

Contents

Contents

Contents.....	I
Figures.....	V
Tables.....	VII
Abbreviations.....	VIII
Summary.....	XIII
Zusammenfassung	XV
1. Introduction	1
1.1. Jasmonic acid and its role in growth and defense	1
1.2. Biosynthesis and perception of jasmonates	2
1.3. Regulation of OPR3 by dimerization.....	7
1.4. Post-translational modification as key regulator of JA-Biosynthesis?... 11	
1.5. Non-OPDA reducing OPRs.....	14
1.6. Objectives of this work.....	16
2. Material and Methods	19
2.1. Chemicals, enzymes and kits.....	19
2.2. Antibodies.....	19
2.3. Oligonucleotides	19
2.4. Plasmids.....	22
2.5. Constructs.....	23
2.5.1. AtOPR3-constructs for complementation of <i>Arabidopsis thaliana</i> . 23	
2.5.2. Constructs in pET21a for heterologous expression of proteins in <i>E. coli</i>	23
2.5.3. Constructs for proximity-dependent biotin identification 2 (BioID2)	24
2.6. Organisms.....	25
2.6.1. Bacteria.....	25
2.6.2. Plants.....	25
2.7. Plant methods.....	26
2.7.1. Growth of <i>Arabidopsis thaliana</i> on soil.....	26
2.7.2. Growth of <i>Arabidopsis thaliana</i> in hydroponic culture	26
2.7.3. Growth of <i>Arabidopsis thaliana</i> under sterile conditions.....	26

Contents

2.7.4.	Growth of <i>Nicotiana benthamiana</i>	26
2.7.5.	Stable transformation of <i>Arabidopsis thaliana</i>	26
2.7.6.	Transient expression of proteins in <i>Nicotiana benthamiana</i>	27
2.8.	Bacterial methods	28
2.8.1.	Heterologous protein expression in <i>E. coli</i>	28
2.8.2.	Cell lysis and Ni ²⁺ -NTA purification of heterologously expressed 6xHis-tagged proteins	28
2.9.	Biochemical Methods	29
2.9.1.	Ion exchange chromatography	29
2.9.2.	Monitoring protein quality via Tycho NT.6.....	29
2.9.3.	Enzyme activity assay	30
2.9.4.	Extraction of reaction products	30
2.9.5.	Sulfotyrosine synthesis	31
2.9.6.	Affinity purification by GFP [®] -Trap.....	32
2.9.7.	Affinity purification by streptavidin sepharose	32
2.10.	Sample preparation for Mass Spectrometry	33
2.10.1.	On-beads proteolytic digestion.....	33
2.10.2.	In-gel proteolytic digestion	34
2.10.3.	On-beads chemical cleavage.....	34
2.10.4.	Desalting of mass spectrometry samples by stop-and-go-extraction tips	34
2.10.5.	Electrospray-Ionisation Mass Spectrometry (ESI-MS)	35
2.10.6.	Analysis and statistics of mass spectrometric data	36
2.10.7.	QTRAP-Mass spectrometry (metabolome-analysis).....	37
2.10.8.	Micro scale thermophoresis	38
2.10.9.	Analytical ultracentrifugation (AUC)	38
2.11.	Basic biochemical techniques	39
2.11.1.	Protein isolation from leaves	39
2.11.2.	Determination of protein concentration by Bradford assay.....	39
2.11.3.	Determination of flavoprotein concentration by measuring FMN cofactor	40

Contents

2.11.4. Separation of Proteins by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)	40
2.11.5. Coomassie Stain.....	40
2.11.6. Western Blot.....	41
2.11.7. Ponceau Stain	41
2.11.8. Isolation of plasmid DNA	42
2.11.9. Isolation of genomic DNA from plant material.....	42
2.11.10. Polymerase chain reaction (PCR)	43
2.11.11. Agarose gel electrophoresis.....	43
2.11.12. Elution of DNA from Agarose	43
2.11.13. Topo-cloning.....	44
2.11.14. Restriction digest and dephosphorylation	44
2.11.15. Ligation	44
2.11.16. Transformation of bacteria	45
2.11.17. DNA sequencing.....	45
3. Results	47
3.1. Characterization of tyrosine-sulfated OPR3	48
3.1.1. Preparation of tyrosine-sulfated OPR3	48
3.1.2. OPR3 and its mutants form dimers during Micro Scale Thermophoresis.....	49
3.1.3. Thermal unfolding profile of AtOPR3 and its mutants.....	51
3.1.4. OPR3 forms a dimer during analytical ultracentrifugation	53
3.1.5. Enzymatic activity of OPR3 and its mutants	58
3.1.6. Conversion of racemic OPDA by OPR3 and Y365SY	64
3.1.7. Summary of biochemical characterization	68
3.2. Is OPR3 posttranslationally modified <i>in vivo</i> ?	70
3.2.1. Expressing OPR3-YFP under its native promotor.....	71
3.2.2. Is tyrosine 365 modified <i>in vivo</i> in response to wounding?.....	72
3.2.3. Is tyrosine 365 modified <i>in vivo</i> in specialized tissues?	81
3.2.4. Post-translational modifications of other amino acids.....	86
3.2.5. Chemical cleavage with formic acid	88

Contents

3.2.6. Summary of mass spectrometric analysis	92
3.3. Identification of interacting proteins by <i>in vivo</i> proximity labeling	94
3.3.1. Selection of candidate proteins and expression.....	95
3.3.2. Biotin supplementation and expression levels in <i>A. thaliana</i>	98
3.3.3. BioID2 screen.....	100
3.3.4. Verification of feasibility	101
3.3.5. Results for OPR3	103
3.3.6. BioID2-results for cytosolic OPR1, OPR2 and OPR4.....	116
3.3.6.1. Proximal proteins of OPR4-BioID2	117
3.3.6.2. Proteins in proximity of OPR2-BioID2	125
3.3.6.3. Proteins in proximity of OPR1-BioID2	131
3.3.7. Summary for BioID2 screen	138
4. Discussion	141
4.1. Does sulfation at position Y365 promote dimerization of OPR3?.....	142
4.2. Is OPR3 posttranslationally modified <i>in vivo</i> ?	145
4.3. On which level is JA-Biosynthesis regulated?	149
4.4. Proximity labeling of OPR interactors by BioID2	151
4.4.1. Objective of BioID2	151
4.4.2. BioID2-candidates for OPR3-interaction	152
4.4.3. Comparison of BioID2- and BIFC-Screen	155
4.4.4. Interacting proteins of other OPRs	159
4.4.4.1. OPR4.....	159
4.4.4.2. OPR1 and OPR2	161
4.4.5. Prospect and limitation of BioID2	164
5. References	169
6. Appendices	195
6.1. Comparison of AtOPR3 and S/OPR3	195
6.2. Dimerization potential of S/OPR3 in different buffers	196
6.3. Monomer-/Dimer percentage calculated from peak area analyzed with Analytical Ultracentrifugation	197
6.4. Catalytic properties obtained with enzyme assays.....	199
6.5. Comparison of catalytic efficiency	199

Contents

6.6. Mass spectrometric analyses of OPDA and OPC-8:0	201
6.7. MS/MS of OPDA and converted OPDA.....	202
6.8. Hypothetical double digest with GluC and AspN of OPR3-YFP	203
6.9. Overlay of <i>S</i> /OPR3 and <i>At</i> OPR3 crystals.....	204
6.10. Fragmentation spectra of standard peptides.	205
6.11. Percentage of localization in BioID2-screen	206
6.12. Perseus/Top30/Unique precipitated proteins.....	206
Danksagung.....	237
Eidesstattliche Erklärung.....	239
Lebenslauf.....	241