

**Characterisation of PpMDHARs and
PpENA1 from the moss, *Physcomitrella patens***

by

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ABSTRACT

Identifying a genetic basis for the tolerance to salinity exhibited by the resilient moss, *Physcomitrella patens*, could provide valuable information for use in the selection or modification of salinity tolerance in crop plants. The overall aim of the work described in this thesis was to identify, express and functionally characterise the protein products of two putative salinity tolerance genes from *Physcomitrella*, namely *PpMdhar* and *PpENA1*. The characterisation of *PpMdhar* and *PpENA1* represents a two-pronged approach into investigating the salinity tolerance of *Physcomitrella* at the biochemical and transport level, respectively. The enzymes encoded by *PpMdhar*s, monodehydroascorbate reductases (MDHARs), are central to the ascorbate-glutathione cycle, and recycle monodehydroascorbate molecules into the antioxidant, ascorbate. Hence, MDHARs play a part in maintaining the capacity of plant cells to remove toxic reactive oxygen species. Given that the production of reactive oxygen species is greatly increased in plants under salt stress, and that *Physcomitrella* is tolerant of high salt, MDHAR enzymes were expressed to determine whether they exhibit increased enzymic activity when compared with MDHARs from higher plants. The protein encoded by *PpENA1* is Na⁺ transporting ATPase, which actively transports toxic Na⁺ ions across the cell membranes, and thereby minimizes the level of Na⁺ that accumulates in the cytoplasm. Thus, in contrast to the mechanism by which MDHARs may help *Physcomitrella* deal with the secondary effects of high salt, the PpENA1 protein could enable the moss to actively exclude Na⁺ ions, and thereby avoid cellular toxicity.

A link between salinity and the transcription of *PpMdhar* and *PpENA1* is reported here, and the function of each gene is investigated. A comprehensive characterisation of the enzymic action of expressed PpMDHAR enzymes is described, demonstrating that the biochemical mechanisms used by *Physcomitrella* in dealing with salt-induced reactive oxygen species are likely to be conserved with vascular plants. The physiological effects of the expression of PpENA1 are investigated *via* complementation experiments in yeast, and the membrane location of the protein is determined. The Na⁺ binding-sites of PpENA1 are predicted using homology modelling and amino acid residues crucial for Na⁺ transport are tested experimentally *via* site-directed mutagenesis. Finally, the introduction of a new, functional Na⁺ binding-site into an inactivated form of the PpENA1 protein demonstrates that a degree of control is possible over the Na⁺ binding-sites in PpENA1.

STATEMENT OF AUTHORSHIP

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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- Drew, D. P., Lunde, C., Lahnstein, J. and Fincher, G. B. (2007). Heterologous expression of cDNAs encoding monodehydroascorbate reductases from the moss, *Physcomitrella patens* and characterization of the expressed enzymes. *Planta*, **225**, 945-954.
- Lunde, C., Baumann, U., Shirley, N. J., Drew, D. P. and Fincher, G. B. (2006). Gene Structure and Expression Pattern Analysis of Three Monodehydroascorbate Reductase (Mdhar) Genes in *Physcomitrella patens*: Implications for the Evolution of the MDHAR Family in Plants. *Plant Mol. Biol.*, **60**, 259-275.
- Lunde, C., Drew, D. P., Jacobs, A. K. and Fincher, G. B. (2007). Exclusion of Na⁺ via the sodium ATPase (PpENA1) ensures normal growth of *Physcomitrella patens* under moderate salt stress. *Plant Physiol.*, **144**, 1786-1796.

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ABBREVIATIONS

A	Absorbance	MDHA	Monodehydroascorbate reductase
ABA	Abscisic acid	MDHAR	MDHA reductase
APX	Ascorbate peroxidase	min	Minutes
ATP	Adenosine triphosphate	mRNA	Messenger RNA
BLAST	Basic local alignment search tool	NADH	Nicotinamide adenine dinucleotide
bp	Base pairs	Ni-NTA	Nickel-nitrilotriacetic acid
CAT	Catalase	OD	Optical density
cDNA	Complementary DNA	PBS	Phosphate buffered saline
CMB	Chloromercuribenzoate	PMSF	Phenylmethylsulphonyl fluoride
d	Days	PCR	Polymerase chain reaction
Da	Daltons	Q-PCR	Quantitative PCR
DHAR	Dehydroascorbate reductase	PEG	Polyethylene glycol
DNA	Deoxyribonucleic acid	RNA	Ribonucleic acid
dNTP	Deoxynucleotide triphosphate	ROS	Reactive oxygen species
DTT	Dithiothreitol	rpm	Revolutions per minute
EDTA	Ethylene diamine tetra-acetic acid	RT	Room temperature (22°C)
ER	Endoplasmic reticulum	SC	Synthetic complete
EST	Expressed sequence tag	SDS	Sodium dodecyl sulphate
FAD	Flavin adenine dinucleotide	SDS-PAGE	SDS-polyacrylamide gel electrophoresis
g	Gram	sec	Seconds
g	Units of centrifugal force	SOD	Superoxide dismutase
GR	Glutathione reductase	TBS	Tris buffered saline
h	Hour	TEA	Triethylamine
HA	Haemagglutinin	TEMED	Tetramethylethylene-diamine
HPLC	High-performance liquid chromatography	Tris	Tris[hydroxymethyl] amino methane
HRP	Horseradish peroxidase	tRNA	Transfer RNA
IMAC	Immobilised metal affinity chromatography	U	Units
IPTG	Isopropylthiogalactoside	UTR	Untranslated region
kb	Kilobase	UV	Ultraviolet
LB	Luria-Bertani	v/v	Volume for volume
M	Molar	w/v	Weight for volume
MCS	Multiple cloning site	YPD	Yeast peptone dextrose