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S-nitrosylation in apoplastic fractions of *Brassica juncea* hints regulation of wide spectrum of stress/redox/metabolic pathways and spatial distribution of targets in cold stress

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Apoplast or the extracellular space is an important site for stress signal perception or initial signaling in response to environmental stimuli. Nitric oxide (NO) is a signaling molecule which modify proteins by S-nitrosylation (a post-translational modification), resulting in the formation of S-nitrosothiols. However, NO mediated signaling in the apoplast remains largely unknown. To decipher this, apoplastic proteins were extracted using vacuum infiltration. Western blotting using anti-RuBisCO antibody and glyceraldehyde 6-phosphate dehydrogenase activity assay, showed purity of the extracts without any chloroplastic and cytosolic contamination. NO was measured in the apoplast of Brassica juncea seedlings using iNO measuring system, which showed that non-enzymatic nitrite reduction was increasing NO production during cold stress. Thiol pool quantification using Ellman's assay showed cold-induced increase in the protein (including S-nitrosothiols) as well as non-protein thiols. About 52 S-nitrosylated spots were resolved using Biotin Switch Technique, neutravidin agarose chromatography and 2-DE in cold stress. Moreover, identification of cold-stress-modulated putative S-nitrosylated proteins by nLC-MS/MS showed that only 38.4% targets with increased S-nitrosylation were secreted by classical pathway, while the majority (61.6%) of these was secreted by unknown/non-classical pathways. About 41% and 38% targets were metabolic/cell-wall-modifying and stress-related, respectively, suggesting the potential role(s) of S-nitrosylation in regulating these responses. S-nitrosylation increased glutathione S-transferase and dehydroascorbic reductase activity, promoted ROS detoxification by ascorbate regeneration and hydrogen peroxide detoxification in cold-stress. A total of 48 putative S-nitrosylated targets were identified, including 25 novels. This study was the first attempt which showed NO mediated cold-stress signaling in the apoplast.

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