

Signaling between Chloroplasts and the Nucleus: Can a Systems Biology Approach Bring Clarity to a Complex and Highly Regulated Pathway?

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Plastids are organelles found in photosynthetic eukaryotes and are best known as sites of photosynthesis. In addition to this role, plastids manufacture and store a myriad of biomolecules, including carbohydrates, amino acids, fatty acids, plant hormones, and nucleotides (Buchanan and Gruissem, 2000). Sulfur and nitrogen are assimilated into biologically available forms in plastids, and antioxidants such as ascorbic acid (vitamin C) and other secondary metabolites are also produced there. Plastids can differentiate into several forms depending on their function in the cell in which they reside. Among the types of plastids found in plants are chloroplasts (photosynthesis), chromoplasts (fruit pigment synthesis and storage), leucoplasts (monoterpene synthesis), amyloplasts (starch storage), and etioplasts (found in dark-grown plants).

In spite of these versatile functions, plastid genomes encode fewer than 100 proteins (Sato et al., 1999). Rather, the vast majority of plastid proteins are encoded by nuclear genes. It has been estimated that approximately 3,000 proteins are transcribed in the nucleus, translated in the cytoplasm, and then imported into plastids. Plastids are believed to have originated from the engulfment of a photosynthetic bacterium by a eukaryotic cell that already contained mitochondria, which are the by-products of a separate endosymbiotic event. After endosymbiosis, most genes were either lost or transferred to the nucleus, where they acquired the regulatory sequences for expression, as well as sequences that encode a transit peptide for targeting proteins to the plastid where they are imported and cleaved (Kleine et al., 2009). Once these proteins reach their proper compartment within the plastid, many are incorporated into multisubunit complexes that include components encoded in plastid genomes. Thus, signaling between plastids and the nucleus is required to maintain plastid biological functions.

Signaling between chloroplasts and the nucleus is bidirectional. In a process called anterograde regulation, the nucleus encodes regulators that convey information about cell type and expresses proteins that

are appropriate for plastid functions within that particular cell type (Pesaresi et al., 2007; Kleine et al., 2009). In anterograde regulation, gene expression in the nucleus and chloroplasts is coordinated so that proper stoichiometry of subunits of plastid protein complexes is achieved. In retrograde signaling, nuclear gene expression is regulated as a result of signals generated from plastids. These signals reflect both the developmental and functional state of the plastid. Once a seedling is established, most retrograde signals are a result of abiotic stresses perceived in the plastid (temperature, high light, etc.). Plastids also communicate with mitochondria, as their metabolism is tightly coordinated.

The pathways of communication between various organelles of a plant cell are quite complex and interdependent (Leister, 2005; Koussevitzky et al., 2007; Pesaresi et al., 2007; Giraud et al., 2009). As such, biochemical and genetic approaches have been slow-going. Recently, systems biology, a multidisciplinary science that uses large data sets to generate hypotheses about a dynamic system, has been used quite effectively to study signaling networks. Systems approaches have provided new insights when conventional reductionism has been unsuccessful (Ahn et al., 2006). Phases of systems biology include collection of high quality, quantitative data, and analysis of these large-scale data sets, followed by computational modeling, hypothesis generation, and testing the model. Here, we summarize the role that systems biology approaches are playing in contributing to our understanding of the molecular mechanisms of signaling between plastids and the nucleus. This *Update* is not meant to be a comprehensive review of the field, but rather points to a few examples where systems biology has provided some useful leads when conventional biochemistry and genetics did not.

SYSTEMS BIOLOGY IN ANTEROGRADE REGULATION

Chloroplast Proteomes: Proteomics and Bioinformatics

The plastid proteome varies with its differentiated form, which is associated with its various functions within plant cells. Some of these plastid types are not very prevalent or are present only in cell types that are difficult to isolate. Thus, the question of how the plastid proteome varies during development or under a specific environmental condition is still a challenge.

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Chloroplasts are the only plastid types for which there is useful proteomic information. Chloroplasts are found mainly in leaf mesophyll cells, with up to 100 copies/cell. Because chloroplasts are green and abundant and mesophyll protoplasts are easy to isolate, the chloroplast proteome has been heavily interrogated (Ferro et al., 2003; Zabrouskov et al., 2003; Friso et al., 2004; Zybailov et al., 2008; Fig. 1). After several false starts owing to lack of sensitivity of mass spectrometers and high false discovery rates of the algorithms used to predict chloroplast transit sequences, the chloroplast proteome is now thought to contain about 2000 proteins (Millar et al., 2006; Yu et al., 2008; Zybailov et al., 2008). To validate these predictions, it will be necessary to test experimentally the localization of at least a subset of the proteins. Several strategies for high-throughput protein localization have been published recently (Koroleva et al., 2005; Marion et al., 2008); thus, we should soon have a near complete catalog of nuclear-encoded genes for plastid-destined proteins in leaf mesophyll cells that can be used as a starting point to interrogate anterograde regulation.

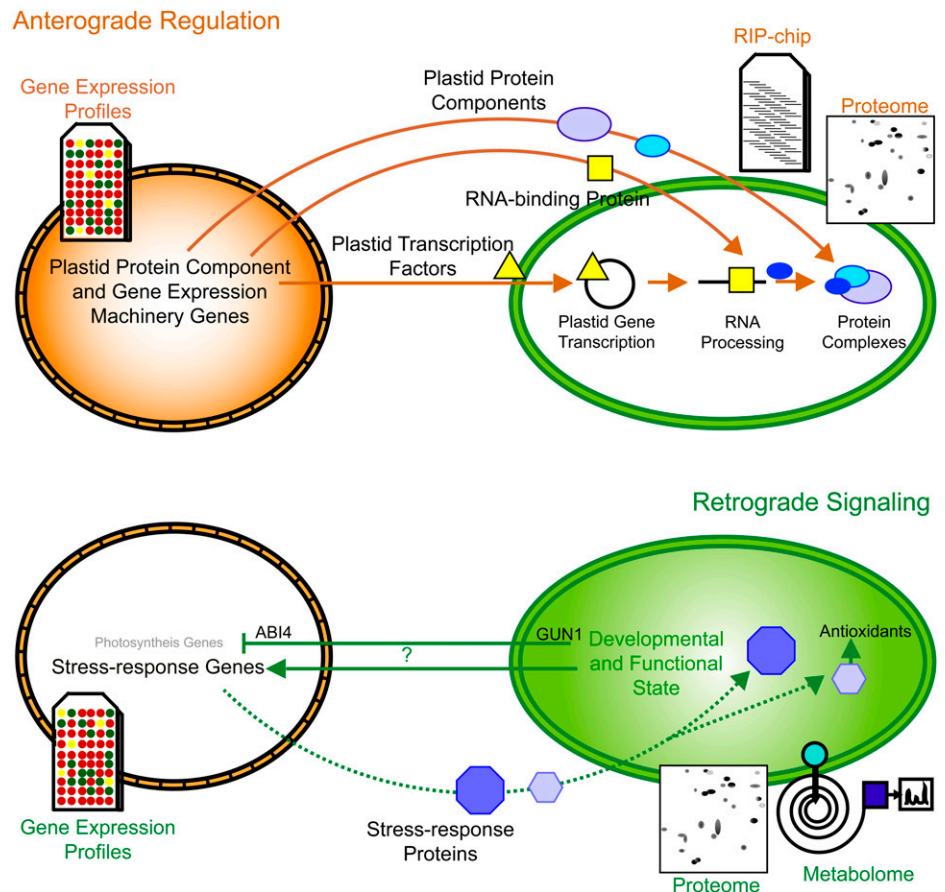
Analysis of Gene Expression and Comparative Proteomics

Considering the fact that most chloroplast proteins are encoded in the nucleus, major questions are, “what

are the regulators of nuclear genes for plastid-destined proteins,” “how do nuclear genes regulate plastid transcription and translation,” and “how are the expression levels of these nuclear genes regulated by the environment?” For instance, when a seedling first encounters light, there is a massive reprogramming of the nuclear genome that must be coordinated with plastid function. After exposure to light, plastids are differentiated from proplastids or etioplasts to chloroplasts. During a short time, photosynthetic complexes are synthesized and assembled into thylakoid membranes, and proteins required for chloroplast DNA replication, transcription, and translation are synthesized and imported into the developing chloroplast. The protein complexes, especially photosynthetic protein complexes, consist of components encoded in the nuclear genome with a small but critical contribution of the chloroplast genome (Leister, 2003). Anterograde regulation coordinates levels of proteins encoded in the chloroplast and nuclear genomes.

To better understand the effects of the environment and genetic background on plastid composition, a set of 3,000 nuclear genes predicted to encode proteins that function in plastids was interrogated on microarrays. Expression levels of these 3,000 genes were determined under 101 different conditions, which included changing environment or genetic backgrounds, and groups of coregulated genes were iden-

Figure 1. Anterograde regulation and retrograde signaling between chloroplasts and the nucleus. Anterograde regulation coordinates expression levels of nuclear genes encoding photosynthetic proteins and plastid gene expression machineries. Gene expression profiling, RNA target identification, and plastid proteomics provide a systematic view of anterograde regulation. Retrograde signaling (solid lines) regulates nuclear gene expression depending on the developmental and functional state of plastids. Gene expression profiling, protein-interacting networks, and changes in the metabolome of chloroplasts will shed light on systems biological analysis of responses (dotted lines) of retrograde signaling.



tified. This study concluded that, in the nucleus, photosynthesis-related gene expression is coregulated with chloroplast gene transcription and translation factors (Biehl et al., 2005). Additionally, expression profiling of nuclear and chloroplast genes encoding chloroplast ribosomal proteins showed that expression levels of these genes in two compartments simultaneously increased during tobacco (*Nicotiana tabacum*) seedling development (MacLean et al., 2008).

Comparative proteomics approaches have compared protein profiles between the wild type and mutants affected in chloroplast development or protein profiles at different stages of chloroplast development (Lonosky et al., 2004; Kleffmann et al., 2007; Kanervo et al., 2008; Rutschow et al., 2008). During the etioplast-to-chloroplast transition, proteins related to plastid protein translation, as well as photosynthesis-related proteins were up-regulated, which is consistent with the gene expression profiling (Lonosky et al., 2004; Kleffmann et al., 2007). Isolation of etioplast protein complexes followed by component identification determined the presence of such complexes as the ATPase, cytochrome *b₆f*, and partially assembled Rubisco (Kanervo et al., 2008). These results indicate that the accumulation of chloroplast- or nuclear-encoded subunits of multisubunit complexes is coordinated from the earliest stages of plant development.

RNA Ligand Identification by RNA Immunoprecipitation-Chip

The nucleus regulates plastid gene expression at both the transcriptional and posttranscriptional levels (Nickelsen, 2003; López-Juez, 2007). It has been reported that the nucleus encodes a number of RNA binding proteins, which are targeted to chloroplasts (Nickelsen, 2003). For example, the largest family encoding RNA-binding proteins in land plants is the pentatricopeptide repeat (PPR) family. There are about 450 family members in *Arabidopsis thaliana*, of which approximately 100 are predicted to be in the plastid (Lurin et al., 2004; Schmitz-Linneweber and Small, 2008). Other well-characterized RNA-binding proteins are also predicted to be plastid localized, including approximately 60 members of the RNA recognition motif and the K-homology domain-containing proteins (Nickelsen, 2003).

Chloroplast localized RNA-binding proteins are known to have a prominent role in chloroplast gene expression (Nickelsen, 2003; Schmitz-Linneweber and Small, 2008). They are required for plastid transcript maturation through splicing of introns, formation of correct 3' ends, and RNA editing. RNA binding also regulates RNA stability and translation initiation of chloroplast mRNA. However, it has been challenging to determine which chloroplast RNAs are bound by nuclear-encoded RNA binding proteins. The identification of these targets may provide a clue for molecular function of the nuclear-encoded factors present in chloroplasts.

Systems levels approaches are allowing the identification of RNAs bound by these proteins. RNA immunoprecipitation-chip (RIP-chip) utilizes a combination of immunoprecipitation of RNA samples followed by hybridization to microarrays (Schmitz-Linneweber et al., 2005). Although true-positives should be verified in independent ways, RIP-chip makes it possible to identify RNA targets bound by RNA-binding proteins. For example, this technique identified RNA targets of the maize (*Zea mays*) PPR proteins CRP1 and PPR4 (Schmitz-Linneweber et al., 2005, 2006). CRP1 binds to the 5' untranslated region of the *petA* and *psaC* RNAs, reflecting translation control by CRP1 in plastids (Schmitz-Linneweber et al., 2005). PPR4 associates with the plastid *rps12* pre-mRNA and mediates its trans-splicing, a mature form of which encodes ribosomal protein S12 (Schmitz-Linneweber et al., 2006).

Besides chloroplast PPR RNA-binding proteins, RNA targets of a member of CRM family member (CFM) RNA-binding proteins were isolated by RIP-chip (Asakura and Barkan, 2007). CFM2 binds to both the group I intron of pre-*trnL-UAA* and group II introns of *ndhA* and *ycf3* pre-mRNA. Furthermore, combined analysis of mass spectrometry and RIP-chip has identified an additional factor involved in chloroplast RNA processing (Watkins et al., 2007). RNC1, a protein containing two ribonuclease III domains, is a protein that interacts with known chloroplast splicing factors, CAF1 and CAF2. RIP-chip determined that RNC1 has broad binding activity to group II intron sequences of several plastid RNAs.

RNA-mediated control of plant development and stress responses is widespread (Lorković, 2009). Thus, it seems likely that cytoplasmic and/or nuclear proteins, which are involved in RNA metabolism, may also contribute to signaling between chloroplasts and the nucleus. In addition, deep sequencing technologies are a powerful tool for global analysis of RNA-binding protein targets (Fox et al., 2009). Instead of microarray hybridization, whole immunoprecipitated RNA samples could be sequenced. Thus, we anticipate studies reporting RIP followed by RNA sequencing (RIP-Seq) for chloroplast RNA binding proteins will be forthcoming. If plastid-, cytoplasmic-, or nuclear-localized RNA binding proteins are implicated in signaling between chloroplasts and the nucleus, RIP-Seq should be able to identify target RNA molecules that may also be involved in signaling.

SYSTEMS BIOLOGY IN RETROGRADE SIGNALING

Chloroplast Development-Dependent Nuclear Gene Expression Profiles

Chloroplasts not only receive signals from the nucleus, but chloroplasts also send signals to the nucleus via chloroplast-to-nucleus retrograde signaling (Fig. 1). In the absence of chloroplast development, expres-

sion of nuclear genes encoding chloroplast proteins, such as the *Lhcb* gene for a light harvesting complex protein, are repressed. To date, molecular genetic studies in *Chlamydomonas reinhardtii* and Arabidopsis have suggested that perturbations in the tetrapyrrole pathway around the Chlorophyll/Heme branchpoint may be involved in generating a distress signal from plastids. The signal may be a tetrapyrrole intermediate as suggested by Strand et al. (2003), or a secondary signal may be generated that exits the plastid (Mochizuki et al., 2008; Moulin et al., 2008). This question has been difficult to resolve, and it is becoming increasingly clear that other approaches are needed to identify the signal and the signal pathway outside of chloroplasts that regulate nuclear gene expression.

Microarray analyses have been used very effectively to analyze retrograde signaling in response to early chloroplast development. To identify a comprehensive set of nuclear genes under the control of retrograde pathways, Koussevitzky et al. (2007) analyzed the global gene expression response of the wild type and two genomes uncoupled (*gun*) mutants defective in retrograde signaling, *gun1* (wild type of which encodes a chloroplast PPR protein) and *gun5* (wild type of which encodes a Mg-chelatase subunit). Affymetrix Arabidopsis ATH1 genome arrays were used to determine the extent of retrograde signaling, identifying >1,000 nuclear genes that were differentially expressed in *gun1* or *gun5* compared with the wild type when grown on norflurazon (an inhibitor of phytoene desaturase that causes photooxidative damage in plastids). Further analysis indicated that about half of these genes were repressed by norflurazon treatment in the wild type, with 330 of these having expression in both *gun1* and *gun5*. This statistical analysis indicates that retrograde signaling regulates a large number of nuclear genes with diverse function.

When the promoters of these 330 genes were systematically interrogated, an element, ACGT, was found to be significantly overrepresented (Koussevitzky et al., 2007; Mockler et al., 2007; Priest et al., 2009). ACGT is the core sequence of ABA response elements, implicating components of the ABA response pathway (or the levels of ABA) in retrograde signaling. Indeed, ABI4, an AP2-type transcription factor, was then shown to be a component of the plastid retrograde signaling pathway. Recently, ABI4 was shown to be a regulator of mitochondrial retrograde signaling as well, providing a point of convergence in the plastid and mitochondrial retrograde signaling pathways (Giraud et al., 2009).

Several pieces of evidence demonstrate the existence of cross talk between chloroplasts and mitochondria. Mutants, either affected in photorespiration activity or having lesions in a gene encoding a component of mitochondrial protein complexes, such as NADH dehydrogenase, showed altered chloroplast functional states, including photosynthetic activity, while, in chloroplast development mutants such as *albostrians*, mitochondria development was also affected (Leister,

2005). Furthermore, chloroplast-mitochondria cross talk seems to contribute to chloroplast-to-nucleus retrograde signaling (Leister, 2005). As ABI4 is involved in both plastid and mitochondria retrograde signaling, it would be interesting to identify regulators of ABI4 gene expression or localization and then test the effects of over- or underexpressing them on the development of chloroplasts and mitochondria.

The *immutans* mutant of Arabidopsis causes a variegated phenotype, having green and albino leaf sectors. In contrast to the complete arrest of chloroplast development seen in norflurazon-treated plants, white and green sectors of *immutans* provide genetically identical sectors of developmentally arrested and wild-type chloroplasts, respectively. Gene expression profiles were compared (Aluru et al., 2009). These experiments demonstrated significant nuclear gene expression changes in the white versus green sectors. Overall, expression of photosynthesis-related genes is repressed, and that of stress response-related genes and of energy metabolism-related genes was induced in the white sectors. Furthermore, gene expression levels in white sectors induced by norflurazon were also compared to those in white sectors of *immutans*. Although there were still differences in gene expression profiles, their transcription profiles were in general similar to each other like their bleached morphological phenotypes (Aluru et al., 2009). It will be of additional interest to compare gene expression profiles between white and green tissues from the same plant treated with reduced concentration of norflurazon as being tried in the study.

Chloroplast Functional State-Dependent Nuclear Gene Expression Profiles

In addition to chloroplast developmental state, nuclear gene expression is regulated by the functional state of chloroplasts via retrograde signaling. In contrast to chloroplast development-dependent signaling studies, intact chloroplasts from mature leaves are the source of tissue in functional state-dependent signaling studies. To alter the chloroplast functional state, environmental conditions, such as very strong light, photosystem-specific light, or electron transport inhibitors, have been used (Rossel et al., 2002; Kimura et al., 2003; Fey et al., 2005; Piippo et al., 2006; Adamiec et al., 2008).

Very strong light results in a reduced state of all photosynthetic components in chloroplasts, and the reduced state changes nuclear gene expression (Rossel et al., 2002; Kimura et al., 2003; Kleine et al., 2007; Adamiec et al., 2008). In general, expression levels of genes encoding proteins involved in photosynthesis are decreased, whereas those of stress response genes are increased. The up-regulated stress response genes encode antioxidant proteins, chaperones, and enzymes involved in biosynthesis of antioxidants. It was proposed that the redox state of the plastoquinone (PQ) pool may regulate the very strong light-driven

gene expression, but only 8% of the induced genes are under the control of PQ redox state (Adamiec et al., 2008).

Chloroplast redox state is altered using light sources specifically exciting photosystem I (PSI light) or photosystem II (PSII light; Fey et al., 2005; Piippo et al., 2006). Shifting conditions between PSI light and PSII light change chloroplast redox state, and 286 genes among 3,292 probe spots specifically respond to reduced redox state of chloroplasts (Fey et al., 2005). In contrast to general up- or down-regulation in the same functional group of genes observed in the very strong light response, the shift experiments caused mixed gene regulation even within the same functional group of genes. Furthermore, under either single PSI or PSII light, the effects of PQ on nuclear gene expression were negligible (Piippo et al., 2006). These results indicate that although both very strong light and the PSI and/or PSII light treatment modulate chloroplast redox state, the effects on nuclear gene expression are different. These differences further reflect that these two conditions might involve separate signaling pathways.

Systems biology attempts combinatorial analysis of multiple microarray data sets. As an example, principles and tools developed for comprehensive comparison of hormone treatments and of reactive oxygen species responses could be considered (Gadjev et al., 2006; Nemhauser et al., 2006). Application of these analyses to the retrograde signaling system requires some modifications. To obtain more comparable and consistent data, microarrays reflecting the whole genome, such as ATH1 chips, should be used (Gadjev et al., 2006; Nemhauser et al., 2006). Application of deep sequencing approaches toward transcript quantification could also be an alternative to a hybridization-based microarray platform (Lister et al., 2009). Additionally, it is recommended that multiple experimental conditions be applied to avoid biased results by side effects of a single treatment (Vandenabeele et al., 2004; Pfannschmidt et al., 2009).

Chloroplast-to-nucleus retrograde signaling may be better understood using models to predict the gene regulatory network. As an initial step, biological networking software can reconstruct a regulatory network using microarray data sets (Nacu et al., 2007). Eventually, a chloroplast developmental or functional state-driven nuclear gene expression network would be built based on genome-wide identification of direct and indirect target genes of so far unidentified positive regulating transcription factors. The direct and indirect target gene data would be obtained using chromatin immunoprecipitation with an antibody against such transcription factors followed by a deep sequencing approach and identification of genes whose expression levels in the loss-of-function mutant background are affected, respectively.

Protein level changes induced by very strong light conditions, however, show drastic differences from gene transcript level changes under comparable con-

ditions. Following very strong light treatment, functional state-driven retrograde signaling induced a number of nuclear genes, including stress response genes (Rossel et al., 2002; Kimura et al., 2003). In contrast, in the thylakoid proteome, a relatively small number of proteins showed significant differences compared to the untreated control under very strong light (Giacomelli et al., 2006). Levels of some stress response proteins, such as Fibrillins family members and PsbS, are up-regulated by very strong light. However, comparative proteome analysis could not detect significant differences in protein levels of other stress response proteins, such as FtsH2 and 2-Cys peroxiredoxin A. More detailed systematic experiments are required to resolve this discrepancy. The length of very strong light treatments should be shortened to hours, instead of days, to make these two analyses comparable. In addition to the thylakoid proteome, for instance, proteomes in whole leaf tissue would be useful given that microarray analysis had identified up-regulated genes that encode cytoplasmic and chloroplast proteins (Rossel et al., 2002; Kimura et al., 2003).

Future Contributions of Systems Biology to Decoding the Signaling Pathway(s) between Chloroplasts and the Nucleus

Protein-protein interactions and subcellular localization play a critical role in signal transduction and are sure to play a role in retrograde signaling. A few proteins from current studies are good candidates to begin biochemical analyses, including the chloroplast-localized PPR protein encoded by *GUN1*, the nuclear-localized transcription factor, *ABI4*, and cryptochrome 1 identified in multiple genetic screens (Kleine et al., 2007; Koussevitzky et al., 2007; Ruckle et al., 2007). It is possible that new signaling proteins will be discovered by determining interactions with known components.

Computational approaches have been employed to identify genome-wide protein-protein interactions (Morsy et al., 2008). In addition, a group of chloroplast-localized proteins were predicted and used to specifically predict protein-protein interactions (Yu et al., 2008). This computational interactome approach should be very informative in studying chloroplast-to-nucleus signaling, although protein-protein interaction predictions require experimental confirmation. To test physical protein-protein interactions, high-throughput experimental tools are being developed. This should yield exciting results soon (Morsy et al., 2008).

Another area that should be developed is metabolomics, which is a highly sensitive measure of all small molecules. Small molecules that accumulate during retrograde signaling might shed some light on the signal from plastids that communicates with nuclear genes (Shulaev et al., 2008). For example, genes involved in synthesizing secondary metabolites were up-regulated in these experiments (Kimura et al., 2003); however, this has not been followed up with

direct measurements of the metabolome (Tian et al., 2007).

Some new studies suggest that development of targeted methods with highly sensitive mass spectrometers is very informative. For instance, tocopherols (vitamin E) and their biosynthetic intermediates accumulate when plants are put into high light stress (Kobayashi and DellaPenna, 2008). In addition, a metabolomics study showed that different levels of thermal dissipation capacity in chloroplasts modulate the abundance of several carbohydrates and amino acids (Frenkel et al., 2009). These results demonstrate the potential of metabolomics in signaling studies as it provides another way of readout for signaling. It would be equally interesting to systematically determine metabolic profile changes during chloroplast development or in different stress conditions. Determination of metabolite changes within chloroplasts or other plastid types will be critical to identify signaling molecules within plastids that trigger retrograde signaling.

CONCLUSION

Systems biology has great potential to better understand signal transduction between plastids and the nucleus. The field has thus far focused on genetics, but recently, genetic approaches are having diminishing returns. There are several reasons why this might be: (1) Core signaling components in the pathway may be encoded by essential genes; (2) there is genetic redundancy in the pathway(s); or (3) new phenotypes need to be incorporated into the screens, which take into account complex feedback mechanisms, etc., that may have been put into place for such a complicated network. Systems biology approaches, including bioinformatics, chloroplast RNA target identification, nuclear gene expression profiling, and proteomics, have begun to contribute to our knowledge of organellar signaling. Most of the data have been analyzed individually, but these massive data sets necessitate integrated analysis to draw greater biological meaning. Interactomics and metabolomics have been underutilized, and more attention should be focused on the use of these tools to studies of signaling between chloroplasts and the nucleus. A combination of approaches, including a systems approach coupled with conventional tools such as genetics and biochemistry, should enhance our understanding of the signaling mechanisms between chloroplasts and the nucleus.

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