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Light as both an input and an output of wound-induced reactive oxygen formation in Arabidopsis leaves

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Abbreviations: DAB, 3,3'-diaminobenzidine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU, 3-(3',4'-dichlorophenyl)1,1-dimethylurea; NBT, nitroblue tetrazolium; PET, photosynthetic electron transport; PQ, plastoquinone; ROS, reactive oxygen species

The wound response of plants is characterized by rapid changes in gene expression, biochemistry and physiology, and is important both in its own right and as a model for studying events elicited by herbivory. We have recently identified links between light and the wound response in Arabidopsis leaves. This includes an influence of the external light environment on the molecular and biochemical response to wounding, and the observation that endogenous bioluminescence (light emission) is a consequence of tissue damage. Here, we show that this link extends to the production of reactive oxygen species. We show that wounding causes rapid, light-dependent production of superoxide and hydrogen peroxide in chloroplasts via disruption of photosynthesis, and that wound-induced bioluminescence is a consequence of the generation of singlet oxygen.

Light is of fundamental importance for plant biology, and is likely to influence a wide range of plant developmental processes and responses to the environment. Light has already been recognized as an important factor during plant pathogen-interactions,[1] and we recently identified aspects of the wound response that were differentially regulated dependent on the external light environment.[2] Prior to that, we and others had identified low level bioluminescence as an early response to wounding and herbivory.[3-5] Hence, light can act as both an input and an output of the wound response in plant leaves. Spontaneous bioluminescence has been reported to occur in many groups of organisms, and is often linked with the generation of reactive oxygen species (ROS).[6,7] ROS are common components of many stress responses,[8] and it is widely accepted that ROS are produced in wounded plant leaves. However, the nature and origin of wound-induced ROS are relatively poorly defined. In tomato, hydrogen peroxide is produced in response to jasmonate signaling and is involved in the regulation of the later stages of the transcriptional response to wounding, including in systemic leaves.[9-11] H_2O_2 is also produced in response to herbivory, where it has been reported to accumulate extracellularly.[12,13] We were therefore interested to characterize the wound-induced production of ROS in plants in more detail.

Work by Chen et al. implicates singlet oxygen, $^1O_2$, as a cause of luminescence produced by wounded soybean cotyledons. We performed experiments to determine whether wound-induced luminescence in Arabidopsis leaves might also be a consequence of $^1O_2$ formation. Firstly, we used Rose Bengal as a photosensitizer to generate $^1O_2$ in detached Arabidopsis leaves. **Figure 1A** shows that strong bioluminescence is emitted following illumination of Rose Bengal-treated leaves, suggesting that $^1O_2$ production can indeed cause bioluminescence. We next examined luminescence in wounded leaves equilibrated in either 10 mM histidine, a scavenger of $^1O_2$, or in deuterium oxide (D_2O; heavy water), which extends the half-life of $^1O_2$ around ten-fold. Consistent with the hypothesis that luminescence is a consequence of $^1O_2$ production, the inclusion of histidine reduced the intensity of wound-induced luminescence relative to controls, whereas luminescence was increased in the presence of D_2O (**Fig. 1B and C**). $^1O_2$ accumulation around wound sites in Arabidopsis was also detected by Flors et al. using a fluorescent reporter.

To further characterize the production of ROS in wounded leaves, we used histochemical staining with nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) to detect superoxide and hydrogen peroxide respectively. Both species were found localized around sites of damage (**Fig. 2A–C**) and could be detected within the first few minutes following wounding. Microscopic examination of stained leaves revealed that the majority of $O_2^{\bullet-}$ and H_2O_2 were restricted to the chloroplasts of mesophyll cells (**Fig. 2D–F**). We recently identified a signal originating from photosynthetic electron transport (PET) that

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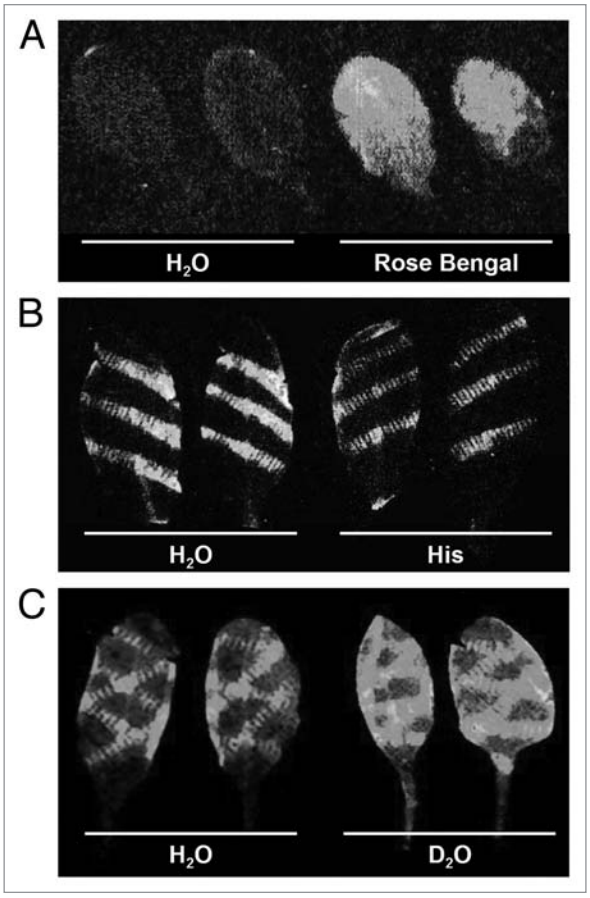

Figure 1. Wound-induced bioluminescence correlates with singlet oxygen production. (A) Luminescence images of Arabidopsis leaves treated with water or the photosensitizer, Rose Bengal. Leaves were vacuum-infiltrated with water or 10 μM Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) and illuminated for 5 min at a PAR intensity of 750 μmolquanta m^{-2} sec^{-1}. (B and C) Luminescence images of wounded Arabidopsis leaves pre-treated with either water (H_2O), 10 mM L-histidine (His) or deuterium oxide (D_2O). Images were taken 5 (A and C) or 10 (B) min after the respective treatments, and are 5 min exposures captured using a fibre optic-coupled CCD system as described previously (Flor-Henry, 2004).[4]

is involved in wound-induced gene expression,[2] and since the most likely source of chloroplast ROS is also PET, we investigated the role of photosynthesis in wound-induced $O_2^{\bullet-}$ and H_2O_2 production. We found that NBT and DAB staining was eliminated in the dark, and that the degree of staining was proportional to the PAR intensity provided following wounding (**Fig. 2A and B**). Pre-treatment of leaves with the PET inhibitors DCMU (3-(3',4'-dichlorophenyl)1,1-dimethylurea) and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone), that respectively prevent the reduction and oxidation of plastoquinone (PQ) in photosystem II, indicated PQ as a likely site for the generation of $O_2^{\bullet-}$, since DCMU but not DBMIB prevented wound-induced NBT staining (**Fig. 2C**). Together, these data suggest that chloroplast ROS are generated as a consequence of perturbation of the light reactions of photosynthesis in wounded leaves. Although $O_2^{\bullet-}$ and H_2O_2 are generated at the wound site coincident with bioluminescence, we were unable to establish any causal link between bioluminescence and either of these ROS.

Taken together, our data identify rapid, localized, light-dependent wound-induced generation of $O_2^{\bullet-}$ and H_2O_2, alongside the generation of singlet oxygen from an unknown source. Furthermore, singlet oxygen is necessary and sufficient to produce luminescence at sites of wounding in plant leaves. Interestingly, Arabidopsis leaves also emit bioluminescence during an R-gene mediated pathogen resistance response, via a mechanism that is independent of $O_2^{\bullet-}$ and H_2O_2.[15] One might therefore speculate that pathogen-induced bioluminescence is also a consequence of $^1O_2$ production. Singlet oxygen is most commonly generated in chloroplasts following perturbation of PET,[16] but there is no evidence for a light requirement for wound-induced bioluminescence and enzymatic lipid peroxidation is perhaps a more likely source of $^1O_2$ in wounded leaves.[4,14] However, our data indicate that wound-induced $O_2^{\bullet-}$ and H_2O_2 do originate from PET. This finding contrasts the view that wound-induced ROS are primarily produced extracellularly, by NADPH oxidase enzymes.[4,17] However, this view is largely based on the use of the NADPH oxidase inhibitor, diphenyl iodonium, which also inhibits photosynthesis.[18] There is clear evidence that NADPH oxidases are important in the late ROS burst produced following wounding,[10,11] but whilst we cannot exclude the possibility that they also contribute to the early response, the data presented here suggest that the majority of early, localized ROS originate from electron transport in the chloroplasts. How wounding might disrupt electron transport during photosynthesis, and what roles, if any, the resultant ROS may play in wound response signaling, remains to be determined.

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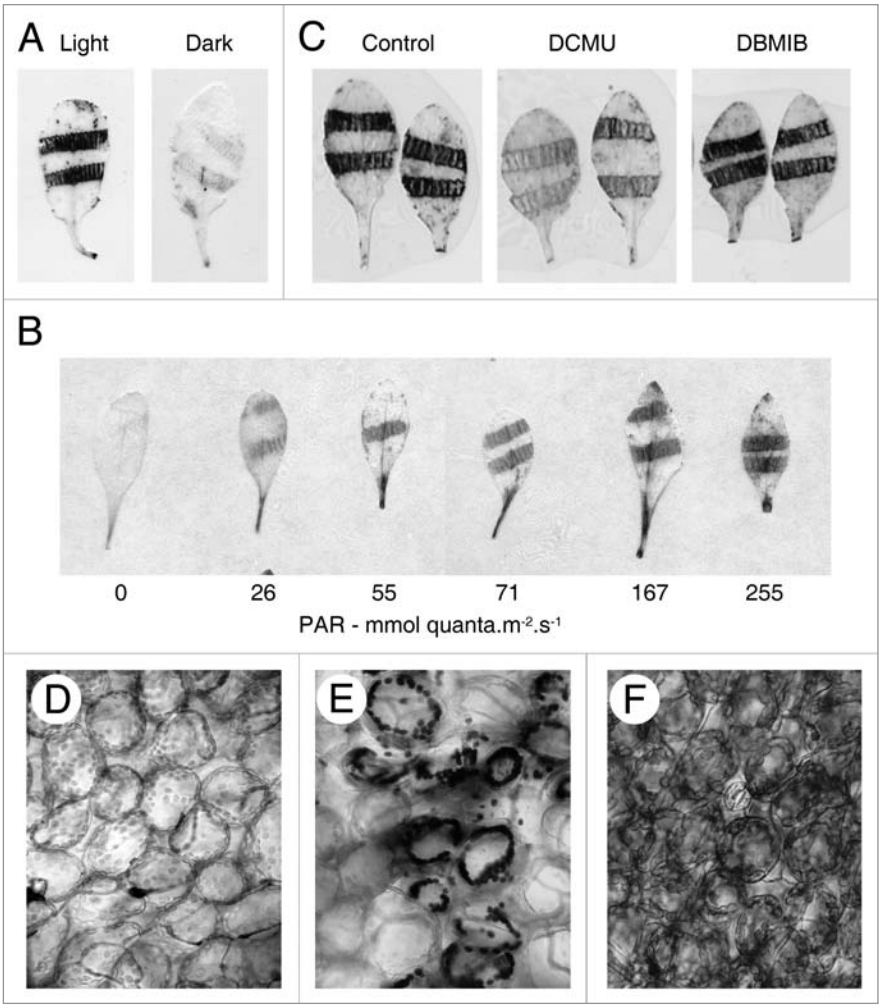

Figure 2. Wound-induced ROS are derived from photosynthetic electron transport. (A) Histochemical localization of superoxide using NBT under ambient light or in the dark. (B) Histochemical localization of wound-induced hydrogen peroxide using DAB at different light intensities. Photon flux densities determined at bench height are indicated below each leaf (PAR; photosynthetically active radiation). (C) Histochemical localisation of superoxide in the presence or absence (Control) of 10 μM DCMU or DBMIB. (D–F) Light micrographs of untreated Arabidopsis leaf tissue (D), and areas surrounding wound sites in leaves cleared of chlorophyll following staining with NBT (E) or DAB (F).