

RESEARCH ARTICLE

A novel aldo-keto reductase gene, *IbAKR*, from sweet potato confers higher tolerance to cadmium stress in tobacco

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Abstract High concentrations of Cd can inhibit growth and reduce the activity of the photosynthetic apparatus in plants. In several plant species, aldo-keto reductases (AKRs) have been shown to enhance tolerance to various abiotic stresses by scavenging cytotoxic aldehydes; however, few AKRs have been reported to enhance Cd stress tolerance. In this study, the gene *IbAKR* was isolated from sweet potato. The relative expression levels of *IbAKR* increased significantly (approximately 3-fold) after exposure to 200 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 or 10 $\text{mmol}\cdot\text{L}^{-1}$ H_2O_2 . A subcellular localization assay showed that *IbAKR* is predominantly located in the nucleus and cytoplasm. *IbAKR*-overexpressing tobacco plants showed higher tolerance to Cd stress than wild-type (WT). Transgenic lines showed a significant ability to scavenge malondialdehyde (MDA) and methylglyoxal (MG). In addition, proline content and superoxide dismutase activity were significantly higher and H_2O_2 levels were significantly lower in the transgenic plants than in the WT. Quantitative real-time PCR analysis showed that the reactive oxygen species (ROS) scavenging genes encoding guaiacol peroxidase (*GPX*), ascorbate peroxidase (*APX*), monodehydroascorbate reductase (*MDHAR*) and peroxidase (*POD*) were significantly upregulated in transgenic plants compared to WT under Cd stress. These findings suggest that overexpressing *IbAKR* enhances tolerance to Cd stress via the scavenging of cytotoxic aldehydes and the activation of the ROS scavenging system.

Keywords cadmium stress, *IbAKR*, *Ipomoea batatas*, sweet potato

1 Introduction

The heavy metal, Cd, is considered to be biologically

nonessential for almost all plants. With rapid industrialization and the widespread use of agrochemicals in agriculture in recent years, very large areas of land have been polluted by Cd. In China, about 7.75% of farmland soil is polluted by Cd^[1]. The effect of Cd toxicity on plants includes the inhibition of growth and a reduction in the activity of the photosynthetic apparatus^[2]. These situations pose a continuously increasing burden to global crop productivity, and hence, there is demand for crop varieties that are adaptive and resistant to various stresses. Genetic engineering has the potential to improve tolerance to Cd stress in plants^[3].

The aldo-keto reductase (AKR) protein superfamily contains > 190 members and is found in all phyla. The AKR superfamily comprises 18 families and includes mainly NAD(P)(H)-dependent oxidoreductases. These enzymes reduce carbonyl substrates, such as lipid peroxidation byproducts, keto-steroids, keto-prostaglandins, retinals and quinones^[4]. In plants, AKRs have been reported to enhance tolerance to various abiotic stresses. The overexpression of a novel alfalfa aldose/aldehyde reductase gene in transgenic tobacco plants enhanced tolerance to oxidative damage caused by paraquat, heavy metals, UV-B (280–320 nm) irradiation, drought and low temperature^[5–7]. The overexpression of a rice AKR increased tolerance to oxidative stress, heat and glyphosate in transgenic rice^[8,9]. An aldose reductase from *Xerophyta viscosa* conferred enhanced drought and salinity tolerance in transgenic tobacco plants^[10]. The expression of an *Arabidopsis thaliana*-encoded AKR protein in transgenic barley increased tolerance to oxidative stress, Cd stress and freezing stress^[11,12]. Salt tolerance increased in *Arabidopsis* transformed with *PpAKR1*, which had been cloned from peach (*Prunus persica*)^[13]. Recently, a novel member of the AKR superfamily was cloned from a soil *Sphingomonas* strain and was responsible for detoxifying the *Fusarium* toxin deoxynivalenol^[14]. Regenerated transgenic tobacco and rice lines expressing *PsAKR1*, an AKR gene from *Pseudomonas*, showed improved tolerance to accelerated aging and glyphosate^[9,15]. However, the role

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of AKRs in Cd stress tolerance has rarely been reported.

Sweet potato, *Ipomoea batatas*, is an important root crop, and the storage root is widely used in the energy industry^[16]. In addition, the concentrations of Cd is lower in the storage root tissue than in the fibrous root, stem and leaves grown in Cd-contaminated farmland^[17], suggesting that sweet potato, as a source of bioenergy, could be a candidate crop for cultivation in Cd-contaminated soil. Therefore, it is of great significance to explore the Cd-stress-responsive genes in sweet potato to improve its Cd tolerance. In this study, a novel aldo/keto reductase gene, *IbAKR*, was cloned from sweet potato cv. Lushu 3, and it was found that the overexpression of *IbAKR* resulted in increased tolerance to Cd stress in transgenic tobacco plants.

2 Materials and methods

2.1 Plant materials

The sweet potato cv. Lushu 3 was used for *IbAKR* gene cloning and expression analysis. Tobacco (*Nicotiana tabacum*) cv. Wisconsin 38 was used to study the functions of *IbAKR*.

2.2 Cloning and sequence analysis of the sweet potato *IbAKR* gene

TransZol UP (TransGen Biotech, Beijing, China) was used to extract total RNA from fresh leaves of 4-week-old *in vitro*-grown Lushu 3 plants. To amplify the full-length *IbAKR* sequence, rapid amplification of cDNA ends (RACE) was performed using a 5'/3' full RACE kit (Takara, Dalian, China). Gene-specific primers (5GSP1 and 5GSP1-2/3GSP1 and 3GSP1-2) were used for 5' and 3' RACE. The open reading frame of *IbAKR* was predicted using ORF finder. The conserved domains of the IbAKR protein were analyzed using NCBI BLAST. For the multiple sequence alignment analysis, the amino acid sequences of IbAKR and other AKR members were retrieved from the AKR superfamily home page and aligned using ClustalW. Phylogenetic analysis was conducted using MEGA6 software. The theoretical molecular weight and isoelectric point (*pI*) were calculated at ProtParam. The primers mentioned above are listed in supplementary Table S1.

2.3 *IbAKR* expression analysis

Total RNA was extracted from different tissues as described above. About 1 µg of total RNA was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocol. The cDNA solution was used as a template for PCR amplification. The sweet potato *Ibactin*

gene was used as an internal control. Quantitative real-time PCR (qRT-PCR) was carried out in a 10 µL volume using SYBR *Premix Ex Taq* (Takara, Dalian, China) and a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Gene expression was quantified using the comparative C_T method^[18]. The primers mentioned above are listed in Table S1.

2.4 Subcellular localization assay

The full-length *IbAKR* coding region without the termination codon was inserted into the pMDC83-GFP vector via PCR with linker primers (pMDC83-AKR-F/R) that contained *PacI* and *AscI* sites, generating a C-terminal fusion with the GFP gene under the control of the CaMV 35S promoter. All plasmid constructs were confirmed by sequencing. The vector *35S:GFP*, which served as the control, and *35S:IbAKR-GFP* were transiently expressed in onion epidermal cells using a biolistic particle delivery system (PDS-1000 Bio-Rad, Hercules, CA, USA). The subcellular localization of the IbAKR-GFP protein was observed with a confocal laser scanning microscope under 488 nm excitation (EZ-C1, Nikon, Tokyo, Japan). The primers mentioned above are listed in Table S1.

2.5 Generation of transgenic plants

The expression cassette P_{35S} -*IbAKR*-*T_{NOS}* was inserted into the plant binary vector pCAMBIA3301 at the *XbaI* and *SacI* sites. The sequence-verified plasmid was transfected into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method. Tobacco leaf explants were used for transformation as described by Horsch et al.^[19]. Both GUS staining and PCR analysis (*Bar*-F/R) were conducted to confirm positive transgenic plants. The primers mentioned above are listed in Table S1.

2.6 Assay for cadmium tolerance

An *in vitro* assay for Cd tolerance was conducted at 28°C using 1/2 MS medium with 200 µmol·L⁻¹ CdCl₂ and a 16/8 h light/dark cycle. Three plants per line were treated. The growth and rooting ability were continuously observed for 4 weeks. In the pot experiment, 8-week-old tobacco plants were planted in a transplanting box and irrigated with Hoagland solution with 100 mmol·L⁻¹ CdCl₂ every three days. Morphological changes in these lines were observed after 2 weeks.

2.7 Analyses of MDA, proline and H₂O₂ content and SOD activity

The malondialdehyde (MDA) content was measured according to the method of Gao et al.^[20]. The proline content and superoxide dismutase (SOD) activity were analyzed as described by He et al.^[21]. H₂O₂ content was

measured according to the method of Alexieva et al.^[22].

2.8 Leaf disc senescence assay and measurement of chlorophyll content

The leaf disc senescence assay was conducted according to Kumar et al.^[10]. To measure the chlorophyll content, 0.2 g of leaf discs were ground in 80% buffered aqueous acetone. The homogenate was filtered through filter paper and diluted to 25 mL with 80% aqueous acetone. The chlorophyll a and b content of the solution was measured at 663 and 645 nm, respectively, using a spectrophotometer. The chlorophyll concentration was calculated as described by Porra et al.^[23].

2.9 Expression analysis of ROS scavenging-related genes

Transgenic and wild-type (WT) plants were treated with 100 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 for 2 weeks, and the third leaves from the top were used to analyze the expression of reactive oxygen species (ROS) scavenging-related genes guaiacol peroxidase (*GPX*), ascorbate peroxidase (*APX*), monodehydroascorbate reductase (*MDHAR*) and peroxidase (*POD*). Tobacco *Ntactin* was used as an internal control, and qRT-PCR analysis was conducted as described above. The qRT-PCR primers are listed in Table S1.

2.10 Statistical analysis

The experiments were repeated three times, the data were analyzed using Student's two-tailed *t*-test, and the results are presented as the mean \pm SE. A value of $P < 0.05$ or < 0.01 was considered statistically significant.

3 Results

3.1 Analysis of *IbAKR* characteristics

The *IbAKR* gene was isolated from the sweet potato

cultivar Lushu 3. It consisted of five exons and four introns and contained a 1053 bp ORF that encoded a 350-amino-acid polypeptide with a calculated molecular weight of 38.32 kDa and a *pI* of 5.85. BLAST showed that the AKR protein contained typical AKR signature motifs (Fig. S1a), including an $(\alpha/\beta)_8$ -barrel motif and catalytic tetrad (Asp-63, Tyr-68, Lys-94, and His-135), which are conserved in the AKR superfamily. The AKR superfamily comprises 18 families and more than 170 members (as of July 2017, families 16–18 had not been included)^[4,14,24]. A phylogenetic analysis of *IbAKR* showed high amino acid identity with *Rauvolfia serpentina* AKR13D1 (AY766462, 51.63%) (Fig. S1b). Based on the nomenclature criteria for the AKR family^[25], *IbAKR* is in family 13 of the AKR superfamily.

3.2 Analysis of *IbAKR* expression

qRT-PCR analysis showed *IbAKR* expression in all organs of sweet potato examined. It was highly expressed in the roots of 4-week-old *in vitro*-grown Lushu 3 plants (Fig. 1a). After 4 weeks growth *in vitro* Lushu 3 plants were treated with 200 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 and 10 $\text{mmol}\cdot\text{L}^{-1}$ H_2O_2 for 0, 3, 6, 12, 24 and 48 h. An increase in *IbAKR* transcripts was observed after exposure to 200 μM CdCl_2 and peaked at 6 h when the expression level was 3 times higher than that at 0 h (Fig. 1b). Under 10 $\text{mmol}\cdot\text{L}^{-1}$ H_2O_2 stress, the expression of *IbAKR* was induced to a higher level (3.5-fold increase) at 12 h (Fig. 1c).

3.3 Subcellular localization of *IbAKR*

The subcellular localization of the *IbAKR* protein was investigated by generating 35S_{pro}-*IbAKR*-GFP and transiently expressing it in onion epidermal cells. The empty vector 35S_{pro}-GFP was used as a control. As shown in Fig. 2, the GFP fluorescence signal of *IbAKR*-GFP was detected in the nucleus and cytoplasm, as was the GFP fluorescence signal of the GFP control.

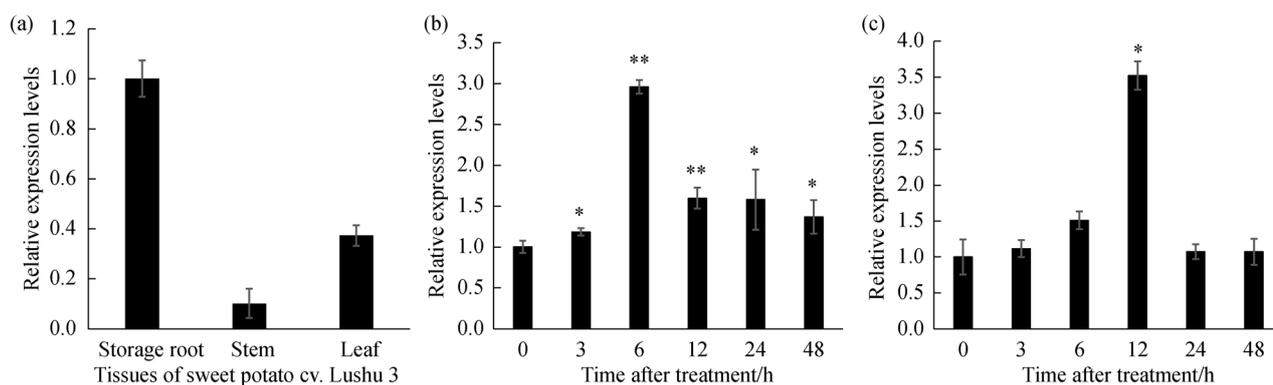


Fig. 1 Analysis of *IbAKR* expression. (a) Organ-specific expression assay of *IbAKR*; (b,c) expression levels of *IbAKR* after treatment with 200 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 (b) or 10 $\text{mmol}\cdot\text{L}^{-1}$ H_2O_2 (c). Data are presented as the mean \pm SE ($n = 3$). * and ** indicate a significant difference from 0 h at $P < 0.05$ and < 0.01 , respectively, by Student's *t*-test.

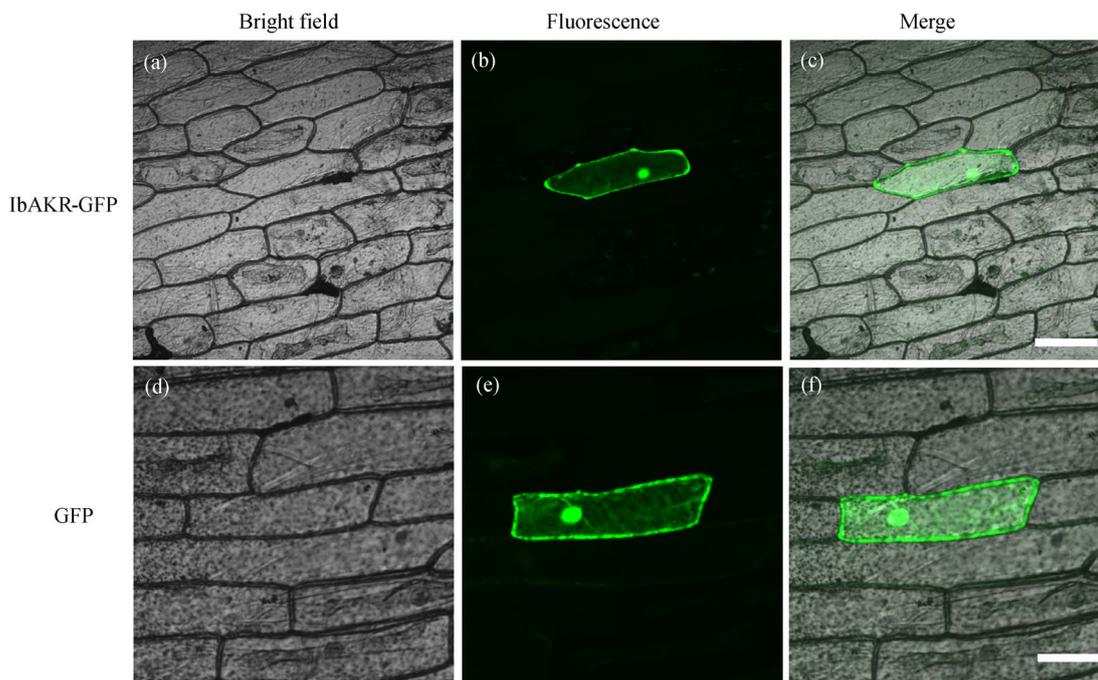


Fig. 2 Subcellular localization of the *IbAKR*-GFP fusion protein in onion epidermal cells. The empty *35S::GFP* vector (GFP) (as a control) and *35S::IbAKR-GFP* (*IbAKR*-GFP) were transiently expressed in onion epidermal cells. Fluorescence from *AKR*-GFP and GFP were observed using a confocal laser scanning microscope under 488 nm excitation. Bar = 100 μm .

3.4 Overexpression of *IbAKR* conferred strong cadmium stress tolerance to transgenic tobacco plants

To elucidate the function of *IbAKR* in plants, six transgenic tobacco lines overexpressing *IbAKR* were generated (L1–L6). The expression level of *IbAKR* was significantly higher in the transgenic plants than in the WT plants. Two lines (L1, L2) were selected for further functional analysis.

To examine the role of *IbAKR* in Cd stress, the WT and transgenic plants were cultured on 1/2 MS medium with 0 or 100 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 for 4 weeks. The growth and rooting of all plants were normal without CdCl_2 . With 100 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 , the growth and rooting development of both the WT and transgenic plants were restrained, but the Cd tolerance was significantly higher in the transgenic plants than in the WT (Fig. 3a–3c). To further test the role of *IbAKR* in Cd stress tolerance, the transgenic plants and WT were grown in a transplanting box and irrigated with a 200 mL of 100 $\text{mmol}\cdot\text{L}^{-1}$ CdCl_2 every 2 d. After 2 weeks, compared to the two transgenic lines, the WT showed more severe chlorosis and necrosis (indicated by red circle in Fig. 4a). The transgenic plants showed significantly lower contents of MDA and H_2O_2 and significantly higher contents of proline than WT (Fig. 4b–4e).

3.5 Overexpression of *IbAKR* reduced the toxic effects of MG

The levels of methylglyoxal (MG), a cytotoxic compound,

increase upon exposing plants to various abiotic stresses. High concentrations of MG are very harmful to plants. The responses of the transgenic lines to MG stress were tested using detached leaf disc senescence assays. Leaf discs from WT and L2 were floated on different concentrations of MG for 2 d. As shown in Fig. 5, the chlorophyll contents of WT and L2 showed no difference with 0 and 5 $\text{mmol}\cdot\text{L}^{-1}$ MG; however, with 10 $\text{mmol}\cdot\text{L}^{-1}$ MG, the leaf discs from L2 retained significantly more chlorophyll than those from WT.

3.6 Overexpression of *IbAKR* upregulated the ROS scavenging-related genes

To investigate the impact of *IbAKR*-overexpression (*IbAKR*-OE) on the transcription of genes related to ROS scavenging, the expression levels of *GPX*, *APX*, *MDHAR* and *POD* under Cd stress were analyzed. The results showed that these genes were significantly upregulated (between 3- and 9-fold) in the transgenic lines compared with the WT after 2 weeks of treatment with 100 $\text{mmol}\cdot\text{L}^{-1}$ CdCl_2 (Fig. 6).

4 Discussion

AKRs encompass a large superfamily of NAD(P)(H)-dependent oxidoreductases^[24]. In plants, a few AKRs have been reported to enhance tolerance to Cd stress^[5,11]. In this

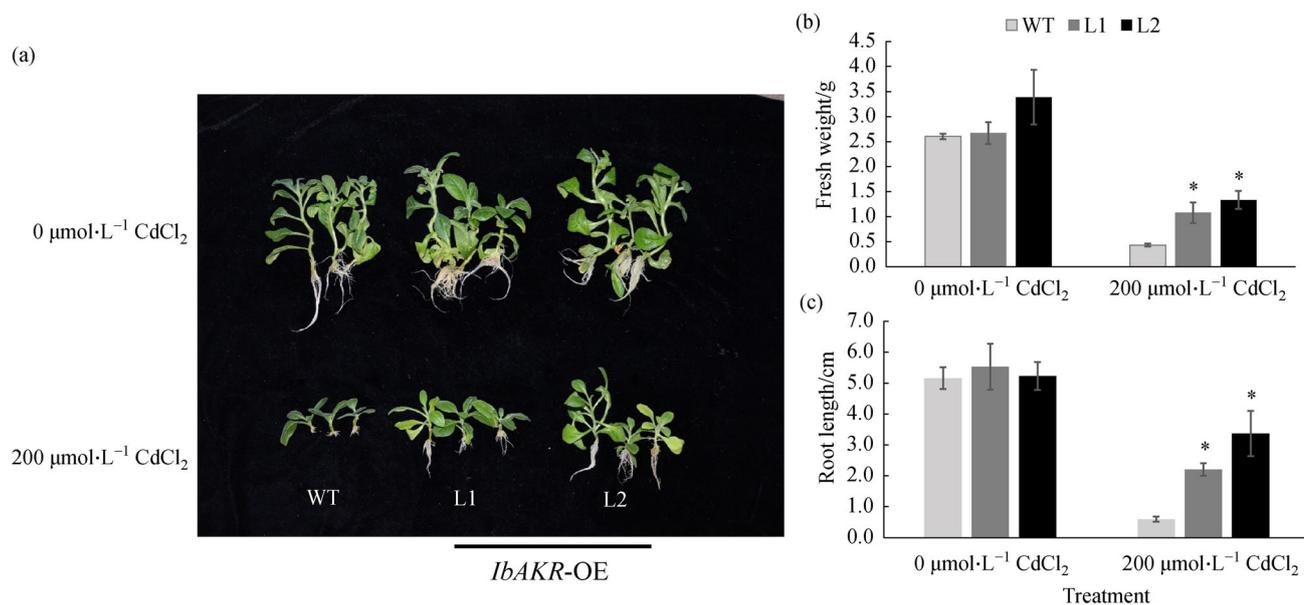


Fig. 3 Elevated cadmium tolerance in *IbaKR*-OE tobacco plants in the *in vitro* assay. (a) *In vitro*-grown tobacco plants were transferred to 1/2 MS medium with or without 200 $\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 . Photographs were taken 4 weeks after the start of treatment; (b, c) fresh weight (b) and root length (c) were measured. Data are presented as the mean \pm SE ($n = 3$). * and ** indicate a significant difference from WT at $P < 0.05$ and < 0.01 , respectively, by Student's *t*-test.

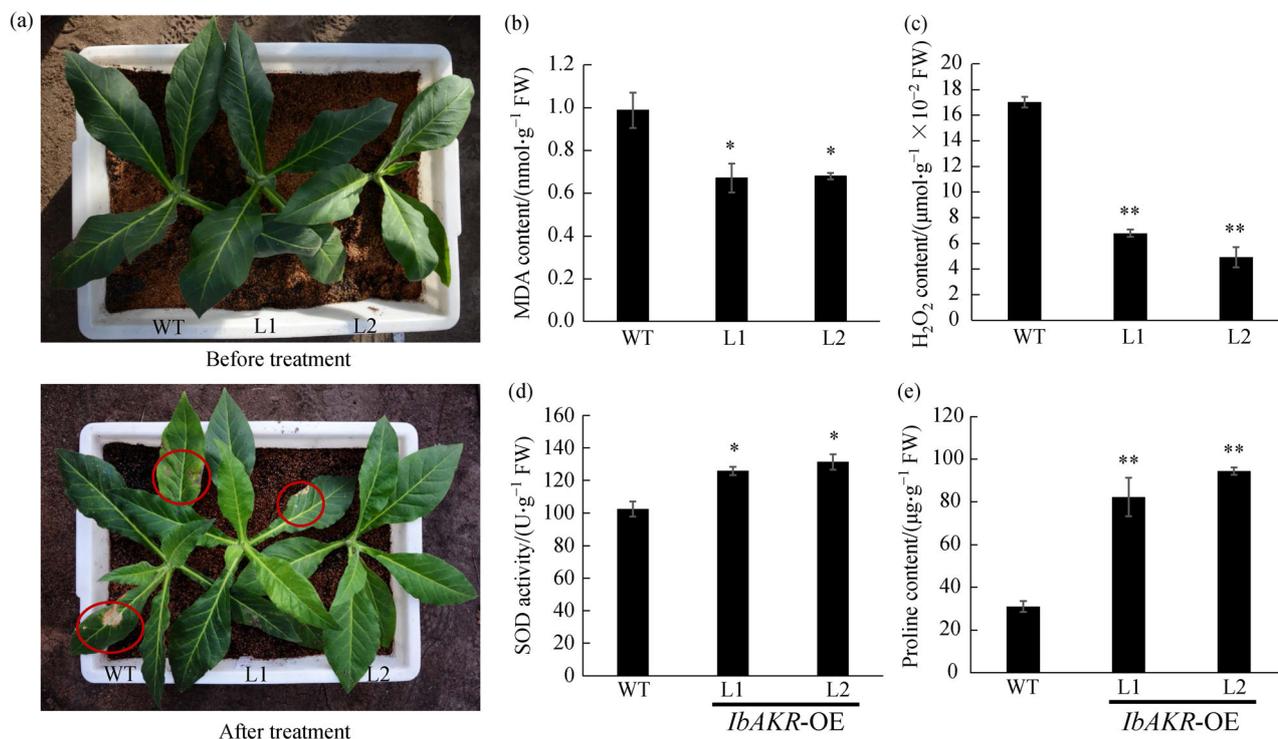


Fig. 4 Elevated cadmium tolerance in *IbaKR*-OE tobacco plants grown in pots. (a) Eight-week-old plants in a greenhouse were irrigated every 3 d with Hoagland solution containing 100 $\text{mmol}\cdot\text{L}^{-1}$ CdCl_2 and morphological changes in the three lines (WT, L1, L2 from left to right) were photographed before and 2 weeks after the start of treatment; red circles indicate necrotic spots; (b–e) content of stress-related metabolites and superoxide dismutase (SOD) in transgenic lines and WT after treatment with 100 $\text{mmol}\cdot\text{L}^{-1}$ CdCl_2 for 2 weeks. Data are presented as the mean \pm SE ($n = 3$). * and ** indicate a significant difference from WT at $P < 0.05$ and < 0.01 , respectively, by Student's *t*-test.

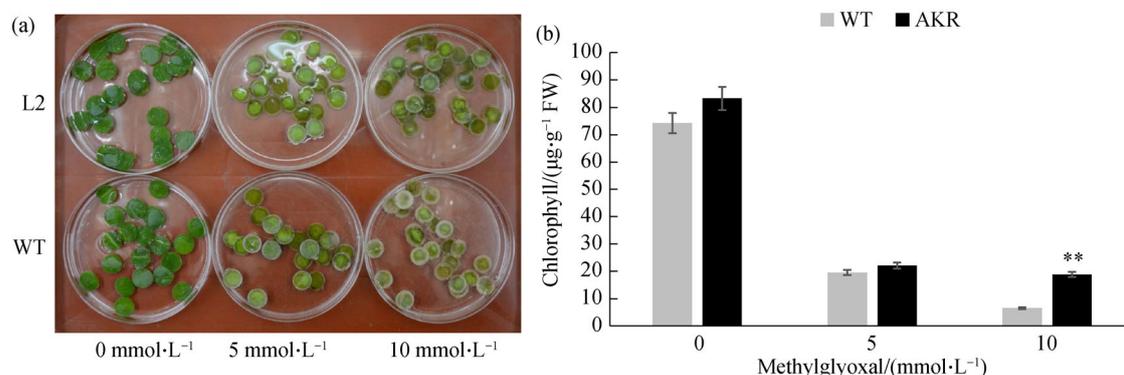


Fig. 5 Leaf disk senescence under methylglyoxal (MG) stress as measured by chlorophyll content. Chlorophyll content ($\mu\text{g}\cdot\text{g}^{-1}$ FW) of leaf discs from WT and transgenic line L2 tobacco plants, kept for 3 d at different concentrations of MG. Data are presented as the mean \pm SE ($n = 3$). * and ** indicate a significant difference from WT at $P < 0.05$ and < 0.01 , respectively, by Student's t -test.

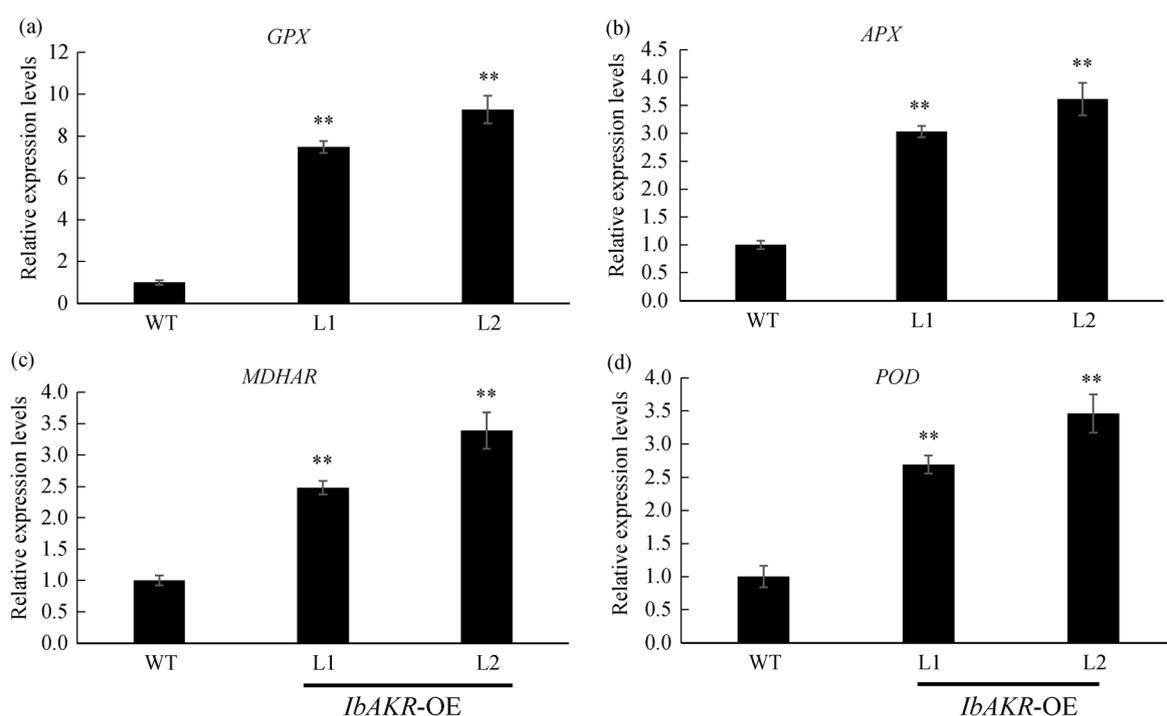


Fig. 6 Analysis of the expression of ROS scavenging-related genes in transgenic plants and WT. The relative expression levels of genes encoding guaiacol peroxidase (*GPX*), ascorbate peroxidase (*APX*), monodehydroascorbate reductase (*MDHAR*) and peroxidase (*POD*) were analyzed after treatment with $100\text{ mmol}\cdot\text{L}^{-1}$ CdCl_2 for 2 weeks. Data are presented as the mean \pm SE ($n = 3$). * and ** indicate a significant difference from WT at $P < 0.05$ and < 0.01 , respectively, by Student's t -test.

study, *IbAKR* was cloned from sweet potato. BLAST showed that *IbAKR* contained typical AKR signature motifs. According to the nomenclature of the AKR superfamily, *IbAKR* shared 51.63% ($> 40\%$) amino acid identity with AKR13D1, suggesting that *IbAKR* belongs to family 13 of the AKR superfamily; however, its amino acid identity with the other members of family 13 was less than 60%, suggesting that *IbAKR* is part of a new subfamily of family 13^[26]. These results showed that *IbAKR* could be a novel member of the AKR superfamily.

The relative expression levels of *IbAKR* in sweet potato increased 3- and 3.5-fold in response to $200\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ CdCl_2 and $10\text{ mmol}\cdot\text{L}^{-1}$ H_2O_2 , respectively, suggesting that *IbAKR* might have a role in Cd stress tolerance. The overexpression of *IbAKR* enhanced tolerance to Cd stress in transgenic tobacco plants. Under Cd stress, transgenic plants showed better growth and rooting than WT (Fig. 3a–3c; Fig. 4a). This result indicates that the *IbAKR* gene may be used to improve plant tolerance to Cd stress.

MDA is often considered a major lipid peroxidation

marker, and its accumulation beyond a threshold level has been reported to cause irreparable damage to the photosynthetic machinery^[27]. MG is a glycolysis-derived toxic aldehyde, and the accumulation of MG to a high concentration is toxic to cells, as it inhibits cell proliferation^[28]. In a previous study, OsAKR1 exhibited high catalytic activity in the reduction of toxic aldehydes, including lipid peroxidation-derived MDA and glycolysis-derived MG, leading to an increased tolerance to oxidative and heat stress^[8]. In our study, we found that overexpressing *IbAKR* could significantly reduce the levels of MDA relative to those in WT under Cd stress (Fig. 4b). Additionally, leaf discs from transgenic plants retained more chlorophyll after MG treatment (Fig. 5). These results suggest that Cd stress tolerance was enhanced in *IbAKR*-OE tobacco plants via scavenging MDA and MG and reduction in membrane damage.

In plants, Cd stress leads to the overproduction of ROS, which are highly reactive and toxic. ROS scavenging systems can detoxify ROS to minimize and/or prevent oxidative damage and cells respond to ROS by increasing the activity of ROS-scavenging enzymes^[29]. As a key enzyme of the ROS scavenging system, SOD is usually induced by various abiotic stresses to promote the rapid dismutation of superoxide into oxygen and H₂O₂, which is subsequently removed by GPX, APX and POD through different pathways; moreover, MDHAR is needed to regenerate the active forms of antioxidants^[30,31]. It has been reported that proline acts as a non-enzymatic ROS scavenger and can scavenge singlet oxygen and hydroxyl radicals^[32]. In our study, *IbAKR*-OE tobacco plants had significantly higher SOD activity and proline levels and significantly lower H₂O₂ levels than WT (Fig. 4c–4e). qRT-PCR showed that the expression of *GPX*, *APX*, *MDHAR* and *POD* was significantly upregulated (Fig. 6), suggesting that the improved Cd stress tolerance of the transgenic tobacco plants is also due to enhanced ROS scavenging.

5 Conclusions

A novel aldo-keto reductase gene, *IbAKR*, has been successfully identified and cloned from the sweet potato cv. Lushu 3. The *IbAKR*-OE tobacco plants possessed higher tolerance than WT to Cd stress. This suggests the transgenic plants have a significant ability to scavenge MDA and MG. In addition, proline content and SOD activity were significantly higher and H₂O₂ levels were significantly lower in the transgenic plants than in the WT. Furthermore, ROS scavenging genes *GPX*, *APX*, *MDHAR* and *POD* were significantly upregulated in transgenic plants compared to the WT under Cd stress. Our results suggest that the overexpression of *IbAKR* enhanced Cd tolerance by reducing cytotoxic aldehydes and increasing the capacity for scavenging reactive oxygen species. This

study indicates that the *IbAKR* gene may be useful for improving tolerance to Cd stress in plants.

Supplementary materials The online version of this article at <https://doi.org/10.15302/J-FASE-2018225> contains supplementary materials (Table S1; Fig. S1).

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Compliance with ethics guidelines Jinxi Huo, Bing Du, Sifan Sun, Shaozhen He, Ning Zhao, Qingchang Liu, and Hong Zhai declare that they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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