Journal of Experimental Botany, Vol. 57, No. 8, pp. 1719–1724, 2006
Oxygen Metabolism, ROS and Redox Signalling in Plants Special Issue
doi:10.1093/jxb/erj183 Advance Access publication 23 May, 2006

Journal of Experimental Botany

A genetic approach towards elucidating the biological activity of different reactive oxygen species in *Arabidopsis thaliana*

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Received 23 December 2005; Accepted 6 March 2006

Abstract

Plants are often exposed to external conditions that adversely affect their growth, development or productivity. Such unfavourable environmental stress factors may result in rapid and transient increases of intracellular concentrations of reactive oxygen species (ROS) that are chemically distinct and impact plants either by being cytotoxic or by acting as a signal. Because different ROS are generated simultaneously in different cellular and extracellular compartments, it is almost impossible to link a particular ROS to a specific stress response and to determine its mode of action. The conditional *flu* mutant of *Arabidopsis* has been used to determine the biological role of singlet oxygen. Immediately after a dark/light shift of the *flu* mutant, singlet oxygen is generated within the plastids activating several stress responses that include growth inhibition of mature plants and seedling lethality. These stress responses do not result from physicochemical damage caused by singlet oxygen, but are attributable to the activation of a genetically determined stress response programme triggered by the Executer1 protein. Singlet oxygen-mediated stress responses at the transcriptional level necessitate a retrograde transduction of signals from the chloroplast to the nucleus that activate distinct sets of genes different from those that are induced by superoxide/hydrogen peroxide. Hence, the biological activities of these two types of ROS are distinct from each other. Whether they act independently or interact is not known yet and is the topic of our current research.

Key words: *Arabidopsis*, *executer1*, *flu*, reactive oxygen species, ROS signalling, singlet oxygen, stress response.

Generation of ROS in chloroplasts

During photosynthesis, reactive oxygen species (ROS) are produced primarily inside chloroplasts and peroxisomes (Foyer and Noctor, 2003). Generation of ROS within chloroplasts either directly or indirectly depends on the interaction of chlorophyll and light. Upon illumination the outer electrons of chlorophylls are elevated to the first and second excited singlet states. De-excitation may occur by emitting fluorescent light or heat or by energy transfer to neighbouring chlorophyll molecules. In the case of the special chlorophyll form of the reaction centre, de-excitation may also include the transfer of the excited electron to a redox partner that forms part of the photosynthetic electron transport chain within the thylakoid membrane. In the case of photosystem II, this electron transfer from the excited chlorophyll P680 is blocked when the pool of plastequinone that acts as secondary electron acceptor of PSII is fully reduced (Krieger-Liszkay, 2005). Under such conditions the primary radical pair $P680^{+}Pheo^{-}$, with P680 being the primary electron donor and pheophytin the primary electron acceptor, recombine, and a high yield of P680 triplet formation is observed (van Mieghem *et al.*, 1989; Barber, 1998). Triplet chlorophyll may transfer its excitation energy onto triplet ground state oxygen giving rise to the highly reactive singlet oxygen ($^1O_2$) (Fig. 1; Durrant *et al.*, 1990).

In principle, all excited chlorophyll molecules may generate $^1O_2$, if exposed to high light intensities. However, the bulk of chlorophyll that forms part of the light-harvesting antenna complexes of photosystems II and I is closely associated with various carotenoids, i.e. within van der Waals distance. In this configuration, carotenoids can either quench chlorophyll triplets and dissipate the excess light energy into heat or, if $^1O_2$ has been formed,

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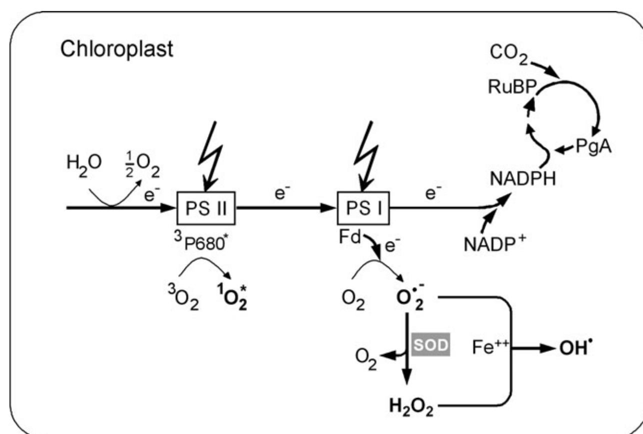

Fig. 1. Generation of ROS within chloroplasts. Under light stress, the excited triplet chlorophyll ^{3}P680* of the PSII reaction centre may transfer its excitation energy onto triplet ground state oxygen $^3O_2$ giving rise to the highly reactive singlet oxygen $^1O_2$. When the light-driven electron transport exceeds the consumption of electron by CO_2 fixation, molecular oxygen can be reduced by PSI to superoxide $O_2^{\bullet-}$ that is rapidly converted to hydrogen peroxide H_2O_2 by superoxide dismutase (SOD). In the presence of transition metal like iron, $O_2^{\bullet-}$ and H_2O_2 can give rise to the production of the highly reactive hydroxyl radical $OH^{\bullet}$. (Adapted from Apel and Hirt, 2004; and reprinted with permission, from the *Annual Review of Plant Biology*, Vol. 55, © 2004 by Annual Reviews www.annualreviews.org).

act directly as $^1O_2$ quenchers (Cogdell and Frank, 1987; Edge *et al.*, 1997). In contrast to the antenna system, in the PSII reaction centre carotenoids are not bound closely to P680 and hence they cannot quench the chlorophyll triplet, thus avoiding a loss in electron transfer efficiency by a competing second light energy-consuming reaction (Ferreira *et al.*, 2004; Kamiya and Shen, 2003; for recent reviews on the topology of β-carotene in the photosystem II reaction centre, see Trebst, 2003; Telfer, 2005). This optimization of electron transfer by the reaction centre of PSII unavoidably leads to the continuous production of $^1O_2$ even at lower light intensities (Keren *et al.*, 1995). The quenching of this $^1O_2$ has been linked to the turnover of the D1 protein of the PSII reaction centre (Hideg *et al.*, 1994). D1 protein degradation and rapid turnover is thought to be essential to detoxify $^1O_2$ directly at the place of its generation and to prevent damage to PSII (Aro *et al.*, 1993*b*). At higher light intensities, when the production of $^1O_2$ exceeds the turnover of D1, photosystem II loses its activity and photoinhibition blocks the electron transport chain (Hideg *et al.*, 1998). This negative impact of high light stress will not occur when the intensity of light-driven electron transport and the consumption of electrons during CO_2 fixation reach equilibrium. However, in their natural habitat plants will only very rarely reach such an equilibrium. The intracellular CO_2 concentration may constantly change and the light intensities also undergo drastic fluctuations throughout the day. A drop in CO_2 concentration or a sudden increase in light intensity may lead to a hyper-reduction of the photosynthetic electron transfer chain that may block electron transport by PSII and enhance the production of $^1O_2$. Plants have evolved two strategies to protect the photosynthetic apparatus against such a photo-oxidative stress: first, the thermal dissipation of excess excitation energy in the PSII antennae (non-photochemical quenching) and, second, the use of additional electron sinks (photochemical quenching). One of these additional electron acceptors is molecular oxygen (Ort and Baker, 2002). It can be reduced by PSI to superoxide (Mehler reaction) (Mehler, 1951) that is rapidly converted to hydrogen peroxide by superoxide dismutases (Fig. 1). In the presence of metal ions, for example, Fe^{2+}, superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) can react and form a third, highly reactive ROS, the hydroxyl radical $OH^{\bullet}$.

When the various protection mechanisms are not sufficient to maintain the acceptor site of PSII in a partially oxidized state, $^1O_2$ production within PSII will increase and will finally lead to the photoinhibition of the electron transport chain (Aro *et al.*, 1993*a*, *b*). If $O_2^{\bullet-}/H_2O_2$ and $^1O_2$ act as signals, their signalling specificity would be expected to be different (Laloi *et al.*, 2004). The enhanced production of $^1O_2$ and $O_2^{\bullet-}/H_2O_2$ occurs under different physiological conditions (Apel and Hirt, 2004). $O_2^{\bullet-}$ and H_2O_2 are produced in varying amounts during photosynthesis. Perception of these fluctuations seems to be involved in controlling the levels of scavengers of H_2O_2 and the up-regulation of ferritin that binds free iron and in this way minimizes the risk of hydroxyl radical formation (Briat *et al.*, 1999). Increased production of $^1O_2$ seems to occur primarily under high light stress conditions that will ultimately result in the inactivation of PSII and photoinhibition (Hideg *et al.*, 1994, 1998) and will activate various stress responses.

Physiological responses to singlet oxygen

In plants under abiotic stress such as drought, high salt, high temperature, or high light stress the concentrations of all major ROS within the chloroplasts are increased simultaneously (Smirnoff, 1993; Fryer *et al.*, 2002; Hideg *et al.*, 2002; Xiong *et al.*, 2002; Apel and Hirt, 2004). Thus, it is almost impossible to attribute a visible stress response to an enhanced level of a particular ROS. To circumvent this problem one would have to find conditions under which only one of the various ROS reaches higher levels and ideally gives rise to a stress response that is easy to score. In earlier work, an experimental system was established that allowed the biological activity of one of these ROS, $^1O_2$, to be analysed.

The conditional *fluorescent* (*flu*) mutant has been identified that is no longer able to suppress the accumulation of protochlorophyllide (Pchlide), the immediate precursor of chlorophyll(ide) (Meskauskiene *et al.*, 2001). In wild-type

plants Pchlide is bound to the NADPH-Pchlide oxido-reductase (POR) that together with Pchlide and NADPH forms a ternary photoactive complex that upon illumination catalyses the reduction of Pchlide to Chlide (Reinbothe *et al.*, 1996). In the dark, excess amounts of free unbound Pchlide accumulate in the mutant that, upon illumination, act as a photosensitizer and by energy transfer generate $^1O_2$ (Rebeiz *et al.*, 1988; Spikes and Bommer, 1991; Shalygo *et al.*, 1998). Etiolated seedlings of the mutant shifted from the dark to the light rapidly bleach and die, whereas etiolated wild-type seedlings green normally (Meskauskiene *et al.*, 2001). The mutant can be rescued, however, by growing it from the very beginning under continuous light. Under these growth conditions Pchlide is immediately reduced to Chlide such that it does not reach toxic levels. Mutant plants grown under continuous light are phenotypically indistinguishable from wild-type plants (Meskauskiene *et al.*, 2001). They can be used to analyse the effect of $^1O_2$ at any developmental stage throughout the life cycle of the plant, simply by transferring plants from continuous light to the dark for 8 h and re-exposing them to light. In this work the effect of $^1O_2$ in young seedlings and mature plants that are ready to bolt was studied. Mutant seedlings grown under non-permissive 16/8 h light/dark cycles bleach, but do not collapse as etiolated seedlings. In mature plants, the response to $^1O_2$ is very distinct from that of seedlings. The release of $^1O_2$ following the shift from the dark to the light leads to a rapid inhibition of the growth rate (Fig. 2). The growth inhibition is maintained as long as these plants are kept under light/dark cycles. The inhibition is reversible, however, after the plants have been returned to the continuous light treatment (op den Camp *et al.*, 2003).

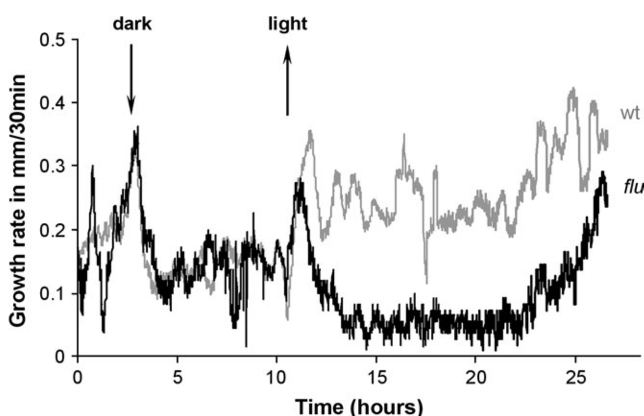

Fig. 2. Growth inhibition in bolting *flu* plants provoked by the release of singlet oxygen following the dark to light shift. Wild-type (wt) and *flu* plants were grown under continuous light until they were ready to bolt. At this stage, plants were transferred to the dark for 8 h then re-exposed to light. Growth rate of *flu* and wild-type plants was recorded continuously during the experiment.

Genetic control of the response to singlet oxygen

These striking stress responses triggered by $^1O_2$ could be due to the cytotoxicity or the signalling role of this ROS. A genetic approach was used to address this question. *flu* plants were mutagenized with EMS and second-site mutants that no longer showed the bleaching of seedlings or the growth inhibition of mature plants, when grown under non-permissive light/dark conditions, were identified. The screening strategy was based on the assumption that the inactivation of components of signal transduction pathways involved in $^1O_2$-mediated signalling leads to the abrogation of either the cell death response of seedlings or the growth inhibition of mature plants or both responses. Two groups of second-site mutants of *flu* were identified, those that are no longer able to overaccumulate Pchlide in the dark and a second group that, in the dark, contains similar excess levels of Pchlide as the parental *flu* line (Goslings *et al.*, 2004; Wagner *et al.*, 2004). Members of this latter group of mutants generated similar amounts of $^1O_2$ as *flu*, when transferred from the dark to the light. Within this group three subgroups could be distinguished that genetically define two different signalling pathways triggering either the bleaching of seedlings or the growth inhibition of mature plants. The third subgroup identifies a common branch point from which the two other response pathways seem to diverge (Fig. 3). Members of this third subgroup seem to have lost the ability to sense the release of $^1O_2$ and they behave like the wild type, despite the release of this ROS after a dark/light shift. These mutants were dubbed *executer* (Fig. 3). Initially, 15 mutants of this type were isolated independently from different batches of M2 plants which later on were shown to be allelic. Thus, there is only a single locus, *Executer1*, which seems to be crucial for triggering $^1O_2$-mediated stress responses (Wagner *et al.*, 2004). The *Executer1* gene was identified by map-based cloning. Executer1 is unrelated to known proteins and has been shown to be a chloroplast protein. Highly conserved homologues of the *Executer1* gene could

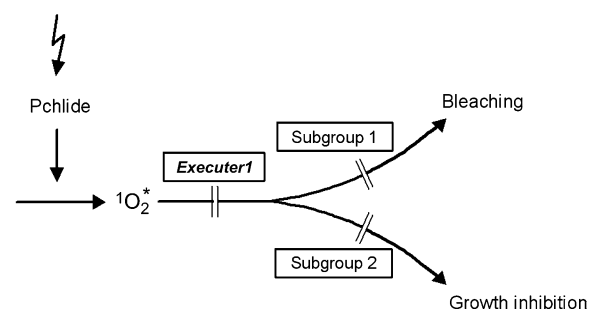

Fig. 3. Suppressor mutants of *flu*. Three different types of second-site mutants of *flu* can be distinguished that genetically define two different signalling pathways (subgroups I, II) and identify a common branch point from which these stress response pathways diverge (subgroup III, *executer1*).

be found in all higher plants, including major crop plants, for which expressed sequence tag sequence data were available. Growth of mature *flu* plants kept under light/dark cycles was blocked, whereas *flu/executer1* double mutants under these conditions grew like the wild type (Wagner *et al.*, 2004). However, double mutants transformed with a wild-type copy of the *Executer1* gene, showed the same severe growth inhibition as the *flu* parental line. Similarly, *flu* mutant seedlings kept under non-permissive light/dark conditions bleached and died, whereas seedlings of the *flu/executer1* double mutant grew like the wild type. However, seedlings of the double mutant complemented with the wild-type copy of the *Executer1* gene bleached and died like seedlings of the *flu* parental line (Wagner *et al.*, 2004). These results clearly demonstrate that the severe growth inhibition of mature plants and seedling lethality are not a consequence of photo-oxidative damage inflicted upon plants by toxic levels of $^1O_2$. Instead, after the release of $^1O_2$ had been perceived by the plant, genetic programs were activated that require the intact Executer1 protein within the plastid. Furthermore, the results of these complementation assays suggest that $^1O_2$ in *flu/executer1* mutant plants reaches similar levels as in *flu* and that, in both mutants, these amounts are too low to cause visible cytotoxic damages.

Under slightly different growth conditions $^1O_2$ in *flu* may also act as a cytotoxin causing severe stress reactions that are not abrogated by the inactivation of Executer1. When 4-d-old etiolated seedlings of *flu* or *flu/executer1* are transferred from the dark to the light, not only *flu* but also *flu/executer1* mutant seedlings collapse and die (D Przybyla, unpublished data). These different responses of *flu/executer1* seedlings to the release of $^1O_2$ can be attributed to different amounts of the photosensitizer Pchlide that, in 4-d-old etiolated seedlings, reach a roughly 3–4-fold higher level than at the end of an 8 h dark period in 4-d-old seedlings that were kept under light/dark cycles. Thus, when analysing the biological activities of ROS in plants it seems crucial to distinguish between conditions that endorse either the cytotoxic or the signalling activity of these molecules. In *flu* plants the *executer1* mutation can be used to define such conditions for $^1O_2$.

Distinct responses of the genome to different ROS

Enhanced levels of $^1O_2$ are generated in plants exposed to severe light stress that may cause inactivation of photosystem II and photoinhibition of the photosynthetic electron transport (Hideg *et al.*, 1994, 1998). On the other hand $O_2^{\bullet-}$ and H_2O_2 are continuously produced in varying amounts during photosynthesis prior to photoinhibition. If these ROS act as signals, enhanced levels of $^1O_2$ may be expected to cause responses that are different from those triggered by $O_2^{\bullet-}/H_2O_2$. This suggestion was tested experimentally by comparing global changes in the expression of nuclear genes of *flu* plants subjected to a dark/light shift with those that occurred after paraquat treatment of *flu* plants kept under continuous light that did not generate enhanced levels of $^1O_2$. The herbicide paraquat acts as a terminal oxidant of photosystem I and, in the light, it reduces oxygen to $O_2^{\bullet-}$ which subsequently dismutates to H_2O_2.

In a first approach, total RNAs were extracted at various times after the beginning of reillumination or following paraquat treatment and then prepared for hybridization to Affymetrix ATH1 gene chips. The transcript levels were expressed relative to those in wild-type controls exposed to a dark/light shift or in mock-treated *flu* plants, respectively. Genes with a 2.5-fold or greater differential expression that were either up-regulated or down-regulated were identified as being affected by $^1O_2$ and/or $O_2^{\bullet-}/H_2O_2$. Based on these criteria, a total of 1206 genes that represent approximately 5% of the total genome were selected (op den Camp *et al.*, 2003). According to the TAIR Gene Ontology annotations (Berardini *et al.*, 2004), 7.4% of them encode transcription factors, 6.8% encode kinase-related proteins, 9.6% are related to responses to stress or abiotic and biotic stimuli, and 14.5% encode plastid proteins. These different classes of genes represent only 5, 4, 5.2, and 12.8% of the total in the *Arabidopsis* genome, respectively. 70 of the genes were specifically up-regulated in *flu* mutants following a dark/light shift, but not during the first 4 h of paraquat treatment. On the other hand, nine genes whose transcript levels were not up-regulated in the *flu* mutant during the first 2 h of reillumination were up-regulated at least 2.5-fold in paraquat-treated *flu* plants. A third group consisted of 31 genes that were up-regulated at all time points under both experimental conditions.

Genes were identified as being activated specifically by $^1O_2$ only, if their expression levels in paraquat-treated plants were equal to or lower than in mock-sprayed control plants at all time points tested. Similarly, genes were considered to be specific for $O_2^{\bullet-}/H_2O_2$ only if their expression level in reilluminated *flu* plants did not exceed that of control plants following a dark/light shift at any time. Because of the stringency of criteria used for the classification of genes, the number of genes that were activated selectively after the release of $^1O_2$ and $O_2^{\bullet-}/H_2O_2$, respectively, is probably much greater. In a less stringent analysis, transcripts were compared that are affected in *flu* 30 min after the beginning of reillumination to those that are affected 4 h following the beginning of paraquat treatment. At these two time points, a similar number of genes was up-regulated more than 2-fold by either $^1O_2$ or $O_2^{\bullet-}/H_2O_2$, i.e. 361 and 357 genes, respectively. Among them, 161 were up-regulated more than 2-fold under both conditions (Fig. 4A; see supplementary Table 1 at JXB online). 200 genes were up-regulated more than 2-fold in *flu* mutants 30 min after a dark/light shift, but not after 4 h

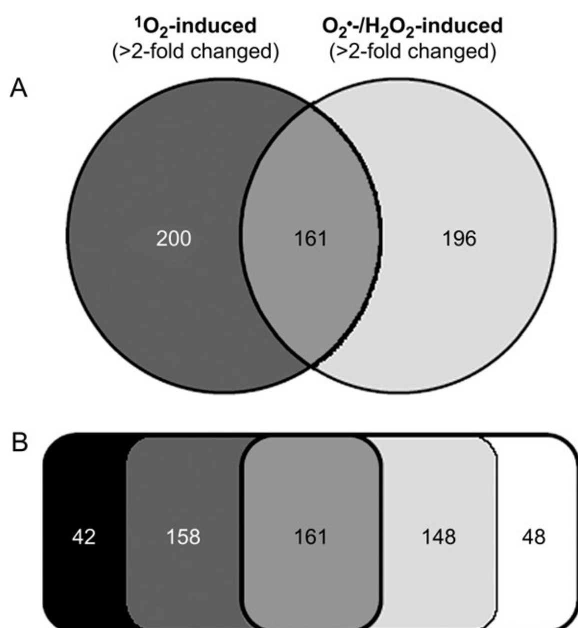

Fig. 4. Relationships of genes up-regulated by singlet oxygen in the *flu* mutant 30 min after reillumination and superoxide/hydrogen peroxide 4 h after spraying treatment with paraquat, based on a Venn diagram. A 2-fold threshold was used for all gene lists. (A) Dark grey: transcript level change >2-fold in *flu* and <2-fold in paraquat treatment. Light grey: transcript level change >2-fold in paraquat treatment and <2-fold in *flu*. Medium grey: transcript level change >2-fold in both conditions. (B) Black: transcript level change >2-fold in *flu* and <1-fold in paraquat treatment. White: transcript level change >2-fold in paraquat treatment and <1-fold in *flu*.

paraquat treatment (Fig. 4A; see supplementary Table 1 at JXB online). Among these 200 genes, 42 have fold-changes below or equal to 1 after paraquat treatment and consequently appear to be highly specific to $^1O_2$; 158 genes have fold-changes between 1 and 2 after paraquat treatment (Fig. 4B; see supplementary Table 1 at JXB online). On the other hand, 196 genes were up-regulated more than 2-fold after 4 h paraquat treatment, but not in *flu* 30 min after the beginning of reillumination (Fig. 4A; see supplementary Table 1 at JXB online). Among these 196 genes, 48 have fold-changes below or equal to 1 in *flu* after reillumination and consequently appear to be highly specific to $O_2^{\bullet-}/H_2O_2$, and 148 genes have fold-changes between 1 and 2 in *flu* (Fig. 4B; see supplementary Table 1 at JXB online). Collectively, these data demonstrate that plants respond rapidly to changes in the concentrations of $^1O_2$ or $O_2^{\bullet-}/H_2O_2$. Because different sets of genes are affected by either $^1O_2$ or $O_2^{\bullet-}/H_2O_2$ their signalling specificity seems to be different.

The changes of nuclear gene expression occur rapidly and very often transiently. Generation of $^1O_2$ and $O_2^{\bullet-}/H_2O_2$ took place within the plastid compartment; hence, these changes in nuclear gene activities imply a rapid exchange of signals between the chloroplast and the nucleus. H_2O_2 has been shown to be freely exchangeable between different intracellular compartments and thus could act as a second messenger that triggers changes in nuclear gene expression outside of the plastid compartment. By contrast, $^1O_2$ is very unstable (Gorman and Rodgers, 1992). Because of its very short half-life it seems unlikely that this ROS leaves the plastid compartment and directly controls nuclear gene activities (Sies and Menck, 1992). Instead, $^1O_2$ may generate a more stable second messenger within the plastid that could disseminate to other subcellular areas, including the nucleus, and trigger changes in nuclear gene expression. Nothing is known yet about the identity of components involved in this $^1O_2$-mediated control of nuclear gene expression. Even though $^1O_2$ and $O_2^{\bullet-}/H_2O_2$-dependent signalling pathways differ with respect to their specificities, it is not clear yet whether they operate independently or may interact. Preliminary data from this laboratory support the latter notion. The overexpression of the plastid-specific thylakoid-bound ascorbate peroxidase reduces the level of H_2O_2 (Murgia *et al.*, 2004) and, at the same time, in the *flu* mutant background, enhances $^1O_2$-mediated stress responses. Thus, $^1O_2$-mediated signalling seems to be part of a more complex ROS-specific signalling network and its effect is antagonized by H_2O_2. At present the analysis of this crosstalk between different ROS continues in our laboratory.

Supplementary data

Supplementary Table 1 containing the microarray data is available at JXB online.

Acknowledgements

This work was supported by grants from the Swiss Federal Institute of Technology (Zurich) and the Swiss National Science Foundation.

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