Review

Natural rubber biosynthesis in plants, the rubber transferase complex, and metabolic engineering progress and prospects

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Summary

Natural rubber (NR) is a nonfugible and valuable biopolymer, used to manufacture ~50 000 rubber products, including tires and medical gloves. Current production of NR is derived entirely from the para rubber tree (Hevea brasiliensis). The increasing demand for NR, coupled with limitations and vulnerability of H. brasiliensis production systems, has induced increasing interest among scientists and companies in potential alternative NR crops. Genetic/metabolic pathway engineering approaches, to generate NR-enriched genotypes of alternative NR plants, are of great importance. However, although our knowledge of rubber biochemistry has significantly advanced, our current understanding of NR biosynthesis, the biosynthetic machinery and the molecular mechanisms involved remains incomplete. Two spatially separated metabolic pathways provide precursors for NR biosynthesis in plants and their genes and enzymes/complexes are quite well understood. In contrast, understanding of the proteins and genes involved in the final step(s)—the synthesis of the high molecular weight rubber polymer itself—is now only beginning to emerge. In this review, we provide a critical evaluation of recent research developments in NR biosynthesis, in vitro reconstitution, and the genetic and metabolic pathway engineering advances intended to improve NR content in plants, including H. brasiliensis, two other prospective alternative rubber crops, namely the rubber dandelion and guayule, and model species, such as lettuce. We describe a new model of the rubber transferase complex, which integrates these developments. In addition, we highlight the current challenges in NR biosynthesis research and future perspectives on metabolic pathway engineering of NR to speed alternative rubber crop commercial development.

Introduction

Over 2500 plant species produce natural rubber (NR; Metcalf, 1967; Bowers, 1990). However, only a small subset produce significant quantities of economically viable high-quality rubber (van Beilen and Poirier, 2007a,b; Mooibroek and Cornish, 2000; Table 1). NR (cis-1,4-polyisoprene) is an irreplaceable high molecular weight biopolymer, which is a critical, nonfugible, raw material vital to industries such as transportation, medicine and defence. Its unique physical properties, which include high elasticity, resilience, impact and abrasion resistance, efficient heat dispersion and malleability in cold temperatures, make NR an important raw material in the manufacture of many different rubber and latex products. However, the para rubber tree (Hevea brasiliensis Müll, Arg.), grown in tropical plantations as clonal, bud-grafted, scions on seedling rootstocks, is the only source of commercially produced NR (Cornish, 2017). The ever-increasing industrial demand for NR coupled with the vulnerability of the H. brasiliensis rubber production system, the current deforestation moratorium, and severe allergic reactions to proteins present in H. brasiliensis latex and rubber products have empowered researchers to domesticate and develop alternative rubber crops amenable to mechanized agriculture in temperate regions (van Beilen and Poirier, 2007a,b; Cornish, 2001a, 2017; Cornish et al., 2015; Schmidt et al., 2010a,b). At this time, a number of established and start-up companies are, or recently have been, involved in alternative rubber products and crops, including the following: (i) P. argentinatum: Apollo, Bridgestone, Cooper, EnergyEne, Ford, Goodyear, Nokian, PanAridus, Guayule Australia; T. kok-saghyz: American Sustainable Rubber, Bridgestone, Continental, Ford, Goodyear, KeyGene, Kultivat, Ling-long, NovaBioRubber, Sumitomo, and there are undoubtedly others.

Despite the emerging strong interest and investment in alternative rubber crops, and a good understanding of NR enzymology (Archer and Audley, 1987; Cornish, 2001a,b; Cornish et al. 2000; Cornish and Scott, 2005; Cornish and Xie, 2012; da Costa et al., 2004, 2006; Espy et al., 2006; Kang et al., 2000a,b), our current understanding of the protein chemistry and the molecular mechanisms involved in rubber biosynthesis is far less complete and has not been recently reviewed. Therefore, here we review the recent biotechnological/metabolic pathway engineering advances made towards improving natural rubber
| Plant Species          | Source and rubber content                        | Rubber Mw (kg/mol) | Production (kiloton/year) (year) | Yield (kg/ha/y) | Rubber properties | Allergenic proteins | Tensile strength | Modulus | Elongation | References                                                                 |
|------------------------|------------------------------------------------|-------------------|---------------------------------|-----------------|-------------------|---------------------|-------------------|---------|------------|----------------------------------------------------------------------------|
| **Rubber tree**        |                                               |                   |                                 |                 |                   |                     |                   |         |            | Tangpaktdee et al. (1996), van Beilen and Poirier (2007b), Cornish (2017) |
| Hevea brasiliensis     | Bark laticifer                                 | 1310              | 12 760 (2017)                   | 500–3000        | Yes               | Yes                | High              | High    | Medium     |                                                                           |
| Guayule                | Bark parenchymal cells                         | 1280              | 10 (1910)                       | 300–2000        | No                | No                 | High              | Low     | High        | McIntyre et al. (2001), van Beilen and Poirier (2007b), Abdel-Haleem et al. (2017), Cornish (2017) |
| Parthenium argentatum  | 3–12 (w/w dwt) of bark                         |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Rubber dandelion       | Root laticifer                                  | 2180              | 3 (1943)                        | 150–900         | Yes               | Yes                | High              | High    | Medium      | Whaley and Bowen (1947), van Beilen and Poirier (2007b), Josefsen (1953), Kreuzberger et al. (2016), Cornish (2017), Bats et al. (2019) |
| Taraxacum kok-saghyz   | 15–30 (w/w dwt) of breeding line root          | 2180              |                                 |                 |                   |                     |                   |         |            |                                                                           |
|                        | 3–9 (w/w dwt) of wild-type root                |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Lettuce                | Stem laticifer                                  | 1380              |                                 |                 |                   |                     |                   |         |            | van Beilen and Poirier (2007b), Bell et al. (2015), McKeon and Britts (2018) |
| Lactuca serriola       | 2–8 (w/w dwt) of latex                         |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Ficus tree             | Bark laticifer                                  | 190               |                                 |                 |                   |                     |                   |         |            | Kang et al. (2000a,b), van Beilen and Poirier (2007b)                     |
| Ficus carica           |                                               | 1500              |                                 |                 |                   |                     |                   |         |            |                                                                           |
| F. bengalensis         | 17                                             | 1–10              |                                 |                 |                   |                     |                   |         |            |                                                                           |
| F. elastica            | 18 (w/w dwt) of latex                          |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Sunflower              |                                               |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Helianthus sp.         | Bark parenchymal cells                         | 69 or 279         |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Goldenrod              | Leaf laticifer                                  | 160–240           |                                 | 110–155         |                   |                     |                   |         |            |                                                                           |
| Solidago lewesorthii   | 5–12 (w/w dwt) of leaf                          |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| S. virgaurea minuta    |                                               |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
| Rubber rabbitbrush     | Shoot cells                                     | 585               |                                 |                 |                   |                     |                   |         |            | Weber et al. (1993), van Beilen and Poirier (2007b)                      |
| Ericameria nauseosa    | 1.5–6.5 (w/w dwt) of shoot                     |                   |                                 |                 |                   |                     |                   |         |            |                                                                           |
yield and quality (specifically high molecular weight NR) in rubber plants. Additionally, we highlight the current challenges and future perspectives in metabolic pathway engineering of natural rubber biosynthesis in these potential rubber crops.

**Alternative natural rubber crops**

Among several rubber-producing species, Parthenium argentatum (Gray; guayule) and Taraxacum kok-saghyz (Radin; TK, rubber dandelion; Russian/Siberian/Kazakh dandelion) have gained increasing attention in recent years, as potentially commercially viable rubber crops (Arias et al., 2016; van Beilen and Poier, 2007a,b; Benedict et al., 2008; Buranov and Elmuradov, 2010; Moobroek and Cornish, 2000). We briefly describe the commercial viability and quality parameters of alternative rubber crops, in comparison with *H. brasiliensis*, in the following paragraphs.

**Guayule, Parthenium argentatum**

*Parthenium argentatum* is a perennial shrub, native to the Chihuahuan desert of Mexico and Texas (Benedict et al., 2008; Coffelt and Ray, 2010). *P. argentatum* NR is high molecular weight and can be used similarly to *H. brasiliensis* rubber, but it combines comparable strength with greater softness and higher stretchiness, and does not contain the proteins present in *H. brasiliensis* latex that can cause severe allergic reactions in human beings (Cornish, 1996; Hamilton and Cornish, 2010; Siler et al. 1996). Its combination of allergy safety and outstanding film properties can be targeted to medical and consumer applications, such as medical gloves, catheters and balloons, as well as other high-margin products like weather balloons and lineman’s gloves (Cornish, 2017). Unlike *H. brasiliensis* and *T. kok-saghyz*, which produce rubber particles in latex of laticifers, *P. argentatum* rubber particles are contained within bark parenchyma cells requiring mechanical extraction to produce latex or solvent extraction to produce rubber (Cornish and Schloman, 2004). NR yield and the overall production system need improvement before *P. argentatum* can be commercially competitive in mainstream markets (Eranki et al., 2017; Soratana et al., 2017). Also, although this is a desert species, *P. argentatum* crops require 1000–2200 mm of total water applied (irrigation plus rainfall) for high NR and biomass yields (Bucks et al., 1985a,b; Foster and Coffelt, 2005; Hunsaker and Elshikha, 2017). The agronomic, technical and economic feasibility of the *P. argentatum* commodity chain has been assessed in Europe, under the EU-PEARLS project (van Loo et al., 2012; Palu et al., 2013; Sfeir et al., 2012, 2014; Snoeck et al., 2011, 2015), and in the United States (Eranki et al., 2017; Soratana et al., 2017). Improved germplasm availability, through molecular and conventional breeding efforts, remains essential (Veatch et al., 2005). Also, established farming practices, processing companies willing to buy *P. argentatum* crops from growers, and the extraction and supply of purified rubber of high quality to rubber product-manufacturing companies, are some of the key bottlenecks still facing *P. argentatum* development as a new industrial rubber crop (Cornish, 2017).

**Rubber dandelion, Taraxacum kok-saghyz**

*Taraxacum kok-saghyz* is an herbaceous plant, native to Kazakhstan and Uzbekistan, and its rubber is very similar in quality to *H. brasiliensis* rubber (Cornish et al., 2015). It can be established from transplants or direct seeding, can be grown as an annual crop and is broadly adapted to temperate regions (Luo et al., 2017; McAssey et al., 2016; Ramirez-Cadavid et al., 2017; Whaley and Bowen, 1947). However, *T. kok-saghyz* is largely undomesticated, and has several inherent problems, such as its need for steady moisture content during germination, slow growth rate, poor competitiveness with weeds, meaningful rubber yield only measurable at maturity, a high degree of heterozygosity and self-incompatibility (Hodgson-Kratky and Woylin, 2015; Warmke, 1943). Therefore, further improvement in rubber yield and agronomic fitness, through molecular and conventional breeding efforts, is essential before *T. kok-saghyz* can become a commercially viable and competitive rubber crop (Cornish, 2017; Kreuzberger et al., 2016, Luo et al., 2017; Ramirez-Cadavid et al., 2017). Burgeoning interest in *T. kok-saghyz* rubber research has led to field-scale trials to evaluate its performance (Arias et al., 2016; Buranov and Elmuradov, 2010; Cornish, 2017; Kreuzberger et al., 2016; Ramirez-Cadavid et al., 2017; Tata et al., 2012).

**Model plants**

The triploid, apomictic *T. brevicomerculatum* Koroleva (TB), a vigorous dandelion often co-located with *T. kok-saghyz*, has proved useful as an analog for *T. kok-saghyz* because it is much less variable and produces some rubber. Both species have been used for rapid genetic analysis and gene function studies in recent years (Collins-Silva et al., 2012; van Deenen et al., 2011; Hilebrand et al., 2012; Lin et al., 2017; Nowicki et al., 2019; Post et al., 2012; Schmidt et al., 2010a; Tata et al., 2012).

*Lactuca sativa* L. (lettuce) also synthesizes high molecular weight NR, especially during bolting, comparable to that of *H. brasiliensis* (Table 1) and, like the *Taraxacum* species, has the advantage of a short life cycle (3–5 months), making it amenable to *in vivo* gene function studies through reverse genetic approaches (Chakrabarty et al., 2015; Table 1). Also, prickly lettuce (*Lactuca serriola* L.), the likely progenitor of cultivated lettuce, may be promising as a potential new crop plant for rubber production (Bell et al., 2015).

**Isoprenoid biosynthesis**

From a metabolic perspective, the isoprenoid biosynthetic pathway involves three phases: (i) formation of isopentenyl pyrophosphate (IPP), (ii) condensation of IPP to synthesize linear isoprenoids, and (iii) condensation, coupled to chain elongation and/or cyclization and modification (Takahashi and Koyama, 2006). In the first phase, the basic isoprenoid unit IPP is formed, which is followed by the isomerization of IPP into dimethylallyl pyrophosphate (DMAPP). In the second phase, linear, usually trans isoprenoids are formed by the sequential condensation of IPP (nonallylic) to DMAPP (allylic) to form longer allylic pyrophosphates (APP) than DMAPP through catalysis by prenyl transferases (Figure 1). In a wider sense, prenyl transferases include all enzymes that catalyse the transfer of allylic prenyl groups to acceptor molecules, such as IPP, aromatic intermediates of quinones or specific proteins (Takahashi and Koyama, 2006). The prenyl transferases responsible for each linear isoprenoid (with a specific number of isoprene units) strictly recognize the prenyl chain lengths of the allylic substrates and regulate the size and stereochemistry of the ultimate products (Takahashi and Koyama, 2006). The carbon chain length specific to naturally occurring linear isoprenoids ranges from C₁₅ in geranyl (trans; Burke et al., 1999) and neryl (cis) pyrophosphate to C₄₅ in...
solanesyl pyrophosphate (Ducluzeau et al., 2012; Hirooka et al., 2003; Ohnuma et al., 1991, 1992). The longer dolichol phosphate is more variable (C55–C100) in size but is still finite (Pan et al., 2000). Rubber, on the other hand, has variable length and can be greater than C100 000 for rubber polymers of 2000 kg/mol or more (Cornish, 2016).

The mevalonate and 2-C-methyl-D-erythritol 4-phosphate IPP biosynthetic pathways

Plants use two pathways, namely the mevalonate (MVA) pathway and the methyleythritol pathway (MEP, plastidic, red arrows and lower case letters) from acetyl-CoA or glyceraldehyde-3-phosphate and pyruvate, respectively. IPP and its stereoisomer dimethylallyl pyrophosphate (DMAPP) condense to form several allylic pyrophosphates (APPs), namely geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15) and geranyl geranyl pyrophosphate (GGPP, C20). These APPs can be used as rubber chain initiators (blue arrow), FPP being the most common initiator, and are also the building blocks for terpenes such as chlorophyll, steroids, plant growth regulators, essential oils and so forth. Natural rubber biosynthesis is catalysed by rubber transferase complexes (magenta) bound to the protoplast unilamella membrane (light blue) of cytosolic rubber particles, and rubber is compartmentalized to the rubber particle interior. Key: MVA enzymes: PDC, pyruvate dehydrogenase complex; AACT, acetyl coenzyme A acetyltransferase; HMGS, hydroxymethylglutaryl coenzyme A synthase; HMGR, hydroxymethylglutaryl coenzyme A reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDC, diphosphomevalonate decarboxylase. MVA substrates: 1. pyruvate; 2. acetyl coenzyme A; 3. acetoacetyl coenzyme A; 4. hydroxymethylglutaryl coenzyme A; 5. mevalonate; 6. phosphomevalonate; 7. diphosphomevalonate. MEP enzymes: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase. MEP substrates: a, pyruvate and D-glyceraldehyde 3-phosphate; b. 1-deoxy-D-xylulose 5-phosphate; c. 2-C-methyl-D-erythritol 4-phosphate; d. 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; e. 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; f. C2-Methyl-D-erythritol 2,4-cyclodiphosphate; g. (E)-4-hydroxy-3-methylbut-2-enyl diphosphate. RP, rubber particle; RT-ase, rubber transferase complex; P, non-RT-ase rubber particle-associated proteins; complexes; PL, protoplast unilamella membrane; NR, natural rubber polymers.

Figure 1 The metabolic route map for natural rubber (cis-1,4-polyisoprene) biosynthesis in plants, including the pathways for substrate synthesis, their locations. Isopentenyl pyrophosphate (IPP), the monomeric subunit for rubber biosynthesis (orange arrow) is synthesized by two pathways, the mevalonate pathway (MVA, cytosolic, green arrows and numerals) and the methyleythritol pathway (MEP, plastidic, red arrows and lower case letters) from acetyl-CoA or glyceraldehyde-3-phosphate and pyruvate, respectively.
flux analysis, is still needed to verify this (Lange et al., 2015; Rodríguez-Concepción, 2006, 2010; Rodríguez-Concepción and Boronat, 2015). The enzyme HMGS catalysing the irreversible conversion of acetacetyl-CoA to HMG-CoA also seems to be a committed step in the MVA pathway in plants (Meng et al., 2017; Suvachitannot and Witsuwannakul, 1995; Tang et al., 2016) and also may be rate-limiting.

In contrast, the plastidic MEP pathway operates eight consecutive enzymes to form IPP and DMAPP from the precursors, pyruvate and D-glyceraldehyde-3-phosphate (Cordoba et al., 2009; Figure 1). All the genes coding the enzymes of this pathway, in plants, are of prokaryotic origin (Cordoba et al., 2009). The first step of the MEP pathway is rate limiting and is catalysed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS, EC 2.2.1.7) converting the precursors pyruvate and glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose 5-phosphate (DXP; Sprenger et al., 1997; Cordoba et al., 2009). A recent study indicates that DXS is the enzyme with highest flux control coefficient (the main rate-determining step) in the Arabidopsis thaliana MEP pathway (Wright et al., 2014). Further condensation of pyruvate and D-glyceraldehyde-3-phosphate leads to IPP production.

In the cytosol, IPP is isomerized to DMAPP by IPP-isomerase. DMAPP is then condensed with IPP in several steps by trans-prenyl-transferases (TPTs) to form geranyl pyrophosphate (GPP, C_{10}, monoterpenoids), farnesyl pyrophosphate (FPP, C_{15}, sesquiterpenoids and triterpenoids) and geranylgeranyl pyrophosphate (GGPP, C_{20}, diterpenoids; van Beilen and Poirier, 2009b). These trans-short-chain prenyl pyrophosphates serve as initiators (allylic primer substrates) for additional IPP condensation and the final product formation (rubber molecules as well as other isoprenoids; Figure 1). The enzyme rubber transferase (RT-ase) or rubber polymerase (EC 2.5.1.20) catalyses such a reaction to polymerize cis-1,4-polyisoprene and can accept trans and cis-allylic pyrophosphates as initiators (e.g. Cornish, 1993; Figure 1; Archer and Audley, 1987; Bushman et al., 2006; van Beilen and Poirier, 2007b; Cornish, 1993, 2001a,b; Cornish and Backhaus, 1990; Cornish and Xie, 2012; Yamashita et al., 2016).

**Cross-talk between the MVA and MEP pathways**

Currently, it is clear that multiple feedback mechanisms regulate both the MVA and MEP pathways (Cordoba et al., 2009; Espenshade and Hughes, 2007; Rodríguez-Concepción, 2006; Rodríguez-Concepción and Boronat, 2015). If IPP can flow from the plastid to the cytosol, plastidal IPP overproduction may be a means of negating IPP substrate limitations for cytosolic RT-ases (see later discussion on IPP pool sizes).

Several studies have demonstrated that metabolic cross-regulation or cross-talk between cytosol and plastids occurs (Angioni et al., 1997; Chow et al., 2012; De-Eknamkul and Potduang, 2003; Kumar et al., 2012; Nagata et al., 2002; Yang and Orihara, 2002). For example, in H. brasiliensis, quantitative real-time polymerase chain reaction (qRT-PCR) analysis of MEP and MVA pathway gene transcripts indicated that the MEP pathway was an alternate source of IPP for NR synthesis in the laticifer cytosol of a clone with low carotenoid production, but not in a clone with high carotenoid levels (Chow et al., 2012; Lau et al., 2016). Similar results were obtained when the plastidic MEP pathway was inhibited by fosmidomycin, a specific inhibitor of DXP reductoisomerase (Kumar et al., 2012; Loreto et al., 2004; Mandel et al., 1996; Sharkey et al., 2001), where some metabolites from the cytosolic MVA pathway flowed into the plastids (Kumar et al., 2012; Nagata et al., 2002). However, the IPP transported from the cytosol into plastids did not fully compensate for lack of a functioning MEP pathway (Bouvier et al., 2005; Kumar et al., 2012). A metabolic engineering study used a multigene approach in order to express all six MVA pathway genes in chloroplasts of Nicotiana tabacum (tobacco) plants (Kumar et al., 2012). The transplastomic plants, expressing all six MVA genes, had unimpeded growth on fosmidomycin (Kumar et al., 2012). Increased levels of mevalonate and cytoplastically synthesized carotenoids also were found in the transplastomic plants, indicating the likely shunting of excess IPP from the chloroplast to the cytosol, augmenting the cytosolic IPP pool.

The importance of chloroplasts in IPP production and accumulation was exploited in a study on artemisinin synthesis and accumulation in N. tabacum (Malhotra et al., 2016) where the compartmental separation of IPP and artemisinin biosynthesis led to significant secondary product increases.

However, cross-talk of isoprenoid intermediates between cytosolic and plastidic compartments was negligible in A. thaliana (Lange et al., 2015). When global gene expression patterns were analysed and pools of isoprenoid metabolites quantified in A. thaliana seedlings subjected to specific inhibitors (lovastatin for the MVA pathway and fosmidomycin for the MVA pathway; Laule et al., 2003), there was no correlation between the patterns of gene expression and metabolite changes.

Apart from IPP, cross-talk also is evident for other common isoprenoid precursors such as GPP, FPP and GGPP (Gutensohn et al., 2013; Hemmerlin et al., 2012; Laule et al., 2003; Mendoza-Poudereux et al., 2015), which can serve as rubber molecule initiators. However, although studies of whole plants and isolated plastids suggest a putative transporter, this has not been identified (Bouvier et al., 2005; Fluge and Gao, 2005; Soler et al., 1993) and the mechanisms regulating the cross-talk are not yet known. This lack of detailed understanding is likely due to confounding effects of multiple factors, including translation, post-translational modification, and other posttranscriptional processes that operate to modulate flux (Kumar et al., 2012; Lange et al., 2015; Laule et al., 2003).

It is also possible that pH gradients may be involved in IPP translocation, as appears to be the case in movement of protonated abscessic acid between compartments (Baier and Hartung, 1988; Cornish and Zeevaart, 1985). A similar protonation of IPP under acidic conditions may allow it similarly to move across the plastidic membrane without a specific transporter.

**Natural rubber biosynthesis**

The basic mechanism of rubber formation is conserved across plant species, but species-specific differences exist in tissue localization, regulation of rubber synthesis, and the molecular weight and composition of the rubber produced (Cornish et al., 1993, Cornish et al. 2000, Cornish, 2001a,b). Synthesis of the nonallylic IPP and the allylic pyrophosphates (APPs) also is ubiquitous (Chen et al., 2011). Biochemical studies have demonstrated that rubber biosynthesis requires a diveralent cation cofactor (magnesium in vivo, although manganese also works in vitro) and integral or tethered membrane localization (Cornish, 2001a,b; Cornish and Xie, 2012; Scott et al. 2003). In general, two mechanisms have been proposed for NR biosynthesis: (i) the de novo formation of NR via sequential cis-1,4-condensation of IPP, via a carbocationic reaction (Puskas et al., 2006), onto short-chain allylic pyrophosphates as priming substrates and (ii) addition of IPP at the α-terminus of partially polymerized rubber molecules.
or polypropenyl pyrophosphates (Cornish, 2001a,b; Espy et al., 2006; Rijruthai et al., 2010; Tong et al., 2017; Wallrapp et al., 2013; Yamashita et al., 2016; Figure 1). It should be noted that the active end of an elongating rubber molecule is an APP moiety.

Proteins and genes involved in NR substrate biosynthesis

MVA, MEP and downstream pathways

Conventionally, the genes involved in the synthesis of IPP and rubber polymer formation are coined “rubber biosynthesis genes” (Chow et al., 2007; Tang et al., 2016) and many have been characterized and manipulated in relation to NR biosynthesis (Table 2).

The recent *H. brasiliensis* genome assembly revealed 94 rubber biosynthesis-related genes belonging to 20 gene families (Tang et al., 2016). Among them, 18 belong to the MVA pathway, 22 to the MEP pathway, 15 to initiator synthesis in the cytosol and 39 to putative rubber particle-associated “rubber elongation” genes [which include 18 REF (rubber elongation factor)/SRPP (small rubber particle protein) genes]. However, of the 22 MEP genes, only two DXS genes (DXS7 and DXS10) showed substantial expression in latex. In contrast, at least one gene for each MVA pathway enzyme was abundantly expressed in latex, suggesting that the MVA pathway is the primary contributor of IPP for *H. brasiliensis* rubber biosynthesis (Sando et al., 2008a,b; Tang et al., 2016). The genome assembly results also found that three of the identified gene families (REF/SRPP, CPT (cis-prenyl transferase) and DXS) each contained more than ten genes (Tang et al., 2016).

All six genes of the *H. brasiliensis* MVA pathway genes have been cloned and their expression levels analysed. However, the multiple genes encoding HMGS and HMGR have different expression patterns. HbAACT1, HMGS1, HbMVK, HbPMK and HbMVD were highly expressed in latex and complemented MVA pathway deletion mutations in yeast (Sando et al., 2008a,b). HMGS activity was positively correlated with the rubber content of latex (Sirirupong et al., 2005), and HMGR1 was reportedly involved in rubber biosynthesis (Chye et al., 1992; Venkatachalam et al., 2009).

The genome assembly of *T. kok-saghyz* revealed a total of 102 candidate rubber biosynthesis-related genes belonging to all six steps of the MVA pathway (40 genes), all eight of the MEP pathway (23 genes), as well as 19 genes for initiator synthesis and 20 genes for rubber particle-associated “rubber elongation” proteins (Lin et al., 2017). Many transcripts (472) shared homology with 49 of the 50 known rubber biosynthesis-related genes and 21 036 potential rubber yield-related SNPs were noted (Luo et al., 2017).

When these genes were compared to their homologs in rubber-producing (*H. brasiliensis*) and nonrubber-producing [*Cynara cardunculus var. scolymus* (Globe artichoke)] plant species, the gene number was similar for enzymes in the MEP pathway and rubber initiator synthesis, but was greater for the MVA pathway and rubber elongation proteins in the rubber-producing plant (Lin et al., 2017).

In *T. brevicorniculatum*, three HMGRs (*TbHMGR1-3*) were identified and their functional analysis indicated that *TbHMGR1* is involved in the regulation of precursors for rubber biosynthesis (van Deenen et al., 2011). *TkHMGR1* and *TkHMGR2* from the *T. kok-saghyz* draft genome were predominantly expressed in roots, with highest expression in root latex (Lin et al., 2017).

Expression of two genes upstream of HMGR, namely the adenosine triphosphate (ATP) citrate lyase (*ACL*) and the ace-tocethyl-CoA thiolase (*AAC*), was correlated with synthesis of precursors of isoprenoid synthesis in *T. brevicorniculatum* latex. Additionally, overexpression of *A. thaliana* ACL, AAC and HMGR in *T. brevicorniculatum* latex caused increased activity and accumulation of all three enzymes, as well as increases in sterol, pentacyclic triterpene, cis-1,4-polyisoprene and squalene to potentially industrially relevant (up to 32 mg/g root dry weight) levels (Pütter et al., 2017). Similarly, the overexpression of full-length or truncated *HbHMGR1* increased sterol accumulation in *N. tabacum* plants (Harker et al., 2003; Schaller et al., 1995).

Recent transcriptome analysis has led to the identification of 1709 new EST (expressed sequence tags) SSRs (simple sequence repeats), and a total of 78 SNP (single nucleotide polymorphisms) markers were validated in the MVA and MEP rubber biosynthesis pathways of *H. brasiliensis* bark tissue (Mantello et al., 2014). In contrast to *H. brasiliensis*, transcriptome analysis (Luo et al., 2017) and marker trait association analysis of rubber yield-related traits in *T. kok-saghyz* (Luo et al., 2018) found more SNP markers related to high rubber concentration in the MEP pathway genes, indicating a stronger involvement of the MEP pathway than the MVA pathway in rubber yield in this species. Also, more SNPs were found in genes involved in inulin production than in rubber biosynthesis (Luo et al., 2017), suggesting a high degree of conservation of rubber biosynthesis genes (Luo et al., 2017).

Parthenium argentatum HMG activity and RT-ase activity increased in cold temperature and were correlated with a rapid increase in rubber formation (Cornish and Backhaus, 2003; Ji et al., 1993). However, overexpression of HMGR only caused increased rubber yield in young *P. argentatum* plants under controlled environment; these increases were not maintained in field-grown transgenics, although an interesting correlation of expression level and heat tolerance was observed (Dong et al., 2013). Transcriptome analysis of cold-acclimated *P. argentatum* ESTs (a total of 11 748) found that most of the ESTs were from genes encoding stress-related proteins, while only just 1% of the ESTs were identified as rubber biosynthesis-related (Ponciano et al., 2012). Also, as in *H. brasiliensis*, a multigene family encodes the *P. argentatum* HMGS and HMGR (Ponciano et al., 2012).

Several TPT genes were overexpressed in two lines of *P. argentatum* and the interspecific hybrid line AZ101 (*P. argentatum* × *P. tomentosum*; Veatch et al., 2005). These included FPP synthase (*FPS*), GGPS synthase (*GGPS*) and a mutated form of GGPP, which produced a mixture of hexa (*C30*) and hepta (*C35*) APPs (*HHPS*; Veatch et al., 2005). Prenyl transferase activity increased in the transgenic plants, but different effects on plant growth and isoprenoid production were observed. In the transgenic *P. argentatum* field plants, more rubber molecules were synthesized than in wild-type plants, but they were of lower molecular weight. Thus, although more molecules were initiated by the increased APP, there appeared to be insufficient IPP to grow them to full length. Rubber content and molecular weight were little affected in the hybrid line by APP overexpression, but total terpene resins nearly doubled, indicating different metabolic regulation in the hybrid and nonhybrid accessions. Heterologous expression of chloroplast-localized GGPS isolated from *Helianthus annuus* (sunflower) in *T. brevicorniculatum*, *N. tabacum* and *A. thaliana* conferred fast plant growth, early flowering and increased seed yield with elevated gibberellin content and
| Gene/protein name | Gene origin | Approach or method | Performance | Pathway or protein | References |
|-------------------|-------------|--------------------|-------------|--------------------|------------|
| ATP citrate lyase (ACL), Acetoacetyl-CoA thiolase (AACT) and 3-hydroxy-methyl-glutaryl-CoA reductase (HMGR) | A. thaliana | Overexpression in T. brevicorniculatum | Overexpression of the three genes resulted in an increase in pentacyclic terpene and cis-1,4-isoprene levels | MVA pathway | Putter et al. (2017) |
| HMGR1 | H. brasiliensis | Overexpression in transgenic Arabidopsis plants | Transgenic plants were morphologically distinct from wild-type plants | MVA | Venkatachalam et al. (2009) |
| REF1, SRP1, REF3, REF7 | H. brasiliensis | Genome assembly | These four genes were highly expressed in latex; four different SRPPs were expressed in the latex | RP-associated Protein | Tang et al. (2016) |
| HbEIN3-1 to HbEIN3-4 | H. brasiliensis | Genome annotation | HbEIN3-1 had high levels of transcript abundance in the latex, and four HbEIN3s responded to ethylene and jasmonic acid treatments suggesting their involvement in ET and JA signalling. | Protein | Yang et al. (2015) |
| CPT, REF, SRP, RBSP, RBIP and others | H. brasiliensis | Late transcriptome qRT-PCR | Ethylene increased cis-polyprenyl synthesis. Hybrid contigs showed 11 classes of metabolic destinations for IPP, including cis-polyprenyl. | CPT and RP-associated proteins | Chow et al. (2012) |
| REF, SRP, HRT1, HRT2 | H. brasiliensis | EST and RT-PCR analysis | These genes were highly expressed as latex-specific genes and cis-acting regulatory elements were identified in promoter regions of these genes. | CPT and RP-associated proteins | Aoki et al. (2014) |
| REF | H. brasiliensis | BAC/SDS-PAGE and MS analysis | Two different forms of REF are mainly and tightly located on the surface of large RPs. | RP-associated proteins | Dai et al. (2017) |
| REF, SRP, HRBP (CBP), CPT | H. brasiliensis | Transient expression in tobacco | SRPP can recruit CPT to the ER and interaction of CPT with HRBP leads to both proteins relocating to the plasma membrane interactions among RP proteins | CPT enzymes | Brown et al. (2017) |
| HRT1, HRT2 | H. brasiliensis | Heterologous expression in yeast and Arabidopsis | The recombinant proteins showed distinct CPT activity and produced polyisoprenoids with chain lengths of C80-C100, but no rubber | RT-ase | Takahashi et al. (2012) |
| HRT1, HRT2 | H. brasiliensis | Overproduction of recombinant protein in E.coli, heterologous expression in yeast, RT-PCR, activity assay | Recombinant HRT2 protein may have synthesized medium-chain polypropenyl diphosphate intermediates as well as long-chain rubber in the presence of RPs. | RT-ase | Asawatreratanakul et al. (2003) |
| HbAAXT1,2, HbHMGS1,2, HbHMGR1,4,5 HbMVK, HbPMK, HbMVD | H. brasiliensis | RT-PCR and loss-of-function complementation in yeast | Many of these genes were highly expressed in latex, and individual functions of the genes were tested in yeast | MVA | Sando et al. (2008a) |
| CPTL2, CPT3 | L. sativa | RNA interference/transient expression in tobacco/expression in yeast microsome | CPTL2 RNAi resulted in reduced rubber content. Yeast microsomes with CPTL2/CPT3 had enhanced synthesis of short cis-polyprenols, but not rubber. | CPT-like enzyme, CPT | Qu et al. (2015) |
| LsCPT3, LsCPTL2, NgBR homologue HRT1-REF bridging protein (HRBP) (CBP) | H. brasiliensis, L. sativa | Cell-free translation-coupled protein introduction system onto WRPs, PR proteomics and interaction network analysis | The results support that the key factor for the reconstitution of RT-ase activity is not an unknown interacting protein for CPT but proper introduction of CPT (HRT1) as a form of complex with REF and HRBP. | CPT-like enzyme/protein | Yamashita et al. (2016) |
| Gene/protein name | Gene origin | Approach or method | Performance | Pathway or protein | References |
|-------------------|-------------|--------------------|-------------|--------------------|------------|
| LsSRPP1-8         | L. sativa   | EST, qRT-PCR and RNA interference | Two LsSRPPs (LsSRPP4 and LsSRPP8) were highly expressed in latex; LsSRPP4 and LsSRPP8 RNAi had no effect on NR amount, molecular weight or polydispersity | RP-associated protein | Chakrabarty et al. (2015) |
| PaFPS             | P. argentatum | Immunoblots and FPS activity assay | Two FPS isoforms were identified, and at least one is on the RPs. | RP-associated protein | Pan et al. (1996) |
| PaCPT1-3 and PaCBP| P. argentatum | Co-expression, yeast mutant complementation, activity assays | Co-expression of PaCBP and PaCPT led to dolichol synthesis but not rubber in yeast. PaCPT3 is a likely catalytic subunit in RTase involving CPT | Protein complexes | Lakusta et al. (2019) |
| PaAOS (PaCYP74)    | P. argentatum | RNAi, overexpression | Involved in RP expansion and phytohormone regulation. | RP-associated protein | Placido et al. (2019) |
| GHS               | P. argentatum | cDNA isolation and heterologous expression in E.coli | The recombinant protein enhanced rubber biosynthetic activity in vitro | RP-associated protein | Kim et al. (2004) |
| TbREF             | T. brevicorniculum | Mass spectrometry/RNA interference | The TbREF was homologous to TbSRPPs, and the promoter was active in laticifers. Rubber content was significantly reduced by TbREF RNAi, correlating with lower TbCPT protein and activity in latex. | RP-associated protein | Laibach et al. (2015) |
| TbRTA (CPTL)      | T. brevicorniculum | Knockdown by RNA interference | TbRTA RNAi inhibited rubber particle formation and prevented rubber biosynthesis, without affecting dolichol accumulation or protein glycosylation in the latex | Rubber transferase | Epping et al. (2015) |
| Tbb2P, TtbSRPP    | T. brevicorniculum | Yeast one-hybrid system/transactivation experiments in tobacco mesophyll protoplasts | Tbb2P regulated TtbSRPP gene expression in response to ABA, reflecting overlapping roles in rubber biosynthesis and stress responses. | Transcription factor protein | Frick et al. (2013) |
| TbCPT1-3          | T. brevicorniculum | RNA interference | TbCPT1-3 RNAi reduced rubber biosynthesis and increased terpenes and inulin levels. | Rubber transferase | Post et al. (2012) |
| TbSRPP3           | T. brevicorniculum | RNA interference, Down-regulation of protein expression | RNA interference of TbSRPP3 affected integrity of rubber particles and rubber content | RP-associated protein | Hillebrand et al. (2012) |
| Tb and Tk 1-SST, 1-FFT and 1-FEH | T. brevicorniculum, T. kok-saghyz | Full-length cDNA isolation and analysis of root mRNA levels (qPCR), overexpression of 1-FEH | Tk 1-SST, 1-FFT gene expression was correlated to amount and degree of polymerization (DP) of inulin during summer. Tk 1-FEH overexpression led to inulin degradation and root rubber increase. | Inulin pathway enzymes | Stolze et al., 2017 |
| TkCPT1-3, TkSRPP1-5 | T. kok-saghyz | Heterologous expression in N. tabacum and yeast, semi-quantitative RT-PCR analysis | CPTs associated with RPs were able to produce polyrenols in yeast; TkCPT1 was predominantly expressed in latex. | CPT enzyme | Schmidt et al. (2010a,b) |
| TkSRPP3           | T. kok-saghyz | Overexpression and RNA interference | TkSRPP3-overexpressing lines have slightly higher levels of root rubber; TkSRPP3 RNAi significantly reduced rubber content and rubber molecular weight. | RP-associated protein | Collins-Silva et al. (2012) |
| TbhHMGR1-3, Tb HMGS, TbMVK, TbMVD | T. brevicorniculum | Full-length cDNA isolation and complementation assay using an IPP auxotroph mutant of E.coli, transient expression assay in Nicotiana benthamiana leaves | High expression of TbhHMGR1, Tb HMGS, and TbMVK in the latex and functionality of TbhHMGR1-3 confirmed in E.coli. Transient assays demonstrated that TbhHMGR1-2 can regulate the MVA pathway by stimulating sterol formation | MVA | van Deenen et al. (2011) |
reduced carotenoid content, but no significant change in rubber content was observed (Tata et al., 2016).

Transcription factor regulatory genes

Transcription factors (TFs) are important genes regulating biosynthetic pathways involving secondary metabolites (Endt et al., 2002; Wang et al., 2013). Studies on transcription factor genes and their roles in regulating rubber biosynthesis in plants appear limited, but negative results suggest that some negative studies have not been reported. A MADS-box TF gene (HbMADS4, inducible by methyl jasmonate (MeJA) and ethylene, and preferentially expressed in latex) was recently isolated from H. brasiliensis. A yeast one-hybrid experiment, which used the HbSRPP promoter as bait, demonstrated that overexpression of HbMADS4 suppressed HbSRPP promoter activity in transgenic N. tabacam plants (Li et al., 2016). Similarly, GUS activity was suppressed when pHbSRPP::GUS was co-expressed with CaMV35S::HbWRKY1 in N. tabacam, making it likely that the transcription factor, WRKY, behaves as a negative regulator of HbSRPP in NR biosynthesis (Wang et al., 2013, 2015).

Transcription factor genes identified from ESTs of H. brasiliensis (e.g., the putative MYB transcription factor from EST clone Hebr-B114) had highly specific late expression (~1000 times higher in latex than stems or leaves; Aoki et al., 2014). The T. brevicorniculatum TbbZIP1 transcription factor gene also was predominantly expressed in laticifers. The activity of this TF, which was dependent upon ABA concentration, promoted the expression of TbsSRPP genes, suggesting a dual role for this gene in rubber biosynthesis and stress adaptation (Fricke et al., 2013; Table 2). The observed effects may have been mediated by ABA-inducible protein kinases. TF gene expression in laticifers suggests that they may play an overarching role in rubber biosynthesis.

Transcriptome analysis of P. argentinum (Ponciano et al., 2012; Stonebloom and Scheller, 2019) has revealed a range of genes more highly expressed in cold-induced tissue in which rubber biosynthesis is synthesized at a greater rate than in plants grown at warmer temperatures (Cornish and Backhaus, 1990, 2003). A cluster of 30 differentially expressed contigs had homologies to DNA-binding proteins or transcription factors and might be involved in cold-induced rubber biosynthesis (Stonebloom and Scheller, 2019).

Rubber particles

Rubber particle architecture

Biosynthesis of NR takes place at the surface of rubber particles produced in the cytosol of cells, whether these are specialized laticifers, as in H. brasiliensis bark and T. kok-saghyz roots, or in adapted bark parenchyma cells, as in P. argentatum. Rubber is compartmentalized in the rubber particle interior as an end product (Cornish, 2001a,b; Schmidt et al., 2010b; Figure 1), which cannot be catabolized during the life of the plant. Rubber particle size (0.08–2 µm in H. brasiliensis, 0.2–8 µm in T. kok-saghyz, 0.2–6.5 µm in Ficus spp and 0.7–2 µm in P. argentinum) and composition vary among different plant species, although ultrastructural analysis indicates a common globular structure (Abdul Ghaffar et al., 2016; Cornish, 2001b; Cornish et al., 1993; Gomez and Hamzah, 1989; Nawamawat et al., 2011; Schmidt et al., 2010b; Siler et al., 1997; Singh et al., 2003; Wood and Cornish, 2000). H. brasiliensis produces large rubber particles (LRP) and small rubber particles (SRP) rather than a continuous size distribution (Singh et al., 2003; Wood and Cornish, 2000). Similarly, two distinct rubber particle morphologies were reported in T. kok-saghyz, namely irregularly shaped cytoplasmic rubber particles and globular and smooth vacuum rubber particles (Abdul Ghaffar et al., 2016).

Three models have been proposed to describe the structure of the rubber particle lipid-protein surface membrane, namely: (i) a general model in which all rubber particles consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane (Cornish, 2001a,b; Cornish et al., 1999), made up of proteins and lipids. Here, hydrophilic amino acids, glycosylated moieties and polar lipid head groups interact with the aqueous cytosol, while hydrophobic protein regions and lipid chains form the interior of the monolayer membrane and are compatible with the hydrophobic rubber polymers. The shell interfacial model is basically the same as this general model (Rochette et al., 2013); (ii) a mixed monolayer (patchy) model, which is similar to the general model but in which the membrane includes patches of rubber polymer (Berthelot et al., 2014; Nawamawat et al., 2011; Siler et al., 1997); and (iii) the H. brasiliensis-specific protein film model, in which the lipid chains of a continuous monolayer of fatty acids interact with the rubber core, their polar head groups interact with amphipathic proteins, while the hydrophilic regions of those proteins interact with the cytosol (Wadeesirisak et al., 2017). This model may be true for particles with high protein content, but cannot be applied to P. argentinum due to lack of sufficient protein to form a contiguous layer (Siler et al., 1997).

The nonrubber components present in rubber particles are highly species-specific and can impact the properties of rubber produced by different plants (Junkong et al., 2017; Monadjemi et al., 2016). For instance, an unusual furanoid fatty acid is a major constituent of H. brasiliensis rubber particle membranes, whereas the membranes of F. elastica particles contain very-long-chain FAs (Siler et al., 1997). These long-chain FAs, combined with integral membrane proteins, form stiff membranes instead of the fluid rubber particle membranes in most other species, including H. brasiliensis and P. argentinum (Cornish et al., 1999; Wood and Cornish, 2000).

Rubber particle proteins

As mentioned above, rubber particles are the location of the enzymes and proteins specifically involved in rubber polymerization (Cornish and Backhaus, 1990). The RT-ase complex likely includes proteins involved in substrate binding, catalysis, molecular weight regulation and correct channelling of the rubber polymer into the rubber particle interior. However, many additional proteins also are associated with rubber particles. These include membrane-bound proteins and other proteins merely rubber particle-associated which can be readily displaced: 186 rubber particle-bound proteins were identified in H. brasiliensis (Dai et al., 2013). In the absence of reproducible solubilized RT-ase activity, it is challenging to unequivocally identify which proteins are directly involved in the regulation of rubber biosynthesis and genetic approaches have played a key role in identification attempts. However, some rubber particle proteins appear to be involved in the structure and integrity of the rubber particle. SRPP and REF do appear to play a significant role in latex coagulation and rubber particle stabilization in H. brasiliensis (Dennis and Light, 1989; Wititsuwannakul et al., 2008). Especially, SRPP has been proposed to stabilize rubber particles by displaying a kind of ‘covering effect’ over the lipid head groups without disturbing membrane integrity (Berthelot et al., 2014;
Wadeesirak et al., 2017). This idea was further supported by studies of lipid droplets that compartmentalize tracylglycerol rather than rubber polymers, in which homologs of HbSRPP were identified as lipid droplet-associated proteins and were required for the maintenance of lipid droplets in stress conditions (Gidda et al., 2013, 2016; Horn et al., 2013; Pyc et al., 2017). SRPP (24 kD) in *H. brasiliensis* rubber particles is one of two major acidic proteins, the other being REF (14.6 kD; Dennis and Light, 1989; Yeang et al., 1996; Oh et al., 1999; Priya et al., 2006; Aoki et al., 2014). The two proteins share significant homology [approximately 72% amino acid homology (Oh et al., 1999)].

P. argentatum and *T. kok-saghyz* rubber particle proteins both contain SRPP (HbSRPP) orthologs, PaGHS and TkSRPPs, respectively (Collins-Silva et al., 2012; Kim et al., 2004).

The most abundant *P. argentatum* rubber particle-bound protein is allene oxide synthase (AOS; also known as rubber particle protein, RPP; Backhaus et al., 1991; Pan et al., 1995; Ponciano et al., 2012) Overexpression of AOS in tobacco created small globules (Backhaus, pers. com.) and RNAi down-regulation of AOS (CPY74) led to smaller rubber particles and increased rubber biosynthesis in *P. argentatum* (Placido et al., 2019), seemingly in contrast to a report (not repeated) that solubilized AOS could synthesize rubber in vitro (Benedict et al., 1989). The expansion of particles may be limited by the amount of particle-bound AOS, leading to increased RT-ase activity. This is analogous to the proposed role of the *H. brasiliensis* REF and SRPP, which associate and may stabilize the particles when they expand as newly synthesized rubber molecules are compartmentalized to the particle interior (Dai et al., 2017). The main SRPP homolog in cold-acclimated *P. argentatum* ESTs has 99.9% and 60% amino acid similarity to a previously isolated *P. argentatum* protein and to *H. brasiliensis* SRPP, respectively (Backhaus et al., 1991; Pan et al., 1995; Ponciano et al., 2012). Five SRPPs (TbSRPP1-TbSRPP5) were identified in *T. brevicorniculatum* (initially designated as *T. kok-saghyz*, due to erroneous identification; Schmidt et al., 2010a). The corresponding genes, except TbSRPP2, were expressed in root laticifers, and the proteins (composed mainly of SRPP3 and SRPP5) were rubber particle-bound (Hillebrand et al., 2012; Wahler et al., 2012). A farnesyl pyrophosphate synthase also appears to be bound to the *P. argentatum* rubber particle membrane (Pan et al., 1996), but this may not be functional in particles with RT-ase activity because kinetic studies could only detect a single IPP using enzyme (Cornish, 2001a,b; Cornish and Scott, 2005).

Both REF and SRPP are allergenic proteins (named HbREF/Hevb3 and HbSRPP/Hevb1) in the context of human type I latex allergy (Yeang et al., 1996). REF is abundantly located on the surface of both small and large *H. brasiliensis* rubber particles (Dennis and Light, 1989; Oh et al., 1999; Schmidt et al., 2010a; Yeang et al., 1996) as well as in the soluble fraction of latex. These proteins also are immunochemically similar to proteins on *T. kok-saghyz* rubber particles (Cornish et al., 2015). In *H. brasiliensis*, SRPP is predominantly localized on small rubber particles, in phloem, and to a lesser degree on large particles but, unlike REF, is not found in the soluble latex protein fraction (Sando et al., 2009). Other factors, like glycosylation, are likely associated with the soluble REF form because the sequence of REF indicates that it, like SRPP, is highly hydrophobic. These proteins have different affinity for the monolayer of rubber particles: a subfraction of REF seems to be an integral membrane protein while another subfraction of REF is readily solubilized, and a third is soluble as mentioned above. In contrast, SRPP binds at the membrane surface and may form a kind of proteinaceous coating (Berthelot et al., 2012, 2014). It also has been proposed that REF may displace membrane lipids forming a type of mixed micelle (Berthelot et al., 2014).

Recent genome analysis of *H. brasiliensis* revealed that SRPP and REF have eight and nine different genes, respectively, the largest numbers reported in any plant genome for single proteins (Lau et al., 2016). Another genome analysis identified 18 REF/SRPP genes in *H. brasiliensis* (SRPP1 to 10 and REF1 to 8, respectively; Tong et al., 2017). REF1 (138 amino acids) and SRPP1 (204 amino acids), the most abundant isoforms (Tong et al., 2017), encode the well-characterized *H. brasiliensis* REF and SRPP particle proteins (Dennis and Light, 1989; Oh et al., 1999).

**Rubber particle proteins implicated in rubber transferase (RT-ase) activity**

RT-ases form a distinct subgroup of the cis-prenyltransferase (CPT) family (Akhtar et al., 2013; Asawatreratanakul et al., 2003; Cornish et al., 2001; Epping et al., 2015; Espy et al., 2006; Hemmi et al., 2001; Kharal and Koyama, 2003; Oh et al., 2000; Schmidt et al., 2010b; Yamashita et al., 2016). RT-ases have low substrate affinity for IPP, which prevents rubber biosynthesis from causing critical IPP deficits—rubber can only be made when IPP is in excess of cellular metabolic requirements (Cornish, 2001a,b; da Costa et al., 2004, 2006). Different types of CPTs are localized differently, possess different substrate-binding requirements and constants, and produce different product sizes. RT-ase is the only CPT capable of producing high molecular weight (>1000 kg/mol) cis-polyisoprene (da Costa et al., 2004, 2006; Espy et al., 2006; Kharal et al., 2001; Kharal and Koyama, 2003; Kharal et al., 2006; Ko et al., 2001; Liang et al., 2002; Post et al., 2012; Shimizu et al., 1998; Shridas et al., 2003; Surmacz et al., 2015; Teng and Liang, 2012), and nonrubber-producing CPTs have been confused with, or misidentified as, RT-ase (Cornish, 1993). The *H. brasiliensis* rubber particle-bound 35-kD CPT (Asawatreratanakul et al., 2003) can be removed from the particles without a concomitant loss of RT-ase activity (Cornish, unpublished data). CPTs are involved insterol synthesis and so are required to form cell membranes (possibly including those of rubber particles). Also, the direct product of most CPTs are relatively short-chain cis-allicylic pyrophosphates, which, if soluble, can function as initiators of rubber molecular biosynthesis. Thus, CPTs can affect rubber biosynthesis without being RT-ase itself (Cornish, 1993; Light and Dennis, 1989; Light et al., 1989).

A CPT-binding protein (CBP) was collectively discovered, in investigations not all related to rubber biosynthesis, via the identification of a new CPT, conserved across the eukaryotic lineages in human, *L. sativa*, *T. kok-saghyz* and *Lycopersicon esculentum* (tomato), by four independent research groups (Brasher et al., 2015; Epping et al., 2015; Park et al., 2014; Qu et al., 2015, respectively). A unique heteromeric CPT protein complex was found to exist in eukaryotes quite unlike the simple prokaryotic CPTs. The heteromeric eukaryotic CPT protein complex was composed of a catalytic CPT and an unusual nonenzymatic protein, designated as NgBR (Nogo-B receptor) in humans, CPTL (CPT-like protein) in *L. sativa*, CPTBP (CPT-binding protein) in *L. esculentum* and RTA (RT-ase activator) in *T. brevicorniculatum* (Brasher et al., 2015; Epping et al., 2015; Kwon et al., 2016; Park et al., 2014; Qu et al., 2015). The fourth nonenzymatic protein (RTA) also is named CBP (CPT-binding protein), and, for clarity, this is the abbreviation we use below as we combine these.
reports. Thus, CBP directly interacts with enzymatically active CPTs and CPT/CBP complexes appear to be involved in both dolichol and NR biosynthesis (Brasher et al., 2015; Epping et al., 2015; Qu et al., 2015; Lakusta et al., 2019; Table 2).

In *T. brevicorniculatum*, CBP interacts with CPTs on the surface of rubber particles (Epping et al., 2015). CBP may perform two important functions: linking CPT to the rubber particle, and also stabilizing the entire complex to protect CPT from degradation, thus ensuring efficient rubber synthesis and accumulation (Epping et al., 2015). The CBP-RNAi transgenic lines generated were unable to synthesize long-chain cis-1,4-polyisoprene (H. brasiliensis), suggesting that assembly of the RT-ase complex requires both the CPT and CBP (Epping et al., 2015).

Likewise, an unusual cis-prenyltransferase-like 2 (CPTL2) protein, with some homology to the human CBP, was needed for rubber biosynthesis in lettuce, but the yeast expressed CPTL2/CPT3 was not active in vitro, and no rubber was made (Qu et al., 2015). The recent *T. kok-saghyz* genome analysis identified eight CPTs and two CPTL genes encoding rubber particle proteins and CPTL1 was highly expressed in latex as expected for rubber particle-bound proteins (Lin et al., 2017). However, the *L. sativa* CPTL2 did not reactivate RT-ase activity in detergent-washed and inactivated *H. brasiliensis* rubber particles (Yamashita et al., 2016) possibly because it was already replaced by CPR (HRBP, Hevea REF Bridging Protein), leaving no room for additional insertion.

Two *H. brasiliensis* CPT-like proteins, designated as putative RT-ases (HRT1 and HRT2), are predominantly expressed in latex (Aoki et al., 2014). HRT2 protein increased new rubber molecule synthesis when added to the washed bottom fraction from centrifuged *H. brasiliensis* latex (Asawatreratanakul et al., 2003). However, rubber particles were still present in this assay and CPT could have stimulated rubber biosynthesis through the initiator system (by producing cis-APP initiators). Also, both HRT1 and HRT2 need latex-specific co-factor(s) for activity (Takahashi et al., 2012). However, partially deproteinized purified rubber particles, which could synthesize very little rubber from exogenous IPP and FPP, regained significant RT-ase activity when HRT1, or *L. sativa* CPT3, was added back to the rubber particles (Yamashita et al., 2016) and high molecular weight rubber was produced. These proteins may have reattached to the rubber particles by interacting with still present SRPP tethered by residual HRBP (a protein binding protein which has a transmembrane domain; Qu et al., 2015).

Other proteinaceous or architectural components of rubber particles are essential to the HRT1-mediated reconstitution of RT-ase activity, because RT-ase activity could not be reconstituted in liposomes (Yamashita et al., 2016). This indicates that *H. brasiliensis* rubber particle-specific components are required for activity, and may include integral proteins, and/or other membrane constituents, such as the unusual furanoid fatty acid, predominant in *H. brasiliensis* rubber particle membranes (Siler et al., 1997). The HRT1, CBP and possibly REF proteins only affected RT-ase activity, not polymer molecular weight, and all the rubber produced, including by the control, was of typically high molecular weight. It is also important to note that several CPTs tested in this system did not lead to RT-ase activity, including the HRT2 (Yamashita et al., 2016). Also, in *H. brasiliensis*, SRPP, REF and CBP are expressed in the endoplasmic reticulum (ER) and appear to be involved in rubber particle ontology. Also, ER-located SRPP interacts with REF and can recruit a cytosolic CPT to the ER membrane (Brown et al., 2017). Although this CPT (CPT6, HRT2) is not as clear a RT-ase catalytic protein candidate as HRT1 (*Yamashita et al., 2016*), this report does provide strong direct evidence for an ER origin of rubber particles.

CPT genes were reported to be required for rubber biosynthesis in *T. kok-saghyz*, *L. sativa* and *H. brasiliensis* (Epping et al., 2015; Qu et al., 2015; Yamashita et al., 2016; Table 2), but their ability to produce allylic pyrophosphate initiators (Cornish, 1993, 201a, b) can make interpretation of such experiments difficult. Four CPT-like sequences were identified in a *P. argentinum* cold-acclimated EST collection (Ponziano et al., 2012), and recently, three CPT genes (PaCPT1-3) and one CBP (PaCBP) also were identified in this species (Lakusta et al., 2019). When PaCBP was co-expressed with each PaCPT in turn they formed a complex which could synthesize dehydrodolichyl-PP in microsomes (Lakusta et al., 2019). Although no rubber was made, PaCPT3 was both highly expressed in rubber-producing stem tissue and was further induced by cold stress, as was PaCBP. Thus, these proteins may be involved in RT-ase activity in this nonlaticiferous species. However, proteomic analysis of *P. argentinum* rubber particles did not find CPT homologues (Kajiura et al., 2018), indicating that the RT-ase catalytic protein is in low abundance.

Also, three CPTs (*TkCPT1-3*) highly homologous to each other (~98%) were expressed in *T. brevicorniculatum* laticifers and root tissue and produced short-chain cis-1,4-polyisoprene (Schmidt et al., 2010a,b). However, these had low homology with other CPTs (~53% with HRT1, ~52% with HRT2, ~31% with REF2 (Sato et al., 1999), and ~21% with ACPT; Lin et al., 2017; Table 2). *T. brevicorniculatum* rubber particle CPTs complemented yeast mutants deficient in CPT activity and maintained activity in *N. tabacum* protoplasts (Schmidt et al., 2010b). Inhibition of CPT by RNAi reduced cis-1,4-polyisoprene content and led to an almost complete absence of long-chain molecules (Post et al., 2012), suggesting a role for this CPT in molecular weight regulation. HMGR (the key rate-limiting enzyme of the MVA pathway) also was inhibited in latex of the *TbCPT-RNAi* plants via an uncharacterized feedback mechanism. Of course, when HMGR activity is reduced, the supply of IPP decreases and the rate of rubber biosynthesis declines, and this is probably the cause of the observed low rubber phenotype (Post et al., 2012). Also, HMGR inhibition caused upstream precursors, such as acetyl-CoA, to accumulate. Feedback regulation effects led to a corresponding increase in the storage carbohydrate, inulin (up to 20%), in transgenic *TbCPT-RNAi* plants (Post et al., 2012). Similarly, *T. brevicorniculatum* rubber and inulin (biosynthesis and degradation) levels were negatively correlated and depended on corresponding enzymatic activities at different periods of the growing season (Hillebrand et al., 2012; Tata et al., 2012). An inverse relationship between inulin and rubber also was observed in *T. kok-saghyz* (Arias et al., 2016; Cornish and Xie, 2012; Kreuzberger et al., 2016).

The role of REF in rubber biosynthesis remains obscure due to conflicting reports. This may partly be caused by the presence of both membrane-associated and soluble forms of REF. Two REF isoforms bind more tightly to large rubber particles of *H. brasiliensis* than the most abundant form of REF (*Dai et al., 2017*). In the HRT-1 study, RT-ase activity was little affected by additional CBP except when this was added to HRT 1 and rubber particles with REF, when activity increased significantly (Yamashita et al., 2016). Cornish also has noted a strong correlation between CHAPS (0%–1%) solubilization of REF and loss of RT-ase activity ($r^2 = 0.948$, df = 5) from purified *H. brasiliensis* rubber particles. Nonetheless, REF does not seem to play a role in rubber
producers or elongation as originally believed (Dennis and Light, 1989). The gel-purified rubber particles used in that study contained many proteins (revealed by silver staining), and the anti-REF IgG was not purified (Cornish, 1993; Dennis and Light, 1989) when used to inhibit rubber biosynthesis: unpurified sera contains components which strongly inhibit RT-ase active (Cornish, 2001b). Although REF and SRPP have multiple isoforms, identified by 2D gels of latex from H. brasiliensis clones of different productivity, few of them were correlated with yield or response to ethylene stimulation (Tong et al., 2017). RNA interference (RNAi) of REF expression in T. brevicorniculatum confirmed that REF was not required for rubber biosynthesis in this species (Laibach et al., 2015). Rubber particle numbers were reduced, however, and REF may provide a needed structural component in rubber particle biogenesis and development. Similarly, RNAi down-regulation of TbSRPP3 severely impacted rubber content, which was affected to a lesser extent by down-regulation of the other TbSRPPs (Collins-Silva et al., 2012; Hillebrand et al., 2012; Table 2). Molecular weight was significantly reduced in all the RNAi lines in one study (Collins-Silva et al., 2012) but not the other (Hillebrand et al., 2012). A role for SRPP in generation of high molecular weight rubber, but not rubber biosynthesis itself, is backed up by an apparent lack of SRPP homologs in Ficus carica and F. benghalensis, which both produce only low molecular weight rubber (Singh et al., 2003). SRPP homologues are present in the high molecular mass rubber producers H. brasiliensis, P. argentatum, T. brevicorniculatum and T. kok-saghyz.

The RT-ase protein complex

The RT-ase enzyme/enzyme complex catalysing rubber biosynthesis (Cornish, 2001b) remains poorly understood. In this section, we have integrated the published reports of proteins apparently involved in RT-ase activity, as discussed above, with structural information either directly obtained or inferred from enzyme kinetics to inform a new schema of the rubber particle-bound RT-ase complex (Figure 2). This model is drawn to assemble the components in such a way that they could interact and perform their putative functions, as discussed below.

Hevea brasiliensis, P. argentatum and F. elastica rubber particles contain very large membrane-associated proteins (Cornish et al., 1993), which have monomeric sizes of 241 000, 287 000 and 360 000 Da, respectively (Cornish et al., 2018). Binding studies, with labelled rubber substrates, demonstrate that these very large proteins are part of the RT-ase complex (Cornish et al., 2018) and the native F. elastica protein was shown to be a dimer (Cornish and Siler, 1996; Cornish et al., 1994). Some interspecific surface commonalities have been demonstrated immunochemically (Cornish et al., 1994; Siler and Cornish, 1993), also supporting a similar function of the large proteins among these species. Thus, it appears that the RT-ase complex contains a large dimeric protein scaffold, half of which is shown in the schema [brown half ring, with grey cross-hatch (Figure 2)]. The scaffold “doughnut-like” dimeric protein tightly, but noncovalently, holds two small specific substrate-binding proteins (Cornish et al., 2018) in position at the entrance of this channel, shown as the blue/red proteins (Figures 2 and 3). The size of the smaller of the two proteins ranges from 1600 to 1800 Da depending on the species, while the larger ranges from 3650 to 3990 Da. It seems likely that the tritated benzophenone allylic pyrophosphates labelled both the allylic and nonallylic pyrophosphate binding sites, because it has been proved that competitive inhibitors of allylic pyrophosphate initiators can bind at both sites (Mau et al., 2003). However, since we only have the minimized structure of the smaller of the two proteins, and it is not yet possible to tell which small protein binds IPP and which binds FPP, the same structure is shown for both in the schemas (Figures 2 and 3).

Enzyme kinetic analysis, and protein purification and quantification studies, demonstrated that there are three of each binding protein per scaffold monomer (Cornish et al., 2018), even though only two of each are shown on the schema for the sake of clarity (Figure 2). However, the tritated benzophenone substrates used to identify the two specific binding proteins did not bind to CPT, SRPP or REF-type proteins in these experiments. This suggests that the catalytic site of the RT-ase complex either is not a CPT (which seems unlikely) or that it is adjacent to the substrate-binding sites, sufficiently close to perform the condensation reaction but not aligned to the benzophenone photoaffinity amino acid labelling moiety at the far terminus of the substrate (see right-hand active site in Figure 2). Thus, the CPT-type catalytic site (shown on the schema as blue amino acids, in the white globular protein representation of the CPT-like protein (Figure 2, and expanded in Figure 3)) is positioned adjacent to these binding sites so that the condensation reaction between IPP and the elongating rubber molecule to take place.

In the schema (Figure 2), an integral membrane CBP (in pink; Qu et al., 2015; Epping et al., 2015; Brasher et al., 2015) is shown integrally attached to the monomer of the dimeric scaffold (in brown) and positioned adjacent to the CPT-like proteins (in white), where it could act as a binding protein.

The rubber particle-bound protein SRPP plays a role in molecular weight regulation (Collins-Silva et al., 2012) and so must also form a subunit of the RT-ase complex (Figure 2, depicted as a blue globular protein). SRPP must be positioned sufficiently close to the RT-ase active site to affect polymer size. Thus, SRPP [blue globular protein (Figure 2)] is placed in the immediate vicinity of both the binding protein and the RT-ase catalytic site where it could affect the chain transfer reaction, that is, speed or impede the displacement of the elongating rubber chain by a new initiator.

However, although the exact position of SRPP and REF, their conformation, and proximity to the growing rubber polymer, is not yet known (Berthelot et al., 2014; Collins-Silva et al., 2012; Guo et al., 2014; Li et al., 2016; Wadeesirak et al., 2017), due to lack of a definitive role in molecular weight regulation or biosynthesis, and the conflicting results discussed above, the integral REF is placed on the schema [green globular protein (Figure 2)] in a position more distant from the active site than SRPP. Membrane-associated and soluble REF forms also are shown because these different isoforms clearly exist (Dai et al., 2017).

The H. brasiliensis HRT1 appears to be part of a surface-bound complex held at the rubber particle surface by a transmembrane tethered CPB protein (HMBR protein, Yamashita et al., 2016) and is indicated on the schema by the CPT-like white globular protein associated with the integral (pink) CPB protein (Figure 2). The P. argentatum rubber particle protein (RPP or AOS) also has a transmembrane tethering domain (Pan et al., 1995) and can form very large multimers (Siler and Cornish, 1994). Electron paramagnetic resonance spin labelling confirmed that neither H. brasiliensis nor P. argentatum have much integral rubber particle membrane protein, but this was in strong contrast to F. elastica and Euphorbia lactiflua rubber particles, both of which
have significant amounts of integral membrane protein (Cornish et al., 1999). Whether or not the RT-ase complex in a particular species is tethered to the rubber particle member surface or is more integrally embedded in the membrane, once the RT-ase has catalysed the condensation reaction, the part of the complex proximal to the active site region plays a significant role in both rubber compartmentalization and rate of reaction. The specific RT-ase binding sites recognize and bind DMAPP and IPP (Figure 3), and the C5 allylic pyrophosphate moiety of initiators larger than DMAPP or of the elongating rubber polymer (which also is an allylic pyrophosphate). The kinetics of RT-ase activity, probed with APP initiators of different size and stereochemistry, using rubber particles purified from Hevea brasiliensis, Parthenium argentatum and Ficus elastica, indicate that the RT-ase complex contains a central channel through which the growing rubber polymers pass as they traverse the uni-lamella membrane of the rubber particles into the rubber particle interior [half ring structure, coloured brown with grey cross-hatches (Figure 2) for a detailed explanation, see Cornish, 2001a,b; Cornish and Scott, 2005;]. The length of the hydrophobic region is slightly shorter in P. argentatum than in H. brasiliensis and F. elastica RT-ase, which was demonstrated kinetically (Cornish, 2001a,b; Cornish and Scott, 2005) and structurally by paramagnetic resonance spectroscopy (Cornish et al., 1999). Also, it has been demonstrated that the rate of rubber biosynthesis increases with the number of RT-ase catalytic sites occupied by elongating rubber molecules (up to six binding sites per dimeric scaffold, as discussed above), probably due to increased hydrophobicity of the channel microenvironment being more favourable to the passage of the hydrophobic rubber chains (Cornish et al., 2018).

The schema (Figure 2) is not an actual RT-ase, but it does provide a predictive model which unifies the majority of the reports on RT-ase. The large scaffold proteins may be a type of
CBP-like proteins and could obviate the need for the smaller CBPs in some rubber-producing species. As described in this review, it is clear that rubber particle composition, RT-ase kinetics and the size of the rubber polymers produced vary considerably among species. The underlying differences in the species-specific RT-ase complexes have not yet been elucidated, and the degree and impact of protein glycosylation, while undoubtedly relevant, has not been touched upon. The precise relationship of these structural and binding proteins to the scaffold protein, small binding proteins, CPT/CBP, SRPP and REF, and other as-yet-unknown subunits, will require both solubilization of active RT-ase complexes and subsequent X-ray crystallographic revelation of their structures.

NR quality and its relationship to molecular weight

The quality of NR is an important determinant for industrial use, and it varies in different plant species. At a molecular level, NR quality is affected by polymer molecular weight and macro-molecular structure, gel content (cross-linked rubber insoluble in organic solvents), and to the composition and amount of nonrubber components like lipids and proteins (Cornish, 2001b; Cornish and Blakeslee, 2011; Schmidt et al., 2010b; Tangpakdee and Tanaka, 1997). Molecular weight is arguably the most important parameter underlying rubber quality, with quality positively correlated to molecular weight. Although a large group of rubber-producing plants are known, only a subset, including H. brasiliensis, T. kok-saghyz, P. argentatum, Cryptostegia grandiflora (Madagascar rubber vine), L. sativa (lettuce) and some Ficus and Euphorbia species produce rubber with an average molecular weight over 1000 kg/mol (van Beilen and Poirier, 2007b; Table 1). RT-ases are a specific family of CPTs with distinct substrate-binding constants (Km’s) and are able to synthesize NR with varying molecular weights depending on the available substrate and cofactor concentrations (da Costa et al., 2004, 2006). All RT-ases investigated to date have high Km IPP’s, preventing rubber (a terminal C sink) from polymerizing IPP in competition with essential IPP-requiring enzymes. Thus, rubber is made when IPP is in excess of their requirements. In vitro studies of H. brasiliensis, F. elastica and P. argentatum rubber particle-bound RT-ases have shown that rubber molecular weight is greatly affected by the ratio of the initiator FPP and IPP as well as their specific concentrations; increasing FPP concentration, under limiting IPP concentrations, causes a reduction in molecular weight, while increasing IPP concentrations under limiting FPP concentration has the opposite effect (Cornish et al. 2000; Cornish, 2001a; Cornish and Scott, 2005; da Costa et al., 2004, 2006). The concentration of the metal ion cofactor/activator also alters the molecular weight of the rubber produced by H. brasiliensis, F. elastica, P. argentatum, L. sativa and T. kok-saghyz (Scott et al. 2003; Bushman et al., 2006; Cornish and Blakeslee, 2011; Collins-Silva et al., 2012). Thus, the laticifer environment may play a significant molecular weight regulatory role. Species-specific in vitro molecular weight regulation studies indicate that additional proteins, such as SRPP and REF, may be involved in the regulation of polymer chain elongation and mature rubber molecular mass (Asawatreratanakul et al., 2003; van Beilen and Poirier, 2007a; Collins-Silva et al., 2012; Cornish, 2001b; Dennis and Light, 1989; Kim et al., 2004; Laibach et al., 2015; Puskas et al., 2006; Wadesiriasik et al., 2017). Only the P. argentatum RT-ase, so far, has been shown to exert some direct control over rubber molecular weight (Cornish and Scott, 2005). It is possible that this ability is related to the lack of laticifers in this species, and other nonlaticiferous rubber plants [e.g. Ericameria nauseosa (grey rabbit brush)] may prove to have similar types of RT-ases.

Involvement of rubber particle proteins and CPTs in functions other than rubber synthesis

In addition to a role in rubber molecular weight regulation, SRPPs also may be induced by stress and play a role in stress tolerance in plants (Balbuena et al., 2011; Fricke et al., 2013). SRPP orthologs isolated from a nonrubber-producing plant, Capsicum annuum (pepper), have significant amino acid homologies to putative stress-related proteins, and when CaSRP1 was overexpressed in A. thaliana, the transgenic plants exhibited enhanced growth rates and drought tolerance (Kim et al., 2010). Similarly, the SRPPs identified in T. brevicorniculatum had high sequence homologies among themselves and with HbSRPP, and also with the A. thaliana stress-related protein, AtSRP (Schmidt et al., 2010a). A SRPP ortholog was differentially expressed in cold-acclimated H. annuus, which is a rubber-producing species (Balbuena et al., 2011). In P. argentatum, RT-ase activity and rubber production are induced by cold temperatures (Cornish and Backhaus, 2003; Madhavan et al., 1989; Ponciano et al., 2012; Salvucci et al., 2010). It is possible that SRPP levels in P. argentatum are post-transcriptionally regulated and SRPP may play a role in cold tolerance (Ponciano et al., 2012).

The recent co-localization of CPTs in rubber particles with SRPPs in T. brevicorniculatum provides further evidence for a link between rubber biosynthesis and stress responses (Hillebrand et al., 2012; Post et al., 2012; Schmidt et al., 2010b). The HbSRPP promoter contains light-dependent and stress-responsive regulatory elements and is upregulated by tapping (Guo et al., 2014; Sookmark et al., 2002). A promoter of HbSRPP with a different sequence to the HbSRPP promoter was functional in the laticifers of transgenic T. brevicorniculatum and also was induced by stresses such as light, cold and tapping (Tata et al., 2012). Thus, the TdSRPPs may be involved in both rubber biosynthesis and plant stress responses. Similarly, as mentioned earlier, a bZIP
transcription factor (TbbZIP.1) in T. brevicorniculatum regulates genes encoding SRPPs in an ABA-dependent manner and contributes to stress tolerance (Fricke et al., 2013; Table 2).

In addition, expression of the AOS (CYP74A) protein in P. argentinum was inversely correlated with biomass and photosynthetic rate in cold temperatures. The phytohormone salicylic acid played a role in the regulation of AOS expression (Placido et al., 2019).

Challenges and future perspectives for metabolic improvement of NR yield

Although great strides have been made in our understanding of isoprenoid biosynthetic pathways, as well as of the genes and proteins involved in rubber biosynthesis, a comprehensive understanding of the molecular mechanisms of natural rubber biosynthesis remains elusive. At present, the simplest explanation given for NR biosynthesis is that specialized CPTs, perhaps evolved from dolichol synthase and/or undecaprenol-PP synthase (Cunillera et al., 2000), lost their specific oligomeric chain termination motifs, allowing them to catalyse the condensation of more than 10 000 IPPs (Kwon et al., 2016). Complementation and heterologous expression studies in bacteria, yeast and plants have not led to production of long-chain rubber molecules and have had limited enhancement of isoprenoid end product levels (Cornish and Xie, 2012; Epping et al., 2015; Kwon et al., 2016; Püttet et al., 2017; Qu et al., 2015; Schmidt et al., 2010b; Stolze et al., 2017; Zhang et al., 2008). Employing cisgenic (host gene sequences only) or marker-free vectors in genetic transformation experiments may reduce adverse effects and improve the final products (Tester and Langridge, 2010). Similar approaches also may be useful for improving rubber yield and molecular weight.

It is clear that the RT-ase has multiple components, some of which are integral membrane components, and some of which are associated with the rubber particles by their strong noncovalent interaction with membrane proteins (Cornish et al., 2018; Yamashita et al., 2016). The complexity of rubber biosynthesis machinery, and its substrates and activator(s) have, thus far, prevented full reconstitution of the RT-ase complex and its RT-ase activity (van Beilen and Poirier, 2007a,b; Cornish et al., 2008). Stolze et al. (2017), 2007b; Zhang et al., 2008). Employing cisgenic (host gene sequences only) or marker-free vectors in genetic transformation experiments may reduce adverse effects and improve the final products (Tester and Langridge, 2010). Similar approaches also may be useful for improving rubber yield and molecular weight.

An in-depth analysis of metabolic flux between the cytosolic MVA pathway and plastidic MEP pathway, including compartmental cross-talk and feedback loops, would be very helpful in guiding choice of the most appropriate IPP-generating pathway to genetically manipulate to create transgenic plants with improved natural rubber content. The failure of overexpressed isoprenoid biosynthesis genes/proteins to increase end product yield may be due to negative feedback mechanisms or inadequate knowledge about additional, or downstream, rate-limiting enzymes (Liao et al., 2016). The advent of new genome editing technology, and other advanced metabolic, proteomic and genomic tools, should allow alternative approaches to investigate the roles of single or multiple rubber-related genes and potentially increase product yields (Gao et al., 2015; Iaffaldano et al., 2016; Piatek et al., 2015).

The current emergence of synthetic biology and its synergy with metabolic engineering offers tremendous opportunities to create tailor-made cell factories for efficient production of desired chemicals (Nielsen and Keasling, 2011; Paddon and Keasling, 2014). The available genomic, transcriptomic and proteomic data from rubber-producing plants (Lin et al., 2017; Luo et al., 2017; Tang et al., 2016; Wang et al., 2015) may inform effective strategies to increase rubber substrate pools and the amount of the catalyst (RT-ase) itself. However, if such studies include determination of the reaction kinetics of RT-ase, this would greatly assist interpretation of the results. Molecular advances, in conjunction with advances at the breeding and agronomic levels, appropriate harvesting techniques and extraction processes (Buranov and Elmuradov, 2010; Cornish, 1996; Ramirez-Cadavid et al., 2017), may increase NR yield to commercially viable levels in alternative rubber crops in the coming years.

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Conflict of interest

The authors have no conflict of interest to declare.

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Rubber biosynthesis and the RT-ase complex

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