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Profiling of phytohormones in rice under elevated cadmium concentration levels by magnetic solid-phase extraction coupled with liquid chromatography tandem mass spectrometry

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ABSTRACT

Phytohormones, a collection of signal small molecules with various structures, regulate a series of physiological processes of plants. For instance, they regulate the growth and development, response to biotic and abiotic stresses. Quantification of trace endogenous phytohormones is essential to elucidate their molecular mechanisms in response to stresses. However, the structural and chemical diversity of phytohormones make it difficult to purify and enrich multiple phytohormones in one-step. In the current study, a method was developed to comprehensively profile phytohormones, including 8 cytokinins (CKs), indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and 10 gibberellins (GAs) by Fe_3O_4@TiO_2-based magnetic solid-phase extraction coupled with ultra-performance liquid chromatography-electrospray tandem mass spectrometry (Fe_3O_4@TiO_2-based MSPE-UPLC-MS/MS). In the proposed method, the phytohormones in the acetonitrile extract of plant tissues were captured and purified by one-step MSPE using Fe_3O_4@TiO_2 as a sorbent prior to UPLC-MS/MS analysis. The sensitivity, accuracy and reproducibility of the proposed analytical method were demonstrated to satisfy the profiling of multiple phytohormones in plant tissue. We then further used the Fe_3O_4@TiO_2-based MSPE-UPLC-MS/MS method to explore the change of phytohormones in rice under Cd stress. The results showed that CKs, IAA, ABA, JA and biological active GAs all increased under Cd stress, suggesting that these phytohormones may take part in response to Cd stress. The study may promote the further understanding of the physiological functions of phytohormones in response to Cd stress.

1. Introduction

Phytohormones play crucial roles in the regulation of plant growth and development as well as in response to biotic and abiotic stress [1]. They have been categorized into several groups based on their structures and physiological functions, including auxins, cytokinins (CKs), abscisic acid (ABA), jasmonates, salicylates, gibberellins (GAs), ethylene (ET) and brassinosteroids (BRs) [1,2]. Multiple plant hormones often co-regulate the development and stress responses [3–6]. For example, indole-3-acetic acid (IAA) and CKs have an antagonistic relationship for the control over apical dominance, lateral bud release, and lateral root branching [7]; ABA and CKs coordinate seed germination, nodulation, and senescence [8].

Heavy metal ions are highly reactive and toxic to living cells, and their accumulation in plants is a major problem in agriculture [9]. Cadmium (Cd), one of the widespread heavy metal pollutants in environment, can affect plant growth and development. Cd is absorbed by the roots from the soil and transported to the shoot, negatively affecting nutrient uptake and homeostasis in plants. In addition, the transcriptome and proteome of plants are changed, resulting in inhibited root and shoot growth and, ultimately, reduced yield [10]. Oxidative stress caused by Cd can induce DNA damage, modify protein side chains and destroy phospholipids [11]. Previous report also suggested that Cd stress may decrease chlorophyll content in rice seedlings, and thus suppress their photosynthesis [12]. Moreover, Cd stress can stimulate plants producing more thiol-containing compounds, such as glutathione, proline and phytochelatins, that can preferentially bind to Cd-ion based on the binding affinities of their donor ligand to neutralize Cd-ion and prevent themselves from Cd stress to some extent [13].

To date, many studies suggest that phytohormones play a role in the adaptation of rice growing under Cd stress [14–18]. For

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instance, rice roots were exposed to 10 mM SA in nutrient solution for 24 h before Cd treatment, which could alleviate Cd-induced oxidative damage and improve Cd tolerance by enhancing the antioxidant defense activities in Cd-stressed rice [15]. Kao et al. used two cultivars of rice seedlings with different tolerance to Cd to examine the role of ABA in Cd tolerance. The results indicated that the increase of endogenous ABA was helpful to improve Cd tolerance of rice seedlings. ABA may exert its regulatory effect on transpiration rate, which reduces the translocation of Cd to the shoot [17]. In 2014, Zhao and co-workers reported that the homeostasis of ABA was important for rice roots growth under Cd stress [19]. However, most of these researches were based on the exogenous application of phytohormones, and few investigations focused on the profiling of multiple endogenous phytohormones in rice seedlings under Cd stress, which may be vitally important for the understanding of the functional roles of phytohormones under Cd stress. In this respect, a simple and reliable method for comprehensive profiling of multiple phytohormones will be beneficial for in-depth studying the roles of phytohormones under Cd stress.

At present, although mass spectrometry based analytical methods have been developed for quantification of multiple phytohormones [2,20–24], the sample preparation is still indispensable in these analytical methods. This is because of low concentration of phytohormones in plant tissues and complex matrices. Furthermore, the structural and chemical diversity of phytohormones make it difficult to purify and enrich multiple phytohormones in one-step. Kojima et al. developed a multi-step strategy for determination of 43 phytohormones in rice belonging to auxins, ABA, GAs and CKs [25]. The phytohormones in rice were stepwise separated into several fractions by multiple solid-phase extraction (SPE), and the fraction containing auxin, ABA, GAs was derivatized with bromocholine as derivatization reagent to increase the MS detection sensitivity. Subsequently, phytohormones in each fraction were, respectively, analyzed using UPLC-MS/MS [25]. Liu et al. described a method for simultaneous analysis of alkaline (CKs) and acidic phytohormones (auxins, IAA and GAs), in which a binary SPE was employed for purification and enrichment of phytohormones. Alkaline and acidic phytohormones were eluted from different SPE cartridges, respectively. The two fractions of elution were combined for UPLC-MS/MS analysis [26]. Obviously, these multiple SPE strategies were tedious and time-consuming. However, one-step sample preparation protocols for simultaneous enrichment of alkaline and acidic phytohormones are still rarely proposed [27].

Magnetic phase extraction (MSPE) has drawn much attention in sample preparation because of rapid and ease of operation. Compared with traditional SPE, the magnetic adsorbents are dispersed in solution and do not need to be packed into a SPE cartridge, thus the column blocking during SPE extraction can be avoided. Moreover, mass transfer of analytes could be facilitated by drastically increasing the interfacial area between adsorbent and sample solution, thus improving sample preparation efficiency [28]. Recently, MSPE is widely used for separation and pre-concentration of analytes in environmental and biological fields. However, the preparation of magnetic adsorbent is relatively tedious [29]. In the current study, Fe_3O_4@TiO_2 magnetic nanoparticles were easily prepared by liquid-phase desorption (LPD). Using the prepared Fe_3O_4@TiO_2, we established a simple and reliable Fe_3O_4@TiO_2-based MSPE-UPLC-MS/MS method for comprehensive profiling of phytohormones, including IAA, ABA, jasmonic acid (JA) and GAs, CKs in rice sample. Magnetic TiO_2 particles were prepared and used for efficient enrichment and purification of phytohormones in acetonitrile extract of rice sample with one-step. Using the developed method, we investigated the concentration variation of multiple phytohormones in rice seedlings (*Oryza sativa* L.) under Cd stress.

2. Materials and methods

2.1. Chemicals and reagents

Phytohormone standards: indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), gibberellins (GA1, GA3, GA4, GA7, GA9, GA12, GA19, GA20, GA24, GA53); N^6-isopentenyladenine (iP), isopentenyladenine riboside (iPR), N^6-isopentenyladenine 9-glucoside (iP9G), *trans*-zeatin (tZ), *trans*-zeatin-riboside (tZR), *trans*-zeatin 9-glucoside (tZ9G), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR) and stable isotope-labeled standards: [$^2H_2$]IAA, [$^2H_6$]ABA, [$^2H_2$]GA1, [$^2H_2$]GA3, [$^2H_2$]GA4, [$^2H_2$]GA12, [$^2H_2$]GA24, [$^2H_2$]GA53, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_3$]DHZ, [$^2H_3$]DHZR were all purchased from Olchemim Ltd. (Olomouc, Czech Republic).

Ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), sodium acetate (NaAc), ethylene glycol (EG), 1,2-ethylenediamine (ETH), ethanol (EtOH), tetraethyl orthosilicate (TEOS), ammonia hydrate ($NH_3{\cdot}H_2O$, 25%, aqueous solution), ammonium hexafluorotitanate ($(NH_4)_2TiF_6$), boric acid (H_3BO_3), formic acid (FA, 88%) were all purchased from Sinopharm Chemical Reagent (Shanghai, China). Acetonitrile (ACN, HPLC grade) was obtained from Tedia Co. (Fairfield, OH, USA). Ultra-pure water used throughout the study was purified with Milli-Q system (Milford, MA, USA).

2.2. Synthesis of Fe_3O_4@TiO_2 magnetic nanoparticles

Firstly, Fe_3O_4 magnetite nanoparticles were synthetized according to previously reported method [30]. Subsequently, Fe_3O_4@TiO_2 magnetic nanoparticles were synthetized by liquid phase deposition according to our previously reported method [31] with minor modification. Briefly, Fe_3O_4 nanoparticles were incubated in 100 mL solution containing of 0.1 M $(NH_4)_2TiF_6$ and 0.3 M H_3BO_3 in a PTFE container. After maintaining the spheres under vacuum for 1 h, the mixture was heated at 35 °C for 16 h under continuous shaking. The resulting composite was thoroughly washed with purified water and dried at 120 °C for 4 h. And then, the dried composites were subjected to heat treatment under air by ramping up to 300 °C at a rate of 1 °C/min and maintained for 2 h.

2.3. Characterizations of Fe_3O_4@TiO_2

The titanium species were characterized by energy-dispersive X-ray analysis (EDX, QUANTA-200, FEI, Eindhoven, Netherlands) by using Mg Kα radiation as the excitation source. The morphology of the particles was observed with a QUANTA-200 scanning electron microscope (SEM, FEI, Eindhoven, Netherlands) and a transmission electron microscope (TEM, JEOL, Kyoto, Japan). Nitrogen adsorption measurement was performed using a JW-BK surface area and pore size analyzer (JWGB Sci. & Tech., Beijing, China). The prepared Fe_3O_4@TiO_2 nanoparticles were pretreated under vacuum and heated at 160 °C for 6 h. The specific surface area value was calculated according to the BET (Brunauer–Emmett–Teller) equation at P/P_0 between 0.05 and 0.35 [32].

2.4. Plant materials

Rice seeds (*Oryza sativa* L. cv. Zhenghan No. 2) were germinated and grown by hydroponic culture in Hoaglandís nutrient solution in a growth chamber (HP400GS, Wuhan Ruihua Instrument & Equipment Co., Ltd., Wuhan, China) at 28 °C with 70–80% relative humidity under a 16-h light:8-h dark photoperiods according to previously described method [9]. Light intensity