Novel Ontogenies Produce *Taraxacum kok-saghyz* Rubber Particles

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Taraxacum kok-saghyz, a temperate, herbaceous crop, produces rubber of very similar quality to that of the tropically-restricted para rubber tree. Well-understood rubber particle ontogeny is fundamental to explaining natural and induced differences in rubber yield. A rubber particle origin within the secretory pathway of the endoplasmic reticulum-Golgi vesicular complex is visualized for the first time in a rubber-producing species. Unexpectedly, in mature plants, we discovered that the cytoplasmic site of rubber particle development bifurcated to also employ laticifer plastids. It will be essential to understand how the two developmentally distinct subsets of rubber particles differentially accumulate in response to external factors to maximize yield as this new industrial crop expands into the commercial sector. Our schema illustrating these processes may be broadly applicable among rubber-producing species.

One-sentence summary

Rubber particles originate in the endoplasmic reticulum-Golgi vesicular complex but develop in two distinct ways.

At least 2,500 plant species are known to make natural rubber (NR) (1), a polyisoprene that occurs in the form of latex. NR is a critical and essential raw material in the modern world currently produced by a single tropical tree species *Hevea brasiliensis* (*para* rubber tree) cultivated as clonal scions on seedling root stocks (2). To diversify the genetic base of NR production and provide rubber supply security, other species are now under development. One of these is *Taraxacum kok-saghyz* (TK, rubber root, rubber dandelion, Russian dandelion, Kazak(h) dandelion) an *Astereacean,* herbaceous plant native to Central Asia. TK roots develop pipe-like multi-nucleate vessels, called laticifers, which produce rubber particles. Histological studies of rubber particles in different species have found glycosylated proteins in the particle membranes suggesting that they originate in the rough endoplasmic reticulum (ER) (3). Particle ontogeny is not known. Therefore, we investigated rubber particle ontogeny in TK seedlings and young plants (6 days to 2 months old) as well as in more mature plants (from 4 to 12 months old) using transmission electron microscopy, with the goal of identifying the subcellular site of rubber particle origination, and possible involvement of vesicular trafficking pathways. We discovered and describe two distinct rubber particle ontogenetic pathways, one of which appears in 6-day-old seedlings and operates throughout root development; the second pathway appears only in mature plants. We present a schema of these processes (Fig.1) and then show the electron micrographs on which it is based to assist the reader's understanding of our interpretation of this complex process. This is the first time that rubber particle ontogeny has been anatomically detailed in a rubber-producing species.

Our micrographs indicate that TK rubber particles originate in the endoplasmic reticulum-Golgi vesicular complex. This observation is in line with known ER functioning as an entry port for protein (as well as complex carbohydrates and lipids) (4) before specific proteins can be trafficked to other cellular locations (5). The conventional secretory pathway between the ER and the Golgi has bidirectional movement (anterograde and retrograde) of membrane traffic, assisted by coat protein complexes (COP) to ensure fidelity and direction (4). The protein complex I (COPI) functions in the retrograde route from the Golgi, whereas protein complex II (COPII) operates in the anterograde pathway from the ER (4). In our work, we observed COPI- and COPII-like vesicles comparable to vesicles reported in Tobacco BY- 2 cells (6), *Scherffelia dubia* (7) and Arabidopsis (8). This anatomical identification is used throughout the paper to refer to the putative COPI and COPII TK vesicles. Thus, we observed COPII vesiculation events (Figs. 2a, 2b and Fig. 1, symbol 1) in which a group of vesicles en route to the *cis*-Golgi (Golgi entry point) eventually merged with the *cis*-Golgi (Figs. 2c and 2d). These vesicles were denser than vesicles budded off the medial-Golgi or the *trans*-Golgi network (TGN) (Fig. 2b). Our observations are consistent with the model of cisternal progression/maturation for Golgi traffic in eukaryotes, where cisternae are viewed as transient carriers for secretory cargoes exported from the ER (9). In this model, the ER export site (ERES) is characterized by local accumulation of COPII proteins (heterodimers of Sec23/24 and heterotetramers of Sec31/13) (10) that later form the vesicles needed to export secretory proteins from the ER. ERESs are associated differently with the Golgi in mammals and plants (10). In mammals, vesicles produced by the ERES are pleomorphic and become the ER-

Golgi intermediate compartment (ERGIC). Together with the microtubules, mammalian ERGIC will tether COPII vesicles to *cis*-Golgi compartments (4). There was debate as to whether COPII vesicles existed in higher plants, as such anterograde events are rarely observed (4). However, in plants, COPII vesicles were proposed to fuse directly to *cis*-Golgi compartments after passing through a cytoplasmic matrix between the closely associated ERES and Golgi (4). In TK, the COPII vesicles fuse to form an IC-like compartment and undergo fusion while being trafficked from the ERES. This report is the first visualization of COPII vesiculation in plants.

Two types of COPI, COPIa and COPIb, have previously been differentiated in plants (11). COPIa was found to exclusively bud from *cis*-Golgi cisternae. COPIa was lightly stained, confined to the ER-*cis*-Golgi interface, and co-localized with COPII vesicles. In contrast, COPIb mostly budded from *medial*-, *trans*- and early TGN cisternae, and exhibited darkly stained luminal content similar to the Golgi cisternae from which it originated (11). We did not detect COPIa budding (Fig. 1, symbol "?") but were able to observe COPIb budding from the *medial* and *trans*-Golgi (Figs. 3a, 3b (arrows) and Fig. 1, symbol 5) in mature plants. COPIb was found near the large electron dense rubber particles in the cytosol, with many small electron dense rubber particles visible in the surrounding area. The nearby COPIb vesicles contained small rubber particles. This led to our interpretation that COPIb vesicles directly contribute to rubber particle formation.

We discovered two pathways of rubber particle ontogeny in TK roots, which are described in detail below, one of which occurs throughout root development and the other of which is only present in mature roots. The common pathway forms rubber particles in the cytosol whereas mature roots also form particles in vesicular plastids. These plastids are very similar to those seen in the laticifers of opium poppies, *Papaver somniferum* (12) and *Papaver bracteatum* (13), which accumulate lipoprotein particles instead of rubber particles. In both TK pathways, mature rubber particles are translocated into the vacuoles. Vacuoles are a common storage compartment for mature rubber particles in other species (14). The two disparate locations of *Taraxacum kok-saghyz* rubber particle ontogeny and development suggest that different vesicular sorting pathways are involved and that the production of plastidic and cytoplasmic rubber particles is determined by location-specific secretory vesicles.

The cytoplasmic pathway forms rubber particles in the cytosol and first appears in 6-day-old seedlings and, based on our observations, is the only ontogenetic pathway apparent in 6-day to 2-month-old plants (young plants). The formation of cytoplasmic rubber particles begins either with the formation of COPIb secretory vesicles by the *medial* and *trans*-Golgi (Figs. 3a, 3b and Fig. 1, symbol 5.) which modify the small rubber particles, or small rubber particles directly originate from the ER cisternae (Figs. 3c, 3d, and Fig. 1, symbol 10). The synthesis of rubber particles from the ER was suggested previously in *Lactuca sativa* (15). The free small particles accumulate near the tonoplast (fig. S2a). In young plants, these small particles either move directly into the vacuole (fig. S4a and Fig. 1, symbol 2), or merge into larger particles in the cytosol before translocation into the vacuole (fig. S4b and Fig. 1, symbol 3). In mature roots, the small particles are produced exactly as in younger roots but all merge into larger particles in the cytosol adjacent

to the tonoplast (fig. S2a and Fig. 1, symbol 10) but do not simply traverse the membrane. Frequently, a dense cytoplasm provides a cup shaped area at the border of the cytoplasm and tonoplast (fig. S4e) surrounded by the ER (fig. S4f). These cup shaped areas appear to be a nurturing habitat for the newly formed rubber particles allowing the particles to increase in size while maintaining a spherical shape. As the rubber particles become mature and are ready to be translocated into the vacuole, the adjacent tonoplast membrane cleaves (figs. S2a and S2d). The rubber particles free themselves from the cytosol and are pinched out or extruded into the vacuole (figs. S2d, S2g and S4e). The tonoplast membrane then reseals (fig. S2j). In both younger and mature roots, where particles are not formed in a cytoplasmic cup, many translocated particles remain tethered to the tonoplast. The tethered particles attract small particles as aggregates in the roots of young plants (fig. S4d and Fig.1, symbol 4), but which form large irregular particles in mature roots (figs. S4g, S4h and Fig. 1, symbol 12).

The second distinct pathway of rubber particle ontogeny occurs only in mature roots (4-12-month-old plants). In this pathway, vesicles from the *medial-*, *trans-*, TGN (Fig. 2b and Fig.1, symbol 6) and the ER cisternae (Fig. 4c and Fig. 1, symbol 8) migrate to the laticifer plastids and release small rubber particles into the plastid. Our micrographs capture TGN producing secretory vesicles which appear to migrate to the ER (figs. S1a, S1b and Fig. 1, symbol 7) and to laticifer plastids (Fig. S1a). This movement of the vesicles from the *medial-* and *trans-* area to the ER is retrograde (like COPIa to ER) and may then transition via the ER to the laticifer plastids. If this interpretation of our electron micrographs is correct, this is a novel observation. The ER cisternae also appear to be directly connected to the laticifer plastids (Figs. 4b, 4c (black arrow) and Fig. 1, symbol 11). Small rubber particles can be translocated directly into the plastid (fig. S1d and Fig. 1, symbol 9) as well as into vacuoles by the ER cisternae.

Once in the plastid, the juvenile particles form layers (Fig. 3e) which merge to form irregular larger particles (Figs. 2a, 4c and figs. S1d). Small particles and irregularly shaped larger particles co-exist (fig. S3g). Mature plastidic rubber particles have a spherical shape (Fig. 3f) and each laticifer plastid produces a single mature particle. Similar electron-dense particles in *T. kok-saghyz* plastids have been observed previously but were interpreted as protein crystals not rubber particles (16). When the latificer rubber particle has matured, the laticifer plastid migrates to the tonoplast (fig. S3m) where the laticifer plastid membrane opens up to release the rubber particle (fig. S3n) while the tonoplast membrane cleaves allowing entry of the rubber particle into the vacuole (fig. S3q). The release of plastidic rubber particles into the vacuole is similar to release of a rubber particle from the cytoplasmic cup described earlier. After release, the tonoplast reseals (figs. S3r and S3s) and the empty plastid dissipates into the cytoplasm (fig. S3q, arrow).

The vacuole serves as the storage compartment for mature rubber particles. Rubber biosynthesis, which is catalyzed by a rubber polymerizing complex embedded in the membrane of the particle (17), cannot occur inside the vacuole due to the presence of acid phosphatases, which would dephosphorylate the active end of elongating rubber chains and any rubber substrates that

might cross the tonoplast. We have found three homologs in *T. kok-saghyz* of the *Lycopersicon esculentum* [AAG40473] and *Arabidopsis thaliana* putative phosphatases [NP 173213, AAM63155] and 10 acid phosphatase genes are known in *T. officinale* (18).

Rubber biosynthetic species occur in four of the six super-orders of dicotyledonous plants (19) suggesting that rubber biosynthesis evolved over 100 million years ago before the divergence of these superorders. Rubber biosynthesis has not been found in the monocotyledonae, Nonetheless, the general architecture of the subcellular compartment and the biochemical requirements for rubber biosynthesis have been conserved even in rubber-producing species without laticifers (17, 20). Thus, the elucidation of rubber particle ontogeny in *T. kok-saghyz* may prove to be broadly applicable among rubber-producing species. In addition, our model of rubber particle ontogeny (Fig. 1) will inform differences in rubber yield among genotypes, or as induced by changes in horticultural, agronomic or post-harvest storage practices. If, for example, cold induced rubber biosynthesis is only mediated by the plastidic rubber particle ontogenetic pathway, then experiments on juvenile plants will not be relevant to the crop system. Similarly, in a proposed 3D farming multiple harvest hydroponic production system, to maximize rubber yield it will be important to know if roots regrown every 8 weeks from mature plants are physiologically juvenile (one pathway) or mature (two pathways).

In conclusion, we have used electron microscopic analysis to investigate the origin of rubber particles in the laticifers of *T. kok-saghyz.* An origin within the secretory pathway of the endoplasmic reticulum-Golgi vesicular complex is visualized for the first time in a rubber-producing species. We discovered an unexpected bifurcation in the development of rubber particles between two subcellular compartments in mature plants not seen in younger ones. It will be essential to understand how these two developmental processes differentially respond to external factors in crop development efforts.

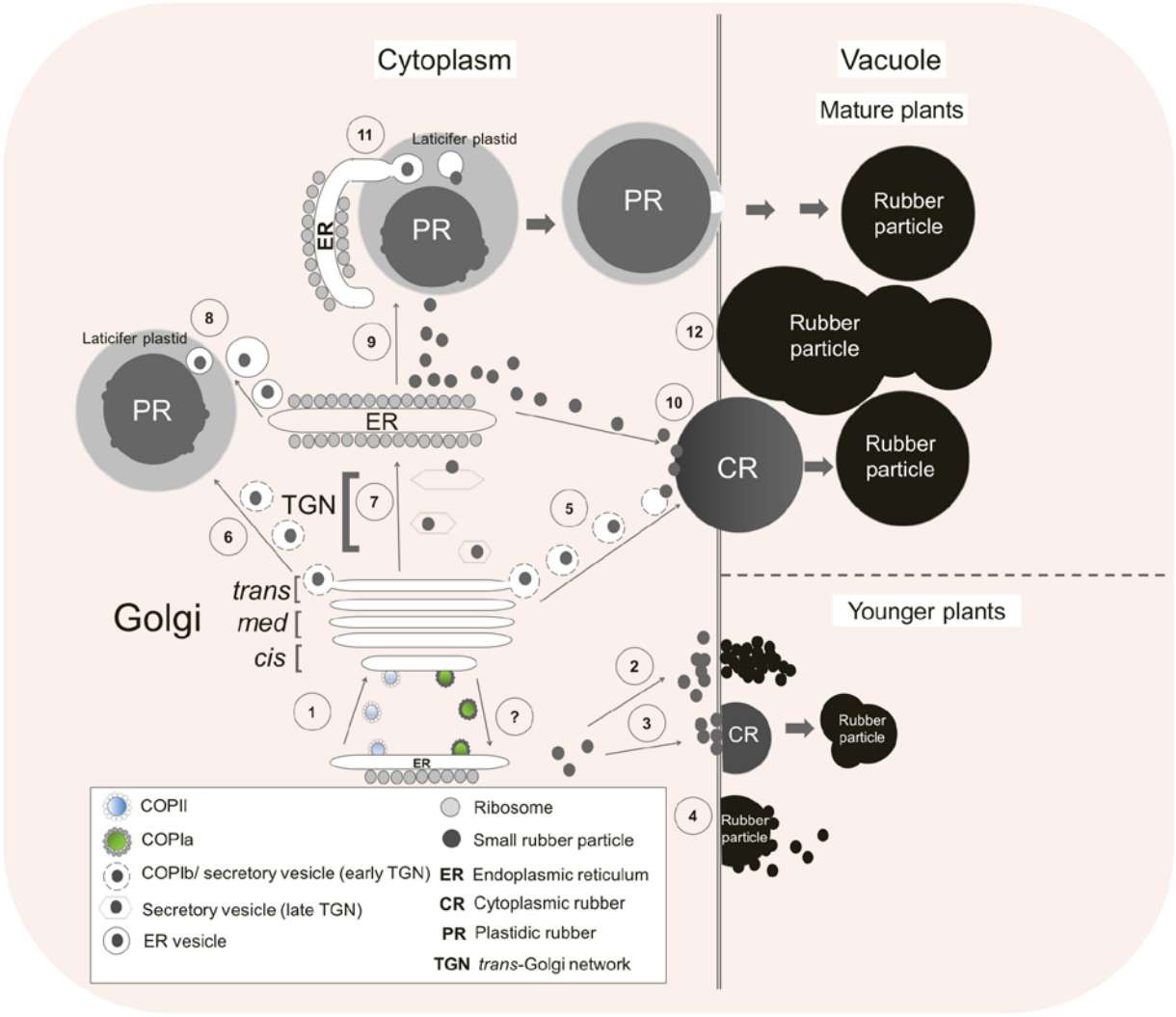


Fig. 1: *Model of rubber particle ontogeny indicating the formation of two of rubber particles, the plastidic and cytoplasmic rubber in TK*

1. The COPII route or the anterograde pathway.
?. The COPIa (the retrograde pathway from the *cis*-Golgi to the ER could not be observed)

In seedlings, small rubber particles either **2**. move directly into the vacuole or **3.** merge into larger particles in the cytosol before translocation into the vacuole.
4. The tethered rubber particles attract small particles as aggregates

In mature plants, the budding of COPIb and the production of secretory vesicles from *medial* and *trans*-Golgi area releases small rubber particle either to
5. directly form cytoplasmic rubber particles or **6**. laticifer plastids rubber
7. Secretory vesicles form in the late TGN and *medial* area of the Golgi and are transported back to the ER.

8. The ER cisternae will form vesicles to move the small rubber particles into the laticifer plastid to form plastidic rubber

The small rubber particles that leave the ER will either **9.** move into laticifer plastids to form plastidic rubber or **10.** directly form cytoplasmic rubber

11. The ER also can connects directly to the laticifer plastid and transfers the small rubber particles into the plastid to form plastidic rubber

12. The tethered rubber particles form large irregular particles

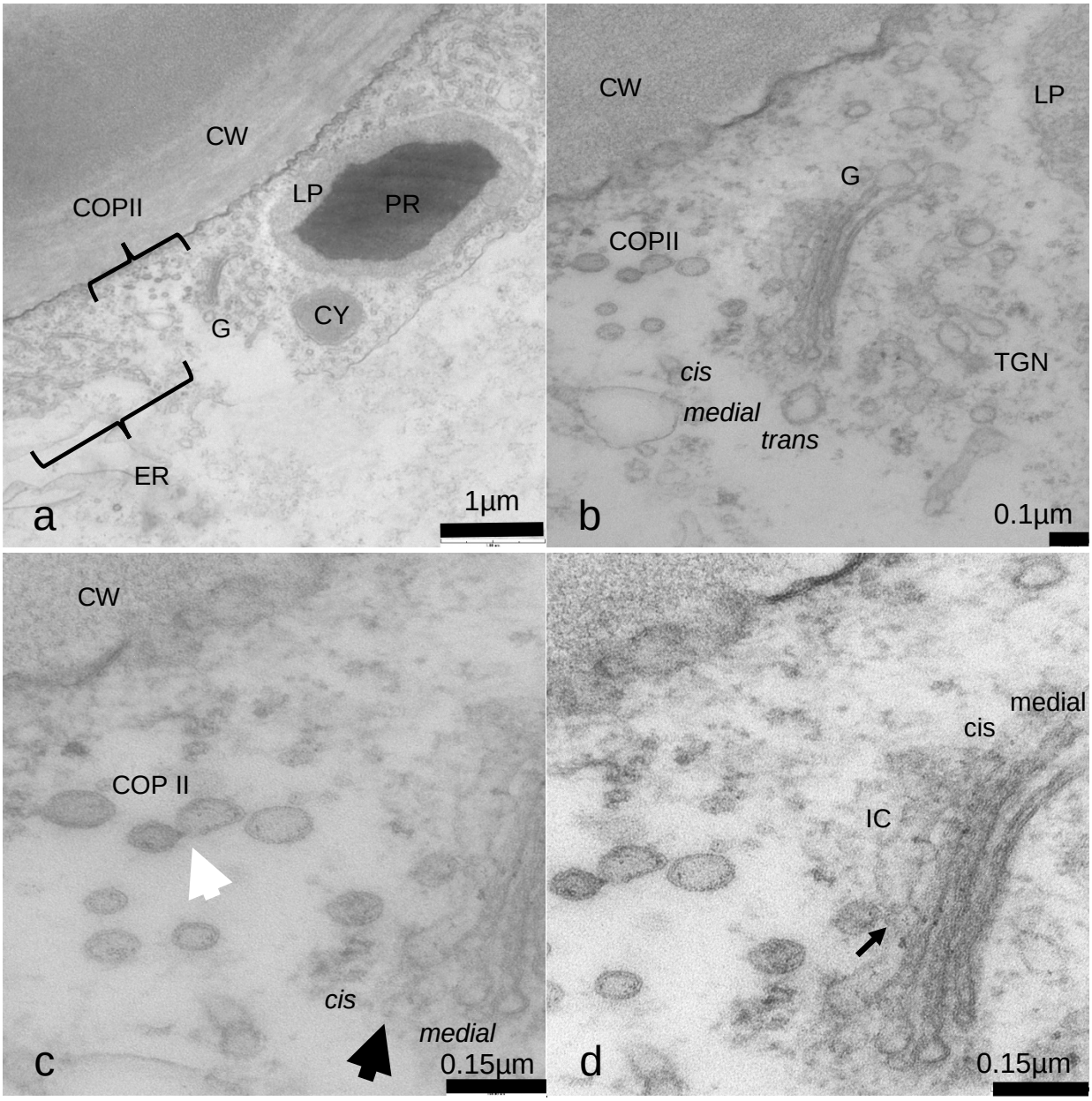
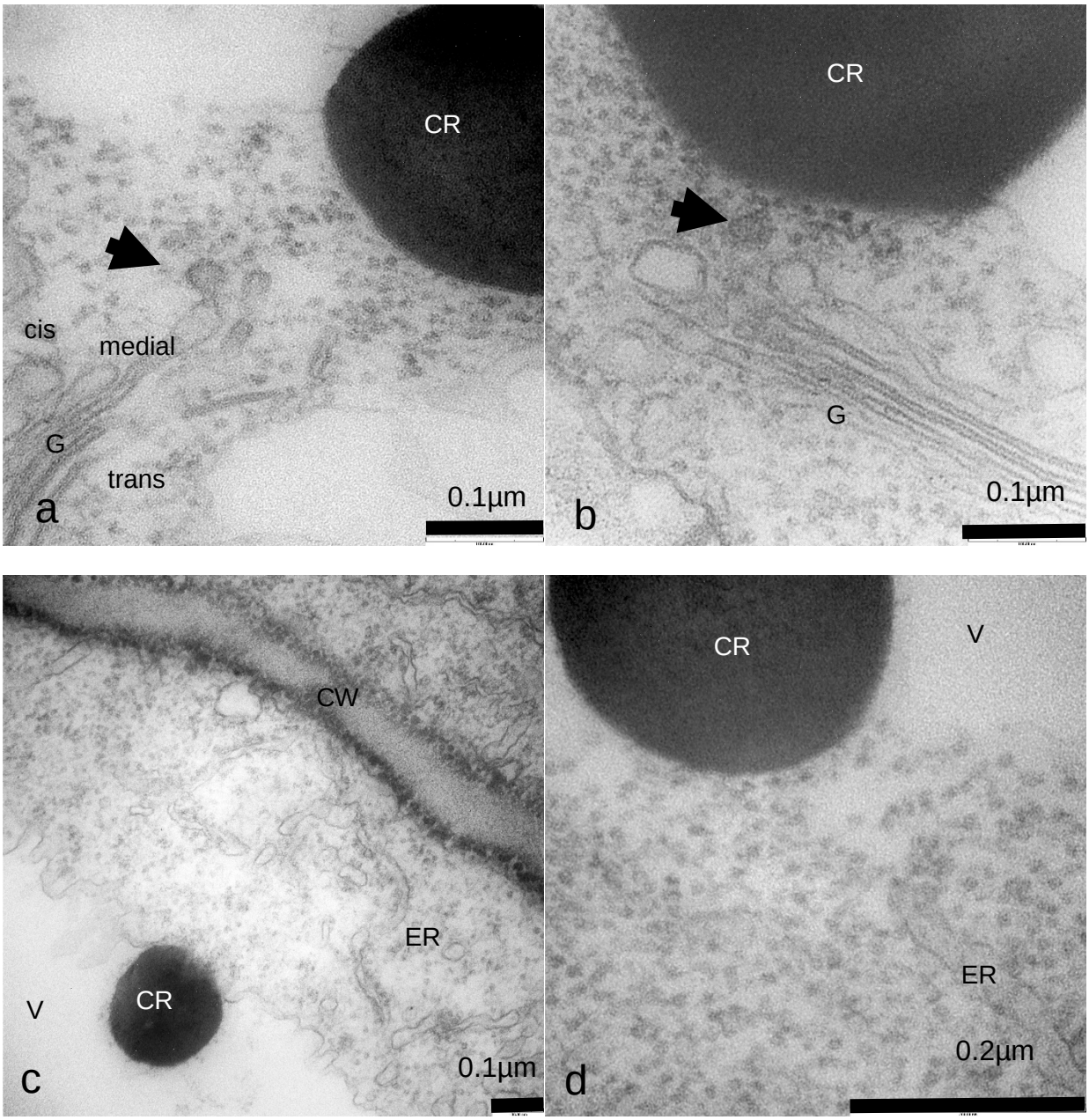


Fig. 2: Longitudinal section of mature root a. overall view of the laticifer cell which shows parts of ER group, COPII group, Golgi and to the laticifer plastid where the small rubber particles merge to become irregular rubber particle and in this case the plastidic rubber; b, c, d. enlargement of (a) which depict b. the COPII entering the cis part of the Golgi c. white arrow is showing the fusion of the vesicles and black arrow indicate the merging of COPII with cis-Golgi and d. arrow indicate the developed IC
Key: Cell wall (CW);crystal (CY); coat protein complex II (COPII); Golgi body (G); intermediate compartment (IC); laticifer plastid (LP);plastidic rubber (PR); endoplasmic reticulum (ER); trans-Golgi network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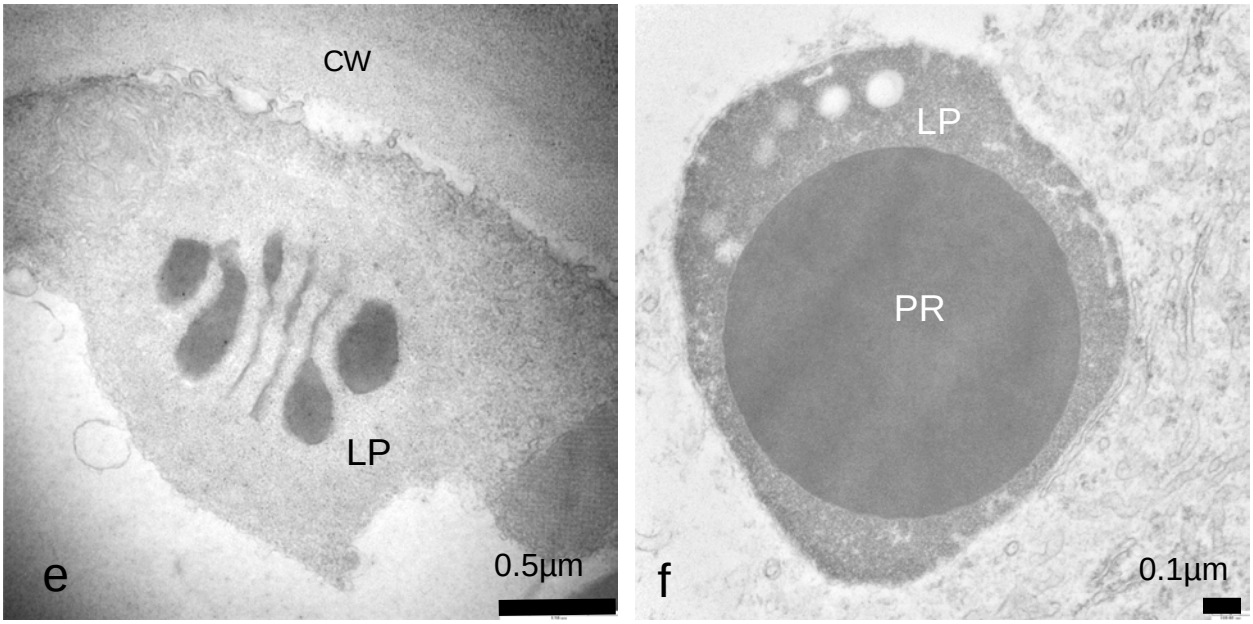

Fig. 3: Cytoplasmic (a, b, c, d) and plastidic (e, f) rubber particles developed from the small rubber particles. a,b. Arrows indicate COPIb captured nearby cytoplasmic rubber c,d. small rubber particles produced from the ER cisternae and vesicle to form cytoplasmic rubber e. longitudinal sectioned of laticifer plastid showing layers formed by the small rubber particles f. longitudinal sectioned of fully developed plastidic rubber in the laticifer plastid
Key: Cell wall (CW); cytoplasmic rubber (CR);endoplasmic reticulum (ER); Golgi body (G); laticifer plastid (LP);plastidic rubber (PR);vacuole (V)

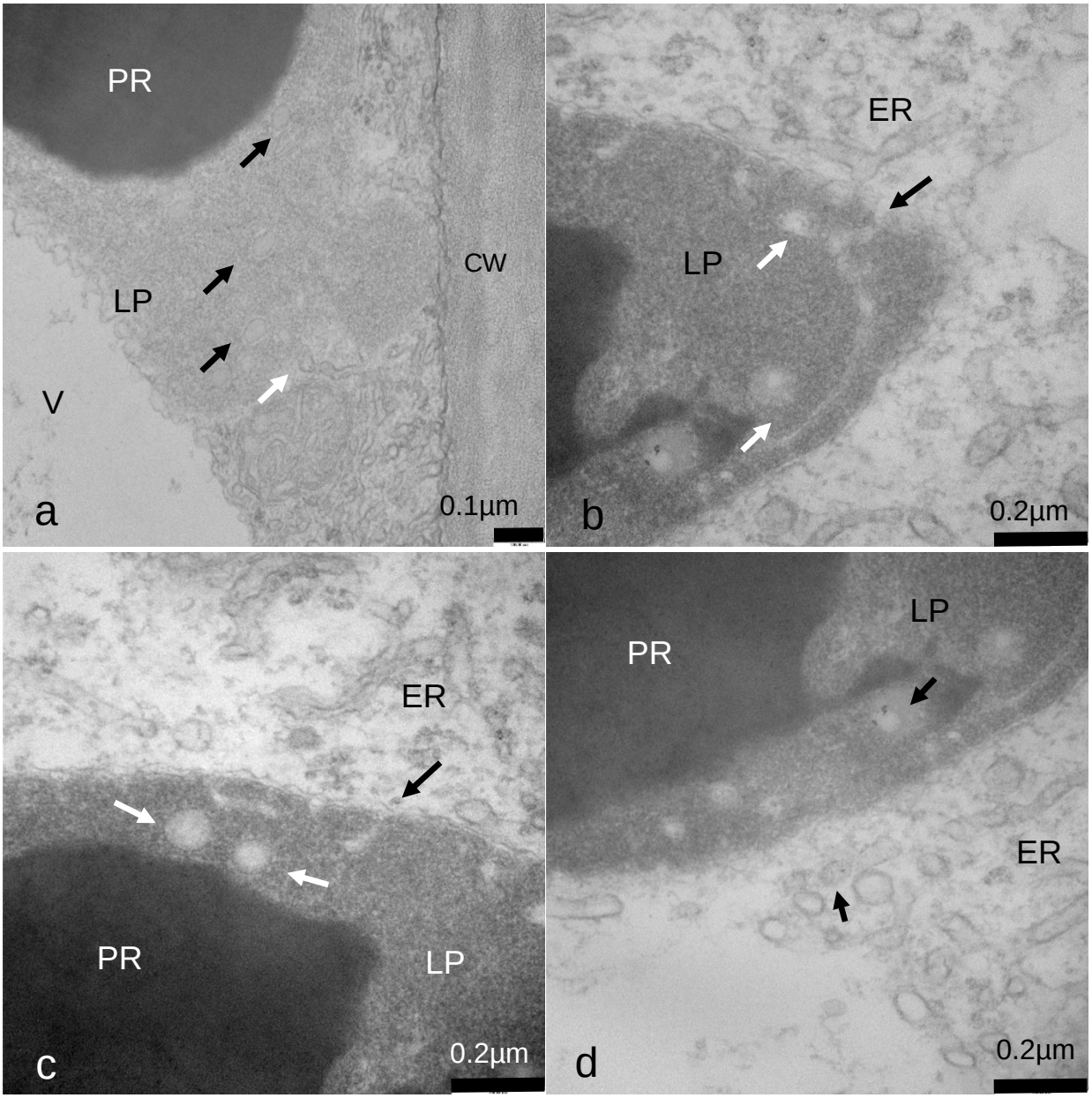

Fig. 4: Longitudinal sectioned of LP with ER and ER secretory vesicles connected to the plastid a. ER (white arrow) connection with the LP and the vesicles (black arrows) can be seen crossing the plastid b. invagination of ER (black arrow) that connect the outside ER with LP as ER vesicles being developed (white arrows) within the LP c. similar observation as in b d. arrows indicate vesicles containing small rubber particles that can be found in both inside and outside (cytosol) of the laticifer plastid suggesting that the vesicle can also be transverse into the laticfer plastid
Key: Cell wall (CW); endoplasmic reticulum (ER); laticifer plastid (LP); plastidic rubber (PR); vacuole (V)

References and notes

1. J. Bonner, “The History of Rubber” in Guayule Natural Rubber: A Technical Publication with Emphasis on Recent Findings (The University of Arizona, Tucson, 1991), pp.1-16

2. K. Cornish, Alternative natural rubber crops: why should we care? Technology and Innovation 18, 245-256 (2017)

3. K. Cornish, Similarities and differences in rubber biochemistry among plant species. Phytochemistry 57, 1123-1134 (2001)

4. F. Brandizzi, C. Barlowe, Organization of the ER–Golgi interface for membrane traffic control. Nature 14, 382-392 (2013)

5. A. Vitale, A. Ceriotti, J. Denecke, The role of the endoplasmic reticulum in protein synthesis, modification and intracellular transport. J. of Exp. Bot. 44, 1417-1444 (1993)

6. C. Ritzenthaler et al, A. Nebenführ, A. Movafeghi, C. Stussi-Garaud, L. Behnia, P. Pimpl, L.A. Staehelin and D.G. Robinson, Reevaluation of the effects of Brefeldin A on plant cells using tobacco bright yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. Plant Cell 14, 237-261 (2002)

7. B.S. Donohoe, B. Kang, M.J. Gerl, Z.R. Gergely, C.M. McMichael, S.Y. Bednarek, L.A.Staehelin, *Cis*-golgi cisternal assembly and biosynthetic activation occur sequentially in plants and algae. Traffic 14, 551-567 (2013)

8. M. Langhans, T. Meckel, A. Kress, A. Lerich, D.G. Robinson, ERES (ER exit sites) and the “Secretory Unit Concept”. J of Microsc. 247, 48-59

9. B.S. Glick, A. Luini, Models for Golgi traffic: a critical Assessment. Cold Spring Harb. Perspect Biol, 3, 1-15 (2011)

10. A. Lerich, S. Hillmer, M. Langhans, D. Scheuring, P. van Bentum, D.G. Robinson, ER import sites and their relationship to ER exit sites: a new model for bidirectional ER-Golgi transport in higher plants. Plant Cell Biol. 3, 1-21 (2012)

11. L. A. Staehelin, B. Kang, Nanoscale architecture of endoplasmic reticulum export sites and of Golgi membranes as determined by electron tomograph. Plant Physiol 147, 1454-1468 (2008)

12. C. L. Nessler, P.G. Mahlberg, Plastids in laticifers of Papaver. I. Development and cytochemistry of laticifer plastids in *P. somniferum* L. (Papaveraceae). Amer. J. Bot. 65, 266-273 (1979)

13. C. L. Nessler, P.G. Mahlberg, Plastids in laticifers of Papaver. II. Enzyme cytochemistry of membrane-bound inclusions of laticifer plastids in *P. bracteatum* Lindl. (Papaveraceae).Amer. J. Bot. 66, 274-279 (1979)

14. R. Goss, "The Morphology, Anatomy, and Ultrastructure of Guayule in Guayule Natural Rubber: A Technical Publication with Emphasis on Recent Findings (The University of Arizona, Tucson, 1991), 33-45

15. Y. Qu, R. Chakrabarty, H.T. Tran, E.G. Kwon, M. Kwon, T. Nguyen, D. Ro, A lettuce (*Lactuca sativa*) homolog of human Nogo-B receptor interacts with *cis*-Prenyltransferase and is necessary for natural rubber biosynthesis. J of Bio. Chem. 290, 1898-1914 (2015)

16. G. Heinrich, Light and electron microscopic investigations on the lactic ducts of *Taraxacum bicorne* D. Flora 158, 413-420 (1967)

17. K Cornish, Biochemistry of natural rubber, a vital raw material, emphasizing biosynthetic rate, molecular weight and compartmentalization, in evolutionarily divergent plant species. Nat. Prod. Rep. 18, 182–189 (2001)

18. A.S. Kashin, V.E. Anfalov, Y.A. Demochko, Studying allozyme variation in sexual and apomictic *Taraxacum* and *Pilosella* (Asteraceae) populations. Russ J of Genet 41,144–154 (2005)

19. V.H. Heywood, "Flowering Plants" in Flowering Plants in Flowering Plants of the World (Oxford University Press, 1978), pp. 7-13

20. K Cornish, D.J. Siler, O. Grosjean, N. Goodman, Fundamental similarities in rubber particle architecture and function in three evolutionarily divergent plant species. J. Nat. Rubber Research 8: 275-285 (1993)

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Supplementary materials

Materials and Methods

Plant materials

The TK seeds were germinated inside a greenhouse at OARDC, Wooster OH on trays using PRO-MIX® BX. The seeds were sown inside a planting hole with 6mm wide and 15 mm depth made on each cells in the germination trays and left uncovered for easy observation when the seeds germinates. The seedlings were harvested at the age of 6, 8, 10 and 12 days based on its first day of germination. Five plants were collected on each day. Meanwhile, mature plant was obtained bi-monthly for a year coming from raised boxes with the density of 400,000 plant/acres.

Tissue preparation, staining for TEM and observation

Roots samples were taken at cotyledonary collars to the hypocotyl area for the seedling (at the age of 6, 8, 10 and 12 days) and roots coming from 2 months and above were sampled in the 2cm area below the leaf crown. In both plant group (young and mature), the samples were taken only at the primary or main roots and not at the lateral roots. Tissue samples were fixed in 3% glutaraldehyde, 2% paraformaldehyde and in 0.1 M potassium phosphate buffer (PB). After three rinses with PB, samples were post-fixed in 1% osmium tetroxide and 1% uranyl acetate, and subsequently dehydrated in an ethanol series, infiltrated and embedded in EM Bed-812 resin. 70 nm sections were collected using the LEICA EM-UC6 Ultra Microtome, stained with 3% aqueous uranyl acetate and Reynold's lead citrate stains. Sections were observed on a Hitachi h-7500 transmission electron microscope and images collected with an Optronics QuantiFire-Model S99835 digital camera.

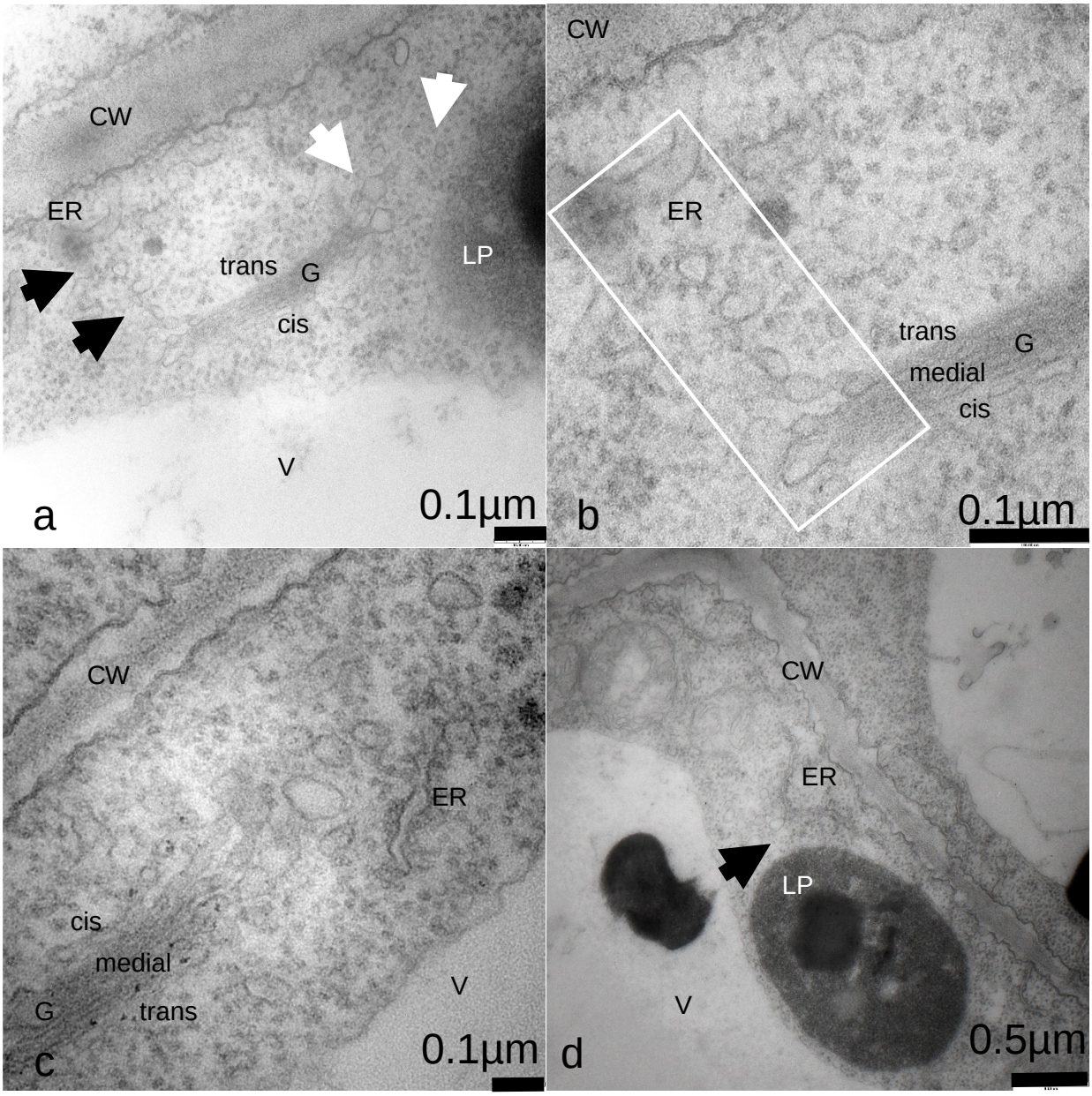


Fig. S1: a. Golgi produced different vesicles for ER (black arrows) and laticifer plastid (white arrows) b, enlargement of a shows vesicles from medial and trans area of the Golgi were directed to the ER c, another example of vesicles were produced from medial-, trans- and early TGN were directed to the ER d, arrow indicate small rubber particle can be translocated into the laticifer plastid

Key: Cell wall (CW); endoplasmic reticulum (ER); Golgi body (G); laticifer plastid (LP); vacuole (V)

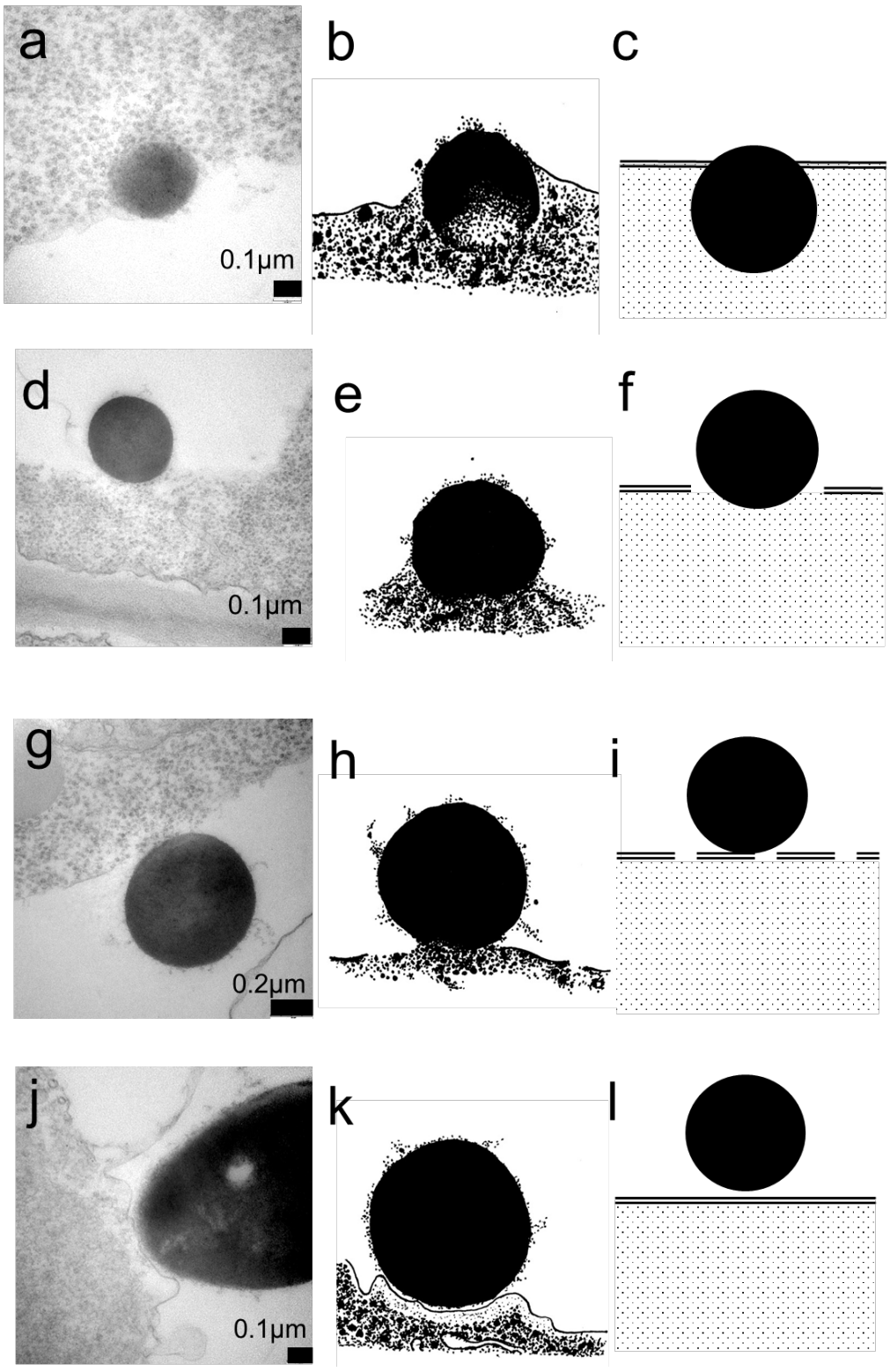


Fig. S2. The formation of cytoplasmic rubber (CR); a,d,g,j, are micrographs images; b,e,h,k, are illustration depicting the stages; and c,f,i,l, the stages by graphic representation a,b,c. small rubber particles accumulate near the tonoplast d,e,f. spherical shape CR traverse into the vacuole

g,h,i. the tonoplast membrane cleaves j,k,l. tonoplast membrane reforms after CR is inside the vacuole

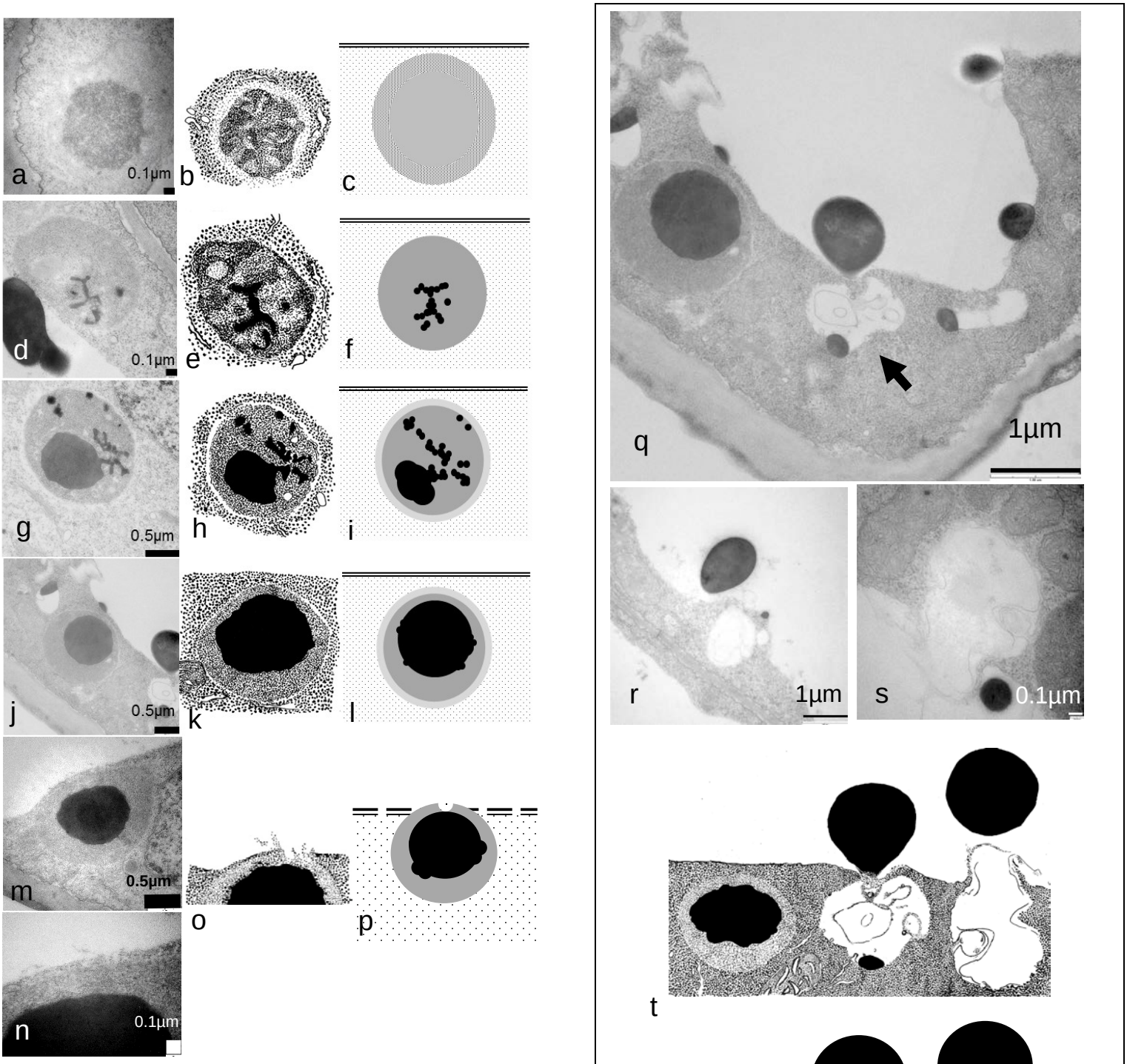
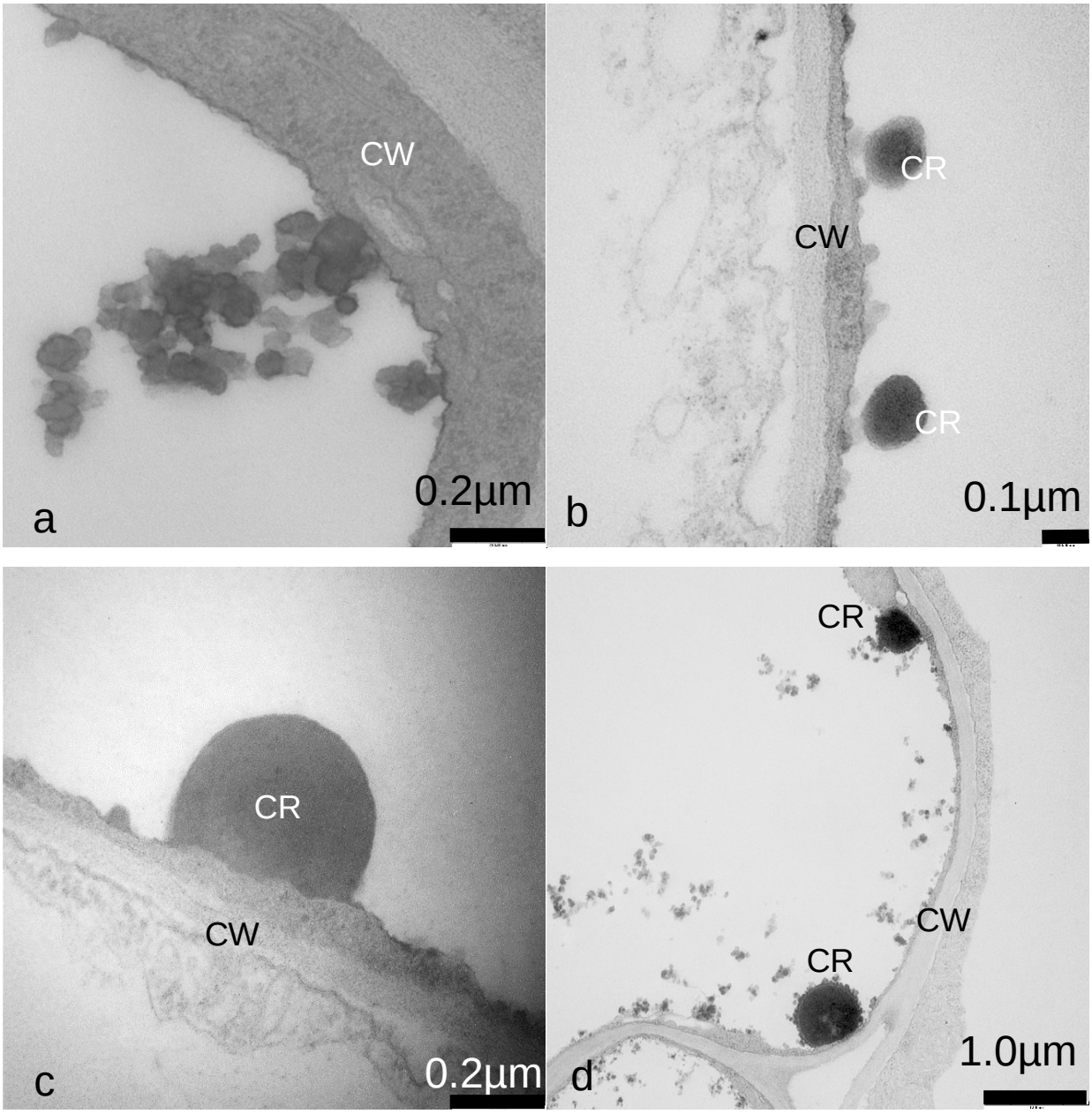


Fig. S3: The formation of plastidic rubber (PR); a,d,g,j,m,n,q,r,s are micrographs images; b,e,h,k,o,t are illustration depicting the stages; and c,f,i,l,p,u the stages by graphic representation. a,b,c. early stage of LP d,e,f. accumulation of small rubber particles in LP g,h.i. development of rubber particle body j,k,l. irregular shape PR m,n,o,p. release of PR q,r,s,t,u. empty plastid will dissipates into the cytoplasm after the release of PR into the vacuole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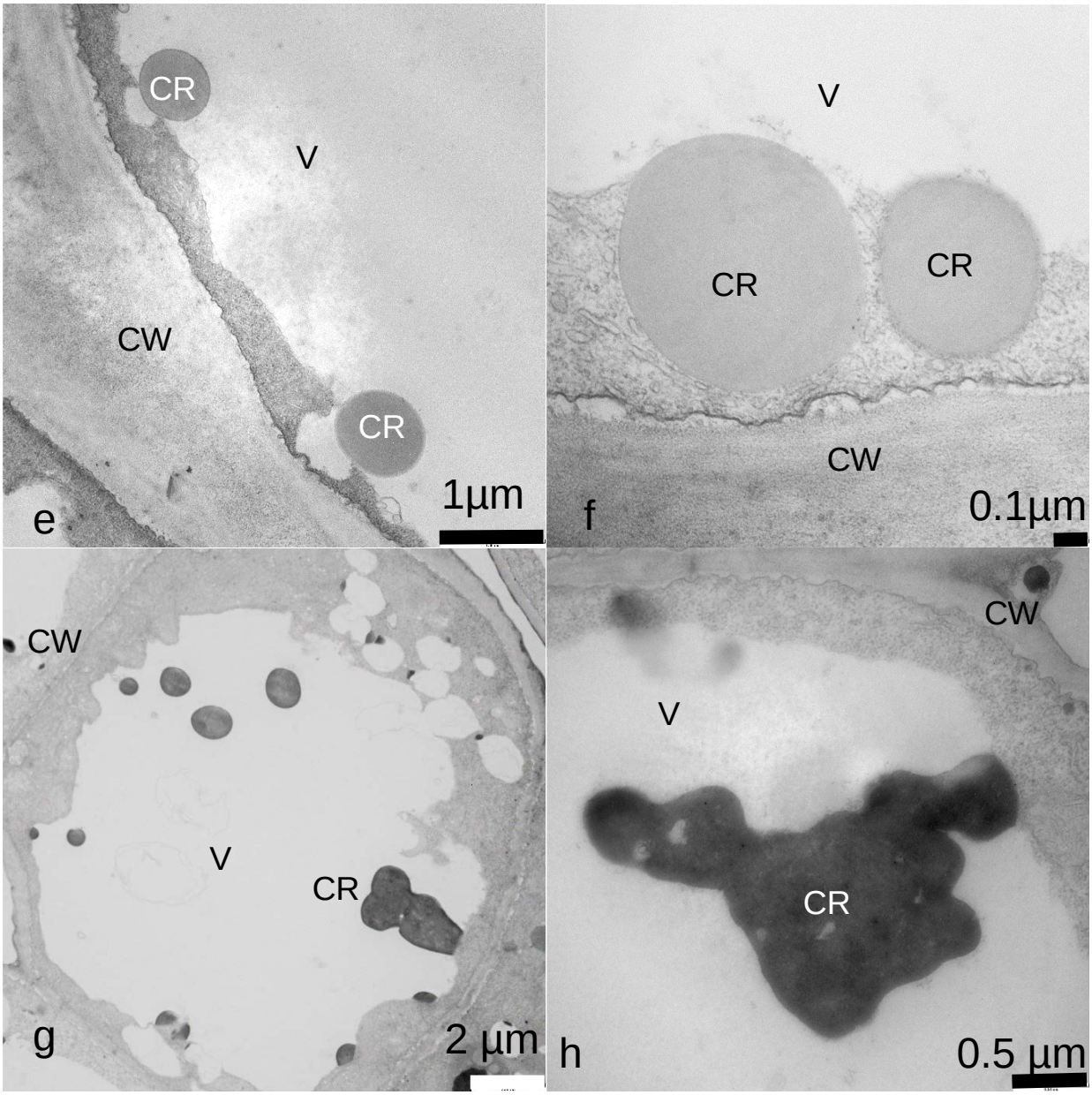

Fig. S4:a,b,c,d. the translocation of CR in the seedlings with a, direct translocation of small rubber particles into the vacuole b, an irregular rubber particles formed by small rubber particles translocated in the vacuole c, CR tethered to the tonoplast d, CR attract small particles as aggregates e,f,g,h, the translocation of CR in mature plant with e, longitudinal sectioned of cup shaped form at the border of the cytoplasm and tonoplast as well as the extrusion process of CR f, the cups surrounded with ER g,h. tethered CR form large irregular roots
Key: Cell wall (CW); cytoplasmic rubber (CR)