Mechanism for Higher Tolerance to and Lower Accumulation of Arsenite in NtCyc07-Overexpressing Tobacco

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Abstract: Arsenite [As(III)] is a highly toxic chemical to all organisms. Previously, we reported that the overexpression of NtCyc07 enhanced As(III) tolerance and reduced As(III) accumulation in yeast (Saccharomyces cerevisiae) and tobacco (Nicotiana tabacum). To understand a mechanism for higher As(III) tolerance and lower As(III) accumulation in NtCyc07-overexpressing tobacco, we examined the expression levels of various putative As(III) transporters (aquaporin). The expressions of putative As(III) exporter NIP1;1, PIP1;1, 1;5, 2;1, 2;2, and 2;7 were enhanced, while the expressions of putative As(III) importer NIP3;1, 4;1, and XIP2;1 were decreased, contributing to the reduced accumulation of As(III) in NtCyc07-overexpressing tobacco. In addition, the levels of oxidative stress indicators (H2O2, superoxide and malondialdehyde) were lower, and the activities of antioxidant enzymes (catalase, superoxide dismutase and glutathione reductase) were higher in NtCyc07-tobacco than in the control tobacco. This suggests that the lower oxidative stress in transgenic tobacco may be attributed to the higher activities of antioxidant enzymes and lower As(III) levels. Taken together, the overexpression of NtCyc07 enhances As(III) tolerance by reducing As(III) accumulation through modulation of expressions of putative As(III) transporters in tobacco.

Keywords: aquaporin; arsenic; Cyc07; NIP; PIP; ROS; tobacco

1. Introduction

Arsenic is present in the land, air and ground water, and acts as a toxic substance to humans, animals and plants [1]. In most cases, arsenic enters humans through drinking water or crops which absorb arsenic that dissolves in rainwater and flows into the environment [2]. The concentration that does not harm humans is less than 10–50 ppb [3], and over than this concentration, the acute and chronic arsenic toxicity will appear [4]. The main toxic effects to humans are cancer [5,6], heart disease (hypertension-related cardiovascular disease) [7], stroke (cerebrovascular diseases) [8], chronic respiratory disease [9] and diabetes [10].

Major forms of inorganic arsenic in the environment, As(III) (arsenite) and As(V) (arsenate), can enter plants through high-affinity Pi transporters and the Nod26-like Intrinsic Protein (NIP) subfamily of aquaporins, respectively [11–13]. Plant aquaporins can be categorized into four subgroups: Plasma membrane Intrinsic Proteins (PIPs), Nod26-like Intrinsic Proteins (NIPs), Tonoplast Intrinsic Proteins (TIPs) and Small Basic Intrinsic Proteins (SIPs). The NIPs are localized at plasma/intracellular membranes, while SIPs are at the endoplasmic reticulum [14]. Aquaporins participate in transportation of water, ammonia, arsenic, boric acid, glycerol, metalloids, nitric oxide, and reactive oxygen.
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species [15–20]. All reported NIPs have As(III) influx activities [11,13,21–26]; the knockout Arabidopsis of AtNIP1;1 and AtNIP7;1 displayed higher tolerance to and lower accumulation of As(III) [13,27]. The nip1;2 and nip5;1 mutants of Arabidopsis accumulated less As(III), while they did not show a higher tolerance to As(III) [27]. In addition, OsNIP3;2 is involved in As(III) uptake in rice since a knockout mutant showed lower accumulations of As(III) in its roots, compared to WT plants [28]. It has been reported that, among NIPs, AtNIP5;1 and AtNIP6;1 of Arabidopsis, OsNIP2;1 (OsLsi1) and OsNIP3;2 of rice, and LjNIP5;1 and LjNIP6;1 of Lotus japonicus are involved in the bi-directional transport (both uptake and export) of As(III) in yeast or plants [11]. Among PIPs, the overexpression of OsPIP2;4, OsPIP2;6 and OsPIP2;7 enhanced As(III) tolerances in Arabidopsis. Interestingly, OsPIP2;6-expressing Arabidopsis exhibited higher activities in As(III) uptake and export in roots in response to short-term (4 h) exposure to As(III) [29]. Recently, 88 aquaporin genes were identified in Nicotiana tabacum genome and assigned into five subfamilies: 34 plasma membrane intrinsic proteins (PIPs); 27 tonoplast intrinsic proteins (TIPs); 20 nodulin26-like intrinsic proteins (NIPs); three small basic intrinsic proteins (SIPs); four uncharacterized X intrinsic proteins (XIPs) [30].

The Cyc07 gene was first reported in Catharanthus roseus [31]. Cyc07 mRNA was detected specifically in the S phase of synchronous cultures as well as in intact plants, and was found mainly in the meristem zone of root tips [32]. Cyc07 has a domain of ribosomal S3Ae [32]. Tobacco Cyc07 (NtCyc07) was cloned and identified to be involved in arsenite tolerance by reducing As accumulation through interacting with ACR1 which is a transcription factor of the arsenite exporter ACR3 in yeast [33]. In addition, the overexpression of NtCyc07 in tobacco enhanced arsenite tolerances by decreasing As accumulation [34]. Tobacco has a single gene of NtCyc07. Regarding genes involved in arsenic tolerance in tobacco, it was only reported that the overexpression of tobacco phytochelatin synthase (NtPCS1) enhances arsenite tolerances in yeast [35] and tobacco [36].

In this report, to understand a mechanism for higher tolerance to and lower accumulation of As(III) in NtCyc07-expressing tobacco, we have examined expressions of putative As(III) transporters NIPs and PIPs, ROS (reactive oxygen species) levels and antioxidant enzyme activities. We found that NtCyc07 enhanced As(III) tolerances by decreasing As(III) accumulations through regulation of expressions of putative As(III) transporters. In addition, NtCyc07-tobacco displayed a lower oxidative stress and higher activities of antioxidant enzymes, which may be ascribed to the lower accumulation of As(III).

2. Results and Discussion

2.1. NtCyc07-Overexpressing Tobacco Displayed Higher Tolerance to and Lower Accumulation of As(III)

We have examined again As(III) tolerance and accumulation in a vector only expressing control (pBI121) and NtCyc07-overexpressing (NtCyc07-3 and NtCyc07-6) tobacco since we challenged plants with 30 µM As(III) which is higher than previously used 15 µM [34] to induce a remarkable difference in As(III) tolerance between control and transgenic plants. As shown in Figure 1a,b, NtCyc07-tobacco exhibited a higher tolerance to As(III) than control plants, and control and transgenic plants were healthy based on green colors/chlorophylls even with enhanced As(III) concentrations. In addition, As(III) accumulation was lower in NtCyc07-tobacco than in control plants (Figure 1c), suggesting that the higher As(III) tolerance in transgenic tobacco is ascribed to the reduced level of As(III).

Supporting our results, many studies have reported that the higher metal tolerance is ascribed to the lower accumulation of metal in plants. Indian mustard (Brassica juncea) with moderate expression of AtPCS1 (Phytochelatin Synthase 1 of Arabidopsis) exhibited increased tolerance to and reduced accumulation of Cd and Zn [37]. Overexpression of tobacco Cyc07 (cell cycle-related protein 7) enhanced As(III) tolerance and reduced As(III) accumulation in tobacco [34]. The overexpression of tobacco PIC1 (Permease In Chloroplast 1) in tobacco enhanced Cd tolerance and reduced Cd content [38]. Overexpression of tobacco Hb1 (non-symbiotic class 1 hemoglobin 1) increased Cd tolerance and decreased Cd level in tobacco [39] and Arabidopsis [40]. The overexpression of CsMTP8 (Metal
Transport Protein 8 of tea plant *Camellia sinensis* enhanced Mn tolerance and reduced Mn accumulation in Arabidopsis [41]. Transgenic tobacco overexpressing tobacco *UBC1* (ubiquitin-conjugating protein 1) displayed higher tolerance to and lower level of Cd [42]. Overexpression of *WRKY13* enhanced a Cd tolerance and reduced Cd accumulation by increasing the expression of Cd exporter PDR8 [43]. Transgenic rice which expresses *TaCNR2* (Cell Number Regulator 2 of wheat *Triticum aestivum*) showed an enhanced Cd tolerance and a reduced Cd level [44]. Overexpression of tobacco *UBQ2* (ubiquitin-extension protein 2) in tobacco and *Arabidopsis* increased a Cd tolerance and reduced a Cd level [45]. Transgenic *Arabidopsis* overexpressing *miRNA156* displayed higher Cd tolerance and lower Cd level [46].

2.2. Expressions of Putative As(III) Transporters Were Modulated in *NtCyc07*-Tobacco

Previously, we have reported that *NtCyc07*-overexpressing tobacco displayed a higher As(III) tolerance and the lower accumulation of As(III), compared with control plants [34]. To understand a molecular mechanism for the higher As(III) tolerance and lower accumulation of As(III) in *NtCyc07*-tobacco, the expression level of putative As(III) transporter aquaporin including *NtNIP1;1* (NP_001312464.1), *NtNIP2;1* (NP_001312464.1), *NtNIP4;1* (AAB81601.1), *NtPIP1;1* (XP_016487110.1), *NtPIP2;1* (XP_016451246.1), *NtPIP3;1* (XP_016460638.1), *NtNIP2;2* (NM_001325404.1), *NtPIP2;7* (NP_001313061.1), *NtPIP2;17* (NP_001312464.1), *NtXIP1;1* (AAB81601.1), *NtXIP2;1* (AF440272.1), *NtXIP2;2* (NP_001325404.1), and *NtNIP1;1* were examined. To date, the involvement of *NtNIP1;1*, *NtNIP2;1*, *NtNIP4;1*, and *NtXIP1;1* in As(III) tolerance and level in *NtCyc07*-expressing tobacco. (*a*) Comparison of morphology and As(III) tolerance in transgenic tobacco expressing pBI121 only (control) and *NtCyc07* (NtCyc07-3 and NtCyc07-6) in response to As(III) stress. All plants were germinated and grown for 3 weeks on 1/2 Murashige and Skoog medium (MS) agar plates without (upper) and with (lower) 30 µM As(III). (*b*) Fresh weights of tobacco shown in (*a*). (*c*) As(III) tolerance rates of tobacco shown in (*a,b*). (*d*) As(III) accumulations in whole seedlings of control and *NtCyc07* tobacco shown in (*a*). 1/2 MS indicates a control (no As(III) treatment). Each value corresponds to the means of three independent experiments, and error bars indicate standard errors. Asterisks indicate significant differences between control and transgenic tobacco (*p* ≤ 0.05).

Figure 1. As(III) tolerance and level in *NtCyc07*-expressing tobacco. (a) Comparison of morphology and As(III) tolerance in transgenic tobacco expressing pBI121 only (control) and *NtCyc07* (NtCyc07-3 and NtCyc07-6) in response to As(III) stress. All plants were germinated and grown for 3 weeks on 1/2 Murashige and Skoog medium (MS) agar plates without (upper) and with (lower) 30 µM As(III). (b) Fresh weights of tobacco shown in (a). (c) As(III) tolerance rates of tobacco shown in (a,b). (d) As(III) accumulations in whole seedlings of control and *NtCyc07* tobacco shown in (a). 1/2 MS indicates a control (no As(III) treatment). Each value corresponds to the means of three independent experiments, and error bars indicate standard errors. Asterisks indicate significant differences between control and transgenic tobacco (*p* ≤ 0.05).
(NP_001312796) and NtXIP2;1 (XP_016489264.1), were examined. To date, the involvement of aquaporin in the As(III) transport has never been reported in tobacco. Therefore, the putative As(III) transporters were selected among NIPs and PIPs based on protein sequences showing differences in homology, substrate specificity determining positions (SDPs), and plasma membrane localization [30].

As shown in Figure 2a–d, among NIPs, NIP1;1 expression was higher and expressions of NIP3;1 and NIP4;1 were lower in NtCyc07-tobacco than in control plants, while the expression level of NIP2;1 was not different from that of control. This suggests that NIP1;1, NIP3;1 and NIP4;1 may be involved in the lower accumulation of As(III) in NtCyc07-expressing tobacco. In support of this, SDP data show that NIP1;1, NIP3;1 and NIP4;1 had a As specificity but NIP2;1 did not [30]. Based on these data, it is assumed that NIP1;1 has a higher activity of As(III) exporter than of As(III) importer, while NIP3;1 and NIP4;1 has a higher activity of As(III) importer. Therefore, the modulated expressions of these aquaporins may contribute to the reduced accumulation of As(III) in transgenic tobacco.

Figure 2. Cont.
Taken together, although all aquaporins were not examined, the overexpression of PIP2;17 plants, while the expressions of putative As(III) exporter NIP1;1, PIP1;1, PIP1;5, PIP2;1, PIP2;2, and PIP2;7, therefore its reduced expression contributes to the lower level of As(III) in tobacco than in control plants. This suggests that XIP2;1 may have a higher activity of As(III) influx, not di characterized yet [30]. As shown in Figure 2k,l, by higher activity of As(III) efflux in transgenic tobacco. Therefore, it is assumed that PIP1;1, PIP1;5, PIP2;1, PIP2;2, and PIP2;7 are involved in decreasing As(III) accumulation by higher activity of As(III) efflux in transgenic tobacco.

As shown in Figure 2e–j, among PIPs which do not have As specificity based on SDP [30], expressions of PIP1;1, PIP1;5, PIP2;1, PIP2;2, and PIP2;7 were higher in NtCyc07-tobacco than in control plants, while PIP2;17 expression did not show a difference from that of control tobacco. Therefore, it is assumed that PIP1;1, PIP1;5, PIP2;1, PIP2;2, and PIP2;7 are involved in decreasing As(III) accumulation by higher activity of As(III) influx in transgenic tobacco.

In addition, we examined expression levels of two XIP in response to As(III) since XIP was not characterized yet [30]. As shown in Figure 2k,l, XIP1;1α expression level in NtCyc07-tobacco was not different from that of control plants, while the expression level of XIP2;1 was lower in transgenic tobacco than in control plants. This suggests that XIP2;1 may have a higher activity of As(III) influx, therefore its reduced expression contributes to the lower level of As(III) in NtCyc07-expressing tobacco. Taken together, although all aquaporins were not examined, the overexpression of NtCyc07 enhances the expressions of putative As(III) exporter NIP1;1, PIP1;1, PIP1;5, PIP2;1, PIP2;2, and PIP2;7, while it
reduces the expression of putative As(III) importer NIP3;1, NIP4;1, and XIP2;1, contributing to the reduced accumulation of As(III) in NtCyc07-overexpressing tobacco. However, gene expressions do not reflect exactly protein amounts and activities, thus it is possible that the involvement and role of presented transporters in As(III) transport may be different from our assumption.

Regarding the involvement of aquaporin in As(III) transport, many NIPs and PIPs have been reported: all these have As(III) import activity, and some (AtNIP5;1, AtNIP6;1, OsNIP2;1, OsNIP3;2, LjNIP5;1, LiNIP6;1, OsPIP2;4, OsPIP2;6, and OsPIP2;7) have both import and export activities. In contrast, the participation of aquaporin in As(III) transport has never been reported in tobacco. Therefore, our study is the first report showing the role of aquaporin in As(III) transport in tobacco. However, until the As(III) import/export activity of tobacco NIPs and PIPs are experimentally proved using oocyte injection or thermodynamic kinetics of As uptake/efflux in plants, our data about roles of NIPs and PIPs in As(III) transport are indirect or assumptive.

2.3. NtCyc07-Expressing Tobacco Displayed a Lower Oxidative Stress and Higher Activities of Antioxidant Enzymes in Response to As(III)

Since As(III) treatments generally induce oxidative stress [47], we compared oxidative stress levels including hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$) and malondialdehyde (MDA, indicator of membrane oxidation) between control and NtCyc07-tobacco (Figure 3a–c). While levels of H$_2$O$_2$, superoxide and MDA were enhanced by As(III) in control tobacco, those were not altered in NtCyc07-tobacco, resulting in lower oxidative stress levels in transgenic tobacco than in control plants. In addition, levels of H$_2$O$_2$ and superoxide were visualized by DAB and NBT staining, respectively (Figure 3d,e). It also confirmed lower contents of H$_2$O$_2$ and superoxide in transgenic tobacco, compared with that of control plants. Considering the lower levels of As(III) and oxidative stress in NtCyc07-tobacco, the lower oxidative stress may be attributed to the lower As(III) level.

In general, antioxidant enzymes ameliorate oxidative stress; therefore, the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) were examined (Figure 4). While the activities of all three enzymes were enhanced by As(III) in NtCyc07-tobacco, those of control plants were not induced, leading to the higher activities of antioxidant enzymes in NtCyc07-tobacco than in control plants. Regarding the lower As(III) level and higher activities of antioxidant enzymes in NtCyc07-tobacco, the higher antioxidant enzyme activity may be ascribed to the lower content of As(III). However, it is also possible that the activity of antioxidant enzyme is enhanced by NtCyc07 itself, in addition to the lower As(III) content. Based on these results, it appears that the lower level of oxidative stress in NtCyc07-expressing tobacco under As(III) stress is ascribed to the higher activities of antioxidant enzymes and the lower level of As(III), when compared with control plants.

![Figure 3. Cont.](image-url)
Figure 3. Cont.
In support of our results, it has been reported that plants with reduced oxidative stress/higher activities of antioxidant enzymes show enhanced metal tolerances. Exogenous application of methyl jasmonate increased a Cd tolerance by reducing Cd-induced oxidative stress in rice [48]. Trehalose treatment increased a Cu tolerance by reducing oxidative stress in rice [49]. The overexpression of tobacco PIC1 (Permease In Chloroplast 1) in tobacco enhanced Cd tolerances and activities of antioxidant enzymes [38]. Application of maleic acid increased Cr tolerances and activities of antioxidant enzymes and reduced oxidative stress in Brassica juncea [50]. Transgenic tobacco overexpressing tobacco UBC1 (Ub-conjugating protein 1) displayed enhanced Cd tolerance and reduced oxidative stress [42]. Overexpression of tobacco UBQ2 (Ub-extension protein 2) in tobacco and Arabidopsis increased Cd tolerances and reduced oxidative stress levels [45]. Overexpression of tobacco Hb1 (non-symbiotic class 1 hemoglobin 1) increased Cd tolerances and decreased oxidative stress in Arabidopsis [40]. Transgenic Arabidopsis overexpressing miRNA156 displayed higher metal tolerance, lower ROS level and higher activities of antioxidant enzymes [46].

Regarding how NtCyc07 modulates expressions of NIPs and PIPs and activities of antioxidant enzymes, it is postulated that NtCyc07 may interact with transcription factors for NIPs, PIPs, and genes of antioxidant enzymes. This may be supported by the report that NtCyc07 interacts with ACR1 which is a transcription factor of ACR3 (arsenite exporter) in yeast [33]. In addition, it cannot be excluded that NtCyc07 may modulate protein amounts and activities of transporters and antioxidant enzymes.
Figure 4. Activities of antioxidant enzymes in response to As(III) in NtCyc07-expressing tobacco. Activities of (a) catalase, (b) superoxide dismutase and (c) glutathione reductase in control (pBI121) and NtCyc07-tobacco. Plants were germinated and grown for 3 weeks on 1/2 MS agar media without or with 30µM As(III). 1/2 MS indicates a control (no As(III) treatment). The data are averages of three independent experiments per each treatment, and error bars indicate standard errors (S.E.). Different letters over columns indicate significant differences ($p \leq 0.05$) between treatments.
3. Materials and Methods

3.1. Plants

In this study, we used the previously reported transgenic tobacco (*Nicotiana tabacum*) lines which overexpress a vector only (*pBI121*) and *NtCyc07* (*NtCyc07-3 and -6*) after harvesting new seeds [34]. Tobacco seeds were sterilized, vernalized, germinated, and grown for 3 weeks on half-strength Murashige and Skoog medium (MS medium, pH 5.7) with or without 30 μM sodium arsenite. The plates were placed in a growth chamber under a 16 h light (cool white fluorescent light at 150 mmol/m²/s) / 8 h dark photoperiod and 23/21 ºC of day/night temperatures.

3.2. As(III) Tolerance and Accumulation

Arsenite tolerance was measured as described in the previous paper [34]. The As(III) tolerance rate (%) was calculated by dividing the fresh weights of the As(III) treated plants (*n* = 30) by the fresh weights of the control plants. To analyze the As(III) accumulation, control (*pBI121*) and transgenic (*NtCyc07*) tobacco seedlings grown for 3 weeks on agar media containing 30 μM As(III) were harvested, washed three times with ice-cold 5 mM CaCl₂ and dried for 72 h at 60 ºC. The dried sample (1 g) was digested with concentrated HNO₃ and HClO₄ in a Teflon Digestion Vessel (Savillex, Eden Prairie, MN, USA). The As(III) concentration was measured in triplicate using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES, Perkin Elmer Optima 4300 DV, San Diego, CA, USA) at a wavelength of 188.98 nm at The National Instrumentation Center for Environmental Management (NICEM, Seoul, Korea).

3.3. Quantitative Real Time PCR (qRT-PCR)

RNA isolation, cDNA synthesis, and qRT-PCR were performed as previously described [51]. Total RNA was isolated from 3 week-old 100 mg seedlings using gDNA removal column and RNA binding column in iQeasy™ Plus plant RNA extraction mini kit (iNtRON biotechnology, Seongnam, Korea). cDNA was synthesized from 2 μg of total RNA using NEXscript™ RT 2X master mix Oligo dT (NEX Diagnostics, Seongnam, Korea). Quantitative RT-PCR was performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each 20 μL reaction mixture contains 10 μL of SYBER Supremix (SsoAdvanced™ Universal SYBR® Green Supremix, Bio-Rad, Hercules, CA, USA), 7 μL of nuclease-free water, 1 μL of cDNA, and 1 μL of gene specific primers. The qRT-PCR reaction conditions were 95 ºC for 30 s, 40 cycles at 95 ºC for 15 s, and 60 ºC for 20 s. Experiments were performed in triplicate (three biological repeats), and each run contained three technical replicates for the cDNA and each primer set. Relative transcript levels were normalized to the internal quantitative control *NtActin* (ACCESSION #U60495.1), and the relative expression level of each gene was calculated using the 2-ΔΔCt method [52]. The gene-specific primers are presented in Supplementary Materials Table S1, and the MIQE check list for qRT-PCR is presented in Supplementary Materials Table S2.

3.4. Analysis of Antioxidant Enzyme Activity

3.4.1. Sample Preparation

Leaf (0.2 g) of tobacco seedlings grown for 3 weeks on 1/2 MS agar media with or without 30 μM As(III) was ground in liquid nitrogen, homogenized in 1.2 mL of potassium phosphate buffer (0.2 M, pH 7.8) containing 0.1 mM EDTA, and centrifuged for 20 min at 15,000×g, 4 ºC. Then, the supernatant was stored at −80 ºC until using it for the enzyme assay.

3.4.2. Measurement of Antioxidant Enzyme Activity

Catalase (CAT; 1.11.1.6) activity was measured as previously described [53]. The reaction was initiated by adding 15 μL of leaf extract to 2.5 μL H₂O₂ (30% solution) prepared in 1 mL 50 mM of...
potassium phosphate buffer (pH 7.5) in a final volume of 1 mL. The extinction coefficient of H$_2$O$_2$
(40 M$^{-1}$ cm$^{-1}$ at 240 nm) was used to calculate the enzyme activity (millimoles of H$_2$O$_2$ per minute per
gram fresh weight).

Activity of superoxide dismutase (SOD; EC 1.15.1.1) was determined using the modified NBT
method [54]. The assay mixture contained 50 mM phosphate buffer (pH 7.8) with 2 mM EDTA, 1 mM
riboflavin, 9.9 mM L-methionine, 0.025% Triton-X100 and 55 µM NBT. After adding 40 µL of leaf
extract to 2 mL of reaction mixture, the reaction was started by illuminating the reaction mixture with
a fluorescent lamp (15 W) for 10 min. The absorbance of the reaction mixture was measured at 560 nm. The
reaction mixture without the leaf extract was used as a control. The enzyme activity (per gram
fresh weight) of the sample was calculated based on the standard curve of pure SOD.

Glutathione reductase activity (GR; EC 1.8.1.7) was determined as described by Smith et al. [55].
The reaction mixture (1 mL) contained 10 µL of leaf extract, 100 mM potassium phosphate buffer
(pH 7.5), 0.1 mM NADPH, 0.75 mM DTNB (2-nitrobenzoic acid), and 1 mM GSSG (glutathione
disulfide). The reaction was initiated by addition of GSSG, and the absorbance at 412 nm was measured
after 3 min when DTNB was reduced to TNB (2-nitro-5-thiobenzoate). The extinction coefficient of
TNB (14.15 M$^{-1}$ cm$^{-1}$) was used to calculate the GR activity (millimoles of TNB per minute per gram
fresh weight). All enzyme experiments were performed in triplicate, and each enzyme activity was
measured three times.

3.5. Measurement of Oxidative Stress

Hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$) and malondialdehyde (MDA) were quantified
in tobacco seedlings grown for 3 weeks on 1/2 MS agar plates as previously described [56–58].
For hydrogen peroxide measurement, 0.3 g of leaf was ground in liquid nitrogen, homogenized with
5 mL of 0.1% trichloroacetic acid (TCA), centrifuged for 15 min at 12,000× g, 4 °C, and the supernatant
was collected. The reaction mixture contained 0.5 mL of leaf extract supernatant, 0.5 mL of 10 mM
potassium phosphate buffer (pH 7.0), and 1 mL of 1 M potassium iodide. The same reaction mixture
lacking the leaf extract was used as a blank. The concentration of H$_2$O$_2$ was determined based on a
standard curve of known concentrations of H$_2$O$_2$.

To measure the concentration of O$_2^-$, 100 mg of leaf was ground to a fine powder in liquid
nitrogen, homogenized in 2 mL of 50 mM potassium phosphate buffer (pH7.8), and centrifuged for
10 min at 4 °C, 10,000× g. Then, 1 mL of the supernatant was mixed with 0.9 mL of 50 mM potassium
phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride, and then incubated for
20 min at 25 °C. Subsequently, 1 mL of 17 mM sulfinilamide and 1mL of 7 mM α-naphthylamine were
added to the incubation mixture, further incubated under the same condition, and the absorbance was
measured at 530 nm. The generation of O$_2^-$ was calculated based on a standard curve of NO$_2^-$ (nitrite).

For the measurement of MDA, 0.5 g of tobacco seedlings was ground in liquid nitrogen,
homogenized with 1.5 mL of 20% (w/v) TCA, and centrifuged for 5 min at 10,000× g. 2 mL of
thiobarbituric acid solution (0.5% (w/v) in 20% TCA) was added to 1 mL of the supernatant, incubated at
95 °C for 15 min, quickly cooled in an ice bath, and centrifuged at 12,000× g for 10 min. The absorbance of
the supernatant was measured at 450, 532 and 600 nm. The concentration of MDA was determined
according to the following formula: concentration (µmol L$^{-1}$) = 6.45 × (OD$_{532}$ – OD$_{600}$) − 0.56 × OD$_{450}$
(OD: optical density). For all measurements, three independent experiments were performed.

3.6. Visualization of H$_2$O$_2$ and Superoxide (O$_2^-$)

H$_2$O$_2$ was visually detected in tobacco leaves as previously described [59]. Leaves of 3-week-old
plants grown on 1/2 MS agar media were immersed and infiltrated under a vacuum with 1.25 mg mL$^{-1}$
3,3ˈ-diaminobenzidine (DAB) (D8001, Sigma-Aldrich, St. Louis, MO, USA) staining solution
(pH 3.8), and incubated at 25 °C for 8 h. Subsequently, stained leaves were bleached in acetic
acid-glycerol-ethanol (1/1/3) (v/v/v) solution for 5 min at 100 °C, preserved in glycerol-ethanol (1/4) (v/v)
solution, and photographed. H$_2$O$_2$ was visualized as a deep brown color due to DAB polymerization.
For each experiment, at least 10 plants were examined per line, and three independent experiments were performed.

$O_2^-$ (superoxide radical) was visually detected by nitro blue tetrazolium (NBT) (N6639, Sigma-Aldrich) staining using the modified method [60]. Briefly, leaves were detached and vacuum-infiltrated for 15 min in 1 mg mL$^{-1}$ NBT solution prepared in 10 mM potassium phosphate buffer (pH 7.8) containing 10 mM NaN$_3$ (sodium azide), incubated for 3 h at 25 °C, bleached as described above, and photographed. The production of $O_2^-$ was visualized as a blue color due to NBT precipitation. For each experiment, 10 plants were examined per line, and three independent experiments were performed.

3.7. Statistical Analysis

Two-way ANOVA was used to analyze the data using SAS software (version 9.1). The means were separated by Tukey’s multiple comparison test with significant differences at $p \leq 0.05$.

4. Conclusions

1. The overexpression of NtCyc07 enhances As(III) tolerance by decreasing As(III) accumulation through the increased expression of putative As(III) exporter NIP1;1, PIP1;1, PIP1;5, PIP2;1, PIP2;2, and PIP2;7, and the reduced expression of putative As(III) importer NIP3;1, NIP4;1, and XIP2;1.

2. NtCyc07-tobacco displays lower levels of oxidative stress and higher activities of antioxidant enzymes. The lower oxidative stress in NtCyc07-tobacco may be attributed to the higher activities of antioxidant enzymes and the lower level of As(III). The higher activities of antioxidant enzymes in NtCyc07-tobacco may be ascribed to the lower content of As(III) and probably the direct effect/function of NtCyc07.

Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/11/1480/s1, Table S1: List of the primer sequences used in this study, Table S2: The MIQE check list for qRT-PCR.

Author Contributions: S.H. designed and supervised whole research, and wrote the article with contributions of all the authors; D.K. and R.B. equally performed all experiments; M.M. contributed to data analysis. All authors have read and agreed to the published version of the manuscript.

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