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Ascorbate deficient semi-dwarf *asfL1* mutant of *Lathyrus sativus* exhibits alterations in antioxidant defense

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Abstract

An ascorbate-deficient semi-dwarf mutant *asfL-1* was detected in 250 Gy γ-ray treated grass pea (*Lathyrus sativus* L.) cv. BioR-231. The mutant contained only 42 % of leaf and 20 % of root ascorbate content of mother control (MC). I investigated the possible causes of ascorbate deficiency and its effect on growth and antioxidant defense in control and 150 mM NaCl-treated seedling after 60-d growth period. Ascorbate deficiency was due to significant reduction in activities of monodehydroascorbate reductase and dehydroascorbate reductase as well as increase in ascorbate oxidase, leading to considerable decrease in redox state. Despite low ascorbate pool and decrease in ascorbate peroxidase activity, shoot and root biomass production in *asfL-1* mutant were similar to MC plants, even at NaCl treatment. High accumulation of glutathione (GSH) coupled with high activities of GSH reductase, catalase, GSH peroxidase and peroxidase in both tissues of the mutant permitted efficient recycling of GSH and scavenging of H_2O_2 through well integrated catalase/peroxidase system, despite high superoxide dismutase activity under NaCl treatment. The collapse of this system led to inhibition of growth in NaCl-treated mother plants. Together, the results suggested that *asfL-1* plants undertook a major reshuffle in its antioxidant defense machinery, which effectively counterbalanced the negative impact of ascorbate deficiency and remained unperturbed by NaCl treatment to maintain normal growth and biomass production.

Additional key words: ascorbate-glutathione cycle, catalase, dehydroascorbate reductase, glutathione reductase, glutathione peroxidase, grass pea, hydrogen peroxide, oxidative stress.

Introduction

One consequence of life in oxygen atmosphere is continuous production of reactive oxygen species (ROS), such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals (Halliwell 2006). Among the ROS-scavenging machinery, ascorbate (ASC)-glutathione (GSH) cycle plays a pivotal role in plant defense where ASC and GSH are the principal antioxidants. The balance between oxidized and reduced forms of both ASC and GSH is crucial in modulating the ROS-antioxidant interactions as it ultimately determines plant growth and development (De Pinto and De Gara 2004).

Synchronized action of antioxidant enzymes is required for successful quenching of free radicals. Superoxide dismutase (SOD) constitutes the first line of defense against ROS, converting superoxide radicals into hydrogen peroxide (H_2O_2) which is reduced to H_2O through ASC-dependent ascorbate peroxidase (APX) in ASC-GSH cycle. The reaction concomitantly generates two oxidized ASC forms monodehydroascorbate (MDHA) and dehydroascorbate (DHA). MDHA is then converted to ASC through NAD(P)H-mediated action of MDHA-reductase (MDHAR). DHA is reduced to ASC by GSH-dependent action of DHA-reductase (DHAR) (Noctor *et al*. 2002). The resultant oxidized form of GSH-disulfide (GSSG) is recycled to GSH by the NADPH-dependent activity of glutathione reductase (GR).

Besides APX in the ASC-GSH cycle, actions of three other prominent H_2O_2-scavenging enzymes are worth

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Abbreviations: AO - ascorbate oxidase; APX - ascorbate peroxidase; ASC - ascorbate, reduced form; ASC-GSH cycle - ascorbate-glutathione cycle; *asfL-1* - ascorbate deficient type-1 mutant in *Lathyrus sativus*; BSO-L - butathione-[S,R]-sulfoximine; CAT - catalase; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DTT - dithiothreitol; GPX - glutathione peroxidase; GR - glutathione reductase; GSH - glutathione, reduced form; GSSG - glutathione, oxidized form; MC - control mother plant; MDA - malondialdehyde; MDHA - monodehydroascorbate; MDHAR - monodehydroascorbate reductase; MT - treated mother plant; MuC, MuT - mutant control and treated plants; POX - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase.

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mentioning. One of them are catalases (CAT) which are exclusively located in peroxisomes and usually degrades photorespiratory H_2O_2 to H_2O without consuming any type of reducing power. Glutathione peroxidase (GPX) can use GSH or thioredoxins as the reducing substrates to metabolize H_2O_2 and to protect cells from peroxide damage (Noctor *et al*. 2002, Navrot *et al*. 2006). Non-specific peroxidases (POX) also function efficiently in H_2O_2 metabolism, utilizing diverse substrates during plant growth (Passardi *et al*. 2004). Increasing evidence suggest that H_2O_2 content is well correlated with rate of lipid peroxidation (malondialdehyde, MDA, content), and NaCl treatment generally increases this rate in salt-sensitive genotypes (Agarwal and Pandey 2004). Therefore, both these compounds are routinely used as biochemical markers for oxidative stress (Cheeseman 2006, Hernández *et al*. 2010).

In contrast to the role of MDHAR and DHAR in regeneration of reduced ascorbate, ascorbate oxidase (AO) oxidizes ASC to MDHA exclusively in the apoplast. MDHA is unstable and rapidly disproportionates to yield DHA and ASC. Apoplastic DHA is then transported to cytosol through plasma membrane to be reduced by DHAR (Noctor *et al*. 2002). The high ratio of ASC to DHA as well as GSH to GSSG is extremely important for maintenance of cellular redox pool in a reducing environment.

Grass pea (*Lathyrus sativus* L.) is a very hardy legume crop, and grows well in Indian subcontinent, South America, the Mediterranean region and Australia for human consumption and animal feedstock (Talukdar 2009). Grass pea can tolerate a wide range of abiotic stresses (Vaz Patto *et al*. 2006, Talukdar 2009), and studies in this aspect have just been initiated (Mahdavi and Sanavy 2007, Talukdar 2011a,b). However, these works are mainly based on analysis of different morpho-physiological parameters in stressed condition, and virtually nothing is known about the intrinsic biochemical mechanisms of antioxidant defense in this hardy crop. Development of stable mutants in recent years open an excellent opportunity to trace the underlying antioxidant defense mechanisms of grass pea (Talukdar 2009, 2011a,c).

The power of mutant analysis in elucidation of the role of different redox components against oxidative stress has been demonstrated in several *Arabidopsis* mutants. Notable among them was *vtc*-mutants, deficient in ASC accumulation in different magnitudes (Conklin *et al*. 1996, Veljovic-Jovanovic *et al*. 2001). The present study began as part of a broader strategy to analyse antioxidant defense of elite grass pea (*Lathyrus sativus*) genotypes including induced mutant lines under various abiotic stresses (Talukdar 2011a,b). One of the mutant lines with characteristic semi-dwarf habit was identified as ascorbate deficient, containing only 42 % of the total leaf and only 20 % of the total root ascorbate of its mother cultivar BioR-231. The mutant line was initially designated as non-winged internode mutant due to absence of normal winged internode (Talukdar and Biswas 2006). The detection of low ascorbate content in a semi-dwarf mutant line is unique in grass pea.

The objectives of the present work were to analyze the alterations in cellular redox status of ASC and GSH and concomitant adjustments made by antioxidant defense systems in leaves and root tissue of ASC-deficient mutant. The fitness of this mutant along with its mother plants was also tested in imposed salinity stress during vegetative stage. I searched the possible reasons behind the ASC-deficiency but nearly normal growth of the mutant by ascertaining the activity of nine enzymes: SOD, APX, MDHAR, DHAR, GR, CAT, GPX, POX and AO in the mutant and in mother plants.

Materials and methods

The non-winged internode mutant was isolated in 250 Gy γ-ray treated M_2 progeny of grass pea (*Lathyrus sativus* L. cv. BioR-231). The mutant was self-fertile and true breeding for its characteristic phenotype of non-winged internode (Talukdar and Biswas 2006, Talukdar 2009). No significant change in its ascorbate content was found in M_3 generation. It was, therefore, renamed as ascorbate deficient type-1 mutant in *Lathyrus sativus* (*asfL-1*).

Fresh and healthy seeds (M_4) of *asfL-1* mutant and its mother plant (cv. BioR-231) were surface sterilized with 10 % (v/v) sodium hypochlorite for 3 min and allowed to germinate in the dark at 25 °C. Seedlings were transferred to earthen pots containing a mixture of soil, *Vermiculite* and farm yard manure (1:1:1). Seedlings were thinned to one per pot after emergence and watered evenly for their uniform growth until 7 d after emergence. The pots were kept under day/night temperature of 27/20 °C, humidity of 70 %, irradiance of 200 μmol m^{-2} s^{-1} and 14-h photoperiod. Salt treatment was commenced on 10-d-old seedlings by watering the plants with equal daily increments of NaCl-supplemented distilled water over 3 d to a final concentration of 150 mM (considered critical for grass pea genotypes). This was applied thrice a week for 7-weeks of continuous treatment. Untreated mutant plants were considered as mutant control (MuC), while the cv. BioR-231 was served as mother control (MC). Mother plants subjected to equal NaCl stress were denoted as treated mother plants (MT). Root length and fresh masses of shoots and roots were measured at harvest (60-d-old plant). Dry masses of the shoots and roots were obtained after drying the samples at 60 °C for 72 h.

The mature fully expanded leaves (grown on primary branches) and roots were collected from MC, MT, MuC and salt-treated mutant plants (MuT) at the beginning (10-d-old seedlings) and after 60-d treatment for biochemical studies. A pool of samples from six plants for each variant was collected, and six independent experiments were performed. All operations were

performed at 0 - 4 °C, except mentioned otherwise. Leaf samples (1 g) were homogenized in an extraction medium containing 50 mM K-phosphate buffer, pH 7.8, 0.1 mM EDTA, 2 mM cysteine, 1 % (m/v) polyvinylpyrrolidone (PVP) and 0.2 % (v/v) *Triton X-100*. For the APX (EC1.11.1.11) activity, 20 mM ascorbate was added to the extraction buffer. The extracts were filtered through two layers of cheesecloth, and the homogenate was centrifuged at 14 000 *g* for 20 min. The supernatant was filtered through a column containing 1 cm^3 of *Sephadex G-50* equilibrated with the same buffer used in homogenization. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm using coefficient of absorbance (ε) 2.8 mM^{-1} cm^{-1} (Nakano and Asada 1981). SOD (EC 1.15.1.1) activity was determined by the nitro-blue tetrazolium (NBT) assay as described by Beyer and Fridovich (1987). The reaction mixture (3 cm^3) contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.1 cm^3 of enzyme extract. One unit of SOD was defined as the amount of protein causing a 50 % NBT photoreduction. MDHAR (EC 1.6.5.4) and DHAR (EC 1.8.5.1) were extracted with 50 mM K-phosphate buffer (pH 7.8), 1 % PVP, 0.2 mM EDTA and 10 mM β-mercaptoethanol. MDHAR activity was determined by following NADH oxidation at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) for 90 s (Dalton *et al*. 1993) and DHAR activity by following ascorbate formation at 265 nm (ε = 14.1 mM^{-1} cm^{-1}) for 3 min (Nakano and Asada 1981). GR (EC 1.6.4.2) was extracted with the same medium as for MDHAR and DHAR but without β-mercaptoethanol and with 0.1 % *Triton X-100*, and its activity was measured by monitoring glutathione-dependent oxidation of NADPH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) for 3 min (Dalton *et al*. 1993). CAT (EC 1.11.1.6) was extracted in 50 mM K-phosphate buffer (pH 7.0) and 0.5 % PVP, and its activity was assayed by measuring the reduction of H_2O_2 at 240 nm (ε = 39.4 M^{-1} cm^{-1}) for 1 min (Aebi 1984). POX (EC 1.11.1.7) was measured by monitoring oxidation of 4 mM guaiacol at 470 nm (ε = 22.6 mM^{-1} cm^{-1}) in 50 mM potassium-phosphate buffer (pH 6.5), following addition of 1 mM H_2O_2 (Veljovic-Jovanovic *et al*. 2001). AO (EC 1.10.3.3) was analysed by monitoring the oxidation of ASC at 290 nm (Takahama and Oniki 1994). Total GPX (EC 1.11.1.9) activity was assayed in three separate sets, using H_2O_2, *tert*-butyl hydroperoxide (for selenium GPX) and cumene hydroperoxide (for Se and non-Se enzyme at 25 °C and pH 8.0) as substrates (Carmagnol *et al*. 1983, Drotar *et al*. 1985). The rate of peroxide removal was measured with respect to the rate of NADPH oxidation at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). Reduced and oxidized form of ascorbate and glutathione were measured following the methods of Law *et al*. (1983) and Griffith (1985), respectively. Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content at 532 nm (ε = 155 mM^{-1} cm^{-1}; Saher *et al*. 2004). H_2O_2 content was measured following the methodology described by Cheeseman (2006).

The results are presented as mean ± SE of at least six replicates. Statistical significance ($P < 0.05$) between mean values of mother plants and those of mutant plants was estimated by *t-test*, while multiple comparisons of means were performed by *ANOVA*, using the statistical software *STATISTICA 6.0* (*Statsoft Inc*., Tulsa, USA).

Results

The whole plant fresh as well as dry mass of 60-d-old MC, MuC and MuT varied non-significantly. The fresh and dry mass of shoot and root and root length were very close in these three plant types. By contrast, MT exhibited 1.5-fold reduction in the fresh mass of shoots and 2.5-fold decrease in the fresh mass of root. Compared to the MC, root length in MT plants was reduced by nearly 3.7-fold, while the dry masses of its shoot and root were reduced by around 1.5-fold and 3.1-fold, respectively (Table 1).

Total ascorbate content (ASC + DHA) in leaves and roots of MuC and MuT was between 37 and 42 % and between 19 and 20 % of the MC plants, respectively. In comparison to MC, ASC content decreased > 6-fold in leaves and 38 to 47-fold in roots, while DHA increased 5.5 to 6-fold in leaves and by nearly1.2-fold in roots of

Table 1. Biomass production in untreated mother control (MC), 150 mM NaCl-treated mother plants (MT), untreated *asfL-1* mutant (MuC) and 150 mM NaCl-treated *asfL-1* mutant (MuT) at age 60 d. Means ± SE ($n = 6$). * denotes the significant differences from MC plants at $P < 0.05$.

Characters	MC	MT	MuC	MuT
Shoot fresh mass [g $plant^{-1}$]	10.7 ± 0.35	7.14 ± 0.39*	9.6 ± 0.18	9.0 ± 0.31
Root fresh mass [g $plant^{-1}$]	11.3 ± 0.19	4.50 ± 0.07*	11.9 ± 0.11	12.1 ± 0.21
Root length [cm]	14.2 ± 0.22	3.84 ± 0.04*	14.8 ± 0.13	15.0 ± 0.23
Shoot dry mass [g $plant^{-1}$]	0.16 ± 0.002	0.11 ± 0.003*	0.16 ± 0.006	0.15 ± 0.009
Root dry mass [g $plant^{-1}$]	0.19 ± 0.003	0.05 ± 0.010*	0.19 ± 0.008	0.20 ± 0.006

Table 2. Changes in leaf and root ascorbate and glutathione content [nmol g^{-1} (f.m.)], and their redox states in untreated mother control (MC), 150 mM NaCl-treated mother plants (MT), untreated *asfL-1* mutant (MuC) and treated *asfL-1* mutant (MuT) at the beginning of the treatment (10-d-old seedling) and after 60-d growth period. Means ± SE ($n = 6$). * and ** denote the significant differences from MC and differences (by *ANOVA*) among genotypes, respectively, at $P < 0.05$.

Organ	Age	Genotype	ASC	DHA	ASC/(ASC+DHA)	GSH	GSSG	GSH/(GSH+GSSG)
Leaf	10 d	MC	1861 ± 27	89 ± 10	0.954 ± 0.05	70.8 ± 2.0	10.7 ± 1.0	0.870 ± 0.01
		MT	1853 ± 19	94 ± 09	0.951 ± 0.07	68.9 ± 1.7	10.7 ± 1.0	0.860 ± 0.03
		MuC	311 ± 11*	508 ± 30*	0.380 ± 0.01*	295.6 ± 11*	22.8 ± 2.0*	0.928 ± 0.06
		MuT	305 ± 19*	495 ± 24*	0.381 ± 0.03*	293.3 ± 7.0*	22.5 ± 1.3*	0.928 ± 0.03
F-value			**	**	**	**	**	**
	60 d	MC	1970 ± 13	90 ± 10	0.956 ± 0.09	70.0 ± 3.0	12.0 ± 4.0	0.850 ± 0.18
		MT	910 ± 15*	390 ± 13*	0.700 ± 0.11*	23.0 ± 2.2*	4.2 ± 1.1*	0.840 ± 0.18
		MuC	300 ± 22*	499 ±20*	0.387 ± 0.01*	328.0 ± 17*	22.5 ± 2.0*	0.929 ± 0.10
		MuT	295 ± 17*	490 ± 11*	0.374 ± 0.08*	329.0 ± 2.0*	23.0 ± 2.0*	0.930 ± 0.18
F-value			**	**	**	**	**	**
Root	10 d	MC	952 ± 28	168 ± 28	0.850 ± 0.03	55.3 ± 4.1	12.7 ± 1.3	0.810 ± 0.07
		MT	949 ± 22	168 ± 25	0.841 ± 0.01	55.3 ± 4.1	12.9 ± 1.3	0.810 ± 0.07
		MuC	25 ± 21*	199 ±28*	0.110 ± 0.11*	371.0 ± 10*	24.8 ± 2.0*	0.933 ± 0.10
		MuT	24 ± 29*	196 ± 25*	0.109 ± 0.10*	373.0 ± 9.0*	22.5 ± 2.0*	0.940 ± 0.12
F-value			**	**	**	**	**	**
	60 d	MC	939 ± 23	157 ± 10	0.850 ± 0.11	51.0 ± 9.0	12.0 ± 3.0	0.810 ± 0.22
		MT	502 ± 25*	229 ± 09*	0.682 ± 0.08*	8.5 ± 2.0*	2.0 ± 0.9*	0.800 ± 0.12
		MuC	23 ± 12*	196 ± 29*	0.105 ± 0.04*	355.0 ± 31*	24.5 ± 6.0*	0.935 ± 0.12
		MuT	20 ± 17*	190 ± 31*	0.095 ± 0.07*	359.0 ± 10*	26.0 ± 2.0*	0.939 ± 0.19
F-value			**	**	**	**	**	**

Table 3. Changes in specific activities of enzymes SOD [U mg^{-1}(protein)]), MDHAR, DHAR, GR and AO [nmol mg^{-1}(protein) min^{-1}] in leaves (L) and roots (R) of untreated mother control (MC), *asfL-1*mutant (MuC) and 150 mM NaCl-treated mother control (MT) and *asfL-1* mutant (MuT) at the beginning of the experiment (10-d-old seedlings) and after 60-d growth period. Means ± SE ($n = 6$). * and ** denote the significant differences from MC and differences (by *ANOVA*) among genotypes, respectively, at $P < 0.05$; ns - not significant.

Enzymes	Age [d]	MC	MT	MuC	MuT	F-value
SOD (L)	10	110.5 ± 3.2	110.5 ± 3.2	92.7 ± 1.8	93.0 ± 2.0	ns
	60	148.0 ± 4.2	222.0 ± 7.0*	133.3 ± 2.2	190.0 ± 3.5*	**
SOD (R)	10	94.5 ± 2.9	94.5 ± 2.9	87.8 ± 1.9	81.8 ± 2.0	ns
	60	151.0 ± 4.0	170.0 ± 5.0*	147.0 ± 3.3	202.8 ± 5.5*	**
MDHAR (L)	10	80.0 ± 2.0	80.0 ± 2.0	17.9 ± 1.0*	18.0 ± 1.1*	**
	60	73.7 ± 6.0	72.8 ± 6.3	13.0 ± 1.5*	13.8 ± 2.0*	**
MDHAR (R)	10	77.0 ± 8.5	77.0 ± 8.5	15.3 ± 1.0*	15.0 ± 1.5*	**
	60	69.0 ± 2.0	68.3 ± 1.6	13.7 ± 1.5*	14.8 ± 1.1*	**
DHAR (L)	10	8.9 ± 2.2	8.9 ± 2.2	1.1 ± 0.1*	1.2 ± 0.1*	**
	60	9.1 ± 3.0	40.1 ± 4.7	1.0 ± 0.1*	1.3 ± 0.1*	**
DHAR (R)	10	8.8 ± 1.9	8.8 ± 1.9	1.0 ± 0.1*	1.3 ± 0.1*	**
	60	8.0 ± 1.3	20.2 ± 3.8	0.9 ± 0.1*	1.0 ± 0.1*	**
GR (L)	10	20.8 ± 2.1	20.8 ± 2.1	60.0 ± 3.9*	57.7 ± 3.3*	**
	60	21.0 ± 0.2	20.7 ± 0.2	64.0 ± 0.37*	65.0 ± 0.5*	**
GR (R)	10	13.9 ± 0.1	13.9 ± 0.1	65.1 ± 2.8*	57.0 ± 2.4*	**
	60	28.8 ± 3.3	30.1 ± 4.0	88.8 ± 7.0*	91.1 ± 7.4*	**
AO (L)	10	207.7 ± 11.8	207.7 ± 11.8	544.0 ± 28.0*	539.9 ± 21.0*	**
	60	175.0 ± 8.6	171.0 ± 11.0	450.0 ± 18.0*	467.7 ± 21.0*	**
AO (R)	10	109.0 ± 6.0	109.0 ± 6.0	420.0 ± 12.0*	427.0 ± 10.9*	**
	60	100.0 ± 3.9	101.8 ± 8.0	310.0 ± 11.0*	293.0 ± 7.3*	**

both MuC and MuT (Table 2). Leaves had a higher content of total ascorbate than roots in MC, MuC and MuT. Compared to MC (ASC 85 - 95%, DHA 5 - 15 %), MuC possessed approximately 38 % ASC and 62 % DHA in leaves and about 11 % ASC and 89 % DHA in roots, exhibiting low redox state of ascorbate in both organs (Table 2). A reverse situation, however, occurred for the glutathione content. Total glutathione (GSH + GSSG) was 3 to 4-fold higher in leaves and was about 6-fold higher in roots of MuC and MuT than in MC plants (Table 2). GSH content increased over MC plants by about 4 to 5-fold in leaves and by 6 to 7-fold in roots, while GSSG content was enhanced by nearly 2-fold in both tissues of the MuC and MuT. The redox state of glutathione remained higher in leaves (0.928 - 0.930) and roots (0.933 - 0.940) of the mutant than in the MC plants (Table 2). In MT, 49-d NaCl treatment caused 1.7-fold decrease in the total ascorbate pool in both tissues, while total glutathione content in relation to the MC was reduced by 3-fold in leaves and by 6-fold in roots. Ascorbate content in MT plants still was considerably higher than that in the mutant plants. ASC redox state in MT plants was lower (1.2 to 1.4-fold) than in MC but was considerably higher (2 to 6-fold) than in MuC and MuT (Table 2). Both ascorbate and glutathione pool and their redox states varied significantly ($P < 0.05$) among the MC, MT, MuC and MuT (Table 2).

Significant variations ($P < 0.05$) were also observed in the activities of different antioxidant enzymes (Table 3). Total SOD activity was comparable in MC and MuC. However, its activity increased by around 1.3-fold and 1.5-fold in MuT and MT, respectively. Considering both leaf and root tissues, the decrease in specific enzyme activities in MuC and MuT ranged approximately between 4.5 and 6-fold for MDHAR and 7 and 9-fold for DHAR in comparison to MC. By contrast, GR activity in MuC and MuT increased by 3 to 5.5-fold over MC plants, showing much higher activity in roots than in leaves of the 60-d-old plants. In MT plants, non-significant change from MC was noticed in activity of MDHAR and GR, but DHAR activity increased by nearly 4.4-fold in leaf and by 2.5-fold in root. AO activity was higher in young

Table 4. Changes in H_2O_2 [µmol g^{-1}(f.m.)] and MDA [mol g^{-1}(f.m.)] content, and APX [nmol mg^{-1} (protein) min^{-1}], CAT [nmol mg^{-1} (protein) min^{-1}], POX [µmol mg^{-1}(protein) min^{-1}] and GPX [nmol mg^{-1}(protein) min^{-1}] activities in leaves (L) and roots (R) of untreated (MC) and 150 mM NaCl-treated mother (MT) plants, and untreated (MuC) and 150 mM NaCl-treated *asfL-1* mutant (MuT) at 10 and 60-d growth period. Means ± SE ($n = 6$). * and ** denote the significant differences from MC and differences (by *ANOVA*) among genotypes, respectively, at $P < 0.05$. ns - not significant.

Enzymes	Age [d]	MC	MT	MuC	MuT	F-value
APX (L)	10	192.8 ± 8.4	192.8 ± 4.4	13.2 ± 4.4*	12.9 ± 3.7*	**
	60	186.7 ± 7.0	181.9 ± 8.8	10.8 ± 4.2*	11.2 ± 1.1*	**
APX (R)	10	129.0 ± 6.0	129.0 ± 6.0	9.7 ± 2.0*	10.0 ± 2.1*	**
	60	109.6 ± 5.7	99.5 ± 3.0	6.9 ± 0.9*	7.2 ± 1.1*	**
CAT (L)	10	39.3 ± 2.5	39.3 ± 2.5	61.0 ± 3.0*	58.3 ± 3.0*	**
	60	38.0 ± 3.3	15.2 ± 1.0*	70.0 ± 6.6*	80.0 ± 4.0*	**
CAT (R)	10	60.8 ± 2.8	60.8 ± 2.8	88.8 ± 3.0*	89.0 ±1.9*	**
	60	64.0 ± 2.0	25.6 ± 0.1*	98.8 ± 3.8*	99.0 ± 5.0*	**
POX (L)	10	230.5 ± 21.2	230.5 ± 21.2	341.5 ± 19.7*	338.0 ± 20.0*	**
	60	233.9 ± 28.0	195.0 ± 20.0*	370.0 ± 29.0*	375.0 ± 31.0*	**
POX (R)	10	265.5 ± 13.0	265.5 ± 13.0	480.0 ± 18.0*	484.5 ± 21.0*	**
	60	288.3 ± 11.0	192.2 ± 9.0*	520.0 ± 31.0*	518.0 ± 29.0*	**
GPX (L)[a]	10	7.0 ± 0.9	7.0 ± 0.9	19.0 ± 1.8*	17.7 ± 2.9*	**
	60	18.0 ± 1.1	30.0 ± 2.0*	28.0 ± 1.4*	33.3 ± 1.7*	**
GPX (L)[b]	10	65.0 ± 7.0	65.0 ± 7.0	90.0 ± 8.0*	89.0 ± 7.7*	**
	60	110.0 ± 6.0	130.0 ± 7.5*	121.6 ± 8.0*	131.0 ± 10.0*	**
GPX (R)[a]	10	7.7 ± 1.0	7.7 ± 1.0	22.0 ± 2.0*	20.7 ± 1.8*	**
	60	14.0 ± 3.9	30.0 ± 4.4*	48.0 ± 5.0*	57.7 ± 6.0*	**
GPX (R)[b]	10	55.0 ± 9.0	55.0 ± 9.0	100.5 ± 13.0*	92.5 ± 11*	**
	60	80.0 ± 7.0	138.5 ± 9.0*	144.8 ± 10.0*	161.8 ± 17*	**
H_2O_2 (L)	10	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	ns
	60	4.7 ± 0.3	10.2 ± 0.1*	4.6 ± 0.3	4.6 ± 0.3	**
H_2O_2 (R)	10	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	ns
	60	3.5 ± 0.1	13.3 ± 0.2*	3.4 ± 0.2	3.5 ± 0.2	**
MDA (L)	10	2.2 ± 1.5	2.2 ± 1.5	2.5 ± 0.9	2.8 ± 1.0	ns
	60	3.4 ± 2.0	7.8 ± 3.0*	3.3 ± 1.9	3.5 ± 2.0	**
MDA(R)	10	2.2 ± 1.1	2.2 ± 1.1	2.2 ± 0.9	2.2 ± 1.0	ns
	60	3.0 ± 2.0	9.0 ± 6.9*	2.8 ± 1.6	2.9 ± 2.2	**

seedlings than in 60-d-old plants. Its activity increased 2.5 to 3-fold in the MuC and MuT, but remained unchanged in MT (Table 3).

Specific activities of CAT, POX and GPX were higher in roots than in leaves of MuC and MuT, while APX followed the reverse trend (Table 4). In relation to MC plants, there was 1.1 to 2.0-fold increase for CAT and 1.5 to 1.8-fold increase for POX activity in the MuC, but APX activity decreased 13 to 17-fold (Table 4). MT plants registered nearly 2.5-fold decrease of CAT activity in both tissues and about 1.2-fold decrease of POX activities in leaf and 1.5-fold in root tissues in comparison with MC. APX activity, however, remained close to MC plants. Compared to 10-d-old seedlings, activities of SOD, CAT (except MT) and POX (except MT) increased in 60-d-old plants. For GPX, the activity was much higher when assayed with cumene-hydroperoxide than with H_2O_2 as substrate. Significant increase of its activity over MC was observed in MuC, MuT and MT plants (Table 4). No activity, however, could be measured with *tert*-butyl hydroperoxide as a substrate.

H_2O_2 content and membrane lipid peroxidation as measured by MDA content were similar in MC, MuC and MuT (Table 4). In MT, significant increases in H_2O_2 and MDA content over MC were observed (Table 4).

Discussion

The semi-dwarf *asfL-1* mutant in grass pea exhibited significant deficiency in total leaf and root ascorbate content, but quite interestingly this feature was accompanied with normal fresh and dry masses of the control mutant plants and under 150 mM NaCl treatment. Low ASC content and concomitant rise of its oxidized form, DHA, resulted in low cellular redox state of ascorbate in the *asfL-1* mutant, and the lowest value (0.095) was recorded in the root. However, Díaz-Vivancos *et al*. (2010) observed positive correlation between shoot growth and the ASC content. Several reports suggested that the root was much less influenced by ascorbate deficiency, and the glutathione played more important role than the ascorbate in promoting signals that permitted cell cycle progression in actively-growing root tissues (Vernoux *et al*. 2000, Maughan and Foyer 2006). Higher increment of both total and reduced GSH content in roots of the *asfL-1* mutant indicated recruitment of a significant amount of glutathione, altering its redox status in roots of the mutant plant. This might help it to maintain normal growth even at 150 mM NaCl for a long period. Interestingly, despite higher content of reduced ascorbate in mother plants than in MuT, mother plants under 150 mM NaCl manifested severe inhibition of both shoot and root biomass accumulation, and the effect was more pronounced on roots. Certainly, low GSH redox pool in MT plants badly impeded its normal growth. Greater role of this thiol-peptide in root development was evidenced in inhibition of root phenotype, but not shoot, in low GSH containing *Arabidopsis rml1* mutant, by addition of GSH inhibitor butathione-[S,R]-sulfoximine (BSO-L) and rescue of root phenotype by addition of GSH (but not ASC or DTT) (Vernoux *et al*. 2000), and also in ASC-deficient roots of salt-treated *Brassica oleracea* (Hernández *et al*. 2010).

Ascorbate deficiency in the *asfL-1* mutant might be due to significant reduction in activities of MDHAR (4.5 to 6-fold) and DHAR (7 to 9-fold) and considerable increase in AO activity. Depletion of MDHAR and DHAR activities severely crippled recycling of reduced ascorbate from its oxidized states, MDHA and DHA, while increased AO activity, particularly in 10-d-old seedlings, overproduced DHA. A positive correlation between AO activity and DHA content was also observed in young seedlings of pea plants, bringing about a decrease in cellular redox state of ascorbate (Díaz-Vivancos *et al*. 2010). The DHA accumulation in the *asfL-1* mutant led to around 2.5-fold decrease in cellular redox state of ascorbate in leaves and about 8 to 9-fold in roots. By contrast, a 2.5 to 4.4-fold increase in DHAR and normal level of MDHAR activity in MT ensured better recycling of ASC. Arrigoni (1994) suggested that DHAR activity was induced when cellular ASC content significantly decreased. Clearly, ASC-deficiency and NaCl-treatment failed to induce this enzyme in *asfL-1* mutant, while the NaCl stress did it in MT plants. This indicated constitutive low activity of ASC-recycling enzymes in the present mutant.

The 4 to 7-fold increase in GSH and 2-fold increase in GSSG content in both MuC and MuT are worth mentioning. Ascorbate deficiency was 5 - 7 times higher in roots than in leaves of the mutant plants, but roots contained higher GSH content along with elevated activity of GR, CAT, POX and GPX than leaves. Increased GR activity reflected steady supply of GSSG, and the efficient recycling of GSSG to GSH, even at NaCl treatment, permitted MuT to maintain higher GSH:GSSG ratio than MC plants. GSSG content in the mutant was increased despite severe depletion of DHAR activity. By contrast, both GSH and GSSG pools in the MT plants were reduced substantially, although GR activity was normal and DHAR activity was increased by 2.5 to 4.4-fold. Together, these results suggested that GSH was continuously consumed by the processes other than ascorbate-glutathione cycle. One possible route is the GSH-dependent GPX activity which functions against H_2O_2 and organic peroxides in the plants (Foyer and Noctor 2011). However, APX is the principal H_2O_2 scavenging enzyme in the plants (Asada 1992), and increased activity of this enzyme is often related to tolerance to salt stress (Lin and Pu 2010, Foyer and Noctor 2011). On the other hand, significant increase in SOD activity and H_2O_2 accumulation were observed in diverse plant types under NaCl treatment (Mallik *et al*.

2011). Despite low APX activity and high SOD activity, H_2O_2 content had not increased in MuT plants. MDA content was also low. Therefore, it seems reasonable to say that H_2O_2 has been scavenged successfully in the mutant due to high GPX, CAT and POX activities. H_2O_2 has the ability to regulate the expression of *GPx* genes and to induce both CAT (Miao *et al*. 2006) and POX activities (De Pinto and De Gara 2004). The over-expression of CAT, GPX and POX in the present MuC plants and their unchanged activity under NaCl treatment suggested constitutive up-regulation of these three enzymes in the *asfL-1* mutant. The elevation of these enzyme activities might also help MuT to maintain low lipid peroxidation. Increased activities of SOD, CAT and POX are generally considered as an adaptive trait against NaCl-induced oxidative stress in plants, bringing about a tight regulation in H_2O_2 content (Agarwal and Pandey 2004, Niknam *et al*. 2006). This system, however, failed to act properly in MT, resulting in higher accumulation of H_2O_2 and MDA than in MuT plants. Presence of a strong compensatory antioxidant enzymatic defense in the removal of H_2O_2 was also reported in CAT-deficient cowpea cultivars under NaCl stress (Maia *et al*. 2010) and in *Arabidopsis* double mutants (Miller *et al*. 2007). Interestingly, no GPX activity could be detected by *tert*-butyl hydroperoxide assay, indicating absence of selenium-containing GPX in the present material.

For the first time, an ascorbate-deficient mutant has been isolated and characterized in grass pea. Data showed significant alterations in the antioxidant metabolism due to ascorbate deficiency in the *asfL-1* plants. Maintenance of normal growth and biomass production under NaCl treatment indicated tolerance of the mutant to salt-induced oxidative stress. Results suggested major alterations in antioxidant defense of the mutant and significant role of the glutathione to maintain normal growth even at high NaCl concentration.

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