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A proteomic analysis of the wound response in *Medicago* leaves reveals the early activation of a ROS-sensitive signal pathway☆

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ABSTRACT

Wounded *Medicago truncatula* leaves produce a burst of O_2^- (phase I) between 1 and 15 min, then of O_2^- and H_2O_2 (phase II) between 1 and 3 h. Our previous results suggest reactive oxygen species (ROS) may provide signals to mobilise early (6 h), apoplastic, wound-responsive proteins (WRPs). 2DE and MALDI-TOF/TOF were used to analyse how the suppression of ROS production at different time points by diphenyleneiodonium (DPI), affects the expression of WRPs. Rapid (≤3 min) DPI inhibition of phase I O_2^- production suppressed the differential regulation of 7 out of 19 WRPs, which were consequently classified as ROS-dependent WRPs. DPI inhibition of only phase II ROS production failed to suppress the wound regulation of 18 out of 19 WRPs, but led to the altered expression of 1 ROS-dependent WRP and 2 non-WRPs (Group B). The data indicates Group B proteins are alternatively targeted via the modulation of phase II ROS production. This reinforces an important role for phase I O_2^- signalling in the early wound response, but indicates that this response is partly regulated by phase II of the oxidative burst. This data provides an informed basis for further proteomic studies aimed at identifying early activated O_2^- signalling components in wounded *Medicago*.

1. Introduction

Reactive oxygen species (ROS {namely superoxide [O_2^-], hydrogen peroxide [H_2O_2] and hydroxyl radical [$^{\cdot}OH$]}) are known to have important roles in a variety of biological processes such as growth and, development [1,2], programmed cell death [3,4], stomatal responses [5,6], hormone signalling and the response to stress [7].

During plant stress, ROS production can arise from a variety of sources, and there is evidence that different plant species can place emphasis on different mechanisms [8,9]. For instance, germin-like oxalate oxidases have been implicated as a major source in wheat and ryegrass [10,11], whereas polyamine oxidases have been implicated in ROS production in tobacco [3]. Moreover, plants can utilise differing mechanisms of ROS production in different phases of the stress-response [11,12]. However, in many plant systems, stress-related production of ROS is thought to be mediated mainly by the activation of a membrane-bound NADPH oxidase complex [4,13,14], or apoplastic peroxidases, such as that described in French bean [15].

Abbreviations: 2-DE, two-dimensional gel electrophoresis; CW, cell wall; DAB, 3,3-diaminobenzidine; DPI, diphenyleneiodonium chloride; ECM, extracellular matrix; NTB, nitroblue tetrazolium; ROS, reactive oxygen species; WRPs, wound-responsive proteins.

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In order to probe the relation of ROS signalling with many biological processes, several studies have utilised pharmacological inhibitors and/or NADPH oxidase knockout mutants for the inhibition of ROS production. Diphenyleneiodonium (DPI) has been frequently utilised for the inhibition of plant NADPH oxidase production of ROS in levels as low as 1–5 μM [16]. DPI is also known to be effective in inhibiting H_2O_2 production from class III peroxidases when used at higher concentrations (>50 μM; [17]).

The knockout of the NADPH oxidase, *Atrboh*C, in the *Arabidopsis* mutant *rhd2* produces an altered root phenotype, which can be phenocopied by inhibiting NADPH oxidase with DPI [1]. In other studies, the silencing of NADPH oxidase genes in Arabidopsis [4], tobacco [18] and tomato [2] led to suppression of ROS production and a diminished defence to pathogen attack. Analogous results were obtained in cultured parsley cells, where DPI inhibited elicitor-stimulated ROS production and blocked defence gene activation and phytoalexin accumulation [19]. Although these studies evidence some of the consequences of repressing ROS production *in planta*, to our knowledge the direct effects of this repression on the plant proteome has yet to be studied.

In plants, a variety of stress stimuli have been seen to induce an oxidative burst, including biotic factors (reviewed in [20,21]) and abiotic stimuli, such as high light stress [22,23], desiccation [24] and mechanical wounding [11,25]. A number of similarities in the cellular responses to these stresses have been reported, indicating that ROS signalling is likely to be a central component of the plant response to a variety of stress conditions [26–28]. Nevertheless, different conditions of stress can induce the formation of different ROS as well as in different intensities, duration and intracellular locations, all of which could provide essential cues for the development of stress-specific, down-stream molecular responses [22,29–31].

The interplay between ROS-producing and ROS-scavenging pathways will determine the intensity, duration and localisation of specific ROS signals. This interplay and the resulting signal transduction requires tight regulation and is likely to employ a variety of factors involved in ROS perception, feedback amplification and/or inhibition loops [29]. The existence of such mechanisms implies that the artificial inhibition of basal or stress-related ROS signalling could trigger altered gene activities designed to compensate for the interrupted signal pathway. Some evidence for this has been obtained in *Arabidopsis* using knock-out mutants deficient in the cytosolic ascorbate peroxidase, Apx1, which is considered essential for the regulation of ROS levels and the maintenance of chloroplastic functions under light stress [32]. Knock-out *Apx1* plants under light stress [32] have been shown to have higher transcript levels of MAPK3, a mitogen activated protein kinase which has been associated with ROS-related signal transduction [33], as well as higher levels of the transcription factors HSF21 and Zat12, both of which are thought to transduce the terminal steps of oxidative stress signalling to target genes under high light conditions [32].

In our earlier work, we have studied how wounding affects the apoplast proteome [34] of *Medicago* leaves [25]. The data suggested that ROS may be an important signal for the mobilisation of early (6 h), leaf apoplastic, wound-responsive proteins (WRPs) [25]. In the current study, we report that the inhibition of wound-related ROS signalling in *Medicago* leaves by DPI at different time points during the oxidative burst markedly alters the range of apoplastic proteins targeted for differential expression. In particular, we report that phase I O_2^- production in *Medicago* is an essential signal for the mobilisation of several early, WRPs in the leaf apoplast. However, further data suggests that the expression of these early WRPs is partly modulated by ROS species produced in phase II of the oxidative burst.

2. Materials and methods

2.1. Plant material

Seeds of *Medicago truncatula* cv. Jemalong were placed on water-soaked filter paper for 48 h and the germinated seedlings then planted in a substrate composed of sand:peat:soil (1:1:1). Growth was in a 19/25 °C, night/day cycle with a photoperiod of 12 h and light intensity of 250 μmol m^{-2} s^{-1}. Leaves were harvested for extraction after 42–45 days.

2.2. Protein extraction

Wounding of leaves employed a scalpel blade to transversally section the leaf into two halves. The wounded leaves were then vacuum-infiltrated for 3 periods of 20 s with either deionised water alone or with 5 μM DPI. The leaves were then placed on water-dampened tissue under light (250 μmol m^{-2} s^{-1}) at 25 °C for 6 h, before being frozen in liquid nitrogen. As the unwounded control, plant leaves were detached from the plant and immediately frozen in liquid nitrogen.

Ionically bound (IB) and soluble proteins of the intercellular fluid (IF) were extracted essentially at 4 °C as described previously [34]. Briefly, for IB proteins, leaves were homogenised in 2 mL/g (fresh weight) of sodium acetate buffer (15 mM, pH 4.5) and centrifuged at 4500 *g* for 5 min. The crude cell wall pellet was washed by centrifugation at 4500 *g* in 5 ml/g (fresh weight) of 1% Triton X-100 followed by three washes in 10 ml/g (fresh weight) of sodium acetate buffer (pH 4.5). The pellet was then resuspended in 1 mL/g (fresh weight) of 1 M KCl for 5 min and centrifuged at 4500 *g* to yield the saline extract. For IF proteins, sectioned leaf pieces were washed with distilled water and vacuum infiltrated with sodium acetate buffer (15 mM, pH 4.5), for three periods of 30 s and carefully blotted before centrifugation at 1480 *g* for 15 min to collect the infiltrate. Both IB and IF extracts were ultrafiltered through a 0.45 μM filter (Schleicher & Schuell, Germany) before being further clarified with the 2-DE Cleanup Kit (GE Healthcare, NJ, USA) as per the manufacturer's instructions.

2.3. The detection of hydrogen peroxide and superoxide in wounded leaves

Wounded leaves placed on water-dampened tissue and left under light (250 μmol m^{-2} s^{-1}) at 25 °C for 0.5, 1,3, 7.5, 15, 30, 60, 120, 180, 240, 300, 320 or 360 min. For the detection of H_2O_2, leaves were infiltrated with 5 mM 3,3 diaminobenzidine (DAB; Sigma, USA), at pH 3.8. For the detection of O_2^-, leaves were infiltrated with 6 mM nitroblue tetrazolium (NBT; Fluka, USA).