CHAPTER 5

Linking Primary and Secondary Metabolism

A Mechanistic Hypothesis for how Elevated CO_2 Modulates Defenses

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INTRODUCTION

Rising atmospheric CO_2 affects insect herbivory by altering both the primary and secondary metabolism of plants (Zavala et al. 2013). In terms of primary metabolism, the portion of plant physiology immediately concerned with growth, elevated CO_2 increases the rate of photosynthesis and thus the accumulation of starch in leaves. The consequences of this effect on insect feeding behavior are two-fold: With a more concentrated supply of carbohydrates, insect herbivores are provided with chemical energy to increase their rates of feeding (Lincoln et al. 1986; for review see Zavala et al. 2013). At the same time, increased starch content dilutes foliar nitrogen and thus obliges insect herbivores to compensate their nitrogen requirements by ingesting more leaf tissue (Lincoln et al. 1986; for review see Zavala et al. 2013). Meanwhile, the effects of elevated CO_2 on plant secondary metabolism—the portion of plant physiology responsible for mediating ecological interactions through chemical defense—are comparatively resistant to generalization. The source of uncertainty in understanding how elevated CO_2 influences insect feeding behavior rests in the regulatory connection between primary and secondary metabolism.

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Soybean (*Glycine max*) grown under elevated CO_2 at the soyFACE field experiment exhibits a pattern of altered responses to common insect crop pests that has no apparent physiological connection to altered carbohydrate metabolism. Leaf herbivory was considerably greater on soybean grown under elevated than ambient CO_2, and increased damage was associate with larger populations of Japanese Beetles (*Papilio japonica*) and soybean aphid (*Aphis glycines*) (Hamilton et al. 2005; Dermody et al. 2008). Increased susceptibility of soybean under elevated CO_2 was not strictly related to increased leaf carbohydrates (O'Neill et al. 2008), but was instead caused primarily by a reduction in chemical defenses. Zavala et al. (2008) report that induction of cysteine protease inhibitors (CysPIs) is significantly slower in soybean grown under elevated CO_2 relative to control. These CysPIs render soybean tissue indigestible to insect herbivores by blocking proteases found in insect guts; they are especially important against coleopteran pests, whose digestive capacity is otherwise robust. Japanese Beetles are common to soybean fields in the American Midwest (Zavala et al. 2009), as are adults of Western Corn Rootworm, *Diabrotica virgifera*, of which a rotation-resistant strain is an increasing cause for concern (Levine et al. 2002). Beyond altered production of CysPIs, O'Neill et al. (2010) report that foliar concentration of the flavonoid quercetin is higher under elevated CO_2 relative to control. Insects feeding on high CO_2 soybean may derive a benefit from consuming this antioxidant, further stimulating herbivore damage. Similar studies by Guo et al. (2012) and Zhang et al. (2015) on tomato, Matros et al. (2006) on tobacco, and Mhamdi and Noctor (2016) on *Arabidopsis*, suggest a tradeoff in plant resistance to pathogens and herbivorous insects, whereby plants become more tolerant to pathogens but also more vulnerable to herbivores with chewing mouthparts.

Growth under elevated CO_2 alters the foliar profiles of secondary metabolites relative to growth under ambient CO_2 (Lindroth 2012). Discerning generalization about the direction of response and classes of compounds affecting such empirical observations, however, is difficult because the chemical defenses produced by plants are idiosyncratic, varying widely from species to species (Berenbaum 1995). Moreover, many such defenses become apparent only upon induction by an insect herbivore; while constitutive expression of secondary metabolites varies by species and CO_2 environment, the element of defense-on-demand, or dynamic induction, adds another dimension of complexity (Meldau et al. 2012). Rather than investigating patterns among chemical phenotypes, a more practical approach to understanding how elevated CO_2 modulates defense may be to consider the physiological mechanisms that underlie plant perception and response to stress. Because the signaling pathways responding to herbivory are more similar among plants than the wide

array of chemical defenses produced, this strategy, implicit in many recent reviews on plant secondary metabolism (e.g., Meldau et al. 2012; Zavala et al. 2013; Schuman and Baldwin 2016), may more readily reveal generalizable responses and underlying mechanisms.

Some generalities in the induction of defense become apparent at the level of plant hormones, although these systems remain quite complex. While plants produce a vast array of secondary metabolites, the induction of chemical defense rests primarily on the activity of two major and highly conserved defense hormones, jasmonic acid and salicylic acid (Thaler et al. 2012). Jasmonic acid and salicylic acid interact with one another as well as with the gamut of other major plant hormones, in particular, gibberellic acid (GA), cytokinins, abscisic acid (ABA), and ethylene (Erb et al. 2012). Moreover, both jasmonic acid and salicylic acid serve functions beyond defense; jasmonic acid is integrated with plant growth while salicylic acid is a determining factor in flowering time (Wasternack and Hause 2013; Vicente and Plascenia 2011). Recently, Zhang et al. (2015) suggest that cross-talk between defense hormones accounts for observed differences in plant resistance to pathogens and insect herbivores under variable CO_2. Thus, though complex, the existence of a regulatory link between the growth and defensive roles of primary and secondary metabolism can be discerned in the concerted activity of plant hormones.

Increasing CO_2 concentration affects both the growth and defense habits of C3 plants, but the regulatory connection between the two roles is not well understood (Zavala et al. 2013). Plants grown under elevated CO_2 exhibit increased foliar concentrations of salicylic acid (Casteel et al. 2012; Zhang et al. 2015; Mhamdi and Noctor 2016). This is thought to suppress the activity jasmonic acid and the expression of its associated chemical defenses (Casteel et al. 2012; Zhang et al. 2015). In turn, the induction of salicylic acid is known to rest on the redox environment within plant cells (Leon et al. 1995; Mateo et al. 2006). Under perception of attack from a biotic agent, the stimulus for a defense response typically begins with a burst of reactive oxygen species (ROS) (Lamb and Dixon 1997). Such 'pulses' of ROS in plant cells can trigger phosphorylation cascades conducted by mitogen activated protein kinases (MAPKs) (Jonak et al. 2002; Apel and Hirt 2004). While the role of CO_2 in affecting either the activity of ROS or mapk's is not well resolved, both photosynthesis and photorespiration are two major sources of CO_2-dependent ROS production in plant cells, and numerous reviews suggest links between ROS production in plant cells and hormonal regulation (e.g., Kerchev et al. 2012; Foyer and Noctor 2009). That exposure to elevated CO_2 affects photosynthesis and photorespiration and consequently ROS produced by these pathways, and that ROS affects defense related hormonal signaling provides a hypothetical link to explain the co-regulation of primary and secondary metabolism.

Although how elevated CO_2 modulates the defense hormone salicylic acid is unknown, decades of research in photosynthesis, signal transduction and physiological ecology have produced a body of literature that imply a connection. The first objective of this chapter is to review the literature that implicates a causal relationship between atmospheric chemistry and plant defense habit. Such a review necessitates emphasis on identifying hypothetical connections that are plausible and informative, but experimentally undefined. Based on a synthesis of available literature, we present the novel hypothesis that: (1) Elevated CO_2 in combination with variable light causes transient excess energy in electron transport to drive increased production of hydrogen peroxide; and, (2) This production of hydrogen peroxide acts as a molecular signal that is transduced through MAPKs to stimulate biosynthesis of salicylic acid. A challenge to testing this hypothesis is that it depends on difficult and highly uncertain measurements of excess energy flow in the light reactions of photosynthesis and intra-cellular concentrations of hydrogen peroxide. Recognizing this methodological challenge, a second objective of this chapter is to propose experimentally falsifiable hypotheses, designed to link previously existing knowledge on plant primary and secondary metabolism. Although this chapter is not intended as a review of experimental methods and protocols, some discussion and explanation of laboratory methods is necessary to describe practically feasible experimental approaches to testing the hypothesis.

How does Elevated CO_2 Affect Primary Metabolism: Setting the Stage

The enzyme responsible for assimilating both CO_2 and O_2 into plant metabolism, Ribulose-1,5-bisphosphate-Carboxylase/Oxygenase (RuBisCO) is thought to be the most abundant protein on Earth (Ellis 1979; Raven 2013). As the predominant entry point for carbon into the biosphere, the active site of RuBisCo represents a transition between atmospheric chemistry and the Earth's ecosystem. In C3 plants, the oxygenation reaction of RuBisCO competes with its crucial carboxylation reaction (Laing 1974). The carboxylation and oxygenation pathways differ in the immediate metabolic fate of their reactants; one product of fixation enters the Calvin-Benson-Basham (CBB) Cycle and the other enters photorespiration, respectively (Weissbach 1956; Ogren and Bowes 1971). While the Calvin Cycle takes place exclusively within the chloroplast, metabolites in the photorespiratory pathway progress through several organelles; from chloroplast to peroxisome, then mitochondria and finally returning to the chloroplast (Fig. 1).

The absence of photorespiration as a 'sink' for energy acquired from light could indirectly favor the reduction of oxygen in the chloroplast. The

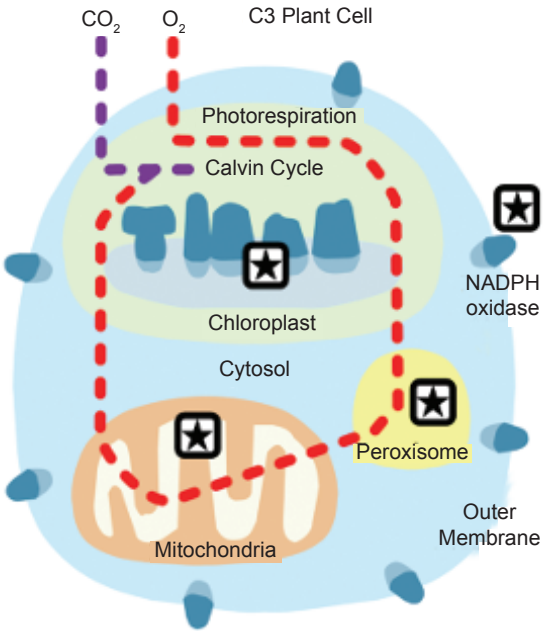

Figure 1. Sources of reaction oxygen species (ROS) in plant cells. The four major sites for the production of ROS are labelled with a star. Metabolic routes followed by the Calvin Cycle and photorespiration are represented as dashed lines.

metabolic pathways that incorporate atmospheric CO_2 and O_2 into plant metabolism differ in their energetic requirements (Farquhar et al. 1980). Per molecule of CO_2 or O_2, respectively, removed from the atmosphere, the Calvin Cycle consumes slightly less ATP and slightly more NADPH relative to photorespiration. Under non-photorespiratory conditions, the ratio of ATP to NADPH consumption by photosynthesis is approximately 21:14, which increases to approximately 21:13 under photorespiratory conditions (Foyer et al. 2012), a small but potentially important change. This difference in stoichiometry means that as an increase in the supply of CO_2 progressively stimulates carboxylation and reduces O_2 consumption by photorespiration, the overall ratio of ATP to NADPH required for both processes declines. Because ATP production, non-photochemical quenching (NPQ), and cyclic electron flow all form a regulatory circuit with one another (Fig. 2; Foyer et al. 2012; Niyogi 1999; Kanazawa and Kramer 2002), one possible consequence of this change in stoichiometry is the exhaust of electrons onto oxygen to form superoxide in the chloroplast stroma (Asada 1999).

To adjust for changes in ATP demand, chloroplasts modulate cyclic electron flow by routing electrons from photosystem I (PSI) through the cytochrome b6f complex (Munekage et al. 2004; Miyake et al. 2005;

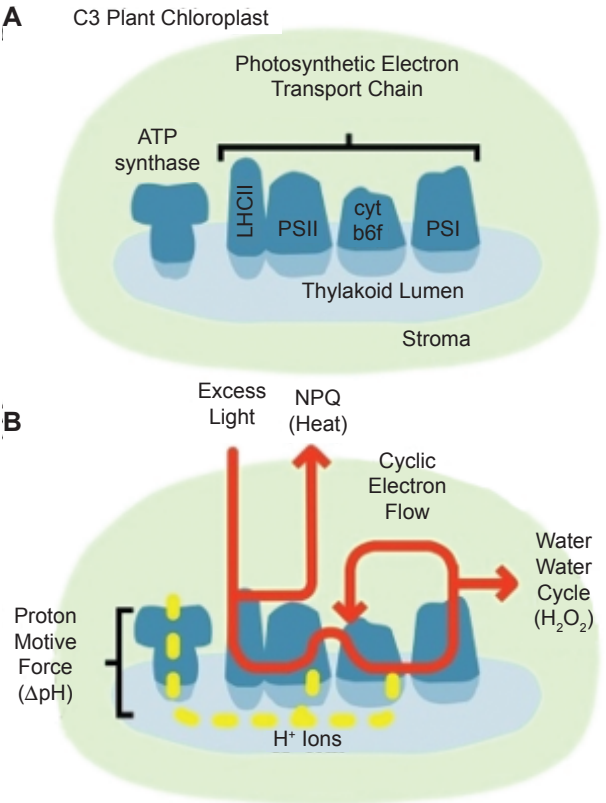

Figure 2A: Schematic of major components of the photosynthetic electron transport chain. 2B: The relationship between electron-flow, proton-motive force and photoprotection (NPQ) in C3 plant chloroplasts. The red line represents flow of energy, either as heat or excitation transfer, while the yellow dashes represent hydrogen ions generated by energy flow through the PETC.

Walker et al. 2014). Electron flow through the cytochrome b6f complex pumps hydrogen ions into the thylakoid lumen, thus generating the proton motive force (pmf) to drive ATP synthase. Although Miyake et al. (2005) and Walker et al. (2013) report that cyclic electron flow increases as pressure of CO_2 declines, to our knowledge no study has addressed the question of how increasing CO_2 affects cyclic electron flow (but see Kanazawa and Kramer 2002). One might suppose that as atmospheric CO_2 increases, the metabolic demand for ATP declines, reducing cyclic electron flow, with the effect that the lumen does not acidify as quickly as it would under conditions of low CO_2.

By lowering pmf, elevated CO_2 relaxes non-photochemical quenching (NPQ) of excess excitation energy in the photosynthetic electron transport chain (PETC) (Miyake et al. 2005; Kanazawa and Kramer 2002). When

plants are exposed to high light, or sudden changes in light intensity, the increased flow of electrons through the PETC rapidly acidifies the lumen beyond the proton motive force required for ATP synthesis (Ort 2001). When the lumen acidifies in this manner, NPQ is induced by the rapid acidification of the thylakoid lumen; the photosynthetic light harvesting complexes (LHCs) change conformation to favor dissipation of excitation energy as heat through xanthophyll pigments in associated light harvesting complexes (Niyogi 1999; Li et al. 2002). The relaxation of NPQ with increasing CO_2 would be the proximate source of additional excess excitation energy in the PETC, under the presumption that the plant loses some efficiency of heat dissipation as an alternative sink for excess energy.

In the absence of a means to dissipate excess excitation energy, it is conceivable that the chloroplast would divert electrons directly to O_2 creating reactive oxygen species (ROS), thereby altering the redox environment within the chloroplast. The Water–Water cycle, also known as the Mehler reaction or pseudocyclic electron flow, describes the transfer of electrons from the PETC to oxygen to form superoxide, followed by antioxidant quenching by ascorbate to glutathione (Asada 1999). Since its core reaction was first identified in the 1950's as the Hill reaction (Hill 1950) the extent of activity and the physiological role played by the Mehler reaction (Mehler 1951) has been a source of debate (for reviews, see: Niyogi 1999; Foyer and Noctor 2012; Heber 2002). To date, there is little experimental evidence that the Water–Water reaction dissipates excess energy, at least not in a manner that could be called a biochemical 'sink' for energy (Ruuska et al. 2000; Driever and Baker 2011). For instance, Heber (2002) argues that the Water–Water cycle prevents transport components of the PETC from becoming over-reduced, particularly under conditions of excess energy, such that cyclic electron flow can properly operate. From this perspective, the Water–Water cycle is perhaps best understood as an exhaust conduit for excess electrons in the PETC. Hence, rather than a true buffer to dissipate excess energy from light, variation in the activity of the Water–Water cycle could be meaningful to intercellular signaling as a source of reactive oxygen species (ROS) in plant cells.

The Linkage between ROS and Defense

Since the late 1990's, reactive oxygen species (ROS) have received field-wide (e.g., Apel and Hirt 2004; Mittler et al. 2011; Foyer and Noctor 2005) and sometimes divisive (see Alpi et al. 2007 vs. Trewavas 2007) discussion as the proximal basis for perception of—and responses to—the surroundings of individual plants. At heart, the redox-based signal transduction consists of the discharge of electrons through a biological substrate; tasked with quenching electrochemical energy, plant cells pass

electrons through complex sequences of reduction and oxidation reactions (Foyer and Noctor 2005). The intracellular sequence of reactions giving rise to ROS spans chloroplast, peroxisome, mitochondria, outer membrane and nucleus (Fig. 1; Cheeseman 2007). By maintaining overall redox homeostasis, plants can sense environmental disturbance as fluctuations in redox state within individual cells (Foyer and Noctor 2005).

Photosynthesis is the first and second largest sources of hydrogen peroxide in plant cells: photorespiration and the Water–Water cycle, respectively (Fig. 1., Foyer and Noctor 2003). During photorespiration, 2-PGA formed in the chloroplast is transported to the peroxisome where glycolate is oxidized to form glyoxalate and hydrogen peroxide (Ogren 1984). The Water–Water cycle describes the production of ROS as a function of electron flow through the PETC and subsequent quenching of hydrogen perxoide by ascorbate within the chloroplast (Asada 1999). Because the Water–Water cycle modulates ROS production in the chloroplast, where the cascade of events leading the production of SA is located, this cycle may play a key role communicating changes in the environment affecting primary metabolism to changes in secondary metabolism that affect susceptibility to herbivory. Beyond photosynthesis, ROS is produced by electron-transport in mitochondrial respiration (Møller 2001), albeit in much lower volume than from photosynthesis and photorespiration (Foyer and Noctor 2003). Unlike photorespiration, photosynthesis and mitochondrial respiration, NADPH-oxidases are activated under specific circumstances. Bound to the outer membrane of plant cells, NADPH-oxidases mediate many growth functions as well as the first lines of defense during invasion from biotic agents (Marino et al. 2012).

Biotic stress universally downregulates photosynthesis genes (Bilgin et al. 2010), but it is unclear whether this regulatory relationship results from retrograde signaling, anterograde signaling, or some combination of the two. Chloroplasts give rise to a variety of signals that influence expression of genes and hormone synthesis in the nuclei of plant cells (Demmig-Adams et al. 2014; Tikkanen et al. 2014; Gollan et al. 2015). Such signaling is considered 'retrograde' because the chloroplast generates the impetus for nuclear gene expression, whereas 'anterograde' signaling occurs when the nucleus manipulates processes occurring in the chloroplast (Woodson and Chory 2008).

The conventional model of nuclear and chloroplast gene expression following recognition of a biotic agent generally assumes that anterograde signaling is responsible for plant responses to stress. Biotic stress—from pathogens or insect herbivores—generates ROS (Torres et al. 2006; Bi and Felton 1995). When plant cells recognize attack through microbial activated molecular patterns (MAMPs), they often trigger adjacent outer-membrane-bound NADPH-oxidases, which release superoxide onto the

cellulose fibers comprising the plant cell wall (Marino et al. 2012). This burst of ROS is recognized as signal event in its own right, because it stimulates salicylic acid biosynthesis, a major plant defense hormone (Lamb and Dixon 1997). Such a signal cascade originating in the outer cell membrane and traveling through the nucleus to regulate chloroplast function would represent an example of anterograde signaling.

In contrast to anterograde signaling, an environmental stimulus originating in the chloroplast and traveling to the nucleus would represent retrograde a signal. An imbalance in the energy distribution in PETC disrupts redox homeostasis, producing ROS, which then influence nuclear gene expression (Gollan et al. 2015). Demmig-Adams et al. (2014) postulate that photoprotective mechanisms modulate this production of ROS and therefore play an important role in physiological regulation, beyond their immediate function in dissipating excess excitation energy. The chloroplast is directly involved in the biosynthesis pathways of two major defense hormones, salicylic and jasmonic acid (Seyfferth and Tsuda 2014; Wasternack and Hause 2013). Because both retrograde and anterograde signal transduction is mediated through the oxidative environment of the plant cell, it is possible that the two types of signals would interact with one another during the induction of defense.

Salicylic acid is an essential mediator between gene transcription and the redox environment within plant tissue (i.e., Leon et al. 1995; Mateo et al. 2006; Tada et al. 2008). NPR1, a master regulating protein responsible for initiating transcription of defense genes, is activated by changes in the redox state of plant cells (Tada et al. 2008). In turn, concentration-dependent reception of salicylic acid by NPR3 and NPR4 determines which defense genes NPR1 activates (Fu et al. 2012). Expression of enzymes driving phenylpropanoid metabolism, in particular, are regulated by salicylic acid (Dixon et al. 2002). During the hypersensitive response (HR) of plant cells, hydrogen peroxide rapidly accumulates in foliar tissue (Levine et al. 1994). When salicylic acid crosses a concentration threshold, the NPR complex activates genetic expression of HR and systemic acquired resistance (SAR) responses (Fu et al. 2012).

Salicylic acid, itself a simple phenolic compound, directs phenylalanine lyase (PAL) metabolism to generate a multitude of phenolic compounds (Dixon et al. 2002). The 6-carbon ring structures common to all such phenolic compounds are characterized by high chemical stability; this core attribute of chemical stability fills a wide variety of advantageous biological functions. Phenolic compounds increase antioxidant capacity of plants because they can accept electrons without losing structural integrity (Dixon and Palva 1995). Similarly, the class of flavonoid compounds absorb UV-radiation, thereby acting as a sunscreen (Dixon and Palva 1995). Lignin, a major component of woody tissue, is a 'super-molecule' created

by linking simple phenylpropanoid compounds in a chaotic repeating fashion (Boerjan et al. 2003). As a defense against herbivorous insects, many plants produce polyphenol-oxidases (PPOs), enzymes which form cross-links between phenolic compounds (Appel 1993). When ingested, PPOs form difficult-to-digest masses of phenolic compounds inside insect guts thereby impeding rate of herbivory (Appel 1993).

When plants perceive stress from pathogens or insects, their chemical defense response frequently rests upon a highly-conserved tradeoff between defense hormones salicylic acid and jasmonic acid (Thaler et al. 2012). Herbivores with chewing mouthparts can possess mechanisms to induce salicylic acid in their host-plants, so as to bypass defenses based on jasmonic acid signaling (Musser et al. 2002; Kästner et al. 2014). Conversely, *Pseudomonas syringae* is a microbial pathogen that inoculates its hosts with coronatine, a chemical mimic of jasmonic acid, thereby suppressing induction of salicylic acid (Zheng et al. 2012). Some studies document that plants grown under elevated CO_2 are more resistant against infection from viral pathogens as a consequence of defenses upregulated by salicylic acid (Matros et al. 2006; Huang et al. 2012). Zhang et al. (2015), find that elevated CO_2 suppresses jasmonic-acid based defense in tomato by upregulating salicylic acid. Moreover, Mhamdi and Noctor (2016) demonstrate that elevated CO_2 upregulates salicylic acid through redox-linked pathways (Mhamdi and Noctor 2016).

Soybean plants grown under elevated CO_2 at the soyFACE field experiment exhibit changes that match characteristics of altered ROS signaling from the chloroplast. Although not isolated to the chloroplast, Cheeseman (2006) found that leaf tissue grown under elevated CO_2 contains more hydrogen peroxide than leaf tissue grown under ambient CO_2, indicating a change in redox state. Qiu et al. (2008) report that protein isolated from leaves of the same experimental treatment exhibit increased carbonylation, a symptom of increased carbonic acid content in leaf tissue that could be caused by greater concentration of hydrogen peroxide. Similarly, foliar concentration salicylic acid is known to track the redox state of plant tissue, and Casteel et al. (2012) observe that foliar concentration of salicylic acid in soybean is higher under elevated CO_2 relative to control. Hence, it is possible that CO_2 modulates the redox environment within soybean tissue and thus influence hormonal regulation of plant chemical defense.

How does the Production of Photochemical ROS Modulate Defense Hormones?

Reactive oxygen species (ROS) signaling controls different biological processes such as responses to biotic and/or abiotic stimuli (Mittler et al.

2011). Although ROS can be accumulated in chloroplast and mitochondria of plants (Apel and Hirt 2004; Mittler et al. 2004), these reactive species are mainly produced by cell wall NADPH oxidases and peroxidases (Apel and Hirt 2004; Nurnberger et al. 2004). Because of the potential toxicity of ROS, non-toxic levels of these species must be maintained in a delicate balancing between ROS production and the metabolic counter-process involving ROS-scavenging enzymes (Mittler et al. 2004). Since plant responses might be regulated by temporal and spatial coordination between ROS and other signals, ROS function as secondary messengers that induce important signaling pathways.

In response to ROS accumulation, early signaling events in plants include increased flux of Ca^{2+} into the cytosol, activation of mitogen-activated protein kinases (MAPKs), and protein phosphorylation (Benschop et al. 2007). MAPKs play major role in signal transduction of diverse stress responses. Three consecutive elements (MAPKKK, MAPKK or MEK and MAPK) compose the signal cascade in which MAPK is finally phosphorylated and activated (Hamel et al. 2006). While the MAPKK4-MAPK3/6 module is known to play role in ROS production by acting upstream of NADPH oxidase, accumulation of H_2O_2 activates MAPK3 and MAPK6 (Kovtun et al. 2000). Although MAPKs are known to be activated by ROS molecules, the mechanism behind the specific activation of MAPK cascade by ROS is not clear (Jalmi and Sinha 2015).

The interactions between ROS and MAPKs have been observed in different plant species. H_2O_2 activates MAPK3 and MAPK6 in rice and gets activated by upstream kinase MAKK6. Pathogen attack induced the accumulation of ROS and activated *Arabidopsis* MAPK3, MAPK4, and MAPK6. Pathogen attack in *Arabidopsis* produced ROS and activated MAPK through the cascade of MEKK1-MEKK4/5-MAPK3/6 (Asai et al. 2002). This cascade induced resistance to a fungal pathogen as well as increasing tolerance to abiotic stresses (Kumar and Sinha 2013; Sheikh et al. 2013; Singh and Jwa 2013). MAPK ultimately phosphorylate and activate several downstream targets like transcription factor, other kinases, phosphatases, and cytoskeleton associated proteins (Hamel et al. 2006; Rodriguez et al. 2010).

MAPK activation and the ROS burst are two early events that trigger plant immunity. Pathogen and herbivore damage are perceived by leaves and lead to rapid transcription and activation of MAPK signaling pathways that induce JA/ET- and SA-regulated defenses in plants (Kandoth et al. 2007; Wu et al. 2007; Liu et al. 2014; Petersen et al. 2000; Liu et al. 2011). Whereas both MAPK3 and MAKP6 redundantly control SA and JA in different plant species, only MAPK6 regulates ET emission (Wu et al. 2007; Kandoth et al. 2007). Silencing MAPK6 but not MAPK3 diminished herbivory-induced ET levels in *N. attenuata* and *Arabidopsis* by

decreasing other kinases expression, like CDPKs (Wu et al. 2007; Schafer et al. 2011). In field-grown soybean, after few minutes of stink bug damage to developing seeds, MAPK6 phosphorylation was enhanced in addition to induction of JA/ET and SA (Giacometti et al. 2016). Although MAPK3 and MAPK6 are involved in the amplification of defensive reactions, MAPK4 takes part in repressing SA biosynthesis. MAPK4 in *Arabidopsis* deters SA signaling pathway by inhibiting EDS1 and PAD4 proteins and liberating the JA/ET pathway (Petersen et al. 2000; Brodersen et al. 2006). GmMAPK4-silenced soybean accumulated SA and SA-regulated genes were up-regulated in leaves (Liu et al. 2011).

A New Hypothesis Linking Primary and Secondary Metabolism

Imbalances Lead to ROS in the Chloroplast Through Water−Water Cycle

Per molecule removed from the atmosphere, CO_2 fixation consumes proportionally less ATP than O_2 fixation (Foyer et al. 2009; Foyer et al. 2012). To adjust for changes in energetic demand caused by variation in gas composition, C3 plants decrease cyclic electron flow with rising CO_2 concentration (Walker et al. 2015; Miyake et al. 2005). Reducing cyclic electron flow relaxes the proton motive force (pmf or ΔpH) across the thylakoid membrane, thereby slowing ATP production in the chloroplast. This relaxation in pmf is known to suppress NPQ mechanisms (Munekage et al. 2004; Miyake et al. 2005), although the effect has not been tested in context of rising atmospheric CO_2. A reduced capacity for NPQ could mean that plants lose heat-dissipation as a means for disposing of excess excitation energy; the remaining question, then, is whether this loss would increase allocation of excess energy to the Water–Water cycle.

Imbalances are Exacerbated in Dynamic Light Environments

Asada (1999) defines 'excess energy' in PETC as any electron flow that cannot be allocated to the combined sinks the Calvin Cycle and photorespiration. Under steady light conditions, plants balance energy intake against the energy demanded by fixation of atmospheric carbon and oxygen. However, during sudden transitions from dark to bright light conditions, such as would occur during cloud-breaks or sunflecks due to moving canopy cover, the sudden influx of light energy into the PETC could outpace demand from its primary sinks (Leakey et al. 2003; Watling et al. 1997). During such times, NPQ plays an important role in dissipating

excess excitation energy as heat from the light-harvesting complexes of the PETC (Watling et al. 1997).

During the Mehler reaction, or Water–Water cycle, excess excitation energy in the photosynthetic electron transport chain (PETC) is transferred to oxygen and then to water to form hydrogen peroxide (Asada 1999). The difference between measured electron transport rates (J) and theoretical rates calculated from gas exchange (J_c or J_g) has been attributed to the Mehler reaction (Krall and Edwards 1991; Ruuska et al. 2000). Thus, if the difference between J and J_g increases when plants are exposed to sudden light variation, the implication is that plants absorb more excitation energy than can be immediately directed to the combined sink of photosynthesis and photorespiration. By the same token, when the PETC departs from redox homeostasis- or non-steady-state photosynthesis—the efficiency of light harvest (Φ_{PSII}) should have a greater rate of change than the efficiency of carbon assimilation (Φ_{CO2}).

Perturbation to redox-homeostasis, or steady-state photosynthesis, is thought to form the basis for retrograde signaling by the chloroplast (for review see Gollan et al. 2015). During chloroplast retrograde signaling, environmental signals originate in the PETC and are transduced through various redox-dependent pathways to ultimately control nuclear gene expression. If the initial signal stimulus in the PETC is modulated by CO_2 concentration, what follows is that retrograde signaling from the chloroplast in response to environmental stress would differ as well. Salicylic acid, in particular, has a well-documented relationship with redox state of plant tissue and expression of genes related to innate immunity (respectively: Mateo et al. 2006; Leon et al. 1995; Vlot et al. 2009).

Are there other Environmental Factors that Drive the Imbalance?

Although this hypothesis focuses on acquisition of light energy, in principle any environmental factor that upsets the balance between energy supply and demand in the PETC would result in an ROS burst from the chloroplast. Under suboptimal temperature conditions, for instance, Fryer et al. (1998) observe that maize plants exhibit a nonlinear relationship between Φ_{PSII} and Φ_{CO2}. Situations that cause rapid closure of stomata could conceivably alter the ratio of CO_2 and O_2 in stomatal cavities, thus restricting CO_2 supply to adjoining cells. The effects of stomata closure during stress deserve careful consideration, as Farquhar and Sharkey (1982) indicate that stress-induced decreases in stomatal conductance rarely influence rates of carbon assimilation. Under dynamic or temporary conditions of stomata closure, however, a rapid shift in intercellular pressure of CO_2 could have the effect of generating differential ROS from chloroplast and peroxisome, an event outlined in more detail by Kangasjärvi et al. (2012).

Considering that some studies observe an influence of insect herbivory on stomatal conductance (e.g., Nabity et al. 2013; Meza-Canales et al. 2017), the role of stomatal closure during stress on redox-based signaling remains an open question.

Why should Photosynthesis and Defense be Linked? Plant Carbon/ Energy Allocation?

The possibility that chloroplast retrograde signaling modifies plant defense responses appeals to the core tenets of optimal defense theories, as described by Zangerl and Bazzaz (1992). Since the 1970's, numerous theories have been proposed to explain how plants allocate energy and material resources between the primary functions of growth and secondary functions of defense (Schuman and Baldwin 2015). The common denominator among such theories holds that plant defense exacts some cost on plant growth, leading to a tradeoff between growth and defense; plants must somehow balance the two functions in order to optimize their evolutionary fitness. Thus, because photosynthesis drives plant growth, the idea that regulation of defense would occur as a function of perturbation to photosynthesis matches ongoing research on the connection between primary and secondary metabolism in plants.

Experimental Approaches to Testing the Hypothesis

The hypothesis that C3 plant chloroplasts could modulate plant secondary metabolism in a CO_2-dependent manner encompasses both proximate photosynthetic mechanisms and ultimate effects on interaction between plants and insects. The two proximate aspects of this hypothesis are that: (1) Elevated CO_2 in combination with variable light causes transient excess energy in electron transport to drive increased production of hydrogen peroxide; and that (2) This increase in production of hydrogen peroxide acts as a molecular signal that is transduced through MAPKs to stimulate biosynthesis of salicylic acid. Hence, *if* increasing CO_2 amplifies the production of ROS by photosynthesis and *if* such an increase in ROS acts as a signal that is meaningful to the biosynthesis of defense hormones, *then* one might predict that the ultimate expression of plant chemical defense against herbivorous insects would likewise depend on concentration of CO_2 in the atmosphere. Hence, tests of this hypothesis are predicated upon measuring interactions between the light and CO_2 environment of plants and their effects on the production of ROS, MAPKs, defense hormones, production of secondary metabolites and plant-insect interactions.

A major obstacle to testing the hypothesis that hydrogen peroxide produced in chloroplasts ultimately influences plant-insect interactions is

the chemical instability of reactive oxygen species: They are very difficult, if not impossible, to isolate and quantify. Both Cheeseman (2006) and Noctor et al. (2016) have published reviews on the topic of measuring ROS in plant tissue, and have concluded that current methods are too error-prone to be trustworthy. Hence, even if one were to use described methods for chemically separating and quantifying ROS in plant tissue, the results would likely meet with implicit distrust from the scientific community. A more effective approach than measuring ROS directly may be to focus on documenting variation in the processes that give rise to ROS, or to seek the immediate effects of the production. Mhamdi and Noctor (2016), for example, use such an indirect measurement strategy by documenting endogenous pools of the antioxidants glutathione and ascorbic acid to demonstrate that the induction of salicylic acid by elevated CO_2 in *Arabidopsis* is redox-dependent.

At the level of photosynthesis, integrated measurements of gas exchange and chlorophyll fluorescence can determine whether elevated CO_2 increases allocation of electrons to oxygen in dynamic light environments. If cyclic electron flow around PSI declines as CO_2 concentrations increase, then induction of NPQ in C3 plants should be relaxed under elevated CO_2 relative to ambient CO_2. Moreover, if the activity of the Water–Water cycle during sudden transitions from steady to non-steady state photosynthesis depends on CO_2 level, then for C3 plants the difference between electron transport rate (J) and theoretical electron transport rate (J_g) should be higher under elevated CO_2 relative to ambient CO_2. In other words, Φ_{PSII} should outpace Φ_{CO2} when plants are exposed to sudden increases in light intensity, especially so with increasing CO_2. The theoretical rate of electron transport (J_g) can be calculated based on a derivation of the Farquhar-von Caemmerer-Berry model of photosynthesis, as in Ruuska et al. (2000). Similarly, when Φ_{PSII} displays a non-linear relationship with Φ_{CO2}, the implication is that the PETC is acquiring more energy than can be immediately directed towards carbon metabolism, as in Fryer et al. (1998).

At the level of intra-cellular signaling, imaging techniques can be used to visualize ROS production in leaf tissue while chromatographic methods can quantify concentrations of major defense hormones. To visualize ROS, diamine-benzidine (DAB) stain reacts with H_2O_2 in the presence of peroxidases to form a brown precipitate. If elevated CO_2 causes differential production of ROS, then leaf tissue grown exposed to sudden dark-to-light transition should appear dark when treated with DAB solution, relative to untreated leaves or leaves treated under ambient CO_2. Similarly, if the induction of salicylic acid by fluctuation in light levels depends on CO_2, then the magnitude of induction of salicylic in C3 plants in dynamic light environments should be increased under elevated

CO_2 relative to ambient CO_2. At the same time, induction of jasmonic acid should be relatively suppressed.

At the level of plant-insect interactions, chemical analysis of secondary metabolites and bioassays with insect herbivores can determine the palatability of plant tissue changes depending on light and CO_2 environment. For instance, if dynamic light and elevated CO_2 interact so as to compromise plant chemical defenses, then insect herbivores would be expected to both prefer and consume more of plant tissue grown under dynamic light and elevated CO_2 than plant tissue grown under steady light and ambient CO_2. Likewise, the profiles of secondary metabolites exhibited by plants grown under differential light and CO_2 regimes should differ from one another.

At all stages of hypothesis testing, genetic manipulation of plants may help to resolve the signaling pathways transduced from initial perturbations of photosynthesis to ultimate effects on plant-insect interactions. To name just a few possibilities, the *Arabidopsis* biological resource center (ABRC) at www.abrc.osu.edu maintains a stock of genetically altered *Arabidopsis* lines that pertain to the hypothesis discussed in this chapter. Among those lines of *Arabidopsis* are NPQ mutants which are deficient in the induction of nonphotochemical quenching (originally developed by Niyogi et al. 1998) and ascorbate mutants which are deficient in production of the antioxidant ascorbate (originally developed by Conklin et al. 2000). By relaxing the induction of NPQ through genetic manipulation, one might expect to replicate the effects of elevated CO_2 on induction of foliar salicylic acid by dynamic light. Similarly, by reducing available pools of ascorbic acid, one might expect to amplify hydrogen peroxide in leaf tissue and thus increase foliar concentration of salicylic acid. Given the broad nature of this hypothesis, many more genetic targets may be possible; the suggestions provided here are intended to serve as examples of how genetic manipulation in plants might be applied to help in testing.

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