



Pauline Moussard (Autor)

# Induced mutagenesis and genetic engineering for the generation and selection of herbicide-resistant Alfalfa

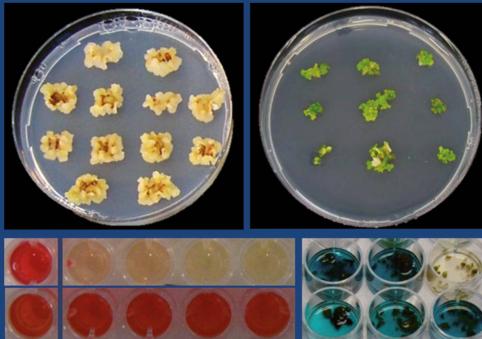
UNIVERSITÄT HOHENHEIM  
SCHRIFTENREIHE ZUR PHYSIOLOGIE UND  
BIOTECHNOLOGIE DER PFLANZEN



Pauline Moussard

Induced mutagenesis and genetic engineering for the  
generation and selection of herbicide-resistant Alfalfa

A. Schaller (Herausgeber) - Band 6



Cuvillier Verlag Göttingen  
Internationaler wissenschaftlicher Fachverlag

<https://cuvillier.de/de/shop/publications/6573>

Copyright:

Cuvillier Verlag, Inhaberin Annette Jentzsch-Cuvillier, Nonnenstieg 8, 37075 Göttingen,  
Germany

Telefon: +49 (0)551 54724-0, E-Mail: [info@cuvillier.de](mailto:info@cuvillier.de), Website: <https://cuvillier.de>



## TABLE OF CONTENTS

<b>TABLE OF CONTENTS</b>	<b>i</b>
<b>ABBREVIATIONS</b>	<b>IV</b>
<b>ZUSAMMENFASSUNG</b>	<b>VI</b>
<b>ABSTRACT</b>	<b>VIII</b>
<b>1 INTRODUCTION AND OBJECTIVES</b>	<b>1</b>
1.1 CHEMICAL WEED CONTROL AND HERBICIDE-RESISTANCE	1
1.2 DEVELOPING A NEW WAY FOR ALFALFA WEED MANAGEMENT	4
1.2.1 Alfalfa importance	4
1.2.2 Alfalfa culture and weed management	6
1.3 INHIBITION OF TARGET ENZYMES BY HERBICIDES AND MECHANISMS OF RESISTANCE	8
1.3.1 Glutamine synthetase	8
1.3.1.1 Enzyme role and structure	8
1.3.1.2 Inhibition of glutamine synthetase	9
1.3.1.3 Resistance to glutamine synthetase inhibitors	10
1.3.2 4-Hydroxyphenylpyruvate-dioxygenase	11
1.3.2.1 Enzyme role and structure	11
1.3.2.2 Inhibition of 4-hydroxyphenylpyruvate-dioxygenase	11
1.3.2.3 Resistance to HPPD-inhibitor herbicides	12
1.3.3 Acetolactate synthase	13
1.3.3.1 Enzyme role and structure	13
1.3.3.2 Inhibition of the acetolactate synthase	15
1.3.3.3 Resistance to acetolactate synthase inhibitor herbicides	16
1.4 CURRENT STATUS OF HERBICIDE RESISTANCE IN <i>MEDICAGO</i> CROPS	17
1.5 OBJECTIVES	18
<b>2 MATERIAL AND METHODS</b>	<b>21</b>
2.1 CHEMICALS	21
2.1.1 Herbicides	21
2.1.2 Antibiotics	21
2.2 PREPARATION AND ISOLATION OF NUCLEIC ACIDS	22
2.2.1 Sequences and accession numbers	22
2.2.2 DNA isolation and quantification	22
2.2.3 Primers	23
2.2.4 PCR	23
2.2.5 Sequencing	25
2.3 CLONING STEPS	25
2.3.1 Restriction digestion	25
2.3.2 Fill-in of 5' overhang of insert	26
2.3.3 Gel elution	26
2.3.4 Ligation	26
2.3.5 Vectors	27

## Table of contents

---

2.4	CULTURE, COMPETENCE AND TRANSFORMATION OF BACTERIA	27
2.4.1	<i>E.coli</i>	27
2.4.2	Vector purification from <i>E.coli</i>	28
2.4.3	<i>A.tumefaciens</i>	29
2.5	CONSTRUCTS AND SELECTION	31
2.5.1	Constructs generated in the thesis	31
2.5.2	Determination of minimal inhibiting concentration for selection	32
2.6	ALFALFA CULTURE AND EXPERIMENTS	32
2.6.1	Alfalfa cultivars	32
2.6.2	Glasshouse culture	33
2.6.3	<i>In vitro</i> culture	34
2.6.3.1	Conditions and material	34
2.6.3.2	<i>In vitro</i> culture media	34
2.6.3.3	Sterilization and germination	35
2.6.3.4	Somatic embryogenesis and regeneration into plantlets	37
2.6.3.5	Adaptation to glasshouse conditions	37
2.6.4	Spontaneous or induced mutations	37
2.6.5	Transformation	38
2.6.5.1	Preparation of the Agrobacteria	38
2.6.5.2	Preparation of the explants	38
2.6.5.3	Transformation method	39
2.6.5.4	Selection	39
2.7	TOBACCO CULTURE AND EXPERIMENTS	40
2.7.1	Glasshouse culture	40
2.7.2	<i>In vitro</i> culture	40
2.7.2.1	<i>In vitro</i> culture media	40
2.7.2.2	Sterilization and germination	41
2.7.2.3	Propagation	41
2.7.2.4	Organogenesis	41
2.7.2.5	Adaptation to glasshouse conditions	41
2.7.3	Mutagenesis	42
2.7.4	Transformation	42
2.7.4.1	Preparation of the Agrobacteria	42
2.7.4.2	Preparation of the explant	42
2.7.4.3	Transformation method	42
2.7.4.4	Selection	43
2.8	ANALYSIS OF THE TRANSFORMANTS	43
2.8.1	PCR	43
2.8.2	GUS-staining	44
2.8.3	Multiplication of transformed plants	44
2.9	PROTEIN CHARACTERIZATION	45
2.9.1	Enzyme assay for ALS expressed in <i>E.coli</i>	45
2.9.2	Enzyme assay for ALS from plant tissue	46
<b>3</b>	<b>RESULTS</b>	<b>47</b>
3.1	<i>IN VITRO</i> CULTURE AND REGENERATION OF ALFALFA	47
3.2	MUTAGENESIS APPROACH TOWARDS HERBICIDE-RESISTANT ALFALFA	49
3.2.1	<i>In vitro</i> minimal inhibiting concentration	49
3.2.2	Alfalfa ALS genes	50
3.2.2.1	Isolation of the genes by PCR	50
3.2.2.2	Comparison with known ALS genes	51
3.2.3	Choice of the mutagenesis conditions	56



## Table of contents

---

3.2.4	Selection of mutant material	57
3.3	GENERATION OF HERBICIDE-RESISTANT ALFALFA BY TRANSGENESIS	58
3.3.1	Test of mutant AtALS genes used for transformation	58
3.3.1.1	<i>E. coli</i> enzyme test	58
3.3.1.2	Tobacco transformation	60
3.3.2	Validation of the alfalfa transformation method by a reporter assay	62
3.3.3	Production of sulfonylurea resistant somatic embryos in alfalfa	64
3.3.4	Resistance to other herbicide classes	67
3.3.4.1	Resistance to glufosinate-ammonium	67
3.3.4.2	Resistance to tembotrione	68
3.4	CONFIRMATION OF THE RESISTANCE	69
3.4.1	Regeneration of new resistant tissues from first selected tissues	69
3.4.2	Sequencing of putative mutant samples	71
3.4.3	Confirmation of the transgenes by PCR	72
3.4.3.1	PCR analysis of pHoe6/Ac transformants	73
3.4.3.2	PCR analysis of AtHPPD transformants	74
3.4.3.3	PCR analysis of pTKVA227:AtALS-W574L transformants	74
3.4.3.4	Marker gene expression in pTKVA227:AtALS-W574L transformants for sulfonylurea resistance	75
3.4.3.5	ALS activity in pTKVA227:AtALS-W574L transformants for sulfonylurea resistance	77
<b>4</b>	<b>DISCUSSION</b>	<b>79</b>
4.1	CHOICE OF MUTAGEN AND MUTAGENESIS CONDITIONS	79
4.2	VALIDATION OF THE MUTAGENESIS PROCESS USING TOBACCO	80
4.3	COMPLICATIONS IN ALFALFA MUTANT SELECTION	81
4.3.1	Regeneration and mutagenesis methods	81
4.3.2	Number of ALS genes, alfalfa polyploidy and mutation frequency	82
4.4	ADVANTAGE OF THE ALFALFA <i>IN VITRO</i> CULTURE AS A GOOD BASIS FOR ALFALFA TRANSFORMATION	85
4.5	INFLUENCE OF THE TRANSFORMATION METHOD, CONSTRUCTS AND SELECTIONS USED ON TRANSFORMATION EFFICIENCY	86
4.6	PROOF OF THE RESISTANCE STATUS	90
4.7	OPPORTUNITY FOR GENE STACKING	91
4.8	TRANSGENIC ALFALFA LINES FOR WEED CONTROL AND FUTURE INTEGRATED WEED MANAGEMENT	92
<b>CONCLUSIONS</b>		<b>97</b>
<b>REFERENCES</b>		<b>101</b>
<b>APPENDICES</b>		<b>IX</b>
<b>AKNOWLEDGMENTS</b>		<b>XX</b>