



Table of Contents

Table of contents.....	I
List of Figures.....	VII
List of Tables.....	XI
Abbreviations.....	XIII
1. Introduction.....	1
1.1 ROS production in different organelles	2
1.2 Oxidative damage	4
1.2.1 Protein oxidative damage.....	4
1.2.2 Lipid peroxidation.....	4
1.2.3 DNA oxidative damage.....	6
1.3 ROS scavenging.....	7
1.3.1 The non-enzymatic antioxidants	7
1.3.2 The enzymatic antioxidants	9
1.4 RES scavenging	11
1.4.1 Flavin-dependent oxidoreductases	11
1.4.2 Yeast Old Yellow Enzymes (OYEs).....	12
1.4.3 Old Yellow Enzyme homologues from bacteria.....	13
1.4.4 12-Oxophytodienoic acid reductases (OPRs)	15
1.4.4.1 OPR substrate specificity.....	16
1.4.4.2 OPR subgroup II: <i>A. thaliana</i> OPR3.....	19
1.4.4.3 <i>A. thaliana</i> OPR subgroup I.....	20
1.5 Aim of the present study	20
2. Materials and methods	23
2.1 Materials.....	23



Table of Contents

2.1.1	Chemicals and research products	23
2.1.2	Equipment	23
2.1.3	Biological materials	25
2.1.3.1	Plants	25
2.1.3.1.1	<i>Arabidopsis thaliana</i>	25
2.1.3.1.2	<i>Nicotiana benthamiana</i>	26
2.1.3.2	Bacterial strains	26
2.1.3.2.1	<i>Agrobacterium tumefaciens</i>	26
2.1.3.2.2	<i>Escherichia coli</i>	26
2.1.3.3	Yeast	27
2.1.4	Culture media and supplements	28
2.1.4.1	Bacterial culture media	28
2.1.4.2	Yeast culture media	29
2.1.4.3	Plant culture media	29
2.1.5	Antibiotics and supplements	30
2.1.6	Oligonucleotides	30
2.1.7	Vectors	33
2.1.8	Antibodies	34
2.2	Methods	34
2.2.1	<i>Nicotiana benthamiana</i> growth conditions	34
2.2.1.1	Agro-infiltration in <i>N. benthamiana</i> leaves	34
2.2.2	<i>Arabidopsis</i> growth and cultivation methods	35
2.2.2.1	Soil culture	35
2.2.2.2	Media culture	36
2.2.3	Stable transformation of <i>Arabidopsis</i>	36



Table of Contents

2.2.4	Preparation of mesophyll protoplasts.....	37
2.2.5	Transient transformation with a particle gun	37
2.2.6	Fluorescence microscopy.....	38
2.2.7	Preparation of <i>E. coli</i> competent cells	38
2.2.8	Preparation of <i>A. tumefaciens</i> competent cells	39
2.2.9	Preparation of <i>Pichia pastoris</i> electrocompetent cells.....	39
2.2.10	Transformation of DNA into bacterial host cells.....	39
2.2.11	Chemical transformation of <i>E. coli</i> Rosetta-gamiB strain	40
2.2.12	Transformation of <i>Pichia pastoris</i>	40
2.2.13	Nucleic acid methods	40
2.2.13.1	Isolation of plasmid DNA.....	40
2.2.13.2	Isolation of genomic DNA from <i>Arabidopsis</i>	41
2.2.13.3	Isolation of total RNA from <i>Arabidopsis</i>	42
2.2.13.4	Polymerase chain reaction (PCR) techniques	42
2.2.13.4.1	Standard PCR method	42
2.2.13.4.2	Reverse Transcription PCR (RT-PCR)	44
2.2.13.4.3	Quantitative RT-PCR (qRT-PCR)	44
2.2.13.5	PCR product purification	45
2.2.13.6	DNA Gel electrophoresis.....	45
2.2.13.7	Isolation of DNA fragments from agarose gels	46
2.2.13.8	Cloning methods	46
2.2.13.8.1	Topo cloning	46
2.2.13.8.2	Restriction digest and analysis	46
2.2.13.8.3	Dephosphorylation of vector ends	46
2.2.13.8.4	Ligation	47



Table of Contents

2.2.13.8.5 Site-directed mutagenesis.....	47
2.2.14 Protein expression and purification.....	49
2.2.14.1 Heterologous expression of <i>AtOPR5</i>	49
2.2.14.2 Preparation of total cell protein for SDS-PAGE.....	49
2.2.14.3 Protein solubility check.....	49
2.2.14.4 Preparation of cleared cell lysate	50
2.2.14.5 Protein extraction from yeast (<i>P. pastoris</i>).....	50
2.2.14.6 Protein purification by affinity chromatography	51
2.2.14.6.1 Ni ²⁺ -NTA affinity chromatography	51
2.2.14.6.2 Glutathione affinity chromatography	51
2.2.14.7 Buffer exchange and desalting of protein solutions.....	51
2.2.14.7.1 Dialysis.....	51
2.2.14.7.2 Ultrafiltration.....	52
2.2.14.8 Size exclusion chromatography	52
2.2.14.9 Denaturation and refolding of proteins	52
2.2.15 Enzyme activity assay.....	52
2.2.16 Purification of inclusion bodies for production of antibodies.....	53
2.2.17 Purification of polyclonal antibodies	53
2.2.18 Protein analysis	54
2.2.18.1 Extraction of plant cellular protein	54
2.2.18.2 Determination of protein concentration	54
2.2.18.3 Preparation of SDS-Polyacrylamide gels.....	55
2.2.18.4 Electrophoresis of protein	55
2.2.18.5 Coomassie staining of proteins separated by SDS-PAGE.....	56
2.2.18.6 Western Blot analysis	56



Table of Contents

2.2.18.7	Immunological protein detection	57
2.2.18.8	Ponceau stain	57
2.2.19	Biological assays.....	58
2.2.19.1	High-light treatment.....	58
2.2.19.2	The variable-to-maximal chlorophyll fluorescence ratio.....	58
2.2.19.3	Methyl Vinyl Keton (MVK) Treatment.....	59
2.2.19.4	Trypan Blue staining for cell death.....	59
2.2.19.5	GUS staining of <i>Arabidopsis</i>	59
2.2.19.6	Bacterial growth curves	60
2.2.20	Statistical analysis	60
3.	Results.....	61
3.1	Molecular characterization of <i>AtOPR5/6</i>	61
3.1.1	Isolation of transformants showing <i>OPR5/6</i> silencing	61
3.2	Phenotypic characterization of transgenic plants.....	66
3.2.1	High-light stress induces photoinhibition in <i>OPR5/6</i> silenced plants.....	66
3.2.2	The high-light stress response correlates with expression levels of <i>OPR5/6</i>	68
3.2.3	Photoinhibition in <i>OPR1-5/6</i> transgenic plants	70
3.2.4	Plant cell death in response to methyl vinyl ketone (MVK).....	73
3.3	Cloning of <i>AtOPR5/6</i> cDNAs	75
3.4	Subcellular localization of <i>AtOPR5/6</i>	78
3.4.1	Expression of <i>AtOPR5/6</i> -GFP fusion protein in <i>Nicotiana benthamiana</i> by agro-infiltration	82
3.4.2	Expression of <i>AtOPR5/6</i> -GFP fusion protein in <i>Arabidopsis</i>	84
3.5	<i>AtOPR5/6</i> promoter activity.....	86
3.6	Biochemical characterization of recombinant <i>AtOPR5/6</i>	88



Table of Contents

3.6.1	Expression of <i>AtOPR5/6</i> recombinant protein in <i>E.coli</i>	88
3.6.2	Extraction and purification of <i>AtOPR5/6</i> recombinant proteins	89
3.7	Overcoming protein aggregation	92
3.7.1	Expression of <i>AtOPR5/6</i> recombinant protein in yeast	94
3.7.2	Improvement of <i>AtOPR5/6</i> solubility in <i>E. coli</i> by co-expression with molecular chaperones.....	97
3.7.3	Enhancement of <i>AtOPR5/6</i> protein solubility through the use of GST as fusion tag.....	100
3.7.4	Refolding of GST-tagged <i>AtOPR5/6</i> recombinant protein.....	103
3.8	Recombinant <i>AtOPR5/6</i> mediate resistance to paraquat.....	105
3.9	Enzyme activity assay.....	108
4.	Discussion	109
4.1	The phenotype of <i>opr1</i> , 2, and 4 mutants and <i>AtOPR5/6</i> silenced plants....	110
4.2	<i>Arabidopsis OPR</i> genes are stress inducible.....	116
4.3	<i>Arabidopsis OPRs</i> are probably RES detoxifying enzymes	117
4.4	Susceptibility to exogenous MVK treatments	118
4.5	<i>AtOPR5</i>	120
4.6	<i>AtOPR5</i> subcellular localization	124
5.	Summary	127
6.	Zusammenfassung.....	129
7.	References	131
8.	Acknowledgements.....	157
9.	<i>Curriculum Vitae</i>	159