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Proteomic analysis of the effect of plant-derived smoke on soybean during recovery from flooding stress

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ABSTRACT

Flooding negatively affects the growth of soybean, whereas the plant-derived smoke enhances seedling growth of crops. To clarify the mechanism underlying the recovery from flooding stress, proteomic analysis was performed based on morphological results. Growth of soybean seedlings was inhibited under flooding stress, but it recovered after water removal following treatment with plant-derived smoke. Sucrose/starch metabolism and glycolysis were suppressed in smoke-treated flooded soybean compared to flooded soybean. The protein abundance and gene expression of *O*-fucosyltransferase family proteins related to the cell wall were higher in smoke-treated flooded soybean than in flooded soybean. Protein abundance and gene expression of peptidyl-prolyl *cis-trans* isomerase and Bowman-Birk proteinase isoinhibitor D-II were lower in smoke-treated flooded soybean than in flooded soybean. Taken together, these results suggest that plant-derived smoke enhances soybean growth during recovery from flooding stress through the balance of sucrose/starch metabolism and glycolysis. Furthermore, the accumulation of cell-wall related protein might be an important factor contributing to recovery of soybean from flooding stress.

Biological significance: Flooding negatively affects the growth of soybean, whereas the plant-derived smoke enhances the seedling growth of crops. To clarify the mechanism underlying the recovery from flooding stress, proteomic analysis of soybean with different treatments including normal conditions, flooding stress, and flooding stress in the presence of plant-derived smoke was performed in this study. Growth of soybean seedlings was inhibited under flooding stress, however, it recovered with plant-derived smoke treatment during recovery from flooding stress. Sucrose/starch metabolism and glycolysis were suppressed in smoke-treated flooded soybean compared to flooded soybean, which suggests altered sucrose/starch metabolism and glycolysis contribute to soybean growth recovery from flood stress. Furthermore, the protein abundance and gene expression of O-fucosyltransferase family proteins related to the cell wall was higher in smoke-treated flooded soybean than in flooded soybean, which might be an important factor contributing to the recovery of soybean from flooding stress.

1. Introduction

Flooding is a serious environmental stress worldwide and is increasing in frequency due to changes in global climate [1]. Flooding significantly reduces the growth and yield of several crops [2]. Water from transient flooding displaces gases in soil pores and induces hypoxia in plants grown on land with poor drainage [3]. Under hypoxic conditions due to flooding, ATP production and energy transformation through oxidative phosphorylation are impaired [4]. Most flooding-stressed plants change from aerobic respiration to glycolysis and fermentation for energy production [3]. Soybean, which is an important economic crop around the world and a major source of protein and oil [5], is particularly sensitive to stress arising from flooding [6]. The germination, growth, and productivity of soybean are markedly reduced under flooding stress [7]. Under flooding stress, soybean displays inhibited supplement uptake, nitrogen fixation, and growth [8]. Because flooding exerts deleterious effects on growth of soybean, elucidation of stress-response mechanisms in soybean exposed to flooding is

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; ABA, abscisic acid; KAR, karrikin; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

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needed.

In soybean, the responses to flooding stress of different plant organs have been reported [9]. Energy metabolic pathways were regulated by protein phosphorylation while metabolism of phytohormones such as ethylene and abscisic acid (ABA) were related to phosphorylation under flooding [9]. Ethylene responses to flooding stress in early-stage soybean were regulated by phosphoproteins [10]. ABA participates in the initial stage of flooding stress by influencing the expression of mRNA encoding nuclear-localized phosphoproteins [11]. Moreover, protein synthesis was impaired by flooding in soybean through a decrease in the amount of mRNA export/pre-ribosome biogenesis-related nuclear proteins [12]. ABA was involved in the enhancement of flooding tolerance of soybean through the control of energy conservation via the glycolytic system and regulation of zinc finger proteins, cell division cycle 5 protein and transducin [13]. Because multiple signal transduction and energy metabolism pathways are affected by flooding, metabolic alterations enhancing flooding tolerance must be investigated.

Smoke is used in traditional farming systems for improving seed germination and seedling vigor [14]. Plant-derived smoke enhances growth of seedlings of various agricultural and horticultural crops [15]. Catechol is a major component of smoke and can induce root growth and suppress root hair elongation of *Nicotiana attenuata* through reactive oxygen species-mediated redox signaling [16]. Karrikins (KAR), other components of plant-derived smoke [17], are a small class of signaling molecules that belong to the butenolide family. Six KAR compounds with similar action were identified in smoke and induced responses in the model plant *Arabidopsis*, promoting seed germination and plant photomorphogenesis [18–20]. Seeds of *Eragrostis tef* treated with various smoke mixtures exhibited higher germination and produced vigorous seedlings under high temperature and low osmotic potential [21]. Plant-derived smoke has great potential to improve yield of *E. tef* [22]. It is proposed that plant-derived smoke might improve soybean recovery after flooding.

To elucidate the mechanisms influencing the positive effects of plant-derived smoke, comprehensive analyses have been performed. Based on transcriptomic analysis, genes related to auxin biosynthesis and redox homeostasis are altered after treatment of *N. attenuata* with catechol [16]. A proteomic technique in *Arabidopsis* demonstrated the involvement of KAR in photosynthesis, carbohydrate metabolism, redox homeostasis, transcriptional control, and protein synthesis/transport/processing/degradation [23]. Furthermore, plant-derived smoke has been found to regulate drought tolerance in *Arabidopsis* [24,25]. Bu et al. [25] reported that the F-box protein MORE AXILLARY GROWTH2 (MAX2), which functions in KAR signaling, can regulate drought tolerance through impairing the expression of stress-responsive genes and ABA biosynthesis, catabolism, transport, and signaling genes in *Arabidopsis* seedlings. Li et al. [24] reported that KAR receptor KARRIKIN INSENSITIVE2 (KAI2) positively regulated drought tolerance of *Arabidopsis* by increasing both drought avoidance and drought tolerance strategies through enhancement of cuticle formation, ABA-induced stomatal closure, cell membrane integrity and anthocyanin biosynthesis. Plant-derived smoke positively regulates stress responses of plants according to its effective components, which variously work on different pathways.

The effects of plant-derived smoke on the germination and growth of several plants have been evaluated, especially under drought stress. Meng et al. [26] reported that KARs delayed soybean germination by mediating ABA and gibberellin biogenesis under shaded conditions, an effect distinct from the observation in *Arabidopsis*. Its effect on soybean growth under flooding stress has not been analyzed. Clarifying the molecular mechanisms underlying the response of soybean to plant-derived smoke during recovery from flooding stress may facilitate the development of flood tolerance. In this study, morphological analysis was performed first through comparisons among treatments, including soybean grown under normal conditions, flooding conditions, and flooding conditions in the presence of smoke. Based on the morphological results, proteomic and bioinformatic analyses were carried out to explore the molecular mechanisms responsible for the effect of smoke treatment on soybean during recovery from flooding stress. Subsequently, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to confirm the proteomic results.

2. Materials and methods

2.1. Plant material and treatments

For preparation of plant-derived smoke, aerial parts of *Cymbopogon jwarancusa* were collected and dried at 25 °C for 2 weeks [27]. A portion (333 g) of dry material was placed in a burner and heated with an electric heater [28]. Smoke from the plant material was bubbled through 1 L of distilled water and converted into concentrated plant-derived smoke, which was stored at 4 °C. It was further diluted to 2000 ppm.

Seeds of soybean (*Glycine* max L. cultivar Enrei) were sterilized with 3% sodium hypochlorite solution, rinsed twice in water, and sown in 450 mL silica sand in a seedling case (150 × 60 × 100 mm^3). A total of 14 seeds were sown at an appropriate planting density in each seedling case. Soybeans were grown in a growth chamber illuminated with white fluorescent light (160 μmol $m^{-2} s^{-1}$, 16 h light period/day) at 25 °C and 60% humidity. To induce flooding stress, water was added above the soil surface to immerse 2-day-old soybeans for 2 days. For plant-derived smoke treatment, the smoke solution was supplied simultaneously with flooding stress, to treat 2-day-old soybeans for 2 days. After 2 days of treatment, the water was removed, both the flooding group and the smoke group of soybeans were moved to normal growing conditions for 4 days recovery period. The total cultivated period is 8 days for each group. In addition, untreated soybeans not exposed to either flooding or smoke were used as the control. Root and hypocotyl were collected for morphological and proteomic analysis. Root, hypocotyl, and cotyledon were collected for gene expression analysis. Three independent experiments were performed as biological replicates for all experiments, meaning that the plants were sown on different days.

2.2. Protein extraction

A portion (300 mg) of each sample was cut into small pieces and ground in a filter cartridge. It was ground after adding 50 μL lysis buffer containing 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine. Then, 50 μL lysis buffer was added and the mixture was ground. The suspension was incubated for 2 min at 25 °C and centrifuged at 15,000 × *g* for 2 min at 25 °C. Afterward, the filter cartridge was removed and the supernatant collected as total protein.

2.3. Protein enrichment, reduction, alkylation, and digestion

Extracted proteins (100 μg) in lysis buffer were adjusted to a final volume of 100 μL. Methanol (400 μL) was added and mixed with each sample before adding 100 μL chloroform and 300 μL water. After mixing and centrifugation at 20,000 × *g* for 10 min to achieve phase separation, the upper phase was discarded, 300 μL methanol was added to the lower phase, and the mixture was centrifuged at 20,000 × *g* for 10 min. The pellet was collected as the soluble protein fraction [13].

Proteins were resuspended in 50 mM NH_4HCO_3, reduced with 50 mM dithiothreitol for 30 min at 56°C, and alkylated with 50 mM iodoacetamide for 30 min at 37°C in the dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase (Wako, Osaka, Japan) at a 1:100 enzyme/protein ratio for 16 h at 37°C. Peptides were desalted with a MonoSpin C18 Column (GL Sciences, Tokyo, Japan). Peptides were acidified with formic acid (pH < 3) and analyzed by nano-liquid chromatography (LC) mass spectrometry (MS)/MS.