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The Changes of Tolerance, Accumulation and Oxidative Stress Response to Cadmium in Tobacco Caused by Introducing *Datura stramonium* L. Genes

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Abstract: Whether it is possible to create suitable plants for cadmium phytoremediation by introducing *Datura stramonium* L. genes into tobacco (*Nicotiana tabacum* L.) remains unclear. Hydroponic experiments were performed on *N. tabacum* L. var. MTLY, a newly developed hybrid variety, and the parents. Seedlings at the six-leaf stage were treated with 0 (control), 10 μM, 180 μM and 360 μM $CdCl_2$ for 7 days, and their differences in Cd tolerance and accumulation and physiological and metabolic responses were evaluated. When subjected to 360 μM Cd, the growth of "MTLY", in terms of the dry weight, plant height and root length, was obviously better than *N. tabacum* L. var. LY2 (female parent). In contrast to *D. stramonium* (male parent) and "LY2", "MTLY" accumulated more Cd in shoots (127.6–3837.1 mg kg^{-1}) and roots (121.6–1167.7 mg kg^{-1}). Moreover, unlike "LY2", "MTLY" could accumulate more Cd in its shoots than roots. Its bioconcentration factor (BCF) and translocation factor (TF) values reached 95.9–149.7 and 1.0–3.5, respectively, which were far greater than those of "LY2". High-dose Cd stress significantly increased reactive oxygen species (ROS) and malondialdehyde (MDA) levels and decreased chlorophyll contents in tobacco seedlings, especially in "LY2". Various enzymatic and non-enzymatic antioxidants in the three materials showed different responses to Cd stress. The change of the phenolic compounds and alkaloids in "MTLY" was basically similar to that in *D. stramonium*, but their levels were apparently higher than those in "LY2". Results indicated that distant hybridization could be one of the effective methods for introducing metal-hyperaccumulator genes into a high biomass species, which contributed to enhancing the Cd tolerance, accumulation and detoxification in tobacco. This study has great significance in obtaining elite germplasm for phytoremediation. The exact mechanisms in molecules and genetics and the practical effectiveness in cadmium-contaminated soil remain to be further elucidated.

Keywords: cadmium; cross breeding; *Datura stramonium* L.; phytoremediation; tobacco

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1. Introduction

Heavy metal pollution of farmland has been a serious problem worldwide. With the rapid development of industry and agriculture, cadmium is regarded as one of the most widely distributed pollutants in China. Cd pollution has severely affected crop yields, food safety and human health [1–5]. As an emerging technology, phytoremediation, the use of green plants to purify the environment, has attracted tremendous attention since the 1990s [1,6]. However, its successful application depends largely on suitable species, such as hyperaccumulating and accumulating plants [3,7–9]. However, to date, only a few species have been recognized as Cd hyperaccumulators [3,9,10]. Although they display an extraordinary capacity to tolerate and accumulate high concentrations of Cd in aboveground tissues, their remediation potential is quite limited because of slow growth, low biomass and climate and geographical restrictions [1,6,7]. Some high-biomass crops such as maize (*Zea mays* L.), sweet sorghum (*Sorghum bicolor* L.) and sunflower

(*Helianthus annuus* L.) have also showed excellent capacities for extracting Cd from soils. However, it has to be considered that they could enter the food chain, influencing food safety and human health [2]. In addition, non-accumulator plants can even be genetically modified to achieve some of the properties of hyperaccumulators. There has been considerable progress in improving their uptake, translocation and/or tolerance so far [3–5,8,10–14]. However, a risk assessment of transgenic plants should be accounted very carefully before there is any field application [9]. The lack of fast-growing, high-biomass hyperaccumulating plants remains a major bottleneck in phytoremediation [2,3].

The conventional breeding approach has been proposed to introduce metal-hyperaccumulation traits into fast-growing, high-biomass plants such as tobacco (*Nicotiana tabacum* L.), breeding plants with superior phytoremediation potentials [8,9,15,16]. Theoretically, distant hybridization can facilitate the introgression of desirable genes between diverse germplasm into the available genetic base, creating new varieties or new types. However, the more taxonomically distant the parental species, the more difficult it is to cross between the two species. This approach has been rarely successful mainly due to the sexual incompatibility between parents [3,17]. Compared to the traditional methods, pollination following grafting could efficiently overcome the barrier of sexual incompatibility between *N. tabacum* L. and some plants and facilitate genes' introgression, exchange and recombination [18–20]. Although the underlying mechanism has not yet been determined, we have successfully obtained new-type tobacco varieties, "MTLY", between *N. tabacum* L. and *Datura* [18,21,22]. In agronomic character, *N. tabacum* L. var. MTLY was generally inclined towards the female parent, *N. tabacum* L., such as plant type, plant height and leaf numbers, but some obvious changes appeared, such as leaf shape and petiole, which resembled *Datura* (male parent) (Figure 1A–C). In addition, there was very obvious difference between *N. tabacum* L. var. MTLY and the female parent tobacco in the major chemical components (including protein, total nitrogen, sugars, reducing sugars, etc.) and the secondary metabolites, such as phenolic compounds and alkaloids. *N. tabacum* L. var. MTLY also produced some new compounds, such as scopolamine and atropine, which are only found in the *Datura* [18,21,22]. The genus *Datura* is widely distributed in the warm regions of the world. They are now a cosmopolitan weed or are cultivated for the production of tropane alkaloids [23]. Some species of *Datura*, such as *D. stramonium* L. and *D. innoxia* Mill., could thrive in a wide variety of soil conditions (such as alkaline, saline, droughty and acid soils) and showed a very strong capacity to tolerate and accumulate various heavy metal(loid)s including cadmium, chromium, copper, lead, manganese, nickel, zinc and arsenic [23–30]. Wild plants of *Datura* could be a valuable resource of genes for phytoremediation. For a long time, tobacco has been known as a promising candidate for the phytoremediation of Cd-contaminated soils because of rapid growth, high biomass and relatively high cadmium accumulation in some genotypes [12,31].

Cadmium has a strong toxicity to plants even at low concentrations, resulting in stunted growth, the inhibition of photosynthesis, perturbation of antioxidant defense, alteration of cell metabolism and even death, which is strongly linked with reactive oxygen species (ROS)-mediated oxidative stress [32–36]. A prerequisite for metal accumulation is the ability to efficiently tolerate high concentrations of metals in tissues and cells [3,4,10,37,38]. The traits of Cd tolerance and accumulation are genetically controlled, which vary greatly, not only among plant species but also among cultivars within the same species, and even among organs and tissues in the same plant [3,26,39–43]. Cd tolerance is governed by a series of complex physiological and biochemical processes [6,42,44–46], among which the intrinsic antioxidant mechanisms, including various enzymatic and non-enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), proline, phenolic compounds and alkaloids, played an important role in reducing, neutralizing and scavenging ROS, as evidenced by studies over a range of plant species [26,34,46–54]. It is not clear whether new-type tobacco will heighten the Cd uptake, accumulation, tolerance and detoxification due to introducing *Datura* genes. In the present study, seedlings were subjected to different Cd concentrations (0, 10, 180 and 360 μM) under hydroponics for 7 days, and the differences of *N. tabacum* L. var. MTLY and the

parents in morphophysiological responses, such as biomass production, oxidative stress and changes of antioxidant components and secondary metabolites and Cd accumulation and translocation in tissues, were evaluated. The potential of *N. tabacum* L. var. MTLY for soil remediation was discussed.

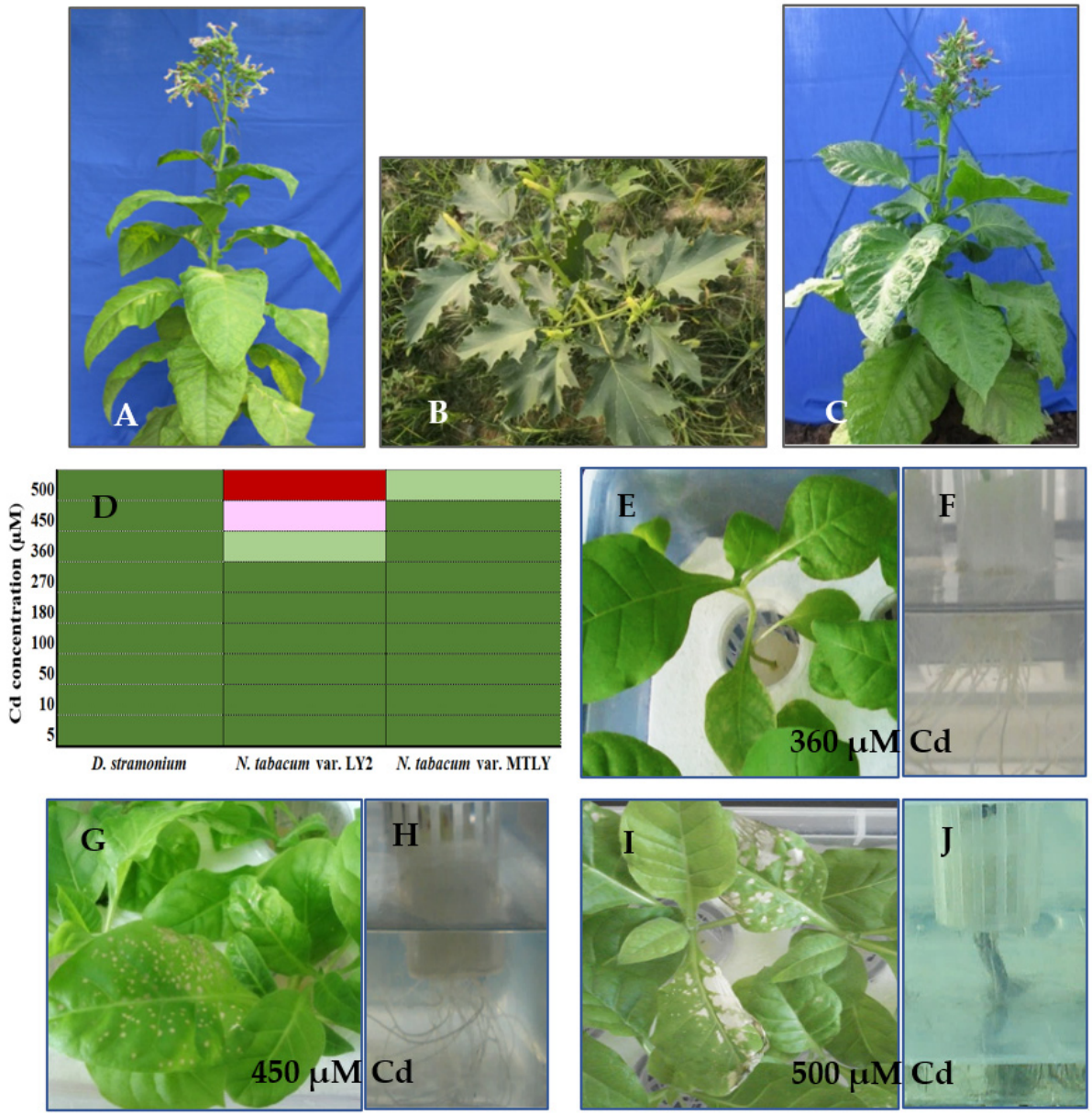

Figure 1. The plant materials and preliminary screening of Cd concentrations. (**A**) *Nicotiana tabacum* L. var. LY2 (pistillate parent). (**B**) *Datura stramonium* L. (pollen parent). (**C**) *Nicotiana tabacum* L. var. MTLY. (**D**) The toxic effects of different Cd concentrations (5–500 μM) on 70-day old seedlings grown in hydroponics. The four colors, i.e., green, pale green, pink and red represented different severities, respectively. Observation showed that Cd tolerance of seedlings was in sequence: *D. stramonium* L. > *N. tabacum* L. var. MTLY > *N. tabacum* L. var. LY2. *D. stramonium* could grow even at 500 μM Cd without visual and conspicuous symptoms. (**E**–**J**) When tobacco seedlings were exposed to 360, 450 and 500 μM Cd, leaves and roots showed varying degrees of toxic symptoms, respectively. (**E**,**F**) Yellow spots on leaves, slight chlorosis of leaves and minor browning of root tips in *N. tabacum* L. var. LY2. (**G**,**H**) *N. tabacum* L. var. LY2 showed obvious phytotoxic symptoms, including leaf chlorosis and root browning. (**I**,**J**) Severe wilting and chlorosis in leaves and brown and necrosis in roots was found in *N. tabacum* L. var. LY2.

2. Materials and Methods

2.1. Plant Materials

Datura stramonium L. was the most common native wild type plant species, which was characterized by a relatively high Cd tolerance and accumulation. Seeds were collected near the Coking Plant in the Jinzhong area of Shanxi Province, China. *Nicotiana tabacum* L. var. LY2, a sun-cured tobacco variety, was developed from "Mulinghuboxiang" and "Mu sun-cured tobacco 93-4-5" in Heilongjiang Province, China. *Nicotiana tabacum* L. var. MTLY, a new-type tobacco variety, obtained from a cross-combination [(*N. glauca* Graham rootstock + *N. tabacum* L. var. LY2 scion) × *D. stramonium* L.], was bred as described previously [18,20–22] (Figure 1A–C). Seeds were kindly provided by Prof. Z. Wei (Shanxi Agricultural University, Taigu, China).

2.2. Hydroponic Experiment

Seeds were soaked in distilled water for 24 h, surface-sterilized in 75% ethanol for 30 s and 10% H_2O_2 for 10 min and thoroughly washed with distilled water. Seeds were germinated in a mixture of peat, perlite and vermiculite (3:1:1) and irrigated every 2 days with 1/10 strength Hoagland nutrient solution. The materials were grown in a plant culture chamber at a temperature of 25 °C, with 75% relative humidity and 16/8 h (light/dark). When plants reached the 6-leaf stage (approximately 66 days old), seedlings were gently washed with deionized water to remove the growth medium before being transferred into plastic containers (29.5 cm × 20 cm × 12.7 cm). Each container was covered with a polystyrol plate with 7 evenly spaced holes (1 plant per hole). Seedlings were grown hydroponically for 7 days in 2 L Hoagland nutrient solution under the same culture condition described above. The nutrient solution was continuously aerated with pumps and renewed every 3 d. One week after transplanting, healthy and uniform seedlings were selected and grown in hydroponic conditions in the presence of 0 (control), 10 μM, 180 μM and 360 μM Cd^{2+}. Cd doses were chosen according to the preliminary screening experiment (Figure 1D–J). Cd was applied as $CdCl_2 \cdot 2.5\ H_2O$. Each treatment was applied in triplicates, and seven plants per plastic container were used as each treatment. Plant health was carefully monitored. After 7 days, plant growth was evaluated by examining the dry weight, plant height and root length. Then, four seedlings in each treatment were harvested and immediately stored at −80 °C for later biochemical analysis, and the other three were separated into shoots and roots and further processed for Cd quantitation and histochemical stain [31,33,35,55].

2.3. Determination of Cd Concentration

Harvested seedlings was thoroughly washed with distilled water, divided into shoots and roots and dried at 70 °C to a constant weight. Dried plant tissues were ground, weighed and completely digested with a solution of HNO_3 and $HClO_4$ (4:1). Then, the mixture was evaporated to dryness and dissolved in 1% HNO_3. The Cd concentrations were determined using a flame atomic absorption spectrometer (240FS AA, Agilent, Santa Clara, CA, USA). The bioconcentration factor (BCF) was defined as the ratio of metal concentration in plant roots and shoot tissues to that in the soil or solution. The translocation factor (TF) indicated the ability of plants to translocate cadmium from the roots to the shoots. BCF and TF were calculated as follows [56–58]:

$$\text{BCF} = [\text{Cd}] \text{ in plant shoots}/[\text{Cd}] \text{ in solution}$$

$$\text{TF} = [\text{Cd}] \text{ in shoots}/[\text{Cd}] \text{ in roots}$$

2.4. ROS and MDA Assay

ROS in leaves were analyzed using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) [59]. In brief, frozen tissue was ground in liquid nitrogen and homogenized in 10 mM Tris-HCl buffer (pH 7.3). After centrifugation at 20,000× *g* for 5 min, the

supernatants were collected. ROS generation was assayed by measuring fluorescence using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, USA). The ROS in the roots was analyzed using the fluorescent probes DCFH-DA [60]. The roots were rinsed, incubated with 10 μM DCFH-DA in darkness at 20 °C for 40 min and then rinsed with deionized water. The tissues were observed and photographed under a fluorescence microscope (Olympus BX51, Tokyo, Japan). The ROS content was expressed by relative fluorescence intensity, calculated using Image J software.

Fresh leaf (approximately 0.1 g) was homogenized in 0.1% cold trichloroacetic acid. The homogenate was centrifuged at 8000× *g* for 10 min at 4 °C. Malondialdehyde (MDA) content was determined by measuring the concentration of thiobarbituric acid-reactive substance (TBARS) as described previously [60,61] and expressed as nmol g^{-1} fresh weight.

2.5. Chlorophyll and Proline Assay

The contents of chlorophylls (chlorophyll a, chlorophyll b and total chlorophyll) were analyzed according to the method of Sun et al. [62]. Fresh tissue (0.1 g) was homogenized in 80% acetone in the dark, and the extract was measured spectrophotometrically at 646 and 663 nm. Chlorophyll content was expressed as mg g^{-1} fresh weight. A total of 0.5 g fresh leaf was homogenized with 5 mL of 3 % sulfosalisylic acid, and the homogenate was cooled after heating for 10 min at 100 °C. After centrifugation at 10,000× *g* for 10 min, the content of free proline in the supernatant was measured using a ninhydrin reagent at 520 nm and expressed as μg g^{-1} fresh weight [63].

2.6. Antioxidant Enzymes Assays

A sample of fresh leaf tissue (0.3 g) was homogenized in an ice-cooled mortar with 3 mL of 50 mM PBS buffer (pH 7.8) containing 1 mM EDTA and 2% PVP. The homogenate was centrifuged at 8000× *g* for 10 min at 4 °C, and the resulting supernatant was used for enzymes determination. SOD activity was assayed by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The activity of CAT and POD was determined using the H_2O_2 and guaiacol substrates, respectively, as described previously [60]. Protein contents were measured as described by Bradford [64] using BSA as a standard. All enzyme activities were expressed as U mg^{-1} protein.

2.7. Histochemical Analysis

Nine seedlings were randomly chosen from each treatment. Samples of the fresh leaf and main root, from the same part of seedlings, were cut off and fixed in 4% paraformaldehyde, dehydrated with a sucrose gradient and embedded in OCT (optimal cutting temperature) compound (Solarbio, Beijing, China). Frozen sections (25 μm thick) were prepared (CM1850, Leica, Wetzlar, Germany) and the following histochemical tests were performed: Fast Blue BB Salt for phenolic compounds and Dragendorff reagent for alkaloids. Control tests were performed simultaneously, according to the recommendations of the respective authors [65–68]. All sections were finally sealed with glycerin, viewed and analyzed under an optical microscope (Olympus BX51, Tokyo, Japan).

2.8. Statistical Analysis

Results were presented as means ± SD. Statistical analyses were performed using the SPSS software (version 25.0). All data were checked for normality using the Shapiro–Wilk test. The general linear model (GLM) was used to perform variance analysis as well as to assess the effects of the plant materials, Cd treatments and their interactions on the test results. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of Cadmium Stress on Seedlings Growth

The growth of *D. stramonium* was almost unaffected by Cd stress. Under normal conditions, there was no difference in the dry weight, plant height and root length between

the two tobacco varieties, but they were significantly inhibited at high Cd doses. At 360 μM Cd, the dry weight, plant height and root length of *N. tabacum* L. var. LY2 decreased by 19.3, 14.3 and 34.6%, while *N. tabacum* L. var. MTLY showed a reduction of 13.8, 6.6 and 20.7%, respectively, when compared to the controls (Figure 2A–D). The growth of *N. tabacum* L. var. MTLY was evidently better than *N. tabacum* L. var. LY2.

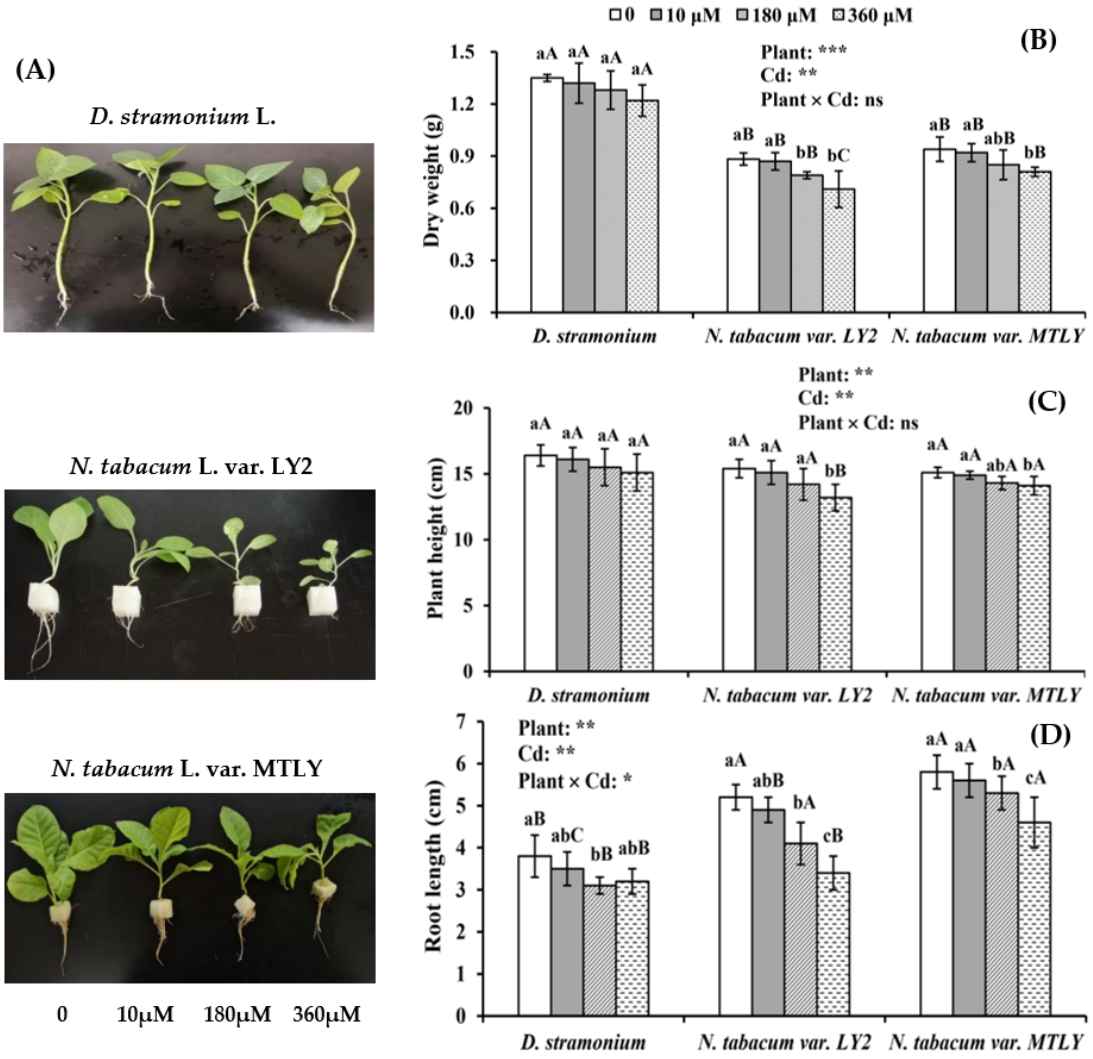

Figure 2. The growth traits of seedlings exposed to different Cd concentrations for 7 days under hydroponic conditions. (**A**) The phenotypic changes of *D. stramonium*, *N. tabacum* L. var. LY2 and *N. tabacum* L. var. MTLY. Dry weight (**B**), plant height (**C**) and root length (**D**). Values were expressed as means ± SD ($n = 9$). Different lowercase letters on the bars indicated significant differences between the same material under different Cd concentrations. Different uppercase letters on the bars indicated significant differences between different materials under the same Cd treatment ($p < 0.05$). GLM was used to assess the effects of the plant materials, Cd treatments and their interactions on test results. The significance was indicated as follows: ns, not significant; * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

3.2. Cd Accumulation in Seedling Tissues

In the control groups, Cd was not detected in the tissues of seedlings. In contrast to *N. tabacum* L. var. LY2, *D. stramonium* and *N. tabacum* L. var. MTLY accumulated more Cd in the shoots than in the roots under Cd treatments. With the increase of the Cd concentration in the culture medium, *N. tabacum* L. var. MTLY accumulated maximum Cd (127.6–3837.1 mg kg^{-1}) in the shoots, but *D. stramonium* (101.0–555.6 mg kg^{-1}) followed by

N. tabacum L. var. LY2 (19.7–49.2 mg kg^{-1}). The contents in roots were in the order as *N. tabacum* L. var. MTLY (121.6–1167.7 mg kg^{-1}) > *N. tabacum* L. var. LY2 (76.1–393.2 mg kg^{-1}) > *D. stramonium* (25.0–136.5 mg kg^{-1}). The BCF and TF values of *N. tabacum* L. var. MTLY was in the range of 95.9–149.7 and 1.0–3.5, respectively, which were far greater than those of *N. tabacum* L. var. LY2 (BCF: 1.2–19.7, TF: 0.13–0.26). These values of *D. stramonium* could reach 13.9–101.0 and 4.0–4.2 (Figure 3A–D). There was apparent difference in the Cd accumulation and translocation ability between the tested materials, and *N. tabacum* L. var. MTLY displayed better traits than *N. tabacum* L. var. LY2.

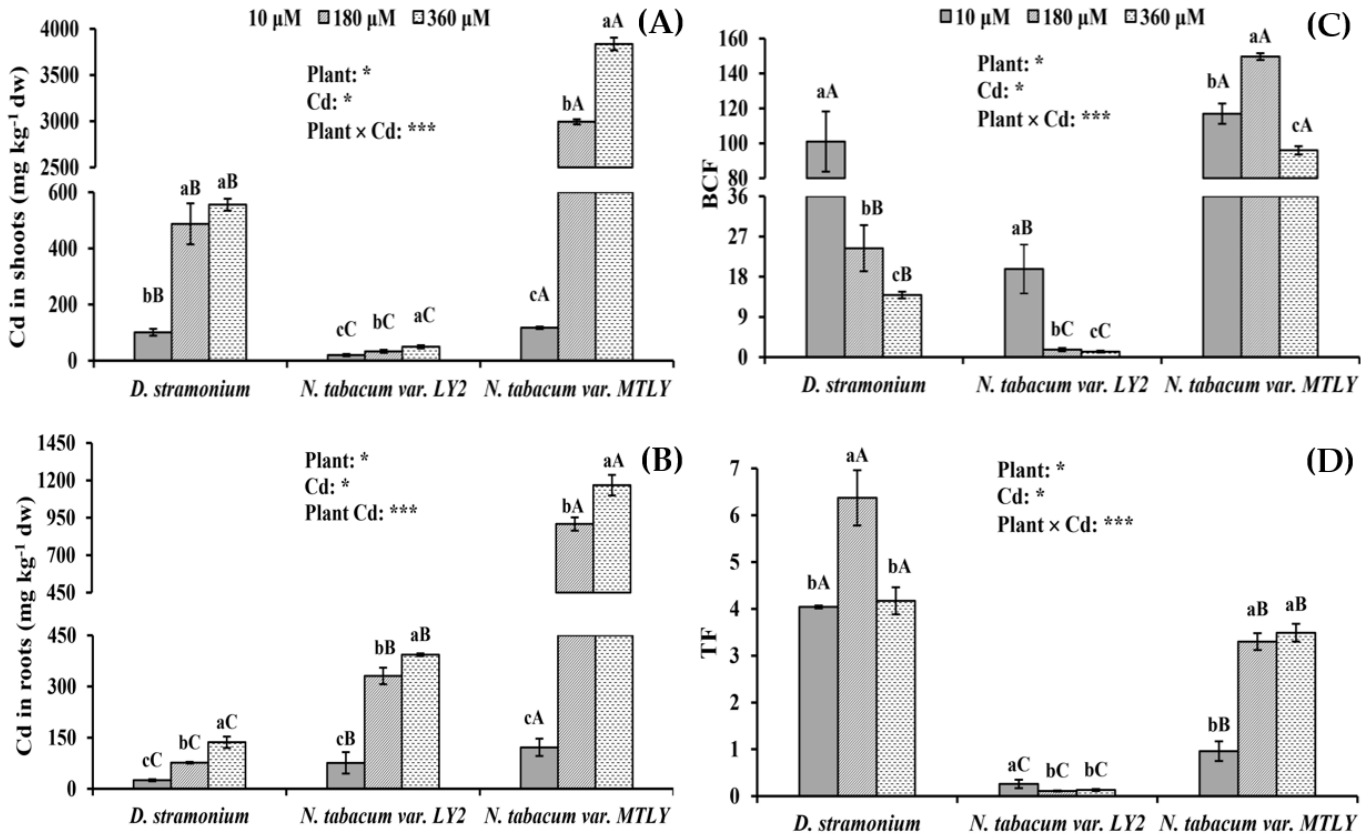

Figure 3. Cd accumulation and translocation in seedling tissues. Cd contents in shoots (**A**) and roots (**B**). Bioconcentration factor (BCF) of Cd in the shoots (**C**). Translocation factor (TF) of Cd from root to shoot (**D**). Values were expressed as means ± SD (n = 9). Different lowercase letters on the bars indicated significant differences between the same material under different Cd concentrations. Different uppercase letters on the bars indicated significant differences between different materials under the same Cd treatment ($p < 0.05$). GLM was used to assess the effects of plant materials, Cd treatments and their interactions on test results. The significance was indicated as follows: * $0.01 < p \leq 0.05$; *** $p \leq 0.001$.

3.3. Cd Exposure Induced Oxidative Stress in Seedlings

Under normal conditions, there was a significant difference in the ROS levels of the leaves and roots between *N. tabacum* L. var. MTLY and the parents. Cd stress significantly increased ROS generation in seedlings compared to the controls, especially in *N. tabacum* L. var. LY2 (Figure 4A–C). Meanwhile, the MDA levels in the leaf tissues obviously increased at high doses, but the chlorophyll contents reduced even at low doses. The increase in MDA and decrease in chlorophyll was most pronounced in *N. tabacum* L. var. LY2, followed by *N. tabacum* L. var. MTLY and *D. stramonium* (Figure 5A–D). These indicated that *N. tabacum* L. var. MTLY was less susceptible to Cd toxicity than *N. tabacum* L. var. LY2.

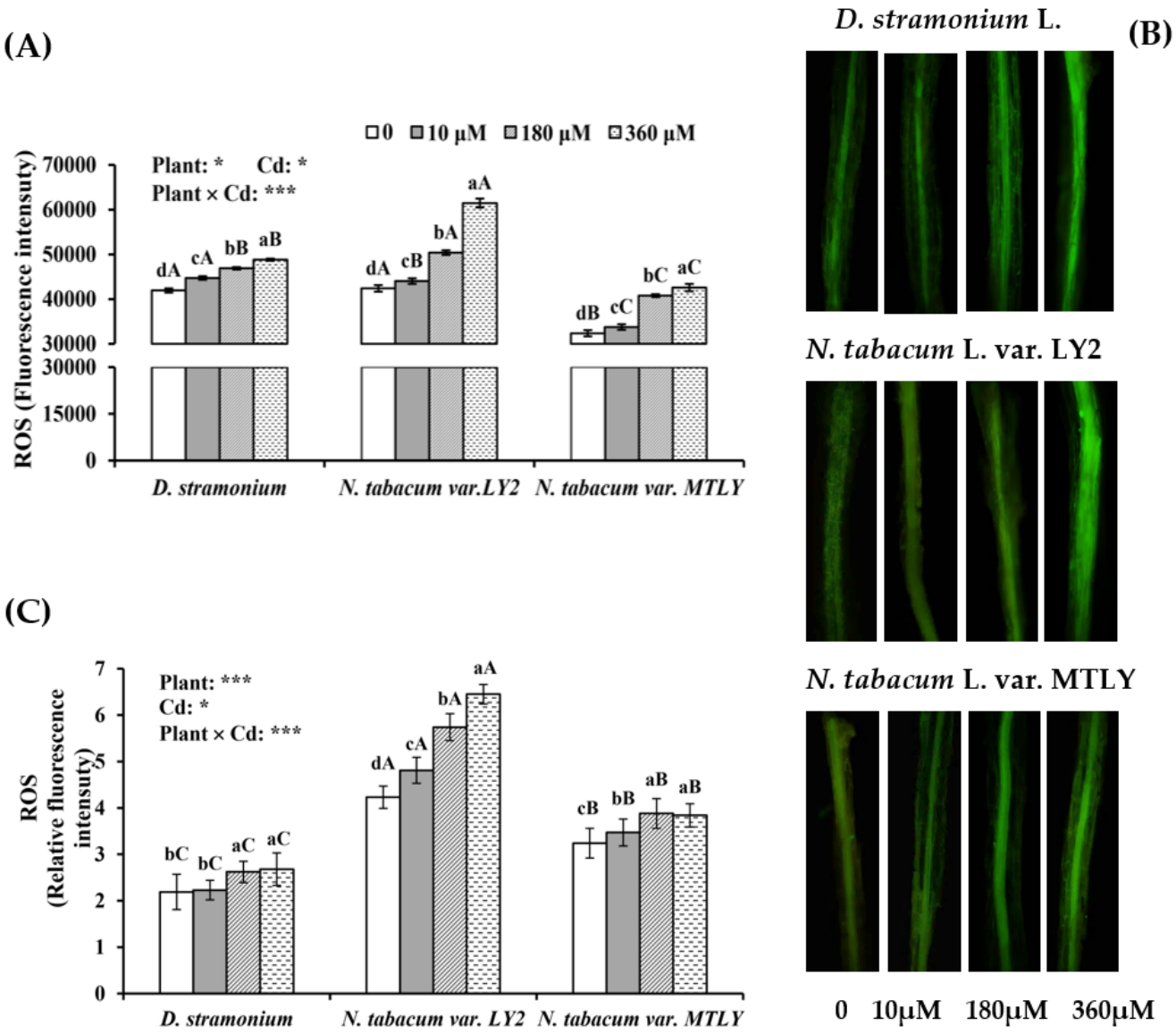

Figure 4. Cd-induced oxidative stress in seedlings. (**A**) Reactive oxygen species (ROS) generation in leaves. DCFH-DA staining of ROS (**B**) and its relative fluorescence intensity (**C**) in root tips. Values were expressed as means ± SD (n = 9). Different lowercase letters on the bars indicated significant differences between the same material under different Cd concentrations. Different uppercase letters on the bars indicated significant differences between different materials under the same Cd treatment ($p < 0.05$). GLM was used to assess the effects of plant materials, Cd treatments and their interactions on test results. The significance was indicated as follows: * $0.01 < p \leq 0.05$; *** $p \leq 0.001$.

3.4. Antioxidant Response of Seedlings to Cd Stress

Under normal conditions, *N. tabacum* L. var. MTLY had higher SOD and POD activities than *N. tabacum* L. var. LY2, but there was no significant difference in the CAT and proline levels between them. Meanwhile, *N. tabacum* L. var. MTLY had higher SOD and CAT activities but lower POD and proline contents than *D. stramonium*. These antioxidant components were increased significantly in response to the high Cd stress compared to the controls. Two tobacco varieties showed higher SOD and CAT activities but lesser POD and proline contents than *D. stramonium*. At 360 μM Cd, there was only no obvious difference in SOD and CAT activities between *N. tabacum* L. var. MTLY and *N. tabacum* L. var. LY2 (Figure 6A–D).

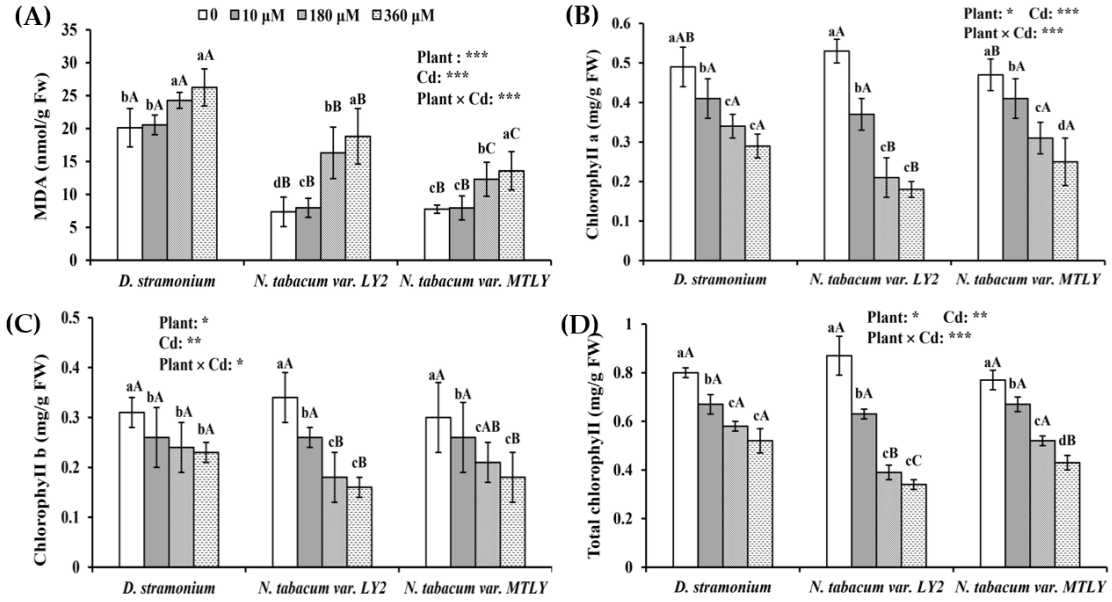

Figure 5. Cd-induced oxidative damage in seedlings. (**A**) Malondialdehyde (MDA) content in leaves. Contents of chlorophyll a (**B**), chlorophyll b (**C**) and total chlorophyll (**D**) in leaves. Values were expressed as means ± SD (n = 9). Different lowercase letters on the bars indicated significant differences between the same material under different Cd concentrations. Different uppercase letters on the bars indicated significant differences between different materials under the same Cd treatment ($p < 0.05$). GLM was used to assess the effects of plant materials, Cd treatments and their interactions on test results. The significance was indicated as follows: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

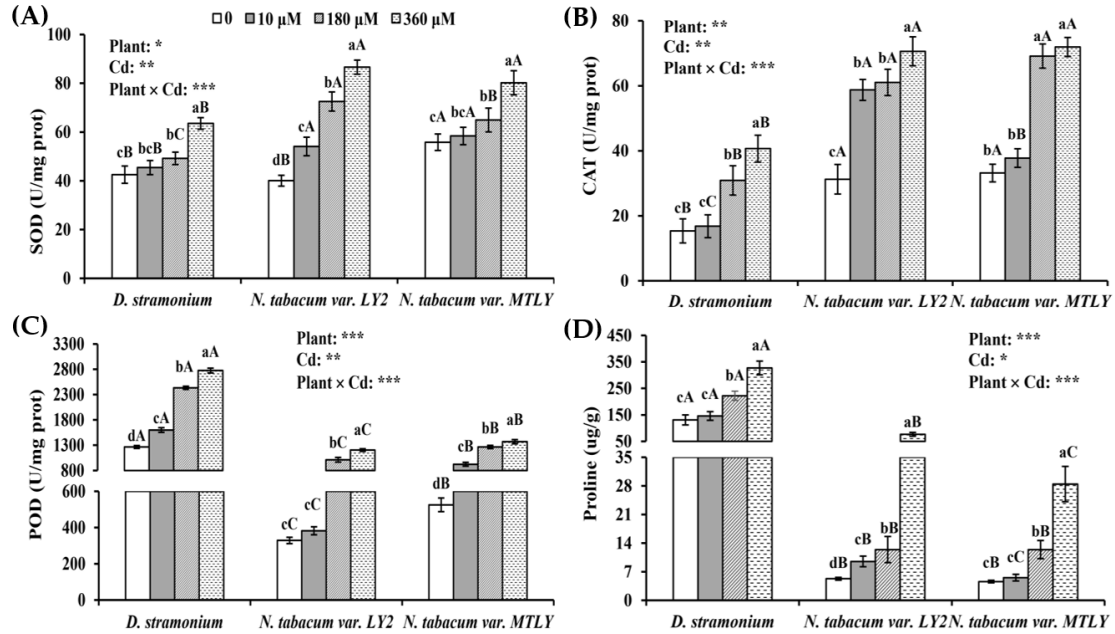

Figure 6. Antioxidant response of seedlings leaves under Cd stress. Superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**) and peroxidase (POD) (**C**) activities. Proline contents (**D**). Values were expressed as means ± SD (n = 9). Different lowercase letters on the bars indicated significant differences between the same material under different Cd concentrations. Different uppercase letters on the bars indicated significant differences between different materials under the same Cd treatment ($p < 0.05$). GLM was used to assess the effects of plant materials, Cd treatments and their interactions on test results. The significance was indicated as follows: * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$.

3.5. Cd Exposure Changed Secondary Metabolism of Seedlings

Fast Blue BB Salt could give a characteristic reddish-brown reaction product, which determined the presence of phenolic compounds. Dragendorff's reagent resulted in saffron yellow staining, indicating the presence of alkaloids. It could be found that phenolic compounds were mainly accumulated in the endodermis, cortex and/or epidermis of the roots, while alkaloids largely accumulated in the endodermis (Figure 7A–L). Additionally, they were distributed widely throughout the mesophyll tissues (Figure 7A–L). Based on their coloring degree and the density distribution, the change of phenolic compounds and alkaloids in *N. tabacum* L. var. MTLY was basically similar to that in *D. stramonium* under cadmium stress, but their levels were apparently higher than those in *N. tabacum* L. var. LY2 (Table 1).

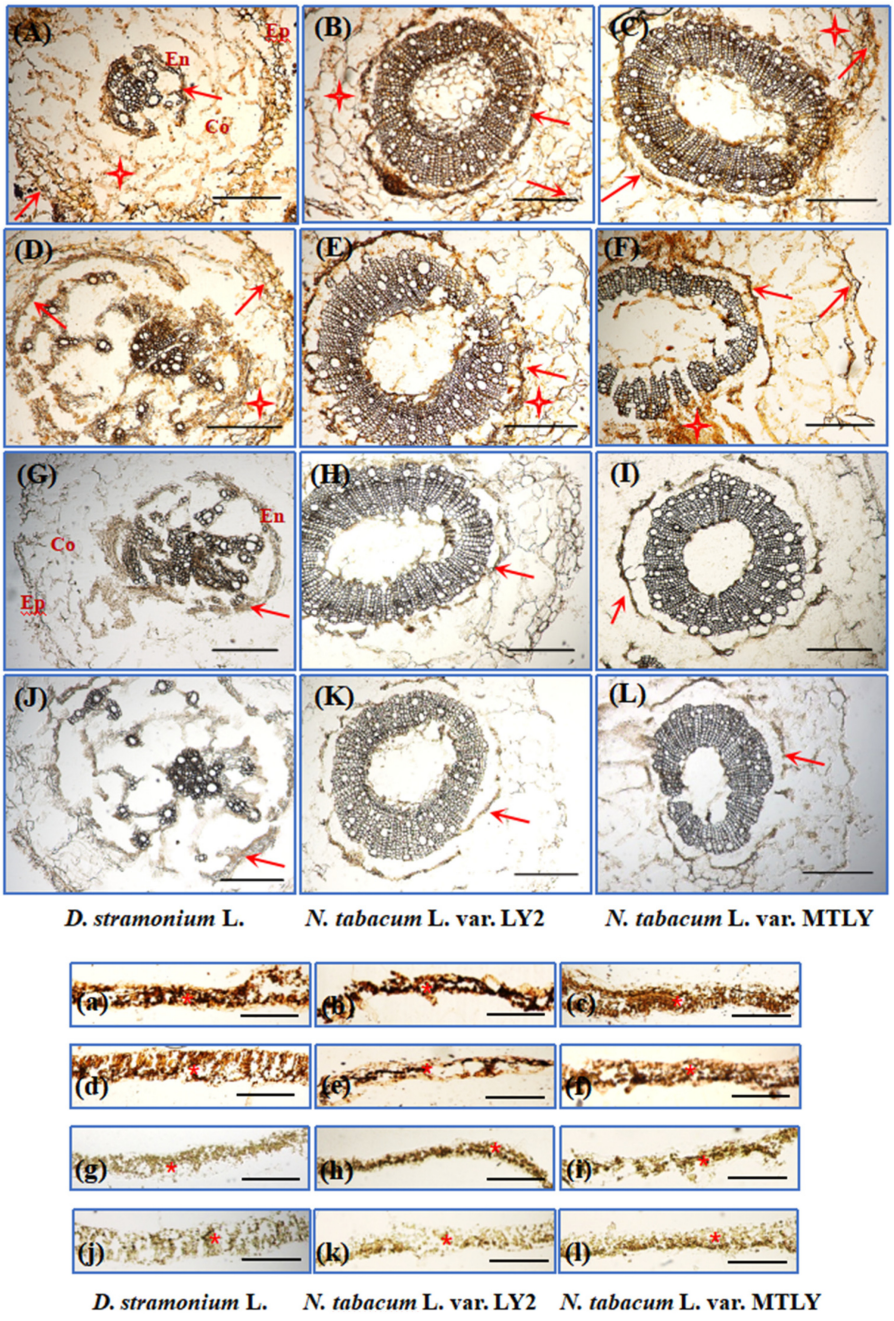

Figure 7. The production and accumulation of secondary metabolites under Cd stress. Representative photomicrographs of cross-sections via histochemical staining. Phenolic compounds were stained reddish-brown with Fast Blue BB Salt. Alkaloids stained saffron yellow with Dragendorff reagent.

Phenolic compounds in roots under control conditions, (**A**) *D. stramonium*, (**B**) *N. tabacum* L. var. LY2 and (**C**) *N. tabacum* L. var. MTLY. Phenolic compounds in roots under Cd stress, (**D**) *D. stramonium*, (**E**) *N. tabacum* L. var. LY2 and (**F**) *N. tabacum* L. var. MTLY. Alkaloids in roots under control conditions, (**G**) *D. stramonium*, (**H**) *N. tabacum* L. var. LY2 and (**I**) *N. tabacum* L. var. MTLY. Alkaloids in roots under Cd stress, (**J**) *D. stramonium*, (**K**) *N. tabacum* L. var. LY2 and (**L**) *N. tabacum* L. var. MTLY. Phenolic compounds in leaves under control conditions, (**a**) *D. stramonium*, (**b**) *N. tabacum* L. var. LY2 and (**c**) *N. tabacum* L. var. MTLY. Phenolic compounds in leaves under Cd stress, (**d**) *D. stramonium*, (**e**) *N. tabacum* L. var. LY2 and (**f**) *N. tabacum* L. var. MTLY. Alkaloids in leaves under control conditions, (**g**) *D. stramonium*, (**h**) *N. tabacum* L. var. LY2 and (**i**) *N. tabacum* L. var. MTLY. Alkaloids in leaves under Cd stress, (**j**) *D. stramonium*, (**k**) *N. tabacum* L. var. LY2 and (**l**) *N. tabacum* L. var. MTLY. Arrows indicated staining in endodermis and/or epidermis, and stars in cortex. Asterisks showed staining in mesophyll tissues. Co, cortex; En, endodermis; Ep, epidermis. Scale bar = 250 μm.

Table 1. A preliminary evaluation for the accumulation of main metabolites in seedling tissues.

Tissues	Secondary Metabolites	Cd Treatments	*D. stramonium*	*N. tabacum* L. var. LY2	*N. tabacum* L. var. MTLY
Root	Phenolic compounds	0	+++	++	+++
		10 μM	+++	+	+++
		180 μM	++	+	+++
		360 μM	++	+	++
	Alkaloids	0	+++	++	+++
		10 μM	++	+++	++
		180 μM	++	++	+++
		360 μM	+++	+	++++
Leaf	Phenolic compounds	0	+++	++	+++
		10 μM	++++	++	++++
		180 μM	+++	+++	++++
		360 μM	+++	+	+++
	Alkaloids	0	+++	++	+++
		10 μM	++++	++	+++
		180 μM	+++	+	+++
		360 μM	+++	+	+++

Note: + weak reaction; ++ moderate reaction; +++ pronounced reaction; ++++ strong reaction. Sample excised from each individual (n = 9 per treatment) was frozen-sectioned, stained with Fast Blue BB Salt and Dragendorff's reagent and observed and analyzed under a microscope.

4. Discussion

A suitable plant for phytoremediation should possess the characteristics of a high accumulation of metals that are preferably in the shoots, a strong tolerance to the metal accumulated, fast growth and high biomass [1,3,37]. Although no plant has yet fulfilled all these criteria, some could be further improved for the desired property either through classic breeding or via genetic engineering [3–5,8–16]. One possible strategy is to heighten metal tolerance and accumulation in high biomass species or to breed large biomass into the existing hyperaccumulators [3,5,10,14–17]. For example, transgenic tobacco expressing genes of various metal chelating molecules and transporter proteins have been achieved, which showed significantly higher Cd tolerance and shoot Cd levels than wild-type plants when grown in pots or hydroponics [12,13,15,69]. A conventional breeding approach has been proposed to introduce excellent foreign genes into crops, broadening the available genetic base and creating new varieties or new types. Unfortunately, progress in this field is rather slow, due mainly to the presence of sexual incompatible barriers between the two distant species [3,9,15–17,41]. Until recently, by a novel approach viz. pollination following grafting, our research group succeeded in developing valuable materials between tobacco (*N. tabacum* L.) and Cd-hyperaccumulators, such as *P. frutescens* and *Ocimum basilicum* L. [18–20]. This technology is worthwhile attempting to breed vari-

eties with superior phytoremediation potential, although the mechanisms are so far not fully understood.

Tobacco has been considered as a promising phytoremediator of Cd-contaminated soil, because it possesses the advantages of high biomass, easy cultivation and wide geographic distribution, which are lacking in most of the known hyperaccumulators [1,10,31,35,69,70]. Out of the 64 recognized species within the genus *Nicotiana*, two species, namely *N. tabacum* L. and *N. rustica* L., are grown commercially in the world. It has been found that there was substantial difference in the Cd uptake, distribution and accumulation among species and cultivars. For example, *N. tabacum* L. is a leaf and root accumulator and *N. rustica* L. is primarily shown to be a root accumulator. Some genotypes of tobacco can accumulate relatively high Cd concentrations in leaves compared to other species [35,38]. The genus *Datura* (Solanaceae) comprises 10–15 annual and perennial herbs and shrubs distributed over tropical and warm temperate regions around the world. However, only four wild species were found in China, including *D. stramonium* L., *D. metel* L., *D. arborea* L. and *D. innoxia* Mill. *D. stramonium* L. is a native weed species with a height of approximately 0.3–1.5 m [7,29]. It exhibited a higher tolerance and accumulation to various metal(loid)s, such as Cr, Cu, Cd, Mn and As [8,24,25,28,30]. In our long-term breeding practice, *N. tabacum* L. var. MTLY, derived from the cross-combination [(*N. glauca* Graham rootstock + *N. tabacum* L. var. LY2 scion) × *D. stramonium* L.], has been obtained via pollination following grafting [18,21]. To evaluate its phytoremediation potential, a hydroponic experiment was performed to characterize the tolerance, accumulation and physiological response of their seedlings to cadmium.

Plants can extract Cd from the soil and transport it via the xylem into shoots where it accumulates. Tolerance means that plant can survive in spite of the toxicity of high metal concentrations. Recent studies indicated that the Cd tolerance and accumulation in plant species are genetically independent properties, and the phytoremediation potential of a given plant is controlled by many genes [3,17,38]. Cd could induce obviously deleterious effects as evident by the phenotypic changes in seedlings, such as stunted growth, chlorosis, necrosis, decrease of chlorophylls, etc., which may be linked to the overproduction of ROS [34,36]. As a result, Cd may lower photosynthetic efficiency through the enhanced degradation of chlorophyll or the inhibition of its biosynthesis, ultimately causing the reduction of plant growth. However, compared to its mother plant tobacco, Cd toxicity has little effect on *N. tabacum* L. var. MTLY when grown hydroponically. Meanwhile, it could accumulate Cd in shoots as high as 127.6–3837.1 mg kg^{-1} when treated with 10–360 μM Cd, and the BCF and TF values reached 95.9–149.7 and 1.0–3.5, respectively, which has exceeded the critical level for a Cd-hyperaccumulator. It was clear that these traits were far better than *N. tabacum* L. var. LY2.

To avoid or combat excessive ROS and the resulting oxidative damage, elaborate antioxidant defense systems comprising of enzymes (such as SOD, CAT, POD, etc.) and antioxidants, such as proline [34,52] and phenolic compounds [53,54,71–73], are necessary. The drastic increase of ROS and MDA contents in the tissues suggested that seedlings have suffered oxidative stress, whereas the degree of damage was much higher in *N. tabacum* L. var. LY2 than in *N. tabacum* L. var. MTLY. This agreed with the results from tobacco and the metal-accumulators *Brassica juncea* L. and *Thlaspi caerulescens* L. [33]. The intracellular ROS levels are regulated by a number of enzymes. SOD is responsible for converting highly reactive superoxide radicals to H_2O_2. The degradation of H_2O_2 to water and oxygen is carried out by CAT in peroxisomes or by POD in vacuoles, the cell wall and the cytosol. The accumulation of ROS in plants could be due to enhanced ROS production and/or decreased capacity for ROS scavenging [33,44]. Extensive studies have showed that the activities of antioxidant enzymes concerning different plant species or cultivars are highly variable in responses to Cd stress. For example, in metal-accumulator species, such as black nightshade (*Solanum nigrum* L.) and Indian mustard (*Brassica juncea* L.), Cd rapidly increased the activities of SOD, CAT and POD. The elevated POD but decreased CAT activities were observed in mung bean (*Phaseolus aureus* Roxb.) seedlings. The activities of

SOD and POD were enhanced, whereas the CAT activity decreased significantly in brahmi (*Bacopa monnieri* L.). In the pea plant (*Pisum sativum* L.), both the SOD and CAT activities were decreased, while the POD did not change significantly [33]. These different results may be related to the genetic background, Cd levels and exposure duration. Proline is very important for Cd tolerance and accumulation in many plant species since it served as a mediator in osmotic adjustment, a stabilizer of sub-cellular structures and a ROS scavenger [26,32,35,46,49]. A higher Pro level was found in the Cd hyperaccumulator *Solanum nigrum* L., more so than in the nonaccumulator plant (*Solanum melongena* L.). It could contribute to alleviates Cd toxicity in *S. nigrum* by detoxifying ROS and increasing the activity of SOD and CAT. Many heavy metal-tolerant plants have also been reported to possess elevated Pro levels when compared with their nontolerant relatives [45,48]. Usually, the levels of ROS and the extent of oxidative damage depend largely upon the whole antioxidant defense system of plants [6]. The accumulation of less ROS in *N. tabacum* L. var. MTLY indicated that its ROS scavenging system might work more effectively as compared to female parents [11].

Besides the well-studied antioxidant systems consisting of low-molecular antioxidants and specific enzymes, some secondary metabolites may play a crucial protective role. Their presence in plants relies upon many factors, such as cultural conditions and genetic background. Phenolic compounds, such as phenolic acids, flavonoids and tannins, could directly scavenge H_2O_2 as antioxidants or act as metal chelators, reducing Cd-induced damage [54,55,71]. Recently, there have been many reports on the biosynthesis and accumulation of phenolic compounds in plants as a response to Cd stress, such as in beans (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.) and blueberries (*Vaccinium corymbosum* L.). When exposed to heavy metals, the roots of many plants exuded high levels of phenolics, and this was considered to be an important biochemical and physiological event in resistance to heavy metals toxicity [51,54,74]. Usually, alkaloids are synthesized in the roots and exported from the roots into the shoots via the phloem and function as a chemical barrier against herbivores and pathogens [68,75]. Studies found that Cd could induce the production and accumulation of indole alkaloids in *Catharanthus roseus* (L.) G. Don, tropane alkaloids in *Atropa belladonna* L. and certain alkaloids in *Senecio coronatus* (Thunb.) Harv. [75,76]. However, the studies on other species and their positive roles in Cd stress are scant. Some scholars suggested that excessive ROS caused by heavy metals oxidized unsaturated fatty acids, initiating the formation of oxylipins, which induced the synthesis of secondary metabolites, such as alkaloids, terpenoids and phenols [75]. Histochemical techniques are fast and cheap methods that can be used to preliminary locate and identify classes of chemical compounds in tissues and cells. Fast Blue BB tests and Dragendorff´s reagent usually indicated the presence of phenolics compounds and alkaloids in tissues [68,74]. Compared with *N. tabacum* L. var. LY2, a higher histochemical staining of metabolites was observed in *N. tabacum* L. var. MTLY and *D. stramonium*, which indicated they may have played an active protective role under Cd stress. Because a complex coordinated defensive mechanism is operating in the tissues, this could improve the seedlings' tolerance against Cd, which is finally reflected in the better growth. The specific ingredients and their contents will be identified by gas chromatography-mass spectrometer (GC-MS) and nuclear magnetic resonance (NMR) in our future experiments. Some research indicated that the combination of metabolomics and histochemistry will better reveal the mechanisms of Cd tolerance and accumulation [24,51,53,54].

5. Conclusions

Compared to *N. tabacum* L. var. LY2, *N. tabacum* L. var. MTLY could fight Cd-caused oxidative stress more effectively, as reflected by the changes of ROS, MDA, antioxidants components, phenolic compounds and alkaloids. The seedlings exhibited obvious tolerance to and more accumulation of Cd than the female parent. The amount of Cd accumulated and the bioconcentration factor (BCF) may entitle it to be considered a hyperaccumulator.

These traits essentially resulted from the introduced *D. stramonium* L. genes. The genotypic difference in response to Cd stress, including plant growth, photosynthesis, enzymatic and non-enzymatic antioxidant defense, has been widely recognized, which may be attributed to the peculiar metabolisms and protective mechanisms that resided in different species and various tissues. How hyperaccumulators are able to tolerate the high concentrations of metals in the interior of their cells has long been a perplexing question. The exact mechanisms in molecules and genetics remain to be further elucidated. This study has great significance in obtaining elite germplasm for phytoremediation, and more field research is required to confirm its potential for this purpose.

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