

## **Chloroplast degradation: ubiquitin pulls the trigger**

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**Diverse proteolytic pathways regulate chloroplasts. Recent studies have revealed significant new roles for chloroplast ubiquitination in stress adaptation, involving targeted protein removal via the ubiquitin-proteasome system, or selective, whole-chloroplast degradation.**

Chloroplasts are essential organelles in green plants, with responsibility for a variety of metabolic and signalling functions in addition to photosynthesis [1]. For example, chloroplasts are heavily involved in stress responses. Harmful reactive oxygen species (ROS) are produced as a by-product of photosynthesis, causing damage to chloroplast constituents, and can become a severe burden in plants experiencing stresses such as salinity, drought or excessive light. To enable proper maintenance of the chloroplast's complex cellular functions, and to cope with stresses, the organelle and its proteome are dynamically controlled. A critical component of such control is proteolytic, involving complex degradation pathways with diverse mechanisms. Two recent studies revealed significant new roles for ubiquitin-dependent protein modification in the regulation of chloroplasts in response to stress [2,3].

Chloroplasts are bounded by a double-membrane envelope which surrounds the aqueous stromal matrix, and have a distinct internal thylakoid membrane system that is the site of photosynthesis. Diverse proteolytic pathways operate specifically in these different sub-compartments, much as they do in the other endosymbiotic organelle, the mitochondrion [4]. In particular, it is well known that internal proteases inherited from the organelle's prokaryotic ancestor are responsible for the removal of damaged or unwanted proteins inside chloroplasts [5].

For outer envelope membrane proteins that lie beyond the reach of the internal proteases, a major cytosolic proteolytic network, the ubiquitin-proteasome system (UPS), is employed [6]. The UPS selectively identifies substrate proteins for removal by labelling them with ubiquitin protein tags through the activity of a series of enzymes, including E3 ligases which mediate substrate recognition [7]. A direct connection between chloroplasts and the UPS was recently revealed by the discovery of an E3 ligase called SP1, a chloroplast outer membrane protein (OMP) that controls chloroplast

biogenesis [8]. Most chloroplast proteins are produced in the cytosol and need to be imported by dedicated translocons in the envelope membranes; the TOC translocon of the outer membrane controls the recognition of such proteins. SP1 directs the UPS-mediated degradation of TOC components, and thereby regulates protein import to control the chloroplast's proteome, functions and developmental fate. Activity of SP1 is particularly important during development phases in which chloroplasts undergo major changes, e.g., during plant greening upon illumination. Recent work revealed that SP1 also plays a critical role in abiotic stress tolerance [3].

By exposing *Arabidopsis* plants to multiple adverse conditions, including salinity, osmotic and oxidative stresses, loss of SP1 was found to increase stress sensitivity whereas SP1 overexpression enhanced stress tolerance [3]. Further observations revealed that SP1 is activated by stress to deplete TOC components and thus regulate protein import: SP1 overexpressor plants under stress conditions displayed reductions in the chloroplast import of new components of the photosynthetic apparatus, in steady-state levels of photosynthetic apparatus components, and in ROS levels, whereas *sp1* mutants displayed opposite effects. Moreover, an import-defective mutant with compromised TOC activity, *ppi1*, displayed stress tolerance similar to that of the SP1 overexpressor. Overall, the data revealed a novel role for ubiquitination in the regulation of the import machinery, enabling suppression of photosynthesis and thus reduced potential for ROS overproduction and photooxidative damage. As major sources of ROS, chloroplasts evolved elaborate protective mechanisms, including ROS scavenging and avoidance [9], of which the latter may include SP1-mediated photosynthetic inhibition. Indeed, when mutations affecting SP1 and ROS scavenging genes were combined, plants exhibited even more severe defects during stress [3].

The bulk degradation of chloroplast proteins occurs during stress or natural senescence, and employs several vacuole-dependent mechanisms [10]: (1) autophagy-dependent processes; (2) a pathway involving Senescence Associated Vacuoles (SAVs), which form in the chloroplasts of senescing leaves for the proteolysis of stromal components; and (3) a pathway involving Chloroplast Vesiculation Protein-Containing Vesicles (CCVs), which acts on both stromal and thylakoidal proteins. While the latter two seem specific to chloroplasts, autophagy is extensively involved in the degradation of other cellular components and is better characterized [11]. Autophagy is controlled by protein modification machinery reminiscent of that which mediates UPS action, and this triggers the formation of a double-membrane autophagosome which engulfs the substrate (e.g., a partial or whole chloroplast) before its transfer to the vacuole for proteolysis. In mitochondria, ubiquitination of OMPs is closely linked to the autophagic, selective clearance of stress-damaged organelles, in a process called mitophagy [12]. However, whether a similar role exists for ubiquitin in chloroplast degradation has been an open question. Recent work by Woodson and colleagues [2] revealed that

ubiquitination is more broadly connected to chloroplasts than previously envisaged, possibly serving to target damaged chloroplasts for vacuolar degradation in response to ROS stress.

So-called retrograde signals relay information on the status of chloroplasts to the nucleus, to regulate the transcription of nuclear genes controlling chloroplast biogenesis and stress adaptation [13]. One such signal is linked to metabolites of the chloroplast-localized tetrapyrrole biosynthetic pathway, which produces heme and chlorophyll. To reveal other controlling mechanisms, Woodson and colleagues [2] made use of a newly-characterized tetrapyrrole pathway mutant with disrupted retrograde signalling, *Arabidopsis ferrochelatase 2 (fc2)*.

Ferrochelatases 1 and 2 (FC1, FC2) are conserved enzymes catalysing the conversion of protoporphyrin IX (Proto) to heme, which serves as a retrograde signal [14,15]. As retrograde signalling is crucial during light-to-dark transitions, Woodson and colleagues [2] investigated the involvement of FC1 and FC2 in such processes. They found that *fc2*, but not *fc1*, mutant plants failed to green during light-to-dark transitions. Transcriptome profiling indicated that *fc2* plants were experiencing oxidative stress under such conditions, while microscopy revealed damage to, and rupture of, chloroplasts in the mutant. The *fc2* mutant displayed over-accumulation of the photosensitizing intermediate, Proto, and of an important ROS, singlet oxygen. Overall, the data indicated that disruption of the tetrapyrrole pathway by *fc2* causes ROS overproduction inside chloroplasts leading to chloroplast degradation, and that this impedes plant greening. This raises an interesting question: How do ROS mediate the degradation of chloroplasts?

To answer this question, Woodson and colleagues [2] sought mutations that restore greening in *fc2* using a second-site suppressor screen, and identified 24 *ferrochelatase-two suppressor (fts)* mutants defining 17 loci. While most suppressors affected the tetrapyrrole pathway and reduced Proto levels, as expected, one (*fts29*) carried a lesion in the *Plant U-Box 4 (PUB4)* ubiquitin E3 ligase gene. The *pub4* mutation specifically suppressed chloroplast damage without affecting Proto or ROS levels, revealing a novel chloroplast quality-control pathway involving ubiquitination. Unlike SP1, PUB4 is localized in the cytosol, and it was reported previously to play roles in cell cycle control [16,17]. Anti-ubiquitin immunoblotting and immunogold labelling provided evidence for chloroplast ubiquitination that was strongly enhanced by *fc2* and suppressed by *pub4* [2]. The data suggested that PUB4 mediates ubiquitination of chloroplast proteins, which might in turn trigger chloroplast degradation. Further analysis implied that the degradation was selective for damaged organelles, dependent on PUB4, and revealed that some damaged chloroplasts were associated with “globular vacuoles”, at the interface with the cellular vacuole, suggesting a potential

quality-control pathway. Such control might enable plants to better cope with ROS damage, as *pub4* plants are hypersensitive to excessive light [2].

These new results raise a number of interesting questions. The first of these concerns the identity of the targets of PUB4. In fact, it is unclear if PUB4 acts directly or indirectly on chloroplast proteins. Although OMPs are likely to be the targets, supporting evidence is missing. In seeking targets, analysis of highly-purified chloroplasts will be necessary to eliminate irrelevant substrates that can be expected based on the cytosolic localization and known other functions of PUB4. Another important question pertains to the mechanism underlying the stimulating of chloroplast ubiquitination. In stressed mitochondria, membrane depolarization inhibits protein import, and this serves as a signal for ubiquitination [18]; but chloroplast protein import does not require a membrane potential, and so presumably a different signal operates. Details on the chloroplast degradation process itself are also lacking. In mitochondria [19], ubiquitination of OMPs can lead to either UPS action or mitophagy, with the UPS-mediated removal of OMPs potentially further facilitating mitophagy. Although *pub4* does not share the general characteristics of autophagy mutants under carbon starvation [2], the possibility exists that it is involved in autophagy only under specific stresses, like the previously reported NBR1 protein [20]. Another puzzling point is that PUB4-dependent chloroplast quality control is not entirely beneficial, because blocking the pathway relieved the conditional lethality of *fc2* mutant.

The relationship between the two recently-described ubiquitin-dependent chloroplast protein degradation pathways, mediated by SP1 and PUB4 [2,3], is another intriguing question. One possible scenario is that ubiquitination has different substrates and consequences depending on the severity of the chloroplast damage experienced, as summarized in Figure 1.

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## Figure Legend

Figure 1. Different modes of ubiquitin-dependent chloroplast protein degradation.

Left: Under mild stress conditions (e.g., high light, salt or osmotic stress), which may be short lived or causative of only limited ROS damage, the chloroplast-localized E3 ligase SP1 mediates the specific degradation of TOC import machinery components by the 26S proteasome (26S). In doing so, the import of new photosynthetic apparatus components is reduced, which in turn suppresses photosynthetic activity and thus avoids the production of more ROS and chloroplast damage.

Right: When the stress is prolonged or severe, high ROS levels inevitably cause damage to chloroplasts. Under such circumstances, the cytosolic E3 ligase PUB4 may extensively ubiquitinate chloroplast outer membrane proteins, which then triggers the vacuole-dependent clearance of whole, damaged chloroplasts via an unknown mechanism. This would provide a chloroplast quality control mechanism to reduce the risk of further ROS accumulation.

