

HINOKININ BIOSYNTHESIS
IN
Linum strictum ssp. corymbulosum

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Ürün Bayındır
from İstanbul

Referee: Prof. Dr. A. Wilhelm Alfermann
Co-referee: Prof. Dr. Rolf Wagner

Berichte aus der Biologie

Ürün Bayindir

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IN
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For the memory of my Father...

Sevgili Babamın anısına...

CONTENTS

Contents	I
Summary	V
Zusammenfassung	VI
List of abbreviations	VII
1. Introduction	1
1.1 Plant secondary metabolism	1
1.2 Lignans and their biosynthesis	1
1.3 Hinokinin and its hypothetical biosynthetic pathways	5
1.3.1 PLR and PLR-like reductases	8
1.3.2 Piperitol-sesamin synthase	9
1.3.3 CytP450 monooxygenases involved in the formation of methylenedioxy bridges	10
1.4 RNA interference (RNAi)	12
1.5 Scope of the thesis	14
2. Material	15
2.1 Oligonucleotides	15
2.2 Vectors	16
2.3 Enzymes and Kits	16
2.4 Genotypes of bacterial strains	16
2.5 Antibiotics	17
2.6 Plant material and <i>in vitro</i> cultivation	17
2.6.1 Cell suspension cultures	17
2.6.2 Shoot cultures	17
2.6.3 Hairy root cultures	17
2.7 Media, buffers and solutions	18
2.8 Chemicals and Materials	19
2.9 Instruments	21
3. Methods	23
3.1 Characterization of suspension cultures of <i>L. corymbulosum</i>	23
3.1.1 Determination of fresh and dry weight	23

3.1.2 Determination of the medium parameters	23
3.1.3 Lignan extraction	23
3.1.4 Lignan analysis with High Performance Liquid Chromatography (HPLC)	23
3.1.5 Elicitation and feeding with matairesinol of the cell cultures	23
3.2 Enzyme preparations from cell cultures of <i>L. corymbulosum</i>	25
3.2.1 PLR and PLR-like (SDR) enzyme preparations	25
3.2.2 SDH enzyme preparations	25
3.2.3 Methylenedioxy bridge-forming enzyme preparations	25
3.2.4 Enzyme assays	25
3.2.5 Determination of protein concentration	26
3.2.6 Calculation of enzyme activity	26
3.3 Molecular biological methods	26
3.3.1 Standard methods	26
3.3.2 RNA isolation	26
3.3.3 RT-PCR	26
3.3.4 PCR conditions and programmes	26
3.3.4.1 Searching for <i>plr</i> and <i>plr</i> -like (<i>sdh</i>) genes	27
3.3.4.2 Searching for <i>sdh</i> and <i>sdh</i> -like (<i>ddh</i>) genes	28
3.3.4.3 Searching for methylenedioxy bridge-forming genes	28
3.3.5 Purification of PCR products	28
3.3.6 Determination of DNA concentration and purity	28
3.3.7 Cloning of PCR products into pGEM-T®	29
3.3.8 Transformation of <i>E. coli</i> (DH5α and BL21(DE3)pLysS)	29
3.3.9 Preparations of plasmid DNA	29
3.3.9.1 Preparation of clean plasmid DNA	29
3.3.9.2 Plasmid preparation with the TENS method	29
3.4 Construction of a cDNA library from <i>L. corymbulosum</i> cell suspension cultures	29
3.4.1 Total RNA isolation	29
3.4.2 Construction of the library and phage packaging	29
3.4.3 Library amplification	30
3.4.4 Transferring of phage DNA to nylon membranes	30
3.4.5 Preparation of probes	30
3.4.5.1 <i>PLR</i> from <i>L. album</i>	30
3.4.5.2 “ <i>G</i> ” from <i>L. corymbulosum</i>	30
3.4.5.3 <i>SDH</i> from <i>Forsythia intermedia</i> and <i>L. album</i>	30
3.4.5.4 <i>CYP81Q1</i> from <i>Sesamum indicum</i>	31
3.4.6 Labeling of the probes	31
3.4.7 Prehybridization, hybridization and detection of positive clones	31
3.4.8 <i>In vivo</i> excision	32
3.4.9 Sequence analyses	32

3.5 Heterologous expression of proteins in <i>E. coli</i> BL21(DE3)pLysS	32
3.5.1 Enzyme assays	32
3.5.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)	33
3.5.3 Determination of the stereospecificity of PLR by chiral HPLC	33
3.6 Construction of <i>ihpRNAi</i> in pHANNIBAL	34
3.6.1 Preparation of competent <i>A. rhizogenes</i> TR105	35
3.6.2 Transformation of competent <i>A. rhizogenes</i> TR105	35
3.6.3 Plasmid preparation from agrobacteria	35
3.6.4 Transformation of <i>L. corymbulosum</i> shoots	35
3.6.5 Extraction of lignans from hairy roots	35
3.6.6 Preparation of protein extracts from hairy roots	35
3.6.7 Molecular analysis of hairy root lines	36
3.6.7.1 PCR analyses	36
3.6.7.2 RNA isolation	36
3.6.7.3 Semiquantitative RT-PCR	37
4. Results	39
4.1 Characterization of two cell lines from <i>L. corymbulosum</i>	39
4.1.1 Growth characteristics	39
4.1.2 Medium parameters	40
4.1.3 Hinokinin content	41
4.1.4 Enhancement of hinokinin accumulation in the cell cultures of <i>L. corymbulosum</i>	42
4.2 Search for enzyme activities in cell cultures of <i>L. corymbulosum</i>	42
4.2.1 PLR and PLR-like (SDR) activity	42
4.2.2 SDH activity	43
4.2.3 Methylene dioxy bridge-forming enzymes	43
4.3 Searching for genes with a PCR-guided strategy	43
4.3.1 <i>Plr</i> and <i>plr</i> -like (<i>sdr</i>) genes	44
4.3.2 <i>Sdh</i> and <i>sdh</i> -like (<i>ddh</i>) genes	44
4.3.3 Methylene dioxy bridge-forming genes	44
4.4 Establishment and screening of a cDNA library of <i>L. corymbulosum</i>	45
4.4.1 Screening the library	45
4.4.1.1 Screening with PLR from <i>L. album</i>	45
4.4.1.2 Screening with "G" from <i>L. corymbulosum</i>	47
4.4.2 An overview on the cDNA library screening	52
4.5 Heterologous expression of <i>plr</i> and <i>plr</i> -like genes in <i>E. coli</i>	54
4.5.1 Enantiospecificity of PLR-Lc	55
4.6 Establishment of hairy root cultures of <i>L. corymbulosum</i>	57
4.7 RNA silencing in hairy roots of <i>L. corymbulosum</i>	57

4.7.1 Molecular analysis of hairy roots	58
4.7.2 RNA silencing of <i>plr</i> -Lc	59
4.7.3 RNA silencing of <i>pcber</i> (G4G)-Lc	61
4.7.4 Comparison of the transcript levels of <i>plr</i> -Lc in <i>pcber</i> (G4G)-ihpRNAi and <i>pcber</i> (G4G)-Lc in <i>plr</i> -ihpRNAi constructs	62
5. Discussion	63
5.1 <i>In vitro</i> cultivation of <i>L. corymbulosum</i>	63
5.2 Biochemical search for enzymes in hinokinin biosynthesis	65
5.3 Cloning the cDNAs of hinokinin biosynthesis	69
5.4 Functional analysis of the cDNA clones	72
5.5 RNA silencing of <i>plr</i> -Lc and <i>pcber</i> -Lc in hairy roots of <i>L. corymbulosum</i>	73
6. Conclusion	77
7. Appendix	79
7.1 Vector maps	79
7.2 DNA-Protein Markers	82
8. References	83
Acknowledgements	93

Summary

Lignans are a class of phenolic compounds that are composed of two phenylpropanoid units. The biologically active and potential drug component lignan (-)-hinokinin was found in hairy root, callus and suspension cultures of *L. strictum* ssp. *corymbulosum* beside other plant species. Two different pathways are plausible for hinokinin biosynthesis. In both pathways (+)-pinoresinol is the central intermediate. In the first pathway, (+)-pinoresinol is reduced enantiospecifically via (+)-lariciresinol to (-)-secoisolariciresinol by a pinoresinol-lariciresinol reductase (PLR). (-)-Secoisolariciresinol is oxidized by secoisolariciresinol dehydrogenase (SDH) to give (-)-matairesinol, from which (-)-hinokinin can be synthesized by the formation of two methylenedioxy bridges. In the second possible pathway, (+)-pinoresinol serves as the substrate for the formation of two methylenedioxy bridges by piperitol-sesamin synthase (PSS) to give (+)-sesamin, which is then converted to (-)-dihydrocubebin with a two-step-reduction reaction similar to the reductions catalyzed by PLR. The last step to form (-)-hinokinin is a dehydrogenation reaction like the one catalyzed by the SDH.

In order to clarify which pathway is involved in (-)-hinokinin biosynthesis, cDNAs have been cloned encoding proteins possibly involved in all described reaction steps. One clone with highest similarity to PLRs, and four clones with highest similarities to phenylcoumaran benzylic ether reductases (PCBERs), which are in the same reductase family with PLRs, were isolated. Incubation of their recombinant proteins with pinoresinol or dehydrodiconiferyl alcohol as substrates proved their functions as PLR and PCBER. The PLR-Lc is enantiospecific for the conversion of (+)-pinoresinol to (-)-secoisolariciresinol, which can be further converted to give (-)-hinokinin. Hairy root lines were transformed with two ihpRNAi constructs to suppress *plr* and *pcber* gene expressions, respectively, to prove the involvement of these genes in (-)-hinokinin biosynthesis. The mRNA level of *plr*-Lc and *pcber*-Lc were significantly reduced in the hairy root lines with the *plr*-ihpRNAi and the *pcber*-ihpRNAi construct, respectively. Hinokinin accumulation was reduced to undetectable levels with the *plr*-ihpRNAi construct, but not with the *pcber*-ihpRNAi construct, proving the involvement of PLR-Lc and the first hypothetical pathway via (-)-matairesinol in the biosynthesis of (-)-hinokinin.

Zusammenfassung

Lignane gehören zu einer Klasse phenolischer Verbindungen, die aus zwei Phenylpropan-Einheiten bestehen. Das Lignan (-)-Hinokinin wurde in Hairy Roots, Kallus- und Zellkulturen von *L. corymbulosum* (Mohagheghzadeh et al., 2006) nachgewiesen. Ausgehend von (+)-Pinoresinol sind zwei Wege zur Biosynthese von (-)-Hinokinin denkbar. Der erste Weg beginnt mit der enantiospezifischen Reduktion von (+)-Pinoresinol über (+)-Lariciresinol zu (-)-Secoisolariciresinol durch die Pinoresinol-Lariciresinol Reduktase (PLR). In einem nächsten Schritt wird (-)-Secoisolariciresinol durch die Secoisolariciresinol Dehydrogenase (SDH) zu (-)-Matairesinol oxidiert, aus dem wiederum (-)-Hinokinin durch die Bildung zweier Methylenedioxy-Brücken entstehen kann. Der zweite mögliche Syntheseweg beginnt mit der Umsetzung von (+)-Pinoresinol zu (+)-Sesamin mit Hilfe einer Piperitol-Sesamin Synthase (PSS) durch die Bildung zweier Methylenedioxy-Brücken. (+)-Sesamin kann in einer zweistufigen Reduktion entsprechend der PLR-katalysierten Reaktion zu (-)-Dihydrocubebin umgesetzt werden. Der letzte Schritt zur Bildung von (-)-Hinokinin würde über eine Dehydrogenierung erfolgen, die der SDH-katalysierten Reaktion ähnelt.

Um herauszufinden, welcher der beiden Wege zur Bildung von (-)-Hinokinin relevant ist, sollten cDNAs kloniert werden, die für die möglicherweise relevanten Proteine kodieren. Hierbei wurden vier Klone isoliert, deren vollständige Sequenzen starke Ähnlichkeit zu Phenylcumaranbenzylether Reduktasen (PCBERs) aufweisen. Ein weiterer Klon hat große Ähnlichkeit zu PLRs. Durch Verwendung der Substrate Dehydrodiconiferylalkohol bzw. Pinoresinol konnte gezeigt werden, dass es sich bei den rekombinanten Proteinen tatsächlich um eine PCBER bzw. PLR handelt. Die PLR ist enantiospezifisch für die Umsetzung von (+)-Pinoresinol zu (-)-Secoisolariciresinol, das dann in (-)-Hinokinin überführt werden kann. Mit Hilfe der beiden Konstrukte *pcber-ihpRNAi* und *plr-ihpRNAi* und durch *Agrobacterium rhizogenes* wurden Sprosse von *L. corymbulosum* transformiert, um den Einfluss dieser Gene auf die (-)-Hinokinin-Biosynthese nachzuweisen. Durch Verwendung dieser Konstrukte in Hairy-Root-Klonen konnte gezeigt werden, dass sich die mRNA Level von *plr-Lc* und *pcber-Lc* signifikant reduzieren ließen. Zudem wurde durch Verwendung des *plr-ihpRNAi*-Konstruktes die Konzentration von Hinokinin unter die Nachweisgrenze reduziert, während sie durch Einsatz des *pcber-ihpRNAi*-Konstruktes nicht beeinflusst wurde. Hierdurch konnte gezeigt werden, dass die PLR-Lc in der Biosynthese von (-)-Hinokinin involviert ist und somit der erste Weg mit Matairesinol als Zwischenprodukt der wahrscheinliche Weg ist.

List of abbreviations

amp	Ampicillin
app.	Approximately
bp	Base pairs
cDNA	Complementary DNA
CytP450	Cytochrome P450
DDC	Dehydrodiconiferyl alcohol
DDH	Dihydrocubebin dehydrogenase
DNA	Deoxyribonucleic acid
DW	Dry weight
e.g.	Exempli gratia, for instance
fupH ₂ O	Filtered ultrapure water
FW	Fresh weight
gDNA	Genomic DNA
h	Hours
HAPLO	Haplomyrfofin
HAS	Haplomyrfofin synthase
HINO	Hinokinin
HS	Hinokinin synthase
IAA	Indole-3-acetic acid
IDDC	Isodihydrodehydrodiconiferyl alcohol
kDa	Kilodalton
KPi	Potassium phosphate buffer
LARI	Lariciresinol
<i>L. corymbulosum</i>	<i>Linum strictum</i> ssp. <i>corymbulosum</i>
MATAI	Matairesinol
max.	Maximum
MDOB	Methylenedioxy bridge-forming
min	minutes
MJ	Methyl jasmonate
NAA	Naphtylacetic acid
NADPH	Nicotinamide-adenine-dinucleotide phosphate (reduced)
OD	Optical density
ORF	Open reading frame
p.A.	pro analysi
PCBER	Phenylcoumaran benzylic ether reductase
PCR	Polymerase chain reaction
pfu	Plaque forming units
PINO	Pinoresinol
PIPE	Piperitol
PLR	Pinoresinol-lariciresinol reductase
PLS	Pluviatolide synthase
PLU	Pluviatolide
PSS	Piperitol-sesamin synthase
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription-PCR
supH ₂ O	Sterile ultrapure water
SDH	Secoisolariciresinol dehydrogenase
SDR	Sesamine-dihydrocubebene reductase
SDS	Sodiumdodecylsulphate
sec	Seconds
SECO	Secoisolariciresinol
SESA	Sesamin
Sol.	Solution
spec	Spectinomycin
Ta	Annealing temperature
Tm	Melting temperature
TAE	Tris-Acetate-EDTA buffer
upH ₂ O	Ultrapure water
V	Volt
v/v	volume/volume
w/v	weight/volume