding voltage-sensitive fluorescent proteins VSFP2.3, VSFP2.4 (color of donor chromophore shown), and VSFP3.1 TagRFP (**Figure 2**) were placed under the control of either the *Arabidopsis* RPS5 promoter (RPS5pro; Weijers et al., 2001) or ubiquitin-10 promoter (Ubi-10 pro; Grefen et al., 2010), both of which are active in root cells, and the 3C transcriptional terminator from the pea rbcS3C gene (Benfey et al., 1989). Constructs were also made that included a WPP domain containing a localization signal for the outer nuclear membrane (Deal and Henikoff, 2011). The constructs were introduced into *Arabidopsis* plants using standard techniques. Around 10–20 transformed lines were obtained with each construct and screened for single locus insertions. Only lines that displayed stable and long-term expression of VSFPs in roots were retained. Images from representative lines are shown in **Figures 3, 4, 5,** and **7**. Constructs not drawn to scale.

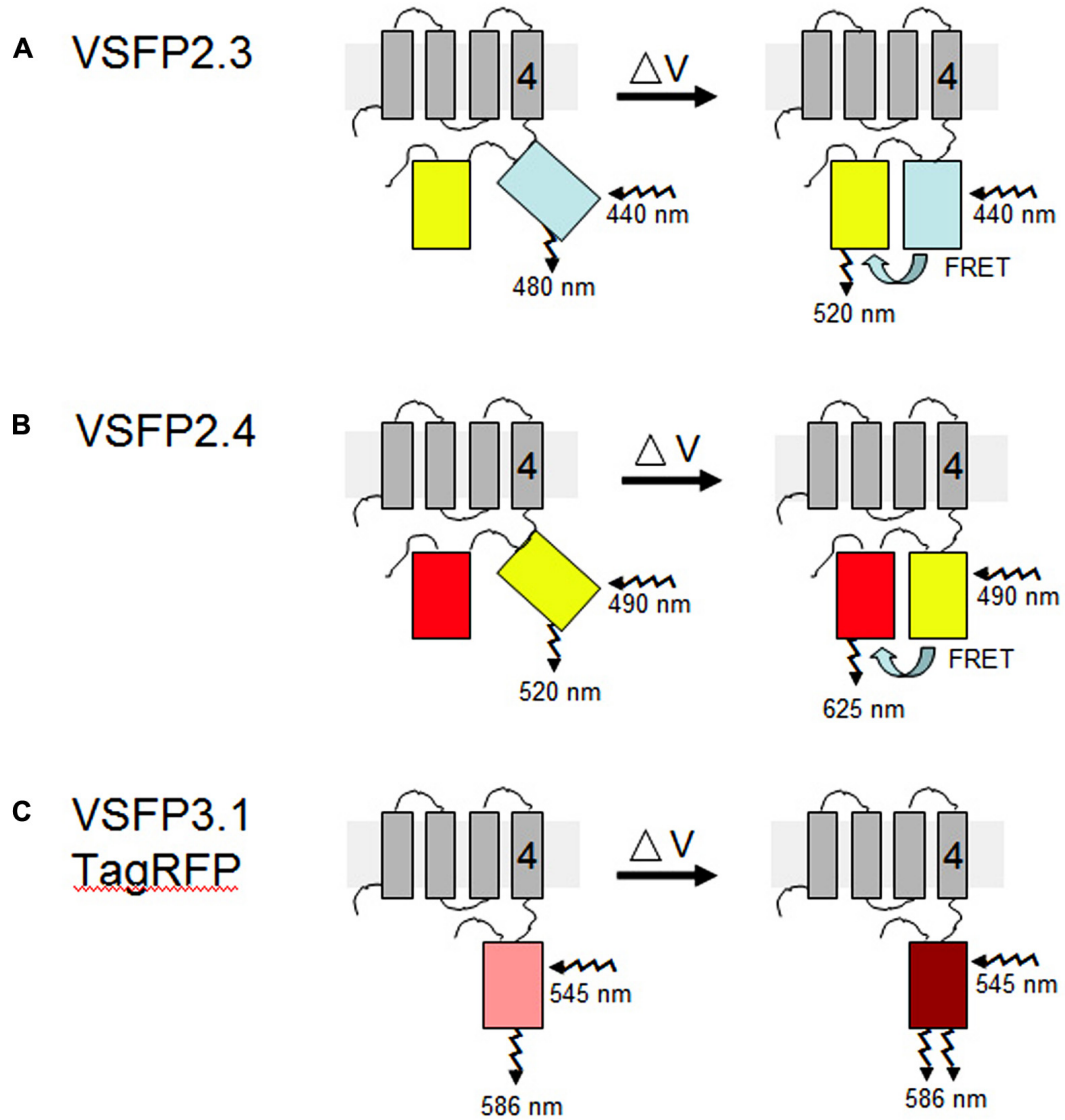

FIGURE 2 | VSFP probes of membrane potential. Different variants of VSFPs contain a combination of a voltage-sensing domain and either a pair of donor and acceptor fluorescent proteins for FRET-based monitoring or a single fluorescent protein for intensity-based recording. One fluorescent protein is attached to the fourth transmembrane segment (gray rectangles, nr. 4) of the *C. intestinalis* voltage sensor-containing phosphatase (Ci-VSP), which comprises transmembrane motifs S1 to S4 that coordinately operate as the voltage-sensing domain. **(A,B)** In the two FRET-based systems, VSFP2.3 and VSFP2.4, illumination with the excitation wavelength of the donor protein results in fluorescence primarily at the donor wavelength under resting conditions (left). A change in the membrane potential (ΔV) allows energy transfer between the two fluorescent proteins, perhaps by aligning them more favorably, such that increased fluorescence of the acceptor protein is observed concomitantly with decreased fluorescence of the donor protein (right). In VSFP2.3, the donor and acceptor fluorescent proteins are mCerulean and citrine, respectively. In VSFP2.4, the donor and acceptor fluorescent proteins are mCitrine and mKate2, respectively (Akemann et al., 2010). The approximate excitation and emission wavelengths of these proteins are shown. **(C)** In the intensity-based probe, VSFP3.1TagRFP, a decrease in membrane potential leads to an enhancement of fluorescence intensity from the fluorescent protein, in this case monomeric TagRFP. The figure is based on previously published ones (Perron et al., 2012; Mutoh and Knoepfel, 2013).

voltage-sensing domain are efficiently targeted to the plasma membrane (Mutoh and Knoepfel, 2013) and this domain appears to work well for plasma membrane localization in root cells in the absence of a targeting signal for a specific internal membrane system (**Figures 3A–D** and **4A,B,E,F**). Long-term expression of VSFPs in the *Arabidopsis* lines we have produced does not seem to have any obvious adverse effects on plant growth, development, or reproduction.

INTENSITY-BASED VSFPs

VSFP3 probes are based on a single fluorescent protein that responds to voltage changes by a variation in fluorescence intensity through a mechanism that is not yet completely understood (**Figure 2**; Perron et al., 2009a,b, 2012). We have adapted a VSFP3.1TagRFP construct for expression in plants by placing it under the control of the ubiquitin-10 promoter (**Figure 1**; Grefen et al., 2010). The TagRFP protein is a monomeric red fluorescent

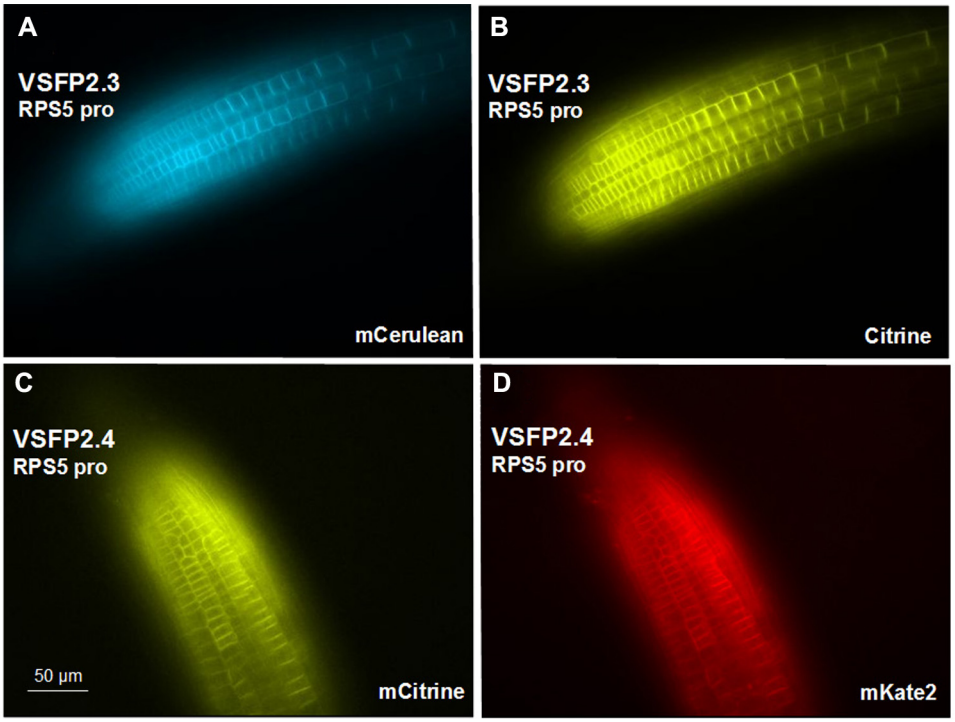

FIGURE 3 | Expression of VSFP2s from the RPS5 promoter in root tips. **(A,B)** VSFP2.3, mCerulean fluorescence (left) and citrine fluorescence (right). **(C,D)** VSFP2.4, mCitrine fluorescence (left) and mKate2 fluorescence (right). Each image was made using the respective excitation and emission wavelengths for the individual chromophores (see below). Note the plasma membrane localization and the relatively uniform and equal signals from the two fluorescent proteins of each FRET pair in this region of the root. In both cases, plants are homozygous for the respective VSFP2-encoding construct. Images shown are fluorescence wide field images acquired after focusing on the top cell layers of the root. Excitation (ex) and emission (em) wavelengths (in nm) of the filter cubes used for this image: mCerulean (ex 436/em 480 nm); citrine (ex 500/em 535 nm); mKate (ex 560/em 645 nm).

protein that is characterized by bright fluorescence, prolonged fluorescence lifetime, high pH stability and reduced tendency to form oligomers (Merzlyak et al., 2007). In an *Arabidopsis* line transformed with the VSFP3.1TagRFP construct, fluorescence signals are localized to the plasma membrane in cells of the root maturation zone (**Figure 5A**).

TARGETING VSFPs TO THE NUCLEAR MEMBRANES

We have a long standing interest in using potential-sensitive probes to study electrical potentials at the nuclear membranes of the nuclear envelope (Matzke and Matzke, 1986, 1991; Matzke et al., 1988, 2010). Although the nuclear envelope is not often considered from an electrophysiological perspective, its electrical properties may influence activities in the nucleus owing to the ion transport capabilities of the nuclear membranes and the ability of electric fields to modulate DNA compaction and interactions with proteins (Musheev et al., 2010, 2013).

The nuclear envelope of eukaryotic cells consists of two membranes, the inner and outer nuclear membranes (INM and ONM, respectively), which are fused at the nuclear pores. The nuclear pores provide the major pathway for nucleo-cytoplasmic transport of macromolecules. The compartment between the two nuclear membranes, referred to as the perinuclear space, is thought to sequester inorganic ions such as calcium for release into the nucleoplasm upon the application of appropriate stimuli (Matzke and Matzke, 1991; Charpentier and Oldroyd, 2013). Because the ONM is continuous with the endoplasmic reticulum (ER), the perinuclear space is contiguous with the lumen of the ER (**Figure 6**).

The possibility of independent control of nuclear calcium and other inorganic ions by channels and pumps in the nuclear membranes is increasingly thought to be an important contributor to the regulation of gene transcription and other processes in the nucleus (Matzke et al., 2010). Plants provide some of the best examples for independent regulation of nuclear calcium in signal transduction cascades that trigger the expression of specific genes. Several nuclear membrane-localized cation channels, CASTOR, POLLUX (Charpentier et al., 2008), and DMI (Riely et al., 2007), have been identified in forward genetic screens for nodulation-deficient mutants in legumes. These channels are essential for perinuclear calcium oscillations that precede activation of nodulation genes in response to bacterial nod factors that interact with cell surface receptors (Capoen et al., 2011).

We are interested in using nuclear membrane-localized VSFPs to monitor changes in nuclear membrane potential that occur in response to various triggering events. Such changes could be independent of the plasma membrane potential but they may also reflect synchronous changes in plasma membrane and nuclear membrane potentials following a given stimulus at the cell surface (**Figure 6**). Conceivably, such an electrically based signal

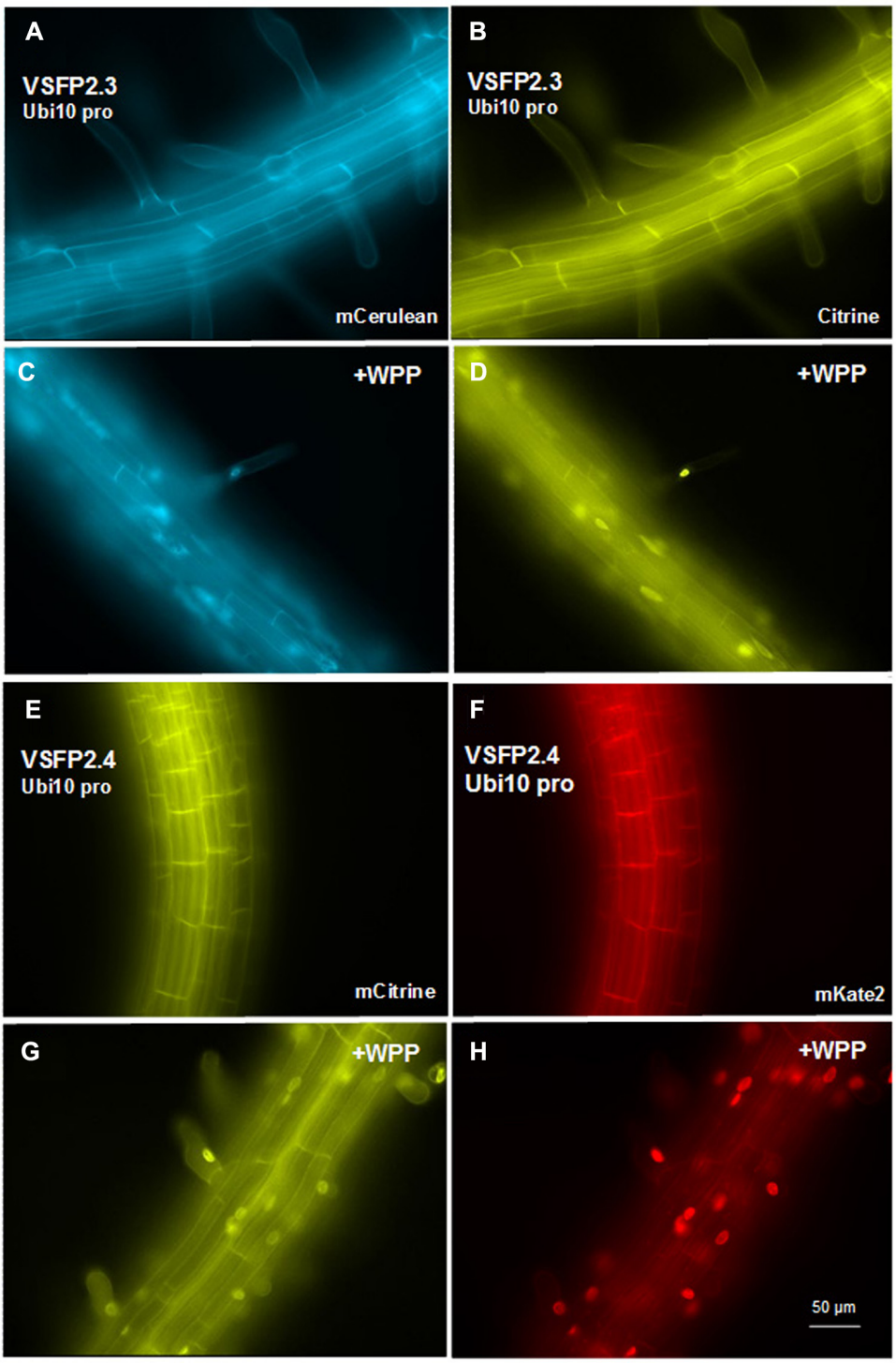

FIGURE 4 | Expression of VSFP2s from the Ubi-10 promoter and targeting to nuclear membrane in root cells. (A–D) When driven by the ubiquitin-10 promoter, the FRET pair in VSFP2.3 (mCerulean and citrine) produces strong and uniform signals at the plasma membrane of cells in the root maturation zone **(A,B)**. When WPP is fused to the VSFP2.3 construct, fluorescence is highly concentrated at the nuclear membrane in cells of the maturation zone and in root hair cells **(C,D**, +WPP**). (E–H)** Similar results are obtained with VSFP2.4 under the control of the ubiquitin-10 promoter (FRET pair mCitrine and mKate2; **E,F**, plasma membrane; **G,H**, +WPP, nuclear membrane). The weaker plasma membrane fluorescence than nuclear fluorescence in **(G)** and **(H)** indicates that ONM targeting of the ubiquitin-10 promoter-driven VSFP2.4 is more specific than the RPS5 promoter-driven VSFP2.4 (**Figure 7**). Images shown are fluorescence wide field images acquired after focusing on the top cell layers of the root or the root hair. Each image was made using the respective excitation and emission wavelengths of the individual chromophores (see **Figure 3** legend).

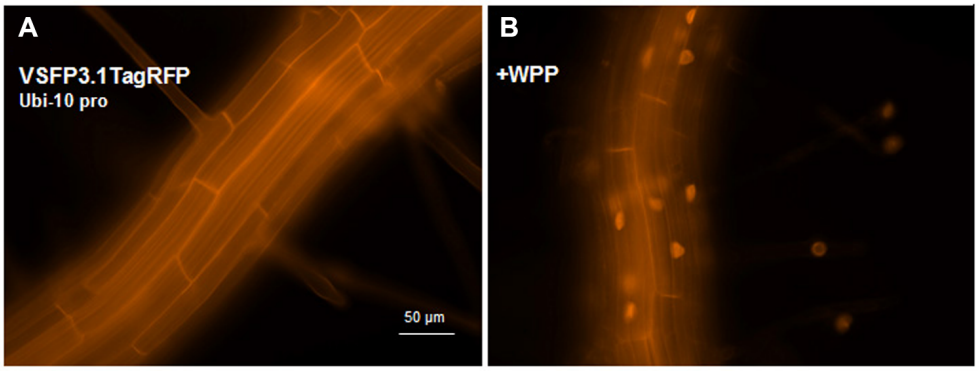

FIGURE 5 | Expression of VSFP3.1TagRFP from the Ubi-10 promoter and targeting to nuclear membrane in root cells. Images show expression of the intensity-based VSFP3.1TagRFP from the Ubi-10 promoter at the plasma membrane **(A)** and the nuclear membrane (**B**, +WPP) in cells of the root maturation zone. In **(B)**, only some background fluorescence is seen in the plasma membrane. The four faint spots to the right in **(B)** are root hair nuclei. The excitation and emission wavelengths of TagRFP are 545 nm and 605 nm, respectively. Images shown are fluorescence wide field images acquired after focusing on the top cell layers of the root or the root hair.

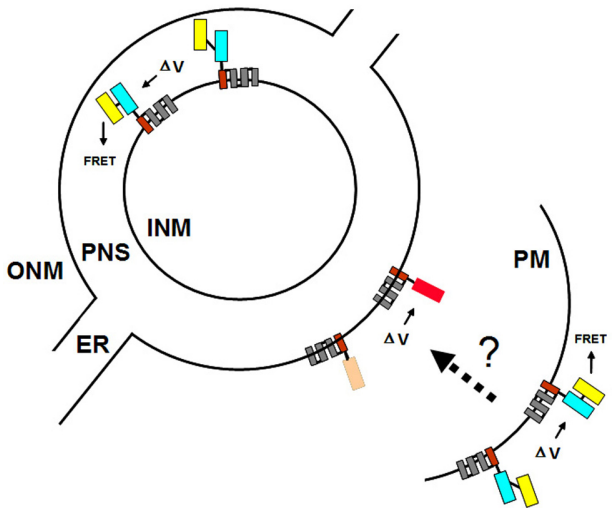

FIGURE 6 | Targeting VSFPs to different membrane systems. The plasma membrane (PM) and the inner and outer nuclear membranes (INM and ONM, respectively) are represented by concentric black partial and full circles. For simplicity, the nuclear pores, at which the INM and ONM are fused, are omitted. The ONM is continuous with the endoplasmic reticulum (ER). The perinuclear space (PNS) between the INM and ONM is contiguous with the lumen of the ER. Targeting of VSFPs to the PM is achieved by Ci-VSP (**Figure 3**). Ideally, targeting signals specific for the INM and ONM can be identified since these two membranes may have distinct and independently regulated potentials. Using different VSFPs in the PM and ONM (or INM) would allow multicolor imaging and simultaneous monitoring of potentials at different membrane systems within a single cell. Shown here is the hypothetical situation of FRET-based VSFP2.3 in the PM (or INM) and intensity-based VSFP3.1TagRFP in the ONM. The dotted arrow and question mark indicate the possibility of detecting synchronous changes in PM and ONM (or INM) potentials using VSFPs.

transduction pathway that couples changes in plasma membrane potential to changes at the nuclear membrane could provoke rapid alterations in gene expression or influence other reactions in the nucleus (Matzke and Matzke, 1991; Shemer et al., 2008).

Given the possibility that the two nuclear membranes maintain distinct membrane potentials that are functionally significant (Matzke et al., 2010), the ideal situation would be to target one VSFP exclusively to either the INM or the ONM and a second VSFP containing a different fluorescent protein or fluorescent protein pair to the plasma membrane (**Figure 6**). To target VSFPs to the ONM, we have used the WPP domain (amino acids 1–111) of the *Arabidopsis* RAN GTPase activating protein (RanGAP1; **Figure 1**), which has been reported to be necessary and sufficient for targeting to the ONM of the nuclear envelope in plants (Rose and Meier, 2001; Deal and Henikoff, 2011).

In transgenic lines containing VSFP2.4_WPP under the control of the RPS5 promoter, expression of the VSFP is visible at the nuclear periphery in cells in the elongation zone and root tip but also at the plasma membrane, indicating that ONM targeting is leaky (**Figure 7**). When the ubiquitin-10 promoter was used to drive VSFP2.3_WPP expression, stronger fluorescent signals were observed at the nuclear rim than at other cell membranes in cells of the maturation zone and in root hairs (**Figures 4C,D**, compare with **Figures 4A,B**). The most specific nuclear localization of the FRET-based probes was observed with VSFP2.4_WPP under the control of the ubiquitin-10 promoter. In these plants, strong signals at the nuclear envelope were observed with little background fluorescence from other parts of the cell (**Figures 4G,H**, compare with plasma membrane staining in **Figures 4E,F**). The intensity-based probe, VSFP3.1TagRFP also displayed relatively strong ONM fluorescence when fused to WPP, with only faint plasma membrane fluorescence still visible (**Figure 5B**). The basis of the observed variations in targeting efficiency by WPP is not known but the findings illustrate that targeting VSFPs to specific membrane systems is not always a straightforward matter. A contributing factor may be differences in background autofluorescence at the distinct excitation wavelengths of the various chromophores. Appropriate background corrections will be necessary for accurate quantitative measurements.

We have tested two localization signals for the INM, one from the mammalian protein nurim (Hofmeister and O'Hare, 2005)

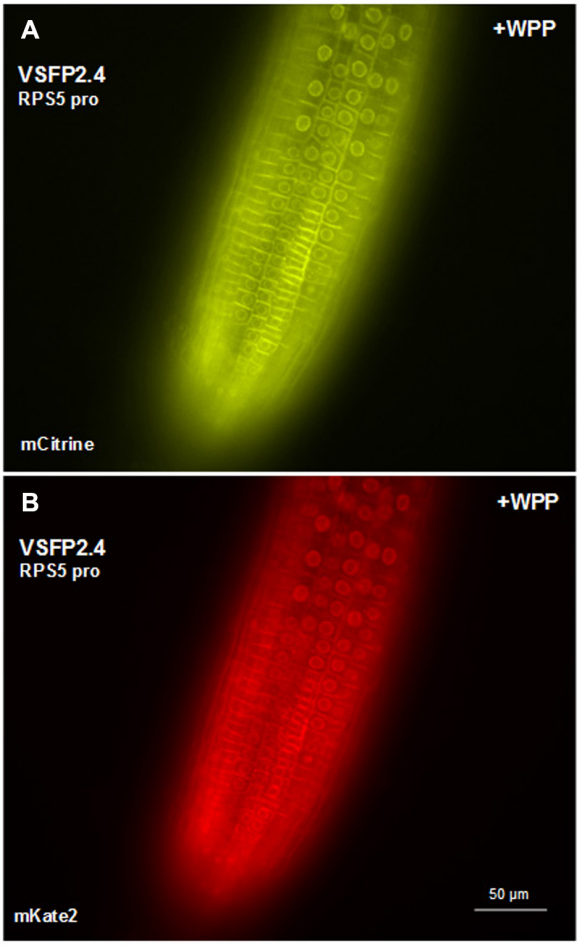

FIGURE 7 | Targeting VSFP2.4 to the outer nuclear membrane in root tip cells. VSFP2.4 (FRET pair mCitrine and mKate) under the control of the RPS5 promoter can be targeted to the outer nuclear membrane (fluorescent circles) when coupled to the WPP domain. Considerable fluorescence is still observed at the plasma membrane, so targeting is not exclusive to the ONM. This may have something to do with the particular VSFP or cell type, since WPP localizes VSFP2.4, under the control of the ubiquitin-10 promoter, preferentially to the ONM in cells of the root maturation zone and root hairs (**Figures 4G,H**). The background autofluorescence may also vary depending on cell type at the excitation wavelength of the donor chromophore. Images shown are fluorescence wide field images acquired after focusing on nuclei in the top cell layer of the root. Each image was made using the respective excitation and emission wavelengths of the individual chromophores (see **Figure 3** legend).

and a second from an *Arabidopsis* SUN protein (Graumann et al., 2010; Oda and Fukuda, 2011). However, neither of these facilitated preferential deposition of VSFPs in the nuclear envelope (data not shown). When the nuclear membrane proteome in plants is determined, it may be possible to identify more efficient targeting signals specific for the INM and ONM. We also tested an ER localization signal (Cutler et al., 2000) but for unknown reasons have been unable to obtain a reliable fluorescence signal enriched in the ER membranes.

DETECTION

The transgenic lines we have developed are suitable for monitoring under a fluorescence microscope equipped with the proper filters and imaging software. We use a Zeiss Axioplan2 equipped with a Quad-view and MetaMorph image analysis software. For imaging, intact living seedlings expressing VSFPs can be mounted in water or buffer on a microscope slide with an indentation for leaves, covered with a 20 mm × 40 mm cover slip, and sealed with rubber cement. It is crucial to immobilize the material to prevent losing the focal plane of the membrane when adding various substances to be tested. Issues concerning signal-to-noise ratio, dynamic range, biological sensitivity, and kinetics of VSFPs have been investigated and discussed for applications in animal cells (Mutoh et al., 2011) but substantial work is still needed in these areas with respect to plant cells.

SUMMARY

We have described the ongoing development of genetically encoded optical probes designed to record coordinated changes in electrical potential at the plasma membrane and nuclear membranes in populations of root cells in living plants. Although the development of these tools is still in the early stages, our preliminary studies and the successful use of VSFPs in animal cells are positive steps toward establishing this innovative technology in a wide range of organisms. The availability of such tools to investigate overarching electrical patterns that transcend single cell boundaries and single membrane systems will contribute needed information on a currently underappreciated dimension of plant systems biology.

ACKNOWLEDGMENTS

We thank Thomas Knoepfel, RIKEN Brain science Institute, for kindly providing the VSFP constructs, Academia Sinica and the Austrian Academy of Sciences for financial support, Johannes van der Winden for technical assistance, and Christine Ying for editorial assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 June 2013; accepted: 24 July 2013; published online: 06 August 2013.

Citation: Matzke AJM and Matzke M (2013) Membrane "potential-omics": toward voltage imaging at the cell population level in roots of living plants. Front. Plant Sci. ***4****:311. doi: 10.3389/fpls.2013.00311*

This article was submitted to Frontiers in Plant Systems Biology, a specialty of Frontiers in Plant Science.