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Chloroplast-associated molecular patterns as concept for fine-tuned operational retrograde signalling

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Chloroplasts compose about one-quarter of the mesophyll cell volume and contain about 60% of the cell protein. Photosynthetic carbon assimilation is the dominating metabolism in illuminated leaves. To optimize the resource expenditure in these costly organelles and to control and adjust chloroplast metabolism, an intensive transfer of information between nucleus–cytoplasm and chloroplasts occurs in both directions as anterograde and retrograde signalling. Recent research identified multiple retrograde pathways that use metabolite transfer and include reaction products of lipids and carotenoids with reactive oxygen species (ROS). Other pathways use metabolites of carbon, sulfur and nitrogen metabolism, low molecular weight antioxidants and hormone precursors to carry information between the cell compartments. This review focuses on redox- and ROS-related retrograde signalling pathways. In analogy to the microbe-associated molecular pattern, we propose the term 'chloroplast-associated molecular pattern' which connects chloroplast performance to extrachloroplast processes such as nuclear gene transcription, posttranscriptional processing, including translation, and RNA and protein fate.

This article is part of the theme issue 'Retrograde signalling from endosymbiotic organelles'.

1. Features of retrograde signalling

Regulatory feedback loops play central roles in biological processes. They are also required to coordinate processes among organelles and cell compartments. The need for communication between the photosynthesizing chloroplast and the extrachloroplast compartments, in particular cytosol, nucleus and mitochondrion, is easily explained by two fundamental requirements: chloroplast metabolism must cover the demand for specific metabolites and energy in development, defence and storage, and unfavourable metabolic states should be avoided. Such states of disequilibria generally are linked to the release of reactive O-, C-, N- and S-species that then may damage essential cell constituents such as nucleic acids and proteins [1].

Considering the richness of chloroplast metabolism, it appears little surprising that recent discoveries have uncovered a high diversity, flexibility and dynamics of signals emanating from chloroplasts. Consequently, the number of pathways and mechanisms of retrograde control of extrachloroplast processes have been expanded over the last 15 years to not only include export of chemical entities, but also surprisingly import for differential processing like signalling via 3′-phosphoadenosine 5′-phosphate (PAP) or release of factors from the envelope like the plant homeodomain transcription factor (PTM), as will be discussed below (figure 1).

Given the increasing complexity, chloroplast retrograde signalling may generally be defined as any mechanism that uses information from chloroplasts to

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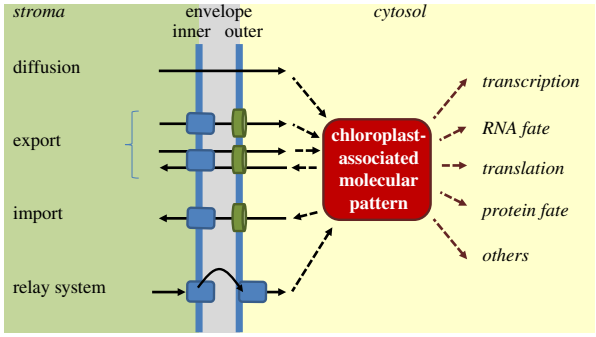

Figure 1. Principal mechanisms of retrograde signal transmission acting on the chloroplast-associated molecular pattern (ChAMP). This scheme shows four possible pathways of retrograde signaling, namely (i) diffusion through the membrane boundary, (ii) export by specific carriers (uni-, sym-, antiporters) through the inner envelope and through porins through the outer envelope membrane (cf. [2]), (iii) specific import as will be discussed for 3′phosphoadenosine 5′-phosphate below, and (iv) relay-type systems, where the signal from the chloroplast does not leave the chloroplast but rather is converted or transmitted at the envelope to another entity, as has been described for the release of the plant homeodomain transcription factor PTM from the outer envelope in response to chloroplast signals [3]. All these chloroplast-dependent retrograde processes define the ChAMP, which in turn determines the downstream adjustment of, e.g., transcription, RNA fate, translation and protein fate. (Online version in colour.)

address extrachloroplast processes. Many signalling pathways involve redox regulatory steps or reactive oxygen species (ROS). This review focuses on such signals that directly or indirectly depend on redox or ROS cues.

2. The central role of chloroplasts in photosynthesizing tissue

Plastids contain more than 3000 nucleus-encoded proteins [4]. The chloroplast includes about 60% of the mesophyll protein and receives about 85% of the de novo synthesized protein in barley primary leaves 10 days post-germination [5]. This figure likely changes with developmental stage of the leaves. Chloroplasts dominate many metabolic pathways of the mesophyll cell, such as carbon-, nitrogen- and sulfur-assimilation, and are involved in most other synthetic pathways.

Photosynthetic carbon assimilation runs with an average rate of 100 µmol CO_2 fixed per mg chlorophyll per hour at ambient CO_2 concentration and saturating photon flux density [6]. This provides a rather precise number for the rate of export of sugar phosphates, particularly of triosephosphates or hexoses, from the chloroplast. As previously estimated, the entirety of the chloroplasts of a mesophyll cell exposes a surface area similar to that of the plasma membrane in *Arabidopsis* [7]. For this estimation, the shape of a chloroplast was taken as an ellipsoid with the dimensions of $8 \times 4 \times 4$ µm^3, which has a surface area of 86 µm^2 per chloroplast. The chloroplast number per mg chlorophyll varies between 1×10^9 and 2×10^9 [8,9]. With the assumption of 10^9 chloroplasts per mg chlorophyll and export of triosephosphates, 33 µmol dihydroxyacetone phosphate (DHAP, which is a triosephosphate) are exported across 860 cm^2 envelope per hour. This rate defines the main transport pathway (bulk transport). All other transport processes will be far below this rate. This implies that carbohydrate metabolism likely dominates the redox state of the cell in the light, though with the constraints of ATP/NADPH-stoichiometry of the consuming reactions. By converting exported DHAP to 3-phosphoglycerate in the cytosol, equivalent amounts of ATP and NAD(P)H are formed. Thus DHAP export provides reduction energy to the cytosol. There are other bulk transport mechanisms at the interface between plastids and cytosol (figure 2).

The second dominant pathway concerns nitrogen reduction. The weight-based C : N ratio of leaves in four crop species ranges between 9.7 in alfalfa, 27.8 in soybean, 30.6 in corn and 35.3 in switchgrass. The corresponding figures for roots are 18.0 (alfalfa), 62.4 (soybean), 37.7 (corn) and 65.6 (switchgrass) [11]. This gives estimates of electron flow into nitrite reduction if exclusively occurring in the leaves. The third important reduction pathway is sulfate reduction. On average, the reduction power directed into C : N : S-assimilation may be estimated to be 40 : 8 : 1 but the variable element composition suggests considerable variation. The estimate also does not consider the contribution of nodules or mycorrhiza to nitrogen supply. In addition, about half of the photosynthetically gained carbon is lost by respiration of shoots and roots in *Ricinus communis* [12], and protein synthesis consumes more than one-quarter of the cell ATP in specialized cells [13]. Thus, in addition to these bulk pathways, many other mechanisms of retrograde signalling coordinate metabolism with development and environmental acclimation.

Plastids take part in many metabolic pathways downstream of the primary C-, N- and S-assimilation, e.g. synthesis of amino acids, hormone precursors, nucleotides, fatty acids, tetrapyrroles, isoprenoids, polyphenols and lignins, and also formation of Fe–S-clusters [14]. The vast metabolism of plastids is not the topic of this review; however, it must be kept in mind since the metabolic state likely affects retrograde processes. A decisive step in retrograde signalling is the information transfer across the boundary membranes of the plastids. Therefore, we will describe transport of some bulk, special and distinctive signalling metabolites in this article.

3. Metabolite transporters with function in cell redox homeostasis and retrograde signalling

As discussed in the previous section, retrograde signalling from plastids requires mechanisms to traverse the envelope. Apart from relay-type systems (figure 1), realized, e.g. by the proteolytic cleavage and activation of the PTM from the outer face of the outer envelope by retrograde signalling [3], retrograde control deploys metabolite transfer. This section presents some selected knowledge on transporters which for the time being can be categorized in those participating in bulk transport of assimilates like triosephosphates (figure 2). Many of them are well characterized and in fact the triosephosphate/phosphate translocator (TPT) was among the first transporter proteins cloned from plants [15]. Another category of transporters catalyse the transfer of more specialized metabolites in smaller quantities, like *O*-acetylserine (OAS), PAP or glutathione (GSH). The nature of members of the third category of transporters, carrying distinctive signalling metabolites, often still awaits clarification.

(a) Triosephosphate/phosphate translocator

All photosynthetic metabolites eventually must leave the fully developed plastid in order to keep a balanced metabolic

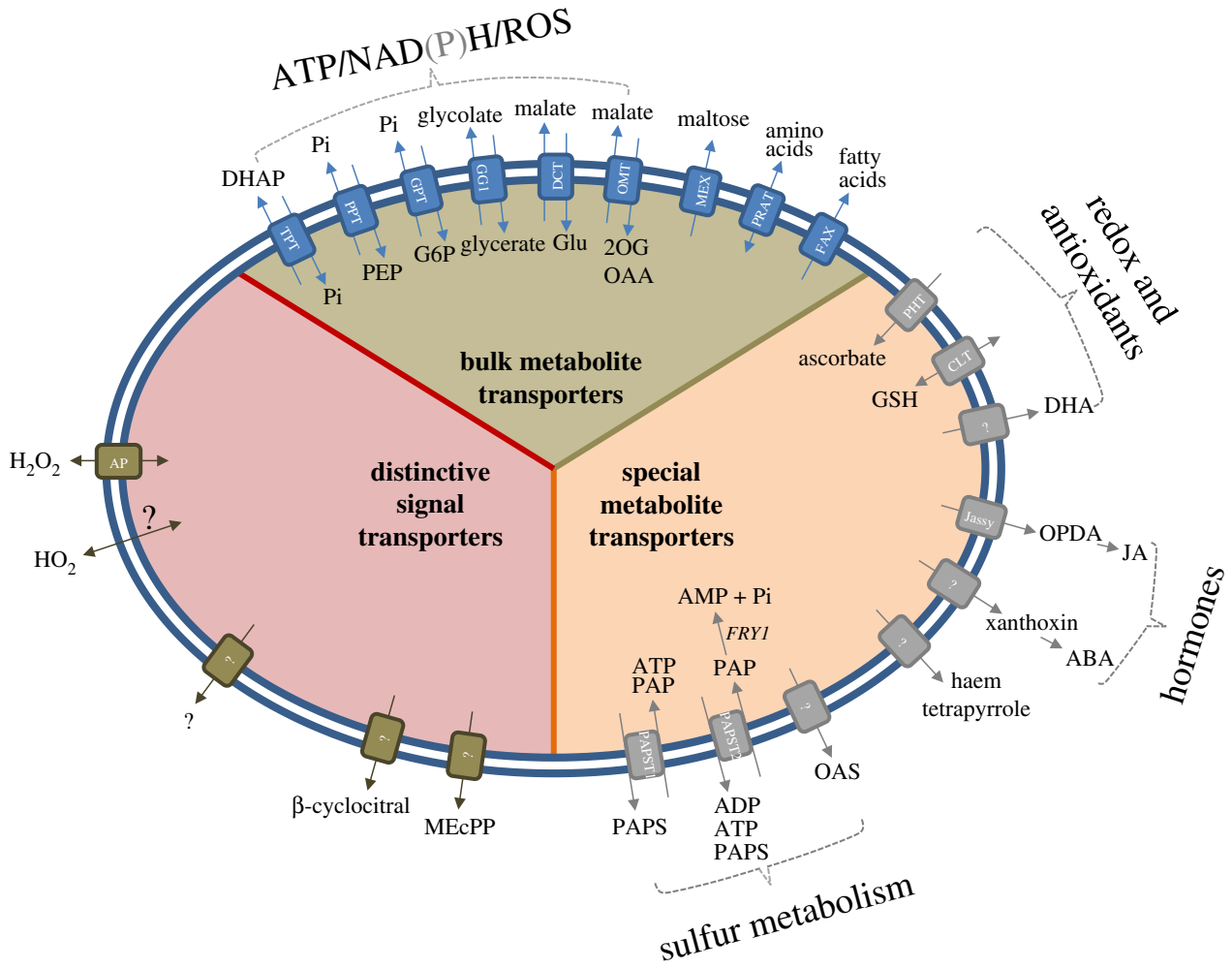

Figure 2. H_2O_2 in the peroxisome. Metabolite transport at the plastid envelope. This scheme distinguishes transporters that transport assimilation products at high rates and with high capacity (bulk metabolite transporters). Other transporters function with lower rate and capacity and play a more specific role in metabolism or signalling, e.g. in hormone, redox signalling and sulfur metabolism. The third category of specific transporters in the context of retrograde signalling of ROS or ROS-derived molecules (distinctive signal transporters) is least understood. Transporter proteins still need to be identified. See text for details. The MEX transporter carries maltose [10]. The first six bulk metabolite transporters are involved in adjustment of extrachloroplast phosphorylation potential (ATP), reduction potential (NAD(P)H) or production of ROS. Glycolate export as part of the photorespiratory cycle releases stoichiometric amounts of H_2O in the peroxisomes. 2-OG, 2-oxoglutarate; ABA, abscisic acid; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; AP, aquaporins; CLT, chloroquinone-like transporters; DCT, dicarboxylate transporter; DHA, dehydroascorbate; DHAP, dihydroxyacetonephosphate; FAX, fatty acid exporter; FRY1, phosphatase fiery 1; GG1, glycolate/glycerate translocator 1; Glu, glutamate; GPT, glucose-6-phosphate/phosphate translocator; G6P, glucose-6-phosphate; GSH, glutathione; H_2O_2, hydrogen peroxide; JA, jasmonic acid; MEcPP, methylerythritol cyclodiphosphate; MEX, maltose transporter; OAA, oxaloacetate; OAS, *O*-acetylserine; OPDA, oxophytodienoic acid; OMT, oxoglutarate–malate transporter; PAP, phosphoadenosine phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PEP, phospho*enol*pyruvate; PHT, H^+/phosphate co-transporter; Pi, inorganic phosphate; PPT, phospho*enol*pyruvate/phosphate translocator; PRAT, amino acid transporter; TPT, triosephosphate/phosphate translocator. (Online version in colour.)

budget. The main role of the TPT in higher plants is the export of triosephosphate and 3-phosphoglyceric acid (3-PGA) and the import of inorganic phosphate (Pi) into the stroma [16]. Upon illumination, the stroma slightly alkalinizes to pH-values above 8, and this pH favours the export of DHAP. The transport capacity of the TPT is very high owing to its protein abundance in the inner envelope membrane [15]. The TPT activity enables efficient carbon fixation, plant growth and storage in distant organs and its molecular mechanism by rocker-switch motion of helix bundles has recently been resolved [17]. Hilgers *et al.* [18] reported the high importance of this translocator in retrograde signalling in *Arabidopsis thaliana* mutants where the blocking of TPT in chloroplasts resulted in a reduction of sucrose biosynthesis and consequently in a high increase of maltose generation as a starch breakdown product. *tpt2* mutants lacking the major TPT show a delayed response of nuclear gene expression in response to low light-to-high light shift [19]. In this work, the readout was the phosphorylation of mitogen-activated protein kinase 6 (MPK6) and the rapid transcript accumulation for the transcription factors ethylene response factors ERF6, ERF104 and ERF105.

Exported DHAP can be converted to 3-PGA by cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), generating NADH and ATP. Thus, the TPT-dependent transport likely contributes the largest share to energy export from the photosynthesizing chloroplast to the cytosol. It should be noted that careful analyses and energetic considerations indicate that the chloroplast NADPH/$NADP^+$ and cytosolic NADH/NAD^+ systems are not in equilibrium, thus establishing unequal redox states in the chloroplast and cytosol [16].

(b) Dicarboxylate transporters and the malate valve

The main role of the malate valves is the maintenance of adequate ATP/NADP(H) ratios in the different cellular compartments. Malate valves are composed of malate dehydrogenases (MDHs) and dicarboxylate translocators responsible for the interconversion of malate and oxaloacetate and their transport [20]. Both proteins are required in photoautotrophic and heterotrophic growth [21]. In chloroplasts, there are two types of malate valves responding to different conditions; one of them is regulated by light through the ferredoxin (FDX)/thioredoxin (TRX) system and the other one plays an important role in dark metabolism to export the NADH originated during glycolysis [21]. The high importance for retrograde signalling of these valves has been addressed in several studies. The lack of chloroplast NADPH-dependent malate dehydrogenase (NADPH-MDH) triggers profound alterations in cell physiology, which include stimulation of photorespiration, increased protein accumulation of NADPH-dependent TRX reductase C (NTRC)/2-cysteine peroxiredoxin (2-CysPRX) and proline accumulation for efficient dissipation of excess reducing power [22]. A connection to chloroplast–mitochondria communication through malate shuttles has been elucidated in *mod1* (mosaic death 1) *A. thaliana* mutants, which accumulate ROS and show enhanced programmed cell death (PCD) [23]. These mutants are deficient in plastid-localized enoyl-ACP reductase. The PCD phenotype of these mutants could be rescued through mutations in plastidial NAD-dependent MDH (plNAD-MDH) and chloroplastic dicarboxylate transporter (DiT1) as well as mitochondrial MDH1 (mMDH1) individually as a consequence of the restored mitochondrial electron transport chain (mETC) complex I activity and involves suppressed ROS production in the mitochondrion. The study also demonstrated the close interconnection between chloroplast and mitochondrion [23].

(c) Transport of precursors and products of sulfur assimilation

O-acetylserine (OAS) functions as substrate in the synthesis of cysteine (Cys) from sulfate [24]. OAS is synthesized in the cytosol, mitochondrion and chloroplast by enzyme complexes consisting of serine acetyl transferase (SAT) and *O*-acetyl serine (thiol) lyase (OASTL). Only unbound OASTL synthesizes Cys. To couple the sulfur assimilation pathways in and outside the chloroplasts there is a need for transport of OAS and Cys across the envelope to balance the demand of the different compartments for Cys in protein and glutathione synthesis. Transporters involved in OAS and Cys translocation across the envelope are so far unknown. But there exist general amino acid carriers in the envelope (figure 2) and Cys transporters have been identified in mitochondria [25,26]. OAS and Cys export likely provides important retrograde information to the extrachloroplast space.

Cys together with glutamate (Glu) and glycine (Gly) serves as substrate for the synthesis of glutathione (GSH) in the plastids. Thus, GSH must be exported from the plastid to the cytosol. Recently, the glutathione transporters were identified as chloroquinone-like transporters (CLT) in *Arabidopsis* [27]. The GSH concentration and redox state strongly affect the redox regulatory network of the cell and retrograde signalling [28].

3′-Phosphoadenosine 5′-phosphosulfate (PAPS) is mainly synthesized in the plastids and used for sulfation reactions in the cytosol or for posttranslational modification of proteins [29]. These sulfation reactions release 3′-phosphoadenosine 5′-phosphate (PAP), which functions as an important retrograde signal, as discussed below. The involved transporters have now been identified as PAPS transporter 1 and 2 (PAPST1, PAPST2) [30]. PAPST1 catalyses the PAPS export to the cytosol in exchange for ATP, or to a lesser extent in exchange for PAP (figure 2). The isoform PAPST2 imports PAP efficiently into the chloroplast in exchange for ADP, ATP or PAPST [30]. PAP is degraded in the chloroplast by the phosphatase fiery (FRY1; also called SAL1), preventing the built-up of a PAP pool in the cytosol. This mechanism is important in stress acclimation, as discussed below.

(d) Plastid-localized preprotein and amino acid transporters

The outer envelope protein 16 (OEP16) is an amino acid transporter in the outer membrane of the chloroplast. OEP16 has a high selectivity, in particular for glutamine and glutamic acid [31]. Rassow *et al.* [32] identified the preprotein and amino acid transporters (PRATs), which are divided into six subfamilies and localize to the mitochondrial and chloroplast inner membranes [14]. PRAT1.1 and PRAT1.2 proteins may be involved in amino acid transport across the inner membrane of the chloroplast [33]. Amino acid export will have strong impact on extrachloroplast metabolism and, therefore, will provide retrograde information on chloroplast metabolic state.

(e) Ascorbic acid transporter

Ascorbate and dehydroascorbate (DHA) transporters in the mammalian system include the Na-dependent vitamin C transporter (SVCT) and glucose transporter (GLUT), respectively [34,35]. While the DHA transporter still awaits its identification, the ascorbate transport into the stroma appears clarified [36]. The phosphate-transporter family (PHT4) consists of four members, which are PHT4;1, PHT4;2, PHT4;3 and PHT4;4 [37,38]. PHT4;1 and PHT4;4 localize in the inner envelope membrane of chloroplasts [39]. Recent studies demonstrated that PHT4;4 functions as ascorbate transporter in the envelope [38]. Cellular ascorbate/DHA homeostasis affects nuclear gene expression, e.g. of defence genes [40,41].

(f) Other transporters

The envelope contains other well-characterized transporters, e.g. for glycolate, fatty acids (FAX), maltose and other phosphorylated metabolites. Recently a protein named Jassy was identified and suggested to function as oxophytodienoic acid (OPDA) transporter [42]. OPDA is the precursor for jasmonic acid (JA) synthesis in the peroxisome and in addition plays roles in JA-independent signalling [43]. The transporter for xanthoxin, the precursor for abscisic acid, is unknown so far, like those involved in the transport of β-cyclocitral (βCC) or methylerythritol cyclodiphosphate (MEcPP) and other distinctive signalling molecules described in §4. Evidence has been published that haem or tetrapyrroles may leave the chloroplast to influence nuclear gene expression [44,45]. However, the nature of the transporters involved is also still elusive. OPDA, xanthoxin, βCC and MEcPP function as retrograde signalling compounds.

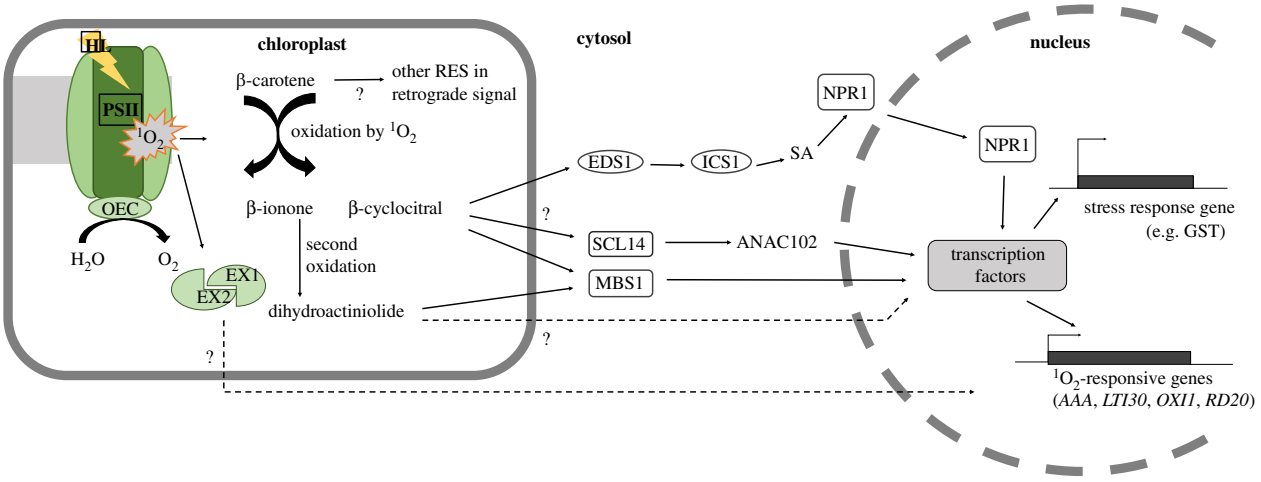

Figure 3. Schematics of signalling pathways related to $^1O_2$-mediated retrograde signalling and triggered by over-reduced PSII. The model summarizes signalling pathways involving carotenoid-derived retrograde signals such as β-cyclocitral and dihydroactiniolide, and addresses regulation of $^1O_2$-responsive genes via EXECUTER proteins, EX1 and EX2, respectively. The pathways are described in detail in the text. Dashed lines indicate signalling connections that are suggested to exist based on circumstantial evidence like transcriptome data. Questions marks denote unknown mechanisms. ANAC102, *Arabidopsis* NAC domain-containing transcription factor; EDS1, enhanced disease susceptibility1; EX, EXECUTER; GST, glutathione-*S*-transferase; ICS1, isochorismate synthase 1; MBS, methylene blue sensitive; NPR1, non-expressor of pathogenesis-related protein; OEC, oxygen evolving complex; PSII, photosystem II; RES, reactive electrophilic species; SA, salicylic acid; SCL14, scarecrow-like 14. (Online version in colour.)

This incomplete compilation shows the richness of transport capacities that link chloroplast with extra-chloroplast metabolism and, thereby, provide retrograde information on the state of the chloroplast to the outside.

4. Reaction products of chloroplast-originated reactive oxygen species as retrograde signals

Chloroplast metabolism generates ROS such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) by reduction of O_2 at photosystem I (PSI) and by excessive excitation of photosystem II (PSII) [46,47]. Metabolic changes in response to environmental stress often increase ROS production in the photosynthesizing chloroplast, foster non-enzymatic lipid peroxidation, and trigger oxidative damage and subsequent degradation of proteins [48]. Oxidative modifications may inactivate enzymes involved in important metabolic pathways or alter signalling cascades. ROS-mediated changes play a fundamental role in cell signalling and this function also includes a direct or indirect role as retrograde signals in the chloroplast–cytosol/nucleus communication [49–51].

ROS function in retrograde signalling by three mechanisms: (i) H_2O_2 may be transferred to the extra-chloroplast space by aquaporin-mediated transport [52] or diffusive leakage; (ii) ROS alter metabolic activities in the chloroplast, leading, e.g., to different metabolite levels and ratios, which then deliver information to the extrachloroplast space; (iii) ROS react with reactive chloroplast molecules like lipids or peptides and the generated reaction products leave the chloroplast and affect extrachloroplast processes like transcription, translation and metabolism to enable the acclimation process [50,51].

$^1O_2$ is unlikely to act as primary retrograde signal owing to its high reactivity and short half-life. $^1O_2$ is produced at PSII under conditions of excess excitation energy. Pigments like the photosensitizer protochlorophyllide absorb photons and transfer the excitation energy to oxygen to generate $^1O_2$ [51]. $^1O_2$ produced by over-reduced PSII engages two different pathways; one of them deploys EXECUTER proteins (EX1, EX2) and the connected signalling pathway and the other one reaction products of $^1O_2$ and β-carotene, namely βCC and dihydroactinidiolide [53].

$O_2^{\bullet-}$ and, after dismutation by superoxide dismutases, H_2O_2 are the rapid ROS signals originating from PSI. Under normal metabolic conditions, e.g. upon changing light intensities, chloroplast ROS are needed for adjusting the redox state of redox-regulated proteins [54]. Under more severe stress, they may trigger oxidative stress responses which in turn liberate retrograde signalling via signalling metabolites such as haem, MEcPP and PAP [55]. Here, we focus on the release of those metabolites that are associated with ROS production in retrograde signals.

5. Retrograde signals from over-reduced PSII

(a) EXECUTER proteins

Molecular and biochemical studies with the *flu* mutant, which accumulates protochlorophyllide in the dark, demonstrated that the nuclear pleiotropic response locus 1 (PRL1) protein and chloroplastic EX1 and EX2 are involved in $^1O_2$-mediated retrograde signalling (figure 3) [56,57]. Both EX1 and EX2 are necessary for full suppression of $^1O_2$-induced gene expression, as revealed from work with double (*ex1/flu*) and triple mutants (*ex1/ex2/flu*) [58,59]. The *chlorina1* (ch1) mutant also accumulates $^1O_2$ at PSII under excess light [60]. These mutants are commonly used for exploring retrograde signalling pathways and revealed another $^1O_2$-dependent signalling pathway, related to the oxidative signal inducible 1 (OXI1) protein, which is a nuclear-localized serine/threonine (Ser/Thr) kinase involved in ROS responses [61]. Furthermore, thylakoid membrane-bound FtsH2 metalloprotease-dependent proteolysis of EX1 has been described to be crucial in $^1O_2$-mediated retrograde signalling [62]. The signal transfer across the envelope remained elusive in the FLU/EX and OXI1 pathways for a long time. Recent studies with *chl1*

and *msbl* mutants have assigned a role to βCC and dihydroactiniolide in this process; however, the precise functions of OX1 and EX1/2 proteins in retrograde signalling remain to be understood.

(b) Retrograde signalling with βCC and dihydroactiniolide

Carotenoids are essential components of photosynthetic reaction centres and light-harvesting complexes and help to quench excited chlorophyll and scavenge $^1O_2$. Carotenoid-derived metabolites play important roles in regulation of plant growth and development, and in environmental acclimation as part of retrograde signalling (figure 3) [63,64].

$^1O_2$ reacts with β-carotene, a 40 carbon compound. Concomitant oxidative cleavage liberates fragments like βCC and β-ionone in the chloroplast [65]. The secondary oxidation of the β-ionone forms dihydroactiniolide. Both βCC and dihydroactiniolide are reactive electrophilic species (RES) containing a highly reactive carbonyl group. Accumulating βCC induces many defence genes, including genes for glutathione-*S*-transferases (GST) and UDP glucosyltransferases [66,67].

Accumulation under high light condition or external application of βCC and dihydroactiniolide up-regulate a large set of $^1O_2$-responsive genes (e.g. *AAA* and *LTI30*), but have little effect on H_2O_2-responsive transcripts [63,65,68]. Both βCC and dihydroactiniolide are volatile compounds that possibly diffuse through the membrane into the cytosol and the nucleus, or use specific transporters that still need to be identified [68]. Their export activates other signalling molecules in the cytoplasm and enhances $^1O_2$-responsive gene expression [66,69]. Candidate signalling elements in the extrachloroplast compartment include methylene blue sensitive (MBS), GST via the salicylic acid (SA) pathway, and the scarecrow-like 14 (SCL14)-dependent xenobiotic detoxifications pathway.

MBSs are small zinc finger proteins first identified in *Chlamydomonas reinhardtii* and *A. thaliana* and play important regulatory roles in expression of $^1O_2$-responsive genes [70]. MBSs locate in the cytosol and nucleus under normal growth conditions. However, experiments using MBS1-GUS reporter constructs indicate their release from cytosolic granules under oxidative stress [70]. Like *flu* and *ch1*, *mbs1* accumulates high concentrations of $^1O_2$ in response to high light [66,70]. Apparently, MBS1 and βCC cross-talk in the cytoplasm and this interaction mediates the transfer of the $^1O_2$ signal to the nucleus [66]. Similarly, dihydroactiniolide induces the gene expression of AAA, OXI1 and RD20, which are $^1O_2$ markers in wild-type *A. thaliana*, but the *mbs1* mutant is unresponsive to this treatment [66]. These pathways are discussed below. Microarray data indicate that H_2O_2-responsive genes (*CAT2* and *APX1*) are innervated by different signalling cascades under high light [65,66].

GST is another important enzyme involved in enhanced $^1O_2$ resistance under light stress [69]. βCC regulates *GST* gene expression by interaction with the SA pathway [65,69]. βCC produced in the chloroplast crosses the envelope [71] and induces expression of enhanced disease susceptibility 1 (EDS1), which is a lipase-like protein involved in SA accumulation [71]. SA accumulation upon βCC treatment and in excess light depends on isochorismate synthase (ICS1) and EDS1 activity [69]. Accumulated SA counteracts chloroplast ROS production and activates the transcriptional co-activator non-expressor of pathogenesis-related genes 1 (NPR1). Using NPR1-GFP reporters, it was demonstrated that βCC promotes NPR1 accumulation in the nucleus [69]. Depending on NPR1 accumulation, transcripts of *GST5* and *GST13* increase upon βCC treatment and in excess light [69]. The results suggest that NPR1 translocation to the nucleus enables its interaction with transcription factors to activate GST gene expression. This response to βCC in young leaves under high light is part of the SCL14-dependent xenobiotic detoxification mechanism [67]. βCC enhances the expression of SCL14 and induces the xenobiotic detoxification response via nascent polypeptide-associated complex domain-containing (ANAC) transcription factors like ANAC102.

6. Retrograde signals from overreduced PSI

(a) Retrograde signalling via methylerythritol cyclodiphosphate

Chloroplast isoprenoids are synthesized via the MEcPP pathway (MEP). This metabolic pathway is involved in plant growth and development and also related to retrograde signalling from the chloroplast [72–75]. In the MEP pathway, the isoprenoid precursor MEcPP is converted to hydroxylmethylbutanyl diphosphate (HMBPP) by L-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HDS) [76]. Isoprenoids play a role as stress signals, and especially the chloroplast MEcPP levels appear critical in retrograde signalling [74]. MEcPP accumulation in the chloroplast was associated with induction of hydroperoxide lyase (HPL), a nuclear stress response gene [72–75].

Previous work with the *ceh1* mutant (constitutively expressing HPL by mutation in the HMBPP synthase (HDS) gene) demonstrated that the *ceh1* mutant accumulates higher levels of MEcPP and HPL transcripts [74]. MEcPP levels correlate with SA contents, transcript amounts of SA genes and the unfolded protein response genes [73–75,77]. In addition, downstream to retrograde signal transduction via MEcPP in *ceh1* mutants, which have elevated levels of MEcPP, highly induced expression of jasmonic acid (JA)-responsive genes is revealed [73]. The retrograde MEcPP pathway also includes interorganellar communication with nucleus, peroxisome and ER [73,77,78]. However, the MEcPP transport mechanism from the plastid to the cytosol and subsequent signal transmission to the nucleus and other organelles awaits clarification.

In bacteria, the MEcPP interacts with chlamydial histone-like proteins [79]. MEcPP-dependent disruption of histone proteins, chromatin remodelling and regulation of HPL and ICS1 gene expression may be part of the regulatory circuitry in plants [80]. MEcPP signalling was linked to transcription factors and histone-based signals [72]. MEcPP may activate gene expression via the *cis*-element RSRE and the calmodulin-binding transcription activator 3 (CAMTA3) [72]. The molecular mechanism of MEcPP-mediated RSRE activation via the transcriptional activator CAMTA3 should be explored further (figure 4).

(b) Retrograde signalling via 3′-phosphoadenosine 5′-phosphate

PAP is a metabolite involved in chloroplast retrograde signalling. Initially, PAP seemed to lack any physiological function in plant cells; however, nowadays it is recognized as

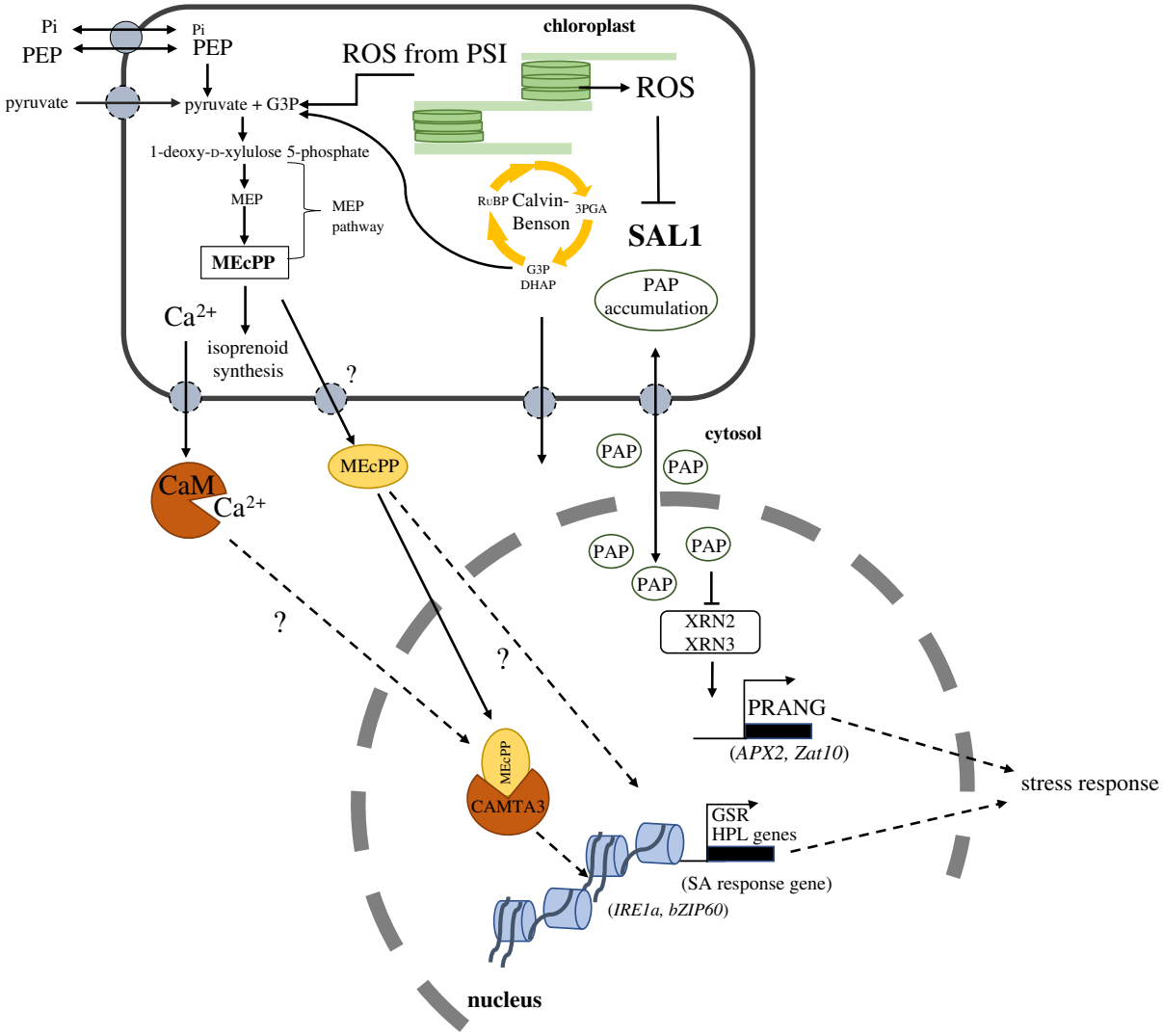

Figure 4. Schematic of signalling pathways related to ROS-mediated retrograde signalling and triggered by over-reduced PSI. This scheme describes signal pathways that involve the MEcPP and PAP-SAL1 pathways. The pathways are described in detail in the text. Questions marks denote unknown mechanisms. Solid lines describe established pathways and dependencies, while dashed lines indicate postulated or unexplored signalling pathways. 3PGA: 3-phosphoglycerate; CaM, calmodulin; CAMTA3, calmodulin-binding transcriptional activator 3; G3P, glyceraldehyde-3-phosphate; GSR, general stress response genes; HL, high light; HLP, hydroperoxide lyase; MEP, methyl-D-erythritol 4-phosphate; MEcPP, methylerythritol cyclodiphosphate; PAP, phosphoadenosine phosphate; PEP, phospho*enol*pyruvate; Pi, inorganic phosphate; PRANG, plastid redox-associated nuclear genes like *Zat10* and *APX2*; PSI/II, photosystem I/II; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; SA, salicylic acid; SAL1, inositol polyphosphate 1-phosphatase; XRN, nuclear 5′–3′exoribonuclease. (Online version in colour.)

important retrograde signal under drought and high light stress [55,81,82]. The pathway includes the chloroplast phosphatase FRY1/SAL1, which regulates PAP levels via its dephosphorylation activity (figure 2) [81]. SAL1 accepts several cellular metabolites as substrates, including inositol 1,4,5-triphosphate (IP3), PAP and PAPS. Thus, SAL1 likely is involved in several metabolic processes in plants [81,83]. Lithium inhibits the 3′(2′),5′-bisphosphate nucleosidase activity of SAL1 [83]. Inhibition of the SAL1 homologue in yeast with lithium increases the cellular PAP levels [84]. Similarly, in plants, chloroplast SAL1 regulates cytosolic PAP levels in a redox-dependent manner by acting as a regulator for PAP import and degradation [81]. Thus here, retrograde signalling involves the inhibited withdrawal of an inhibitory signalling compound (figure 1).

Work with redox and ROS mutants like *alx8*, *xm2* and *xm3* demonstrated that SAL1 functions as an oxidative sensor in the chloroplast. ROS accumulation under stress alters the redox poise of the stroma and oxidizes thiols of target proteins either directly [85] or, e.g., via TRX oxidation [54]. Oxidized SAL1 is inhibited and this triggers PAP accumulation in the chloroplast and cytosol. The transport system for import of cytosolic PAP into the chloroplast for degradation by SAL1 has just been elucidated as PAPST1 and PAPST2 (figure 2). Accumulating PAP moves to the nucleus and inhibits nuclear 5′–3′ exoribonucleases (XRNs) (figure 4). This inhibition leads to expression of plastid redox-associated nuclear genes (PRANGs) such as *APX2*, *ELP2* and *Zat10* [55,81,86]. The SAL1–PAP retrograde signalling pathway is connected with abscisic acid (ABA) and JA signalling [82,87].

XRNs control RNA catabolism and play a role in rRNA processing [88,89]. In *Arabidopsis*, two of the three nuclear-encoded XRNs localize in the nucleus (XRN2, XRN3) while XRN4 is detected in the cytosol [90]. Overall, the SAL1–PAP–XRN pathway may be the best understood retrograde signalling mechanism so far and illustrates the cross-talk between the phosphorylated metabolite PAP, ROS, redox, ABA and oxylipin signalling [55,81,86].

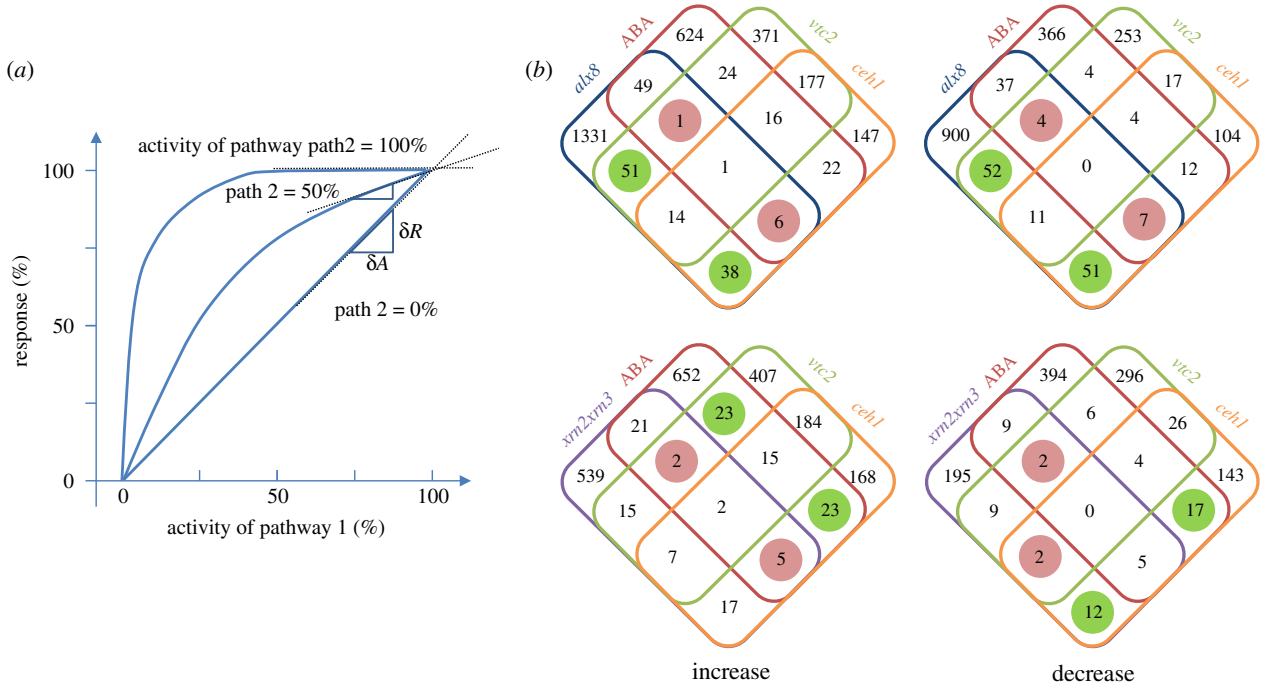

Figure 5. Interference between signalling pathways and application of the control theory. (*a*) Depiction of the control strength exerted by signalling pathway 1 on the response of the system. The normalized slope of the response-versus-activity plot ($\delta R/\delta A$) defines the control coefficient *c*, which regularly ranges between $c = 0$ (no control) and $c = 1$ (full control). The summation theorem states that the sum of *c*-values of all involved components cannot exceed 1. A theoretical example could be the MEcPP pathway versus high light acclimation. Reducing the activity of the pathway 1 will not affect the response until more than 50% of the pathway activity is lost. However, impeding pathway 2 will increase the control coefficient of pathway 1. (*b*) Venn diagrams of overlapping transcriptomic changes (upregulation and downregulation) for multiple retrograde signalling pathways. Microarray-based expression data were taken from [75,81,103–105]. Transcript amounts of mutants or treatments were compared with wild-type and filtered for fold change ≥ 2, ≤ -2 and $p \leq 0.05$. The filtered datasets were compared for possible overlaps using the online tool DRAW VENN DIAGRAM (bioinformatics.psb.ugent.be/webtools/Venn/).

Interestingly, this pathway may rely on removal of a signalling compound from the cytosol, and thus the opposite direction of metabolite transfer as discussed above.

7. The concerted action of signalling pathways in retrograde control

It is now widely accepted that plant responses to combined stresses usually cannot be predicted from studies of responses to single stresses [91,92]. One cause for this deviation is that specific signalling pathways often are affected by interfering pathways. Synergistic or antagonistic cross-talk has also been observed for retrograde signalling. Thus, short term high light acclimation involves multiple pathways, which include oxylipins, mitogen-activated protein (MAP) kinases, ROS and thiol homeostasis. Despite the predominance of mitochondrial cysteine synthesis under regular growth conditions, plants lacking the chloroplast SAT2;1 or its regulator cyclophilin 20-3 were unable to fully activate the high light acclimation response [93]. Likewise, *Arabidopsis* mutants with decreased endogenous amounts of ascorbate (*vtc1*) or glutathione (*pad2*) showed strong alterations in high light-triggered transcript accumulation [28].

There exist many other established cross-talks in retrograde signalling, as also pointed out above. Thus, βCC-dependent signalling is linked to salicylic acid (SA) [69]. Signalling during PSI photoinhibition affects oxylipin metabolism [94]. Amounts of OPDA-responsive transcripts are increased in high light-treated *Arabidopsis*, and their expression further increases during recovery from high light stress in *Arabidopsis* mutants like *proton gradient5* (*pgr5*) or *nonphotochemical quenching4* (*npq4*) [94]. This particular study also showed strong interference with the gene ontology groups of SA, ethylene and H_2O_2 signalling.

Jiang *et al*. [95] linked MEcPP signalling to auxin function. The MEcPP signal uses transcriptional and post-translational mechanisms to modulate auxin amounts and transport. MEcPP reduces the protein amounts of the auxin-efflux carrier pin-formed1 (PIN1) at the plasma membrane [96].

OPDA functions as precursor in JA biosynthesis, but also acts as a signal molecule associated with redox state [96,97]. The studies with *stn7*, *tap38* and *npq4* mutants indicate two different pathways of OPDA signalling, namely either independent or dependent on excitation energy transfer. The latter pathway is related to JA signalling and regulates excitation energy transfer and distribution through STN7 protein kinase, TAP38/PPH1 phosphatase and the PSII subunit PSBS protein [97]. Oxylipin signalling regulates the photo-oxidative stress response by addressing antioxidant enzymes, heat shock proteins, transcription factors, and protein kinases [98–100]. Moreover, integrated 'omics analyses in *hydroperoxide lyase 1* (*ceh1*), SA-defective mutant *eds16*, and a *coronatine-insensitive1* (*coi1*)/*ceh1* double mutant demonstrated that MEcPP signalling affects SA signalling and cross-talks with the JA pathway depending on OPDA [73,77].

A meta-transcriptome analysis from multiple studies on retrograde signalling identified sugar- and ROS/redox-related signals at the beginning of many plastid-triggered signalling cascades [101]. The authors defined lateral modules associated with the core module, and these included ABA and auxin signalling components. All pathways revealed strong interactions and were suggested to constitute a complex and integrated network.

8. Retrograde pathways versus networks and metabolite patterns

The given examples demonstrate the tight and multifaceted connections between the various signalling pathways in retrograde regulation. Interestingly, these interferences often are identified long after the initial identification of the signalling components and pathways. This shows the strength and drawback of genetic approaches when screening for loss or gain of function mutations. Applying control theory [102] to retrograde signalling, a change in the activity of a participating component of pathway 1 will cause a change in the response strength (figure 5). In the given example, more than 50% of the pathway 1 could be inactivated without effects on the response. The response collapses only upon almost complete deletion of pathway 1, as in a loss of function mutant. Pathway 1 has an infinitely small control coefficient c in the wildtype ($c = 0$ at 100% pathway activity). Let us consider a second interfering pathway 2 which also participates in the response. In this case, 50% inactivation of pathway 2 increases the control coefficient of pathway 1 to $c = 0.25$. Pathway 1 takes over full control ($c = 1$) upon deletion of pathway 2.

According to this scenario, loss of function mutations directly lead from 100% pathway activity to 0% and this may be interpreted as $c = 1$; however, the contribution may be small or insignificant near wild-type levels of the pathway component. Naturally, it is difficult to experimentally establish the broad range dependency of, e.g., light acclimation on MEcPP concentration. But it should be noted that the control theory has been expanded and can be applied to local and global dependencies [106].

This example illustrates cross-talk and the existence of networks of multiple components also in retrograde signalling. Figure 5*b*, for instance, indicates such an overlap in transcriptomic changes for multiple signalling pathways, i.e. *alx8*/*xrn2*/*xrn3* mutants (affected in the SAL1–PAP signalling pathway) [81,103], ABA-dependent signalling (ABA treatment; [104]), *vtc2* mutants (low ascorbic acid; [105]) and *ceh1* (defective in salicylic acid signalling; [75]). There was strong overlap in transcript responses among the signalling pathways. As expected, the groups of co-regulated transcripts were large for any comparison of two signalling pathways (green), and smaller for three or four (red). For example, the overlap was higher for increased transcripts in *vtc2* and *ceh1* mutants, as was the case for *alx8* or *xrn2xrn3* with ABA. These co-regulated transcripts surely indicate the interdependence of the included signalling pathways.

As compiled in this review, the exchange of many metabolites and messengers between the chloroplast and the cytosol creates a complex chemical framework concerning energization, reduction power, oxidants, ions, specific metabolites, fluxes and so on. Strong or weak synergistic or antagonistic effects occur, and thus network performance can hardly be predicted. In order to account for this complexity, we propose the use of the term 'Chloroplast-Associated Molecular Patterns' (ChAMP) as concept for fine-tuned retrograde signalling.

The analysis and understanding of the ChAMP state depends on subcellular metabolomics which can be achieved by using non-aqueous fractionation of rapidly freeze-quenched tissue and isolation of subcellular fractions that represent the previous *in vivo* metabolite concentrations [107,108]. Such datasets will have to be linked to transcriptome analyses. Another important parameter is the time-dependency of retrograde signalling and response [109], because the ChAMP will have strong time-dependent dynamics. Computational biology will be needed to dissect the dependencies to understand the operational retrograde signalling in its entirety.

Data accessibility. This article has no additional data.

Authors' contributions. Each author wrote specific sections of the article, and discussed and improved the whole manuscript.

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