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 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.
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

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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.
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

Table 1. Reticulated Leaf Mutants of Arabidopsis thaliana.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein name</th>
<th>Protein function</th>
<th>Names</th>
<th>Leaf expression pattern</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g33320</td>
<td>Phosphoenolpyruvate/Phosphate Translocator 1 (PPT1)</td>
<td>Import of Phosphoenolpyruvate into the chloroplast</td>
<td>cue1/nox1</td>
<td>Vasculature-specific</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>At4g34740</td>
<td>Glutamine Phosphoribosyl Pyrophosphate Aminotransferase (ATase2)</td>
<td>First step in de novo purine biosynthesis</td>
<td>dov1/cia1/atd2/alx13</td>
<td>n.d.</td>
<td>5, 6, 7, 8, 9</td>
</tr>
<tr>
<td>At5g54810</td>
<td>Tryptophan Synthase β1 (TSB1)</td>
<td>Tryptophan biosynthesis</td>
<td>trp2/trp3/smo1</td>
<td>n.d.</td>
<td>10, 11</td>
</tr>
<tr>
<td>At1g15710</td>
<td>Arogenate Dehydrogenase (TyrAAT2)</td>
<td>Tyrosine biosynthesis</td>
<td>n.d.</td>
<td>n.d.</td>
<td>12, 13, 14</td>
</tr>
<tr>
<td>At2g41680</td>
<td>NAD(P)H-Thioredoxin Reductase (NTRC)</td>
<td>Regulation of multiple plastid metabolic pathways</td>
<td>ntrc</td>
<td>Strong expression throughout leaf, highest in vasculature</td>
<td>15, 16, 17, 18</td>
</tr>
<tr>
<td>At3g27740</td>
<td>Carbamoyl Phosphate Synthetase α (CarA)</td>
<td>Arginine and pyrimidine biosynthesis</td>
<td>ven6</td>
<td>Vasculating and neighboring mesophyll cells</td>
<td>19, 20, 21</td>
</tr>
<tr>
<td>At1g29900</td>
<td>Carbamoyl Phosphate Synthetase β (CarB)</td>
<td>Arginine and pyrimidine biosynthesis</td>
<td>ven3</td>
<td>Vasculating and neighboring mesophyll cells</td>
<td>19, 20, 21</td>
</tr>
<tr>
<td>At1g09780</td>
<td>Phosphoglycerate Mutase 1 (iPGAM1)</td>
<td>Interconversion of 3-phosphoglycerate and 2-phosphoglycerate</td>
<td>ipgam1</td>
<td>n.d.</td>
<td>22</td>
</tr>
<tr>
<td>At3g08590</td>
<td>Phosphoglycerate Mutase 2 (iPGAM2)</td>
<td>Interconversion of 3-phosphoglycerate and 2-phosphoglycerate</td>
<td>ipgam2</td>
<td>n.d.</td>
<td>22</td>
</tr>
<tr>
<td>At2g24120</td>
<td>Nuclear-Encoded Plastid RNA Polymerase (RpoTp)</td>
<td>Transcription of the chloroplast genome</td>
<td>scabra3</td>
<td>n.d.</td>
<td>23</td>
</tr>
<tr>
<td>At2g37860</td>
<td>Reticulata (RE)</td>
<td>n.d.</td>
<td>rel/ven2/lcdd1/rcd2</td>
<td>Proximal region of leaf primordia and vasculature of mature leaf</td>
<td>19, 24, 25, 26, 27</td>
</tr>
<tr>
<td>At3g08640</td>
<td>Alpha-tandem</td>
<td>n.d.</td>
<td>ven5/rer3</td>
<td>Distal region of leaf primordia and vasculature of mature leaf</td>
<td>19, 27</td>
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<tr>
<td>At5g12470</td>
<td>MEP3</td>
<td>n.d.</td>
<td>rer4</td>
<td>n.d.</td>
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<tr>
<td>At5g27010</td>
<td>ARM-domain protein</td>
<td>n.d.</td>
<td>ven1</td>
<td>n.d.</td>
<td>14</td>
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<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>ven4</td>
<td>n.d.</td>
<td>19</td>
</tr>
<tr>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>ven4</td>
<td>n.d.</td>
<td>19</td>
</tr>
<tr>
<td>Various</td>
<td>Ribosomal subunits</td>
<td>Ribosome scaffolding and translational regulation</td>
<td>rpl7b/den5/rpl10ab/de n12/rpl18c/rpl38b/rpl39c/den30/rps6a/rps21b/rps28b/rps15ab/rps28a/iden29</td>
<td>n.d., data have not been determined.</td>
<td>28</td>
</tr>
</tbody>
</table>

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) Streafeld 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) Pérez-Pérez et al., 2013; (24) Horiguchi et al., 2011.
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 point 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 H₂O₂ 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 phosphoenolpyruvate (PEP) pool were also decreased, including cinnamic acid, sinapate, and indole-3-acetonitrile, suggesting misregulation of the plastid 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; Streitfield 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) (Streitfield 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; Streitfield 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 (PDPK) 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; Voll et al., 2003). This could 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.

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 Pro35S::NPTIII and Transcriptional Silencing Information (TIS) in a repressor of silencing 1 (rost) 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 PEP 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/ipgam2 mutants, as with the cue1 mutants, are hampered in the provision of PEP for the plastid. Follow-up experiments on ipgam1/ipgam2, 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 semidominant 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 Monomethylster 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, which 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 following 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 atase2 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 isopenetyl 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, 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.
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).
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\(_2\)O\(_2\) (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 highlighted, 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\(_2\)O\(_2\)-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\(_2\)O\(_2\) 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\(_2\)O\(_2\)-mediated signal from BS chloroplasts to activate Ascorbate Peroxidase 2 (APX2) expression, which subsequently drives H\(_2\)O\(_2\) 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 (Greveiling 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 *Arabidopsis 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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