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SILENCING OF THE *PHYTOENE DESATURASE* GENE MITIGATES OXIDATIVE STRESS THROUGH THE ACCUMULATION OF FREE AMINO ACIDS

MD SARAFAT ALI[1] AND KWANG-HYUN BAEK*

Department of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 38541, Republic of Korea

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Abstract

Phytoene desaturase (PDS) is a rate-limiting enzyme involved in the biosynthesis of carotenoids, which converts phytoene to zeta-carotene in a two-step desaturation reaction. Transiently blocked carotenogenesis by silencing the *PDS* gene in *Nicotiana benthamiana* (*NbPDS*) using the virus-induced gene silencing (VIGS) technique was used. Silencing of *NbPDS* induced dwarfism and an albino-type leaf trait in *N. benthamiana*. The *NbPDS*-silenced leaves accumulated free amino acids in amounts 9.5-folds greater than those of the *GFP*-silenced control leaves, but contained only 59.6% of total soluble proteins. When treatment with 10 and 100 μM paraquat was carried out to induce oxidative stress, *NbPDS*-silenced *N. benthamiana* demonstrated more resistance at both concentrations compared to the control plants. These data strongly suggest that high concentrations of free amino acids occur because they are inadequately incorporated into proteins of the *NbPDS*-silenced plants, but reduce injury inflicted by oxidative stress even without the assistance of important antioxidants like carotenoids.

Introduction

Plants have evolved to develop a large number of physiological and biochemical strategies to cope with stresses (Liang *et al.* 2018). All environmental and biotic stresses trigger a generalized oxidative stress in plants (Xie *et al*. 2019). The stress is induced by over-production and accumulation of oxidative molecules, such as the reactive oxygen species (ROS) including peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen (Kanojia and Dijkwel 2018)**.** Attack of ROS on biological molecules induces deoxyribose oxidation, removal of nucleotides, DNA-protein crosslinking, and strand breakage in the DNA (Halliwell 2006). Particularly, changes in the nucleotides of one DNA strand due to oxidative stress can lead to mismatches with nucleotides of the other strand. This phenomenon can lead to mutations and, in consequence, synthesis of unwanted or damaged proteins within the cell.

The ROS produced extensively in plants due to stresses are scavenged by activating the antioxidative systems, which are categorized into two groups, those with enzymatic and non-enzymatic components (Pandey *et al.* 2017). The enzymatic components include several antioxidant enzymes, such as superoxide dismutase, catalase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, guaiacol peroxidase and ascorbate peroxidase (Abogadallah 2010). Non-enzymatic components comprise a number of low-molecular weight antioxidant compounds, such as carotenoids, tocopherols, ascorbates, glutathione, and phenolic compounds (Soares *et al.* 2018).

*Author for correspondence: <khbaek@ynu.ac.kr>. [1]Department of Biotechnology and Genetic Engineering, Bangabandhu Sheikh Mujibur Rahman Science & Technology University, Gopalgonj 8100, Bangladesh

Carotenoids belonging to the family of tetraterpenes can function as potent non-enzymic antioxidants in addition to their roles of accessory light-harvesting pigments and precursors for phytohormones. Carotenoids are involved in diverse but critical plant processes, such as photosynthesis, photomorphogenesis, photoprotection, and development (Nisar *et al.* 2015). Under conditions of oxidative stress, carotenoids are involved in the photoprotection of the reaction-center chlorophylls against ROS and prevent the formation of chlorophylls with triplet excited states (^{3}Chl*) in the chloroplasts (Ramel *et al.* 2013). Stabilization of the plasma membrane against membrane lipid peroxidation is another contribution of the carotenoids towards protective function (Ademowo *et al.* 2017).

Synthesis of carotenoids takes place from two molecules of geranylgeranyl diphosphate and requires the enzymes phytoene synthase, phytoene desaturase (PDS), and lycopene cyclase (Sun *et al.* 2018). PDS, an intrinsic membrane protein found in both chloroplasts and chromoplasts, is the rate-limiting molecule in the biosynthesis of carotenoids (Brausemann *et al.* 2017). It converts phytoene to zeta-carotene in a two-step desaturation reaction and silencing of the *PDS* gene in higher plants produces photobleaching symptoms in the leaves (Srinivasan *et al.* 2017).

Free amino acids accumulate in plants as compatible solutes under stressful conditions. The accumulation of free amino acids can increase the ability of the plant cells to retain water without affecting their normal metabolism. The free amino acids, either directly or indirectly, stabilize the quaternary structure of proteins and membranes protecting them against the adverse effects of drought, high salinities and extreme temperatures (Sakamoto and Murata 2000).

The potential protective roles of the *PDS* gene in response to abiotic and multi-stresses were demonstrated in *Nicotiana benthamiana* (Anaraki *et al.* 2017). However, there is no report on the roles of the *PDS* gene in the accumulation of free amino acids and protection against oxidative stress. In the present study, virus-induced gene silencing (VIGS) to silence the *PDS* gene in *N. benthamiana (NbPDS)* was applied and measured the differential accumulation of free amino acids induced by it. Furthermore, the roles of accumulated free amino acids were identified by measuring their tolerance to oxidative stress induced by paraquat treatment.

Materials and Methods

Seeds of *Nicotiana benthamiana* were sown and the plants were grown in plastic pots (12 cm diameter × 10 cm height) containing 70% coco peat, 17% peat moss, 5% zeolite, and 8% perlite. The plants were regularly watered and grown under fluorescent lights at 120 µ Einstein/m^2/s with a regime of 16/8 hrs light/dark cycles at 22 ± 2^{o}C in the walk-in chamber at the Molecular Physiology lab in the Department of Biotechnology, Yeungnam University, Republic of Korea.

For transient silencing of the *NbPDS* gene, *Agrobacterium* with a VIGS construct of 369 bp nucleotide sequence derived from the *N. tabacum PDS* gene (NCBI accession No. AJ616742) was inoculated into the leaves of *N. benthamiana* (Hasan *et al.* 2014). The silencing of *NbPDS* was confirmed by conducting a semi-quantitative RT-PCR using the appropriate primer sets (Ali *et al.* 2015). *N. benthamiana* leaves were also inoculated with a construct of the green fluorescent protein (*GFP*) gene as the control. The *GFP* control construct was prepared by inserting a 983 bp nucleotide sequence from the *GFP* gene of *Aequorea victoria* (Hasan *et al.* 2014).

The leaves of *N. benthamiana* were analyzed for the contents of free amino acids and total soluble proteins, as described by Ali *et al*. (2015). The levels of free amino acids and total soluble proteins were analyzed using the leaves that were 6 positions above those infiltrated for *GFP*- or *NbPDS*-silencing in the respective *N. benthamiana* plants. To observe the effects of oxidative stress on the *NbPDS*-silenced plants, 28-day-old infiltrated plants were sprayed thoroughly with different concentrations of paraquat (methyl viologen, 1,1'-dimethyl-4,4'-bipyridinium dichloride;

Sigma, St Louis, MO, USA) in 0.05% Tween 80 and those sprayed only with 0.05% Tween 80 were used as the control. Each plant was sprayed with 40 ml of the solution in total to ensure even and complete coating of the plant with paraquat.

All experiments were conducted thrice, using samples in triplicates. The data are presented as the mean ± standard deviation of the three replicates. Differences among groups were evaluated by ANOVA using the Statistical Analysis Software (SAS) version 9.4 (SAS Inc., Cary, USA).

Results and Discussion

By silencing the *NbPDS* gene, its role in the differential accumulation of free amino acids and tolerance of the plants to variations in oxidative stress was identified. Due to silencing of the *NbPDS* gene, as confirmed by the semi-quantitative RT-PCR (data not shown), and a consequential reduction of PDS enzyme, the *NbPDS*-silenced *N. benthamiana* demonstrated a photo-bleached phenotype and retarded growth (Figs 1 and 2). As the loss of PDS activity led to the accumulation of phytoene in the white tissues of *Arabidopsis immutans* mutants and blocking of the carotenogenesis step (Wstzel *et al.* 1994), that diminished levels of carotenoids also resulted in the characteristic photobleaching phenotype observed in *NbPDS*-silenced *N. benthamiana* were elucidated

Fig. 1. The *GFP*- and *NbPDS*-silenced *N. benthamiana* sprayed with 10 μM paraquat. Arrows indicate injuries caused by paraquat treatment. Injured leaves showed burning symptoms on the leaves. Pictures of the plants were taken 24 and 48 hrs after treatment with 10 μM paraquat.

The levels of free amino acids and total soluble proteins were analyzed in the *NbPDS*-silenced plants. Compared to the control plants, the profile of free amino acid contents in *NbPDS*-silenced *N. benthamiana* was significantly different, and found to be greater by 9.5-folds (Table 1, Fig. 3a). It was found that, upon *NbPDS*-silencing, the amount of accumulation of polar positively charged

amino acids such as lysine, histidine and arginine was the highest. A maximum increase in the amount of accumulated arginine was observed, which was 89.2-folds higher than that of the control plant (Table 1). The polar uncharged amino acids such as serine and threonine demonstrated the second-highest accumulation amounts resulting from the *NbPDS*-silencing. In contrast to the enhanced accumulation potential of free amino acids, the amount of total soluble proteins in *NbPDS*-silenced *N. benthamiana* was lower, only 59.6%, compared to that of the control plants (Fig. 3b).

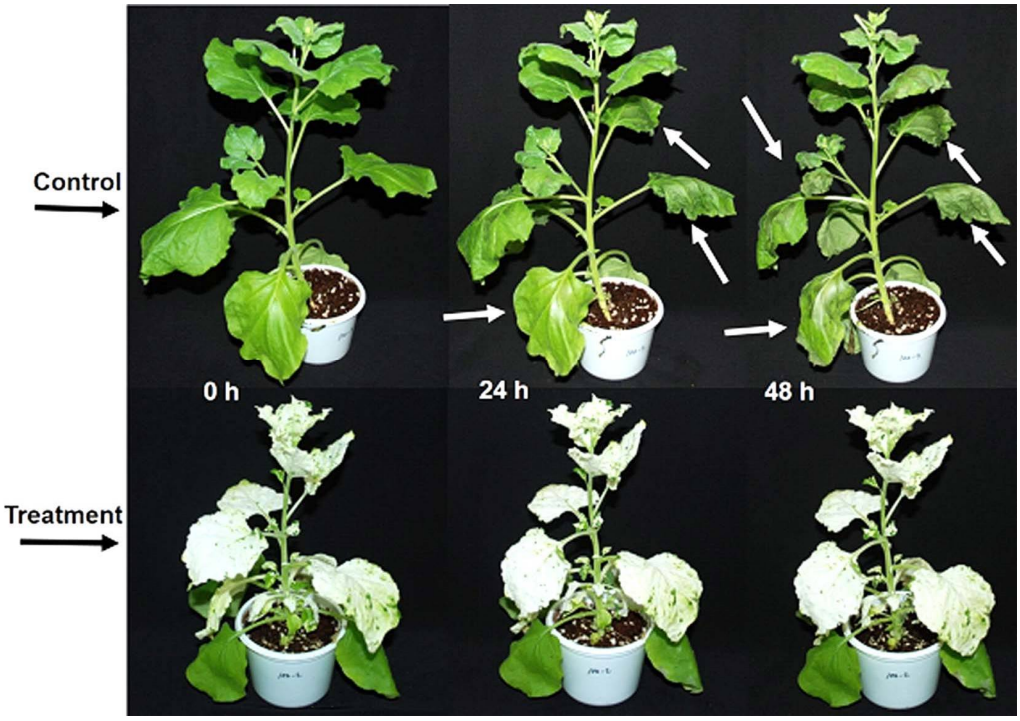

Fig. 2. The *GFP*- and *NbPDS*-silenced *N. benthamiana* sprayed with 100 μM paraquat. Arrows indicate injuries caused by paraquat treatment. Injured leaves showed burning symptoms on the leaves. Pictures of the plants were taken 24 and 48 hrs after treatment with 100 μM paraquat.

The ROS-generating compound paraquat was used to investigate the response of *NbPDS*-silenced plants to oxidative stress. Treatment with both 10 and 100 μM paraquat injured the control, and the extent of injury was particularly severe in plants subjected to the 100 μM concentration (Figs 1 and 2). Contrarily, no severe symptoms of injury were observed in the *NbPDS*-silenced plants subjected to both 10 and 100 μM paraquat spraying (Figs 1 and 2). Paraquat inhibits electron transport and CO_2 assimilation in the cells and also triggers the production of ROS, thereby disrupting cellular structures and inhibiting cell growth (Qian *et al.* 2009). The *immutans* (*im*) mutants of *Arabidopsis* were more tolerant to oxidative stress due to the presence of abnormal chloroplasts lacking colored carotenoids in their white leaf tissues, formed as a result of a defect in the phytoene desaturase activity (Aluru *et al.* 2009). Although diminished amounts of carotenoids were evident in the photobleached white tissue in both the cases, it is not indicative of increased oxidative stress because carotenoids are antioxidant compounds. Therefore,

in the present study, significantly high concentrations of free amino acids due to *NbPDS*-silencing can increase the tolerance of silenced plants to oxidative stress and protect them from injury after paraquat spraying.

Table 1. The contents of the amino acids in the 6th leaf of *N. benthamiana* silenced with the *GFP* gene or *NbPDS* gene.

Amino acids	Concentration (μg/g dw)		Ratio (folds)
	GFP-silenced	*PDS*-silenced	
Nonpolar aliphatic			
Glycine	0.5 ± 0.1b[z]	1.5 ± 0.1a	2.9
Alanine	2.0 ± 0.3b	19.0 ± 5.0a	9.7
Proline	11.0 ± 5.4b	40.9 ± 20.1a	3.7
Valine	ND	23.2 ± 1.1	
Leucine	1.7 ± 0.1b	22.3 ± 1.4a	13.2
Isoleucine	1.0 ± 0.2b	12.6 ± 1.1a	12.3
Methionine	0.7 ± 0.0a	1.0 ± 0.9a	1.5
Nonpolar aromatic			
Phenylalanine	1.2 ± 0.3b	2.3 ± 0.5a	1.9
Tyrosine	0.9 ± 0.0b	11.1 ± 0.7a	12.8
Polar uncharged			
Serine	1.3 ± 0.2b	24.1 ± 2.4a	19.2
Threonine	1.6 ± 0.3b	17.3 ± 0.7a	10.9
Polar positively charged			
Lysine	0.9 ± 0.1b	18.3 ± 1.6a	19.3
Histidine	0.2 ± 0.1b	10.2 ± 1.4a	58.1
Arginine	0.3 ± 0.1b	31.0 ± 4.4a	89.2
Polar negatively charged			
Aspartate	13.0 ± 1.6b	150.0 ± 3.6a	11.5
Glutamate	2.7 ± 1.3b	36.1 ± 4.0a	13.3
Non-protein			
GABA	13.0 ± 1.6b	72.4 ± 10.3a	5.6
Total	52.0 ± 9.6b	493.4 ± 32.8a	9.5

[z]Different letters in the same row indicate a significant difference ($p < 0.05$). ND: not detected.

After 24 hrs, *NbPDS*-silenced plants subjected to 10 μM paraquat treatment accumulated 10.4-folds higher levels of total free amino acids and 81.5% total soluble proteins than those of the control (Table 2, Fig. 3c and d). After 24 hrs, in *NbPDS*-silenced *N. benthamiana* subjected to 100 μM paraquat treatment, the content of total free amino acids and total soluble proteins was 6.9 -fold higher and 56.1% compared to that of the control, respectively (Table 3, Fig. 3c and d). After 48 hrs, *NbPDS*-silenced plants subjected to 10 and 100 μM paraquat treatment contained 63.3 and 73.8% total soluble protein contents compared to those of the controls, respectively (Fig. 3e).

In the controls, paraquat treatment significantly changed the contents of free amino acids such as arginine. Due to the changes in the levels of amino acids by paraquat treatment, the ratio of increase of the amino acid content in the *NbPDS*-silenced plants to that of the control at 0 hr was 9.5-fold (Table 1), whereas those of plants treated with 10 and 100 μM paraquat at 24 hrs was

10.4- and 6.9-folds, respectively (Tables 2 and 3). This indicates that 100 μM paraquat treatment induced severe injury in the controls increasing their content of free amino acids but did not induce damage in the *NbPDS*-silenced plants, which were protected by the presence of high levels of the free amino acids. As reported previously, increase in the levels of free amino acids can provide resistance to plants under conditions of both abiotic and biotic stresses (Hildebrandt *et al.* 2015). Free amino acids accumulated due to *NbPDS*-silencing may contribute in increasing the plant tolerance to oxidative stress under normal growth conditions and upon paraquat treatment.

Table 2. The contents of the amino acids in the 6th leaf of *N. benthamiana* silenced with the *GFP* gene or the *NbPDS* gene after 24 hrs of 10 μM paraquat treatment.

Amino acids	Concentration (μg/g dw)		Ratio (folds)
	GFP-silenced	*PDS*-silenced	
Nonpolar aliphatic			
Glycine	0.5 ± 0.1b[z]	1.4 ± 0.2a	3.0
Alanine	2.1 ± 0.3b	22.1 ± 6.1a	10.3
Proline	8.5 ± 1.6b	36.2 ± 7.2a	4.3
Valine	1.7 ± 0.3b	28.2 ± 4.4a	16.6
Leucine	1.7 ± 0.5b	27.3 ± 5.9a	16.0
Isoleucine	1.2 ± 0.2b	17.5 ± 3.3a	14.9
Methionine	0.5 ± 0.1b	1.6 ± 0.6a	3.3
Nonpolar aromatic			
Phenylalanine	0.9 ± 0.4b	2.7 ± 0.3a	3.0
Tyrosine	0.8 ± 0.3b	13.4 ± 4.2a	16.8
Polar uncharged			
Serine	1.5 ± 0.4b	32.3 ± 8.5a	21.0
Threonine	1.6 ± 0.3b	20.3 ± 4.4a	13.1
Polar positively charged			
Lysine	1.1 ± 0.3b	18.5 ± 4.5a	16.7
Histidine	0.4 ± 0.1b	11.1 ± 2.2a	25.9
Arginine	0.3 ± 0.1b	33.5 ± 6.4a	99.1
Polar negatively charged			
Aspartate	13.1 ± 1.5b	153.6 ± 16.9a	11.7
Glutamate	3.3 ± 1.1b	38.3 ± 3.2a	11.6
Non-protein			
GABA	11.1 ± 1.2b	66.8 ± 13.9a	6.0
Total	50.4 ± 5.4b	524.9 ± 56.0a	10.4

[z]Different letters in the same row indicate a significant difference ($p < 0.05$).

Total soluble protein contents in the *NbPDS*-silenced plants were significantly lower than those of the control (Fig. 3b). From 24 to 48 hrs, the ratio of total protein contents in the control to the *NbPDS*-silenced plants sprayed with 10 μM paraquat decreased from 81.5 to 63.3%, whereas, that of the plants subjected to 100 μM paraquat treatment increased from 56.1 to 73.8%. All data indicated that a considerable quantity of free amino acids might not have been incorporated into the proteins in the *NbPDS*-silenced *N. benthamiana*, but ironically, the increased content of total

free amino acids reduced injury by oxidative stress, as indicated by reduced total soluble protein contents.

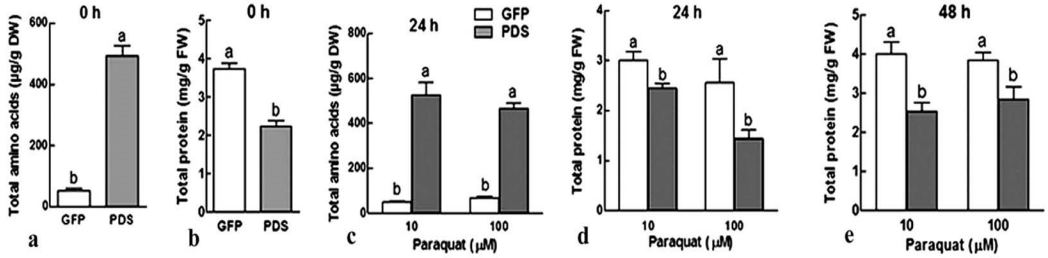

Fig. 3. Amounts of total free amino acids and total soluble proteins in leaves of *GFP*-silenced control and *NbPDS*-silenced *N. benthamiana*. (a) Total amino acids and (b) total soluble proteins from *GFP*- and *NbPDS*-silenced plants without paraquat treatment. (c) Total amino acids and (d) total soluble proteins after 24 hrs of paraquat treatment. (e) Total soluble proteins after 48 hrs of paraquat treatment. Different letters on bars indicate significant differences at $p < 0.05$.

Table 3. The contents of the amino acids in the 6th leaf of *N. benthamiana* silenced with the *GFP* gene or *NbPDS* gene after 24 hrs of 100 µM paraquat treatment.

Amino acids	Concentration (µg/g dw)		Ratio (folds)
	GFP-silenced	*PDS*-silenced	
Nonpolar aliphatic			
Glycine	0.5 ± 0.1b[z]	1.5 ± 0.1a	3.2
Alanine	6.4 ± 0.3b	16.5 ± 0.9a	2.6
Proline	10.7 ± 1.9b	18.1 ± 9.5a	1.7
Valine	2.0 ± 1.8b	28.0 ± 2.2a	13.7
Leucine	3.2 ± 0.7b	26.8 ± 4.0a	8.5
Isoleucine	2.1 ± 0.6b	16.7 ± 1.3a	7.9
Methionine	0.4 ± 0.1	ND	
Nonpolar aromatic			
Phenylalanine	1.3 ± 0.1b	2.8 ± 0.4a	2.1
Tyrosine	1.4 ± 0.2b	15.6 ± 1.1a	11.5
Polar uncharged			
Serine	2.7 ± 0.5b	30.6 ± 0.8a	11.3
Threonine	2.6 ± 0.4b	19.3 ± 2.7a	7.5
Polar positively charged			
Lysine	2.0 ± 0.2b	20.7 ± 3.3a	10.3
Histidine	0.6 ± 0.3b	12.6 ± 2.0a	21.8
Arginine	1.1 ± 0.7b	36.2 ± 10.1a	34.4
Polar negatively charged			
Aspartate	15.5 ± 5.3b	144.7 ± 20.7a	9.3
Glutamate	3.4 ± 1.3b	29.8 ± 2.6a	8.7
Non-protein			
GABA	11.5 ± 1.5b	47.5 ± 6.9a	4.1
Total	67.5 ± 10.4b	467.5 ± 21.2a	6.9

[z]Different letters in the same row indicate a significant difference ($p < 0.05$). ND: not detected.

The present study identified that silencing of the *NbPDS* gene in *N. benthamiana* produced albinism in the plants but increased the content of their free amino acids and their capacity to withstand severe oxidative stress. Silencing the *PDS* gene induced a phenotypic disadvantage by the manifestation of dwarfism and albinism, but alternatively increased the amount of free amino acids which can mitigate the oxidative stress conditions and possibly be used for amino acid capsule formation.

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