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

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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, executor1, 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/C255 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 ($^1$O$_2$) (Fig. 1; Durrant et al., 1990).

In principle, all excited chlorophyll molecules may generate $^1$O$_2$, 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 $^1$O$_2$ has been formed,
act directly as 1O2 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 1O2 even at lower light intensities (Keren et al., 1995). The quenching of this 1O2 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 1O2 directly at the place of its generation and to prevent damage to PSII (Aro et al., 1993). At higher light intensities, when the production of 1O2 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 CO2 fixation reach equilibrium. However, in their natural habitat plants will only very rarely reach such an equilibrium. The intracellular CO2 concentration may constantly change and the light intensities also undergo drastic fluctuations throughout the day. A drop in CO2 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 1O2. Plants have evolved two strategies to protect the photosynthetic apparatus against such a photooxidative 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 PSII 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, Fe2+, superoxide anion (O2−) and hydrogen peroxide (H2O2) can react and form a third, highly reactive ROS, the hydroxyl radical OH•.

When the various protection mechanisms are not sufficient to maintain the acceptor site of PSII in a partially oxidized state, 1O2 production within PSII will increase and will finally lead to the photoinhibition of the electron transport chain (Aro et al., 1993a, b). If O2−/H2O2 and 1O2 act as signals, their signalling specificity would be expected to be different (Laloi et al., 2004). The enhanced production of 1O2 and O2−/H2O2 occurs under different physiological conditions (Apel and Hirt, 2004). O2− and H2O2 are produced in varying amounts during photosynthesis. Perception of these fluctuations seems to be involved in controlling the levels of scavengers of H2O2 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 1O2 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, 1O2, to be analysed.

The conditional fluorescent (flu) mutant has been identified that is no longer able to suppress the accumulation of protoclorophyllide (Pchlide), the immediate precursor of chlorophyll(ide) (Meskauskiene et al., 2001). In wild-type
Plants Pchlide is bound to the NADPH-Pchlide oxi-do-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 $^1$O$_2$ (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 $^1$O$_2$ 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 $^1$O$_2$ 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 $^1$O$_2$ is very distinct from that of seedlings. The release of $^1$O$_2$ 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).

**Genetic control of the response to singlet oxygen**

These striking stress responses triggered by $^1$O$_2$ 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 $^1$O$_2$-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 $^1$O$_2$ 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 $^1$O$_2$ 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, Executor1, which seems to be crucial for triggering $^1$O$_2$-mediated stress responses (Wagner et al., 2004). The Executor1 gene was identified by map-based cloning. Executor1 is unrelated to known proteins and has been shown to be a chloroplast protein. Highly conserved homologues of the Executor1 gene could

![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.](image)

![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, executor1).](image)
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 \( \text{O}_2^\cdot \). Instead, after the release of \( \text{O}_2^\cdot \) 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 \( \text{O}_2^\cdot \) 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 \( \text{O}_2^\cdot \) 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 \( \text{O}_2^\cdot \) 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 executor1 mutation can be used to define such conditions for \( \text{O}_2^\cdot \).

**Distinct responses of the genome to different ROS**

Enhanced levels of \( \text{O}_2^\cdot \) 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 \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) are continuously produced in varying amounts during photosynthesis prior to photoinhibition. If these ROS act as signals, enhanced levels of \( \text{O}_2^\cdot \) may be expected to cause responses that are different from those triggered by \( \text{O}_2^\cdot /\text{H}_2\text{O}_2 \). This suggestion was tested experi-

mentally 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 \( \text{O}_2^\cdot \). The herbicide paraquat acts as a terminal oxidant of photosystem I and, in the light, it reduces oxygen to \( \text{O}_2^- \) which subsequently dismutates to \( \text{H}_2\text{O}_2 \).

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 \( \text{O}_2^\cdot \) or \( \text{O}_2^- /\text{H}_2\text{O}_2 \). 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 plastidic 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 \( \text{O}_2^\cdot \) 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 \( \text{O}_2^- /\text{H}_2\text{O}_2 \) 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 \( \text{O}_2^\cdot \) and \( \text{O}_2^- /\text{H}_2\text{O}_2 \), 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 \( \text{O}_2^\cdot \) or \( \text{O}_2^- /\text{H}_2\text{O}_2 \), 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
Collectively, these data demonstrate that plants respond to singlet oxygen and consequently appear to be highly specific to 1O2; 158 genes have fold-changes below or equal to 1 after paraquat treatment and 42 genes were up-regulated more than 2-fold in both conditions. (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.

The changes of nuclear gene expression occur rapidly and very often transiently. Generation of 1O2 and O2−/H2O2 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. H2O2 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, 1O2 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, 1O2 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 1O2-mediated control of nuclear gene expression. Even though 1O2 and O2−/H2O2-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 H2O2 (Murgia et al., 2004) and, at the same time, in the flu mutant background, enhances 1O2-mediated stress responses. Thus, 1O2-mediated signalling seems to be part of a more complex ROS-specific signalling network and its effect is antagonized by H2O2. 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.

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References


