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Singlet oxygen-mediated signaling in plants: moving from *flu* to wild type reveals an increasing complexity

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Abstract

Singlet oxygen ($^1\text{O}_2$)-mediated signaling has been established in the conditional *fluorescent* (*flu*) mutant of *Arabidopsis*. In the dark, the *flu* mutant accumulates free protochlorophyllide (Pchl_{id}), a photosensitizer that in the light generates $^1\text{O}_2$. The release of $^1\text{O}_2$ leads to growth inhibition of mature plants and bleaching of seedlings. These $^1\text{O}_2$ -mediated responses depend on two plastid proteins, EXECUTER (EX) 1 and 2. An *ex1/ex2/flu* mutant accumulates in the dark Pchl_{id} and upon illumination generates similar amounts of $^1\text{O}_2$ as *flu*, but $^1\text{O}_2$ -mediated responses are abrogated in the triple mutant. The $^1\text{O}_2$ - and EX-dependent signaling pathway operates also in wild type placed under light stress. However, it does not act alone as in *flu*, but interacts with other signaling pathways that modulate $^1\text{O}_2$ -mediated responses. Depending on how severe the light stress is, $^1\text{O}_2$ - and EX-dependent signaling may be superimposed by $^1\text{O}_2$ -mediated signaling that does not depend on EX and is associated with photo-oxidative damage. Because of its high reactivity and short half-life, $^1\text{O}_2$ is unlikely to be a signal that is translocated across the chloroplast envelope, but is likely to interact with other plastid components close to its site of production and to generate more stable signaling molecules during this interaction. Depending on the site of $^1\text{O}_2$ production and the severity of stress, different signaling molecules may be expected that give rise to different $^1\text{O}_2$ -mediated responses.

Keywords

Singlet oxygen-mediated signaling; Executer; Light stress; *flu* mutant; *Arabidopsis thaliana*

The effect of environmental changes on the production of reactive oxygen species in plants

Plants, being sessile, encounter a wide range of environmental stress factors that they cannot avoid by moving away. These stress factors may adversely affect their growth, development, or productivity (Bray et al. 2000). The combination of different stress factors and their relative impact may frequently change. Plants have adapted to these challenges by continuously monitoring their environment, sensing multiple environmental factors, and transforming these cues into signals that need to be integrated before activating gene expression changes to shape a response appropriate for the prevalent environmental condition. How the integration of these different environmental signals takes place and determines the plant's responses to adversity is largely unknown. During the perception and

integration of environmental cues, chloroplasts play important roles as sensors of environmental stress factors that interfere with the photosynthetic electron transport chain such as high light, high or low temperature, or drought (Foyer and Noctor 2009). Under these stress conditions, the absorbed light energy exceeds the plant's capacity to assimilate CO₂ and leads to the hyper-reduction of the electron transport chain that blocks the transfer of electrons from the excited photosystem II (PSII) reaction center (RC) to photosystem I (PSI) (Niyogi 1999; Li et al. 2009). One of the consequences of these stress conditions is an enhanced production of reactive oxygen species (ROS) within chloroplasts (Apel and Hirt 2004).

Plants have evolved two main strategies to avoid hyper-reduction of the photosynthetic electron transport chain and to maintain the acceptor site of PSII in a partially oxidized state. Non-photochemical quenching dissipates excess light energy absorbed by the antenna pigment protein complexes as heat (Müller et al. 2001; Li et al. 2009). Chlorophyll (Chl) in light-harvesting Chl-protein complexes is in close contact with various xanthophylls, a group of carotenoids that efficiently quench and scavenge excess light energy (Codgell and Frank 1987; Ramel et al. 2012a). The second strategy, photochemical quenching, operates by directing electrons away from the acceptor site of PSII to various electron sinks (Barber and Andersson 1992; Asada 1999; Li et al. 2009) (Fig. 1). This may happen for instance through the reduction of oxygen to superoxide by reduced electron transport components associated with PSI (Mehler 1951; Asada 1999; Rizhsky et al. 2003) (Fig. 1B) or by reactions linked to the photorespiratory cycle (Kozaki and Takeba 1996) (Fig. 1C). In both cases, photochemical quenching leads to the enhanced production of superoxide radical O₂[•] – and hydrogen peroxide (H₂O₂).

When these quenching mechanisms are not sufficient to sustain a partial oxidation of the acceptor site of PSII, the absorbed light energy may remain trapped within the excited PSII and promote the generation of a third ROS, singlet oxygen (¹O₂) (reviewed by Krieger-Liszkay et al. 2008; Li et al. 2009) (Fig. 1D). Formation of ¹O₂ occurs within chloroplasts by energy transfer from the excited triplet state of Chl to ground state triplet oxygen (³O₂) either within light-harvesting antenna complexes or the RC of PSII (Apel and Hirt 2004; Krieger-Liszkay et al. 2008). ¹O₂ production in light-harvesting complexes happens under severe light stress, when the absorbed light exceeds the plant's capacity to dissipate excess light energy as heat. ¹O₂ produced under these conditions may lead to non-enzymatic peroxidation of lipids and carotenoids and cause photo-oxidative damage (Reinbothe et al. 1996; Apel 2001; Triantaphylidès and Havaux 2009; Ramel et al. 2012a). ¹O₂ production in the RC of PSII occurs not only under high light stress as in the case of light-harvesting complexes, but also at a reduced rate under dim light (Szilard et al. 2005). Unlike carotenoids of antennae complexes, carotenoids in the RC of PSII are localized away from the RC P680 Chl and thus are unable to quench directly the excited triplet state of this special Chl that may be formed when the electron acceptor site of PSII is reduced and unable to oxidize the excited P680 Chl (Telfer 2005; Ferreira et al. 2004; Loll et al. 2005). In case the carotenoids were close enough to quench triplet Chl of the RC, they would compete for light energy and thus reduce the efficiency of light-driven electron transport by the RC Chl. ¹O₂ formation by this special Chl seems to be a trade-off, optimizing the performance of the RC and becoming an inherent property of PSII (Vass and Cser 2009; Kim et al. 2012). There are several ways of how ¹O₂ produced during the quenching of the excited triplet state of the RC Chl by oxygen can be scavenged. The core of the RC of PSII contains the two closely related membrane proteins D1 and D2 that are encoded in the chloroplast and bind various co-factors including the P680 Chl (Ferreira et al. 2004; Loll et al. 2005). They are required for charge separation and electron transport. Being close to the site of ¹O₂ formation in the RC of PSII, the D1 protein is a primary target of ¹O₂ and seems to act as a major scavenger of ¹O₂ (Vass and Cser 2009). Its high turnover rate has been

attributed to the rapid degradation of the oxidized D1 protein after its interaction with $^1\text{O}_2$ and its replacement by newly synthesized D1 polypeptides (Aro et al. 1993; Lindahl et al. 2000; Nixon et al. 2004). Besides the D1 protein, α -carotene, plastoquinol, and α -tocopherol have also been implicated with scavenging $^1\text{O}_2$ and protecting PSII against photo-oxidative damage (Telfer et al. 1994; Trebst et al. 2002; Kruk and Trebst 2008).

$^1\text{O}_2$ -mediated signaling in the conditional *flu* mutant of *Arabidopsis thaliana*

Enhanced levels of $\text{O}_2^{\bullet-}$, H_2O_2 , and $^1\text{O}_2$ within chloroplasts correlate with responses of plants to stress (Apel and Hirt 2004; Gechev et al. 2006). All three ROS may interact directly with various molecules and in this way irreversibly destroy or alter the function of the target and cause visible damage (Sies and Menck 1992; Halliwell and Gutteridge 1989; Girotti 2001; Davies 2004; Watabe et al. 2007). As the accumulation of these ROS in chloroplasts correlates with changes in nuclear gene expression, they have been also perceived as signaling molecules that may initiate stress responses (Forman and Cadenas 1997; Klotz 2002; Finkel 2003; Apel and Hirt 2004). The physiological consequences of an enhanced production of either $^1\text{O}_2$ or $\text{H}_2\text{O}_2/\text{O}_2^{\bullet-}$ in chloroplasts are different. While an enhanced production of $\text{H}_2\text{O}_2/\text{O}_2^{\bullet-}$ in chloroplasts helps to sustain PSII activity, an enhanced release of $^1\text{O}_2$ is associated with photoinhibition of PSII (Krieger-Liszkay et al. 2008) (Fig. 1). As the accumulation of these ROS signifies different physiological states of the chloroplast, their signaling specificities are likely to be different from each other.

Environmental stress impeding photosynthetic electron transport leads to the enhanced production of all three ROS. Thus, it is difficult to establish a causal link between a specific stress response of the plant and a particular ROS and to exclude the cytotoxicity of this ROS and confirm its signaling role. For the analysis of $^1\text{O}_2$ -mediated stress responses, these obstacles have been overcome in the conditional *fluorescent* (*flu*) mutant of *Arabidopsis*. In the dark, the *flu* mutant accumulates the immediate precursor of chlorophyllide, protochlorophyllide (Pchlde), in plastid membranes due to the impairment of negative feedback control of tetrapyrrole biosynthesis (Meskauskiene et al. 2001; op den Camp et al. 2003; Przybyla et al. 2008). In the light, Pchlde acts as a potent photosensitizer and generates $^1\text{O}_2$. By varying the length of the dark period, the level of Pchlde can be modified and conditions can be defined that minimize the cytotoxicity of $^1\text{O}_2$ and reveal the signaling capacity of this ROS (Kim et al. 2012). Following reillumination of pre-darkened *flu* mutants, a multitude of $^1\text{O}_2$ -mediated stress responses are induced that include at the cellular level drastic nuclear gene expression changes, enzymatic lipid peroxidation that gives rise to an enhanced synthesis of oxylipins such as jasmonic acid and 12-oxo-phytodienoic acid (OPDA), and the accumulation of other stress hormones and signaling molecules such as salicylic acid and ethylene (Danon et al. 2005; Ochsenbein et al. 2006; Baruah et al. 2009a). At the whole plant level, the release of $^1\text{O}_2$ in chloroplasts of the *flu* mutant leads to growth inhibition of mature plants, lesion formation in leaves, and the bleaching of *flu* seedlings. These pleiotropic $^1\text{O}_2$ -mediated responses depend on the activity of the two nuclear-encoded plastid proteins EXECUTER1 (EX1) and EXECUTER2 (EX2) (Wagner et al. 2004; Lee et al. 2007). An *ex1/ex2/flu* triple mutant lacking these two proteins overaccumulates Pchlde in the dark and upon re-illumination generates similar amounts of $^1\text{O}_2$ as the parental *flu* line, but $^1\text{O}_2$ -mediated responses of the *flu* mutant are abrogated in the triple mutant, suggesting that they are due to $^1\text{O}_2$ -mediated and EX-dependent signaling rather than $^1\text{O}_2$ directly (Kim et al. 2012).

Initially, $^1\text{O}_2$ -mediated responses of the *flu* mutant had been attributed to the activation of a single retrograde plastid-to-nucleus signaling pathway that was thought to control directly the multitude of changes seen in the *flu* mutant after the onset of $^1\text{O}_2$ production (op den Camp et al. 2003). However, based on more recent studies of the *flu* mutant, this seemingly

simple picture of a singular $^1\text{O}_2$ -dependent signaling pathway needs to be corrected (Kim et al. 2012). The onset of $^1\text{O}_2$ production in the *flu* mutant is rapidly followed by a loss of chloroplast integrity that occurs prior to expression changes of most of the $^1\text{O}_2$ -responsive genes identified in earlier studies (op den Camp et al. 2003; Gadjev et al. 2006). Chloroplast leakage is the first $^1\text{O}_2$ -mediated intracellular change visible to the eye that precedes the subsequent rupture of the central vacuole and the final collapse and death of the cell. During the first 15 min of re-illumination, a subset of $^1\text{O}_2$ -responsive nuclear genes is up-regulated, while the chloroplast integrity is still maintained. It is not clear yet whether these very early gene expression changes cause the loss of chloroplast integrity or chloroplast leakage is triggered by a separate $^1\text{O}_2$ -dependent mechanism. A majority of $^1\text{O}_2$ -responsive genes are activated only after widespread chloroplast leakage has been initiated. Disruption of intracellular integrity, e.g., by the $^1\text{O}_2$ -mediated leakage of chloroplasts or the rupture of the vacuole, is likely to activate additional signaling pathways that may evoke drastic nuclear gene expression changes. Hence, many $^1\text{O}_2$ -responsive genes reported earlier for the *flu* mutant (op den Camp et al. 2003; Gadjev et al. 2006) seem to be only indirectly affected by the release of $^1\text{O}_2$ and are probably controlled by signaling events that are activated during the loss of cellular integrity. This notion is in agreement with the fact that the expression of a major part of $^1\text{O}_2$ -responsive genes is triggered by various hormones and in response to pathogens (Baruah et al. 2009a).

$^1\text{O}_2$ -mediated signaling in wild type placed under light stress

Due to its unique properties, the *flu* mutant has proven to be an excellent experimental system to reveal a signaling capacity of $^1\text{O}_2$ and to identify constituents necessary for the transfer of $^1\text{O}_2$ -dependent signals from the plastid to the nucleus and the induction of stress responses. However, as the mechanisms underlying $^1\text{O}_2$ production in the *flu* mutant differ from those responsible for an enhanced release of $^1\text{O}_2$ in wild type, one may question whether $^1\text{O}_2$ -mediated signaling and EX-dependent signaling in *flu* and wild type are the same, and if so trigger similar responses. Genetically controlled traits such as $^1\text{O}_2$ -mediated growth inhibition of mature plants and programmed cell death in seedlings must have evolved under selective pressure and thus should be beneficial to plants. The $^1\text{O}_2$ -mediated growth inhibition of mature *flu* plants resembles a stress-induced stunted growth response implicated with helping plants to withstand stress by slowing down their metabolic activity (Netting 2000) and thus conceivably could confer a selective advantage. On the other hand, the possible benefits of the $^1\text{O}_2$ -mediated suicidal response of seedlings as seen in the *flu* mutant are less obvious. To address this seeming paradox, recent work has been aimed at demonstrating $^1\text{O}_2$ -mediated signaling in wild type and analyzing its physiological significance. To enhance production of $^1\text{O}_2$ and to study its biological activity, either plants were infiltrated with dyes such as rose Bengal that in the light acts as a $^1\text{O}_2$ -specific photosensitizer (Triantaphylidès et al. 2008), or wild-type (Kim et al. 2012) and light-sensitive mutants such as *chl1*, *vte1*, *npq1*, and *lut2* (Triantaphylidès et al. 2008; Alboresi et al. 2011) were subjected to light stress. Plants exposed to light stress enhance the production not only of $^1\text{O}_2$ but also of H_2O_2 and $\text{O}_2^{\bullet-}$, but unlike plants infiltrated with dyes, they generate $^1\text{O}_2$ only within the plastid and not in other sub-cellular compartments. Generation of $^1\text{O}_2$ triggers a wide range of responses such as nuclear gene expression changes, enzymatic and non-enzymatic lipid peroxidation, and the bleaching of seedlings that resemble $^1\text{O}_2$ -mediated stress responses of the *flu* mutant. An EX-dependent up-regulation of $^1\text{O}_2$ -responsive genes in plants placed under mild light stress (Kim et al. 2012) suggests that the $^1\text{O}_2$ - and EX-dependent signaling pathway as described in the *flu* mutant operates also in wild type. However, $^1\text{O}_2$ -mediated and EX-dependent responses of *flu* and wild type differ in several ways (op den Camp et al. 2003; Kim et al. 2012).

In wild type, Chl rather than Pchl_{ide} acts as the photosensitizer that generates $^1\text{O}_2$ (Fig. 2). While in *flu* seedlings, only $^1\text{O}_2$ -mediated and EX-dependent signaling is induced following a dark/light shift, in wild type exposed to light stress, an H_2O_2 -dependent signaling pathway is also activated independently of EX1 and EX2 (Fig. 2). Similar to the *flu* mutant, in wild type loss of chloroplast integrity precedes the $^1\text{O}_2$ -mediated and EX-dependent cell death, but these responses do not immediately follow the light stress-induced accumulation of $^1\text{O}_2$ as in *flu*, but occur only after an extended light stress treatment of several days (Kim et al. 2012). Hence, in wild type, $^1\text{O}_2$ -mediated and EX-dependent signaling does not seem to act alone as a default pathway as it does in *flu*, but interacts with other signaling pathways that converge with the $^1\text{O}_2$ -dependent signaling pathway and delay or modify some of the responses triggered in the *flu* mutant by the release of $^1\text{O}_2$ (Fig. 2).

Modulation of $^1\text{O}_2$ -mediated signaling in wild type

Several modulators have been identified during second-site mutant screens of the *flu* mutant (Baruah et al. 2009a; Meskauskienė et al. 2009; Simkova et al. 2012a, b). These modulators interact with $^1\text{O}_2$ signaling and seem to be responsible for the delay or suppression of the $^1\text{O}_2$ -mediated chloroplast leakage and lesion formation. In several of these second-site mutants, perturbations of cellular homeostasis prior to $^1\text{O}_2$ production confer an enhanced stress resistance by activating acclimation that suppresses $^1\text{O}_2$ -mediated cell death without blocking up-regulation of $^1\text{O}_2$ -responsive marker genes (Fig. 2B). Most of the perturbations identified thus far disturb chloroplast protein homeostasis by inactivating components required for the synthesis or degradation of plastid proteins (Meskauskienė et al. 2009; Coll et al. 2009; Saini et al. 2011). The underlying mechanisms for establishing stress acclimation and suppressing $^1\text{O}_2$ -mediated and EX-dependent cell death in these mutants are not known yet. Another modulator, acting outside of plastids, provides clues as to how $^1\text{O}_2$ -mediated signaling may possibly interact with and be controlled by other signaling pathways. One of the second-site mutations of *flu* that in the absence of $^1\text{O}_2$ generation leads to the constitutive activation of the $^1\text{O}_2$ -responsive marker gene *AAA-ATPase* resides within the *PLEIOTROPIC RESPONSE LOCUS1* (*PRL1*) gene (Baruah et al. 2009b) (Fig. 2B). *PRL1* forms part of the nuclear PRP nineteen complex (NTC) that has been found in a wide range of eukaryotes (Nemeth et al. 1998; Palma et al. 2007). Inactivation of *PRL1* de-represses $^1\text{O}_2$ -responsive gene expression in light-grown *flu* seedlings (Baruah et al. 2009b). Upon a dark-to-light shift, the *prl1/flu* double mutant generates $^1\text{O}_2$ and enhances further the expression of $^1\text{O}_2$ -responsive genes, indicating that *PRL1* is not part of the $^1\text{O}_2$ -dependent signaling pathway, but rather controls this pathway independently of $^1\text{O}_2$. While the expression of $^1\text{O}_2$ -responsive nuclear genes is still activated in *prl1/flu* seedlings following a dark/light shift, the $^1\text{O}_2$ -mediated cell death response of the *flu* mutant is suppressed. Similarly, in wild-type seedlings exposed to light stress, cell death is suppressed in plants lacking active *PRL1* (Baruah et al. 2009b). As signaling pathways associated with sugar and hormone responses and pathogen defenses converge on *PRL1*, the *PRL1*-containing NTC complex may play a role as a central hub and modulate responses of plants to environmental changes for instance by interconnecting $^1\text{O}_2$ -mediated signaling with other stress-related signaling pathways, thereby controlling the $^1\text{O}_2$ -mediated cell death response (Nemeth et al. 1998; Baruah et al. 2009b) (Fig. 2B).

Another suppressor of $^1\text{O}_2$ -mediated stress responses that operates under light stress seems to be H_2O_2 (Laloi et al. 2007; Simkova et al. 2012b). Soon after the beginning of light stress, generation of $\text{H}_2\text{O}_2/\text{O}_2^{\bullet-}$ is induced in wild-type seedlings as indicated by the up-regulation of the H_2O_2 -specific marker gene *FERRITIN1*. Light stress-induced expression of *FERRITIN1* is preceded by the rapid up-regulation of $^1\text{O}_2$ -responsive gene expression. At the same time, enzymatic lipid peroxidation prevails suggesting that during this initial stage of stress treatment, $^1\text{O}_2$ and $\text{H}_2\text{O}_2/\text{superoxide}$ act primarily as signals and do not cause

extensive oxidative damage (Kim et al. 2012). In contrast to $^1\text{O}_2$ -responsive genes, *FERRITIN I* expression is not affected in an *ex1/ex2* background suggesting that H_2O_2 - and $^1\text{O}_2$ -dependent signaling occurs via two distinct pathways (op den Camp et al. 2003; Kim et al. 2012). An antagonistic effect of H_2O_2 on $^1\text{O}_2$ -mediated stress responses has been discovered in plants overexpressing the thylakoid-specific ascorbate peroxidase, an H_2O_2 -scavenging enzyme (Murgia et al. 2004). The reduction of H_2O_2 concentrations in plastids accelerates the intensity of $^1\text{O}_2$ -mediated stress responses, suggesting that H_2O_2 either directly or indirectly suppresses $^1\text{O}_2$ -mediated signaling (Laloi et al. 2007) (Fig. 2B). This negative control by H_2O_2 may help to explain why in wild type, chloroplast leakage and cell death are not rapidly induced, but are separated temporally by several days from the onset of $^1\text{O}_2$ production at the beginning of light stress (Kim et al. 2012). In the *flu* mutant, $^1\text{O}_2$ -mediated signaling is triggered without a concomitant activation of H_2O_2 -dependent signaling (op den Camp et al. 2003) and the beginning of $^1\text{O}_2$ generation after a dark-to-light shift is followed almost immediately by chloroplast leakage and the initiation of cell death (Kim et al. 2012) (Fig. 2A).

$^1\text{O}_2$ itself may also act as a modulator and modify consequences of $^1\text{O}_2$ -mediated signaling through an auto-regulatory feedback control that induces stress acclimation. In *Chlamydomonas*, sub-lethal stress leads to a transient or moderate elevation of $^1\text{O}_2$ and results in the activation of a subset of $^1\text{O}_2$ -responsive genes and protection against a subsequent more severe $^1\text{O}_2$ -mediated stress (Ledford et al. 2007) (Fig. 2B).

Different ways of how $^1\text{O}_2$ affects signaling in wild type

$^1\text{O}_2$ -mediated signaling has been analyzed in wild type under different light stress conditions. In seedlings placed under mild light stress, up-regulation of $^1\text{O}_2$ -responsive marker genes, chloroplast leakage, and lesion formation are abrogated in the absence of active EX1 and EX2, indicating that these mild light stress-induced responses are controlled by the $^1\text{O}_2$ - and EX-dependent signaling pathway. Under more severe light conditions, however, the activation of this signaling pathway in wild type is less clear. A majority of genes up-regulated in high light stress-treated plants or cell cultures were found to be similar to $^1\text{O}_2$ -responsive genes described earlier in the *flu* mutant (Alboresi et al. 2011; Gonzalez-Pérez et al. 2011; Ramel et al. 2012b). This striking overlap of $^1\text{O}_2$ -responsive genes activated in *flu* in response to $^1\text{O}_2$ and in wild-type and light-sensitive mutants in response to severe light stress was taken as evidence to infer a role of the $^1\text{O}_2$ - and EX-dependent signaling pathway during a response of wild type to severe light stress. There are several findings that are difficult to reconcile with such a conclusion. An enhanced expression of $^1\text{O}_2$ -responsive genes reported earlier in wild type exposed to severe high light stress may indicate an impairment of cellular integrity rather than an activation of the $^1\text{O}_2$ - and EX-dependent signaling pathway (Kim et al. 2012). The up-regulation of $^1\text{O}_2$ -responsive genes in the *flu* mutant described by op den Camp et al. (2003) and Gadjev et al. (2006) had been measured between 30 min and 2 h after a dark-to-light shift (op den Camp et al. 2003). By this time, $^1\text{O}_2$ -mediated and EX-dependent chloroplast leakage and a rupture of the central vacuole had been already initiated (Kim et al. 2012). Thus, it seems likely that the expression of most of these genes is under the control of other signaling pathways activated as a consequence of a loss of cellular integrity in the *flu* mutant. Such an interpretation is in line with the extensive non-enzymatic peroxidation of polyunsaturated fatty acids and carotenoids by $^1\text{O}_2$ in plants exposed to severe light stress that is indicative of photo-oxidative damage (Triantaphylidès et al. 2008; Ramel et al. 2012b). Under such stress conditions, $^1\text{O}_2$ -mediated and EX-dependent signaling seems to be masked and superimposed by the cytotoxicity of $^1\text{O}_2$ (Wagner et al. 2004; Kim et al. 2008, 2012).

To find out which of the $^1\text{O}_2$ -responsive genes activated in *flu* prior to the onset of chloroplast leakage is also activated in wild type subjected to mild light stress via EX-dependent signaling, the following analysis was carried out. First, early $^1\text{O}_2$ -responsive genes in the *flu* mutant were identified by comparing RNA-seq-based transcriptomes of light-grown *flu* and wild-type seedlings shifted to the dark for 8 h and re-exposed to light for 15 and 30 min and 2 h. Differentially up-regulated genes of the *flu* mutant after 15 min of re-illumination were considered to represent $^1\text{O}_2$ -responsive genes that are activated by the release of $^1\text{O}_2$ prior to the loss of chloroplast integrity. A total of 394 genes belong to this group. The number of differentially up-regulated genes in the *flu* mutant rapidly increased to 1,172 (30 min of re-illumination) and 3,187 (2 h of reillumination) after chloroplast leakage and collapse of the central vacuole had been initiated. The 394 early $^1\text{O}_2$ -responsive genes of *flu* seedlings were then compared to light stress-induced and EX-dependent gene activities of wild type. In wild type exposed to a mild combined higher light/lower temperature stress for up to 24 h, the expression of 64 of the light stress-induced genes was suppressed in *ex1/ex2* seedlings. Only five of these genes were found among the early $^1\text{O}_2$ -responsive genes of *flu* (Kim et al. in preparation). Thus, only a minute fraction of the early $^1\text{O}_2$ -responsive genes of the *flu* mutant is up-regulated in wild type placed under mild light stress that activates $^1\text{O}_2$ -mediated and EX-dependent signaling without causing detectable oxidative damage (Fig. 3). Conversely, most of the EX-dependent genes activated in wild type under mild light stress are not up-regulated in the *flu* mutant following a dark-to-light shift (Fig. 3).

The identification of signaling molecules

$^1\text{O}_2$ -mediated and EX-dependent signaling has been established not only in the *flu* mutant but also in wild type (Kim et al. 2012; Kim and Apel 2013). In the past, $^1\text{O}_2$ had been largely considered to be detrimental to cells due to its high reactivity and potential toxicity (Sies and Menck 1992; Girotti 2001; Davies 2004). It is not known yet how such a highly reactive molecule may initiate signaling rather than causing oxidative damage. It has been proposed that $^1\text{O}_2$ generated within chloroplasts acts also as a signal in the surrounding cytoplasm (Krieger-Liszkay et al. 2008). However, because of its high reactivity and short half-life, it seems more likely that $^1\text{O}_2$ is confined to the plastid compartment and interacts with other plastid components close to its site of production (Ohad et al. 2011) giving rise to more stable signaling molecules that cross the chloroplast envelope membrane and modulate nuclear gene expression. Inactivation of the two plastid proteins EX1 and EX2 abolishes cellular responses to $^1\text{O}_2$, further supporting the notion that the primary reactions of $^1\text{O}_2$ initiating signaling must take place within the plastid. Thus far, two targets within the plastid have been identified that react with $^1\text{O}_2$ and release oxidation products that may act as a potential signal. Both these targets are localized close to the site where $^1\text{O}_2$ is generated. One of them is β -carotene, the other one is the D1 protein of the RC of PSII.

As discussed earlier, β -carotene within the core of PSII is not close enough to the RC P680 Chl to quench the excitation energy of the triplet state of this Chl and to prevent generation of $^1\text{O}_2$, but it reacts with $^1\text{O}_2$ giving rise to several non-enzymatic oxidation products (Telfer et al. 1994; Ramel et al. 2012a, b). Elevated levels of these products have been found in plants exposed to severe light stress. When applied exogenously to the plant, one of them, β -cyclocitral, confers an enhanced stress resistance and leads to an up-regulation of nuclear genes, most of which had been also activated in the *flu* mutant following the release of $^1\text{O}_2$. Based on these biological activities, β -cyclocitral has been proposed to form part of the $^1\text{O}_2$ -dependent signaling pathway (Ramel et al. 2012b) first described in the *flu* mutant (op den Camp et al. 2003; Wagner et al. 2004). However, β -cyclocitral-induced responses do not seem to require the activity of EX proteins. Up-regulation of most of the β -cyclocitral-responsive genes that are also up-regulated in the *flu* mutant following a dark-to-light shift is

indicative of cellular damage and does not seem to be under control of the $^1\text{O}_2$ - and EX-dependent signaling pathway. One of the hallmarks of $^1\text{O}_2$ -mediated and EX-dependent signaling is the activation of programmed cell death. Such a response has not been reported for plants under severe light stress or treated with 8-oxo-dGTP and thus programmed cell death in plants exposed to mild light stress is probably controlled by signals different from 8-oxo-dGTP.

The second target that interacts with $^1\text{O}_2$ and releases fragments that potentially act as signals is the D1 protein. The D1 protein of the RC of PSII functions as a major scavenger of $^1\text{O}_2$ (Telfer 2005; Vass and Cser 2009). Following its reaction with $^1\text{O}_2$, the D1 protein is proteolytically cleaved to smaller fragments and replaced during the repair of PSII RC by a newly synthesized D1 polypeptide (Aro et al. 1993; Nixon et al. 2004). While in cyanobacteria, one of these proteolytic fragments has been suggested to be biologically active (Stelljes and Koenig 2007), in plants, no such activity has been reported yet. If indeed breakdown products of the D1 or other protein constituents of the core of PSII function as signals that trigger $^1\text{O}_2$ -mediated and EX-dependent signaling, one would expect the EX proteins to be localized close to the RC of PSII. Currently, we are testing possible interactions of EX1 and EX2 with components of the RC of PSII.

Collectively, available experimental evidence suggests that production of $^1\text{O}_2$ within chloroplasts may activate at least two signaling pathways (Kim et al. 2008). Under severe light stress, $^1\text{O}_2$ causes photo-oxidative damage and non-enzymatic lipid peroxidation. Some of the non-enzymatic oxidation products such as 8-oxo-dGTP are biologically active and may act as signals (Mueller et al. 2008; Ramel et al. 2012b). Signaling under such severe stress conditions triggers responses to insults detrimental to the plant not only in wild type but also in *ex1/ex2* mutant plants (Kim et al. 2012). On the other hand, under mild light stress that does not lead to detectable photo-oxidative damage and does not promote non-enzymatic lipid peroxidation, $^1\text{O}_2$ -mediated and EX-dependent signaling triggers the expression of $^1\text{O}_2$ -responsive genes that are distinct from genes up-regulated in plants under severe high light stress and known to enhance stress resistance. If indeed EX proteins operate close to the RC of PSII and EX-dependent $^1\text{O}_2$ -mediated signaling is initiated during light-dependent turnover of PSII, this signaling might be considered to form an integral part of photosynthesis that translates environmental variability affecting photosynthetic electron transport into signals that control the readjustment of the whole organism to environmental changes.

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Fig. 1.

Generation of reactive oxygen species (ROS) by the photosynthetic electron transport chain of higher plants in response to environmental stress. **A** A hypothetical equilibrium between light-driven electron transport by photosystem II (PSII) and the consumption of electrons during CO₂ fixation would minimize the generation of ROS by the photosynthetic apparatus. **B–D** However, the continuously changing environment of plants impedes the establishment of such equilibrium. Under excess light (**B**) or under drought conditions that result in stomata closure and reduced CO₂ fixation (**C**), hyper-reduction of the electron transport chain blocks electron transport by PSII and keeps absorbed light energy trapped within PSII and may cause photo-oxidative damage. To keep the acceptor site of PSII partially oxidized, the water–water cycle (**B**) and photorespiration (**C**) may function as additional electron sinks and lead to an enhanced production of superoxide and hydrogen peroxide. **D** When under severe stress, these photochemical quenching mechanisms are not sufficient to keep the acceptor site of PSII partially oxidized, the excited P680 chlorophyll of PSII reaction center may act as photosensitizer and transform ³O₂ to the highly reactive ¹O₂ by energy transfer. Key enzymes involved in these processes are shown in encircled

numbers: (1) superoxide dismutase, (2) ribulose 1,5-bisphosphate carboxylase, (3) glycolate oxidase, (4) catalase, and (5) ascorbate peroxidase

Fig. 2.

Schematic diagrams comparing $^1\text{O}_2$ -mediated and EX-dependent signaling in light-grown *flu* mutants following a dark-to-light shift (**A**) and in wild type exposed to a mild higher light/lower temperature stress (**B**). **A** The *flu* mutant suffers from an impairment of negative feedback control of tetrapyrrole synthesis. In the dark, the mutant overaccumulates Pchlode within chloroplast membranes. During re-illumination, excited Pchlode acts as a photosensitizer and generates highly reactive $^1\text{O}_2$ by energy transfer to ground state $^3\text{O}_2$. $^1\text{O}_2$ -mediated and EX-dependent signaling in the *flu* mutant operates as a default pathway that leads to an almost immediate loss of chloroplast integrity and a subsequent cell death. As indicated by question marks, it is not clear yet whether $^1\text{O}_2$ -responsive genes cause the loss of chloroplast integrity or chloroplast leakage is triggered by a separate $^1\text{O}_2$ -dependent mechanism. In *flu*, $^1\text{O}_2$ -mediated changes depend on EX1 and EX2 and are suppressed in *flu/ex1/ex2* triple mutants. **B** In wild type exposed to mild light stress, chlorophyll associated with PSII rather than Pchlode acts as a photosensitizer. Besides $^1\text{O}_2$, wild type under mild light stress produces enhanced amounts of H_2O_2 . It is not known yet whether H_2O_2 antagonizes $^1\text{O}_2$ -mediated responses directly by suppressing $^1\text{O}_2$ production or indirectly via gene expression changes. $^1\text{O}_2$ -mediated chloroplast leakage and programmed cell death are controlled by several modulators that in addition to H_2O_2 include the PLEIOTROPIC RESPONSE LOCUS (PRL) 1 protein (Baruah et al. 2009b) and stress acclimation triggered by pre-stress or $^1\text{O}_2$ -mediated gene expression changes. $^1\text{O}_2$ -mediated responses of plants exposed to mild light stress depend on EX1 and EX2 and are suppressed in *ex1/ex2* plants (Kim et al. 2012)

Fig. 3.

Genome-wide transcriptional analysis of $^1\text{O}_2$ - and EX-dependent transcript changes in *flu* and wild type. The *Venn diagram* identifies a total of 5 genes that are up-regulated in the *flu* mutant during 15 min of light following 8 h of dark treatment and that are down-regulated in *ex1/ex2* mutant plants versus wild type exposed to a combined mild higher light/lower temperature stress (Kim et al. 2012). Total transcripts of wild-type and *ex1/ex2* mutant were analyzed and differentially regulated transcripts identified after 0, 15, and 30 min and 2 h of stress treatment. More than 2,000 transcripts were up-regulated in wild type exposed to the mild light stress for up to 24 h, but only 69 of them were suppressed in *ex1/ex2* seedlings (Kim et al. in preparation). $^1\text{O}_2$ -mediated up-regulation of transcripts in the *flu* mutant after 15 min of re-illumination occurs prior to chloroplast leakage. These transcripts are considered to represent genes controlled by $^1\text{O}_2$ -mediated and EX-dependent signaling. A majority of $^1\text{O}_2$ -responsive genes reported earlier (op den Camp et al. 2003; Gadjev et al. 2006) are activated only after the onset of chloroplast leakage and the collapse of the central vacuole. Most of these genes are probably not controlled by EX-dependent signaling, but by other signaling pathways that are activated during the loss of cellular integrity