Endogenous ABA alleviates rice ammonium toxicity by reducing ROS and free ammonium via regulation of the SAPK9–bZIP20 pathway

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Received 26 September 2019; Editorial decision 31 January 2020; Accepted 11 February 2020

Editor: Hideki Takahashi, Michigan State University, USA

Abstract

Ammonium (NH₄⁺) is one of the principal nitrogen (N) sources in soils, but is typically toxic already at intermediate concentrations. The phytohormone abscisic acid (ABA) plays a pivotal role in responses to environmental stresses. However, the role of ABA under high-NH₄⁺ stress in rice (Oryza sativa L.) is only marginally understood. Here, we report that elevated NH₄⁺ can significantly accelerate tissue ABA accumulation. Mutants with high (Osaba8ox) and low levels of ABA (Osphs3-1) exhibit elevated tolerance or sensitivity to high-NH₄⁺ stress, respectively. Furthermore, ABA can decrease NH₄⁺-induced oxidative damage and tissue NH₄⁺ accumulation by enhancing antioxidant and glutamine synthetase (GS)/glutamate synthetase (GOGAT) enzyme activities. Using RNA sequencing and quantitative real-time PCR approaches, we ascertain that two genes, OsSAPK9 and OsbZIP20, are induced both by high NH₄⁺ and by ABA. Our data indicate that OsSAPK9 interacts with OsbZIP20, and can phosphorylate OsbZIP20 and activate its function. When OsSAPK9 or OsbZIP20 are knocked out in rice, ABA-mediated antioxidant and GS/GOGAT activity enhancement under high-NH₄⁺ stress disappear, and the two mutants are more sensitive to high-NH₄⁺ stress compared with their wild types. Taken together, our results suggest that ABA plays a positive role in regulating the OsSAPK9–OsbZIP20 pathway in rice to increase tolerance to high-NH₄⁺ stress.

Keywords: ABA, ammonium assimilation, antioxidant activity, high ammonium stress, OsbZIP20, OsSAPK9.

Introduction

Nitrate (NO₃⁻) and ammonium (NH₄⁺) are the two major inorganic nitrogen (N) forms accessed by plant roots. Although NH₄⁺ is frequently absorbed by roots more readily, and its assimilation requires less energy than that of NO₃⁻, excessive NH₄⁺ is toxic to plants (Britto et al., 2001; Kronzucker et al., 2001; Britto and Kronzucker, 2002; Li et al., 2014). Under
high-NH$_4^+$ stress, the roots, as the site of initial stress perception, undergo a series of physiological, cellular, and morphological changes, including inhibition of root growth and gravitropism (Li et al., 2010; Zou et al., 2012, 2013; Di et al., 2018). Several physiological mechanisms have been proposed to explain NH$_4^+$ toxicity, and these have included rhizosphere acidification, ionic imbalance, carbon metabolism disturbance, energy consumption, and hormone alteration (Britto and Kronzucker, 2002; Di et al., 2018). Many components of the stress syndrome, however, remain unclear.

NH$_4^+$ toxicity takes place when plants accumulate high tissue levels of free NH$_4^+$, resulting from both excessive NH$_4^+$ exposure and disturbance of NH$_4^+$ assimilation in plant cells (Barker and Corey, 1991; Bittsánszky et al., 2015). Therefore, the capacity for NH$_4^+$ assimilation, mediated by glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH), is viewed as critical in the detoxification of excessive NH$_4^+$ (Miflin and Habash, 2002; Hoa et al., 2003; Skopelitis et al., 2006). Knockout of the genes encoding these enzymes results in increased free NH$_4^+$ accumulation in plant tissue (Tabuchi et al., 2005; Tamura et al., 2010, 2011; Funayama et al., 2013). Plant species with higher GS activity accumulate less free NH$_4^+$ and are indeed more tolerant to high NH$_4^+$ (Cruz et al., 2006; Omari et al., 2010). A recent study in Arabidopsis thaliana revealed that AtNRT1.1 negatively regulates NH$_4^+$ tolerance by inhibiting the activities of GS, GOGAT, and GDH through a nitrate-independent pathway, and knockout of AtNRT1.1 enhances NH$_4^+$ assimilation and reduces free NH$_4^+$ accumulation in nrt1.1 mutants (Jian et al., 2018). Clearly, therefore, the regulation of NH$_4^+$ assimilation under high NH$_4^+$ warrants further study.

It is well known that reactive oxygen species (ROS) are generated under various environmental stresses, and that high levels of ROS induce oxidative damage and then injury and programmed cell death (Mittler and Blumwald, 2015). Excessive NH$_4^+$ accumulation in plant cells has been reported to induce high levels of H$_2$O$_2$ and oxidative stress in the roots of A. thaliana, tomato, and rice (Patterson et al., 2010; Fernández-Crespo et al., 2015; Xie et al., 2015). Moreover, NH$_4^+$ also up-regulates the activities of antioxidant enzymes, including catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD), all active in scavenging ROS and relieving oxidative stress (Nimptsch and Pflugmacher, 2007; Wang et al., 2008; Patterson et al., 2010; Xie et al., 2015). Recently, the heme–hemes oxygenase OsSE5 has been reported to regulate root growth under high-NH$_4^+$ stress by activating antioxidant enzymes, namely ascorbate peroxidase (APX), CAT, and SOD, and overexpression of OsSE5 in A. thaliana increases NH$_4^+$ tolerance (Xie et al., 2015). Concurrent overexpression of OsGS1.1 and OsGS2 leads to increased accumulation of glutathione (GSH), a powerful non-enzymatic antioxidant, and reduced ROS accumulation under high NH$_4^+$ (James et al., 2018).

The phytohormone abscisic acid (ABA) plays a pivotal role in coordinating responses to environmental cues (Yamaguchi-Shinozaki and Shinozaki, 2005, 2006). Several studies have suggested a possible interaction between NH$_4^+$ and ABA. In rice, NH$_4^+$ supply enhances ABA content under drought stress, which, in turn, increases water uptake and is associated with increased drought tolerance (Ding et al., 2016). Moreover, the latter report also showed that ABA transport from roots to shoots increased under NH$_4^+$ supply (Peuke et al., 1998). Our previous study in A. thaliana identified a plastid metalloprotease AMOS1/EGY1 as an important intersection point of NH$_4^+$ and ABA (Li et al., 2012). Transcriptome analysis shows that 90% of NH$_4^+$-activated genes are regulated by AMOS1/EGY1, and a large portion of them carry a core motif of an ABA-responsive element in their promoters (Li et al., 2012). Thus, the ABA signaling pathway is deeply involved in the NH$_4^+$ response.

Under environmental stress, ABA content is elevated, which consequently activates the ABA signaling pathway and triggers physiological reactions affecting stress resistance (Mittler and Blumwald, 2015). ABA binds to the receptor RCAR/PYR/PYLs, and then binds to protein phosphatase 2C (PP2C), inhibiting its enzymatic activity and dissociating the PP2C–SNF1–related protein kinase 2 (SnRK2) complex. Subsequently, auto–phosphorylated SnRK2s can activate downstream transcription factors (TFs) and then induce transcription of key genes including those coding for ion channels and late embryogenesis abundant (LEA) proteins (Ma et al., 2009; Soon et al., 2012; Zhang et al., 2019). Of the regulated TFs, the basic leucine zipper (bZIP) TF family has been investigated with special attention in rice and Arabidopsis, due to the synergistic regulation between ABA and environmental stress (Kim, 2006; Nijhawan et al., 2008). In rice, there are 89 bZIPs, and these have been classified into 13 groups (A, B, C, D, E, F, G, H, I, J, K, L, and S) (Nijhawan et al., 2008). Of these, OsZIP66 (Hobo et al., 1999), OsZIP23 (Xiang et al., 2008), OsZIP46 (Tang et al., 2012), OsZIP72 (Lu et al., 2009), OsZIP12/OsABF1 (Hossain et al., 2010), OsAB15 (Zou et al., 2008), and OsZIP71 (Liu et al., 2012) have been reported to regulate osmotic stress responses via ABA signal transduction. Nevertheless, whether the interaction of ABA signaling and OsZIP is involved in the response to high-NH$_4^+$ stress remains unclear.

Here, we analyzed gene expression, enzyme activities, metabolites, and the physiological processes in ABA-related mutants and their wild types under high-NH$_4^+$ stress. We aimed to explore: (i) whether ABA functions in response to high-NH$_4^+$ stress; (ii) which physiological processes are involved; and (iii) which members of the ABA signaling pathway are involved. Our results provide new understanding of the involvement of ABA signaling in response to high-NH$_4^+$ stress.

**Materials and methods**

**Plant materials and growth conditions**

Seeds were surface-sterilized with 1% sodium hypochlorite for 10 min, washed extensively with distilled water, and then germinated in distilled water at 28 °C for 2 d. The treatment solution was applied as described in Sun et al. (2017), and the solutions containing 1 mM and 7.5 mM (NH$_4$)$_2$SO$_4$ were designated as normal ammonium (NA) and high ammonium (HA), respectively. Solutions were exchanged every 12 h to ensure that plants remained at a nutritional steady state in the hydroponic system. Samples (three biological replicates) of roots were taken after the imposition of N excess treatments 12 h later, frozen immediately, and stored at −80 °C for associated analyses.
Determination of H$_2$O$_2$ content

Determination of H$_2$O$_2$ content was performed according to Liu et al. (2010). Roots used in measurements of H$_2$O$_2$ content were stored in liquid nitrogen immediately after harvesting. H$_2$O$_2$ concentrations were calculated using a standard curve prepared with known concentrations of H$_2$O$_2$.

Free NH$_4^+$ content determination

Roots were collected and desorbed with 10 mM CaSO$_4$ for 5 min, to remove extracellular NH$_4^+$. Free NH$_4^+$ contents were determined as previously described (Sun et al., 2017).

Determination of glutamine and glutamate contents

A 0.5 g aliquot (FW) of roots was frozen in liquid nitrogen immediately after each treatment. Then, 10 ml of 50% ethanol solution (containing 0.01 mM HCl) was added and placed in a water bath at 4 °C and subjected to ultrasound for 30 min. Then, centrifugation took place at 12000 rpm at 4 °C for 5 min, and ~1 ml of extract was filtered using a 0.22 μm filter membrane and then placed in a SYKAM Amino Acid Analyzer for further analysis (Sykam, Germany).

Malondialdehyde (MDA) contents and antioxidant enzyme assays

MDA contents were measured according to the method of Heath and Packer (1968). The SOD activity in roots was estimated by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). CAT activity of root was assayed from the rate of H$_2$O$_2$ decomposition as measured by the decrease of absorbance at 240 nm, following the procedure of Aebi (1984). APX activity in roots was assayed according to Chance and Maehly (1955), and the activity was determined by monitoring the increase of absorbance at 470 nm.

Estimation of proline and soluble sugar contents

Root samples (1 g) were extracted in 3% sulfoisocrylic acid, and the proline content was estimated following the method of Dey et al. (2016), using acid-ninhydrin reagent. The proline content was calculated from a standard curve plotted against t-proline (0–100 μg). Total soluble sugar content was estimated following the method described by Dey et al. (2016). The total sugar content was estimated from a standard curve plotted using 0–100 μg of glucose.

NH$_4^+$ assimilation enzyme analysis

For the GS, GOGAT, and GDH enzyme activities, crude root extracts were collected and enzyme activities were determined according to previous methods (Jian et al., 2018).

Transactivation assay

The transactivation assay was performed according to the methods described by Hossain et al. (2010). The yeast strain Y2HGold (Clontech) was used to test for the presence of an activation domain in the gene. For the transcription activation assay, sequences containing the full-length cDNA of OshZIP20 were fused in-frame with pGBK T7 to construct pGBK T7-OshZIP20. The construct was transformed into yeast strain Y2HGold. The empty pGBK T7 vector was also transformed into yeast cells as a negative control. The transformants were incubated on SD/Leu-/Trp-/His+2 mM 3-amino triazole (3-AT) at 30 °C for 2–3 d.

Yeast one-hybrid assay

To analyze the G-box– or ABRE-binding activity of OshZIP20, the construct was inserted into pHIS2 vectors (Clontech). In addition to the HIS2 minimal promoter in the pHIS2 expression vector, we synthesized oligonucleotide sequences that fused four tandem repeat copies of the G-box (5’-CACGTG-3’) or ABRE (5’-GTACGTGTC-3’). This G-box or ABRE was annealed and ligated to form four tandem copies and inserted into the pHIS2 vector that had been digested with the same enzymes, and the fusion construct was pHIS2::G-box or pHIS2::ABRE. The full-length cDNA of OsbZIP20 was synthesized and introduced into pGADT7 (Clontech) to construct the pGADT7-OsbZIP20 vector. These plasmids (pGADT7-OsbZIP20 and pHIS2::G-box or pHIS2::ABRE) were transformed into the yeast strain Y2HGold (Clontech) carrying the reporter gene HIS2 and used for the binding experiment with yeast. Samples were grown on selective medium plates SD/Leu-/Trp-/His+2 mM 3-AT for 2–3 d at 30 °C.

Two-hybrid system assay

The full-length cDNAs of OshZIP20 and OsSAPK9 without stop codons were synthesized and introduced into pGADT7 and pGBK T7, respectively (Clontech). The plasmids pGBK T7 and pGADT7 were each transformed into Y2HGold acting as the control. pGBK T7-OsSAPK9/8/9/10+pGADT7-OsbZIP20 were transformed into Y2HGold. Empty pGADT7 and pGBK T7 vectors were also transformed into yeast cells as negative controls. The transformants were incubated on SD/Leu-/Trp-/His+2 mM 3-AT at 30 °C for 2–3 d.

BiFC analyses

The full-length cDNAs of OshZIP20 and OsSAPK9 without stop codons were synthesized and introduced into pGB D11 and pGB D12 vectors and expressed in Escherichia coli BL21 (DE3) pLysE strain (Invitrogen) upon induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Similarly, first the 893 bp (encoding 297 amino acids) from the OsbZIP20 and the 1086 bp coding sequence (CDS) of OsSAPK9 in XhoI and EcoRI restriction sites allowed expression of 6×His N-terminal tagged OsSAPK9 protein in Esherichia coli BL21 (DE3) pLysE strain (Invitrogen) using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The expressed proteins were purified in the native condition and used for the in vitro kinase assay. In vitro phosphorylation of the generic substrate histone III (Sigma) was performed as described previously (Hou et al., 2010). Three-week-old Nicotiana benthamiana leaves were agroinfiltrated with agrobacterial cells containing the indicated constructs. Two days after incubation, fluorescence was analyzed by confocal microscopy.

In vitro kinase assay

The in vitro kinase assay was performed according to the methods described by Dey et al. (2016). The recombinant pRSET plasmid (Invitrogen) containing the 1086 bp coding sequence (CDS) of OsSAPK9 in XhoI and EcoRI restriction sites allowed expression of 6×His N-terminal tagged OsSAPK9 protein in Esherichia coli BL21 (DE3) pLysE strain (Invitrogen) upon induction by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Similarly, first the 893 bp (encoding 297 amino acids) from the OshZIP20 CDS of Oryza nataegron were cloned into a pPR SET vector in BamHI and EcoRI restriction sites, transformed, and expressed in pLysE cells with induction by 0.5 mM IPTG. The expressed proteins were purified in the native condition and used for the in vitro kinase assay. In vitro phosphorylation of the generic substrate histone III (Sigma) was performed as described previously. In vitro phosphorylation of OshZIP20 was performed by incubating the individual reaction mixture for 40 min at 25 °C following the above-mentioned protocol. The products were fractionated by 12% SDS–PAGE and visualized by autoradiography.

Quantitative real-time PCR

Total RNA was extracted from shoots and roots harvested at the specified time points with TRIzol reagent (Invitrogen, USA) and treated with RNase-free DNase I (Promega). Total RNA (2 μg) was used for reverse transcription with M-MLV Reverse Transcriptase (Promega), and the cDNA samples were diluted 2-fold. For quantitative real-time PCR (qRT-PCR), triplicate quantitative assays were performed on each cDNA dilution with ChamQ SYBR qPCR MasterMix (Q311-02, Vazyme Biotech Co., Ltd), and a CFX Manager sequence detection system according to the following protocol: denaturation at 95 °C for 30 s; for initiation; denaturation at 95 °C for 10 s; and 40 cycles of amplification, annealing, and extension at 55 °C/60 °C for 30 s. The specificity of the amplification was ascertained using a melting curve performed from 65°C to 95 °C, as
well as sequencing of the amplification. Three independent replicates were performed per experiment, and the means and corresponding SEs were calculated. The OsActl gene was used as a normalization control. Primer sequences are as listed in Supplementary Table S1 at JXB online.

Phos-tag SDS–PAGE assays

The peptide fragment (CTGLDYAGDPPFTGLSP) of hZIP20 was used to immunize rabbits, and antisera was collected to determine the titer. For phos-tag SDS–PAGE assays, the nuclear protein of roots (200 mg FW) was obtained using a Nuclear Extraction Kit (BB-3154-1, BestBio). Nuclear protein was then used for phos-tag SDS–PAGE by the SuperSep™ Phos-tag™ Kit (198-17981, Wako). All experiments were completed following the supplier’s operation manual. Coomassie Brilliant Blue staining was used to normalize protein amounts.

Statistical analysis

All statistical analyses were performed using Prism 6 software (GraphPad Software), and one-way or two-way ANOVA was performed. P<0.05 was set as the significance cut-off. All values were presented as means ±SD.

Results

Higher endogenous ABA enhances NH4+ tolerance in rice

Our previous RNA sequencing (RNA-seq) analysis showed that the transcript levels of ABA biosynthesis genes in roots were up-regulated by high NH4+ (Sun et al., 2017). Here, we show, using qRT-PCR, that genes involved in ABA biosynthesis were up-regulated 3.5- to 9.7-fold following high-NH4+ treatment (Fig. 1A). To investigate the role of ABA under high-NH4+ stress in rice, a pharmacological investigation was carried out by using exogenous ABA and fluorine (Flu; ABA biosynthesis inhibitor), and then root length and fresh weight were determined (Fig. 1B–D). Our results reveal that maximum primary root length and root fresh weight when grown in high-NH4+ media were 57.5% and 45.8% of those grown on control (1 mM NH4+) media; they were 93.5% and 96.8% following addition of ABA, and 40.1% and 21.3% after addition of Flu (Fig. 1B–D). Moreover, measurements of ABA content also showed that high NH4+ promoted ABA accumulation, and that ABA and Flu could increase or decrease this promotion (Fig. 1E). These results indicate that high NH4+ stimulates ABA biosynthesis and that ABA plays a positive role under high-NH4+ stress.

To further examine the function of ABA, a mutant high in endogenous ABA, aba8ox3, and a mutant low in endogenous ABA, phs3, were used (Fig. 2A; Supplementary Fig. S1) (Fang et al., 2008; Cai et al., 2015). Endogenous ABA content determination showed that aba8ox3 and phs3 contained more or less ABA compared, respectively, with their backgrounds under control and high-NH4+ conditions (Fig. 2B). Similar to ABA or Flu supplementation, the relative maximum primary root length and root fresh weight under the high-NH4+ treatment in aba8ox (98.9% and 97.3%) and in phs3 (41.9% and 35.7%) were higher or lower, respectively, compared with their backgrounds (60% and 57.8% in ZH11; 57.1% and 57.2% in Nip) (Fig. 2). To sum up, ABA plays a protective role in the root growth inhibition brought about by high NH4+.

Endogenous ABA improves NH4+ tolerance by strengthening NH4+ assimilation

To dissect the reason for improved NH4+ tolerance by increasing endogenous ABA, we first determined free NH4+ contents in roots. Our data show that high NH4+ induces free NH4+ accumulation in wild-type roots, and that it increases in phs3 and decreases in aba8ox3 (Fig. 3A). Subsequently, we tested the enzyme activities of GS, GOGAT, and GDH under control and high NH4+ (Fig. 3B). T o sum up, ABA plays a positive role under high-NH4+ stress.

Higher endogenous ABA reduces cell damage by up-regulating antioxidant activities at high NH4+

Stresses are frequently associated with the generation of ROS, such as H2O2, and ROS build-up, in turn, leads to lipid peroxidation, which results in the production of MDA (Dey et al., 2016). MDA is a stress-specific molecular marker that is indicative of the extent of membrane injury and cell and tissue damage. To determine whether high-NH4+ stress also induces ROS generation and whether ABA is involved in this, we measured H2O2 and MDA contents in aba8ox3, phs3, and their backgrounds under control and high-NH4+ stress. Our data show increases in H2O2 and MDA contents in all tested materials under high NH4+. However, increases were less in aba8ox3 and more in phs3 after NH4+ treatment, indicating that higher endogenous ABA can reduce oxidative damage by decreasing ROS content under NH4+ stress (Fig. 4A–D).

Furthermore, we also determined the enzyme activities of several antioxidant enzymes, including SOD, APX, and CAT, in aba8ox3, phs3, and their backgrounds under control and high-NH4+ stress. Our results show that the SOD, APX, and CAT enzyme activities can be induced in the wild types (ZH11 and Nip) by high NH4+. This induction was strengthened in aba8ox3, and was weakened in phs3 (Fig. 4E–J). Taken together, the data clearly reveal that ABA can reduce oxidative damage induced by ROS by increasing antioxidant enzyme activities.

Endogenous ABA up-regulates OsbZIP20 under high-NH4+ stress

Our previous reports revealed that a key gene involved in ABA signaling, namely OsbZIP20, was up-regulated by high NH4+ (Sun et al., 2017). To further unravel the function of endogenous ABA under high NH4+, we analyzed the transcription...
of OsbZIP20 under NH$_4^+$ stress by qRT-PCR. Our data show that the relative transcription levels of OsbZIP20 under high NH$_4^+$ were 6-fold higher compared with control, and transcription was further induced at higher concentrations of NH$_4^+$ (Fig. 5A). Moreover, the induction of OsbZIP20 was increased in the aba8ox3 mutant, and was decreased in the phs3 mutant (Fig. 5B). These results indicate that OsbZIP20 is involved in the response to high-NH$_4^+$ stress by an ABA-dependent pathway.

OsbZIP20 combines with the G-box and ABREs and interacts with OsSAPK9 in vitro and in vivo

To test whether OsbZIP20 is an effective OsbZIP TF, the yeast one-hybrid (Y1H) system was used to determine the transactivation activity of OsbZIP20. The AREB/ABF TF-binding sites, ABRE (ACGTGG/TC) and G-box (CACGTG), were introduced into the pHIS2 vector, and the full-length coding sequence of OsbZIP20 was introduced into the pGADT7 vector (Fig. 6A). The yeast strain Y1HGold co-transformed with pHIS2-G-box or pHIS2-ABRE+pGADT7 (negative controls) failed to grow on SD/Leu-/Trp-/His+2 mM 3-AT plates (Fig. 6B). The results indicate that OsbZIP20 can bind to the G-box and ABREs in yeast. Then, we used the yeast two-hybrid (Y2H) system to identify the transactivation activity of OsbZIP20. The transformants grew well on the SD/Trp-/Leu- plates (Fig. 6C). However, when transferred to SD/Leu-/Trp-/His+2 mM 3-AT plates, both pGBK7-OsbZIP20 and negative control failed to grow, indicating that OsbZIP20 had no transactivation activity (Fig. 6C).

The activation of many AREB/ABF proteins requires the phosphorylation by SnRK2 (also referred to as ABA-activated protein kinase, SAPK, in rice) protein kinases (Fujita et al., 2013). To identify the interaction between OsbZIP20 and OsSAPKs in plants, we then performed Y2H system assays. Our results show that, when cultured on the SD/Leu-/Trp-/His+2 mM 3-AT plates, only transformants of pGBK7-OsSAPK9+pGADT7-OsbZIP20 grew well compared with pGBK7-OsSAPK8+pGADT7-OsbZIP20, pGBK7-OsSAPK10+pGADT7-OsbZIP20, and the negative control (Fig. 6D). To confirm the interaction, bimolecular fluorescence complementation (BiFC) analyses were performed in tobacco
cells (Fig. 6E). Our data show that YFP was reconstituted when the CDSs of OsSAPK9 and OsbZIP20 were co-expressed (Fig. 6E). In contrast, OsSAPK9–nYFP and cYFP, or nYFP and cYFP–OsbZIP20, did not result in fluorescence (Fig. 6E), suggesting that OsSAPK9 and OsbZIP20 interaction is specific. These results suggested that only OsSAPK9 could interact with OsbZIP20 in vivo and in vitro.

OsSAPK9 can phosphorylate OsbZIP20 in vivo and in vitro

To further investigate how OsSAPK9 interacts with OsbZIP20, we purified the OsSAPK9 and OsbZIP20 proteins in the E. coli system (Fig. 7A). Our results verified that OsSAPK9 can phosphorylate OsbZIP20 (Fig. 7A). Furthermore, to investigate
the role of ABA in the phosphorylation of OsbZIP20 by OsSAPK9, we analyzed the phosphorylation of bZIP20 by phos-tag SDS–PAGE in Nip and in the OsSAPK9 knockout mutant (Figs 7B, 8A). Our results show that ABA can induce the expression of OsbZIP20, and a new phosphorylated band of OsbZIP20, and that these inductions do not occur in the knockout mutant sapk9 (Figs 7B, 8A). Overall, these data reveal that the activity of OsbZIP20 can be activated by the...
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phosphorylation of OsSAPK9 and that ABA can promote this phosphorylation in an OsSAPK9-dependent manner.

*bzip20* and *sapk9* mutants show higher sensitivity to high NH$_4^+$, accompanied by higher free NH$_4^+$ and H$_2$O$_2$ accumulation in tissue

To further analyze OsZIP20 and OsSAPK9 function under high-NH$_4^+$ stress, knockout mutants (*sapk9* and *bzip20*) were constructed by clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (Fig. 8A). Sequence analysis showed there was a single base C deletion in the first exon of *sapk9* and a 20 base deletion in the third exon of *bzip20*, respectively, which resulted in gene inactivation (Fig. 8A). We then observed the phenotypes of *sapk9* and *bzip20* under control and high-NH$_4^+$ conditions. There was no difference among Nip, *sapk9*, and *bzip20* under control conditions, whereas the high NH$_4^+$ inhibited root growth
in all genotypes, with greater inhibition in sapk9 and bzip20 (Fig. 8B–D). Exogenous ABA could rescue root growth in Nip, but not in sapk9 and bzip20 (Fig. 8C, D). These results indicate that OsSAPK9 and OsbZIP20 were involved in high NH$_4^+$ response via an ABA-dependent pathway.

To further test the roles of OsSAPK9 and OsbZIP20 in response to high-NH$_4^+$ stress, we then measured the free NH$_4^+$ content in all genotypes under control and high NH$_4^+$ conditions. All genotypes grown in control solution exhibited similar free NH$_4^+$ accumulation. However, the free NH$_4^+$ accumulation increased 1.18-fold in Nip, 4.23-fold in sapk9, and 4.15-fold in bzip20 compared with the control condition (Fig. 8E). With the addition of exogenous ABA to high-NH$_4^+$ solutions, compared with the high-NH$_4^+$ condition, the free NH$_4^+$ accumulation decreased 37.7%, but only 5.4% and 13.4% in sapk9 and bzip20, respectively (Fig. 8E).

H$_2$O$_2$ contents in roots varied slightly among the Nip and mutants in the control condition and were 0.53, 1.81, and 1.52 times higher in Nip, sapk9, and bzip20, respectively, under high NH$_4^+$ compared with control (Fig. 8F). Consistently, the MDA concentrations were also increased in sapk9 and bzip20 compared with Nip under high-NH$_4^+$ stress (Fig. 8G). Exogenous ABA inhibited the accumulation of H$_2$O$_2$ and MDA in Nip roots, but not in sapk9 and bzip20 roots (Fig. 8F, G). To sum up, OsSAPK9 and OsbZIP20 were involved in the high-NH$_4^+$ response through regulating free NH$_4^+$ and H$_2$O$_2$ accumulation in roots.
OsSAPK9 and OsbZIP20 function under high-NH₄⁺ stress by enhancing NH₄⁺ assimilation and antioxidant activity

Under control conditions, GS and GOGAT activities were slightly lower in sapk9 and bzip20 than in Nip, in roots (Fig. 9A, B). The GS and GOGAT activities decreased in Nip roots under high-NH₄⁺ stress and more so in sapk9 and bzip20 mutants under the same high-NH₄⁺ conditions (Fig. 9A, B). The activity of GDH was slightly higher in Nip than in sapk9 and bzip20 roots under control conditions (Fig. 9C). High NH₄⁺ reduced GDH activity by 5.2% in Nip but to a greater extent in sapk9 and bzip20, where it decreased by 17.1% and 23.3%, respectively (Fig. 9C). Consistently, the accumulation of Glu and Gln induced by high NH₄⁺ was also reduced in the sapk9 and bzip20 mutants (Supplementary Fig. S3).

Only mild differences in SOD, CAT, and APX activities were observed in roots of Nip, sapk9, and bzip20 (Fig. 9D–F). However, the activities of SOD, CAT, and APX were increased under high-NH₄⁺ conditions, respectively, while they were not increased in sapk9 and bzip20 (Fig. 9D–F). Furthermore, exogenous ABA application can obviously promote the activities of these enzymes, including GS, GOGAT, GDH, SOD, CAT, and APX under high-NH₄⁺ stress in Nip, and this promotion was reduced or even disappeared in sapk9 and bzip20 (Fig. 9). Taken together, these results suggest that OsSAPK9 and OsbZIP20 have a positive role in the activities of NH₄⁺ assimilation and antioxidant enzymes in response to high-NH₄⁺ stress via an ABA-dependent pathway.

Discussion

ABA is involved in the response to NH₄⁺ stress by enhancing NH₄⁺ assimilation and antioxidant enzyme activities

The plant hormone ABA plays a key role in a broad array of adaptive stress responses to environmental stimuli in plants (Cutler et al., 2010; Raghavendra et al., 2010; Weiner et al., 2010). Many studies have revealed that NH₄⁺ can regulate ABA biosynthesis and transport (Peuke et al., 1998; Li et al., 2012; Ding et al., 2016). In A. thaliana, transcriptome analysis revealed that many genes induced by high NH₄⁺ contain the core motif of the ABARE in their promoters, indicating that ABA signaling is involved in the response to high NH₄⁺ (Li et al., 2012). However, how ABA signaling regulates NH₄⁺ stress in rice has remained unknown. Here, we identify that ABA positively regulates NH₄⁺ tolerance. This conclusion is supported by physiological, pharmacological, and genetic evidence.

First, in addition to root growth inhibition (Fig. 1B–D), it was observed that high NH₄⁺ up-regulated transcription of ABA biosynthesis genes (Fig. 1A). Subsequent results revealed that high NH₄⁺ increased ABA content in roots (Fig. 1E). Similar induction was also observed in A. thaliana (Li et al., 2012). To unravel the mechanism of ABA action under high-NH₄⁺ stress, we adopted a pharmacological approach using applications of exogenous ABA and Flu, an inhibitor of ABA biosynthesis (Yoshioka et al., 1998). As expected, the treatment with ABA and Flu resulted in a decrease or increase in the inhibition of root growth induced by high NH₄⁺ stress, respectively (Fig. 1B–D). Consistent with this, a mutant with higher endogenous levels of ABA, aba8ox3, and one with lower levels, phs3, were tolerant and sensitive to high-NH₄⁺ treatment, respectively (Fang et al., 2008; Cai et al., 2015) (Fig. 2A–C). To sum up, our results suggest that ABA plays a significant positive role in the response to high-NH₄⁺ stress.

Secondly, it has been proposed that a plant’s tolerance to high NH₄⁺ is related to its capacity for NH₄⁺ assimilation (Cruz et al., 2006; Liu and von Wirén, 2017). However, our results show that high-NH₄⁺ stress can inhibit the activities of key enzymes of NH₄⁺ assimilation, including GS, GOGAT, and GDH in roots; that is, primary NH₄⁺ assimilation is functionally impaired in roots under high NH₄⁺ (Fig. 3). Our results also show, however, that high-NH₄⁺ treatment up-regulates the transcription of genes encoding NH₄⁺ assimilation enzymes in roots, namely OsGS1.2 and OsNADH–GOGAT1, and that these inductions are enhanced in aba8ox3 and weakened in phs3, while they do not occur in sapk9 and bzip20 (Supplementary Figs S4, S5). This shows that differential regulation of NH₄⁺ assimilation takes place transcriptionally and post-transcriptionally in rice and that the OsSAPK9–OsSAPK20 pathway is involved in this regulation. The results are in agreement with previous reports in A. thaliana, showing that the regulation of GS/GOGAT enzyme by NH₄⁺ can occur differently at levels of transcription, post-translation, and post-translation (Thomsen et al., 2014; Guan et al., 2016; Jian et al., 2018).

In rice roots, OsGS1.2 and OsNADH–GOGAT1 are expressed abundantly in the surface cell layers of roots, namely in the cells of the epidermis and the exodermis, and this can protect the remainder of the root tissue from high-NH₄⁺ toxicity (Kronzucker et al., 2001; Ishiyama et al., 2003, 2004; Tabuchi et al., 2007; Hachiya et al., 2012). Gln, the major product of NH₄⁺ assimilation and the principal organic N form involved in long-distance N transport in the xylem, can be transported to the cortex and the central cylinder (Fukumori and Chino, 1982; Lea et al., 2007). While ABA biosynthesis in roots mainly occurs in the pericycle (Bourias et al., 2013), in such cases, once NH₄⁺ is taken up by the roots, ABA may be rapidly transported to the surface of roots and enhance the catalytic activities of OsGS1.2 and OsNADH–GOGAT1, mediated by the OsSAPK9–OsSAPK20 pathway, protecting roots from the effects of high NH₄⁺. In line with this assumption, our results show that addition of exogenous ABA in roots can increase GS and GOGAT activities in roots, especially under high-NH₄⁺ stress, and that the effect of exogenous ABA on GS/GOGAT enzyme activities was weakened or even disappeared (Fig. 9; Supplementary Fig. S2). Furthermore, overexpression of OsGS1.1 and OsGS1.2 under the control of the Cauliflower mosaic virus (CaMV) 35S promoter resulted in increased GS activities but also increased sensitivity to abiotic stress and poor plant growth, revealing that higher expression of OSGS1.1 and OSGS1.2 in all tissues would disturb the normal nutrient metabolism and signaling pathway (Cai et al., 2009). In contrast, co-overexpression of OsGS1.1/OsGS2 was associated with enhanced tolerance to abiotic stresses and yield improvement.
These incompatible phenotypes might be due to an imbalance in C/N metabolism (Bao et al., 2014). It is to be expected that the abiotic tolerance and yield improvement resulting from overexpression of GS furthermore depends on higher GOGAT activity, sufficient supply of ATP and NADH, and 2-oxoglutarate from C metabolism. Taking these results together, manipulating ABA signaling to enhance GS/GOGAT and GDH enzyme activities in situ appears a promising strategy to increase NH$_4^+$ assimilation under abiotic stresses.

Thirdly, it is well known that environmental stresses induce oxidative stress and lead to higher accumulation of ROS (Mittler et al., 2011). Moreover, high-NH$_4^+$ treatment has also been reported to augment ROS, especially H$_2$O$_2$ in roots, pointing to a role for ROS in high-NH$_4^+$-induced root growth inhibition (Liu and von Wiren, 2017; Li et al., 2019). To protect cells from oxidative damage by overaccumulated ROS, plants have evolved a sophisticated antioxidant defense apparatus to scavenge ROS, including enzymatic and non-enzymatic systems (Apel and Hirt, 2004; Mittler et al., 2004). In rice, an OsSE5-mediated antioxidant defense alleviates root elongation from NH$_4^+$ inhibition, indicating that an up-regulated antioxidant apparatus can offset NH$_4^+$ toxicity in roots (Xie et al., 2015). In the past two decades, the crosstalk between ABA and ROS signals has been intensively studied (Gill and Tuteja, 2010). ABA-enhanced stress tolerance has been associated with the induction of antioxidant defense systems, including enzymatic and non-enzymatic components, protecting plant cells against oxidative damage (Zhang et al., 2014; Yang et al., 2015). ABA acts as a key blocker of H$_2$O$_2$ production during exposure to osmotic stress and increases the activities of antioxidant enzymes against oxidative stress (Lee and Luan, 2012). In our study, the aba8ox3 mutant, endowed with high endogenous ABA, accumulated less, and the phs3 mutant, endowed with low endogenous ABA, accumulated more H$_2$O$_2$ under high-NH$_4^+$ stress (Fig. 4A, B). Consistent with this, the MDA content, as a biomarker of cell membrane injury, was significantly increased in phs3 compared with its wild type, especially in osaba8ox3, indicating that higher ABA can reduce the oxidative damage normally encountered under high-NH$_4^+$ stress (Dey et al., 2016) (Fig. 4C, D). The lower MDA content implies a higher reductive ability, reflecting higher abiotic stress resistance (Marcińska et al., 2013). To cope with the overproduction of ROS, plants also employ scavenging enzymes such as SOD, APX, and CAT, to adjust ROS homeostasis (Apel and Hirt, 2004; Mittler et al., 2004; Conde et al., 2011). In our study, high-NH$_4^+$ stress-induced SOD, APX, and CAT activities in osaba8ox3 were significantly higher than those in the wild.
type and in osph3 (Fig. 4E–J). These results reveal that ABA can enhance the activities of antioxidant enzymes, including SOD, APX, and CAT, followed by reduced ROS accumulation, which results in decreased oxidative damage to roots.

In addition, many plants accumulate compatible solutes for maintaining membrane integrity and scavenging ROS in response to osmotic adjustment (Hong et al., 2000; Couée et al., 2006). Soluble sugars act as osmo-protectants, maintaining cell turgor, and protect the integrity of cell membranes (Couée et al., 2006). Endogenous sugar availability can feed the oxidative pentose phosphate pathway, leading to additional ROS scavenging (Couée et al., 2006). Moreover, exogenous sucrose feeding has been shown to increase SOD, CAT, and APX activities in wheat under salt stress conditions (Yan et al., 2012) and in A. thaliana following atrazine treatment (Ramel et al., 2009). Upon NH4+ stress imposition, higher contents of soluble sugars were accumulated in aba8ox3 compared with osph3 (Supplementary Fig. S6). Another important class of smaller molecules known as ‘compatible osmolites’ includes proline. Proline has a key role in protecting against oxidative stress by scavenging of ROS (Hong et al., 2000; Sorkheh et al., 2012; Yan et al., 2012). Proline accumulation was closely associated with an increase in ammonium concentration, while high-NH4+–induced proline accumulation was also involved in regulating antioxidative activity and osmotic adjustment in white clover (Trifolium repens L.) (Kim et al., 2004). In our study, higher contents of proline were accumulated in osaba8ox3 compared with the wild type and phs3 under high-NH4+ stress. To sum up, ABA-induced osmolite accumulation in roots is another possible ABA-mediated resistance mechanism to high NH4+ (Supplementary Fig. S6). Additionally, GS overexpression also resulted in a reduction of ROS and MDA levels under abiotic stress by enhancing the activities of antioxidative enzymes, including SOD (Lee et al., 2013; Molina-Rueda et al., 2013; Molina-Rueda and Kirby, 2015; James et al., 2018). Glu is required for the synthesis of proline and GSH, while co-overexpression of OsGS1.1 and OsGS2 in rice led to greater accumulation of proline and less MDA under drought and salt stresses (James et al., 2018). Mutation of GS in L. esculentum plants led to significantly lower proline levels under drought stress (Diaz et al., 2010). Hence, NH4+ assimilation is clearly linked to antioxidant activity under high-NH4+ stress, and ABA appears to be involved in this.

The OsSAPK9–OsbZIP20 pathway is an important positive regulatory system for ABA signaling under NH4+ stress

ABA-responsive gene expression is directly regulated by TFs that recognize and bind to cis-elements in the promoter regions upstream of their target genes (Fujita et al., 2011). bZIP TFs play important roles in the ABA/stress signaling pathway (Kim, 2006; Nijhawan et al., 2008), and these have been designated as ABFs or AREBs (Yamaguchi-Shinozaki and Shinozaki, 2005), but little is known about their functions in rice under high-NH4+ stress. Of the 13 rice bZIP TF groups, Groups A, C, and S participate in abiotic stress signaling (Nijhawan et al., 2008). Nevertheless, rice has six members in Group C, namely OsbZIP15, OsbZIP20, OsbZIP33, OsbZIP52, OsbZIP58, and OsbZIP88, and, of these, OsbZIP20, OsbZIP33, and OsbZIP88 responded to salt and drought stress, while only OsbZIP20 was induced under high NH4+, indicating that OsbZIP20 plays a special role under NH4+ stress (Fig. 5; Supplementary Figs S7, S8) (Nijhawan et al., 2008; Sun et al., 2017). OsbZIP20 is a typical member of the Group C bZIP family, but has no transactivation activity (Fig. 6C), while it can bind the G-box or ABRE (Fig. 6B), which are ubiquitously found in the promoters of plant genes regulated by environmental signals (Nijhawan et al., 2008). Moreover, OsbZIP20 transcript was significantly higher or lower in aba8ox3 or phs3 under high-NH4+ stress, respectively, compared with their wild types (Fig. 5B), while the OsbZIP20 transcript was significantly induced under different high-NH4+ conditions, suggesting that OsbZIP20 is involved in the response to high NH4+ via an ABA-dependent pathway (Fig. 5A). Furthermore, the promotion of NH4+ assimilation and antioxidant enzymes by exogenous ABA was decreased or even disappeared in bzip20 (Fig. 9). However, when we analyzed the promoters (~2000 bp) of genes encoding NH4+ assimilation enzymes in roots, there were no bZIP20-binding cis-elements. Thus, we conjectured that OsbZIP20 participates in the NH4+ stress pathway indirectly, by regulating the activities of NH4+ assimilation and antioxidant enzymes, and the detailed mechanism affecting the enzyme activities of GS/GOGAT by OsbZIP20 should be investigated in future studies. The ABA-dependent phosphorylation of the ABF/AREBs is indispensable for its activation. The ABA-dependent activation of ABF/AREB proteins requires phosphorylation by SnRK2 protein kinases (Furihata et al., 2006; Fuji et al., 2007). In rice, 10 SnRK2 members, designated as OsSAPK1–OsSAPK10 (ABA-activated protein kinase 1–10), have been identified, which are activated by hyperosmotic stress, and Subclass III SnRK2s, including OsSAPK8, 9, and 10, which are also induced by ABA (Kobayashi et al., 2004; Dey et al., 2016). OsSAPK8, OsSAPK9, and OsSAPK10, which possess both autophosphorylation and transphosphorylation activities, control AREB/ABFs in ABA-responsive gene expression under osmotic stress conditions (Fujita et al., 2011; Dey et al., 2016). As OsbZIP20 does not possess transactivation activity and is involved in the ABA response, we analyzed the interaction of OsbZIP20 and OsSAPK8, 9, and 10, which function in response to ABA (Fig. 6C). We demonstrate that only OsSAPK9 can interact with OsbZIP20 in vitro and in vivo, and that OsSAPK9 also phosphorylated OsbZIP20, and subsequently might activate its function (Fig. 6D–F). Furthermore, when we analyzed the phosphorylation of OsbZIP20 in vivo, we found that there were four phosphorylated protein bands in the control condition, and exogenous supply of ABA could induce a new phosphorylated protein band, showing that ABA can further induce the phosphorylation of OsbZIP20 (Fig. 7B). Moreover, this new induced phosphorylated protein band disappeared in the sapk9 mutant, revealing the role of OsSAPK9 in ABA-induced phosphorylation of OsbZIP20 (Fig. 7B). Previous work had shown that OsSAPK9 is located in the cytosol and the nucleus, similar to other SnRK2 family members (Mao et al., 2010; Tian et al., 2013; Dey et al., 2016), and we also detected the nuclear localization signal (NLS) in the OsbZIP20 amino acid
sequence, indicating nuclear localization of OsbZIP20 and suggesting that the interaction between OsSAPK9 and OsbZIP20 occurs in the nucleus. However, a significant signal was also detected in the cytosol, as shown in Fig. 6E, indicating that the interaction between OsSAPK9 and OsbZIP20 may also occur in the cytosol. Another working hypothesis is that OsSAPK9 phosphorylates and activates OsbZIP20 in the cytosol, and then the activated OsbZIP20 is transferred to the nucleus to regulate the transcription of downstream genes involved in acclimation to environmental stress. A similar phenomenon has been reported in previous studies, showing that phosphorylation was responsible for light-modulated GBF2 translocation from the cytosol to the nucleus (Harter et al., 1994; Terzaghi et al., 1997). Clearly, future studies will have to be designed to unravel the possible role of phosphorylation of OsbZIP20 by OsSAPK9 and whether a transfer of activated OsbZIP20 to the nucleus takes place. Our results also suggest that ABA can induce the expression of OsbZIP20 and that OsSAPK9 is involved in this process. Taken together, our data suggest that the initiation of the interaction between OsSAPK9 and bZIP20 can occur without ABA or NH₄⁺, but that exogenous ABA can significantly enhance the transcription of SAPK9 and bZIP20 and phosphorylation of bZIP20.

In addition, OsSAPK9 was induced by high NH₄⁺, and the induction was further intensified by applications of endogenous ABA (Supplementary Fig. S9). OsSAPK9 has also been reported to phosphorylate OsbZIP23 in response to drought stress (Dey et al., 2016), implying that OsSAPK9-mediated antioxidant activity augmentation is a more general mechanism in response to abiotic stress (Battaglia et al., 2016), suggesting that OsSAPK9-mediated antioxidant activity is increased or even disappeared in sapk9 and bzip20 under high-NH₄⁺ stress (Figs 8, 9; Supplementary Fig. S3). Moreover, ABA-induced NH₄⁺ assimilation and antioxidant activity enhancement decreased or even disappeared in sapk9 under high-NH₄⁺ stress (Figs 8, 9), indicating a strong role for OsSAPK9 in ABA-dependent NH₄⁺ resistance. Furthermore, the increased or decreased antioxidant activity was also found in overexpression and knockdown lines of OsSAPK9 (Fig. S10), supporting previous findings, these observations establish that ABA-dependent phosphorylation of OsbZIP20 by OsSAPK9 protein kinases is involved in the activation of AREB/ABFs in rice under high-NH₄⁺ stress. Thus, the SnRK2–AREB/ABF system contributes to the presence of a high-NH₄⁺ adaptive stress response in rice.

The activated ABF/ABRE system can induce the expression of LEA genes, which are novel ABRE-dependent AREB/ABF target genes, and these play crucial roles in cellular tolerance in response to abiotic stress (Battaglia et al., 2008; Yoshida et al., 2010). The expression of OsLEA4 genes can be induced by the application of ABA and by various abiotic stresses (Duan and Cai, 2012). There are 34 OsLEA genes in the rice genome, while only 16 of them are induced by exogenous ABA (Wang et al., 2007). As shown in Supplementary Fig. S10, upon imposition of high-NH₄⁺ stress, much higher expression levels of these genes are induced in osaba8ox3 compared with the wild type and phs3. The up-regulated OsLEA genes belonged to group 3, which has been suggested to function in the preservation of protein and membrane structure during various abiotic stresses (Duan and Cai, 2012). Hence, the regulation of the OsLEA3 group by OsSAPK9 and OsbZIP20 warrants further study in the future.

In conclusion, our study shows that the increase of antioxidant enzyme activities and of NH₄⁺ assimilation are active responses to a high-NH₄⁺ challenge and are associated with higher endogenous ABA. Moreover, in the process, the OsSAPK9–OsbZIP20 pathway plays a critical regulatory role (Fig. 10). The understanding of the tolerance mechanism to high NH₄⁺ involving the OsSAPK9–OsbZIP20 pathway induced by higher endogenous ABA is of great importance to confronting this significant agronomic problem.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Relative transcription of ABA8OX3 in aba8ox3 and CRTISO in phs3.

Fig. S2. Exogenous ABA and an ABA inhibitor alter the activities of NH₄⁺ assimilation enzymes.

Fig. S3. Glu and Gln contents in sapk9 and bzip20 mutants after high-NH₄⁺ treatment.

Fig. S4. Transcript expression analysis of genes encoding NH₄⁺ assimilation enzymes by qRT-PCR in aba8ox3 and phs3 mutants.

Fig. S5. Transcript expression analysis of genes encoding NH₄⁺ assimilation enzymes by qRT-PCR in sapk9 and bzip20 mutants.
Fig. S6. Proline and soluble sugar contents of roots in osbaRox3 and osph3 under high NH$_4^+$.

Fig. S7. Sequences analysis of OsbZIP20 with OsbZIP52 and AthZIP9.

Fig. S8. Phosphorylation site prediction in OsbZIP20 by the GPS 2.1 program.

Fig. S9. OsSAPK9 is involved in the response to high NH$_4^+$.

Fig. S10. Relative transcript levels of LEA genes in abbaRox3 and phs3 under normal and high-NH$_4^+$ conditions

Table S1. Primers used in this study

Acknowledgements

abbaRox3 and phs3 mutants are from Professor Guohui Zhu (South China Agricultural University) and Professor Chengcai Chu (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), respectively. We thank Professor Luxing Yuan (China Agricultural University) for technical support and helpful discussions. This research was financially supported by the National Natural Science Foundation of China (31430095, 31572205, and 31601823) and the University of Melbourne.

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