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Plastid Signals and the Bundle Sheath: Mesophyll Development in Reticulate Mutants

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ABSTRACT The development of a plant leaf is a meticulously orchestrated sequence of events producing a complex organ comprising diverse cell types. The reticulate class of leaf variegation mutants displays contrasting pigmentation between veins and interveinal regions due to specific aberrations in the development of mesophyll cells. Thus, the reticulate mutants offer a potent tool to investigate cell-type-specific developmental processes. The discovery that most mutants are affected in plastid-localized, metabolic pathways that are strongly expressed in vasculature-associated tissues implicates a crucial role for the bundle sheath and their chloroplasts in proper development of the mesophyll cells. Here, we review the reticulate mutants and their phenotypic characteristics, with a focus on those in *Arabidopsis thaliana*. Two alternative models have been put forward to explain the relationship between plastid metabolism and mesophyll cell development, which we call here the *supply* and the *signaling* hypotheses. We critically assess these proposed models and discuss their implications for leaf development and bundle sheath function in C3 species. The characterization of the reticulate mutants supports the significance of plastid retrograde signaling in cell development and highlights the significance of the bundle sheath in C3 photosynthesis.

Key words: reticulate; mesophyll; bundle sheath; development; intercellular signaling; leaf variegation; plastid.

INTRODUCTION

Variegation mutants display vegetative tissue containing patches of differing colors that arise from differential chloroplast development (Yu et al., 2007). While most variegation mutants develop their variegation pattern independently of cell type, a subset of variegated mutants develops mesophyll-specific defects, resulting in leaves with pale-green interveinal tissue superimposed on a normal-green vasculature. The prominent, web-like vascular pattern in these mutants has led them to be named reticulate mutants (Figure 1A). Reticulate mutants have been described in a number of plant species, and offer a powerful method to investigate leaf development and cell-specific function and regulation (Table 1). In this regard, reticulated mutant screens of *Arabidopsis thaliana* have been utilized to isolate candidate genes involved in cell-specific leaf development processes (Kinsman and Pyke, 1998; Berná et al., 1999).

As the number of cloned reticulate mutants has grown, interesting patterns have emerged. Nearly all reticulated mutants are affected in nuclear-encoded, plastid-localized genes that are central to primary metabolism. This reinforces the significance of plastid homeostasis and retrograde signaling in cell and tissue development. Perhaps more surprisingly, promoter::GUS experiments of several of the affected genes have uniformly revealed dominant (or exclusive) expression in vasculature or bundle sheath (BS) cells rather than the mesophyll (M) cells where the mutant phenotype is manifested.

In higher plants, the leaf organ develops from a leaf primordia consisting of three transcriptionally distinct cell layers which give rise to the epidermis, vasculature, and internal parenchymatous tissue (Barton, 2010). Their differentiation into the various cell types is driven by a complex interaction of transcription factors, small interfering RNAs, metabolites, and phytohormones (Yanai et al., 2005; Zhao et al., 2010; Yoshida et al., 2011; Byrne, 2012).

The mature leaf is enclosed on the top and bottom by a single-cell layer of epidermal tissue, between which are the parenchymatous M cells, the primary photosynthetic cells of the leaf (Figure 1B). Species such as *A. thaliana* harbor two distinct M cell types: the palisade M, which serves as the

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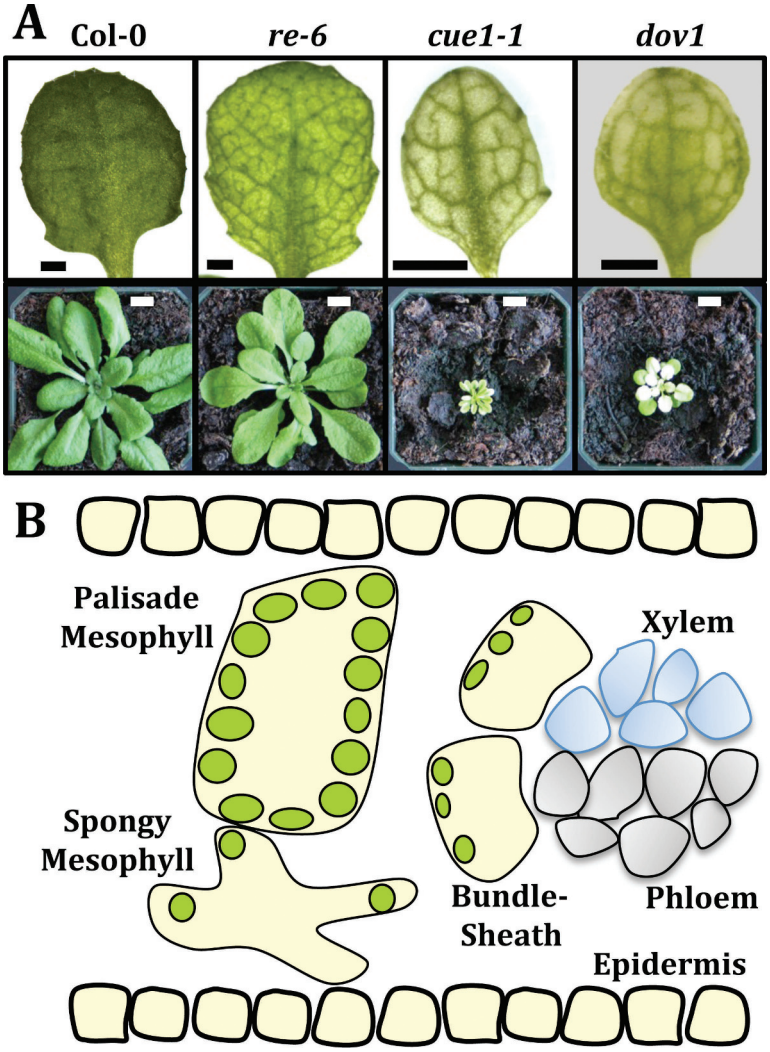

Figure 1. The Reticulate Phenotype Is Caused by Specific Disruption of the Mesophyll Cells in Leaf Tissue.

(A) Reticulated leaves, such as seen here in the *re-6*, *cue1-1*, and *dov1* alleles, display a prominent, web-like, vascular pattern on a pale-green lamina. Black scale bars indicate 1 mm and white scale bars indicate 3 mm.

(B) Schematic cross-section illustrating the various cell types of C3 leaf tissue. Chloroplasts are represented by green ovals and are used to indicate the chloroplast-containing cells of the leaf. The unique chloroplast morphology, as seen in *A. thaliana* leaf tissue, is reflected here. Although bundle sheath chloroplasts display normal internal ultrastructure and are photosynthetically active, they are smaller, fewer in number, and are positioned along the cell wall distal to the vasculature.

primary site of photosynthesis and is characterized by elongated cellular structure; and the spongy M, which is loosely packed and contains fewer chloroplasts. Embedded within the M are the leaf veins, consisting of phloem, xylem, and associated cells that conduct water, minerals, assimilates, and many other compounds throughout the plant. The veins are surrounded by one or more layers of chlorenchymatic BS cells that act as an interface between the M and veins, regulating fluxes of compounds into and out of the vasculature. The BS cells have been the subject of extensive investigation in plants performing C4 photosynthesis, where a special distribution of functions has been established between the M and BS cells to facilitate carbon concentration (Langdale, 2011). BS cells of C3 plants have received relatively less attention, but are also photosynthetically active and relevant to proper development and function of the plant (Fryer et al., 2002; Leegood, 2008).

Corresponding to their distinct functions, BS and M cells of *A. thaliana* also display unique chloroplast morphology. While the internal ultrastructure, including thylakoid structure, is comparable, the chloroplasts of the BS are smaller and fewer in number compared to those in the M (Kinsman and Pyke, 1998). Furthermore, BS chloroplasts are often placed along the cell wall distal to the vascular strand and adjacent to the M tissue (Figure 1B).

The reticulate mutants provide a tool to elucidate cell-specific development and function in C3 leaf tissue, which benefits our understanding of intercellular signaling and

Table 1. Reticulated Leaf Mutants of *Arabidopsis thaliana*.

Accession number	Protein name	Protein function[a]	Names[b]	Leaf expression pattern	References[c]
At5g33320	Phospho*enol*pyruvate/ Phosphate Translocator 1 (PPT1)	Import of Phosphoenolpyruvate into the chloroplast	*cue1/nox1*	Vasculature-specific	1, 2, 3, 4
At4g34740	Glutamine Phosphoribosyl Pyrophosphate Aminotransferase (ATase2)	First step in *de novo* purine biosynthesis	*dov1/cia1/atd2/alx13*	n.d.[d]	5, 6, 7, 8, 9
At5g54810	Tryptophan Synthase β1 (TSB1)	Tryptophan biosynthesis	*trp2/trp3/smo1*	n.d.[d]	10, 11
At1g15710	Arogenate Dehydrogenase (TyrAAT2)	Tyrosine biosynthesis	n.d.[d]	n.d.[d]	12, 13, 14
At2g41680	NAD(P)H-Thioredoxin Reductase (NTRC)	Regulation of multiple plastid metabolic pathways	*ntrc*	Strong expression throughout leaf, highest in vasculature	15, 16, 17, 18
At3g27740	Carbamoyl Phosphate Synthetase α (CarA)	Arginine and pyrimidine biosynthesis	*ven6*	Vasculature and neighboring mesophyll cells	19, 20, 21
At1g29900	Carbamoyl Phosphate Synthetase β (CarB)	Arginine and pyrimidine biosynthesis	*ven3*	Vasculature and neighboring mesophyll cells	19, 20, 21
At1g09780	Phosphoglycerate Mutase 1 (iPGAM1)	Interconversion of 3-phosphoglycerate and 2-phosphoglycerate	*ipgam1*	n.d.[d]	22
At3g08590	Phosphoglycerate Mutase 2 (iPGAM2)	Interconversion of 3-phosphoglycerate and 2-phosphoglycerate	*ipgam2*	n.d.[d]	22
At2g24120	Nuclear-Encoded Plastid RNA Polymerase (RpoTp)	Transcription of the chloroplast genome	*scabra3*	n.d.[d]	23
At2g37860	Reticulata (RE)	n.d.[d]	*re/ven2/lcd1/rcd2*	Proximal region of leaf primordia and vasculature of mature leaf	19, 24, 25, 26, 27
At3g08640	Alpha-tandem	n.d.[d]	*ven5/rer3*	Distal region of leaf primordia and vasculature of mature leaf	19, 27
At5g12470	MEP3	n.d.[d]	*rer4*	n.d.[d]	27
At5g27010	ARM-domain protein	n.d.[d]	n.d.[d]	n.d.[d]	14
n.d.	n.d.[d]	n.d.[d]	*ven1*	n.d.[d]	19
n.d.	n.d.[d]	n.d.[d]	*ven4*	n.d.[d]	19
Various	Ribosomal subunits	Ribosome scaffolding and translational regulation	*rpl7b/den5/rpl10ab/den12/rpl18c/ rpl38b/rpl39c/den30/rps6a/rps21b/rps28b/rps15ab/rpl28a/den29*		28

[a] If an enzymatic activity or other function is known or hypothesized by sequence homology.

[b] All mutant names reported in the literature.

[c] References are as follows: (1) Li et al., 1995a; (2) Streatfield et al., 1999; (3) Voll et al., 2003; (4) He et al., 2004; (5) Kinsman and Pyke, 1998; (6) Van der Graaff et al., 2004; (7) Hung et al., 2004; (8) Woo et al., 2011; (9) Rosar et al., 2012; (10) Barczak et al., 1995; (11) Jing et al., 2009; (12) Rippert and Matringe, 2002; (13) Rippert et al., 2009; (14) Myouga et al., 2013; (15) Serrato et al., 2004; (16) Pérez-Ruiz et al., 2006; (17) Rintamäki et al., 2008; (18) Lepistö et al., 2009; (19) Berná et al., 1999; (20) Potel et al., 2009; (21) Mollá-Morales et al., 2011; (22) Zhao and Assmann, 2011; (23) Hricová et al., 2006; (24) Conklin et al., 1996; (25) Barth and Conklin, 2003; (26) Overmyer et al., 2008; (27) Pérez-Pérez et al., 2013; (28) Horiguchi et al., 2011.

[d] n.d., data have not been determined.

cell differentiation. Such insight into the state of functional differentiation in C3 plants is also constructive in the efforts towards engineering C4 photosynthesis into C3 plants. In this review, we summarize the characteristics of reticulate mutants described in the literature. As the majority of mutants described or cloned come from *A. thaliana*, we focus on mutants of this species and draw on mutants from other species where relevant. Notably, no reticulate phenotype has been described in a C4 species, which may be a reflection of the unique division of labor between BS and M within this

photosynthetic system. We note that a number of striped mutants, reminiscent of the reticulate phenotype, have been described in C4 and C3 plants, such as the *bundle sheath defective* and *yellow stripe* mutants of Maize (Langdale and Kidner, 1994; Curie et al., 2001). However, these mutants do not demonstrate M-specific defects as seen in the true reticulate phenotype. While these mutants are informative, they are beyond the scope of this review.

The surprising expression patterns of reticulate genes points to a role for the vasculature/BS (whose formation precedes that of the M) in control of M development. Two hypotheses, which we call here the *signaling* and *supply* hypotheses, have previously been put forth to explain the M-specific defects in reticulate mutants. We describe and evaluate these hypotheses in light of the most recent results from reticulate mutants.

A. THALIANA RETICULATED LEAF MUTANTS

The *reticulata-related* (*rer*) Family

The first reticulated leaf mutant described in the literature was the *reticulata* (*re*) mutant identified by George Rédei in 1964 (Rédei and Hironyo, 1964). *re* has been used for decades as a visible marker for genetic mapping due to its easily identifiable phenotype and low degree of pleiotropy (Figure 1A). Recently, reticulate leaf mutants have been described in two additional genes of the reticulata-related (rer) gene family, named *reticulata-related 3* (*rer3*, *alpha-tandem*) and *reticulata-related 4* (*rer4*, *mep3*) (Pérez-Pérez et al., 2013). RE, RER3, and RER4 all localize in the chloroplast outer and inner envelope membranes, and contain the domain of unknown function (DUF) 3411 (González-Bayón et al., 2006). Although no precise functions for the RER family have been discovered, they are plant-specific and gene co-expression analysis reveals strong association with a number of genes of amino acid (AA) and nucleotide metabolism (Pérez-Pérez et al., 2013).

The *re* mutant alleles are notable for their low pleiotropic effects. Morphological defects are limited to the reticulate leaf phenotype in cotyledons and leaves—a manifestation of dramatically reduced M cell numbers, which still maintain wild-type size and are unaffected in plastid development (González-Bayón et al., 2006). In contrast, the *rer3* alleles (*rer3-1*, *rer3-2*, and *rer3-3*) display embryogenesis defects, mild leaf hyponasty, and leaf serration in addition to their reticulate phenotype. M cell numbers are reduced, as for *re* mutants, but epidermal cell expansion is also impaired, likely related to the stunted growth of the leaves. The *rer3-3* embryogenesis defects are correlated with elevated auxin signaling in developing *rer3-3* embryos, compared to wild-type and *re-3*. The *rer4-1* mutant also displays stunted growth along with weak leaf reticulation (Pérez-Pérez et al., 2013).

As for many reticulated leaf mutants, the magnitude of reticulation in *re* positively correlates with prevailing light intensity (Barth and Conklin, 2003; Overmyer et al., 2008). More surprisingly, the leaf reticulation in *re*, *rer3*, and *rer4* mutants—but not the *cue1* reticulate mutant (see below)—could be rescued by growth under short-day conditions (Pérez-Pérez et al., 2013). Only under long-day photoperiods, localized cell death and H_2O_2 accumulation were elevated in the perivascular M tissue of *re*, *rer3*, and *cue1* reticulate mutants compared to wild-type, leading to the hypothesis that the reticulate phenotype arises from photo-oxidative stress and cell death, which attenuates M differentiation.

The reticulate phenotype of *re* also becomes less apparent as leaves age, which is consistent with the higher expression of *RE* in young and meristematic leaf tissues (González-Bayón et al., 2006). Indeed, *RE* and *RER3* expression is dominant in leaf primordia, particularly in the BS, whereas lower expression of these genes is detected in mature leaves and is restricted to the vasculature-associated tissue (Pérez-Pérez et al., 2013). Expression of *RE* and *RER3* shows very little overlap. RER3 is expressed in the proximal half while RE is expressed in the distal half of the leaf primordia. Further demonstrating the unique roles of the two genes, overexpression of RER3:GFP failed to complement the *re* mutant phenotype and overexpression of RE failed to complement the phenotype of *rer3*.

Metabolic profiling of *re* and *rer3* indicated both mutants were impaired in branched chain AA and aromatic AA metabolism (Pérez-Pérez et al., 2013)—a pattern that is repeated in many of the reticulate mutants. In addition to decreased levels of aromatic AAs, several other products downstream of the plastidic phospho*enol*pyruvate (PEP) pool were also decreased, including cinnamic acid, sinapate, and indole-3-acetonitrile, suggesting misregulation of the plastidic shikimate pathway or insufficient supply of PEP to the plastid (Pérez-Pérez et al., 2013). PEP levels, however, were not quantified in the mutants.

The *ppt1* Mutants—*cue1*/*nox1*

ppt1 mutants (*cue1*/*nox1*) are affected in the PEP/Phosphate Translocator (PPT1) of the chloroplast envelope membrane, responsible for importing PEP into the chloroplast stroma (Fischer et al., 1997; Streatfield et al., 1999; Voll et al., 2003). The *A. thaliana* genome harbors two PPT isoforms: PPT1, expressed along the leaf veins and throughout the roots, and PPT2, expressed throughout the whole leaf (Knappe et al., 2003). The *ppt1* mutants suffer from a stunted growth phenotype and reticulated leaves (Figure 1A) (Streatfield et al., 1999; Voll et al., 2003). Despite their vasculature-specific expression in leaves, the *ppt1* mutants are primarily affected in the development of palisade M cells, which are fewer in number, smaller, and contain aberrant chloroplasts (Li et al., 1995a; Streatfield et al., 1999; He et al., 2004). Conversely, a PPT2 knockout has no visible phenotype (Li et al., 1995a).

The *cue1* allele of *PPT1* was originally identified from a screen for defective light-regulated genes, and found to under-express chlorophyll a/b-binding (*CAB*) genes in M, but not BS or epidermis, further highlighting the M-specific phenotype of the *ppt1* mutants and implicating PPT1 in

retrograde signaling (Li et al., 1995a). As with the *re* mutants, the magnitude of the visible phenotypes in *ppt1* mutants is correlated with the light intensity. Furthermore, reticulation and *CAB* under-expression are more pronounced in young leaf tissue. The *nox1* allele of *PPT1* was identified in a screen for nitric oxide (NO) over-accumulation and displays the same reticulated, stunted growth phenotype found in *cue1* (He et al., 2004). The authors proposed that the elevated levels of NO derive from the increased accumulation of Arg (see below), which serves as N donor in the proposed NO biosynthetic pathway. What role, if any, NO plays in BS-M signaling or M development is unclear.

PEP is synthesized in the cytosol and is provided to the plastid by the PPT1/2 transport proteins. The plastidic PEP serves as substrate for numerous plastid biosynthetic pathways, including the shikimate pathway, responsible for biosynthesis of many primary and secondary metabolites including the aromatic AA Phe, Tyr, and Trp (Maeda and Dudareva, 2012). Overexpression of PPT1, or targeting heterologous pyruvate/orthophosphate dikinase (PPDK) to *cue1* plastids, complemented the visual and biochemical phenotypes, indicating that shortage of PEP in the plastid stroma is the primary cause of the *cue1* phenotype (Voll et al., 2003).

Interestingly, the *cue1* leaf reticulate phenotype was rescued by collectively adding the three shikimate-derived aromatic AAs (Phe, Tyr, Trp) to the growth medium, but not by adding AAs individually (Streatfield et al., 1999; Voll et al., 2003). This might be explained by the fact that the branch points in the shikimate pathway are tightly regulated by feedback inhibition from the aromatic AA end-products.

The *cue1* mutant suffers from a number of metabolic imbalances. AA levels are deregulated in the *cue1* mutants. *cue1* has a decreased ratio of aromatic AAs to non-aromatic AAs (Voll et al., 2003). In particular, Arg is increased 10-fold and Phe is reduced to 50% relative to wild-type (Streatfield et al., 1999). Consistent, phenolic compounds derived from Phe, including flavonoids, anthocyanins, hydroxycinnamic acids, and simple phenolics, are diminished (Streatfield et al., 1999; Voll et al., 2003). Interference in phenolic acid metabolism has also led to abnormal leaf morphology in tobacco plants, as discussed further below (Elkind et al., 1990; Tamagnone et al., 1998a, 1998b). Accumulation of soluble sugars (glu, fru, suc) is also substantially reduced in the mutant (Streatfield et al., 1999). PEP is also channeled into fatty acid metabolism in the plastid; however, fatty acid levels were not changed in *cue1* relative to wild-type.

Chl fluorescence measurements indicate *cue1* is impaired in electron transport and non-photochemical quenching. Fluorescence-induction kinetics indicated a reduced pool of the photosynthetic electron transporter, plastoquinone, which is derived from the shikimate pathway. The impaired photosynthetic activity presumably accounts for the reduced levels of soluble sugars.

A role for *PPT1* in nuclear epigenetic modifications was implied by identification of two novel *ppt1* mutants that repressed transcriptional silencing of Pro_{35S}::NPTIII and *Transcriptional Silencing Information* (*TIS*) in a *repressor of silencing 1* (*ros1*) background (Shen et al., 2009). Remarkably, application of aromatic AAs (Phe, Tyr, Trp) restored the transcriptional silencing. These observations provide additional evidence that provision of PEP to the plastid by PPT1 impinges on plastid retrograde signaling, and that retrograde signaling affects the epigenetic state of the nucleus, in addition to nuclear gene transcription.

ipgam Double Mutants (*ipgam1/ipgam2*)

Interconversion of 2-phosphoglycerate and 3-phosphoglycerate in glycolysis is catalyzed by Phosphoglycerate Mutase (PGAM) (Fothergillgilmore and Watson, 1989). Multiple isoforms of this enzyme exist, which are divided into two classes based on dependence (dPGAMs) or independence (iPGAMs) of 2,3-bisphosphoglycerate as co-factor (Jedrzejas, 2000). Based on homology, only two iPGAM genes (iPGAM1—At1g09780 and iPGAM2—At3g08590) were predicted in the *A. thaliana* genome (Zhao and Assmann, 2011). Indeed, while single mutants of these genes had only slightly reduced levels of iPGAM activity, no activity could be detected in two iPGAM double mutants, indicating iPGAM1 and iPGAM2 jointly contribute all iPGAM activity in *A. thaliana* (Zhao and Assmann, 2011). Correspondingly, the *ipgam1/ipgam2* double mutants, but not single mutants, display reticulated leaves, severely stunted growth, and a male sterile phenotype (Zhao and Assmann, 2011). The *A. thaliana* iPGAM proteins are plastid-localized, where they regulate the levels of 2-phosphoglycerate, a direct substrate for PEP biosynthesis via enolase (Voll et al., 2009; Zhao and Assmann, 2011). Thus, *ipgam1/2* mutants, as with the *cue1* mutants, are hampered in the provision of PEP for the plastid. Follow-up experiments on *ipgam1/2*, such as complementation tests with aromatic AAs or PEP, or metabolite profiling, have not been reported in the literature.

The *carbamoyl phosphate synthetase* Mutants—*ven3* and *ven6*

A genetic screen for altered leaf phenotypes yielded the reticulated mutants *venosa* (*ven*) 1–6 (Berná et al., 1999). *ven2* was found to be allelic to *re*, and *ven5* allelic to *rer3*, both described above, while *ven1* and *ven4* have yet to be genetically mapped. *ven3* and *ven6*, however, were recently shown to encode the large (β) and the small (α) subunit of Carbamoylphosphate Synthetase (CPS), respectively (Mollá-Morales et al., 2011). The *ven3* and *ven6* mutants are semi-dominant and suffer from stunted growth and reticulated leaves—a manifestation of smaller and fewer M cells (Mollá-Morales et al., 2011). Although the vasculature-associated and BS cells appeared unaffected in *ven3* or *ven6*, GUS-staining revealed dominant expression in vasculature-associated cells,

with weaker expression in the immediately adjacent M cells (Potel et al., 2009).

Localized to the plastid, CPS catalyzes the synthesis of carbamoylphosphate, necessary for conversion of ornithine to citrulline, used in Arg and pyrimidine biosynthesis. In plants, a single CPS is involved in both Arg and pyrimidine biosynthesis, requiring tight coordination between the pathways (Slocum, 2005). The co-eluting pool of citrulline and Arg was diminished in *ven3* and *ven6*, concomitant with strong increases in ornithine and N-acetyl ornithine, consistent with a block in CPS activity upstream of Arg and pyrimidine biosynthesis (Potel et al., 2009). Additionally, exogenous application of the CPS product, citrulline, suppressed the reticulation and dwarf phenotypes in *ven3* and *ven6* (Mollá-Morales et al., 2011).

Gene silencing of the CPS large subunit in *Nicotiana tabacum* also led to a reticulated phenotype and stunted growth (Lein et al., 2008). Metabolite levels were not quantified in this mutant; however, in the same study, another mutant identified in the final step of pyrimidine biosynthesis, UMP synthase, lacked the reticulate or stunted growth phenotypes, indicating impairment of Arg rather than pyrimidine biosynthesis causes the leaf reticulation in *ven3* and *ven6*.

Tryptophan Biosynthesis—*trp2-301*

A reticulated leaf phenotype has been reported in the leaky mutant allele *trp2-301* (*smo1*) of Tryptophan Synthase β1 (*TSB1*, *TRP2*). TRP2 is the predominantly expressed isoform catalyzing the final step in Trp biosynthesis, converting indole to Trp (Jing et al., 2009). Mutants in the other five steps of Trp biosynthesis have been identified (*trp5*, *trp4*, *trp1*, *trp3*) or developed by antisense repression (*pai* and *igs*), but a reticulated leaf phenotype has not been reported (Last and Fink, 1988; Rose et al., 1992; Niyogi et al., 1993; Li et al., 1995b; Li and Last, 1996; Radwanski et al., 1996; Rose et al., 1997). Among an allelic series of more than a dozen *trp2* mutants, *trp2-301* (*smo1*) is among the weakest alleles (Barczak et al., 1995; Jing et al., 2009). While most *trp2* alleles have an absolute requirement for Trp to be viable, and thus are grown on Trp-containing soil, *trp2-301* is viable in the absence of Trp supplementation. It is, however, stunted in growth and displays reticulated leaves, particularly in cotyledons and younger leaves, as reported in many other reticulated mutant classes.

The phenotype of *trp2-301* has been the most extensively characterized of the *trp2* alleles. Cross-sections of leaf tissue revealed reduced epidermal and M cell size (~30% of wild-type) but no effect on cell number, concomitant with decreased endoreduplication, indicative of impaired cell elongation (Jing et al., 2009). *trp2-301* also suffered from delayed chloroplast development, with smaller chloroplasts and underdeveloped thylakoids, although this phenotype was largely suppressed at later stages of growth or by Trp supplementation to the growth media.

Trp is synthesized in the plastids of plants (Zhao and Last, 1995) and serves dual purposes as a precursor for secondary metabolism (e.g. biosynthesis of auxins, glucosinolates, alkaloids, or phytoalexins) and as an integral component of protein synthesis (Kutchan, 1995; Zhao and Last, 1996; Tzin and Galili, 2010; Maeda and Dudareva, 2012). Surprisingly, metabolite measurements in several *trp2* and *trp3* alleles indicated elevated levels of auxin (as free indole acetic acid (IAA)) (Normanly et al., 1993; Ouyang et al., 2000). However, instability of indole-3-glycerophosphate, the substrate of the TRP2–TRP3 protein complex, leads to formation of free IAA during standard sample handling, thus IAA measurements from the *trp2* and *trp3* mutants are dubious (Muller and Weiler, 2000). Furthermore, application of exogenous auxin to plants failed to complement the reticulated phenotype in *trp2-301* (Jing et al., 2009). Since Trp is essential in protein biosynthesis, which itself is linked to cell expansion, protein levels were also measured in *trp2-301*, but were wild-type-like (Jing et al., 2009). Furthermore, the impairment of protein biosynthesis in wild-type plants by the translation inhibitor cycloheximide failed to replicate a leaf reticulate phenotype (Jing et al., 2009). Thus, the reticulate phenotype does not appear to be caused by impaired protein biosynthesis.

ntrc (*NADPH-thioredoxin reductase*)

Redox regulation, mediated via thioredoxin (Trx) proteins, is responsible for the control of many metabolic activities and environmental adaptations (Montrichard et al., 2009). The catalytic cycle of Trxs requires a continuous supply of electrons from the reducing power of Ferredoxin (Fd) or NADPH, mediated by Fd-Trx Reductases (FTRs) and NADPH-dependent Trx Reductases (NTRs), respectively (Schurmann and Buchanan, 2008; Spinola et al., 2008). A single C-type NTR (NTRC) has been identified, which is unique to photosynthetic organisms and is the only NTR in plastids of autotrophic and heterotrophic tissue (Serrato et al., 2004; Kirchsteiger et al., 2012). *ntrc* T-DNA knockout alleles display a stunted growth phenotype with smaller, misshapen M cells and fewer, aberrant M chloroplasts, manifested at the whole-leaf level as reticulate leaves (Serrato et al., 2004; Pérez-Ruiz et al., 2006; Rintamäki et al., 2008). Remarkably, these characteristics are strongly dependent on photoperiod (Lepistö et al., 2009). The reticulated leaf and stunted growth phenotypes are most severe under short-day conditions (8h light, 16h dark) but are reduced with successively longer light periods, and continuous light largely rescues the *ntrc* mutant.

Although plastid development is impaired in *ntrc* and carbon assimilation rates are reduced, the assembly of the photosynthetic complexes is unaffected (Lepistö et al., 2009). Consistently with most other reticulate mutants, *ntrc* is impaired in regulation of shikimate-derived aromatic AA accumulation, as well as the downstream product, auxin (IAA). These patterns are amplified under short-day

conditions when the phenotype is strongest, as reported in the *re* and *rer3* mutants. Supplementation of growth media with Phe, Trp, or IAA partially complemented the phenotype (Lepistö et al., 2009). Thus, we hypothesize that the *ntrc* phenotype may be caused by a constraint in the regulation of aromatic AA metabolism.

NTRC is unique among NTRs in that, along with an NTR domain, it also contains a functional Trx domain (Serrato et al., 2004; Pérez-Ruiz et al., 2006), thereby coupling the electron transfer from NADPH to regulatory targets into a single polypeptide. NTRC has been shown to regulate a number of diverse plastidic functions, including chlorophyll biosynthesis, aromatic AA metabolism, starch metabolism, and ROS scavenging and signaling (Pérez-Ruiz et al., 2006; Rintamäki et al., 2008; Spinola et al., 2008; Stenbaek et al., 2008; Kirchsteiger et al., 2009; Michalska et al., 2009; Kirchsteiger et al., 2012; Richter et al., 2013). The chlorophyll biosynthetic enzymes, Glutamyl-tRNA Reductase (GluTR) and Mg-protoporphyrin IX Monomethylester Cyclase (MgPMME) and the plastidic 2-Cys Peroxiredoxin (2-Cys Prx) have been conclusively demonstrated to be direct substrates of *A. thaliana* or rice NTRC (Pérez-Ruiz et al., 2006; Richter et al., 2013).

Redox homeostasis in the plastid is maintained under light by reducing power from photosynthetic electron transport to Fd and NADPH, via FTR and NTR enzymes (Hanke and Mulo, 2013). However, in darkness, the sole source of reducing power in plastids is NADPH from the plastidic oxidative pentose phosphate pathway; thus, the elimination of NTRC would be expected to have an especially noticeable effect on redox homeostasis and signaling in the dark, when it serves as the sole mediator of reduction. This is consistent with the photoperiod-dependent phenotype of the *ntrc* mutant and the observation that prolonged exposure to darkness followed by return to standard growth conditions led to elevated accumulation of peroxides and lipid peroxidation in *ntrc* compared to the wild-type (Pérez-Ruiz et al., 2006). Although the relationship between NTRC and M-specific development ultimately remains unclear, NTRC is crucial for maintaining plastidic redox homeostasis, especially in the absence of photosynthesis, and is a redox switch controlling diverse signaling and metabolic processes (Kirchsteiger et al., 2012).

ATase2 Mutants—*dov1*, *cia1*, *atd2*, and *alx13*

The committed step of *de novo* purine biosynthesis is catalyzed by glutamine phosphoribosyl pyrophosphate aminotransferase (ATase), responsible for deamination of glutamine into glutamate (Mok and Mok, 2001; Hung et al., 2004; van der Graaff et al., 2004; Zrenner et al., 2006; Rosar et al., 2012). *A. thaliana* harbors three homologs of ATase: ATase1, 2, and 3 (Rosar et al., 2012). Several mutant alleles of the dominantly expressed isoform, *ATase2* (*dov1*, *cia1-2*, *atd2*, *alx13*), display stunted growth and leaf reticulation patterns, whereas investigation of an *atase2* knockout mutant in *A. thaliana* shows no visible phenotype (Hung et al., 2004). While the *dov1* allele displayed strong reticulation (Figure 1A), the *cia1-2* and *atd2* alleles typically displayed variegated patterns and chlorotic patches on leaf tissue, and the *alx13* allele showed chlorotic patches at the lateral margins with occasional weak reticulation, indicating a variable phenotypic penetrance among *atase*2 mutants, dependent on the strength of the allele, the plant age, and environmental conditions (Kinsman and Pyke, 1998; Hung et al., 2004; Woo et al., 2011; Rosar et al., 2012). However, the defects in the *atase2* alleles are restricted to M cells of true leaves, with normally developed BS cells and chloroplasts. As for the other reticulate mutants, the extent of reticulation in *dov1* decreased with leaf age. This phenomenon may be due to the purine salvage pathway, which is functional in the ATase2 mutants, and can remobilize purines released during senescence in older leaf tissue (Woo et al., 2011).

De novo purine biosynthesis is shared between the plastid and the cytosol in *A. thaliana*, with the initial steps, including the committed step by ATase, localized to the plastid (Hung et al., 2004; van der Graaff et al., 2004; Zrenner et al., 2006). The mutations in *atase2* alleles can impair biosynthesis of purines and accumulation of their downstream products: (1) nucleic acids, (2) the energy currencies, ATP, and GTP, and related nucleoside phosphates, (3) the reducing equivalents, NAD(P) and FAD(P), (4) cytokinins, and (5) enzymatic co-factors (Smith and Atkins, 2002). Indeed, decreased levels of ATP, ADP, GTP, and GDP, concomitant with increased levels of their building block AAs, Asp, and Gly, were found in the *cia1-2* allele (Hung et al., 2004; Rosar et al., 2012). Further, the mutant phenotype was biochemically complemented by exogenous AMP, though not by the related purine derivatives GMP, IMP, FAD, and NADH or by a synthetic cytokinin analog (Hung et al., 2004; van der Graaff et al., 2004; Rosar et al., 2012).

Cytokinins drive cell mitosis and are abundant in meristematic tissues (Mok and Mok, 2001; Matsuo et al., 2012; Murray et al., 2012). Consistently with lowered purine levels, total cytokinin contents in *dov1* leaves were decreased, and cytokinin and auxin reporter::gus assays revealed that perception and/or biosynthesis of both phytohormones were diminished in *dov1* (Rosar et al., 2012). This agrees with the growth retardation and lowered mitotic activity. However, the bioactive cytokinins (*trans*-zeatin, *cis*-zeatin, and isopentenyl adenine) were, in fact, elevated in *dov1*, and application of exogenous cytokinins to *dov1* or *cia1-2* did not revert the stunted growth or reticulate phenotypes (Hung et al., 2004; Rosar et al., 2012). Hence, the direct cause of the *atase2* phenotypes remains unclear.

The Plastid RNA Polymerase—RpoTP

A. thaliana encodes three nuclear-encoded T7 phage-type RNA polymerases (RpoTs) that drive expression of subsets of

the organellar genomes. One is targeted to the mitochondria (RpoTm), one to the plastid (RpoTp), and one is dually targeted (RpoTmp). The *scabra3* (*sca3*) mutants are perturbed in the plastid-specific RNA polymerase, RpoTp, which impedes chloroplast biogenesis. Characterization of two *sca3* mutants (*sca3-1* and *sca3-2*) revealed a reticulate phenotype, stunted growth, and a wrinkled, serrated leaf lamina (Hricová et al., 2006). While epidermal cells were indistinguishable from wild-type plants, interveinal tissue revealed dramatically reduced M density and irregularly shaped M cells. Although the above mutant phenotype was reported to a variable extent in both alleles, the weaker allele (*sca3-1*) was found to contain chloroplasts indistinguishable from wild-type, except for fewer starch grains. Conversely, the stronger allele (*sca3-2*) contained a dramatically reduced number of chloroplasts, which were smaller with less developed thylakoids. Significantly, the chloroplasts in the vicinity of the vasculature were more like wild-type.

The Plastid Envelope DNA-Binding (PEND) protein from pea localizes to the plastid inner envelope membrane where it is thought to tether the plastid nucleoid via direct binding of plastid DNA (Sato et al., 1998; Sato and Ohta, 2001). It is dominantly expressed in young seedlings and developing plastids, when expression of the plastid genome is most active. Thus, PEND is suggested to regulate replication and transcription of the plastid genome through control of nucleoid morphology and localization. It is interesting that a *Brassica napus* PEND homolog, also demonstrating DNA-binding capability and plastid membrane localization, produces a reticulate phenotype and impaired palisade M development when heterologously overexpressed in tobacco (Wycliffe et al., 2005). Together, the *sca3* and *pend* mutants indicate that interference of plastid DNA transcription primarily affects M cell development over that of the BS.

Cytosolic Ribosomal Subunits

The only *A. thaliana* reticulate mutants not affected in plastid-localized gene products are mutants in cytosolic ribosome subunits. These mutants display various leaf developmental defects including leaf reticulation. Phenotypic analysis of 13 mutants in 11 ribosomal subunit genes demonstrated smaller and narrower leaves, most of which also displayed a mild reticulate pattern (Horiguchi et al., 2011). Further investigation of a subset of eight of these mutants showed that reductions in both cell division and cell expansion account for the stunted growth defects. Consistently with the reticulate pattern, most mutants suffered from disorganized palisade M cells and increased air space. In these mutants, the number of M cells was generally reduced, whereas the M cell size was increased, indicating impaired cell division (Horiguchi et al., 2011). Collectively, the phenotypes of ribosomal subunit mutants indicate varying contributions to leaf development, many of which include defects in M-specific cell proliferation.

Uncharacterized Mutants

A search of the Chloroplast Function Database II (http://rarge.psc.riken.jp/chloroplast/), which compiles and curates nuclear-encoded chloroplast mutants in *A. thaliana*, identified two additional reticulated mutants affected in At5g27010 and At1g15710 (Myouga et al., 2013). Although At5g27010 is predicted to encode an Armadillo-repeat (ARM) domain, its localization remains unclear and no literature has been published on this protein or corresponding mutant lines, so its function remains unknown. On the other hand, At1g15710 is localized to the plastid stroma and encodes one of two isoforms of Arogenate Dehydrogenase (TyrAAT2), responsible for synthesizing Tyr from arogenate and NADP (Rippert and Matringe, 2002; Zybailov et al., 2008; Rippert et al., 2009), which again links a reticulate leaf phenotype to plastidial aromatic AA biosynthesis.

MOST RETICULATE MUTANTS SHARE DEFECTS IN PLASTID PRIMARY METABOLISM

Taken together, genes affected in reticulated mutants are predominantly involved in plastidic primary metabolic pathways (Figure 2). The affected metabolic pathways are related either to (1) the shikimate pathway and provision of its central precursor, PEP, or (2) metabolism of purine and pyrimidine nucleotides. Even mutants of the *rer* gene family—although their functions remain unclear—show significant co-expression with genes involved in plastidic AA and nucleotide metabolism. In fact, among the top 35 co-expressed genes of *re*, 11 are known or hypothesized to function in AA and/or nucleotide metabolism, and show a Pearson Correlation Coefficient greater than 0.63 (P.K. Lundquist, C. Rosar, A.P.M. Weber, unpublished results). Further supporting the importance of the shikimate pathway in M development, supplementing wild-type *A. thaliana* (Ws-2) with 4 mM Phe or Tyr and 2% Suc, which is expected to alter the distribution of flux between the Tyr and Phe/Trp branches of the shikimate pathway, also induces a reticulate leaf phenotype (Voll et al., 2004).

These affected metabolic pathways are functionally related, jointly providing compounds required for gene expression and protein synthesis. However, they also feed intermediates for numerous and diverse downstream products serving many functions, from photosynthetic light harvesting to herbivore defense (Tzin and Galili, 2010; Maeda and Dudareva, 2012). The aromatic AAs Phe, Tyr, and Trp products of the shikimate pathway serve as precursors for many primary and secondary metabolites, including phenylpropanoids, lignins, prenyl-lipids, glucosinolates, alkaloids, and phytohormones (Tzin and Galili, 2010; Maeda and Dudareva, 2012). The central role of these pathways likely increases the pleiotropy in the reticulate mutants and drives the challenge to discover the cause(s) of M-specific developmental defects.

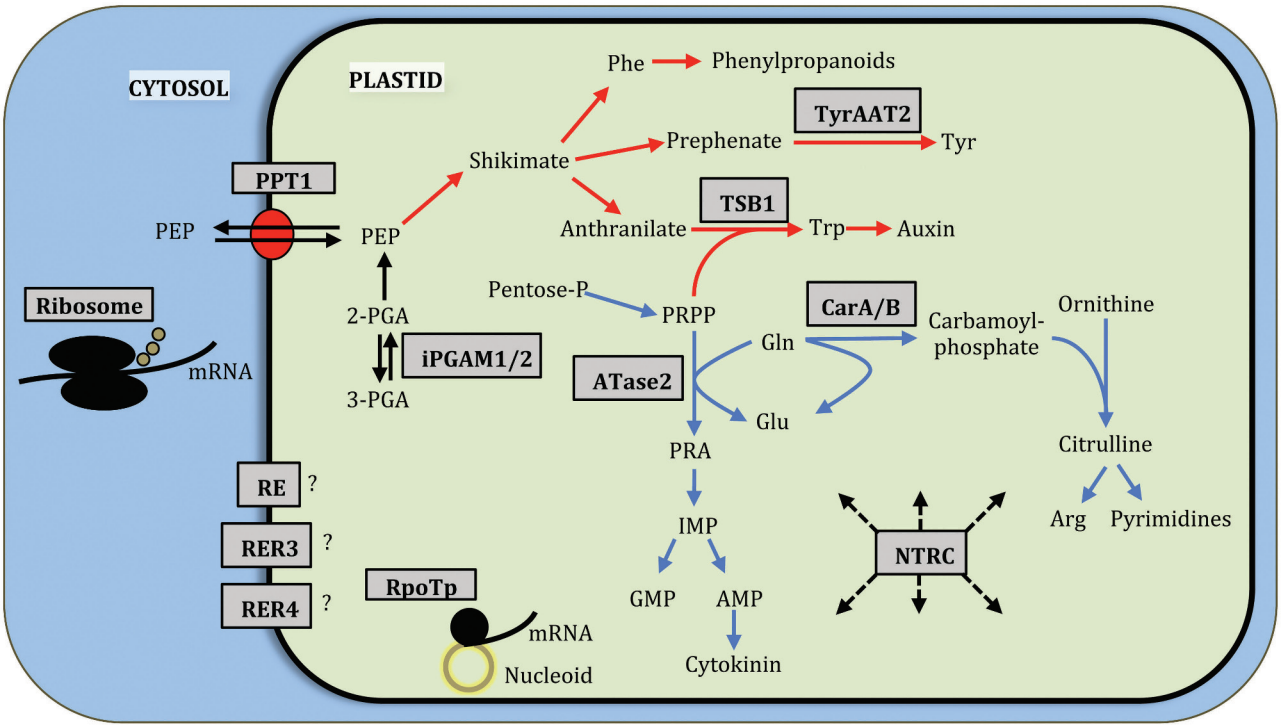

Figure 2. Reticulate Mutants Are Preferentially Affected in Plastid Metabolic Pathways.

The affected genes of all *A. thaliana* reticulate mutants are illustrated here in gray boxes, indicating their localization and function (if known). All genes are involved in primary metabolic steps of the plastid, with the exception of the cytosolic ribosome subunits. Metabolic pathways related to the shikimate pathway and downstream products are highlighted with solid red lines, and the pathways related to nucleotide biosynthesis and downstream products are highlighted with solid blue lines. Supply of Phospho*enol*pyruvate (PEP) via transport (PPT1) or metabolism (iPGAM1/2) is highlighted with solid black lines. The regulatory nature and diverse targets (many of which likely are unknown) of NADPH-dependent Thioredoxin Reducates C (NTRC) are depicted with dashed lines. The function of the Reticulata protein family (RE, RER3, and RER4) are not known, but are localized in the plastid envelope. Refer to the text for further details about functions of each gene. All amino acids are designated by their three-letter code. AMP, adenosine-5′-monophosphate; iPGAM, 2–3-bisphosphoglycerate-independent phosphoglycerate mutase; GMP, guanosine-5′-monophosphate; IMP, inosine-5′-monophosphate; PGA, phosphoglyceric acid; PPT1, PEP-phosphate translocator 1; PRA, 5-phosphoribosylamine; PRPP, 5-phosphoribosyl-1-pyrophosphate; RpoTp, plastid-localized RNA polymerase.

Metabolic perturbations in AA or nucleotide metabolism have been confirmed in a number of the reticulate mutants; yet analysis of the reticulate mutants does not indicate that a common metabolic defect is responsible for reticulation in all of the mutants. While many of the mutants are affected in AA metabolism, altered AA levels do not show consistent changes among all the reticulate mutants. For example, the *cue1* mutant of *PPT1* required supplementation with all three aromatic AAs to chemically complement the reticulate phenotype, indicating broad metabolic perturbations within the shikimate pathway were responsible for the reticulate phenotype in the *cue1* mutant. Metabolic defects consistently target primary metabolism, suggesting that perturbations in the plastid are widespread in most or all of the reticulate mutants. Complicating the characterization of metabolic defects, all prior metabolite analyses of the reticulate mutants have used whole-leaf tissue sampling, obscuring possible cell-specific deficiencies. Thus, analysis of cell-specific samples may provide greater clarity to the metabolic defects in reticulate mutants.

THE SUPPLY AND SIGNALING HYPOTHESES

Based on the observations of the reticulate mutants, two hypotheses have been presented in the literature to explain their unique M-specific phenotype (Streatfield et al., 1999; Rosar et al., 2012). We call these hypotheses the *supply* hypothesis and the *signaling* hypothesis, and develop them here in light of the most recent metabolic and morphologic characteristics of the reticulate mutants (Figure 3). Both hypotheses are grounded in the observation that many of the mutated genes are expressed predominantly around the vasculature, where the phenotype is not manifested. Because vasculature tissue (including the BS) precedes development of the M, thus positioning it for the subsequent control of the M, both the *supply* and *signaling* hypotheses propose vasculature control of M development (Pyke et al., 1991; Kinsman and Pyke, 1998; Candela et al., 1999).

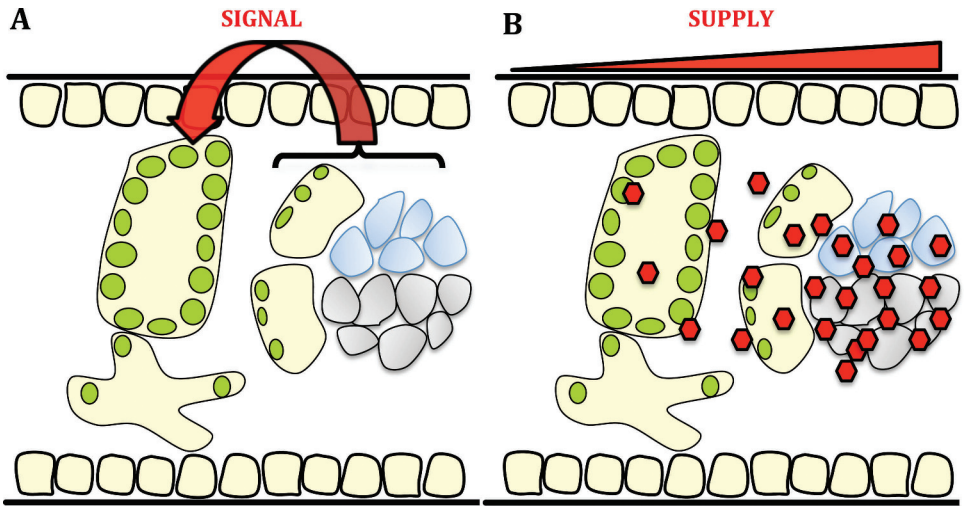

Figure 3. The Signaling and Supply Hypotheses.

The schematic cross-section has been presented in Figure 1 and identifies the cell types illustrated here.

(A) The signaling hypothesis states that a metabolic signal is generated in the vasculature or bundle sheath and is transported to the mesophyll. This signal triggers development of the mesophyll. If the signal is disturbed, as hypothesized for the reticulate mutants, the mesophyll does not develop properly, affecting cell proliferation and/or size, as well as chloroplast morphology.

(B) The supply hypothesis proposes that metabolites transported or synthesized in the vasculature are shuttled to the developing mesophyll in young plants. If the supply of metabolites (depicted here as red hexagons) is insufficient, as suspected in the reticulate mutants, the mesophyll cannot properly develop.

The *supply* hypothesis proposes that primary metabolites are provided to the developing M tissue in insufficient supply, thus restricting M development (Rosar et al., 2012). Metabolites would be amply supplied to the vasculature, which predates differentiation of the M, but are depleted by the time later-developing cells, such as the M, differentiate. Consistently with this hypothesis, it is observed that *ppt1*, *trp2*, *atase2*, *re*, *rer3*, *ven3*, and *ven6* mutants are reduced in certain proteinogenic AAs or nucleotides, but exogenous application of the affected metabolites or specific downstream products reverts the phenotype in most of these mutants. The joint interpretation points to a limited supply of AA(s) or nucleotides specifically to the M, interfering with proper cell development.

In a number of the reticulate mutants, it was found that leaves forming later in the life of the plant, when the oldest leaves had presumably begun to senesce, displayed less reticulation. This is consistent with a remobilization of limited metabolite to the newly developing leaf tissue—a point made by Woo et al. (2011) to explain the *alx13* phenotype and accords with the supply hypothesis. Pérez-Pérez et al. (2013) suggest that decreased malate levels in *re* and *rer3* mutants might specifically interfere with M tissue, as malate is supplied to the leaf M for photosynthesis via the vasculature. Again in line with the supply hypothesis, the authors alternatively suggested that the higher expression of the genes in BS might permit sufficient production of the limiting metabolite(s) in this tissue, even though it is equally important for both tissue types. This suggestion, in particular, could neatly explain why the phenotype is restricted to M tissue, where expression is not as great. However, the genes affected in a number of reticulate mutants have homologous genes that are expressed at higher levels in the M.

Conversely, the *signaling* hypothesis suggests that molecular signals are transmitted from the vasculature to govern M development (Figure 3) (Knappe et al., 2003; Voll et al., 2003). If transmission of the signal(s) is impaired by perturbations at the site of synthesis, proper M development is impaired. Dehydrodiconiferyl alcohol glucoside (DCG), a phenylpropanoid-derived secondary metabolite, displays strong growth-promoting activity and is thought to act in a cytokinin-mediated pathway controlling cell division and expansion (Teutonico et al., 1991; Tamagnone et al., 1998b). Voll et al. (2003), in first describing the *signaling* hypothesis, suggested that the dramatic reduction of DCG may account for the reticulate phenotype in the *cue1* mutant. They proposed that DCG is produced in *PPT1*-expressing vasculature tissue, possibly in the roots where PPT1 but not PPT2 is expressed, and acts in a signaling pathway regulating M development (Knappe et al., 2003; Voll et al., 2003). This is consistent with the reticulate phenotype found in tobacco plants expressing the AmMY308 transcription factor from *Antirrhinum majus*, which disrupts phenolic acid and DCG synthesis (Tamagnone et al., 1998b).

There are many other molecules that could also be plausible molecular signal(s) in the signaling hypothesis, including ROS, siRNAs, small molecules (e.g. phytohormones, or

metabolites), or proteins. Dunoyer et al. (2010) demonstrated cell-to-cell mobility of exogenous siRNA duplexes emanating from vasculature into the M tissue. A phloem-companion cell-specific promoter, driving expression of a *SULFUR* double-stranded RNA transgene, generated an inverse reticulate phenotype resulting from chlorosis in siRNA recipient cells, extending 10–15 cell layers into the M tissue. This inverse reticulate phenotype illustrates the capability of RNA-mediated signaling in tissue-specific developmental control. Signaling via the extracellular matrix is another feasible route to convey developmental signals. Secretion of small peptides is established as an important mechanism of cell-to-cell communication and can regulate cell proliferation and organ growth (Amano et al., 2007; Matsubayashi, 2011). Similarly, extracellular pools of ATP or various AAs reportedly induce Ca^{2+}-influx spikes at the plasma membrane and alter cellular gene expression patterns (Chivasa et al., 2005; Stephens et al., 2008; Chivasa and Slabas, 2012; Vincill et al., 2013).

THE ROLE OF THE C3 BUNDLE SHEATH

Due to its central position within the leaf, at the interface of the M and vasculature, the BS is well placed to regulate information flux. Characterization of reticulate mutants has supported a crucial role for the metabolic state of the BS in regulating M development. The C3 BS holds key roles in production of fixed carbon, directing sulfur and nitrogen assimilation, and is the site of synthesis of metabolites and systemic signals such as H_2O_2 (Hibberd and Quick, 2002; Leegood, 2008; Janacek et al., 2009). In the *supply* and the *signaling* hypotheses presented here, the BS and associated vasculature are proposed to provide essential primary metabolites or metabolic signals to the developing M tissue (Figure 3).

A division of labor between BS and M tissues in C3 plants is well known (Leegood, 2008). As the reticulate mutants highlight, a number of enzymes of AA metabolism are enriched in the vasculature/BS. In cucumber, the M and phloem sap composition differs during the day/night cycle, suggesting that AA metabolism predominantly occurs within vasculature or neighboring cells (Mitchell et al., 1992). Furthermore, several AA transporters are expressed preferentially in the BS of *A. thaliana* (Kwart et al., 1993; Fischer et al., 1995; Rentsch et al., 1996; Hirner et al., 1998). It is proposed that Gln is produced in higher amounts in vascular cells (BS, xylem parenchyma, mestome sheath, and epidermis) of wheat and transported to the M (Kichey et al., 2005). However, similar AA compositions of phloem and M sap in spinach, barley, and sugar beet indicate that AA metabolism may not be compartmentalized in these species (Riens et al., 1991; Winter et al., 1992; Lohaus et al., 1994).

Cells around the vein of C3 plants also play a crucial role in carbohydrate metabolism (Janacek et al., 2009). Because diffusion of atmospheric CO_2 from the stomata to the BS is expected to be slow, the majority of photosynthetic carbon fixation at the BS should use carbon provided from the vasculature (Hibberd and Quick, 2002). In C3 plants, high activities of decarboxylases, which are also found in the C4 BS, are abundant in vein-associated tissues (Hibberd and Quick, 2002). With these decarboxylases, BS cells in stems and petioles are able to utilize malate, transported through the vascular stream, for photosynthetic carbon fixation (Hibberd and Quick, 2002). The malate, a four-carbon organic acid, is produced in root tissue by fixation of CO_2, and thus represents a form of C4 photosynthesis in which the vasculature takes the place of the mesophyll for provision of four-carbon organic acid (Hibberd and Quick, 2002).

The BS is an important site of carbon fixation for the plant. Targeted knockdown of chlorophyll synthesis indicated that photosynthesis in vasculature-associated cells plays a prominent role in providing carbon backbones to the shikimate pathway, leading to aromatic AA biosynthesis (Janacek et al., 2009). Additionally, sucrose produced from these cells around the vascular bundles is loaded into the phloem and translocated to growing apices (Hibberd and Quick, 2002). In light of this knowledge, impairment of BS chloroplast homeostasis may disrupt provision of critical carbohydrate to the growing leaf apices of the plant, consistently with our *supply* hypothesis of reticulate mutants.

The BS plays a pivotal role in generating ROS signals that are involved in plant development. For example, the BS chloroplast is responsible for a H_2O_2-burst involved in systemic acquired acclimation (Karpinski et al., 1999). The synthesis of abscisic acid (ABA) is concentrated in the vascular parenchyma and may be a key regulator of the H_2O_2 generation (Karpinski et al., 1999; Cheng et al., 2002; Fryer et al., 2003; Koiwai et al., 2004; Christmann et al., 2005; Nambara and Marion-Poll, 2005; Kanno et al., 2012). ABA generated in the vascular parenchyma is necessary to initiate a H_2O_2-mediated signal from BS chloroplasts to activate Ascorbate Peroxidase 2 (APX2) expression, which subsequently drives H_2O_2 synthesis (Galvez-Valdivieso et al., 2009). Distinct characteristics of antioxidant metabolism in *A. thaliana* BS cells suggest that ROS from BS cells may be part of a wider signaling network (Fryer et al., 2003; Kangasjärvi et al., 2009). We point out that the reticulate mutant *alx13* was found in a screen for mutations that alter regulation of the APX2 gene (Woo et al., 2011).

The distribution of enzymes between BS and M show similar patterns in C3 and C4 plants, suggesting C3 BS cells are pre-disposed to C4 photosynthesis (Christin et al., 2010; Langdale, 2011; Sage et al., 2011). The sulfate metabolism, predominantly occurring in the C4 BS, is also predominant in the C3 BS (Leegood, 2008). Light-dependent acclimation processes in C3 plants, mainly mediated by ROS, are likely compartmentalized between M and BS (Kangasjärvi et al., 2009).

RETROGRADE SIGNALING

Plastids are semi-autonomous organelles that have developed a bidirectional communication network with the nucleus, to coordinate gene expression for proper growth and

development (Chi et al., 2013). The evidence from the reticulate mutants, as well as other variegated mutants, strongly suggests that defects in plastid physiology are responsible for the developmental defects in the M and other cell types, emphasizing the significance of retrograde signaling in the reticulate mutants. The reticulate and variegated mutants are almost universally affected in plastid-localized gene products. Further, many of the other variegated leaf mutants, such as *pac-2*, *cla1-1*, *atd2*, and *im*, demonstrate abnormal or absent palisade M cells only in white or yellow sectors, where chloroplast development is blocked, while green sectors, containing normally developed chloroplasts, maintain normal palisade M development (Grevelding et al., 1996; Estévez et al., 2000; Aluru et al., 2001; van der Graaff et al., 2004).

The observations from reticulate and variegated mutants are wholly consistent with a dependence of photosynthetic cell development on the physiological state of its plastids, mediated by retrograde signaling (Rodermel, 2001; Yu et al., 2007). However, it remains unclear whether the defective plastids of reticulate mutants, (1) interfere with a necessary signal to guide M development or (2) transmit a novel signal inhibiting M development. Studies of retrograde signaling mutants, however, support the latter possibility (Rodermel, 2001). Higher-order mutants, resulting from crosses of reticulate mutants, indicate distinct signaling pathways are triggered from specific plastidic defects. More specifically, it was found that *re* operates in the same signaling pathway as *cue1*, which is distinct from that used by *dov1*, *rer3*, or *ven3/6.* Furthermore, *rer3* was found to operate in a pathway distinct from *ven3/6*, indicating at least three different signaling pathways are employed among the reticulate mutants (González-Bayón et al., 2006; Pérez-Pérez et al., 2013).

SUMMARY

Both the *signaling* and the *supply* hypotheses are interwoven with the role of the BS and associated vasculature of C3 plants. However, relatively little is known about this tissue type (Leegood, 2008). Reticulated mutants are thus candidates for understanding internal leaf development and deciphering the physiological role of the BS.

The recent cloning and molecular characterization of a number of reticulate mutants from *A. thaliana* and other C3 species emphasize the significance of primary metabolism in the BS. We propose that the inhibition of AA and/or purine biosynthesis around the veins is causal for the reticulated phenotype, as reflected in the *signaling* and *supply* hypotheses. These hypotheses have been presented to explain the reticulate phenotype in light of the characterized reticulate mutants, and provide a framework for further investigation of cell-specific development and intercellular signaling. To deepen the understanding of the BS's role in C3 plants, tissue-specific profiling techniques, such as laser-capture micro-dissection, will be of great utility (Schmid et al., 2012).

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