Does singlet oxygen activate cell death in Arabidopsis cell suspension cultures? Analysis of the early transcriptional defence responses to high light stress

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**Abstract**

Can Arabidopsis cell suspension cultures (ACSC) provide a useful working model to investigate genetically-controlled defence responses with signalling cascades starting in chloroplasts? In order to provide a convincing answer, we analysed the early transcriptional profile of Arabidopsis cells at high light (HL). The results showed that ACSC respond to HL in a manner that resembles the singlet oxygen ($^{1}\text{O}_2$)-mediated defence responses described for the conditional *fluorescent (flu)* mutant of *Arabidopsis thaliana*. The *flu* mutant is characterized by the accumulation of free protochlorophyllide (Pchlide) in plastids when put into darkness and the subsequent production of $^{1}\text{O}_2$ when the light is on. In ACSC, $^{1}\text{O}_2$ is produced in chloroplasts at HL when excess excitation energy flows into photosystem II (PSII). Other reactive oxygen species are also produced in ACSC at HL, but to a lesser extent. When the HL stress ceases, ACSC recovers the initial rate of oxygen evolution and cell growth continues.

We can conclude that chloroplasts of ACSC are both photosynthetically active and capable of initiating $^{1}\text{O}_2$-mediated signalling cascades that activate a broad range of genetically-controlled defence responses. The up-regulation of transcripts associated with the biosynthesis and signalling pathways of OPDA (12-oxophytodienoic acid) and ethylene (ET) suggests that the activated defence responses at HL are governed by these two hormones. In contrast to the *flu* mutant, the $^{1}\text{O}_2$-mediated defence responses were independent of the up-regulation of *EDSI* (enhanced disease susceptibility) required for the accumulation of salicylic acid (SA) and genetically-controlled cell death.
Interestingly, a high correlation in transcriptional expression was also observed between ACSC at HL, and the *aba1* and *max4* mutants of Arabidopsis, characterized by defects in the biosynthesis pathways of abscisic acid (ABA) and strigolactones, respectively.

**TEXT**

1O₂ photoproduction in thylakoids of wild type plants and the *flu* mutant of *Arabidopsis thaliana*

Plants exposed to HL produce reactive oxygen species (ROS) in chloroplasts when the electron transport chain of thylakoid membranes becomes over-reduced and the ground state molecular oxygen participates as an electron or energy acceptor of photosystem I (PSI) and PSII.¹ The antioxidant systems in chloroplasts can cope with ROS production if the light stress is moderate or does not perdure; however, ROS can have a detrimental effect on cells if the ROS production eventually overwhelms the antioxidant systems. Out of all ROS, 1O₂ has been proposed to be the major ROS produced at HL.²³ In the conditional fluorescent (*flu*) mutant of *Arabidopsis thaliana*, the chlorophyll precursor Pchlide accumulates in thylakoids and low/medium light irradiance catalyzes the 1O₂ production at high amounts.⁴⁵ Interestingly, 1O₂ can be both a very cytotoxic ROS and a signalling molecule. Thanks to several research studies on the *flu* mutant over the last decade,⁶⁻⁸ a better understanding of the 1O₂-mediated defence responses in plants has emerged. On the one extreme, a genetically acclimatory response is proposed when the 1O₂ production is low in the *flu* mutant. With a greater rate of 1O₂ production, programmed cell death (PCD) is activated, a defence process that is genetically controlled by two chloroplast-located proteins denoted Executer1 (EX1) and Executer2 (EX2).⁹ On the other extreme, when free Pchlide is highly concentrated in plastid membranes 1O₂ is
produced massively during illumination, causing chemical oxidative damage. In this latter case, cell death is no longer under genetic control.

In wild type plants, although $^{1}\text{O}_2$ is constitutively produced in chloroplasts, its production can be enhanced in PSII, through the radical pair mechanism,\textsuperscript{10} when its acceptor side becomes over-reduced due to HL conditions; a situation that differs significantly from the way $^{1}\text{O}_2$ is produced in the \textit{flu} mutant. Whether this makes a difference or not in the $^{1}\text{O}_2$-mediated defence responses of plants has not been addressed yet, but queries about this point have already been raised.\textsuperscript{11} In an attempt to shed more light on this issue, we performed an early transcriptional analysis in ACSC.\textsuperscript{12} Our main finding was that the defence responses activated in ACSC at HL closely resembled those described for several abiotic and biotic stimuli, similarly to what had been previously observed for the \textit{flu} mutant. However, significant differences were pinpointed in the induction of transcripts with key roles in the genetically-controlled cell death of the \textit{flu} mutant as those corresponding with the biosynthesis and signalling cascade of ET, SA and jasmonic acid (JA). In what follows, we outline the main factors that might explain the differences in the $^{1}\text{O}_2$-mediated defence responses between ACSC at HL and the \textit{flu} mutant.

**Are chloroplasts functional in ACSC and, if so, can they initiate defence responses under HL stress?**

Chloroplasts in ACSC proved to be photosynthetically active, although they did not fully mature during cell growth on the basis of several physiological features.\textsuperscript{12,13} This conclusion was further supported when the microarray data of nine-day-old ACSC (\textit{i.e.}, control ACSC growing continuously at a light irradiance of 50 µE m$^{-2}$ s$^{-1}$) and microarray data of light-adapted, seven-day-old seedling plants\textsuperscript{14} were normalized and analysed together.
The results showed that there were approximately 3,500 down-regulated transcripts and 2,700 up-regulated transcripts (with an adjusted \( p \)-value < 0.05) in control ACSC when compared with the seven-day-old seedling plants. In spite of the huge number of transcripts showing significant differential expression in ACSC, the functional enrichment analysis using FatiGo\(^{15,16} \) revealed that only three Gene Ontology (GO) biological process terms were significantly over-represented in the 3,500-down-regulated transcript list with an adjusted \( p \)-value < 0.05 (i.e., GO:0051276, chromosome organization; GO:0006334, nucleosome assembly and GO:0009408, response to heat shock). In contrast, 26 GO biological process terms were significantly over-represented in the 2,700 up-regulated transcript list with an adjusted \( p \)-value < 0.005. It is worth noting that about two-thirds of the GO biological processes over-represented in the up-regulated transcript list could be divided into two groups: one consisting of nine GO terms associated with abiotic stimuli (i.e., phototropism, blue and (far) red photoreceptors and cold) and the other consisting of nine GO terms with chloroplast organization, photosynthesis and chlorophyll biosynthesis (Figure 1).

The 6,200-differentially-expressed transcripts suggested that the metabolic stage of control ACSC (i.e., chloroplast redox state, sugar content, chlorophyll intermediates, etc.) could potentially trigger additional changes in the transcriptional expression, apart from those expected to be induced by ROS production. This issue had already been an object of concern and a matter of debate in several scientific fora before the microarray data became publicly available. However, our functional classification of the 449-differentially-expressed transcripts in ACSC after the dark-to-HL shift (i.e., \( 1500 \mu \text{E m}^{-2} \text{s}^{-1} \) for 30 min) did not exhibit biological processes corresponding with cell organization, development or biogenesis with any statistical significance that could cast doubt on using ACSC as a model system.
to investigate genetically-controlled responses to HL stress. Instead, biological processes associated with responses to abiotic and biotic stimuli, and PCD were over-represented (see below). Additionally, a hierarchical clustering analysis revealed a high correlation between the transcriptional profiles of ACSC at HL and the *flu* mutant,\textsuperscript{6,12} suggesting $^{1}\text{O}_2$ production in ACSC. Particularly, we could infer from the photodamage of the D1 protein that $^{1}\text{O}_2$ was photosensitized in the reaction centre (RC) of PSII.\textsuperscript{12} The over-reduction of the thylakoid membranes of ACSC at HL induced the $^{1}\text{O}_2$ production in active PSII complexes. However, a percentage of $^{1}\text{O}_2$ production was also envisaged to have its origin in non-reducing Q$_B$ PSII complexes (with an inactive acceptor side), largely present in thylakoid membranes of nine-day-old ACSC on the basis of the experimentally-determined low ratio of $F_v/F_M$ (~0.51); where $F_v$ and $F_M$ stand for variable and maximum fluorescence, respectively. In summary, although chloroplasts in ACSC were not fully mature organelles, they were capable of both sensing HL stress and initiating a signalling cascade that activated a broad range of genetically-controlled defence responses.

**Acclimatory or cell death responses?**

The physiological response of ACSC after the HL treatment indicated that the stress caused was moderate. The initial oxygen evolution rate was recovered after the treatment and the cellular growth rates of control and 30-min, HL-treated ACSC remained similar after several days until eventually they both died. Based on the above results, we could draw the conclusion that the applied stress treatment did not cause cytotoxic cell death in ACSC. The functional enrichment analysis revealed that most of the significantly over-represented biological processes corresponded with defence responses to abiotic and biotic stresses (*i.e.*, chitin, water deprivation, hormone stimuli, pathogens, wounding, etc.)
and cell death (*i.e.*, hypersensitive response, host PCD induced by symbiont, innate immune response, etc.). Biological process terms such as PCD and ET-, SA- and JA-mediated signalling pathways hinted that genetically-controlled cell death should take place in ACSC after the 30-min, HL treatment. This would have been our conclusion if physiological cell death had been observed and transcripts with a prominent role in PCD had appeared up-regulated in the differential transcript expression analysis; however, this was not the case.

In the first instance, there was no evidence whatsoever for the early up-regulation of \textit{EDS1} encoding the enhanced disease susceptibility protein 1. This gene is known to be required for the resistance to pathogens, the biosynthetic activation of SA, and the modulation of the \textit{1}O\textsubscript{2}-mediated cell death response. Either the dependence of the SA accumulation on the \textit{EDS1} expression or the fact that SA does not accumulate at HL\textsuperscript{18,19} led us to conclude that the \textit{1}O\textsubscript{2}-mediated defence responses in ACSC were not controlled by SA. The H\textsubscript{2}O\textsubscript{2} production would also have been an indicator of SA accumulation in ACSC; however, we only detected a very slight production of H\textsubscript{2}O\textsubscript{2} when ACSC were subjected to HL stress for 45-min or longer.\textsuperscript{12} These results pointed to the activation of an EDS1/SA-independent signalling pathway in ACSC under HL stress.

In the second instance, several transcripts involved in either the biosynthesis or the signalling pathway of JA and ET were up-regulated in ACSC at HL. Danon and co-workers\textsuperscript{20} proposed both that the SA and ET signalling pathways function additionally during the \textit{1}O\textsubscript{2}-mediated cell death and that the oxylipins OPDA and dinor-OPDA (enzymatically-synthesized in chloroplasts after the release of \textit{1}O\textsubscript{2}) antagonize the cell death-inducing activity of JA in the \textit{flu} mutant. There were several up-regulated ET
transcripts in ACSC at HL that closely tallied with those reported in the flu mutant. Two of them were of particular interest: ERF1, encoding ET responsive factor 1, is directly induced by ET (and also JA)\textsuperscript{21} and ACS6, encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase 6, catalyzes the biosynthesis of ACC, a precursor of ET. Both products, ACC and ET, were suggested to play a prominent role in the \textsuperscript{1}O\textsubscript{2}-mediated cell death of the flu mutant.\textsuperscript{20} Likewise, we identified up-regulated transcripts encoding chloroplast- and cytosolic-located enzymes (LOX3, lipoxygenase 3; AOC3, allene oxidase cyclase 3; GSTU10, GSTU12, GSTF6 and GSTF7, tau and phi glutathione $S$-transferases) with key roles in the biosynthesis and glutathione-conjugation of ODPA. Strikingly, we found no evidence for the up-regulation of transcripts encoding peroxisome-located ABC transporters for OPDA or enzymes that were responsible for the later steps in the biosynthesis of JA.\textsuperscript{22} OPDA has been demonstrated to play several roles in the early response to oxidative stress in plants, the induction of Ca\textsuperscript{2+} and wounding signals, and the inhibition of cell death.\textsuperscript{20,22,23} Taki and co-workers\textsuperscript{23} proposed that there was a group of transcripts that responded to OPDA, but not to JA (or methyl jasmonate, MeJA). Using the lists of the OPDA- and JA-specific up-regulated transcripts provided by the cited authors, we found that ACSC at HL exhibited higher correlation with the OPDA treatment than with the JA or MeJA treatments (Figure 2).

Taking together all the pieces of information extracted from the differential expression analysis on hormone stimuli, we can reach the conclusion that the EDS1/SA and JA signalling cascades were not activated in ACSC at HL. Both signalling cascades were shown to be responsible for the \textsuperscript{1}O\textsubscript{2}-mediated cell death in the flu mutant. Likewise, the role of ET on cell death would only be partial if an additional effect of ET and SA was indeed required.\textsuperscript{20} Although higher expression of transcripts involved in the OPDA
biosynthesis, but not in the JA biosynthesis, cannot be directly associated with OPDA biosynthetic alterations in ACSC, several examples of prompt accumulation of OPDA and late accumulation of JA have been reported for SA-deficient mutants in response to light or pathogen treatments.\textsuperscript{17,24} We thus infer that $^{1}$O$_{2}$ production during the HL treatment induce lipid peroxidation in chloroplasts of ACSC that presumably ends up with the temporal accumulation of OPDA, supporting the view that cell death is inhibited in ACSC during the early stage of the HL treatment. Why EDS1/SA and JA signalling cascades are not activated in ACSC at HL is not known yet. One speculative explanation might be that the level of $^{1}$O$_{2}$ production in ACSC at HL was low, when compared with the flu mutant. Here, it is worth noting that oxygen evolution was only inhibited 30\% in ACSC during the HL treatment. Consequently, if the $^{1}$O$_{2}$ production was kept low, the EX1/EX2 signalling pathway would remain inactive and, instead, an oxylipin-dependent signalling pathway would be activated, where OPDA (and presumably other oxylipins) had a prominent role in the activation of transcripts associated with oxidative stress. Alternatively, it would be possible that the perception of $^{1}$O$_{2}$ production and the activation of the EX1/EX2 signalling pathway depend on the specific site of $^{1}$O$_{2}$ production in thylakoid membranes (\textit{i.e.}, in surface-exposed regions of the thylakoid membranes, where free Pchlide accumulates in the flu mutant, or in the membrane-hidden region of PSII RC of ACSC or wild type plants). Whatever the reason might be, the $^{1}$O$_{2}$ production in ACSC mediated defence responses whose immediate effect was not the activation of PCD (at least under our experimental conditions). Figure 3 shows a schematic diagram summarizing the signalling cascade of the $^{1}$O$_{2}$-mediated defences in ACSC at HL.

\textbf{Is }$^{1}$O$_{2}$\textbf{ produced in max4 and aba1 mutants of Arabidopsis?}
In a hierarchical clustering analysis, we unexpectedly observed a high correlation between the transcriptional profiles of ACSC at HL, and the max4 and aba1 mutants of Arabidopsis, characterized by being defective in strigolactones and ABA, respectively. These two hormones are (apo)carotenoid-derived products whose absence produces alterations in both the photosynthetic apparatus25,26 and the induction of non-photochemical quenching (NPQ).25,27 When the differential transcript expression profiles of max4 and aba1 were compared with the list of transcripts specifically up-regulated by $^1$O$_2$, H$_2$O$_2$ and O$_2^{ullet-}$,28 we found that several tens of transcripts specifically up-regulated by $^1$O$_2$ were significantly represented in max4 and aba1, in contrast to the other two ROS. Does this mean that max4 and aba1 produce $^1$O$_2$? We cannot give an accurate answer yet; however, based on the recent study carried out with the double xanthophyll mutant of Arabidopsis named npq1lut2,29 we might envisage that (the single xanthophyll mutant) aba1 could also produce $^1$O$_2$. All the single or double xanthophyll mutants of Arabidopsis are characterized by exhibiting an inhibited induction of NPQ,25,27,30 which means that their ability to cope with excess energy excitation is impaired. This is clearly manifested in the npq1lut2 mutant, where $^1$O$_2$ production has been demonstrated to occur at HL.29 The inhibition of the NPQ is less severe in aba1 than in npq1lut2 and, consequently, we should probably expect a lower production of $^1$O$_2$. Intriguingly, $^1$O$_2$ in npq1lut2 is perceived as a signalling molecule (rather than a cytotoxic molecule) that activates an acclimatory response, but not PCD, similarly to what we have observed in ACSC at HL. At present, we are working on max4 and the results (to be published elsewhere) indicate that the expression of $^1$O$_2$ markers such as At5g64870, encoding a nodulin-like protein, and lipid peroxidation are both enhanced.

Conclusions
In summary, we can conclude that chloroplasts in ACSC are functional and able to initiate signalling cascades that activate a broad range of defence responses under HL stress. Based on our early transcriptional profile analysis, we can infer that the differential transcript expression is mediated by $^{1}\text{O}_2$ photosensitized in PSII. The induction of transcripts associated with the biosynthesis and signalling cascade of OPDA, but not of SA and JA, also leads us to conclude that PCD is not the immediate response in ACSC at HL. Finally, the high correlation between the transcriptional profiles of ACSC at HL, and max4 and aba1 indicates that $^{1}\text{O}_2$ production ought to take place in these mutants.

Acknowledgements

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References


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Figure Legends

Figure 1

Over-represented biological processes in control ACSC when compared with light-adapted, seven-day-old seedling plants of Arabidopsis Col-0. For the sake of clarity an adjusted p-value of ≤ 0.001 was chosen.

Figure 2

Venn diagram representing the number of up-regulated transcripts in ACSC at HL that are included in the lists of OPDA- and JAs- (JA plus MeJA) responsive genes described in the study by Taki and co-workers.

Figure 3
Hypothetical model of the cellular events associated with the $^1\text{O}_2$-mediated defence responses in ACSC at HL. The dotted line in the square inset indicates the level of $^1\text{O}_2$ production.
Figure 2

ACSC at HL (416)

OPDA-specific responsive genes (157)

JA- and MeJA- responsive genes (371)

343

54

103

19

0

0

352
1O2-mediated defence responses in ACSC

- Activation of defence responses to: chitin, water deficit, wounding, etc.
- Inhibition of programmed cell death