Flavonolignan Biosynthesis in *Silybum marianum* – Potential Regulatory Mechanisms and Candidate Genes

Dissertation zur Erlangung des

Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

dem Fachbereich der Pharmazie

der Philipps-Universität Marburg

vorgelegt von

Lennart Poppe

aus Lüdenscheid/Germany

Marburg/Lahn 2017

Vom Fachbereich Pharmazie der Philipps-Universität Marburg

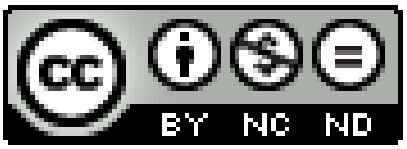
als Dissertation am 04.10.2017 angenommen.

Erstgutachterin: Prof. Dr. Maike Petersen

Zweitgutachter: Prof. Dr. Arnold Grünweller

Tag der mündlichen Prüfung: 04.10.2017

Hochschulkennziffer: 1180



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Acknowledgments

After five years of exciting research and joyful moments I have reached the end of my time preparing for my PhD.

I hereby like to thank my PhD supervisor Prof. Dr. Maike Petersen for her support during this thesis. I will miss the many pleasant conversations about pharmaceutical and biological topics which could broaden my scientific horizon and mostly satisfy my natural curiosity. Her guidance, encouragement and advice throughout this research are greatly appreciated.

I am very grateful to Prof. Dr. Arnold Grünweller for being the co-examiner of my thesis.

Furthermore, I would like to thank the current and former colleagues in the Petersen working group for their support and help, including Marie-Claire Badouin, Elke Bauerbach, Tobias Busch, Agus Chahyadi, Sandra Dietzler, Olga Haag, Simona Riedel, Jennifer Robinson, Thanh Son Ta, Victoria Werner, Stefan Wolters and especially Dr. Soheil Pezeshki for his friendship and inspiring conversations and also my life partner Julia Wohl.

While not directly involved in my projects, I would also like to express my thanks to the employees of the Institute of Pharmaceutical Biology and the former and current colleagues of the Li working group for the good companionship and joyful atmosphere. I will miss all the fun times together.

Special thanks go to my family, especially my parents, who have always supported me and particularly invested so much time and efforts in my education over so many years. I am truly grateful that I could pursue my scientific goals. This would not have been possible without you.

Publications and Presentations

Publication	Poppe, L., Petersen, M. Variation in the flavonolignan composition of fruits from different *Silybum marianum* chemotypes and suspension cultures derived therefrom. Phytochemistry 2016, 131, 68–75.
Scientific lectures	Flavonolignan biosynthesis in *Silybum marianum* – potential regulatory mechanisms and candidate genes. PhD student and postdoc conference of the Deutsche Pharmazeutische Gesellschaft (DPhG), Sanofi-Aventis, Frankfurt, 2017.
	Candidate genes involved in flavonolignan biosynthesis in *Silybum marianum*. Section of Natural Products of the Deutsche Botanische Gesellschaft (DBG), Meisdorf, 2016.
Poster presentations	Candidate genes involved in flavonolignan biosynthesis in *Silybum marianum*. International Plant Science Conference, *Botanikertagung*, Freising, 2015.
	Identification of candidate genes involved in flavonolignan biosynthesis in *Silybum marianum*. International Plant Science Conference, *Botanikertagung*, Tübingen, 2013.

I. Table of contents

II. Abbreviations

Measures and units

aa = amino acid
bp = base pair(s)
kDa = kilo-Dalton
M = molar, mol l^{-1}
OD_{600} = optical density at 600 nm
rpm = revolutions per minute
S = siemens
T_m = melting point in °C
U = units (enzyme activity)

Chemicals

ABTS = 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)
AcOH = acetic acid
APS = ammonium persulphate
BCIP = 5-bromo-4-chloro-3-indolyl phosphate
CA = caffeic acid
Con. alc. = coniferyl alcohol
DTT = dithiothreitol
EDTA = ethylenediaminetetraacetic acid
EGTA = ethyleneglycol-bis(aminoethylether)-N,N,N',N'-tetraacetic acid
EtOH = ethanol
EtOAc = ethyl acetate
FA = formic acid
ISBN = isosilybin
KA = kojic acid
KPi = potassium phosphate buffer
LiAc = lithium acetate
MeJA = methyl jasmonate
MeOH = methanol
NBT = nitro-blue tetrazolium chloride
PABA = *para*-aminobenzoic acid
PEG = polyethylene glycol
PMSF = phenylmethylsulfonyl fluoride
SBN = silybin
SCN = silychristin
SDN = silydianin
SDS = sodium dodecyl sulphate
Tax. = taxifolin
TCA = trichloroacetic acid
TEMED = tetramethylethylenediamine
TRIS = tris(hydroxymethyl)aminomethane

Databases and bioinformatics programs

EBI = European Bioinformatics Institute
EC = Enzyme commission number
GBIF = Global Biodiversity Information Facility
GlycoEP = Prediction of Glycosites in Eukaryotic Glycoproteins
NCBI = National Center for Biotechnology Information
SignalP = Prediction of Signal Peptides

Species names and abbreviations

Ar = *Armoracia rusticana*, horseradish, Brassicaceae

E. coli = *Escherichia coli*, Enterobacteriaceae

Fi = *Forsythia x intermedia*, forsythia, Oleaceae

Lu = *Linum usitatissimum*, flax, Linaceae

Mm = *Micania micrantha*, bitter vine, Asteraceae

P. pastoris = *Pichia pastoris*, Saccharomycetaceae

Rv = *Rhus verniciflua*, Chinese lacquer tree, Anacardiaceae

S. cerevisiae = *Saccharomyces cerevisiae*, Saccharomycetaceae

Sl = *Solanum lycopersicum*, tomato, Solanaceae

Sm = *Silybum marianum*, milk thistle, Asteraceae

Genes and proteins

3H = 4-coumaroylshikimate 3-hydroxylase

4CL = 4-coumarate:CoA ligase

BSA = bovine serum albumine

C4H = cinnamate 4-hydroxylase

CAD = cinnamyl alcohol dehydrogenase

CCoAOMT = caffeoyl-CoA *O*-methyltransferase

CCR = cinnamoyl-CoA reductase

CHI = chalcone isomerase

CHS = chalcone synthase

DIR = dirigent protein

F3H = flavanone 3'-hydroxylase

FHT = flavanone 3β-hydroxylase

HRP = horse radish peroxidase

HST = 4-hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase

LAC = laccase

lacZ = β-galactosidase

PAL = phenylalanine ammonia-lyase

POD = peroxidase

Nucleic acids and nucleotides

A = adenine
C = cytosine
cDNA = complementary DNA
G = guanine
K = G or T (keto group)
M = A or C (amino group)
mRNA = messenger RNA
N = unspecific nucleotide
R = A or G (purine)
S = G or C (strong H-bridge bonds)
T = thymine
U = Uracil
W = A or T (weak H-bridge bonds)
Y = C or T (pyrimidine)

Proteinogenic amino acids

A = alanine (Ala)
C = cysteine (Cys)
D = aspartate/aspartic acid (Asp)
E = glutamate/glutamic acid (Glu)
F = phenylalanine (Phe)
G = glycine (Gly)
H = histidine (His)
I = isoleucine (Ile)
K = lysine (Lys)
L = leucine (Leu)
M = methionine (Met)
N = asparagine (Asn)
P = proline (Pro)
Q = glutamine (Gln)
R = arginine (Arg)
S = serine (Ser)
T = threonine (Thr)
V = valine (Val)
W = tryptophan (Trp)
Y = tyrosine (Tyr)

III. Introduction

1. Classification, morphology and etymology of Silybum marianum

Silybum marianum (L.) Gaertn. belongs to the family Asteraceae (Compositae) or daisy family, an extremely large and widespread family of flowering plants (Angiospermae) - more precisely, the magnoliopsida (dicotyledons) with its subclasses of asterids and further on the order Asterales (Angiosperm Phylogeny Website; GBIF Backbone Taxonomy).

Milk thistle is a native of Southern Europe through to Asia (Fuchs, 1543), but it also occurs in the Canaries, the Azores and Madeira and extends eastward to South Russia and Iran. It is as well naturalised in the Americas and in South Australia as an invasive plant (Popay, 2013). For medicinal supply *Silybum marianum* is cultivated on large fields in Austria, Hungary, Germany, China and Argentina. The presence in botanical gardens throughout the world, because of its pharmaceutical interest, is noteworthy as well.

There are many common names given to milk thistle, including Our Lady's thistle, Marian thistle, St. Mary's thistle, Sow thistle, variegated thistle or Wild artichoke and many more (Quattrocchi, 1999). Its botanical name *Silybum marianum* is prioritised by most scientific sources, in contrast to the uncommonly used name *Carduus marianus*.

Milk thistle usually is an annual or biannual plant with relatively typical purple thistle flower-heads and shiny green leaves with white lines/marbling. According to an old legend these milky-white veins of the leaves originated from the milk of the Virgin Mary which once dropped upon a plant and led to the names St. Mary' thistle and Our Lady's thistle, as well as the names *marianus* and *marianum*. Its genus name "*Silybum*" is originally derived from the Greek word "silybon" meaning tassel. The spherical purple flower-heads are supposed to look like tassels. The stately plant reaches a height of 20 to 150 cm, rarely up to 200 cm with an overall conical shape. The big shiny green leaves with its thorny serrated margins make this beautiful thistle easy to recognise (Fig. 1). During the first year it develops its basal leaf rosettes. Later on or rather the next year the stems grow upwards and the first terminally branched flower heads form (Fig. 2). The stem is grooved and mostly branched and carries a fluffy hair coat. The mature plants have hollow stems. The fruits (achenes) develop from the fertilised inflorescences. The outer appearance of mature milk thistle fruits varies from greyish white to brown and almost shiny

black. The white, long pappus at the top is surrounded by a yellowish basal ring (Fig. 3) (Rose, 1981; Wichtl, 2009).

Fig. 1: Illustration of *Silybum marianum* - Flora von Deutschland, Österreich und der Schweiz, Otto Wilhelm Thomé, 1885 - www.BioLib.de

Fig. 2: Inflorescence of *Silybum marianum* – Wikimedia commons: H. Zell, 2009

Fig. 3: Achenes from milk thistle. Marchart, Waldland Vermarktungsgesellschaft

The family Asteraceae plays an important role in modern herbal medicine with pharmaceutical interesting active compounds from plants like chamomile (*Matricaria chamomilla*), wolf's bane (*Arnica montana*), absinthe (*Artemisia absinthium*), pot marigold (*Calendula officinalis)*, purple coneflower (*Echinacea purpurea*) and many more (Wichtl, 2009). Additionally, members of the daisy family are of economic relevance, providing products such as herbal teas, lettuce or cooking oils (Watson and Dallwitz, 1992 onwards). With about 1.700 genera and about 24.000 species the plants belonging to this family present high complexity and diversity in their chemical and biological profiles (Funk et al., 2009). According to Zdero and Bohlmann (1990), about 7000 different compounds were isolated and chemically identified from up to 5000 species until the early 1990s, including numerous di-, tri- and sesquiterpenoids, sesquiterpene lactones, flavonoids, alkaloids, coumarins, polyacetylenes and many other polycyclic organic compounds. This calls for exceptional diversity in connection with extensive bioactivity.

Almost all higher plants have in common that they produce a broad spectrum of specialised metabolites. Although these substances are not directly involved in the development or growth of the organism like primary metabolites, they play a crucial role in increasing the plants' chances of long-term survival, persistence and fertility. Among other functions they are essential for defence against herbivores and pathogens and communication with other organisms. They further support in enduring abiotic stress such as increased UV-radiation or environmental challenges. Furthermore, these organic compounds are of special pharmaceutical interest since they can serve as medicines. Therefore they should be referred to as specialised metabolites/compounds, because they are anything but secondary. On account of their pharmaceutical potential, the specialised compounds identified from Asteraceae species can be regarded as promising natural substances for future medicinal discoveries. More specifically, their anti-inflammatory and antitumoral properties could be an interesting approach for further potential applications in case of cardiovascular disorders or in cancer therapy (Lajter, 2016).

2. Specialised compounds from *Silybum marianum*

The fruit wall of the achenes of *Silybum marianum* contains the largest amount of the pharmaceutically interesting flavonolignan mixture silymarin (3-6%). In addition, flavonoids like quercetin, kaempferol, taxifolin as well as fatty oils (20-30%) with linoleic acid, tocopherols and sterols have been identified in the fruits (Hohmann et al., 2001). Silymarin has low water solubility and bioavailability, as well as poor intestinal absorption. Therefore, new soluble silybin derivatives (e.g. silybin-*N*-methyl-glucamine, silybin bis-hemisuccinate or a β-cyclodextrin complex) have been designed (Loguercio and Festi, 2011). Silymarin itself represents a mixture of polyphenolic molecules of more than seven different compounds (silybin A/B, isosilybin A/B, silydianin, silychristin, isosilychristin and others), among which silybin represents the major compound of the extract (Fig. 4; Kuki et al., 2012; Lee and Liu, 2003). All silymarin components are derived from the precursors taxifolin and coniferyl alcohol. However, *Silybum marianum* is not the only species that can synthesise these special types of flavonolignans. A Chinese group identified several identical substances in *Euonymus acanthocarpus* (Celastraceae), congeneric to our indigenous European spindle (*Euonymus europaeus*). Silybin A+B, isosilybin A+B and silychristin could be isolated from bark material and positively confirmed by analytical methods (Zhu et al., 2012).

During the early 1980s, it has been observed, that flavonolignan constituents obtained from a white flowering milk thistle variety differed greatly from those of the purple-flowered type. The deoxyflavonolignans thus obtained have been elucidated and identified as silandrin (3-deoxyisosilybin), silymonin (3-deoxysilydianin), silyhermin and neosilyhermin (Szilági et al., 1981; Fiebig and Wagner, 1984). Another flavonolignan found in tincture preparations of milk thistle fruits, worth to be mentioned, is silyamandin. It is an artificial structure probably formed by degradation and conversion of silydianin favoured by temperature and prolonged ageing (MacKinnon et al., 2007). Until today, 23 flavonolignans have been identified in *Silybum marianum* (Csupor et al., 2016). Most of the common silymarin structures are positional isomers/regioisomers and thus contain the same number of atoms of each element, but bond together in different ways (e.g., silybin, silydianin, silychristin…). The exception are silybin A/B and isosilybin A/B which are stereoisomers, isomeric molecules that have the same molecular formula and sequence of bonded atoms, but differ in the three-dimensional orientations of their atoms. To be more exact, these constituents are diastereomers. They have different configurations at two stereocenters and are not mirror images of each other.

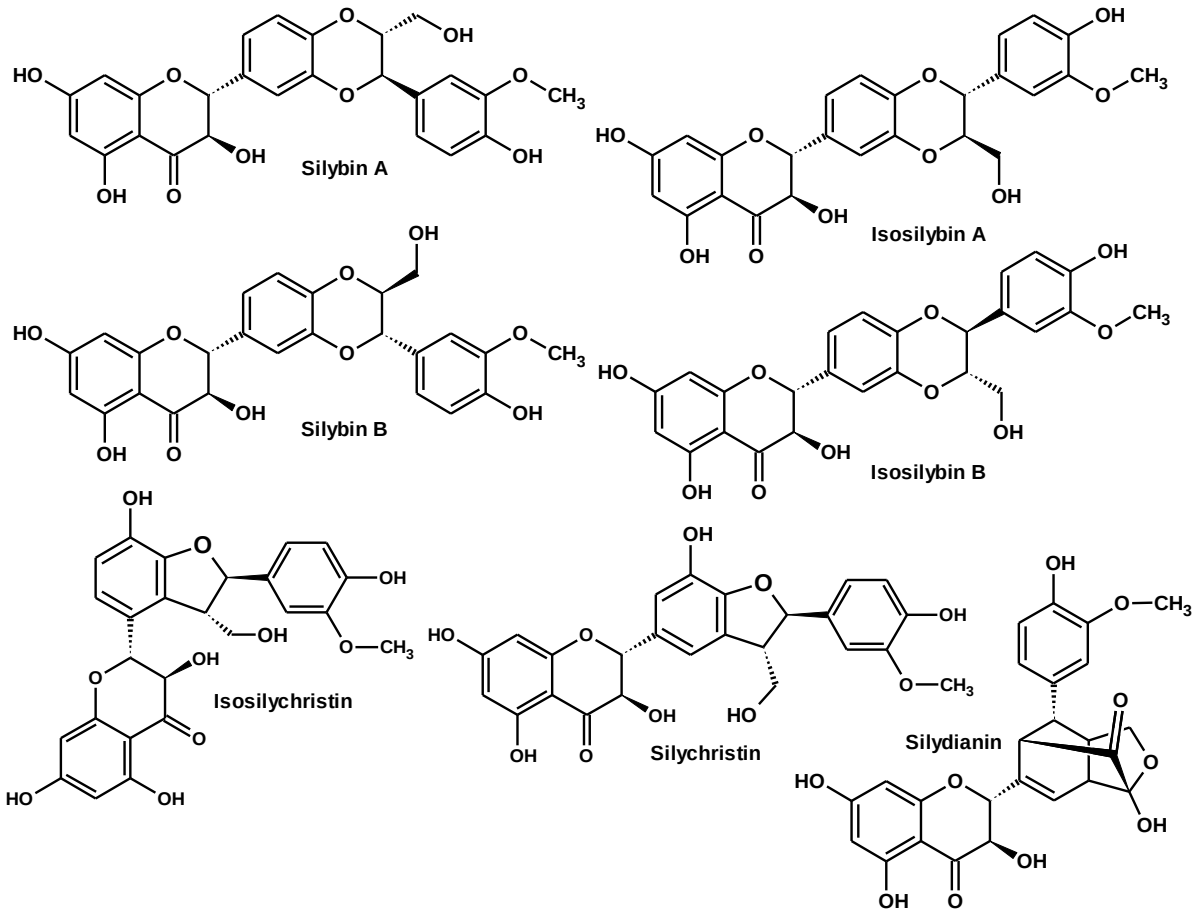


Fig. 4: Constituents of silymarin, a mixture of different isomers. Mainly regioisomers occur, except silybin A+B and isosilybin A+B, which are diastereomers.

3. Medicinal application and clinical research

Milk thistle has been used in traditional medicine for centuries mainly for the treatment of hepatic disorders (Benedum et al., 2000). This dates back to the first century when the Roman naturalist, Pliny the Elder, wrote about the plant's juice stimulating the bile flow and Dioscorides, who used the seeds as a remedy for poisoning from snake bites. The 16th century physician and botanist Leonhart Fuchs praised its effectiveness against poisons in his book "New Kreüterbuch" (1543). One century later, Nicholas Culpeper, a famous British botanist and herbalist, recommended its use in the treatment of disorders affecting the liver and other bodily fluids (Grieve, 1971). In the beginning of the 19th century milk thistle was recommended and used for the treatment of blood and liver problems as well as for intestinal cleansing. It was found that the fruits contain the active principle that has the protective effects on the liver. The formula "Tinctura Cardui Mariae Rademacher" still is listed in some pharmacopoeias and the German "Synonymverzeichnis". More recently, with the evidence of its hepatoprotective effects in animal experiments (1949) and the isolation of the flavonolignan mixture silymarin (1969) as well as the systematic research of ingredients and preparations of milk thistle fruits, the first finished medicinal product called Legalon® was commercially produced (Schadewaldt, 1969).

Nowadays the application of silymarin mainly is focused on oral treatment of toxic liver damage and for supportive therapy in chronic inflammatory liver diseases and in case of liver cirrhosis (Leng-Peschlow, 1996; Wellington and Blair, 2001). In most instances, adjusted dry extracts come to use [e.g. Legalon® Madaus or Silimarit® Bionorica (Fig. 5)]. There is evidence, that silymarin increases protein synthesis in hepatocytes by stimulating RNA polymerase I activity. This leads to increased stability of biological membranes and increased protein biosynthesis (Vargas-Mendoza, 2014). Furthermore, due to its phenolic structure, silymarin functions as a scavenger of radicals that can be formed during metabolisation of toxic substances (e.g. ethanol or xenobiotics) and it has been shown to positively enhance the hepatic glutathione system (Karimi et al., 2011; Bergheim et al., 2005; Kwon et al., 2013). In addition, intravenously given high doses of the constituent silybin can serve as a first "antidote" in case of mushroom poisoning with death cap (*Amanita phalloides*). It prevents the uptake of amatoxins by liver cells and thus protects undamaged liver tissue (Leschert et al., 2006). Its antioxidative, antifibrotic, anti-inflammatory, immunomodulatory, antiviral and lipid peroxidation preventive properties have been the subject of various investigations in recent years (Dixit et al., 2010; Ghosh et al., 2010; Parmar and Gandhi, 2008). Recently, beneficial effects in cancer therapy and

chemoprotective effects could be proven as well (Abarikwu et al., 2009; Faezizadeh et al., 2012; Milic et al., 2013), on the one hand, by its toxicity reducing, protective effects during chemo- or radiotherapy and on the other hand by positively influencing the misbalance between intact surviving cells and apoptotic cell death, by inducing several genes involved in apoptosis or suppressing the proliferation of tumour cells as well as by inhibition of epidermal growth factor receptors (EGFR). Even the anti-inflammatory effects of silymarin could sensitise tumour cells to apoptosis. This could be related to the inhibition of the transcription factor nuclear factor-κB (NF-κB) and thus the formation of interleukins and the tumour necrosis factor (TNF)-α (Ramasamy and Agarwal, 2008).

To summarise, silymarin is among the most commonly used and well-studied natural compounds used for therapy of liver diseases worldwide. There already have been various promising approaches and investigations confirming its broad spectrum of activity and application potentials. Since there is a high demand for natural products in the modern healthcare market, further experiments are to be expected.

Fig. 5: Historical Silimarit® package (19xx)

4. Biosynthesis of silymarin components

4.1 Phenolic compounds (phenylpropanoids)

In addition to the terpenoid metabolism the polyphenolic pathway ranks among the most widespread secondary metabolic pathways of plants (Emiliani et al., 2009). The starting compounds of the phenolic pathway are the two aromatic amino acids phenylalanine and tyrosine. These emerge from the shikimate pathway starting with the coupling of erythose-4-phosphate and phosphoenolpyruvate and leading via the intermediary shikimic acid to chorismic acid. Chorismate is then transformed to L-phenylalanine and L-tyrosine (Herrmann and Weaver, 1999).

The structures and properties of polyphenols are very diverse. Among them range all compounds with two or more hydroxyl groups or hydroxy derivatives linked to a benzene ring. Consequently, the group includes specialised metabolites like anthocyanins, catechins, coumarins, flavonoids, isoflavonoids, lignans and other phenylpropanoids. As lignin, which consists of polymerised monolignol monomers (coniferyl alcohol, 4-coumaryl alcohol and sinapyl alcohol), they are crucially involved in the process of lignification. Lignin is one of the most frequently occurring biopolymers along with cellulose (Freudenberg and Nash, 1968). Phenylpropanoids are named after their six-carbon, aromatic phenyl group substituted with a three-carbon propene side chain. The key enzyme of the phenylpropanoid biosynthesis responsible for the formation of *t*-cinnamic acid from L-phenylalanine is phenylalanine ammonia-lyase (PAL) (Koukol and Conn, 1961; Vogt, 2010). The reaction takes place in the cytosol of the plant cells. Cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) catalyse the formation of the intermediate 4-coumaric acid and finally the central metabolite 4-coumaroyl-CoA is formed, which is the key compound in the biosynthetic pathway of flavonoids and the precursors of stilbenoids and lignans, such as the monolignol coniferyl alcohol, as well as many other phenolic compounds (Fig. 6).

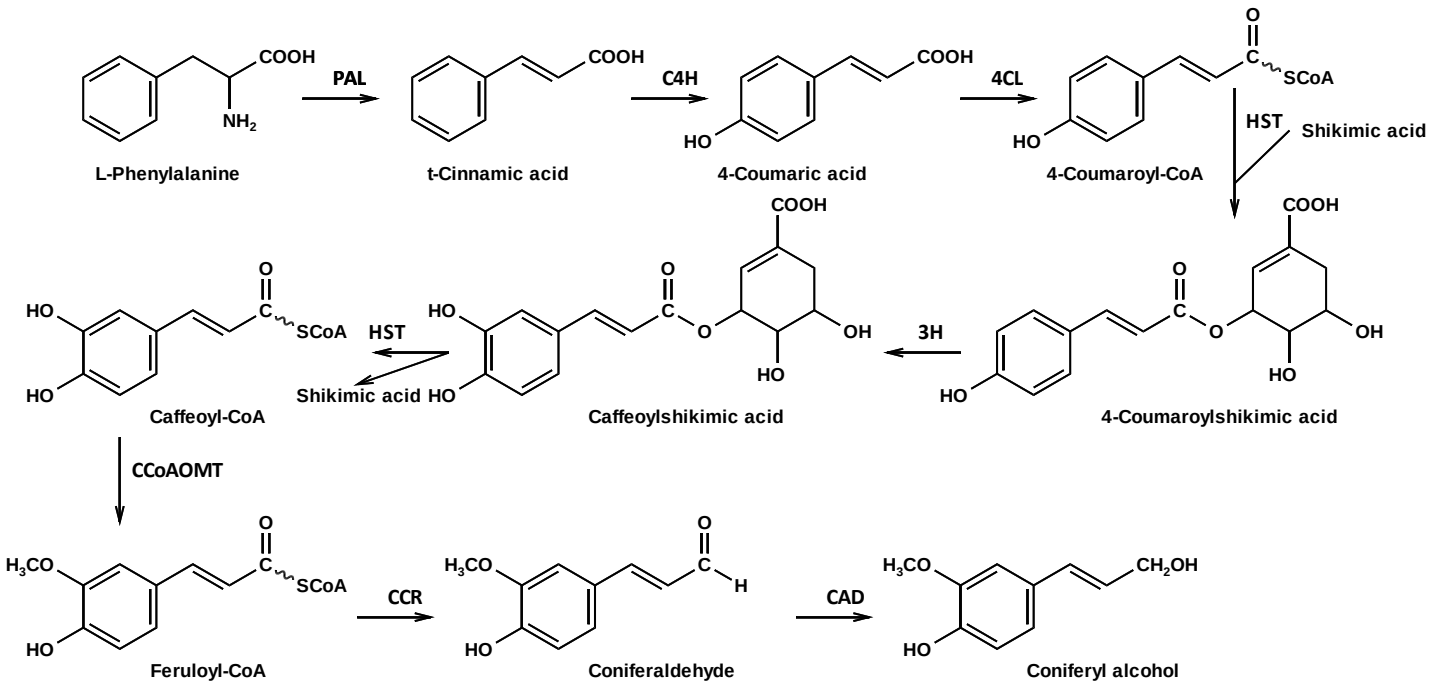


Fig. 6: The main biosynthetic pathway towards the monolignol coniferyl alcohol. PAL = phenylalanine ammonia-lyase, C4H = cinnamate 4-hydroxylase, 4CL = 4-coumarate:CoA ligase, HST = 4-hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase, 3H = 4-coumaroylshikimate 3-hydroxylase, CCoAOMT = caffeoyl-CoA *O*-methyltransferase, CCR = cinnamoyl-CoA reductase, CAD = cinnamyl alcohol dehydrogenase.

a) The flavanonol 2,3-dihydroquercetin (taxifolin)

The flavanonol **taxifolin** belongs to the flavonoids. Based on their core structures, flavonoids can be subdivided into different subgroups that are in one form or the other present in almost all higher plants (Fig. 7; Andersen and Markham, 2006). Taxifolin takes position as the key intermediate between flavonols and flavonolignans. The chemical compound 4-coumaroyl-CoA, formed during the phenylpropanoid pathway, is the starting substance for flavonoid biosynthesis. If combined with 3 acetyl units from 3 malonyl-CoA, chalcone, containing two aromatic rings, is formed. Chalcone synthase or naringenin-chalcone synthase (CHS) catalyses the conversion of the above-mentioned precursors under decarboxylation (3x) and release of 4 coenzyme A to yield naringenin chalcone. CHS belongs to the polyketide synthases and is omnipresent in higher plants. In the next step, chalcone isomerase (CHI) converts chalcones to flavanones and vice versa. For the biosynthesis of flavanonols, the resulting naringenin can be hydroxylated twice, once at the C-3 position and again at the C-3' position to yield 2,3-dihydroquercetin (Fig. 8; Jiang et al., 2015).

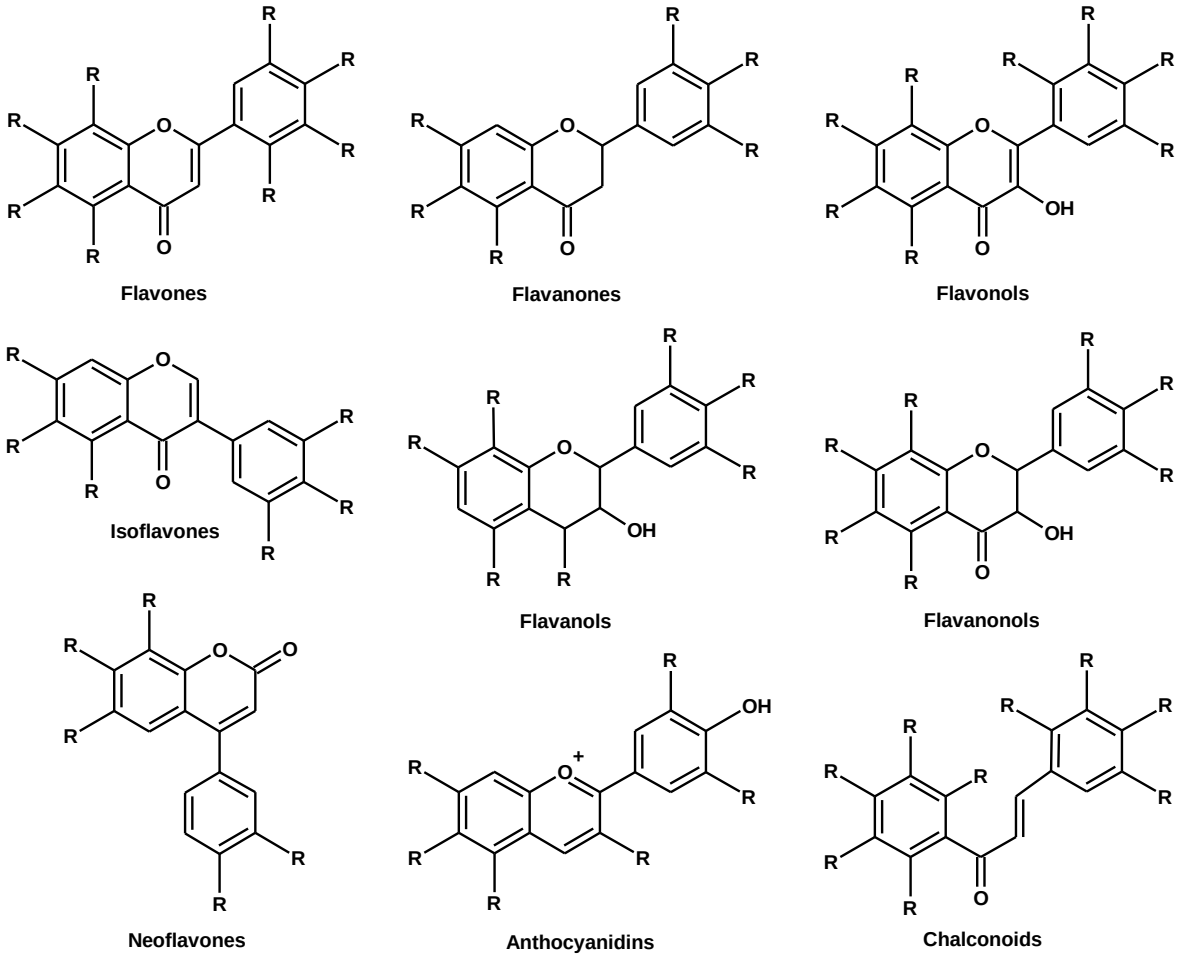


Fig. 7: Structures of flavonoid subgroups. Substituents are marked with –R. Most common substitutents are either hydroxy (-OH), methoxy (-OCH_3) or sugar residues (e.g. rhamnose or rutinose).

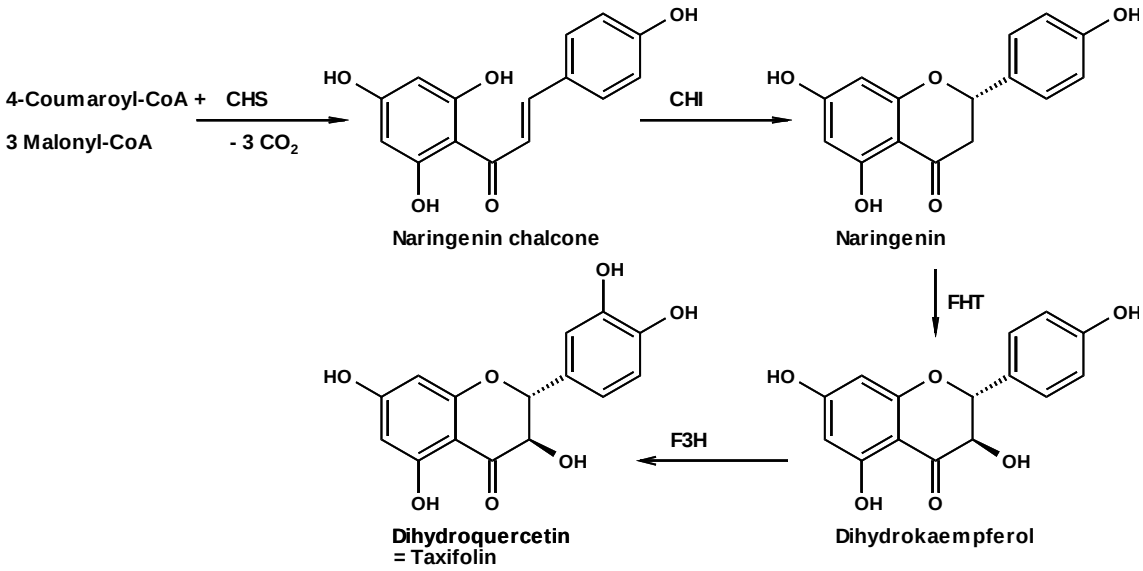


Fig. 8: Biosynthetic pathway to the flavanonol taxifolin. CHS = chalcone synthase, CHI = chalcone isomerase, FHT = flavanone 3β-hydroxylase, F3H = flavanone 3'-hydroxylase.

4.2 **Flavonolignans (silymarin)**

Flavonolignans are natural phenols composed of a flavonoid and a phenylpropanoid part. In case of the more popular compounds extracted from the purple flowering milk thistle varieties these are taxifolin and coniferyl alcohol. The biosynthesis of the deoxy forms, like silandrin and silyhermin, however, should involve eriodictyol or naringenin and the biosynthesis of the two 2,3-deoxy compounds apigenin or chrysoeriol instead of taxifolin (Fiebig and Wagner, 1984). Moreover, more "non-taxifolin-based" flavonolignans like hydnocarpin and hydnowightin from *Hydnocarpus wightiana* seeds, scutellaprostin from *Scutellaria prostrata* or flavonolignans derived from the flavone tricin have been isolated and described as well (Chambers et al., 2015).

Most studies focus on the pharmacological use and the chemistry of silymarin, whereas the biosynthesis has been poorly investigated. The protein(s) responsible for the coupling and thus the formation of the typical flavonolignan components have yet to be identified. The reaction is supposed to take place via a radical coupling mechanism by radical-forming oxidative enzymes like peroxidases or laccases, similar to the formation of monolignol radicals and polymerisation during lignification (Poppe and Petersen, 2016).

Lignin **peroxidases** (EC 1.11.1.14; diarylpropane:O_2,H_2O_2 oxidoreductase) have molecular weights between 38 and 43 kDa and manganese peroxidases (EC 1.11.1.13; Mn(II):H_2O_2 oxidoreductases) molecular weights between 45 and 47 kDa (Gold and Alic, 1993). Both are heme-containing glycoproteins with one protoporphyrin IX as a prosthetic group and two conserved calcium-binding sites as well as four conserved disulphide bridges. While lignin peroxidases are better known for lignin degradation, special focus is given to the class III heme peroxidases, which consist of the secretory higher plant peroxidases (Passardi et al., 2005). These display much discussed functions and reactive capacity. This includes tissue-specific functions comprising cell wall biosynthesis (lignification), cell elongation, participation in plant defence mechanisms, detoxification and seed germination (Almagro et al., 2008; Shigeto and Tsutsumi, 2015). A peroxidase potentially involved in the formation of flavonolignans could be similar structured as class III secretory peroxidases like the commonly used horseradish peroxidase (HRP, EC 1.11.1.7). In that case, post-translational modification and extended glycosylation patterns would be highly probable and should contribute to its function and activity (Wuhrer et al., 2005; Palm et al., 2014; Baker et al., 2016).

Laccases (EC 1.10.3.2; benzenediol:O_2 oxidoreductases) are commonly extracellular copper-containing glycoproteins with molecular weights between 60 and 80 kDa (Thurston, 1994). The active site of laccases contains four copper ions, from which one is a mononuclear "blue" copper ion. Therefore they are referred to as "blue" laccases (Morozova et al., 2007). They play a role in the degradation, but also the formation of lignin polymers, by promoting the oxidative coupling of monolignol units. In addition to the obvious differences in structure, laccases are generally considered to have a lower oxidation potential (Heinzkill et al., 1998).

Both types of enzymes catalyse one-electron oxidations of phenolic substrates leading to radical formation (Kersten et al., 1990). Peroxidases use hydrogen peroxide, laccases molecular oxygen as the electron donor. In the special case of silymarin biosynthesis, the radicals formed by the oxidising enzymes will couple in a non-stereospecific way (Becker and Schrall, 1977; Gavezzotti et al., 2014) leading to regioisomers and diastereomers as disparate mixtures. Furthermore, both enzyme classes are known to have multiple isoforms which vary in enzymatic activity, substrate specificity, functions, stability or other biochemical properties (Krainer et al., 2014; He et al., 2014; Sergio et al., 2007). For instance, with regard to milk thistle, Greimel and Koch (1977) discovered several peroxidase isoenzymes and divided them into four groups, based on their charge and migration speed during discontinuous electrophoresis. They concluded that the different activity distribution of the peroxidases is closely related to the organ specificity of its isoenzymes.

There are two different proposed mechanisms for the radical formation and coupling of the two substrates in silymarin formation. The more common one hypothesises the formation of two separate radicals, one at the phenoxy group of taxifolin and another at the side chain of coniferyl alcohol, leading to a very reactive quinone methide intermediate. The final step of the biogenesis is a thermodynamically controlled nucleophilic attack of an intramolecular free hydroxyl group to the monolignol part with subsequent rearrangements and cyclisation, finally leading to the stable lignin-like structures of interest (Fig. 9; Nyiredy et al., 2008). The O-β coupling step was shown to be neither regio- nor enantioselective (Mascher et al., 1993; Kim et al., 2003).

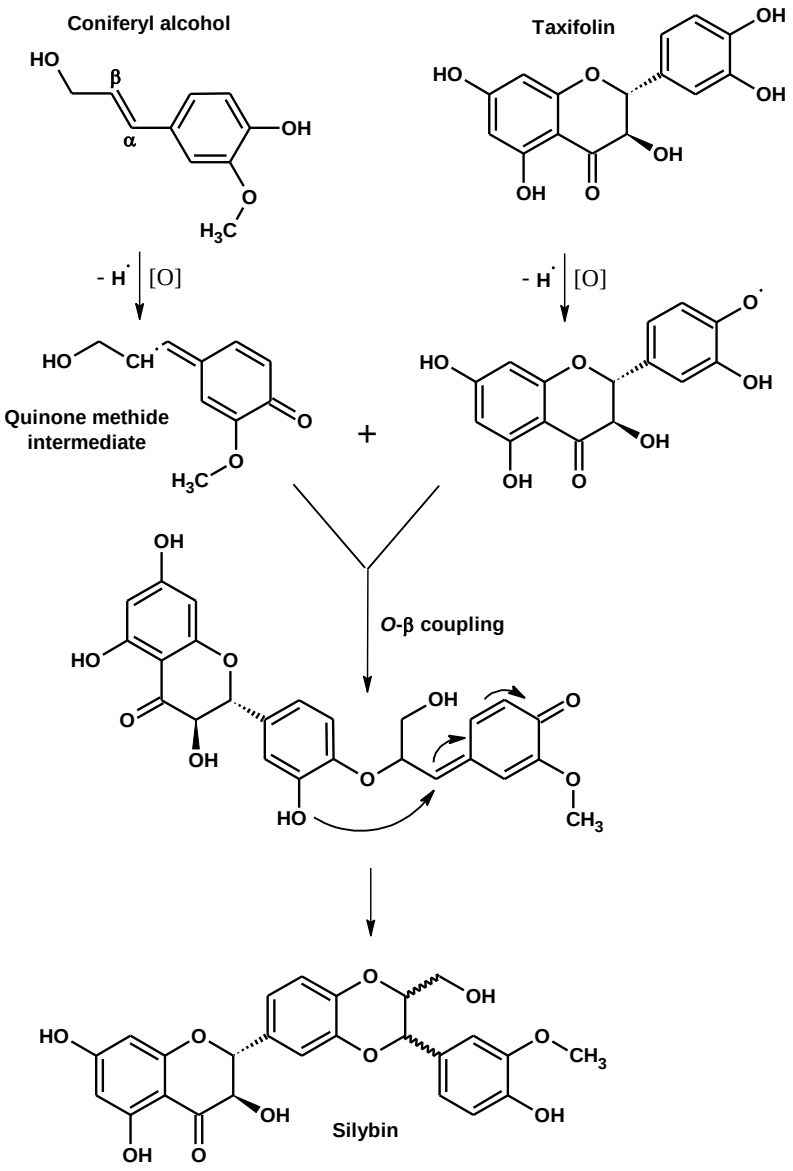


Fig. 9: Biosynthesis of silymarin constituents (e.g. silybin) according to Nyiredy et al. (2008). [O] = oxidation.

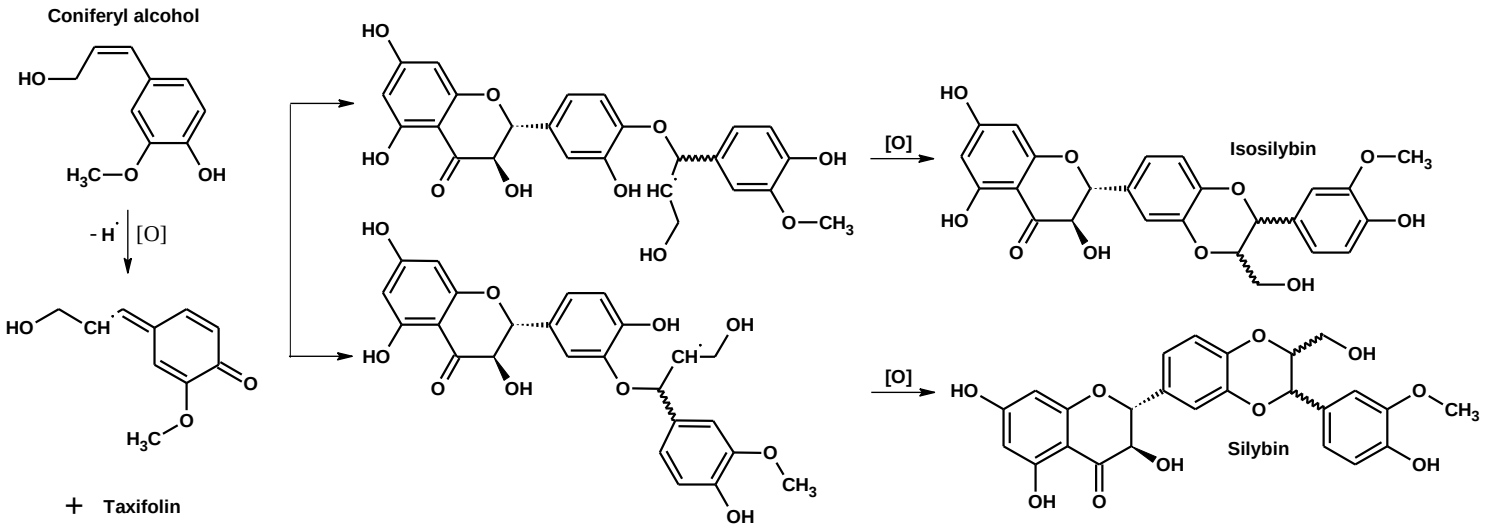


Fig. 10: Biosynthesis of silymarin constituents (e.g. silybin/isosilybin) according to Althagafy et al. (2013). [O] = oxidation.

Another possibility is the formation of only one radical by single electron oxidation of coniferyl alcohol with subsequent addition to one of the hydroxy groups of taxifolin's catechol part, and finally a second oxidation to yield silybin or isosilybin (Fig. 10; Althagafy et al., 2013). However, the study only presented oxidative coupling with silver(I)oxide (Ag_2O) instead of enzymes to form the silymarin constituents. Nevertheless, a similar reaction mechanism could also take place in flavonolignan biogenesis *in vivo*.

A further important question arises by looking into the relative amounts of the different constituents of flavonolignans, particularly in fruits of milk thistle plants harvested from different origins. It can be observed that there are distinct chemotypic variations in different ecotypes/cultivars and genotypes (Hasanloo et al., 2005; Hetz et al., 1995; Martin et al., 2006; Shokrpour et al., 2007; Poppe and Petersen, 2016). It could thereby be commercially profitable, to increase and stabilise specific fruit yields and genotypic varieties with a preferred regioisomer profile. From a scientific perspective, clarifying the underlying mechanism behind the distribution of the positional isomers is particularly interesting. So far, very little is known about why these distinctions in positional isomer proportions occur and how the plant discriminates between them. Possible causes could be on the proteomic and genomic levels, the stage of fruit development, transport systems or environmental influences.

At this point it is worth considering whether the occurrence of varying regioisomer and diastereomer ratios might be due to the presence of specialised proteins driving the formation of specific compounds of the silymarin mixture. So-called specifier or **dirigent proteins** might direct regio- and/or stereoselectivity and thus play an active role in coupling the phenoxy radicals during flavonolignan biogenesis. Around 1995, the group of Davin and Lewis was the first who discovered this new type of proteins which could determine precisely the biochemical outcome of phenoxy radical coupling of monolignols during lignan biosynthesis and lignification without having an own catalytic activity (Davin and Lewis, 2000). These specialised proteins could direct the stereoselective biosynthesis of (+)-pinoresinol from coniferyl alcohol monomers (Fig. 11; Davin et al., 1997). Recently, however, due to extensive mutational analysis, the thesis about no catalytic activity was challenged. The group of Schaller et al. proposed a catalytic function for dirigent proteins in the cyclisation step of the quinone methide intermediate. This was attributed to active proton donation by hydrogen bond formation or acid catalysis (Gasper et al., 2016). Another dirigent protein was identified in *Arabidopsis thaliana*, responsible for enantioselective synthesis of (-)-pinoresinol (Pickel et al., 2010). The first 3D structure of a dirigent protein resulted in a trimeric structure with an eight stranded β-barrel topology for each monomer (Kim et al., 2007); the molecular mass of the monomers is about 20 kDa, rarely up to 40 kDa. Meanwhile, more dirigent proteins involved in lignan/lignin biosyntheses, for instance from *Arabidopsis* and *Schisandra* species (Kim et al., 2012; Pickel et al., 2010) or *Isatis indigotica* plants (Li et al., 2014) have been described. Other dirigent proteins are involved in gossypol biogenesis in cotton species (Fig. 12; Effenberger et al., 2015). Since the reaction mechanism between the phenoxy radical of taxifolin and the monolignol part (coniferyl alcohol) might take place in a similar way, the assumption of the involvement of dirigent proteins in silymarin formation seems reasonable.

Often proteins with a similar structure are also referred to as disease resistance-responsive or dirigent-like proteins. These mostly serve as a plant defence mechanism by inducing a set of responses that can help against the effects of invading pathogens (Martin et al., 2003). In many cases, their direct biochemical functions or a possible dirigent activity have not yet been fully investigated (Seneviratne et al., 2014).

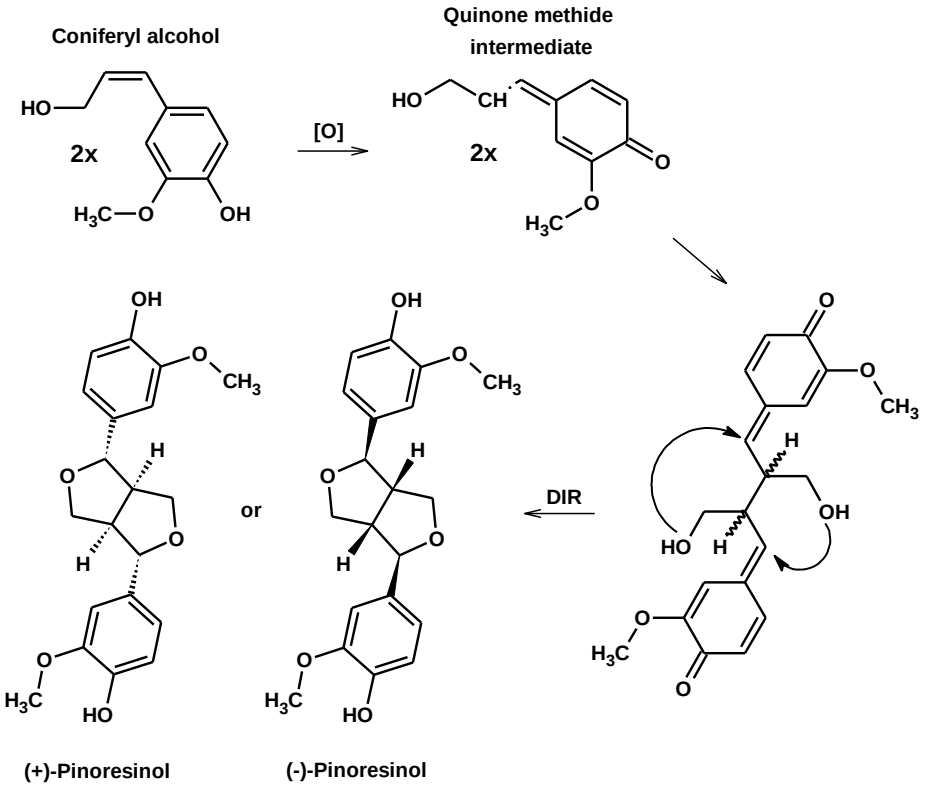


Fig. 11: Biosynthesis of (±)-pinoresinol according to Davin et al. (1997). [O] = oxidation, DIR = dirigent protein.

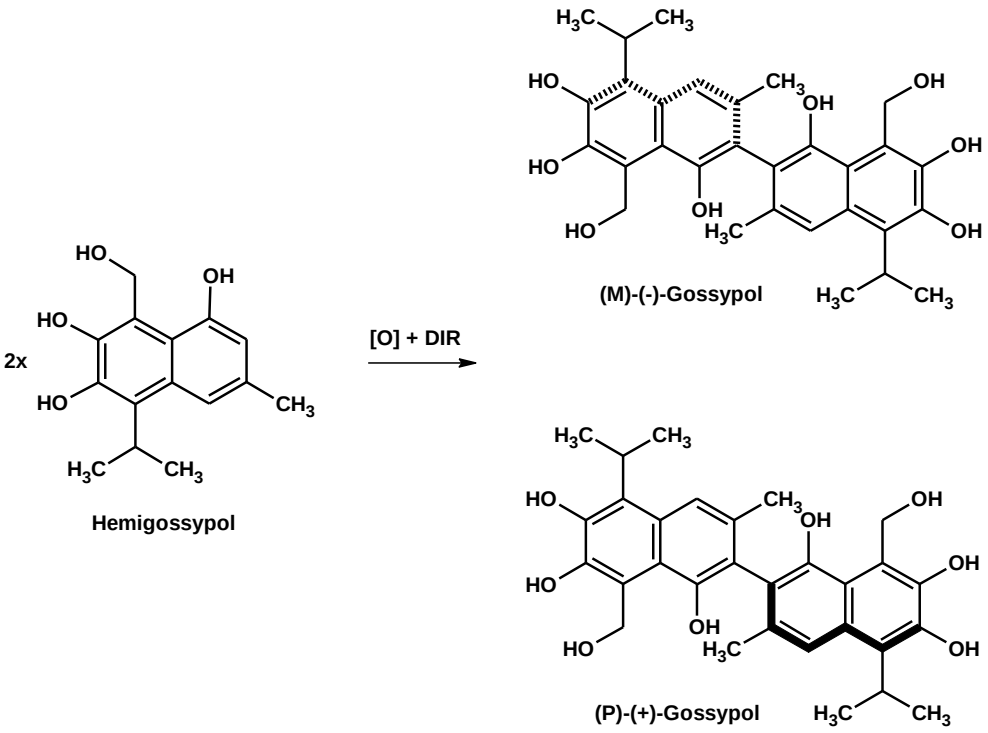


Fig. 12: Biosynthesis of (±)-gossypol according to Effenberger et al. (2015). [O] = oxidation, DIR = dirigent protein.

5. Cell cultures

Plant cell suspension cultures are widely used in biology as a useful tool for scientific research of plant processes. A major benefit is gained avoiding the structural complexity of the organism from wildlife populations or greenhouses (Rao and Ravishankar, 2002). In addition, problems with plant protection, supply or species occurring only to certain seasons can be easily prevented.

An *in vitro* cell population guarantees a high rate of cell growth, thus an unlimited availability of material. Furthermore, its controllable growing conditions ensure a good opportunity for the analysis of complex physiological processes at cellular and molecular levels (Moscatiello et al., 2013). Due to optimisation of the medium parameters and harvest conditions or manipulations on a molecular biological level the production of high-value secondary metabolites can be influenced, for example through the supply of precursors or the application of elicitors, resulting in possible cost-efficient production of commercially interesting substances (Smetanska, 2008). By means of culture characterisations useful information about optimal growing conditions, enzyme activities and secondary metabolite accumulation can be collected.

However, there are also some drawbacks, since cell cultures lack biotic and abiotic stress like UV exposure, drought or herbivorous attacks and infections, unless intentionally caused (elicitors). As a consequence, a decrease in the secondary metabolism with a negative effect on the substance profile is probable, because the organism is no longer dependent on these special compounds. Moreover, the missing selection pressure can lead to increased susceptibility towards contaminations (Pezeshki and Petersen, 2016). Because cell suspension cultures are designed for high cell mass accumulation, thus more cell divisions, mutations at the DNA level or changes of epigenetic nature are plausible as well. There even are special natural compounds, like volatile oils from the family of Asteraceae or Lamiaceae, which only are present in specialised compartments (secretory glands). In suspension cells, which are mainly undifferentiated cells, these special structural elements are often missing, so that hardly any oil is produced (Mulder-Krieger et al., 1988). Unfortunately, this is similar to silymarin accumulation of *in vitro* cell cultures of *Silybum marianum*. Since the plant accumulates almost all flavonolignans in the pericarp of mature fruits, its production in undifferentiated cell cultures is very low and even disappears with increasing subcultures (Becker and Schrall, 1977; Hasanloo et al., 2008); this makes biotechnological production hardly commercially viable. Similar effects are known from proanthocyanidins and their precursor (-)-epicatechin in *Arabidopsis thaliana*. In contrast to

flavonols and anthocyanins, which for the most part are present in all plant organs except the seeds, these compounds are specifically accumulated in the seed coats (Lepiniec et al., 2006; Routaboul et al., 2006). Several transport proteins are involved and responsible for subcellular translocation of flavonoids and accumulation of specific substances in different plant storage locations (Saito et al., 2013).

Another important aspect worth pointing out is the extracellular accumulation and secretion of silymarin and other flavonoid compounds due to active transport systems (Prieto and Corchete, 2014; Sánchez-Sampedro et al., 2005). Additionally, the release of proteins into the culture medium, especially proven for extracellular peroxidases (Quiroz-Figueroa and Loyola-Vargas, 2001; Ravi et al., 1986), along with the precursors of flavonolignans, can lead to active formation of the characteristic silymarin components. If now one considers the suspension cell culture medium as apoplastic space, thus resembling the missing fruit wall, interesting relations and possibilities for further scientific investigations, concerning the biosynthesis, distribution and final storage location of silymarin, come to mind.

6. Working objectives

The overall objective of this dissertation was to provide further insight into the final step of flavonolignan biosynthesis and its underlying mechanisms responsible for the formation of the active substance mixture silymarin in *Silybum marianum*. For this purpose, special focus was set on two types of enzymes; proteins catalysing one-electron oxidations of phenolic substrates leading to radical formation, like peroxidases (PODs) and laccases (LACs), and dirigent proteins (DIRs), potentially involved in regulating the stereo- and/or regioselectivity leading to distinct characteristic distributions of the individual silymarin components. By means of molecular methods, full length sequences coding for putatively involved genes, have been identified. In order to evaluate the function of the encoded proteins attempts to actively express these in various host cells were undertaken.

In another approach, suspension cell cultures of different *Silybum marianum* cultivars varying in the composition of their flavonolignan mixture have been established to get useful information about enzyme activities, growing parameters and possible plant processes taking effect in flavonolignan biosynthesis. This includes an attempt to enhance the *in vitro* silymarin production via elicitation.

IV. Material and Methods

1. Plant cell cultures

In the focus of this PhD thesis were plant cell cultures from *Silybum marianum* (milk thistle). Plants from different origins and ecotypes were of special interest. Initially, we started with plant material collected from the Old Botanical Garden in Marburg. The mature achenes of milk thistle plants were the starting material for callus and cell suspension cultures (see below). These cultures were characterised first and an attempt to increase the silymarin content by means of elicitation was undertaken. Later on, cell cultures from two more varieties of *Silybum marianum* (fruits from milk thistle plants collected in the Botanical Garden in Frankfurt and fruits obtained from a pharmaceutical wholesaler, harvested from cultivation areas in Poland) with differing flavonolignan distribution were established and investigated as well.

1.1 Establishment of callus and suspension cultures

The mature achenes were washed with 70% EtOH for 1-2 min, followed by treatment in 5% chlorine disinfectant solution for 20 min and three subsequent washing steps in sterile dist. H_2O. After this sterilisation step the fruits were germinated on MSOH (MS-medium without addition of hormones; Murashige and Skoog, 1962) agar plates. After germination, parts of the developed cotyledons were placed on MS-Li, B5 (Gamborg et al., 1968) and CB-2 (Gertlowski and Petersen, 1993) medium with agar. For composition of media see IV.8.

Two weeks later callus grown on B5 and CB-2 medium was immersed in 50 ml liquid B5 medium (without agar); additionally some callus was always retained as a safe stock and transferred to fresh medium once per month. The suspension cultures were transferred into fresh medium (50 ml) once every week, using 5.0 g cell wet weight, and shaken at 220 rpm and 26 °C in the dark. Cells grown in B5 medium showed better growth parameters and were chosen for future experiments. On MS-Li medium small roots could be excised from the leaf margin and were cultivated in liquid MS-Li medium, transferred into fresh medium every 3 weeks using 0.4 g root tips (~1 cm).

2. Culture characterisation

A culture characterisation serves to determine the optimal growth conditions. This includes various growth parameters, enzyme activities and expression levels or the accumulation of secondary metabolites, which were of particular interest in our case. The experiment was carried out with established suspension cultures from milk thistle fruits harvested in the late summer of 2012 in the Old Botanical Garden Marburg. At the start of this experiment, the suspension cultures were in their 5^{th} subculture. At first, 5.0 g cells (wet weight) of *Silybum marianum* suspension cultures grown for seven days were transferred into fresh sterile culture medium (B5) with a heat-sterilised perforated spoon in a laminar flow workbench. This corresponds to day 0. In total, 15 flasks were inoculated. Every day for 13 consecutive days, 1 flask was taken for the determination of cell fresh and dry weight, silymarin and protein content plus enzyme activities and medium parameters. Harvesting and processing of the cells preferably took place at the same time of day. With help of a suction filter and a water-jet vacuum pump cells and medium were separated. 3 ml medium were filled in two 1.5 ml reaction tubes immediately and placed on ice or frozen at -20 °C. Remaining medium was preserved for further determinations. The cells were weighed (fresh weight per flask), 2 x 0.5 g of the moist cells were filled in pre-weighed plastic centrifuge tubes and frozen at -20 °C for later determinations of dry weight and silymarin content. The remaining cells were saved for preparation of enzyme crude extracts (IV.5.1.a) and determination of enzyme activities.

2.1 Determination of growth parameters

The following medium parameters were determined:

a. pH value

After calibration with adequate buffer solutions the pH value of the medium was determined at room temperature with a pH-electrode.

b. Conductivity

For the determination of the conductivity the medium was diluted with dist. H_2O in a 1:4 ratio. The electrode was first calibrated and the measurement took place at room temperature.

c. Sugar content

The total sugar content was measured with a refractometer after calibration with H_2O.

2.2 Enzyme extraction from cells and medium

Crude extracts from plant suspension cells can be used as a source for enzymes. The cells must first be shredded in a suitable buffer system and possible oxidation prevented. This procedure is explained in detail in IV.5.1.a.

Since the necessary enzyme(s) for the coupling reaction between taxifolin and coniferyl alcohol are also released to the medium of milk thistle suspension cells a specific preparation took place here, as well. For a detailed explanation see IV.5.1.b.

2.3 Determination of dry weight and flavonolignan components

Frozen cells prepared for this step were lyophilised for 48 h, followed by weighing and subtraction of the tare weight of the plastic tubes. Cell dry weight per 0.5 g moist cells also enables calculation of dry weight per flask. Further on these cells were used for determination of the silymarin content. In addition, 0.5 g of the root cultures developed on solid MS-Li medium were analysed for their silymarin content once.

The lyophilised cells or roots in the plastic tubes were squelched with a stirring rod and 5 ml of MeOH added, the tubes sealed with a perforated lid and shaken vigorously. Subsequently, tubes were placed in a 70 °C pre-heated ultrasonic bath for 10 min. After shaking once more the ultrasonic treatment was repeated. Tubes were centrifuged at 3.000 g for 10 min and supernatants carefully collected. The solvent was evaporated with help of a rotary evaporator. Residues were re-dissolved in 0.5 ml MeOH each and transferred to 1.5 ml reaction tubes.

Before HPLC measurement 100 µl of these samples were diluted with 100 µl of eluent (47.5% MeOH, 52.5% H_2O, 0.01% phosphoric acid) and centrifuged at 16.000 g for 10 min.

2.4 Determination of protein concentrations

To determine the protein content of the samples, they were measured photometrically using the assay according to Bradford (1976) (IV.5.6).

2.5 Enzyme activity

The activity of crude protein extracts and medium samples were determined by performing enzyme assays as described in IV.5.10. After desalting via PD-10 column 20 µl of the gained sample were added to a mixture of the educts taxifolin and coniferyl alcohol. With subsequent HPLC measurement the formed flavonolignan products could be determined qualitatively and quantitatively.

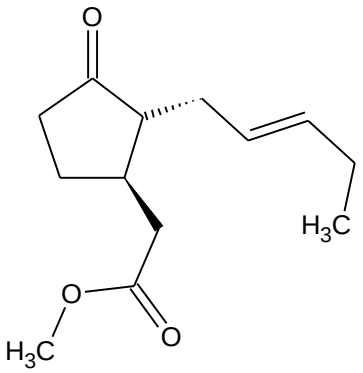
3. Elicitation of a suspension culture

Although the best culture conditions were determined before, some secondary metabolites are produced in low concentrations and in some cases not at all, for which it is necessary to develop strategies such as elicitation to stimulate the production of the desired component (Zhao et al., 2005). Compared with the whole fruit, silymarin production in plant cell cultures is very low (0.01-0.04% of the dry weight versus 1-5% in fruits) (Cacho et al., 1999; Andrzejewska and Sadowska, 2008; Hasanloo et al., 2008).

An elicitor is a compound (natural or synthetic) that can simulate a stress condition in the culture, triggering the same response in the cells that would be induced in the natural environment, usually resulting in the accumulation of secondary metabolites (Zhao et al., 2005; Vasconsuelo and Boland, 2007). Additionally recent results suggest that excretion of silymarin and its precursors is a transporter-dependent active transport and that yet another mechanism involving a vesicle trafficking system seems to participate in driving this class of secondary metabolites to the extracellular compartment (Prieto and Corchete, 2013).

In this experiment a suspension culture of milk thistle was tested whether it can be induced with an elicitor to produce more components of the flavonolignan mixture silymarin. Cultures were treated with either methyl jasmonate (MeJA), a known elicitor for secondary metabolites in plants, or EtOH as a control.

Jasmonate is a volatile organic compound used in plant defence and many diverse developmental pathways such as seed germination, root growth, flowering, fruit ripening, and senescence. It is biosynthesised from linoleic acid by the octadecanoid pathway. Plants produce jasmonic acid and MeJA in response to many biotic and abiotic stresses (in particular, herbivory and wounding), resulting in a transient accumulation in the whole plant (Halitschke and Baldwin, 2005). Its broad effectiveness is explained by the fact that this molecule acts as a second messenger in a wide spectrum of signalling pathways (Van der Fits and Memelink, 2000; Zhao et al., 2005).



Methyl jasmonate (MeJA)

3.1 Sterile inoculation and elicitation of cultures

At the beginning of the elicitation experiment 5.0 g of seven days grown suspension cultures of *Silybum marianum* were transferred into fresh sterile culture medium (B5) with a heat-sterilised perforated spoon in a laminar flow workbench. At this time the cells had been subcultured for about fifteen times. 12 flasks were inoculated in B5 medium. 50 µl filter-sterilised EtOH (control) or 50 µl 100 mM (end concentration in flask: 100 µM) ethanolic MeJA were added to six flasks each at day three. Cultures were incubated on a rotary shaker (220 rpm) at 26 °C in the dark. Suspension cultures were harvested at the following time-points and analysed in parallel. The experiment was repeated a few weeks later.

3.2 Determination of flavonolignan components

After 0/12/24/48/96/216 h cells and medium were separated with a suction filter and a water-jet vacuum pump. Remaining medium was kept for further determinations. 2.0 g of these moist cells were filled in pre-weighed plastic centrifuge tubes and frozen at -20 °C for later determination of the silymarin content.

The extraction method was as described in IV.3.3. However this time, 100 µl of the samples were diluted with 400 µl HPLC eluent before measurement via HPLC. Unfortunately, the cell samples for the first test series got unusable due to a technical problem in the lyophilisation system.

3.3 Accumulation and release of silymarin to medium

In addition to its enzymatic activity, medium of suspension cultures of *Silybum marianum* contains small amounts of silymarin and its precursors taxifolin and coniferyl alcohol. They could be released through some passive or active mechanisms or silymarin formation takes place there due to the presence of secreted peroxidases. It was investigated whether more silymarin products could be found in the medium after elicitation with MeJA. The extent of this "accumulation" was determined by extracting the whole culture medium with EtOAc.

The cell-free medium was first filtered through filter paper, sucked through a membrane filter (0.8 mm pore size) and centrifuged for 5 min at 3.000 g in order to remove all particulate cell residues. Afterwards it was extracted twice with an equal volume of EtOAc. After merging of the two organic extracts it was evaporated with a rotary evaporator. Residues were re-dissolved in 0.15 ml (1^{st} experiment) or 0.25 ml (2^{nd} experiment) MeOH each and transferred to 1.5 ml reaction tubes. The elicitation experiment was repeated under almost the same conditions. Measurement and evaluation was done via HPLC without further dilution steps.

3.4 Degradation of silymarin

It was also tested whether the silymarin components are stable in the medium of the suspension cells or whether degradation occurs. Degradation due to temperature, the medium pH and potentially present enzymes could influence flavonolignan stability to a certain extent.

In a first experiment, medium, in which *Silybum marianum* suspension cells had grown for seven days, was compared to TRIS-HCl buffer. Both were adjusted to pH 10 and the medium was additionally filtered and desalted with a PD-10 column (IV.5.4). Then 200 µl of the medium/buffer were mixed with 50 µl of a 10 mM silymarin standard (~240 µg). In each case three samples were shaken at 26 °C at 120 rpm for 24 h in the dark and two more samples were directly (0 h) extracted with EtOAc to serve as a reference.

The second experiment was very similar. However, fresh medium at two different pH values was compared to adjusted TRIS-HCl buffers. The extraction method and incubation time were the same.

All samples were extracted with 250 µl EtOAc three times each. The combined extracts were then evaporated under the hood overnight, re-dissolved in 250 µl HPLC eluent and measured via HPLC (IV.5.13).

4. Flavonolignan composition from varying milk thistle chemotypes

While looking for factors possibly regulating the positional isomer or diastereomer ratio of the flavonolignans in *Silybum marianum*, the investigation of milk thistle fruits collected from plants grown in various habitats in Europe, should provide further new insights. For this purpose, mature fruits from two different German and Polish cultivars each, as well as fruits from Hungary and Bulgaria have been compared with respect to their ratio of flavonolignans. Furthermore, callus and suspension cultures have been established (IV.1.1) from the varieties with clear distinctions in their regioisomer distribution. These were then as well analysed for silymarin content, especially the positional isomer ratios, in cells and released to the outer compartment. Additionally, crude enzyme extracts and concentrated cell culture media were fractionated and purified via FPLC for further investigation of proteins involved in silymarin formation. Enzyme assays for dirigent proteins and possible differences in the enzyme(s) responsible for radical

formation and the coupling reaction of taxifolin and coniferyl alcohol were performed. Most of the methods and results, which will be further explained, have been published previously (Poppe and Petersen, 2016).

4.1 Origin of milk thistle fruits

As a starting material for this experiment, six batches of milk thistle fruits from different origins were purchased. Two-thirds among them were acquired from pharmaceutical wholesalers. Some of these also differed in the drying process and time of harvest. This can of course also affect the silymarin composition. More detailed information can be found in Table 1. The other two ripe fruit samples were from the Old Botanical Garden Marburg (BGMr) and the Botanical Garden Frankfurt (BGF). Both air-dried and harvested between August and September 2012.

Pharmaceutical wholesaler	Abbreviation	Country of origin	Batch number	Drying process	Time of harvest
Heinrich Klenk GmbH & Co KG Schwebheim (Germany)	Klenk	Poland	7180 A 120207	air-dried	09/2011
Alfred Galke GmbH Bad Grund (Germany)	Galke	Hungary	23731	35-40 °C 48 h	08/2013
Bombastus Werke AG Freital (Germany)	Bombastus (Bomb.)	Bulgaria	282034	35-40 °C 48 h	07-09/2012
Caesar & Loretz GmbH Bonn (Germany)	Caelo	Poland	13326201	35-40 °C 48 h	08/2012

Table 1: Information about achenes from *Silybum marianum* purchased from pharmaceutical wholesalers with special regards to drying process and harvesting times.

4.2 Extraction method for silymarin from fruits

In order to get further information about the amount and ratio of flavonolignans in fruits of milk thistle it is important to apply a good extraction method. The pharmacopoeia describes standardised dry extracts for silymarin with acetone, EtOAc or EtOH as extractant.

To test the effectivity of varying extractants, exactly 1 g fruits of a batch of *Silybum marianum* (Galke / Hungary) were pulverised in liquid N_2 and the powder suspended in 10 ml acetone, EtOAc or MeOH in duplicate samples. After 2 x 10 min in an ultrasonic bath at 70 °C and temporary shaking followed by centrifugation at 3.000 g for 10 min the supernatants were collected and evaporated to dryness. The residue was re-dissolved in 0.5 ml MeOH. Finally, samples were prepared for analysis via TLC (IV.5.12) and HPLC (IV.5.13). Before evaluation by HPLC, 100 µl of the obtained samples were diluted with 400 µl eluent, followed by centrifugation at 16.000 g for 5 min. Samples were analysed twice.

Two further extraction steps were added in order to find out whether the single extraction is quantitative. All three extracts were then analysed separately by HPLC.

4.3 Establishment and cultivation of *in vitro* cultures

As described in IV.1.1, callus and suspensions cultures were then established from seedlings of the fruit samples with the most striking differences in their regioisomer pattern. In addition to the already existing cultures from the Marburg variety, the decision was done in favour of the variant from Poland (Klenk) and the one received from Frankfurt, which shared its pattern with the remaining, not further used, fruit samples.

4.4 Determination of flavonolignan composition

a. Suspension cells

After 7 days of growth in B5 medium, cells and medium were separated with a suction filter and a vacuum pump, the medium was kept for further analyses. 2.0 g medium-free cells were filled in pre-weighed plastic centrifuge tubes and frozen at -20 °C.

The extraction was performed as described in IV.2.3. Before HPLC analysis 100 µl of each sample was diluted with 400 µl eluent and centrifuged at 16.000 g for 5 min.

b. Cell culture medium

Extraction of flavonolignans from the cell culture medium was done as described in IV.3.3. In this case, however, the volumes of two culture flasks were combined (60-80 ml) and extracted two times with an equal volume of EtOAc in order to obtain higher flavonolignan amounts. The organic solvent phases were combined and evaporated with a rotary evaporator. Residues were re-dissolved in 0.5 ml MeOH each and transferred to 1.5 ml reaction tubes. After addition of the same volume eluent and centrifugation at 16.000 g for 5 min the extracts were analysed by HPLC (IV.5.13).

4.5 Enzyme preparation and purification

a. Crude extracts

Crude protein extracts were prepared as described in IV.5.1.a. 7 day old suspension cultures from all three varieties served as starting material. After successful extraction, samples were desalted with a PD-10 column (IV.5.4) and stored at -20 °C until they were tested in enzyme assays. In case of further purification steps via FPLC, the desalting step was omitted. Protein concentration was determined according to IV.5.6.

b. Concentrated medium

Cell-free media were kept for further concentrating steps as described in IV.5.3.c. This time the medium was purified even more carefully by means of three additional filtration steps through membrane filters with a pore size of 0.8 (1x) and 0.2 (2x) µm. During this process the medium was kept around 0 °C. Each medium was concentrated via Centricon® centrifugal devices at 8.000 g and 4 °C until the desired concentration was reached. The media were concentrated by 10-fold, based on the starting volume from two mixed suspension cultures. The concentrated media were stored at -20 ° C until purification by FPLC.

c. Fractionation, purification and identification

For fractionation and purification, the protein extracts from suspension cells of the three varieties with different regioisomer patterns in the fruits, were loaded into a super loop connected to an ÄKTA purifier system with a column for anion exchange chromatography (IV.5.2.a) after filtration to remove particles. After successful fractionation and storage on ice, a test for peroxidase activity was performed (IV.5.10.f). The active fractions then were pooled for further concentration steps. The remaining fractions were stored at -20 °C. These were tested later for dirigent proteins (IV.5.10.c).

The pooled peroxidase-positive fractions were concentrated with Centricon® tubes. Afterwards, the concentrated protein solution (250-500 µl) was loaded to a capillary loop connected to the ÄKTA purifier system. The proteins were separated by molecular mass with help of a gel filtration column (IV.5.2.b). The resulting fractions were again tested for peroxidase activity (IV.5.10.f). The active fraction(s) were stored on ice. With help of the established calibration curve the approximate molecular mass of the protein could be determined. Determination of the protein concentration was carried out in a modified form of the Bradford assay (IV.5.6).

4.6 Enzyme assays

Initially, the purified peroxidases of the three respective types of suspension cultures, extracted from either the cells or the corresponding medium, were tested in standard enzyme assays (IV.5.10.a) for possible differences in their activity or even regiospecificity.

Afterwards, the fractions gained during the separation with the anion exchange column were tested for dirigent proteins, possibly regulating the coupling reaction between taxifolin and coniferyl alcohol. This might manifest in either quantity shifts of the individual flavonolignan regioisomers or even only changes in the diastereomeric distribution of silybin or isosilybin. With this in mind, several enzyme assays were carried out as described in IV.5.10.c.

5. Enzymology

5.1 Plant enzyme sources

a. Crude extracts

An easy way to gain active protein is the extraction from shredded fresh plant suspension cells, even though naturally a mixture of all present enzymes and proteins is the result. For that purpose 5 g fresh, medium-free cells were weighed into a plastic centrifuge tube and 5 ml Tris(hydroxymethyl)aminomethane-HCl buffer (0.05 mol l^{-1} pH 10.0 with 1 mmol l^{-1} DTT) and 1 g Polyclar 10 (1/5 of the cell weight) were added. These quantities could also be adjusted by the amount of available cells. The mixture was stirred and homogenised three times with a blender (UltraTurrax), using a protocol of 30 sec of blending, followed by 30 sec of cooling on ice. This homogenate was centrifuged at 10.000 g at 4 °C for 20 min. The supernatant was decanted and desalted with help of PD-10 columns (IV.5.4). Most analyses of enzyme parameters were performed with protein preparations from cultures, which were grown in B5 medium until day 7. In case of the culture characterisation a sample was prepared every day (IV.2.2). Since fewer cells were available in the first few days and the determination of the silymarin content had priority, this also could have negative effects on the respective activity of the crude extract. Otherwise, exactly 5 g cells were used.

b. Medium

In order to use the medium from the same B5 cell cultures as an enzyme source it had to be properly cleaned up. For this purpose, all cells were separated from the medium in the first step. This was done by repeated vacuum filtration through a membrane filter with 0.8 µm pore size and subsequent centrifugation at 4 °C and 8.000 g for 5 min. This was followed by a desalting step with PD-10 columns (IV.5.4). Afterwards, the samples could be used as an enzyme source for assays (IV.5.10).

Frequently, the obtained samples were further concentrated for increased enzyme activity via Centricon® tubes (IV.5.3.c). Most medium samples were concentrated 10-fold. This also enabled the visualisation of enzymes after additional TCA precipitation (IV.5.3.b) and SDS-PAGE separation. In addition, the entire medium from two combined flasks could be loaded into the super loop of the FPLC system in one go for further fractionation and purification (IV.5.2).

c. Cell wall protein extraction (CWPE)

In order to obtain typically secreted proteins that are still attached to the cell walls, suspension cells of the three different origins (IV.4.3) were treated successively with increasing concentrations of an ionic solution according to Pickel (2011). For this purpose, the cells were first separated completely from the medium by sucking off through a membrane filter with 0.8 µm pore size. About 40 g cell fresh weight from two flasks per cultivar was thus obtained as starting material. Then shaking at 4 °C and 200 rpm in extraction buffer A (half of the culture volume) for 15 min and repeated separation bz vacuum filtration followed. The aqueous solution was saved and the remaining cells were extracted again in buffer B under the same conditions for quantitative extraction. Both cell free solutions then were combined and 10-fold concentrated with the help of Centricon® tubes (IV.5.3.c) before application in enzyme assays or additional concentration by TCA precipitation (IV.5.3.b) for SDS gel electrophoresis.

Extraction buffer A	Extraction buffer B	0.5 M KPi (pH 6.0, 100 ml)
0.1 M KPi (pH 6.0)	0.1 M KPi (pH 6.0)	87.7 ml 0.5 M KH_2PO_4
75 mM KCl	150 mM KCl	12.3 ml 0.5 M K_2HPO_4

In the further course a hybrid extraction method according to Printz et al. (2015), has been tried as well. In this protocol, adapted from Verdonk et al. (2012) and Feiz et al. (2006), a sequential three-step extraction based on low ionic strength buffers with additional $CaCl_2$, EGTA, and LiCl is described. Using this procedure should lead to good purity of the wall fraction and a high yield of protein extraction. In our special case, however, since this method did not lead to particularly good results, a further detailed explanation of the experiment will be omitted.

5.2 Fast protein liquid chromatography (FPLC)

Another option to gain high recovery rates of the protein(s) of choice is the additional purification and separation by chromatographic methods, e.g. ion exchange chromatography, followed by size exclusion chromatography. Ideally, a chromatography system like ÄKTA, designed for increased productivity and reliable results, is used for that purpose. This chromatography system functions according to similar principles as in high-performance liquid chromatography (HPLC). Different affinities of various components for two materials enable focused separation. Here as well, a mobile phase (buffer) and a stationary phase (column packing material) ensure fractionation.

a. Ion exchange chromatography (IEC)

Ion exchange chromatography belongs to the most common chromatographic methods for protein purification. The proteins of interest, dissolved in the running buffer, will bind to the resin due to their charge. Non-binding components will be washed out. The elution buffer then increases in its ionic strength (e.g. salt concentration, gradient), slowly displacing the bound proteins. Depending on the ionic strength of the protein, they dissociate sooner or later and can be collected in fractions in the eluent. Conductivity (salt concentration), pH and UV-absorption at 280 nm (protein concentration) can be monitored by appropriate detectors.

For membrane-bound or secretory peroxidases, fractionation by anion exchange chromatography (AEC) seemed appropriate. Other groups already achieved good results with this kind of separation (Al-Senaidy and Ismael, 2011; Pérez-Galende et al., 2014). All runs with the ÄKTA-purifier system were carried out at room temperature. Buffers were also brought to room temperature beforehand and filtered particle-free.

The sterile-filtered protein extracts (IV.5.1) were applied to a Q-Sepharose column (1.6 cm x 11 cm) equilibrated with equilibration buffer (0.05 M TRIS–HCl, pH 8.0) at a flow rate of 1 ml min^{-1}. Q-Sepharose is a strong anion exchange material. Protein samples were loaded to the system with help of a super loop (max. 10 ml) at a flow rate of 0.5 ml min^{-1}. Subsequently, the column was washed with one column volume of equilibration buffer. Proteins were eluted with two column volumes of a linear gradient of 0-0.5 M NaCl in equilibration buffer. 2 ml fractions were collected and placed on ice for activity tests.

b. Size exclusion chromatography (SEC)

Size exclusion chromatography separates proteins almost only by their size or rather molecular weight. In addition, form factors such as the three-dimensional structure and glycosylations can make slight differences. The column (stationary phase), usually consists of very small porous polymer beads. Typically the proteins are transported through an aqueous phase (gel filtration chromatography). Whereas smaller proteins get entangled in the pores, bigger molecules cannot enter and simply pass by. The larger the protein, the shorter its retention time and vice versa.

In addition to its short and clearly defined separation times another benefit is the possibility to determine the approximate molecular weight of the protein of interest with help of calibration proteins.

For further purification, the peroxidase-positive protein samples from AEC were pooled and concentrated by ultrafiltration (Centricon®). Concentrated enzyme solutions were loaded onto a gel filtration column (Superdex™ 200 Increase, 10/300) previously equilibrated with 0.01 M NaH_2PO_4, 0.14 M NaCl buffer, pH 7.4 at a flow rate of 0.75 ml min^{-1} via capillary loop (200-500 µl). Elution was carried out in the same buffer and at the same flow rate. Fractionation started after 0.3 column volumes. Fractions (2 ml) were collected, placed on ice and tested for peroxidase activity. Only the most active fraction was used as enzyme source for further experiments.

The approximate molecular weight of the protein was determined with the aid of a previously established calibration curve with proteins of known mass. For this purpose, proteins of different molecular weights are loaded to the gel filtration column under the same running conditions as described in the size exclusion method above. The calibration proteins were transferrin (80 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (26 kDa) and cytochrome c (12.3 kDa). These were adjusted to 1 mg ml^{-1} each and 200 µl of the mixed solution was loaded to the gel filtration column via capillary loop. The individual substances can then be assigned to a respective fraction by UV-absorption. The molecular weight of the unknown protein can then be determined by its elution time.

c. Purification of histidine-tagged proteins

The purification of histidine-tagged recombinant proteins can also be performed by FPLC. It is also known as immobilised metal ion affinity chromatography (IMAC). The polyhistidine tag of the protein, usually consisting of six histidine residues (His) linked to the *N*- or *C*-terminus of the protein, forms a reversible complex with immobilised nickel atoms of a Ni-Sepharose column. With increasing imidazole concentration of the elution buffer, it is displaced and washed out. This leads to a selective separation of the target protein from non-tagged proteins. After detection via UV-absorption it can be easily fractionated and collected for further studies.

The sterile-filtered protein solution in His-binding buffer (3 ml) was injected to the column (HisTrap™ FF, bed volume 1 ml) via a capillary loop (500 µl) in several steps at a flow rate of 0.25 ml min^{-1}. The flow-through was collected. Before, the column had been equilibrated with binding buffer (0.02 M NaPi, 0.5 M NaCl, 0.02 M imidazole, pH 7.4) at a flow rate of 1 ml min^{-1} until conductivity and absorption were constant. After repeated washing with binding buffer until constant values were reached the elution step was initiated. This was done with a linear gradient of increasing concentration of the elution buffer (0.02 M NaPi, 0.5 M NaCl, 0.25 M imidazole, pH 7.4) at a flow rate of 1 ml min^{-1}. At the same time, 2 ml fractions were collected. Eluting protein was detected by UV-absorption (280 nm) and subjected to activity assays (IV.5.10).

Purification of His-tagged proteins can also be done by gravity elution from self-packed columns. 0.5-1.0 ml of a suspension of nickel-NTA(nitrilotriacetic acid) material were loaded into an empty disposable column and washed with H_2O several times. 1 ml of His-Tag binding buffer (50 mM KPi, 300 mM NaCl, 10 mM imidazole pH 8.0) was added and the nickel-NTA equilibrated for 30 min and then drained. 500 mM KPi, 3 M NaCl, 100 mM imidazole pH 8.0 in a 1:10 ratio was added to the protein sample and mixed with the column material. The column was sealed tightly, placed in ice and rocked gently for 1 h. Afterwards the fluid was drained, mixed and 200 µl kept for SDS-PAGE (flow-through). The column material was washed eight times with 2 ml His-Tag washing buffer 1 (50 mM KPi, 300 mM NaCl, 20 mM imidazole pH 8.0) in order to remove unbound proteins. If required, two additional washing steps with 1 ml washing buffer 2 (50 mM KPi, 300 mM NaCl, 50 mM imidazole pH 8.0) were added. Aliquots of the washing fractions were saved for SDS-PAGE analysis. The elution of bound proteins was done in three steps with 1 ml elution buffer (50 mM KPi, 300 mM NaCl, 250 mM imidazole pH 8.0) each. These fractions were combined and desalted with PD-10 columns. The nickel-NTA-column

was cleaned with 1 ml 10% AcOH and washed with H_2O several times. Until further use, the columns were stored at 4 °C in 1 ml 20% EtOH.

5.3 Concentration of protein solutions

a. Ammonium sulphate precipitation

An ammonium sulphate precipitation is an effective and simple method for enrichment and partial purification of proteins (Cooper, 1981). In this process, salt ions compete with the hydration shell of the hydrated protein. The ammonium sulphate concentration affects the protein surface charge at which the protein is dehydrated to a sufficient degree for precipitation. Hydrophobic proteins precipitate faster (earlier) than hydrophilic ones. This method can either be used for the precipitation of all proteins present in the solution or for fractionation by using different ammonium sulphate saturation steps. This can result in a partial purification of the protein of interest.

An ice-cold saturated ammonium sulphate solution is needed. The pH of the solution should correspond to the sample pH in order to prevent pH change during the salting-out process. In these experiments pH 10 was used. At first, the volume of the crude protein extract was defined to determine the required amount of saturated ammonium sulphate solution. The sample was stirred on ice. Within 20 min the required volume of ammonium sulphate solution was added dropwise to the crude extract. After mixing another 20 min on ice, centrifugation at 4 °C 30.000 g for 20 min followed. Pellet and supernatant were separated. The pellet was dissolved in 3.0 ml 0.05 M TRIS-HCl buffer pH 10 and stored at -20 °C. The supernatant could be used for further precipitations.

The following precipitation steps have been performed:

Saturation of ammonium sulphate:	0 – 20%
	20 – 40%
	40 – 60%
	60 – 80%

The obtained fractions were desalted with a PD-10 column (IV.5.4). For total precipitation of all proteins at once the ammonium sulphate concentration was adjusted to 80% saturation.

b. Trichloroacetic acid (TCA) precipitation

Precipitation with trichloroacetic acid (TCA) is a simple and quick way to gain concentrated denatured proteins. This can be useful for SDS gel electrophoresis or sample preparation prior to mass spectrometry.

The sample solution was mixed with the same volume of 40% TCA (final concentration 20%) and incubated on ice for 1 h. After centrifugation at 4 °C and 13.000 g for 20 min, the pellet was washed with ice-cold 80% acetone and the solvent evaporated. Finally, the pellet was dissolved in 0.1 M $NaHCO_3$ with 10% sucrose or another suitable buffer for further investigations.

c. Concentration by centrifugal filters

Centrifugal filters (e.g. Centricon®) can be used for protein purification. On the one hand these filter systems reduce the volume of protein solutions while increasing the protein concentration and on the other hand can remove or replace buffer ingredients.

For example, 5 day old habituated medium (B5) from suspension cultures of *Silybum marianum* was concentrated. For this, two Centricons® were washed with 14 ml dist. H_2O and repeatedly filled with the same amount of particle-free medium (IV.5.1.b), whirling up the liquid with a pipette from time to time. In the end, the media samples were concentrated to 5 ml, aliquoted, desalted with a PD-10 column and stored at -20 °C. All centrifugation steps were performed at 4 °C for 15-30 min at 8.000 g. The concentrated protein samples could be applied in enzyme assays.

Furtermore, these concentrated protein samples could also be used for further purification and fractionation steps by means of FPLC (IV.5.2). In doing so, the desalting step was omitted.

5.4 Desalting via PD-10 columns

All protein extracts, except the ones purified with the FPLC system, were desalted with PD-10 columns. It was done based on the manufacturer's instructions (GE Healthcare). 25 ml TRIS-HCl pH 8-10 buffer with 1 mM DTT were used to equilibrate the column with elution buffer, followed by addition of 2.5 ml protein sample and discarding the flow-through. In the last step the PD-10 column was eluted with 3.5 ml buffer, while collecting the flow-through. Afterwards the columns were washed with 25 ml H_2O and stored at 4 °C until further usage. The collected protein fractions were aliquoted and stored at -20 °C.

5.5 Isolation of recombinant proteins

a. *E. coli* strains (genetically modified)

Proteins were expressed overnight in *E. coli* BL21(DE3)pLysS and BL21-CodonPlus (DE3)-RIPL strains (IV.7.7.a). The bacterial cultures were transferred in portions into 50 ml falcon tubes and centrifuged at 4 °C for 5 min at 3.000 g. The supernatant was discarded, the pellet frozen in liquid N_2 and stored at -80 °C. The cells were suspended in TRIS-HCl buffer pH 8.5 (4 ml per 1 g pellet), in case of the second strain mixed with lysozyme (1 mg ml^{-1}), and thawed on ice for about 30 min. Homogenisation was done on ice with a sonifier with an intensity setting of 100% and 0.3 amplitude for 60 sec each. Afterwards the homogenates were centrifuged at 4 °C and 6.000 g for 10 min. The supernatant was used for purification with His-Tag columns (IV.5.2.c). In each case a sample of the pellet (after soluble protein extraction) was re-dissolved in H_2O and used as the insoluble fraction for SDS-PAGE (IV.5.8), likewise 20 µl of the supernatant was used as the soluble fraction in SDS-PAGE.

b. *Pichia pastoris* / *Saccharomyces cerevisiae* strains (genetically modified)

Proteins were synthesised in either *P. pastoris* X-33 or *S. cerevisiae* (CB018, InvSc1) for 12-72 h. The cultures were transferred in portions into 50 ml Falcon tubes and centrifuged at 4 °C for 5 min at 1.500 g. The cell pellet was frozen in liquid N_2 and stored at -80 °C. In case of *P. pastoris* the supernatant was saved for precipitation with ammonium sulphate with subsequent PD-10 desalting. For disintegration the cells were resuspended in 1 ml breaking buffer (50 mM Na_3PO_4, 1 mM EDTA, 5% glycerol, 10 mM imidazole and 1 mM PMSF, pH 8.5) per 1 g cell pellet, thawed on ice and centrifuged at 4 °C for 5 min at 1.500 g. After decanting the supernatant the pellet was resuspended in breaking buffer (1 µl mg^{-1}) and the same volume acid-washed glass beads (~0.5 mm ø) added. Disintegration was done by vigorously shaking in a benchtop homogeniser (Minilys®) at 4.000 rpm for 30 sec, followed by cooling on ice for 30 sec and 2-3 repetitions. Afterwards the suspensions were centrifuged at 4 °C and 13.000 g for 10 min and the supernatant transferred to new tubes. In each case a sample of the pellet was resuspended in H_2O and used as the insoluble fraction for SDS-PAGE. Likewise 20 µl of the supernatant was used as the soluble fraction.

5.6 Determination of protein concentration

Protein concentration was measured by using an assay according to Bradford (1972). It serves for the photometric quantification of a protein solution using calibration with a protein of known concentration. The Bradford solution is composed as follows:

Coomassie Brilliant Blue G250	100 mg
96% ethanol	50 ml
85% *o*-phosphoric acid	100 ml
Dist. H_2O	850 ml

The solution was filtered twice and stored at 4 °C until used. 20 µl of a 1:1 diluted sample (with TRIS-HCl buffer pH 10.0) was incubated with 2 ml Bradford reagent in disposable macro cuvettes for 15 min. Absorbance was measured at 595 nm against Bradford reagent with 20 µl

buffer as reference value. Calibration was performed with standard samples of 0.25, 0.5 and 1 mg ml^{-1} bovine serum albumin (BSA) measured in duplicate. The protein concentration of the unknown sample was calculated with help of the straight calibration line.

In the case of very small amounts of protein (e.g. proteins purified by FPLC), the test had to be slightly modified. Here 50 µl of the protein sample were used. Accordingly, the calibration curve had to be adjusted with appropriately diluted BSA standards (1:50).

5.7 Protoplast isolation

Protoplasts are cells whose cell wall has been removed with the help of enzymes. In case of plants, cellulase digests the cellulose of the cell walls and pectinase decomposes the pectin of the middle lamella. Without cell wall the resulting protoplasts are of spherical shape. To prove the hypothesis that silymarin mostly is stored in the intact cell walls of suspension cells and not the protoplast, a complete digestion of the outer layer should lead to increased amounts of silymarin in the corresponding medium, whereas the protoplasts should be largely free of silymarin. Otherwise the result would indicate a presence of flavonolignans and precursors in the interior of the cells.

For protoplast isolation suspensions cells of *Silybum marianum* (5-8 days old) were filtered and squeezed through a tea strainer. 5 g drained cells were weighed and suspended in 50 ml protoplast medium "P5" [B5 with extra 0.5 M mannitol (4.57 g) and 1.4 times the amount of Ca^{2+} (210 mg l^{-1} $CaCl_2 \cdot 2\ H_2O$)] with 2% cellulase "Rohament CT" (1 g) and 0.8% pectinase "Rohament P" (0.4 g). Cell wall digestion proceeded in darkness at 25 °C on a shaker (100 rpm). After 24 h the suspension was filtered through nylon tissue for removal of possible cell aggregates. All further steps were performed at 4 °C. Protoplasts were sedimented by centrifugation at 100 g for 5 min. The supernatant was carefully removed with a glass pipette. The sediment was resuspended in enzyme-free protoplast medium. This washing process was repeated three times. Finally protoplasts were suspended in 1-2 ml "P5".

Protoplasts were examined with a microscope and looked similar to plant protoplasts isolated by other groups.

5.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins according to their molecular weight, based on their differential rates of migration through a sieving matrix (a gel) under the influence of an applied electrical field. SDS (sodium dodecyl sulphate) is a detergent that is present in the SDS-PAGE sample where, after boiling in presence of a reducing agent (β-mercaptoethanol), it disrupts the tertiary structure of proteins. This brings the folded proteins down to linear molecules. It also coats the protein with a uniform negative charge, which masks the intrinsic charges of the protein. SDS binds fairly uniformly to the linear proteins (around 1.4 g SDS per 1 g protein), meaning that the charge of the protein is now approximately proportional to its molecular weight. SDS is also present in the gel and the electrophoresis buffer.

The system consisted of a lower separating gel and an upper stacking gel layer, called a discontinuous system (Laemmli, 1970). The gel formation takes place by a radical reaction mechanism. The polymerisation starter ammonium persulphate (APS), together with tetramethylethylenediamine (TEMED) as a catalyst, leads to polymerisation of the acrylamide/bisacrylamide (37.5:1) mixture.

First, the glass plates were thoroughly cleaned with 70% EtOH and placed into the gadget with an appropriate spacer in between. Subsequently the separating gel was mixed and quickly poured between the glass plates (see table for composition). This was directly overlaid with a small layer of *n*-butanol to straighten the line between separating and stacking gel. This was removed after polymerisation of the separating gel. Then the stacking gel was mixed and poured on top of the separating gel layer and a comb for ten pockets added. After complete polymerisation the gel was fixed in the electrophoresis chamber and the buffer reservoir filled with running buffer (192 mM glycine, 25 mM TRIS und 0.1% SDS, pH 5.3). The comb was removed carefully and the pockets repeatedly cleaned with running buffer to remove gel residues or air bubbles.

Pipetting scheme for two separating and stacking gels:

	separating gel		stacking gel	
TRIS-HCl-buffer	pH 8.8, 1.5 M	2.50 ml	pH 6.8, 0.5 M	1.25 ml
(Bis-)/Acrylamide (30%)		4.10 ml		0.75 ml
Dist. H_2O		2.90 ml		2.80 ml
SDS (10%)		400 µl		200 µl
TEMED		16 µl		10 µl
APS (10 %)		70 µl		40 µl

Samples from yeast expression cultures (IV.7.7) were centrifuged for two min at 8.000 g before the obtained pellet was re-dissolved in 100 µl 0.2 M NaOH and incubated for at least 30 min at room temperature. After centrifugation at 8.000 g for two min pellets were re-dissolved in 20 µl Roti-Load™ and heated to 95 °C for 10 min before they were carefully pipetted into the gel pockets. Fractions after His-Tag purification (IV.5.2.c) of recombinant protein extracts or (concentrated) cell suspension media (20 µl) samples were directly mixed with 10 µl Roti-Load™ and denatured under the same conditions. Running times for the gels were between 1.5 and 2 h at 150 V and 100 mA. After the blue band (bromophenol blue) crossed the lower edge of the gel, the run was finished. As a standard, size markers between 10-250 kDa were used.

a. Coomassie-Brilliant Blue staining

After electrophoresis the stacking gel part was carefully removed and the separating gel stained with 0.3 mM Coomassie-Brilliant Blue R-250 in MeOH:AcOH:H_2O (4.5:1:4.5; v/v) for 0.5-1 h; short heating in the microwave at 800 W could accelerate this process. The subsequent double destaining process was done in the same solvent mixture (without Coomassie reagent) for 1.5-2 h until the optimal result was achieved. Gels were finally wrapped in plastic film, photographed and digitalised for later evaluation.

b. Silver staining

If staining with Coomassie reagent was not sensitive enough, the gels were additionally stained with silver.

After completely destaining the Coomassie stained gel in MeOH-AcOH-H_2O (4:1:5; v/v), silver staining was done according to the Silver Stain Plus™ kit manual from Bio-Rad. It describes a simple four-step process, starting with fixation in an enhancer solution for 20 min with subsequent washing in frequently renewed dist. H_2O for 20-30 min. The following staining and developing step was done in a freshly prepared staining solution, containing the silver complex, a reduction moderator, an image development reagent and Na_2CO_3 as an accelerator. As soon as the desired staining intensity was reached, usually after 10-20 min, the reaction was stopped in 5% AcOH for at least 15 min and then washed in dist. H_2O. Silver stained gels then were wrapped in plastic film, photographed and digitalised for later evaluation. In some cases, bands were cut out accurately and used for protein identification after tryptic digestion. The determination was done by mass spectrometry.

5.9 Western Blot

Western blotting, also known as protein immunoblot, is a core technique in cell and molecular biology. It is used to detect the presence of a specific protein in a complex mixture extracted from cells. The separation by size using gel electrophoresis, efficient transfer to the surface of a membrane and the specific detection of a target protein by specific antibodies belongs to the three key elements of this technique. The target protein can be visualised as band on the blotting membrane with help of colour or chemoluminescence detection systems.

After successful SDS-PAGE the gel was removed from the chamber and the stacking gel part detached, followed by equilibration in transfer buffer (Towbin buffer; 25 mM TRIS, 192 mM glycine, pH 8.3, 20% MeOH) for 10-30 min.

Pre-cut filter paper and the sponge material for the blotting chamber were briefly wetted in transfer buffer. The membrane (PVDF, Immobilion™-P from Millipore; 0.45 µm pore size) was put in MeOH for about 15 sec, washed in dist. H_2O for 2 min and equilibrated in transfer buffer for at least 5 min. The single layers were assembled in the correct order, air pockets removed and

installed in a tank blotting system filled with transfer buffer. After blotting for 1.5 h at 100 V the membrane was carefully removed and analysed by immunodetection. In the later course of this work, a semi-dry blotting system was used. For this, blotting was done with a constant current, depending on the membrane size (length*width*2 mA), for about 1 h.

The membrane was washed three times in TBS buffer (10 mM TRIS-HCl, 0.9% NaCl, pH 7.4) for 5 min. The used SDS-gel was dyed as described in IV.5.8, to check for transfer efficiency. Blocking of the membrane was achieved in TBS-T buffer (10 mM TRIS-HCl, 0.9% NaCl, 0.05% Tween 20, pH 7.4) with 5% milk powder for 1.5 h, followed by two washing steps in TBS-T for 5 min. After that the membrane was incubated with the first antibody (Pierce™ 6x-His Epitope Tag Antibody), diluted 1:10000 in TBS-T with 1% milk powder overnight at 4 °C. The next day the membrane was washed eight times for 5 min in TBS-T and then incubated with the second antibody (anti-mouse IgG alkaline phosphatase), diluted 1:20000 in TBS-T for 1 h at room temperature. Finally the membrane was washed five times in TBS-T for 5 min and was dyed with freshly prepared NBT-BCIP solution. The membrane was equilibrated in substrate buffer (100 M TRIS-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) for 5 min and then dyed in 10 ml substrate buffer mixed with 80 µl BCIP (20 mg/ml in 100% dimethylformamide) and 60 µl NBT (50 mg/ml in 70% dimethylformamide) for 15-30 min and finally stopped by washing in dist. H_2O for three times. This detection method leads to an intense deep purple coloration when it reacts with alkaline phosphatase. The alkaline phosphatase catalyses the cleavage of a phosphatase group of BCIP to yield an indigo dye. In turn, the indoxyl group reduces a tetrazolium salt (NBT) to produce an insoluble formazan that combines with the indigo dye to form a colored precipitate (Altmann, 1976).

In order to check the protein transfer the membrane was dyed with amido black staining solution (0.1% in MeOH:AcOH:H_2O, 4:1:5) for 10 sec with subsequent destaining in MeOH:AcOH:H_2O (4.5:1:4.5) for 10-15 min.

5.10 Enzyme assays

In vitro enzyme activity tests were performed in the course of this work mainly as endpoint assays, which in turn were measured by HPLC. The reaction is terminated after a certain incubation period and the resulting product or reporter molecule can be detected. Assays were directly stopped with 6 N HCl and put on ice as zero control samples. Furthermore, boiling (95 °C for 10 min) or omitting the enzyme can provide information about the actual involvement of an enzyme responsible for the formation of the investigated product.

Enzyme assays were performed in duplicate. The assay components were pipetted into 1.5 ml test tubes as described below.

a. Standard assays

10 mM taxifolin (in 20% EtOH)	25 µl
10 mM coniferyl alcohol (in 20% EtOH)	25 µl
0.03% H_2O_2	25 µl
Enzyme solution	5-15 µl
0.05 mol l^{-1} TRIS-HCl buffer pH 10.0	*ad* 400 µl

The reaction was started with addition of the enzyme, immediate shaking and incubation at 30 °C and 600 rpm in an Eppendorf Thermomixer® for 15 min. Addition of 75 µl 6 N HCl and shaking stopped the reaction. Afterwards the products were extracted twice with 500 µl EtOAc each. The combined EtOAc extracts were evaporated under the hood overnight or in a centrifugal evaporator (SpeedVac) and the dry residues re-dissolved in 150 µl of the HPLC solvent mixture and centrifuged at 16.000 g for 5 min prior to HPLC analysis.

b. Modified assays

For the determination of the optimal reaction conditions different pH values, reaction times and enzyme quantities were tested. Additionally, modified substrate concentrations and ratios (taxifolin:coniferyl alcohol 1:4, 1:1 and 4:1) have been applied in enzyme assays to check for possible effects on the flavonolignan composition. Later, assays were also performed in a 3:1 ratio in favor of taxifolin, since they already yielded well-visible flavonolignan peaks in the evaluation. Possible differences based on variations between the enzyme sources or the varying preparation methods have been investigated in numerous experiments as well. Including mainly preparations from suspensions cells (crude extracts), the used medium from suspension cultures or extracts purified or concentrated with the help of Centricon® tubes or chromatographic methods.

Additionally, different commercially available enzymes (peroxidase from horseradish (*Armoracia rusticana*), laccase from Chinese lacquer tree (*Rhus verniciflua*); both 1 mg ml^{-1} in 0.1 M KPi buffer pH 7.0) or reagents [silver(I)oxide (Ag_2O)] (Althagafy et al., 2013) for an enzyme-free coupling reaction were tested for their effect on the formation of flavonolignans. Assay conditions remained the same except for Ag_2O where 15 µl 100 mM Ag_2O in conc. NH_3 were applied and the reaction was stopped with additional freezing in liquid N_2.

c. Assays for chromatography fractions

The protein samples extracted from the suspension cells of *Silybum marianum* were fractionated via AEC (IV.5.2.a). This applies to the crude extracts and the respective concentrated medium of the cell cultures specially established for this purpose, in which the differences in the distribution of positional isomers were most striking. Every second fraction eluting from the column was tested for the possible presence of dirigent proteins and analysed by HPLC (IV.5.13). As enzyme source *Silybum marianum* peroxidase after purification by SEC (IV.5.2.b) was used.

The enzyme assay was handled as described above and consisted of:

10 mM taxifolin (in 20% EtOH)	75 µl
10 mM coniferyl alcohol (in 20% EtOH)	25 µl
0.03% H_2O_2	25 µl
Peroxidase (after SEC)	10 µl
Fraction from AEC (2, 4, 6…)	15 µl
0.05 mol l^{-1} TRIS-HCl buffer pH 10.0	*ad* 400 µl

d. Inhibition assays

Another approach to obtain information on possibly involved proteins are experiments with inhibitors. Finding and applying specific inhibitors for each enzyme type might give information about the responsible enzyme type in protein extracts from *Silybum marianum*.

Laccases can be very strongly inhibited by various reagents. Commonly used inhibitors are dithiothreitol (DTT), thioglycolic acid (TGA), cysteine, diethyldithiocarbamic acid (DDC), EDTA, sodium fluoride and sodium azide (NaN_3), but also kojic acid or hydroxyglycine have been reported to inhibit laccases (Johannes and Majcherczyk, 2000; Alcalde, 2007).

Peroxidases are inhibited by sodium azide, cyanide, L-cystine, dichromate, ethylene thiourea, hydroxylamine, sulfide, vanadate, 4-aminobenzoic acid and several metallic cations (Sigma-Aldrich database).

Inhibitors were dissolved in 1 ml dist. H_2O and adjusted to a concentration of 10 mM:

compound	**abbreviation**
Dithiothreitol	DTT
Ethylenediaminetetraacetic acid	EDTA
Potassium fluoride	KF
Potassium ferricyanide	$K_3Fe(CN)_6$
Tetrabutylammonium hydrogen sulphate	$C_{16}H_{36}N^+HSO^{4-}$

Cobalt chloride	$CoCl_2$
Tin(II) chloride	$SnCl_2$
Zinc chloride	$ZnCl_2$
Kojic acid	KA
4-Aminobenzoic acid	PABA

Inhibition assays were performed as described above with crude extract or laccase (1 mg ml^{-1}) / peroxidase (1 mg ml^{-1}) at a reaction time of 15 min:

10 mM taxifolin (in 20% EtOH)	25 µl
10 mM coniferyl alcohol (in 20% EtOH)	25 µl
0.03% H_2O_2	25 µl
Peroxidase, laccase or crude extract	15 µl
Inhibitor (1.25 mM final conc.)	50 µl
0.05 mol l^{-1} TRIS-HCl buffer pH 10.0	*ad* 400 µl

e. Removal or omission of electron donors

Laccases require oxygen as second substrate in order to catalyse one electron oxidations of phenolic substrates for radical formation. Oxygen is present in every standard enzyme assay, thus no further addition is necessary.

To get an idea about the possible enzyme(s) responsible for linking taxifolin and coniferyl alcohol in *Silybum marianum* it is good to know which electron donor is required. Therefore enzyme assays were performed in which we tried to remove the electron donor (O_2). This was achieved by the reaction of glucose oxidase in a glucose-containing buffer system (15% D-(+)-glucose in TRIS-HCl buffer) without shaking for 3 min before the actual assay was performed.

$$\text{ß-D-glucose} + O_2 + H_2O \xrightarrow{glucose\ oxidase} \text{D-gluconic acid} + H_2O_2$$

Glucose oxidase was dissolved in 1 ml 0.05 mol l^{-1} TRIS-HCl buffer and adjusted to 266.6 U μl^{-1}.

Assays were performed as described above with a reaction time of 20 min:

10 mM taxifolin (in 20% EtOH)	25 µl
10 mM coniferyl alcohol (in 20% EtOH)	25 µl
0.03% H_2O_2	25 µl
Laccase or crude extract	15 µl
Glucose oxidase (10 U final conc.)	15 µl
0.05 mol l^{-1} TRIS-HCl buffer pH 10.0	
(+ 15% D-(+)-glucose)	*ad* 400 µl

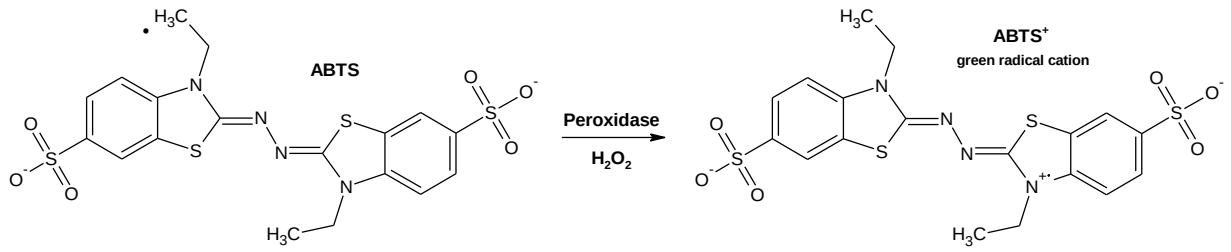
In the case of the reaction with a peroxidase no special pretreatment was necessary. Assays were done as always with one change: By omitting H_2O_2 the electron donor crucial for a peroxidase reaction was removed.

Additionally, substitution experiments with NADPH have been tried.

10 mM taxifolin (in 20% EtOH)	25 µl
10 mM coniferyl alcohol (in 20% EtOH)	25 µl
50 mM NADPH (5 mM final conc.)	40 µl
Peroxidase or crude extract	15 µl
0.05 mol l^{-1} TRIS-HCl buffer pH 10.0	*ad* 400 µl

f. Peroxidase activity assay

Oxidative enzymes like peroxidases can be easily identified and tested for their activity using colour reactions. In the presence of an electron donor (e.g. H_2O_2), these react with a specific substance, which leads to the change of colour due to shifts in its wavelength. Substances like DAB (3,3'-diaminobenzidine) or ABTS (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) are well suited for that purpose. In the case of ABTS, its oxidation leads to a color change from a bright transparent green to a deep dark green. This can then be measured photometrically and thus the activity can be determined. In our case, it usually was only important whether peroxidase activity could be detected at all, specific activity was rather incidental.



ABTS and H_2O_2 solutions had to be freshly prepared. The enzyme assay was composed of a 0.1 mol l^{-1} KH_2PO_4 buffer pH 5.0 with added ABTS (0.4 mM) and 0.01% H_2O_2. From this solution, 400 µl were added to test tubes for each sample to be analysed. Mainly samples resulting from chromatographic fractionation (IV.5.2) or protein samples from heterologous expression (IV.7.7) were tested using this method. The reaction was started with addition of 20 µl of the respective fraction or protein sample and vigorous shaking. A positive test for peroxidase activity could quickly be detected by the change of colour. After 10 min at the latest the assays were photographed and analysed.

5.11 Spectrophotometrical detection of silymarin

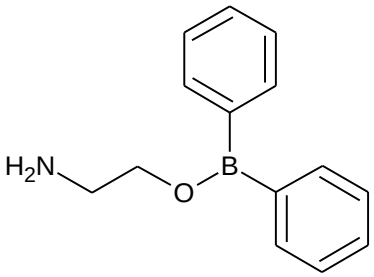
An attempt has been made to develop a quantitative optical test to measure the silymarin concentration formed in standard enzyme assays. Special attention was given to the distinct dark yellow colouration at the end of the reaction time. The assay components were pipetted into 1.5 ml test tubes as described below:

	1	2	3	4	5
10 mM taxifolin	25 µl	25 µl	0 µl	25 µl	0 µl
10 mM coniferyl alcohol	25 µl	0 µl	25 µl	0 µl	25 µl
0.03% H_2O_2	25 µl	25 µl	25 µl	25 µl	25 µl
Enzyme crude extract	5 µl	5 µl	5 µl	0 µl	0 µl
0.05 mol l^{-1} TRIS-HCl buffer pH 10.0	320 µl	345 µl	345 µl	350 µl	350 µl

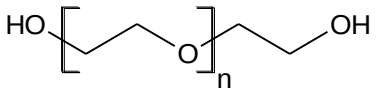
After 15 min reaction time and suitable dilution (1:10 or 1:5) with TRIS-HCl buffer pH 10.0 samples were measured photometrically in the UV-visible spectral range (185-700 nm). The same buffer acted as reference.

5.12 Thin-layer chromatography (TLC)

Extracts from different milk thistle plant parts and corresponding reference compounds were applied to a thin-layer plate coated with silica gel. Caffeic acid served for orientation. For this purpose a silica $60F_{254}$-plate with $CHCl_3$-acetone-FA (37.5:8.25:4.25, v/v) as mobile phase was used. Afterwards the thin-layer plate was sprayed with 1% diphenylboryloxyethylamine ("Naturstoffreagenz A") in MeOH with subsequent spraying with 5% polyethylene glycol (PEG 4000) in MeOH. The respective plant material was extracted very similar to that described in IV.4.2.



Diphenylboryloxyethylamine



Polyethylene glycol

10 µl of the following references dissolved in MeOH were applied at a time:

10 mM	silymarin mixture
10 mM	silydianin, silychristin, silybin or isosilybin
10 mM	taxifolin or coniferyl alcohol
555 µM	caffeic acid

The starting material included various parts of *Silybum marianum:*

0.5 g	lyophilised root cell cultures (MS-Li plates)
2.0 g	fresh leaves
1.0 g	whole unripe fruits (very early stage of development)
1.0 g	whole fully mature fruits
0.5 g	fruit coat (pericarp and testa) separated from the embryo
4.5 g	embryo separated from the fruit coat

5.13 High-performance liquid chromatography (HPLC)

HPLC measurements during the first years of this work were all performed under isocratic conditions. The composition of the mobile phase remained stable throughout the time. A mixture of 85% H_3PO_4–MeOH–H_2O (0.01:46.5:53.5, v/v) at a flow rate of 1 ml min^{-1} served as the mobile phase. The stationary phase was an Equisil ODS-column (4 mm ø, 250 mm length with 20 mm precolumn). Exactly 20 µl sample were injected onto the column by a sample loop. Flavonolignan elution was recorded at 288 nm for 40 min.

In the later course, flavonolignan analysis was carried out on a new HPLC system, including an autosampler and a column oven. Advantages were automated measurement, gradient elution and better reproducibility. The new system was capable of running complex gradients. Thus the run times could be halved but nevertheless more efficient separation of the individual silymarin peaks be achieved. Column, flow rate, injection quantity and recording wavelength remained the same.

The solvent gradient was as follows:

Time [min]	H_2O [%]	MeOH [%]
0	55	45
5	60	40
10	62.5	37.5
12	50	50
18	55	45
25	55	45

The two substrates coniferyl alcohol and taxifolin, as well as the flavonolignans silychristin, silydianin, silybin, isosilybin and a silymarin mixture at concentrations of 0.1 to 5 mM dissolved in eluent or MeOH were used as standards for identification and quantification.

6. Molecular biology

Molecular biological techniques were applied to isolate three types of cDNAs/genes: peroxidase (POD), laccase (LAC) and dirigent proteins (DIRs).

6.1 RNA extraction

Total RNA extracts from fresh fruits of *Silybum marianum* were prepared using a phenol-chloroform extraction essentially according to Chomczynski and Sacchi (1987).

All materials needed for RNA extraction were autoclaved twice or heated at 200 °C for 2 h. In addition, all working steps with RNA were performed with gloves. Unripe fruits collected and separated from the inflorescence of the plant growing in the old botanical garden of Marburg were frozen and pulverised in liquid N_2 in a mortar. It was crucial to keep all material cold to prevent any enzymatic activity, which could destroy the RNA. Approximately 50 mg of this powder was mixed and incubated at room temperature with 500 µl "Solution D", a 4 M guanidinium thiocyanate solution in 25 mM citrate buffer pH 7.0 and 0.5% laurylsarcosine. After adding 50 µl 2 M sodium acetate pH 4, the solution was mixed gently. In the next step 500 µl of phenol saturated with citrate buffer pH 2 was added. The content of the tube was mixed gently and 100 µl ice-cold chloroform were added. The reaction tube was vigorously mixed for 10 sec, incubated on ice for 15 min and centrifuged for 15 min at 12.000 g and 4 °C. 400 µl of the aqueous supernatant were transferred to a new tube and 400 µl ice-cold 2-propanol (1x volume) were added. The RNA was precipitated for 15 min at -20 °C with subsequent centrifugation for 10 min at 12.000 g and 4 °C. The supernatant was collected. 400 µl 70% EtOH (1x volume) were added to wash the pellet and the tube was centrifuged for 5 min at 7.500 g and 4 °C. After removing the supernatant, 400 µl 100% EtOH were added. Again the tube was centrifuged for 5 min at 7.500 g and 4 °C. The supernatant was removed and the remaining pellet was dried at 37 °C.

The RNA was dissolved in 20 µl dist. H_2O at 50 °C. The amount and purity of RNA was determined photometrically and the integrity was controlled on an agarose gel. Samples with high amount (> 3.000 ng μl^{-1}) of pure RNA (ratio of 260/280 nm > 1.6) were chosen for further experiments.

6.2 cDNA synthesis

The cDNA was synthesised with the RevertAid First Strand cDNA Synthesis Kit.

1 µg RNA and 1 µl oligo(dT)$_{18}$ primer were adjusted to a total volume of 12 µl with sterile H_2O in an Eppendorf® tube. The content of the tube was mixed gently and centrifuged for a few sec. It was heated in a water bath for 5 min at 70 °C and then cooled on ice. After short centrifugation, 4 µl 5x reaction buffer, 1 µl RiboLock RNAse inhibitor and 2 µl 10 mM dNTP mix were added. After gentle mixing and centrifugation, the tube was incubated at 37 °C for 5 min. 1 µl RevertAid reverse transcriptase was added and the tube was incubated for 1 h at 42 °C. The reaction was stopped by heating for 5 min at 70 °C.

6.3 Polymerase chain reaction (PCR)

a. PCR with degenerate primers

Since the genome of *Silybum marianum* is not sequenced yet, genes had to be identified by PCR (Mullis et al., 1987; Saiki et al., 1988). Initially degenerate primers were used for this purpose. They allow the recovery of genes from organisms where genomic information is not available. The primers were designed by aligning homologous gene sequences of other organisms from the NCBI database with help of a bioinformatics tool (CLC sequence viewer 7.6). Therefore, (partial) DNA sequences of similar proteins from preferably closely or distantly related organisms are listed vertically and compared with each other. When using enough sequences, regions with very high similarity can be identified. Assuming that the nucleic acid to be amplified also contains these conserved regions, it is possible to generate primer pairs that bind with a high probability to the unknown sequence.

In the case of the reference sequences, attention was first paid to as high as possible species relationship. Thus, all sequences coding for peroxidases originate from the family Asteraceae. For the laccase and dirigent protein sequences this was not feasible. Due to lack of sequences of closely related species, the required primers relied on distinct families with even some gymnosperms among them. All primers necessary for this work were ordered from Eurofins Genomics and can be found in the specific list (IV.9).

The gene library numbers of sequences used for the design of degenerate primers are as follows:

Peroxidase (POD):

Artemisia annua	(AY208699)
Cynara scolymus	(ADP37429; GU145301)
Helianthus annuus	(DQ889869)
Mikania micrantha	(FJ872511)
Senecio squalidus	(AJ810531)
Zinnia elegans	(AB023959; AJ880395)

Dirigent proteins (DIRs)

Dysosma tsayuensis	(DQ418765)
Forsythia x intermedia	(AF210061; AF210062)
Podophyllum peltatum	(AAK38666)
Schisandra chinensis	(ADR30610)
Sesamum indicum	(AY560651)
Sinopodophyllum hexandrum	(DQ414685)
Thuja plicata	(AAL92120; AF210064)

Laccase (LAC):

Glycine max	(AAM54731)
Medicago truncatula	(AES71245)
Pisum sativum	(ABC59623)
Rosa hybrid cultivar	(ACC78283)

A standard PCR protocol was used for a final volume of 25 µl:

cDNA	1.0 µl
GoTaq® buffer (5x)	5.0 µl
10 mM dNTP mix	0.5 µl
25 mM $MgCl_2$	3.0 µl
Primer 1 (10 or 100 mM)	0.5 µl
Primer 2 (10 or 100 mM)	0.5 µl
GoTaq® polymerase	0.1 µl
Dist. H_2O	14.4 µl

Usually a master mix was prepared for up to 5 samples. In some instances other polymerases were used as well, such as DreamTaq, PhusionTaq or HotStart/TrueStart Taq. Buffer and total volume were then adjusted accordingly. These ensured lower error rates due to advantages such as proofreading, high fidelity and good PCR specificity. The PCR cycles were the following:

1st cycle:	94 °C, 120 sec / GSP T_m -5 °C, 60 sec / 70 °C, 90 sec
2nd - 39th cycle:	94 °C, 30 sec / GSP T_m -5 °C, 60 sec / 70 °C, 90 sec
40th cycle:	94 °C, 60 sec / GSP T_m -5 °C, 60 sec / 70 °C, 10 min
End:	cooling at 6 °C.

T_M is the temperature at which binding of the primer to the melted template sequence is expected (primer hybridisation, "annealing"). Usually, the melting temperature T_m -5 °C was chosen as starting point with a gradient of up to 6 °C with steps of 1-2 °C above and below. The melting point of primers was calculated with a tool on the Eurofins website. In case of additional method-specific overhangs, these additional nucleotides were omitted in the calculation. Touchdown PCRs were performed as well. Here, a temperature just above T_M is chosen and the annealing temperature is reduced by 0.5 °C per cycle. This is repeated 10-15 cycles, followed by a further 20-30 cycles at a fixed annealing temperature T_m -5 °C. By doing this, a highly specific primer bonding to the sequence and efficient amplification could be expected.

The product of the PCR was analysed by agarose gel electrophoresis (IV.6.4). Products of the expected size were cut out and purified by gel extraction (IV.6.5).

b. RACE PCR

RACE (rapid amplification of cDNA ends) PCR is a complex method that allows identifying full-length sequences of a partially known gene with nontranslated regions. The 3' end is often generated via the poly(A) tail of the mRNA. For the usually more challenging 5' RACE, an adapter must be ligated to the 5' end of the mRNA.

Classical RACE PCR was performed according to a modified protocol of Scotto-Lavino et al. (2006a, 2006b). Unfortunately, this method only resulted in two incomplete 3’ ends without stop codon. The 5’ RACE method led to no results at all. Therefore, this method is only described briefly and will not be mentioned further.

To generate 3' end partial cDNA clones, mRNA is reverse transcribed using a 'hybrid' primer (Q_{total}; Q_T) that consists of two mixed bases (GATC or GAC followed by (T)17 followed by a unique 35-base oligonucleotide sequence (Q_I-Q_O). Amplification is then performed using a primer containing part of this sequence (Q_{outer}; Q_O), which now binds to each cDNA at its 3' end, and using a primer derived from the gene of interest (GSP1). A second set of amplification cycles is then carried out using 'nested' primers (Q_{inner}; Q_I) and GSP2 to quench the amplification of non-specific products.

To generate 5' end partial cDNA clones, reverse transcription (primer extension) is carried out by using a gene-specific primer (GSP-RT) to generate first-strand products. Following this, a poly(A) tail is appended using terminal deoxynucleotidyl transferase (TdT) and dATP. Amplification is then achieved using the hybrid primer Q_T to form the second strand of cDNA, the Q_O primer, and a GSP upstream of the one used for reverse transcription. Finally, a second set of PCR cycles is carried out using nested primers (Q_I and GSP2) to increase the yield of specific product.

SMART-RACE PCR is an important modification of the classic RACE PCR, whereas SMART stands for "switching mechanism at 5' end of RNA transcript". The experiments were carried out using the SMARTer® RACE 5'/3' Kit from Clontech (Chenchik et al., 1998; Bower and Johnston, 2010). A supplied special modified oligonucleotide and a reverse transcriptase should ensure better sensitivity and higher specificity.

The principle behind this is that the reverse transcriptase used, attaches a poly(C) tail to the 3' end of the cDNA (corresponding to the 5' end of the template RNA), which then serves as a template for a G-rich second primer. This second primer contains an adapter sequence, which is also used by the reverse transcriptase as a template for the same cDNA ("switching mechanism"). Thus, the completed cDNA contains two adapter regions, at the 3' and 5' ends. 5’ and 3’ gene-specific primers can then be used in long-distance PCR to generate full-length cDNA. Additionally, the gene specific primers of the kit contain an adapter sequence for easy incorporation into the included pRACE vector.

According to the SMARTer® RACE Kit protocol, 4 µl 5x First-Strand Buffer, 0.5 µl 100 mM DTT and 1 µl 20 mM dNTP mix were pipetted into a reaction tube (solution A). In another tube, 1 µl RNA, 1 µl 5' CDS primer A and 9 µl water were mixed. The reaction tube was incubated at 72 °C for 3 min, cooled for 2 min at 42 °C and finally put on ice. To this sample 1 µl SMARTER II A oligonucleotide was added (solution B). Solution A was mixed with 0.5 µl of RNase inhibitor and 2 µl SMARTScribe™ reverse transcriptase and then completely added to solution B. The reaction tube was incubated for 90 min at 42 °C and heated to 70 °C for 10 min afterwards. The 5' cDNA was diluted with 90 µl TE buffer.

The subsequent PCR had a total volume of 50 µl and was composed as follows:

SeqAmp buffer (2x)	25.0 µl
SeqAmp DNA Polymerase	1.0 µl
SMARTer RACE cDNA	2.5 µl
Universal Primer Mix (10x) [UPM]	5.0 µl
10 µM 5' or 3' GSP	1.0 µl
Dist. H_2O	15.5 µl

The gene specific primers (GSPs) had preferably a $T_M > 70$ °C, which enabled the use of touchdown PCR, but at least ≥ 65 °C. Furthermore, they had 15 bp overlaps with vector sequences at their 5' ends, necessary for successful In-Fusion cloning. The PCR cycles were the following:

Touchdown PCR (if GSP $T_M > 70$ °C):

1^{st} - 5^{th} cycle:	94 °C, 30 sec / 72 °C, 3min
6^{th} - 10^{th} cycle:	94 °C, 30 sec / 70 °C, 30 sec / 72 °C, 3 min
11^{th} - 40^{th} cycle:	94 °C, 30 sec / 68 °C, 30 sec / 73 °C, 3 min
End:	cooling at 6 °C.

Normal PCR (if GSP T_M between 60-70 °C)

1^{st} - 39^{th} cycle:	94 °C, 30 sec / GSP T_m - 5 °C, 30 sec / 72 °C, 3 min
40^{th} cycle:	94 °C, 30 sec / GSP T_m - 5 °C, 30 sec / 72 °C, 10 min
End:	cooling at 6 °C.

If necessary, the PCR product was diluted 1:100 with dist. H_2O or TE buffer and a nested PCR was performed. This method was used to generate the 5' ends of the DIRs. Otherwise difficulties in distinction would have been likely because of a high similarity of gene sequences. In that case, the included Nested Universal Primer (NUP) was used during PCR instead of the Universal primer mix (UPM). Furthermore, a second 5' GSP, binding further inside in the sequence, was applied. For amplification, the simpler PCR protocol was chosen.

The resulting promising PCR products of appropriate sizes were not inserted into the pRACE vector by simple ligation, but by a recombination. This In-Fusion cloning was carried out with specific enzymes included in the kit. The manufacturer's instructions were followed, but in half of the indicated volumes:

Linearised pRACE vector	0.5 µl
Gel-purified RACE product	3.5 µl
In-Fusion HD Master Mix	1.0 µl

Samples were incubated for 15 min at 50 °C and transferred to ice until transformation of frozen Stellar™ competent cells (IV.7.2.a).

c. PCR for amplification of full length sequences

After successful generation of the complete coding sequence by assembly of the 5 'and 3' end, full length primers were used for expression experiments in different pro- and eukaryotic cell lines. For this purpose, the necessary primer restriction sites were matched to those also present in the plasmids. The complete primer list can be found in chapter IV.9. The necessary PCR was performed with a polymerase with proofreading activity to obtain a high accuracy of replication. Two different enzymes were used:

High Fidelity PCR Polymerase Mix:

HiFi buffer + $MgCl_2$ (10x)	5 µl
2 mM dNTP Mix	5 µl
10 mM primer f	0.25 µl
10 mM primer r	0.25 µl
cDNA (template)	1.5 µl
HiFi Polymerase	0.25 µl
Dist. H_2O	*ad* 50 µl

Phusion® High Fidelity DNA Polymerase:

HF buffer + $MgCl_2$ (10x)	10 µl
2 mM dNTP Mix	5 µl
10 mM primer f	0.5 µl
10 mM primer r	0.5 µl
cDNA (template)	0.5 µl
Phusion® Polymerase	0.25 µl
Dist. H_2O	*ad* 50 µl

The PCR cycles were the following:

1st cycle:	94 °C, 120 sec / GSP T_m -5 °C, 60 sec / 72 °C, 90 sec
2nd - 39th cycle:	94 °C, 30 sec / GSP T_m -5 °C, 60 sec / 72 °C, 90 sec
40th cycle:	94 °C, 60 sec / GSP T_m -5 °C, 60 sec / 72 °C, 10 min
End:	cooling at 6 °C.

The PCR products were analysed by agarose gel electrophoresis. Matching products were cut out, purified via gel extraction and ligated into pDrive until further transformation of *E. coli* and confirmation by sequencing.

d. Colony PCR

Colony PCR is a convenient method for determination of the presence or absence of insert DNA in plasmid constructs introduced into organisms (*E. coli*, *Saccharomyces cerevisiae*). This method was mainly used for the conformation of successful transformation of yeast expression cell lines. The respective colony, which was used for expression later on, was picked from the agar plate and suspended in 100 µl 200 mM LiAc, 1% SDS solution and incubated for 5 min at 70 °C. This heating step causes the release of the plasmid DNA from the cell, after which it can serve as template for a PCR reaction. For further purification 300 µl of 100% EtOH were added and mixed vigorously. The DNA and cell debris were sedimented by centrifugation at 15.000 g for 3 min and the resulting pellet washed in 70% EtOH. It was re-dissolved in 100 µl dist. H_2O and centrifuged again at 15.000 g for 15 sec. 1 µl of the resulting supernatant was used as template for a standard PCR reaction with specific primers for the respective protein. Colonies verified in this way can then be re-inoculated, multiplied and stored at 4 °C until further expression analysis.

6.4 Agarose gel electrophoresis

For the separation of DNA and RNA samples agarose gels were used as a matrix. 1.4 g agarose were dissolved in 200 ml of either 0.5x TBE-buffer (44.5 mM boric acid, 1 mM Na_2-EDTA and 44.5 mM TRIS) or in 1x TAE-buffer (20 mM acetic acid, 1 mM Na_2-EDTA, 40 mM TRIS), by heating in a microwave (0.7% agarose gel). Approximately 50 ml were used to prepare a gel.

While still hot, 0.7 µl of 1% ethidium bromide solution were added and mixed well. Accordingly, the electrophoresis chamber contained either 0.5x TBE-buffer or 1x TAE-buffer. The slots of the gel were loaded with a mixture of RNA or DNA with 6x loading dye (0.03% bromophenol blue, 0.03% xylene cyanol, 60 mM EDTA in 60% glycerol). Usually, 8 µl sample were mixed with 2 µl dye when using PCR products and 3 µl sample and 3 µl dye when using RNA. As a marker, 3 µl of a DNA ladder mix (GeneRuler™) was used. The DNA was separated with 110 V for about 45 min until the lower blue band (bromophenol blue) had reached two thirds of the gel. The gels were then checked under UV at 254 nm or digitally by means of blue-green LED light and an amber filter.

6.5 Purification of DNA fragments from agarose gels

Once a DNA fragment with the correct calculated length (given as base pairs, bp) was obtained, all PCR samples, which contained this band, were separated completely by agarose gel electrophoresis and the respective bands were cut out of the gel. For gel extraction and purification a NucleoSpin Extract II kit was used.

Firstly, the gel matrix was dissolved, using the binding buffer NT in a ratio of 200 µl buffer/100 mg gel matrix. The tube was incubated at 50 °C until the gel was liquefied (5-10 min.). The solution was centrifuged through a NucleoSpin silica filter for 1 min at 11.000 g. The flow-through was discarded. 600 µl of the wash buffer (NT3) was added and again the flow-through was discarded after centrifugation at 11.000 g for 1 min. The silica membrane was then centrifuged for 2 min at 11.000 g to dry the silica membrane. Finally 15 µl of dist. H_2O were added into the centre of the silica filter. After an incubation time of 5 min, the flow-through was centrifuged into a reaction tube and the silica filter was cleaned with dist. H_2O for further use. The DNA concentration of the sample was measured photometrically.

6.6 Ligation

Ligation is the covalent linkage of an insert sequence with a vector.

a. TA-ligation

TA-ligation is a simple way of inserting DNA fragments into vectors. It is based on the attachment of an adenosine nucleotide (A) at the end of the PCR reaction of the DNA polymerase type A to the finished strand. This A can interact with an overhanging thymidine nucleotide (T) of the linear plasmid, thus facilitating the incorporation of the insert into the vector. Since both ends of the insert as well as the vector are identical, half of the vectors result in a reverse incorporation of the insert. Ligation reactions into the TA-cloning vector pDrive were performed with the Qiagen PCR cloning kit.

0.25-1.0 µl DNA fragment solution and 0.5 µl pDrive cloning vector were pipetted to 2.5 µl 2x ligation buffer with ligase in a total volume of 5 µl. The mixture was incubated overnight at 4-10 °C in the refrigerator or at least for half an hour at 16 °C. Then the ligase was denatured for 5-10 minutes at 65 °C, prior to transformation.

b. Ligation into restriction sites

The second applied method is ligation with T4 DNA ligase into the sticky ends of DNA fragments previously cut with restriction endonucleases. For this ligation, a DNA fragment digested with restriction enzymes is introduced into a plasmid. This was also pre-digested with the respective enzymes and, if necessary, additionally purified. Thus, a directed incorporation of the insert can occur if different restriction endonucleases have been used for the two restriction sites. Insert and plasmid were used in a ratio of 5-10:1 in favour of the insert. The respective amounts were determined photometrically and the quantities to be used calculated accordingly. In addition, the concentration of the DNA fragment could previously be increased by evaporation. The ligation mix had a total volume of 10 µl. First the insert was re-dissolved in the required volume of water.

Plasmid	1.0 µl
T4 Ligase buffer (10x)	1.0 µl
T4 DNA Ligase	1.0 µl
DNA fragment solution	7.0 µl

The mixture was incubated for 20 h at 4 °C and the reaction stopped at 65 °C for 10 min. The cooled solution was used for the transformation of pro- and eukaryotic expression strains.

6.7 Restriction enzyme digest

Digestion with restriction endonucleases is an important method of molecular biology. The restriction digest is used to characterise DNA on the basis of the resulting characteristic DNA fragments and to prepare DNA for cloning attempts. The standard digestion assays were performed in a total volume of 15 µl. The choice of the final buffer concentration was dependent on the enzyme(s) used. The aim was to obtain maximum enzyme activity with simultaneous suppression of unspecific digestion (star activity). The choice of the enzyme(s) was directed to the restriction sites in the gene and/or the plasmid. If there were variations in the pipetting scheme, it is explicitly pointed out in the respective method.

Otherwise the assays were composed as follows:

2x yellow buffer (10x) or	3.0 µl	EcoRI buffer (10x)	1.5 µl
1x orange buffer (10x) or	1.5 µl	EcoRI	0.5 µl
1x red buffer (10x)	1.5 µl	Plasmid	3.0 µl
NdeI | EcoRI | HindIII | HindIII	0.5 µl	Dist. H_2O	10.0 µl
XhoI | NotI | XhoI | EcoRI	0.5 µl		
Plasmid	3.0 µl		
Dist. H_2O	*ad* 15.0 µl		

For digestion of high amounts of DNA the final volume was extended to 20 µl. In addition, the concentration of restriction enzymes was tripled (1.5 µl each) and only plasmid DNA sample used instead of further dilution with dist. H_2O. Samples were incubated overnight (~16 h) or at least for 3 h at 37 °C. If only information about the actual presence of an insert had to be gathered quickly, short heating in the microwave (30 sec) was enough to detect bands by agarose gel electrophoresis.

7. Genetic engineering

7.1 Chemically competent *E. coli* cells

For the genetic engineering work safety strains of *E. coli* were used: EZ from Qiagen, BL21(DE3)pLysS from Novagen, BL21-CodonPlus (DE3)-RIPL from Agilent and Stellar™ from Clontech. The EZ and BL21 strains were multiplied and made chemically competent in the laboratory. The EZ cells were used for plasmid multiplication and verification, whereas the BL21 strains were used solely for expression studies. Competent Stellar™ cells were only used for transformation with pRACE constructs (IV.6.3.b).

In order to get competent bacteria, cells of a stock culture were picked with a sterile toothpick and inoculated in a test tube containing 2 ml liquid LB medium and 2 µl tetracycline (12.5 mg ml^{-1} Tet in EtOH). In case of the BL21-CodonPlus (DE3)-RIPL cells, tetracycline was replaced with chloramphenicol (50 mg ml^{-1} Cam in EtOH). The test tubes were closed with a lid and shaken overnight at 220 rpm and 37 °C. The next morning, 0.75 ml of fresh sterile LB was taken from a 250 ml Erlenmeyer flask, acting as a reference for the determination of the optical density at 600 nm (OD_{600}). The rest (~100 ml) was mixed with 90 µl of the tetracycline solution. 2 ml of the overnight culture were then added to the flask and cultivated at 220 rpm and 37 °C until an optical density (OD_{600}) of 0.6 was reached. The suspensions were then centrifuged in two sterile 50 ml tubes at 3.000 g and 4 °C for 10 min. The supernatant was discarded and the pellet resuspended in 10 ml cold 100 mM $CaCl_2$. The suspensions were combined and centrifuged at 2.500 g and 4 °C for 12 min. Again, the supernatant was discarded and the pellet resuspended in 10 ml cold 100 mM $CaCl_2$, followed by incubation on ice for 20 min and centrifugation under the same conditions once more. Finally, the supernatant was discarded and the cells resuspended in 2 ml 100 mM $CaCl_2$ with 15% glycerol. The bacteria were distributed in aliquots of 150 µl in sterile 1.5 ml reaction tubes, frozen in liquid N_2 and stored at -80 °C.

7.2 Transformation of pro- and eukaryotic cell lines

a. *E. coli* strains (heat shock transformation)

Tubes with 150 µl frozen competent *E. coli* cells were thawed on ice. 5 µl of ligation mix (IV.6.6.a) were added to 70 µl of these, mixed very carefully and put back on ice for 30 min. The tubes were then incubated in a water bath at 42 °C for exactly 90 sec and cooled again on ice for 2 min. 150 µl SOC medium were added and incubated at 37 °C for 45 min with gentle shaking in an Eppendorf Thermomixer (650 rpm). The mixture was added to a pre-warmed LB agar plate (containing the appropriate amount of antibiotic) in a Petri dish and plated with a sterile Drigalski scoopula. Clones containing pDrive could additionally be selected for integrated DNA fragments via blue-white screening. For this purpose, X-Gal (80 µg ml^{-1}) and IPTG (12 µg ml^{-1}) were added to the LB medium for plates. After drying of the liquid film, the dishes were closed, sealed and incubated overnight (~18 h) at 37 °C.

b. *Pichia pastoris* (electroporation)

To promote integration, the pPICZα constructs were linearised in the 5' AOX1 promoter region. In case of the laccase and both dirigent proteins SacI was used for this purpose (overnight at 37 °C). The peroxidase construct was linearised with BstXI overnight at 55 °C. Both restriction enzymes were heat-inactivated after complete linearisation (SacI at 65 °C and BstXI at 80 °C for 20 min).

Enzyme buffer (10x)	2.0 µl
SacI | BstXI	5.0 µl
Plasmid	10.0 µl
Dist. H_2O	3.0 µl

The constructs were purified by phenol/chloroform extraction (Sambrook and Russell, 2006) once. The sample was added to an equal volume of phenol and vortexed vigorously. After centrifugation (16.000 g; 3 min) the upper phase (containing the DNA) was transferred to a new Eppendorf tube. An equal volume of $CHCl_3$ was added, vortexed and centrifuged again. The aqueous phase was transferred into a new Eppendorf tube with 1/10 volume 3 M sodium acetate

and 2.5 volumes of 100% ice-cold EtOH, vortexed and kept at -20 °C for 15 min. After centrifugation at 16.000 g and 4 °C for 3 min the DNA pellet was washed with 80% EtOH, air-dried, resuspended in 10 µl dist. H_2O and finally stored at -20 °C until further use.

Transformation of *P. pastoris* was done by means of electroporation. 4 ml of the *P. pastoris* X-33 strain were grown in YPD medium in a small tube at 30 °C overnight. The next day 250 ml of fresh medium were inoculated with 1 ml of the overnight culture in a 1 liter flask and grown overnight again to an OD_{600} of ~1.5. The cells were centrifuged at 1.500 g for 5 min at 4 °C and resuspended in 250 ml ice-cold sterile H_2O. This step was repeated twice, but cells were resuspended in 125 ml ice-cold sterile H_2O the second time and in 10 ml ice-cold 1 M sorbitol after the third centrifugation step. Finally the cells were centrifuged as described above, the pellet resuspended in 1 ml ice-cold 1 M sorbitol in a final volume of approximately 1.5 ml. Cells were kept on ice and used the same day.

80 µl of these *P. pastoris* X-33 cells were mixed with 10 µg of the linearised pPICZα A constructs and transferred to an ice-cold 0.2 cm electroporation cuvette. The cuvette was incubated with the cells on ice for 5 min. They were pulsed with a BioRad MicroPulser™ according to the parameters for yeast (*P. pastoris*) as suggested by the manufacturer. Afterwards 1 ml ice-cold 1 M sorbitol was added immediately and the cuvette contents transferred to a sterile 1.5 ml Eppendorf tube. This was incubated at 30 °C without shaking for 1-2 h. 200 µl of the content were spread on YPDS plates containing the appropriate concentration of Zeocin™ (100 µg ml^{-1}) and incubated for 2-3 days at 30 °C.

c. Saccharomyces cerevisiae

Transformation of *S. cerevisiae* strains was done with the lithium acetate method according to Gietz and Schiestl (2002). All steps were done with autoclaved buffers and toothpicks. At first, carrier DNA (fish sperm) was dissolved in TE buffer (10 mM TRIS-HCl, 1 mM Na_2EDTA pH 8.0) by stirring at 4 °C for 1-2 h and then heated to 99 °C for 5 min and stored on ice until further use. The plasmid DNA was diluted with dist. H_2O to a concentration of 50 ng $µl^{-1}$. An amount of yeast cells about as large as a pinhead was picked from a fresh YPD plate and resuspended in 1 ml dist. H_2O. The suspension was centrifuged at 16.000 g for 30 sec and the supernatant removed. The following was pipetted on the cell pellet in the given order and mixed afterwards.

Polyethylene glycol (50% w/v)	240 µl
1 M lithium acetate	36 µl
Carrier DNA (2 mg ml^{-1})	50 µl
Plasmid (50 ng $µl^{-1}$)	34 µl

The samples were incubated for at least 1 h at 42 °C, subsequently centrifuged at 16.000 g and resuspended in 1 ml dist. H_2O. Finally, 200 µl of these were spread on SCD plates and incubated at 30 °C for 3-4 days.

After formation of successfully transformed *S. cerevisiae* cells, colonies were streaked out on fresh SCD plates and checked for the correct insert via colony PCR (IV.6.3.d).

7.3 Overnight cultures

After successful transformation of *E. coli* EZ cells and streaking on solid media plates, colonies were picked with the help of sterile toothpicks and placed in a tube containing 4 ml LB medium with ampicillin (0.1 mg ml^{-1}). The tubes were closed, sealed and put into an incubator at 37 °C with 220 rpm rotation for 18 h. The next day, the cells were centrifuged, plasmids extracted by plasmid preparation (IV.7.4) and a control digest performed, before further sequencing or recloning steps.

7.4 Plasmid preparation

Plasmid preparation is a simple method to separate and extract plasmids from bacteria. These were then either sequenced for verification, or the insert was cut by restriction enzymes and ligated into another vector. Also, multiplied empty vectors could be obtained this way.

1.5 ml of the cell suspension from overnight cultures were pipetted into test tubes followed by centrifugation at 1.500 g for 5 min. The supernatant was discarded and the procedure was repeated with another 1.5 ml cell suspension. After the second centrifugation step the supernatant was removed quantitatively with a pipette and the protocol from the QIAprep® Spin Miniprep Kit by Qiagen was followed:

The pellets were resuspended in 250 µl buffer P1 (50 mM TRIS-HCl pH 8.0, 10 mM EDTA, 100 µg ml^{-1} RNase A). 250 µl buffer P2 (200 mM NaOH, 1% SDS) were added and the tubes were mixed by inverting them 5 times. 350 µl buffer N3 (4.2 M guanidinium-HCl, 0.9 M CH_3CO_2K, pH 4.8) were added and the tubes were again mixed by inverting them a few times. Afterwards, the tubes were centrifuged for 10 min at 16.000 g. The supernatants were pipetted carefully to QIAprep® spin columns and centrifuged for 1 min at 16.000 g. The flow-through was discarded and the columns were washed with 500 µl buffer PB (5 M guanidinium-HCl, 30% 2-propanol) by centrifugation at 16.000 g for 1 min. After discarding the flow-through the columns were washed again with 750 µl buffer PE (10 mM TRIS-HCl pH 7.5, 80% EtOH) by centrifugation at the same conditions. The flow-through was discarded again and the columns were dried by short centrifugation. To elute the DNA, 50 µl sterile water were applied to the column. After incubation for 5 min at room temperature, the columns were centrifuged for 1 min at 11.000 g and the flow-through containing the plasmid-DNA collected in 1.5 ml test tubes.

7.5 Sample verification and pre-expression measures

The vectors were subjected to a restriction digest (IV.6.7) and the product was examined by gel electrophoresis. For the sequencing, 20 µl were added to new reaction tubes and send to Eurofins Genomics or adjusted to a concentration of ~80 ng $µl^{-1}$ with a total volume of 12 µl and sent to Seqlab. From colonies with correct sequences, glycerol stocks were prepared. Full-length sequences were used for cloning into expression vectors.

For cloning into an expression vector after successful sequencing, correspondingly more plasmid DNA sample was digested, to generate more insert. The inserts were ligated to the plasmid with T4 DNA ligase (IV.6.6.b) and then brought into the strain *E. coli* EZ first. After verification of correct clones, transformation of the appropriate expression strain followed.

7.6 Glycerol stocks

When the sequencing results of plasmids were satisfying, glycerol stocks for cryopreservation were prepared. For this 75 µl sterile glycerol and 425 µl liquid bacteria culture (overnight cultures) were thoroughly mixed. Subsequently, these were frozen in liquid N_2 and stored at -80 °C for stable long-term storage. Additionally, crucial constructs were stored at -20 °C in form of plasmids dissolved in dist. H_2O.

7.7 Expression of recombinant proteins

a. *E. coli* BL21 strains

For synthesis of heterologous proteins expression vectors were used, that contained the gene in the correct reading direction. These were introduced into *E. coli* BL21(DE3)pLysS or BL21-CodonPlus (DE3)-RIPL by transformation. For this 2 ml LB medium with 10 µl ampicillin (20 mg ml^{-1}) were inoculated with a bacterial colony and incubated over night at 37 °C and 220 rpm. The next day, the overnight culture was added to 100 ml LB medium (0.75 ml were saved for the photometrical reference) and cultivated at 37 °C and 220 rpm to an OD_{600} of about 0.4. With addition of 100 µl 1 M IPTG (final concentration 1 mM) induction of protein expression was started. After cultivation overnight at 37 °C cells were collected by centrifugation for 5 min at 5.000 g in 50 ml tubes. Finally they were frozen in liquid N_2 and stored at -80 °C until further homogenisation and purification steps (IV.6.5.a).

b. *P. pastoris* X-33 strain

For expression of recombinant proteins in *Pichia* strains special media were used (BMGY/BMMY). Because adequate aeration during MeOH induction is an important parameter for efficient expression in *P. pastoris* baffled flasks covered with cheesecloth were used. Furthermore cultures were never allowed to be more than 30% of the total flask volume. All expression studies were done at 28-30 °C in an incubation shaker.

For this 25 ml BMGY was inoculated with a single colony of the respective *Pichia* clone in a 500 ml baffled flask. It grew at 230 rpm at 30 °C until the culture reached an OD_{600} ~10 (18 h). The cells were harvested by centrifugation at 1.500 g for 5 min at room temperature. The cell pellet was resuspended in 200 ml BMMY in a 1 l baffled flask to an OD_{600} of 1.0 to induce expression. To maintain induction 100% MeOH was added every 24 h to a final concentration of 0.5-1%.

After 0, 12, 24, 48 and 72 h at 28 °C and 160-180 rpm 1 ml of the expression culture was transferred to a 1.5 ml tube and centrifuged shortly for expression level analysis later on. For expression of secreted proteins the supernatant was transferred to a separate tube. All samples were frozen quickly in liquid N_2 and stored at -20 °C. Supernatants (extracellular) and cell pellets (intracellular) were analysed for protein expression via SDS-PAGE and Western blot. Cell disruption and protein extraction were performed as described in section IV.5.5.b.

c. *S. cerevisiae* CB018 and InvSc1 strains

In the case of the successfully transformed yeast cells mainly two different media were used for expression of recombinant proteins. SCD medium was used for cell mass accumulation and SCG medium for induction. Each time a single colony of the CB018 or InvSc1 strain containing the correct pYES2/NT C construct was inoculated and grown overnight at 30 °C in 3 ml SCD as the pre-culture. It was further transferred to a 500 ml baffled flask and shaken at 200 rpm at 30 °C overnight in about 100 ml total volume of SCD. The next day the cells were harvested by centrifugation at 1.500 g for 5 min at room temperature. The resulting cell pellet was resuspended in 200 ml SCG medium to an OD_{600} of ~1.5 in a 1 l baffled flask to induce expression. Flasks were covered with 2 sterile layers of cheesecloth.

After 0, 6, 12 and 24 h at 30 °C and 200 rpm 1 ml of the expression culture was transferred to a 1.5 ml tube, its OD_{600} measured and centrifuged shortly for expression level analysis later on. Finally cells were harvested after 24 h (OD_{600} ~2.75) by centrifugation at 1.500 g for 5 min at 4 °C. To test for secretion of expressed proteins 1.5 ml of the supernatant was transferred to a separate tube. All samples were frozen quickly in liquid N_2 and stored at -20 °C until further homogenisation and purification steps (IV.6.5.b). Cell pellets were analysed for protein expression by SDS-PAGE and Western blot.

8. Culture media

8.1 B5 medium (Gamborg et al., 1968)

As a standard medium for the cultivation of callus and suspensions cells of *Silybum marianum*, B5 was used. It was composed as follows:

Macroelements:		Microelements:	
KNO_3	2500.0 mg l^{-1}	H_3BO_3	3.00 mg l^{-1}
$MgSO_4 \cdot 7\ H_2O$	250.0 mg l^{-1}	$ZnSO_4 \cdot 7\ H_2O$	3.00 mg l^{-1}
$NaH_2PO_4 \cdot H_2O$	172.0 mg l^{-1}	$MnSO_4 \cdot H_2O$	1.00 mg l^{-1}
$CaCl_2 \cdot 2\ H_2O$	150.0 mg l^{-1}	KI	0.75 mg l^{-1}
$(NH_4)_2SO_4$	134.0 mg l^{-1}	$Na_2MoO_4 \cdot 2\ H_2O$	0.25 mg l^{-1}
$FeSO_4 \cdot 7\ H_2O$*	25.6 mg l^{-1}	$CuSO_4 \cdot 5\ H_2O$	0.25 mg l^{-1}
Na_2-EDTA*	34.3 mg l^{-1}	$CoCl_2 \cdot 6\ H_2O$	0.25 mg l^{-1}

* These two components were combined in a solution which was autoclaved before use in order to obtain the Fe-EDTA complex.

Vitamins:		Hormones:
Thiamine-HCl	10 mg l^{-1}	2,4-D (final conc. 1 mg l^{-1}; stock sol. 0.5 mg ml^{-1})
Pyridoxine-HCl	1 mg l^{-1}	Kinetin (final conc. 0.1 mg l^{-1}; stock sol. 0.2 mg ml^{-1})
Nicotinic acid	1 mg l^{-1}	

Further components:	
Myo-inositol	100 mg l^{-1}
Sucrose	20 g l^{-1}

Macro- and microelements were each prepared as 10x stock solutions. Vitamins were aliquoted into portions according to 1-2 l medium and stored at -20 °C. Stock solutions for hormones were stored at 4 °C. After mixing all components the missing volume was adjusted with dist. H_2O and the pH value was adjusted to 5.8 with 0.5 M HCl. Finally, 50 ml medium were filled into 250 ml Erlenmeyer flasks each, closed with cellulose stoppers and autoclaved.

8.2 CB-2 (Gertlowski and Petersen, 1993)

The medium CB-2 differs from B5 almost only in terms of added hormones. A highly soluble source of peptides and amino acids in the form of N-Z-amines® is also added (2 g l^{-1}).

Hormones:

2,4-D (final conc. 2.0 mg l^{-1}; stock sol. 200 mg l^{-1})
IAA (final conc. 0.5 mg l^{-1}; stock sol. 50 mg l^{-1})
Kinetin (final conc. 0.2 mg l^{-1}; stock sol. 20 mg l^{-1})
NAA (final conc. 0.5 mg l^{-1}; stock sol. 50 mg l^{-1})

8.3 MS-Li (Empt et al., 2000)

MS-Li medium is a variant of the popular MS medium (Murashige and Skoog, 1962) with additional 0.4 mg l^{-1} NAA specially adapted to cell cultures of *Linum* (flax) species.

8.4 Lysogeny Broth (LB)

The standard medium for the cultivation of bacteria like *Escherichia coli* is LB (Sezonov et al., 2007):

Tryptone / peptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	10 g l^{-1}
± Agar	10 g l^{-1}

The solution was adjusted to pH 7.0 with 0.5 M NaOH and autoclaved. Solid medium for plates additionally contained 1% agar. If a selection marker for antibiotic resistance was present on the plasmid, the medium was adapted accordingly, by adding ampicillin (100 mg l^{-1}) or other suitable antibiotics after cooling to ~55 °C.

In case of transformed *E. coli* cells with the pPICZα A plasmid for Zeocin™ resistance, the amount of added NaCl had to be adjusted, because the antibiotic is very sensitive to high salt concentration. Zeocin™ (25 µg ml^{-1}) was added after cooling to ~55 °C. The Low Salt LB medium was composed as follows:

Tryptone / peptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	5 g l^{-1}
± Agar	10 g l^{-1}

8.5 Super Optimal broth with Catabolite repression (SOC)

SOC medium is a nutritious bacterial growth medium adjusted from the LB medium. It was used to regenerate bacteria after heat shock because it should provide higher transformation efficiency (Hanahan, 1983). It is composed as follows:

Tryptone / peptone	20 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	0.5 g l^{-1}

The solution was adjusted to pH 7.0 with 1 M NaOH and autoclaved. After cooling, sterile solutions of 1 M KCl (2.5 ml), 1 M $MgCl_2$ (10 ml) and 1 M glucose (20 ml) were added.

8.6 Yeast extract Peptone Dextrose medium ± Sorbitol (YPD/YPDS)

These specially adapted media were used for *P. pastoris* and *S. cerevisiae* cell lines for simple cultivation purposes. The main focus was on the accumulation of cell mass of pre-cultures. The extra addition of adenine was only necessary for the CB018 yeast strain. Inclusion of sorbitol was only required for the *P. pastoris* X-33 strain after transformation via electroporation (IV.7.2.b), since it stabilised the cells as they appeared to be osmotically sensitive.

YPD		YPDS	
Tryptone / peptone	20 g l^{-1}	Tryptone / peptone	20 g l^{-1}
Yeast extract	10 g l^{-1}	Yeast extract	10 g l^{-1}
Glucose	20 g l^{-1}	Glucose	20 g l^{-1}
± L-adenine-sulphate	55 mg l^{-1}	1 M sorbitol	182.2 g l^{-1}
± Agar	20 g l^{-1}	± Agar	20 g l^{-1}
± Zeocin™	100 µg ml^{-1}	± Zeocin™	100 µg ml^{-1}

Most solutions were already prepared as autoclaved stock solutions. If needed, agar was added prior to autoclaving. The pH value was adjusted to 5.6 with 1 M NaOH. The glucose solution was sterile-filtered and added after autoclaving to prevent possible heat-induced degradation processes. This also applied to the amount of antibiotic (Zeocin™) used for selection of pPICZα A containing cells. For a quick, direct way to select putative multi-copy recombinants the Zeocin™ concentration could be raised up to 1 mg ml^{-1}. YPD(S) media and plates were stored at 4 °C in the dark.

8.7 Minimal Dextrose/Methanol medium (MD/MM)

These media served the sole purpose to find out the Mut phenotype of the successfully transformed and plated *P. pastoris* cells. This special feature describes the ability to utilise MeOH. Mut^S refers to a slow MeOH metabolising phenotype, caused by the loss of alcohol oxidase activity. Transformation of the X-33 wild type strain with plasmid DNA linearised in the promoter region usually will yield Mut^+ transformants.

To be safe, this was tested by streaking single Zeocin™ resistant colonies on both media types with subsequent incubation for 2 days at 30 °C. Mut^+ strains successfully grew on both plates, while Mut^S only grew on the MD plates and showed little or no growth on the MM plate.

MD		MM	
Yeast nitrogen base	13.4 g l^{-1}	Yeast nitrogen base	13.4 g l^{-1}
Biotin	4x 10^{-5}%	Biotin	4x 10^{-5}%
Glucose	20 g l^{-1}	MeOH	0.5%
Agar	15 g l^{-1}	Agar	15 g l^{-1}

8.8 Buffered Glycerol/Methanol-complex medium (BMGY/BMMY)

For expression of recombinant *P. pastoris* strains BMGY/BMMY (buffered complex glycerol or MeOH) medium was used. These media contain yeast extract and peptone to stabilise secreted proteins and prevent or decrease proteolysis of secreted proteins. Additionally, they should provide better growth and biomass accumulation. The medium containing glycerol served mainly for that purpose, while the medium with MeOH was used to initiate the induction.

BMGY		BMMY	
Tryptone / Peptone	20 g l^{-1}	Tryptone / Peptone	20 g l^{-1}
Yeast extract	10 g l^{-1}	Yeast extract	10 g l^{-1}
Yeast nitrogen base	13.4 g l^{-1}	Yeast nitrogen base	13.4 g l^{-1}
Biotin	4x 10^{-5}%	Biotin	4x 10^{-5}%
100 mM KH_2PO_4	13.6 g l^{-1}	100 mM KH_2PO_4	13.6 g l^{-1}
Glycerol	10 g l^{-1}	MeOH	0.5-1%

In all cases, 10x stock solutions were prepared and autoclaved. The KH_2PO_4 solution was adjusted to pH 6.0 with 1 M KOH before use. The necessary volumes of each component were then mixed together accordingly. Only the required amount of MeOH was added through a sterile filter after autoclaving to the otherwise complete solution.

8.9 Synthetic Complete minimal defined medium (SC)

For expression of recombinant *S. cerevisiae* strains SCD/SCG (synthetic complete dextrose or galactose) medium was used. The former was used primarily for biomass accumulation of pre-cultures and the medium containing galactose for expression. Again, the addition of adenine was only required for the CB018 yeast strain. The pH value was adjusted to 5.6 with 1 M NaOH.

SCD		SCG	
Yeast nitrogen base	6.7 g l^{-1}	Yeast nitrogen base	6.7 g l^{-1}
Glucose	20 g l^{-1}	Galactose	20 g l^{-1}
± L-adenine-sulphate	40 mg l^{-1}	± L-adenine-sulphate	40 mg l^{-1}
± Agar	20 g l^{-1}	± Agar	20 g l^{-1}

The yeast nitrogen base was prepared as 10x stock solution. Agarose was only added for pouring of plates. In addition, depending on the used strain, small amounts of defined essential amino acids were added prior to autoclaving. These were prepared as stock solutions as well, divided into individual portions of 50 ml and stored at -20 °C. Uracil was explicitly omitted to make selective plates for growing pYES2/NT C transformants. Sterile-filtered glucose and galactose were added later to ensure their stability. Plates and media were stored at 4 °C.

Amino acid composition for media (necessary for the InvSc1 yeast strain):

0.01% adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan
0.005% aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine

Amino acid composition for media (necessary for the CB018 yeast strain):

0.005% phenylalanine, adenine
0.003% isoleucine, leucine, serine, threonine, tyrosine, valine
0.002% tryptophan, methionine, lysine, histidine, arginine

9. Primer list

9.1 Degenerate primers

name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
Perox2_f	CATGATTGYTTYGTTMADGG	52.9		Degenerate PCR primer for POD
Perox2_r	TKRCYYADCTTRAYCATTGC	53.9		Degenerate PCR primer for POD
SmLac-f	GGVACWSTKTGGTGGCAYGC	60.4		Degenerate PCR primer for LAC
SmLac-r	CCATGMAGRTGVATDGGRTG	58.3		Degenerate PCR primer for LAC
DP-1.1-f	AATGCMACHKCHKCMATWGT	54.6		Degenerate PCR primer for DIRs
DP-1.2-f	GAYCCNATTACYYTWGACAACAA	57.1		Degenerate PCR primer for DIRs
DP-2.2-r	GCCATGAARAARTCMCCHGT	57.0		Degenerate PCR primer for DIRs

9.2 RACE PCR primer

a. According to Scotto-Lavino et al. (2007)

name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
RACE-Q_T	CCAGTGAGCAGAGTGACGAGGACTC GAGCTCAAGCTTTTTTTTTTTTTTTT	73.4		cDNA synthesis primer (3' RACE)
RACE-Q_O	CCAGTGAGCAGAGTGACG	58.2		1st primer
RACE-Q_I	GAGGACTCGAGCTCAAGC	58.2		2nd primer
Perox-3GSP1	GTTAATGGATGTGATGCGTCG	57.9		gene specific 3' RACE primer for POD
Perox-3GSP2	GGATGATACTACAAATTTCGCTGG	59.3		gene specific 3' RACE primer for POD
Perox-5GSP2	GAACCGTAAGTTCTCACTTGG	57.9		gene specific 5' RACE primer for POD
Perox-5GSP1	CAAAGTCTGTCGAAAAGGTTGC	58.4		gene specific 5' RACE primer for POD
Perox-5GSPRT	GGCTCAACTTGATCATTGCG	57.3		gene specific 5' RACE primer for POD

3’-Lacc-GSP1	GGAACACTTTGGTGGCACG	58.8		gene specific 3’ RACE primer for LAC
3’-Lacc-GSP2	GCTTAGAGCCACTGTCTATGG	59.8		gene specific 3’ RACE primer for LAC
5’-Lacc-GSP2	CCTGTAGACTTTTGTTCCACG	57.9		gene specific 5’ RACE primer for LAC
5’-Lacc-GSP1	CCTTGCAACACAATCTGCACC	59.8		gene specific 5’ RACE primer for LAC
5’-Lacc-GSPRT	CCATGAAGGTGGATTGGATGG	59.8		gene specific 5’ RACE primer for LAC
Dir1-3GSP1	CAACTGCCGCCATTGTAGG	58.8		gene specific 3’ RACE primer for DIR1
Dir1-3GSP2	CCAGCTTGGGGTAATAACAC	57.3		gene specific 3’ RACE primer for DIR1
Dir1-5GSP2	GCAAAGTTAATGCTACCCTTATG	57.1		gene specific 5’ RACE primer for DIR1
Dir1-5GSP1	CTTGTCTTGTTCATCAAAGGATCG	59.3		gene specific 5’ RACE primer for DIR1
Dir1-5GSPRT	CAGTTCCACCAATCACAGAAATG	58.7		gene specific 5’ RACE primer for DIR1
Dir2-3GSP1	GACAACAATTTGCATTCCTCTCC	58.9		gene specific 3’ RACE primer for DIR2
Dir2-3GSP2	CTCGGGGTCAATACATCTATG	57.9		gene specific 3’ RACE primer for DIR2
Dir2-5GSP2	CTTGAGTGGATGTGGATTAGC	57.9		gene specific 5’ RACE primer for DIR2
Dir2-5GSP1	CCACCAACAACTGATATATCCC	58.4		gene specific 5’ RACE primer for DIR2
Dir2-5GSPRT	CCATGAAGAAGTCCCCTGTC	59.4		gene specific 5’ RACE primer for DIR2

Underlined nucleotides stand for the part of the primer corresponding to the sequence.

b. SMARTer® RACE PCR kit

name	**sequence (5’–3’)**	**T_m (binding part) [°C]**	**restriction site**	**comment**
UPM Long	TAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGAGT	73.2		Universal Primer Mix (Long)
UPM Short	CTAATACGACTCACTATAGGGC	58.4		Universal Primer Mix (Short)
3POX	GATTACGCCAAGCTTGGTTGTAG CACTTGGTGGCCCGAGTTGG	71.0		gene specific 3’ RACE primer for POD
5POX	GATTACGCCAAGCTTCTCACTTG GGCATCGGTTGAACCACCG	69.5		gene specific 5’ RACE primer for POD

3Lacc	GATTACGCCAAGCTTCCTACCCG TTCCCTAAGCCCAACCGTG	71.0		gene specific 3' RACE primer for LAC
5Lacc	GATTACGCCAAGCTTCTGGAGGT TTCACGGGGAAGTCAGTGGTG	70.9		gene specific 5' RACE primer for LAC
3' DIR1_SMART	GATTACGCCAAGCTTGCCACAGC TGCCATTGTAGGTGCTCC	69.5		gene specific 3' RACE primer for DIR1 (1)
3' DIR2_SMART	GATTACGCCAAGCTTGCATTCCT CTCCGGTTGGTCGGGCTC	71.1		gene specific 3' RACE primer for DIR2 (1)
5DIR	GATTACGCCAAGCTTGCCATGAA GAAGTCMCCWGTYCCACC	68.0		common gene specific 5' RACE primer for DIRs
DIR1-5'	GTCTTGTTCATCAAAGGATCGG	58.4		gene specific nested PCR primer for DIR1
DIR2-5'	CCCTAAACTTGAGTGGATGTGG	60.3		gene specific nested PCR primer for DIR2
3-DIR1	GATTACGCCAAGCTTCCTAGAAA GAACATTCGCTCTCGACGTCC	68.1		gene specific 3' RACE primer for DIR1 (2)
3-DIR2	GATTACGCCAAGCTTGCCAATTC GACAGCAGCTATTGTGGCTGC	69.5		gene specific 3' RACE primer for DIR2 (2)

Underlined nucleotides stand for the part of the primer corresponding to the sequence.

9.3 Full length primers / gene expression primers

a. Expression in pET-15b

name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
5'-POX-FL	ATA**CATATG**GCTCGTTCGTCGTCG TCTTAC	64.4	NdeI	full length primer for POD
3'-POX-FL	ATA**CTCGAG**TTAATTAGTTCTTCT GCAATTCGTCCG	60.4	XhoI	full length primer for POD
5'-LAC-FL	ATA**CATATG**GATCGTGTGTTCACC ACCATTGC	64.8	NdeI	full length primer for LAC
3'-LAC-FL	ATA**CTCGAG**TCAACACACGGGTA AGTCTAGTGGTG	64.8	XhoI	full length primer for LAC
SmDIR1-VL-f	ATA**CATATG**AGTTCTAAGTATCAA AACTCAACAC	59.3	NdeI	full length primer for DIR1
SmDIR1-VL-r	ATA**CTCGAG**TCACCAACATTCATA GAACTTAATGTC	58.9	XhoI	full length primer for DIR1
SmDIR2-VL-f	ATA**CATATG**AGTGTAAAAACAAGT TCAAATATAG	56.3	NdeI	full length primer for DIR2
SmDIR2-VL-f	ATA**CTCGAG**TTACCAACACTCATA GAACTTTATGTC	58.9	XhoI	full length primer for DIR2

Restriction sites are written in **bold** letters. Underlined nucleotides stand for the part of the primer corresponding to the sequence.

b. Expression in pPICZα A (with signal peptide)

name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
5-POX-FL	ATA**GAATTC**ATGGCTCGTTCGTCGTCGTCTTAC	64.4	EcoRI	full length primer for POD
3-POX-FL	ATA**GCGGCCGC**ATTAGTTCTTCTGCAATTCGTCCGAACC	63.7	NotI	full length primer for POD
5-LAC-FL	ATA**GAATTC**ATGGATCGTGTGTTCACCACCATTGC	64.8	EcoRI	full length primer for LAC
3-LAC-FL	ATA**GCGGCCGC**ACACACGGGTAAGTCTAGTGGTGG	64.4	NotI	full length primer for LAC
5-DIR1-FL	ATA**GAATTC**ATGAGTTCTAAGTATCAAAACTCAACAC	59.3	EcoRI	full length primer for DIR1
5-DIR2-FL	ATA**GAATTC**ATGAGTGTAAAAACAAGTTCAAATATAG	56.3	EcoRI	full length primer for DIR1
3-DIR1+2-FL	ATA**GCGGCCGC**CCAACAYTCATAGAACTTWATGTCAAC	59.7	NotI	common full length primer for DIRs

Restriction sites are written in **bold** letters. Underlined nucleotides stand for the part of the primer corresponding to the sequence.

c. Expression in pPICZα A (without signal peptide)

name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
5POXoS-f	ATA**GAATTC**CAACTTTCGGCAAACTTTTACGCGACC	65.0	EcoRI	full length primer for POD
3-POX-FL	ATA**GCGGCCGC**ATTAGTTCTTCTGCAATTCGTCCGAACC	63.7	NotI	full length primer for POD
5LACoS-f	ATA**GAATTC**ATGCCAAATGCAAAGACTCACTACCATG	63.7	EcoRI	full length primer for LAC
3-LAC-FL	ATA**GCGGCCGC**ACACACGGGTAAGTCTAGTGGTGG	64.4	NotI	full length primer for LAC
5DIR1oS-f	ATA**GAATTC**TCACCTAGAAAGAACATTCGCTCTC	61.3	EcoRI	full length primer for DIR1
5DIR2oS-f	ATA**GAATTC**TCATACAATCATGAAGGAAACAATAAAC	57.8	EcoRI	full length primer for DIR1
3-DIR1+2-FL	ATA**GCGGCCGC**CCAACAYTCATAGAACTTWATGTCAAC	59.7	NotI	common full length primer for DIRs

Restriction sites are written in **bold** letters. Underlined nucleotides stand for the part of the primer corresponding to the sequence.

d. Expression in pYES2/NT C

name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
5POX_HindIII	ATA**AAGCTT**ATGGCTCGTTCGTCGTCGTCTTAC	64.4	HindIII	full length primer for POD
3POX_XhoI	ATA**CTCGAG**ATTAGTTCTTCTGCAATTCGTCCGAACC	63.7	XhoI	full length primer for POD
5LAC_HindIII	ATA**AAGCTT**ATGGATCGTGTGTTCACCACCATTGC	64.8	HindIII	full length primer for LAC
3LAC_XhoI	ATA**CTCGAG**ACACACGGGTAAGTCTAGTGGTGG	64.4	XhoI	full length primer for LAC
5DIR1_HindIII	ATA**AAGCTT**ATGAGTTCTAAGTATCAAAACTCAACAC	59.3	HindIII	full length primer for DIR1
3DIR1_XhoI	ATA**CTCGAG**CCAACATTCATAGAACTTAATGTCAAC	58.9	XhoI	full length primer for DIR1
5DIR2_HindIII	ATA**AAGCTT**ATGAGTGTAAAAACAAGTTCAAATATAG	56.3	HindIII	full length primer for DIR2
3DIR2_XhoI	ATA**CTCGAG**CCAACACTCATAGAACTTTATGTCAAC	60.4	XhoI	full length primer for DIR2

Restriction sites are written in **bold** letters. Underlined nucleotides stand for the part of the primer corresponding to the sequence.

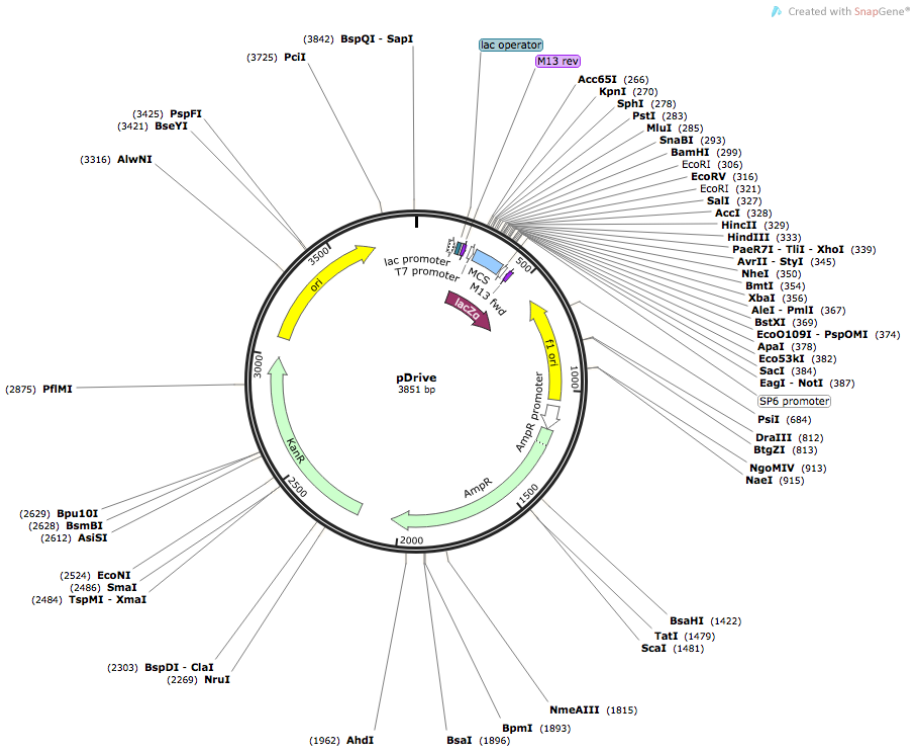
9.4 Sequencing primers

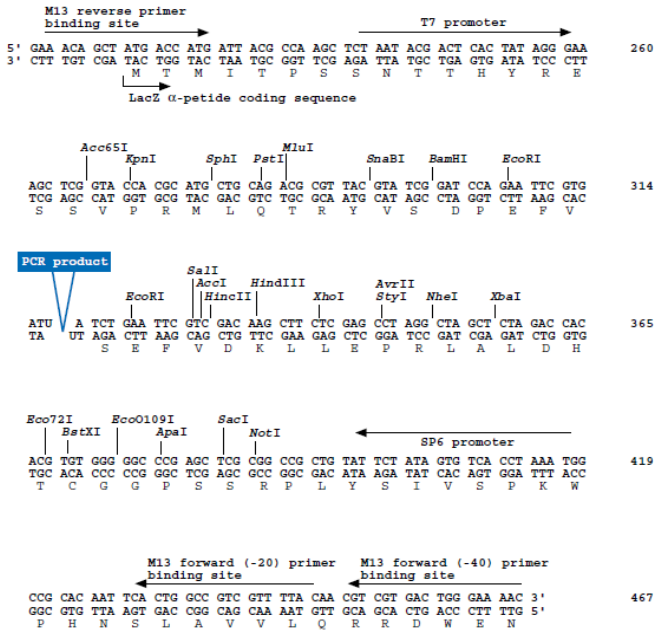
name	sequence (5'–3')	T_m (binding part) [°C]	restriction site	comment
LacSeq1	CCTTCACAACCAGCTTCAGG	59.4		sequencing primer for LAC
LacSeq2	GCCATGAACTATGTGCATGCC	59.8		sequencing primer for LAC

10. Vector sequences, maps and features

All information regarding the following vector maps can also be taken from the manufacturers' manuals.

10.1 pDrive (Qiagen)

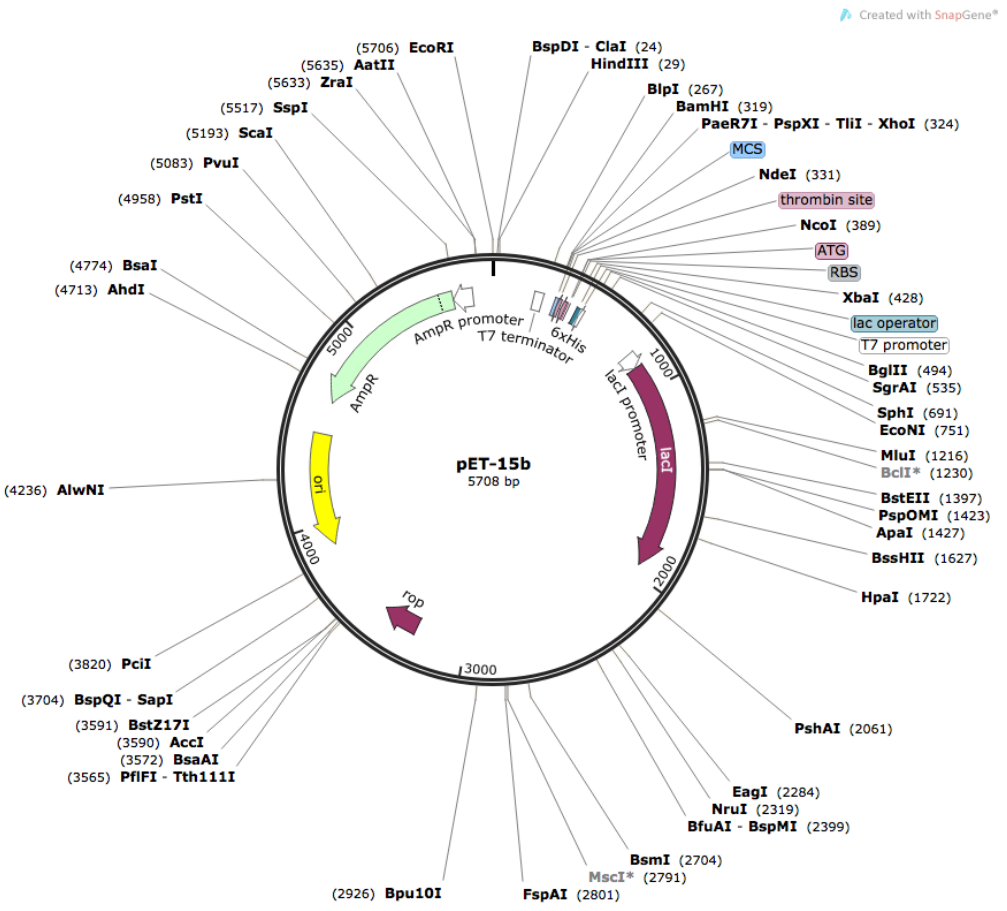


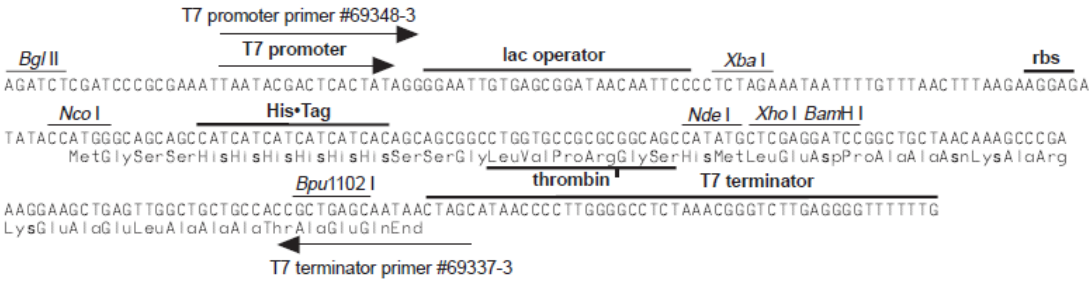


Location of specific vector features

Vector size (bp): 3851
Multiple cloning site: 266–393
LacZ α-peptide: 216–593
T7 RNA polymerase promoter: 239–258
T7 transcription start: 256
SP6 RNA polymerase promoter: 398–417
SP6 transcription start: 400
Ampicillin resistance gene: 1175–2032
Kanamycin resistance gene: 2181–2993
pUC origin: 3668
Phage f1 origin: 588–1043
Primer binding sites:*
M13 forward (–20): 431–447
M13 forward (–40): 451–467
M13 reverse: 209–224
T7 promoter primer: 239–258
SP6 promoter primer: 400–418

10.2 pET-15b (Novagen)

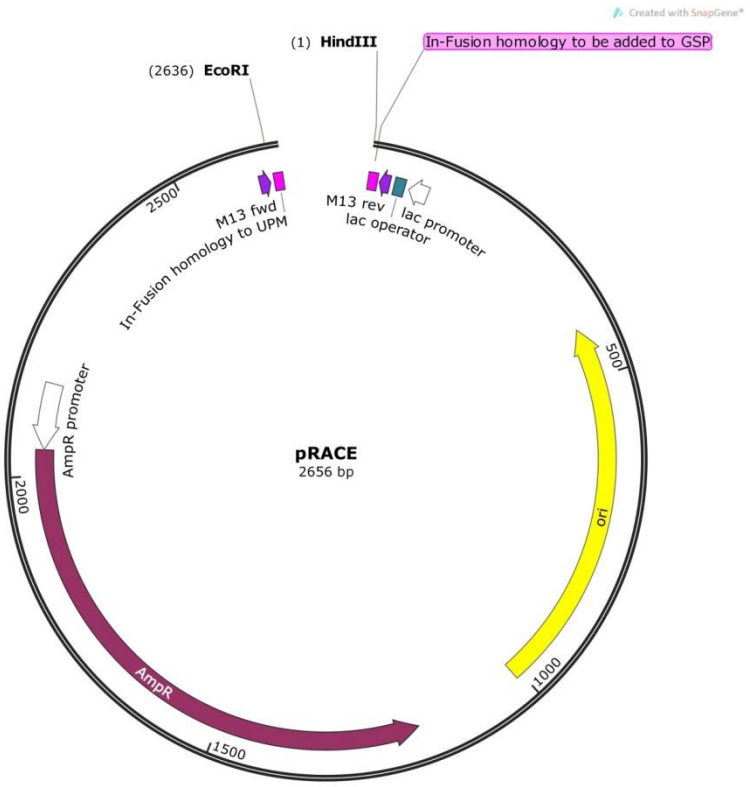


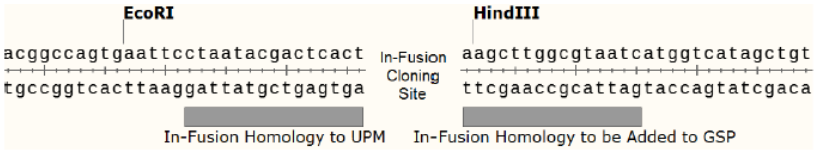


Location of specific vector features

T7 promoter: 463-479
T7 transcription start: 452
His-Tag coding sequence: 362-380
Multiple cloning sites (Nde I - BamH I): 319-335
T7 terminator: 213-259
lacI coding sequence: (866-1945)
pBR322 origin: 3882
bla coding sequence: 4643-5500

10.3 pRACE (Clontech)

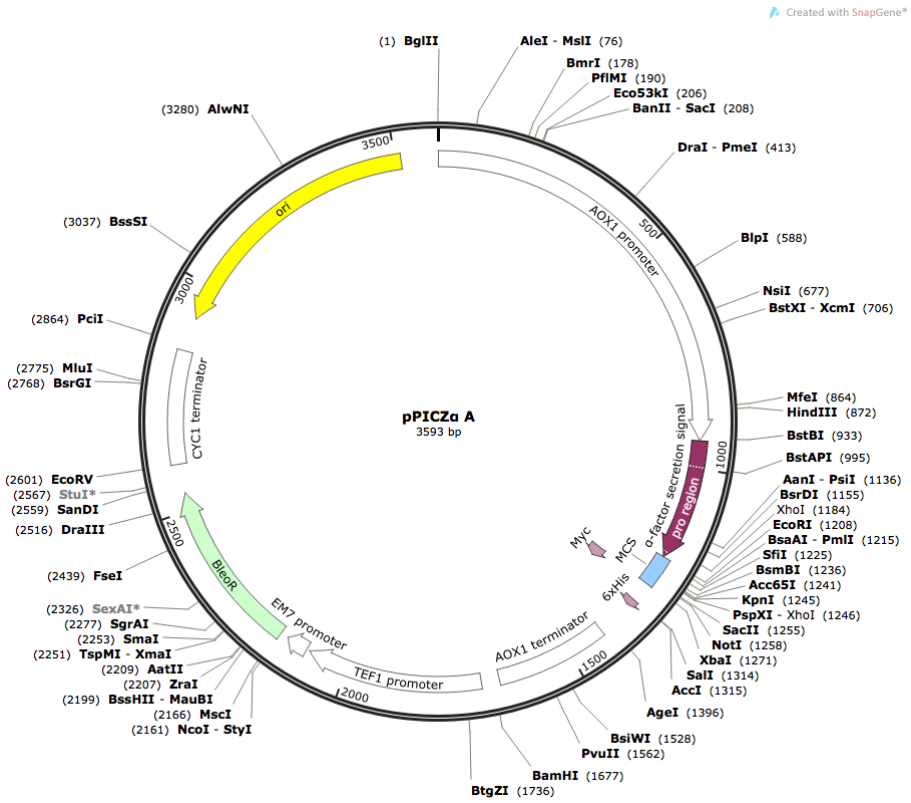


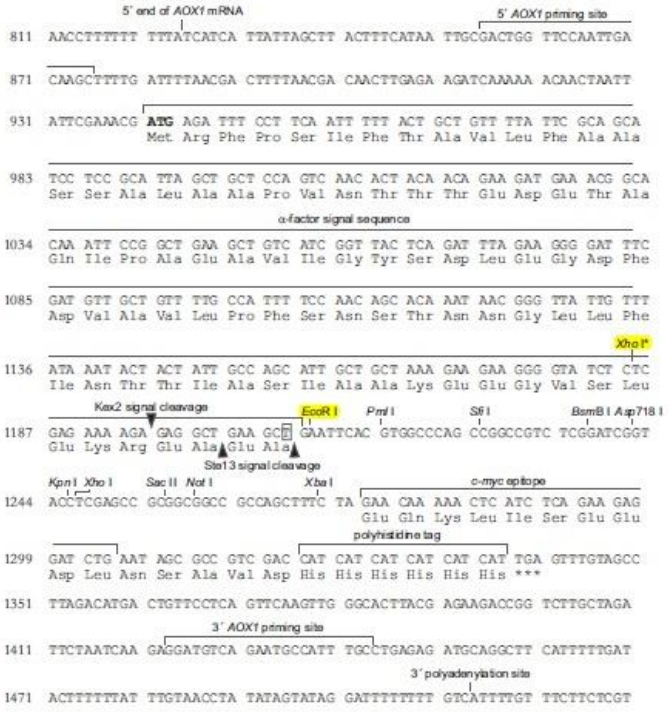


Location of specific vector features

In-Fusion Cloning Site (blunt ends, generated by inverse PCR): 1
In-Fusion Homology to be added to Gene-Specific Primer (GSP): 1–15
M13 rev (sequencing primer): 19–35 (complementary)
lac promoter: 67–97 (complementary)
ori (high-copy-number ColE1/pMB1/pBR322/pUC origin of replication): 421–1009 (complementary)
AmpR (ampicillin resistance gene): 1180–2040 (complementary)
AmpR promoter: 2041–2145 (complementary)
M13 fwd (sequencing primer): 2619–2635
In-Fusion Homology to Universal Primer Mix (UPM): 2642–2656

10.4 pPICZα A (Invitrogen, provided by Prof. Dr. A. Schaller, Hohenheim)

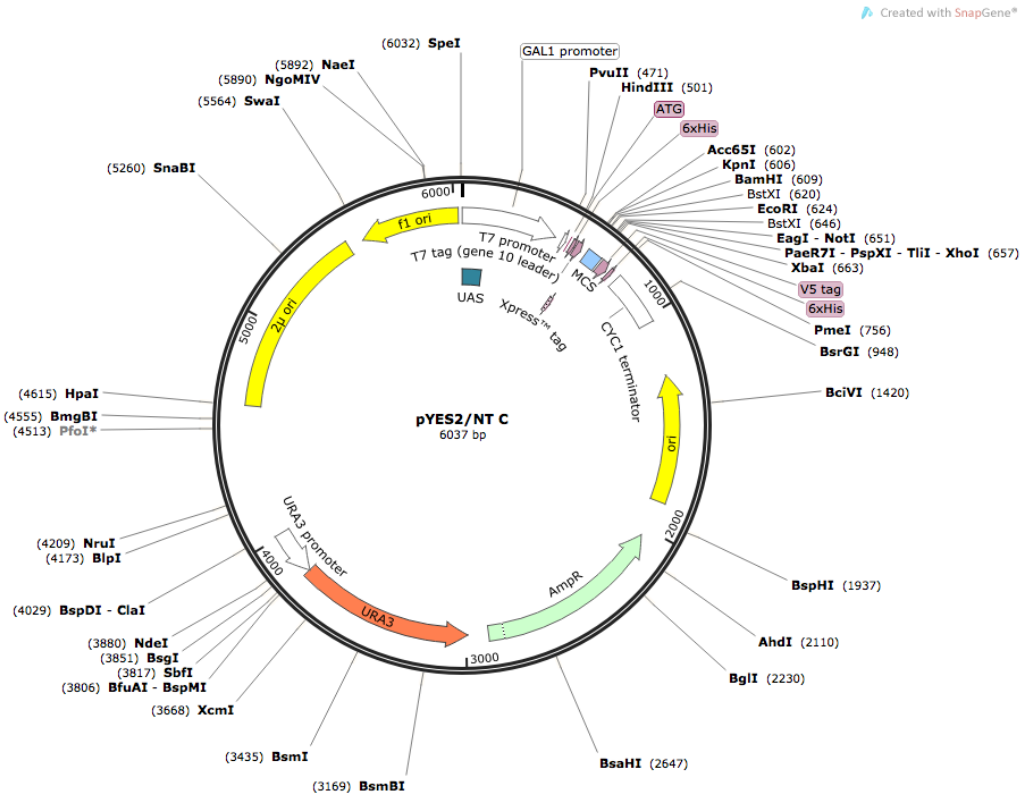




Location of specific vector features

5’ AOX1 promoter region: 1-941
5’ AOX1 priming site: 855-875
α-factor signal sequence: 941-1207
α-factor priming site: 1144-1164
Multiple cloning site: 1208-1276
c-myc epitope: 1275-1304
Polyhistidine (6xHis) tag: 1320-1337
3’AOX1 priming site: 1423-1443
AOX1 transcription termination region: 1341-1682
TEF1 promoter: 1683-2093
EM7 promoter: 2095-2162
Sh ble ORF: 2163-2537
CYC1 transcription termination region: 2538-2855
pUC origin: 2866-3539 (complementary)

10.5 pYES2/NT C (Invitrogen)




```
                GAL1 promoter
                    TATA box
300 TTAACAGATA TATAAATGCA AAAACTGCAT AACCACTTTA ACTAATACTT TCAACATTTT

                                                start of transcription
360 CGGTTTGTAT TACTTCTTAT TCAAATGTAA TAAAAGTATC AACAAAAAAT TGTTAATATA

    GAL1 forward priming site   3′ end of GAL1 promoter
420 CCTCTATACT TTAACGTCAA GGAGAAAAAA CCCCGGATCG GACTACTAGC AGCTGTAATA

    T7 promoter/priming site          Hind III                Polyhistidine region
480 CGACTCACTA TAGGGAATAT TAAGCTTACC ATG GGG GGT TCT CAT CAT CAT CAT
                                     Met Gly Gly Ser His His His His

534 CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT
    His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp

                Xpress™ epitope          Asp718 I  Kpn I  BamH I        BstX I*  EcoR I
582 CTG TAC GAC GAT GAC GAT AAG GTA CCC GGA TCC AGT GTG GTG GAA TTC
    Leu Tyr Asp Asp Asp Asp Lys Val Pro Gly Ser Ser Val Val Glu Phe
            Enterokinase recognition site  ▲ EK cleavage site
                                BstX I*  Not I    Xho I    Xba I
630 TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC GAG TCT AGA GGGCCCTTCG
    Cys Arg Tyr Pro Ala Gln Trp Arg Pro Leu Glu Ser Arg

                        V5 epitope
679 AA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT
       Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg

                Polyhistidine region          Pme I
726 ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTAAACCC GCTGATCCTA
    Thr Gly His His His His His His ***

                                        CYC1 reverse priming site
773 GAGGGCCGCA TCATGTAATT AGTTATGTCA CGCTTACATT CACGCCCTCC CCCCACATCC
```

Location of specific vector features

GAL1 promoter: 1-451
GAL1 forward priming site: 414-437
T7 promoter/priming site: 475-494
ATG initiation codon: 510-512
Polyhistidin (6xHis) region: 522-539
Xpress™ epitope: 579-602
Enterokinase (EK) recognition site: 588-602
Multiple cloning site: 602-669
V5 epitope: 682-723
Polyhistidine (6xHis) region: 733-750
CYC1 transcription termination signal: 783-1036
CYC1 reverse priming site: 800-818
pUC origin site: 1220-1893
Ampicillin resistance gene: 2038-2898 (complementary)
URA3 gene: 2916-4023 (complementary)
2µ origin: 4027-5498
f1 origin: 5566-6021 (complementary)

11. Genotypes of laboratory strains (bacteria and yeast)

Information regarding the following genotypes was either taken from the respective manuals of the bacterial strains or in case of *S. cerevisiae* CB018 requested.

E. coli EZ (Qiagen)

E. coli str. [F'::Tn10(Tc^r) $proA^+B^+$ $lacI^qZ\Delta M15$] recA1 end A1 hsdR17(r_{K12}^- m_{K12}^+) lac glnV44 thi-1 gyrA96 relA1

E. coli Stellar™ (Clontech)

E. coli str. F^-, ara,Δ(lac-proAB) [Φ80d lacZΔM15], rpsL(str), thi, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, dam, dcm

E. coli BL21(DE3)pLysS (Novagen)

E. coli str. B F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) $[mal_B^+]_{K-12}(\lambda^s)$ pLysS[T7p20 $orip_{15A}$](Cm^r)

E. coli BL21-CodonPlus (DE3)-RIPL (Agilent Genomics, provided by Prof. Dr. Klebe, Marburg)

E. coli str. B F^- ompT $hsdS(r_B^- m_B^-)$ dcm^+ Tet^r gal λ(DE3) endA Hte [argU proL Cam^r] [argU ileY leuW $Strep/Spec^r$]

Pichia pastoris X-33 (Invitrogen, provided by Prof. Dr. A. Schaller, Hohenheim)

P. pastoris str. wild-type, $HIS4^+$, Mut^+

Saccharomyces cerevisiae CB018 (provided by Dr. Backhaus, Marburg)

S. cerevisiae str. Matα his3-11,15 leu2-3,112 trp1-1 ura3-1 $ade2\text{-}1^{ochre}$ can1-100 pep4Δ::HIS3 prb1Δ::hisG prc1Δ::hisG

Saccharomyces cerevisiae InvSc1 (Invitrogen)

S. cerevisiae str. MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52

12. Reagents and kits

product	company	product	company
5‘-Full RACE Core Set	TaKaRa	PD-10 Columns Sephadex G-25M	GE Healthcare
BlueStar Prestained Protein Marker	Nippon Genetics	pectinase “Rohament P”	Röhm GmbH
cellulase “Rohament CT”	Röhm GmbH	Phusion® Polymerase (2 U μl^{-1})	NEB
DreamTaq (5 U μl^{-1})	Fermentas	Pierce™ 6x-His Epitope Tag Antibody (HIS.H8)	Fisher Scientific
ExactStartTM Eukaryotic mRNA 5‘-& 3‘-RACE Kit	Epicentre	Qiaprep® Spin Miniprep Kit	Qiagen
goat anti-Mouse IgG Fc	Fisher Scientific	restriction enzymes: BstXI, EcoRI, HindIII, NdeI, NotI, PstI, SacI, XhoI	Fermentas
horseradish peroxidase, HRP (560 U mg^{-1})	Fluka	Revert Aid First Strand cDNA Synthesis Kit	Fisher Scientific
laccase (Chinese lacquer tree) ($\geq$ 50 U mg^{-1})	Sigma	RNase H (5 U μl^{-1})	Fermentas
GeneRuler™ 1 kb DNA Ladder	Fisher Scientific	Roti®-Mark Standard	Roth
GeneRuler™ DNA Ladder Mix	Fisher Scientific	Roti®-Mark TRICOLOR Protein marker, prestained	Roth
GoTaq® Flexi DNA Polymerase Kit (5 u μl^{-1})	Promega	Silver Stain Plus Kit	Bio-Rad
Hifi Polymerase Mix (5 U μl^{-1}	Fermentas	SMARTer® RACE 5‘/3‘ Kit	Clontech
Ni-NTA His-Bind® Superflow™	Novagen	T4 DNA Ligase (5 U μl^{-1})	Fermentas
NucleoSpin®-Extract II Kit	Macherey-Nagel	T4 RNA Ligase (10 U μl^{-1})	Fermentas
PageRuler™ Protein Ladder	Fermentas	terminal deoxynucleotidyl transferase (TdT) (20 U μl^{-1})	Fermentas
PCR Cloning kit	Qiagen	TrueStart Polymerase (5 U μl^{-1})	Fisher Scientific
glucose oxidase (117.2 U mg^{-1})	Sigma	Vivaspin® 20 Centrifugal Concentrator	Sartorius AG

13. List of chemicals

product	company	product	company
1-naphthaleneacetic acid (NAA)	Duchefa	lithium acetate	Sigma
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)	Sigma	lithium chloride	Roth
2,3-dihydroquercetin / (+)-taxifolin	Roth	L-leucine	Roth
2,3-dihydroquercetin / (+/-)-taxifolin	Sigma	L-lysine	Serva
2,4-dichlorophenoxyacetic acid (2,4-D)	Duchefa	L-methionine	Roth
4-aminobenzoic acid	Sigma	L-phenylalanine	Roth
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Roth	L-proline	Roth
5-bromo-4-chloro-3-indolyl phosphate (BCIP)	Roth	L-serine	Roth
6-benzylaminopurine	Duchefa	L-threonine	Roth
acetic acid, glacial	Roth	L-tryptophan	Roth
acetone	Roth	L-tyrosine	Fluka, Merck
acrylamide/bisacrylamide (30%, 37.5:1)	Roth	L-valine	Roth
agar-agar	Cero	lysozyme	Fluka
agarose (SeaKem® LE)	Biozym	magnesium chloride hexahydrate	Roth
ammonium nitrate	Roth	magnesium sulphate heptahydrate	Merck
ammonium persulphate (APS)	Sigma	manganese(II) chloride heptahydrate	Roth
ammonium sulphate	Roth	manganese(II) sulphate pentahydrate	Duchefa
ampicillin	Roth	methanol	Fisher Scientific
ascorbic acid	Roth	methyl jasmonate (MeJA)	Fluka
biotin	Sigma	myo-inositol	Sigma
boric acid	Merck / Roth	$NADPH+H^+$, sodium salt	Roth
bovine serum albumin (BSA)	Roth	n-butanol	Alfa Aesar
bromophenol blue	Merck	nickel(II) sulphate hexahydrate	Roth
caffeic acid	Serva	nicotinic acid	Duchefa
calcium chloride dihydrate	Roth	nitro-blue tetrazolium chloride (NBT)	Roth

chloramphenicol	Serva	N-Z-Amine®, casein hydrolysate	Sigma
chloroform	Roth	phenol (citrate buffer saturated)	Sigma
cobalt(II) chloride	Merck	phenol/chloroform (1:1)	Roth
coenzym A-trilithium salt dihydrate	AppliChem	phenylmethylsulfonyl fluoride (PMSF)	Roth
coniferyl alcohol	Fluka	phosphoric acid (85%)	Roth
Coomassie Brilliant Blue G250	Fluka	pinoresinol	donation by Norman G. Lewis
Coomassie Brilliant Blue R250	Fluka	Polyclar™ 10	Ashland Inc.
copper(II) sulphate pentahydrate	Fluka	polyethylene glycol 4000 (PEG)	Roth
D-(+)-galactose	Acros Organics / Roth	potassium acetate	Acros organics
D-(+)-glucose	Roth	potassium chloride	Roth
diethyl ether	Roth	potassium dihydrogen phosphate	Roth
dimethylformamide (DMF)	Merck	potassium ferricyanide	Sigma
diphenylboryloxyethylamine (2-APB), "Naturstoffreagenz A"	Roth	potassium fluoride	Roth
dipotassium hydrogen phosphate	Roth	potassium hydroxide	Merck
disodium hydrogen phosphate dihydrate	Roth	potassium iodide	Merck
disodium ethylenediaminetetraacetate dehydrate (EDTA-Na_2)	Roth	potassium nitrate	Roth
dithiothreitol (DTT)	Roth	pyridoxine hydrochloride	Duchefa
dNTPs (dATP, dCTP, dGTP, dTTP)	Fermentas	silver(I) oxide	Roth
D-sorbitol	Fluka	silybin	Sigma
ethylenediaminetetraacetic acid (EDTA)	Merck	silychristin	Dr. Madaus & Co
egtazic acid (EGTA)	Roth	silydianin	Bionorica
ethanol	Roth / Sigma	silymarin	Sigma
ethidium bromide	AppliChem	sodium acetate trihydrate	Merck
ethyl acetate	Roth	sodium chloride	Roth
ethylenediaminetetraacetic acid ferric, sodium salt [EDTA-Fe(III)-Na]	Sigma	sodium citrate	Roth
fish sperm (carrier DNA)	Serva	sodium dihydrogen phosphate monohydrate	Merck
formic acid (98%)	Roth	sodium dodecyl sulphate (SDS)	Roth

glycerol	Roth	sodium hydrogen carbonate	Roth
glycine	Merck	sodium hydroxide	Merck
guanidine thiocyanate	Roth	sodium molybdate dihydrate	Fluka
guanidine-HCl	Roth	sodium tetraborate decahydrate, borax	Merck
hydrochloric acid (37%)	Roth	sucrose	Aldi Nord
hydrogen peroxide (30%)	Sigma	tetrabutylammonium hydrogen sulphate	Sigma
indole-3-acetic acid (IAA)	Duchefa	tetracycline	Sigma
iron(II) sulphate heptahydrate	Fluka	tetramethylethylenediamine (TEMED)	Roth
isopropanol	Roth	thiamine hydrochloride	Roth
isopropyl-β-D-thiogalactopyranoside (IPTG)	Roth	tin(II) chloride	Roth
isosilybin	Bionorica	trichloracetic acid	Roth
kinetin	Duchefa	tris(hydroxymethyl)-aminomethane (TRIS)	Roth
kojic acid	Alfa Aesar	tryptone/peptone	Roth
L-adenine	Roth	Tween 20	Sigma
L-arginine	Roth	xylene cyanol	Fluka
L-aspartic acid	Roth	yeast extract	Roth
L-cysteine	Roth	yeast nitrogen base	Conda
L-histidine	Roth	Zeocin™	Fisher Scientific
liquid nitrogen	Linde	zinc chloride	Merck
L-isoleucine	Roth	zinc(II) sulphate heptahydrate	Merck

All chemicals were of p.a. or purest available quality.

14. Instruments and materials

instrument	product	manufacturer/distributor
autoclaves	Systec VX-150	Systec GmbH
	Systec VX-95	
	AL02-02-100	Advantage–Lab
benchtop homogeniser	Minilys®	Bertin Instr.
Bunsen burner	Flammy S	Schütt
cell culture shakers	Certomat SII	B. Braun Biotech.
	RS-306	Infors AG
	TR-150	
centrifuges	Biofuge 17RS	Heraeus Sepatech
	Fresco 17	
	Pico 17	
	Centrifuge 5415D	Eppendorf
	3-30KS	Thermo Scientific
	Sorvall RC6+	
conductometer	Conmet 1W	HANNA
ultrapure water	OmniaPure	Stakpure GmbH
electroporation apparatus	BioRad MicroPulser™	Bio-Rad
exposition cassette	24x30	Rego
homogeniser	Ultra Turrax T25 Basic	IKA
FPLC columns	HisTrap™ FF, 1 ml	GE Healthcare
	Superdex™ 200 Increase 10/300 GL	
FPLC system	ÄKTA pure 25 L1	GE Healthcare
freeze dryer	Christ L1	B. Braun Biotech
freezer	C585 Innova	New Brunswick Scientific
gel documentation systems	Quick Store „plus“	RS-Laborgeräte
	FAS-Digi	Nippon Genetics
HPLC columns	Equisil ODS	Dr. Maisch HPLC GmbH
HPLC systems	Chromaster 5160 Pump + Organizer	VWR/Hitachi
	Chromaster 5280 Auto Sampler	
	Chromaster 5310 Column Oven	
	Chromaster 5430 Diode Array Detector	
	Spectra System P 400	Thermo Scientific
	Spectra System SN 4000	
	Spectra System SCM 1000	
	Spectra System UV 1000	
ice machine	AF 80	Scotsman
	RF-0244A	Manitowoc
magnetic stirrer	MR 3001	Heidolph Instr.
oven	U40	Memmert
PCR thermocycler	Eppendorf Mastercycler gradient	Eppendorf
	MyCycler	Bio-Rad

pH-electrode	Accumed Basic	Fisher Scientific
photometer	BioPhotometer	Eppendorf
	UVIKON Spectrophotometer 930	Kontron Instruments
	Specord 200 plus	Analytik Jena
pipettes	P10ml, P1000, P200, P20, P10N, P2N	Gilson
refractometer	HR-18	A. Krüss Optronic GmbH
rocking platform	Duomax 1030	Heidolph Instr.
rotary evaporator	Rotavapor RE120	Büchi
scales	PT 310	Sartorius
	EG 300-3M	Kern
	440-35A	
	440-47	
	H64	Mettler
	Explorer EX2250	OHAUS
shaking incubator	Ecotron	Infors HT
	10X 400	Gallenkamp
laminar flow bench	Gelaire Laminar Air Flow Class 100	Gelman Instrument
	Laminar Flow Workstation	Microflow
thermomixer	Thermomixer Comfort	Eppendorf
transfer membrane	Immobilon-P IPVH00010	Millipore
ultrasonic bath	Sonorex Super RK 510 H	Bandelin
ultrasonic processor	UP 200S	Dr. Hielscher
UV-hand lamp	HL-6-KM	Bachofer GmbH
vacuum centrifuge	Univapo 100 H	UniEquip
	RVC 2-18 CDplus	Christ
vacuum pump	MZ 2C NT	Vacuubrand
voltage controller	E835	Consort
	E143	
	EV2310	
	EV3020	
mixer	Vortex-Genie 2	Scientific Industries
	Vortex Mixer	VELP Scientifica
water bath	SW	Julabo
	Thermomix ME	B. Braun Biotech.
	Thermomix 7P	

V. Results and Discussion

1. Culture characterisation

The aim of the characterisation of a suspension culture during a cultivation period is to determine growth phases for optimal harvesting times. Of particular interest was the accumulation of the flavonolignan mixture silymarin as well as enzyme activities of proteins involved in the biosynthesis. In addition, parameters regarding growth and nutrient consumption, such as protein or sugar content, can provide useful information about possible adjustments to the nutrient content of the medium. Furthermore, the accumulation of secondary substances after elicitation, possible commercial utilisation or just simplified handling of ongoing working processes can be discussed.

Since the medium itself is a kind of apoplastic space for cell cultures, many interesting aspects also arise here. Metabolites and enzymes can be secreted to the apoplastic space and reactions can take place here. Conductivity measurement of the medium allows a direct, rough insight into possible changes and physiological processes of the suspension cultures. More detailed investigations of enzyme activities and determination of secondary metabolites can provide further information on similar processes taking place in the natural plant.

For observations during the culture characterisation, it should be noted that natural fluctuations can occur in the individual samples. These can appear in the form of "outliers" in the evaluation and are therefore inconclusive in critical evaluation. In addition to natural fluctuations, minor errors or differences in the processing of the samples are unavoidable. The age of the suspension cultures also plays an important role, since it is known that the metabolism of suspension cultures changes in the long term.

1.1 Medium parameters

In the following, the observed changes of medium parameters like pH-value, conductivity and sugar content will be described. In addition a small change in colour of the medium from light yellow to brownish could be discovered. It can be assumed that this occurs because phenol oxidases and phenolic compounds are released into the medium, also by increasing cell lysis.

The initial pH-value of the culture medium is about 6.0. Within 14 days it increases up to a maximum of pH 7.7 on day 11 and then decreases again to a relatively constant level of pH 7.3. Towards the end of the characterisation period beginning cell lysis and thus increased release of cell plasma and organelles to the medium led to a higher pH-value.

The conductivity represents the amount of dissolved charged compounds. A clear trend could be observed. At the start of the cultivation period values around 900 µS cm^{-1} could be measured, descending towards the end to values about 500 µS cm^{-1}. Since plant cell growth depends, inter alia, on ions from micro and macro elements of the medium to maintain their primary and secondary metabolism, the decrease in conductivity could be explained by this.

The daily measured sugar content followed this downward trend. Before autoclaving, 2% sucrose where added to the B5-medium. Measured values were between 2.3% and 0.2%. However, it should be noted that the sugar content was recorded by refractive index and thus other dissolved substances with light-refracting properties could influence the total value. The added sugar from the medium was consumed as nutrient, from which the plant cells gained their necessary energy. This addition was necessary, because the cell cultures were cultivated in the dark, thus cannot live photoautotrophically. The outlier at day 10 can be seen as a minor exception.

The change of the parameters pH, conductivity and sugar content over time proceeded mostly as expected. After inoculation of plant cell cultures in fresh medium and a small familiarisation phase (lag phase), the cells could adjust to their particular conditions and pass into a strong growth phase. As a result, conductivity and sugar content decreased almost linearly and can be seen as a sign of active metabolism. After a week of growth most essential nutrients were consumed. This lack of resources led to the start of upcoming cell lysis (dying-off phase), resulting in continuously rising pH values. Similar processes have also been described for an *in vitro* culture of *Coleus blumei* (Petersen, 1994) or a *Forsythia* × *intermedia* cell suspension culture (Schmitt and Petersen, 2002).

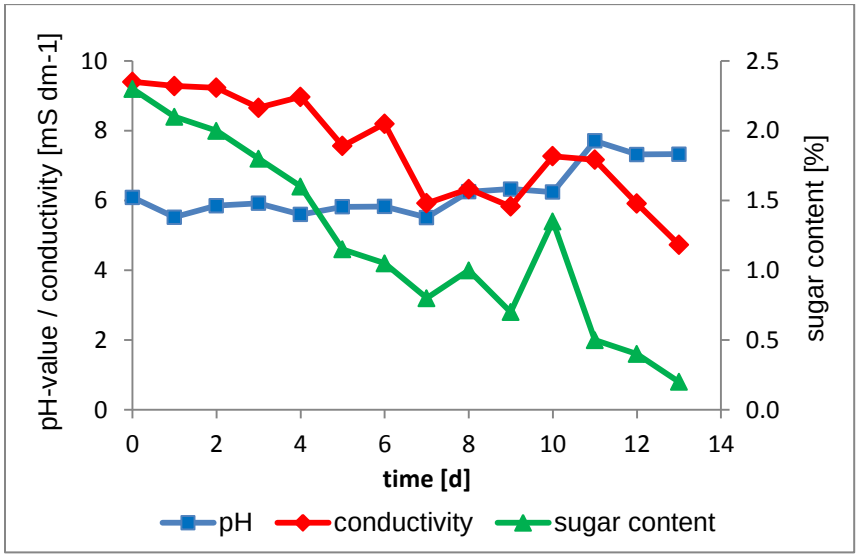


Fig. 13: Medium parameters (pH-value, conductivity and sugar content) of a *Silybum marianum* suspension culture.

1.2 Growth parameters

The dry weight of the cells, after lyophilisation, enables statements about the development of the cells. Its peak of growth (~0.9 g dry weight per flask) was reached after about a week (day 6-7), also correlating with its maximal fresh weight. The minimal cell dry weight at day 4 (about 0.28 g/flask) and the slightly decreased pH-value on day 1 and 4 can be explained with a probable bacterial contamination, resulting in an alcoholic smell and negative effects on growth parameters. This is reflected particularly in the increased protein content in the medium on day 4. After day 8 no further obvious cell growth could be determined (Fig. 14).

This observation also correlates with the protein content in the suspensions cells. The outlier at day 11 can be seen as a minor exception. The protein concentration in the medium is delayed and really only apparent from day 6 onward with its peak at day 10, which appears to also make sense in this context (Fig. 15). Only after reaching maximal cell growth and protein concentration within the cells, an increased release of substances and proteins to the outer compartment takes place. The starting cell lysis certainly also contributes to this.

However, determination of the protein concentration with the Bradford (1976) assay has to be seen critically, as it can only measure soluble proteins with nonpolar residues and cationic charge. As a consequence it is impossible to make a clear statement about the overall protein concentration in cell cultures. Nevertheless the measured value can be taken as a reference for protein content.

In summary, it can be said that after inoculation of plant cell cultures in fresh medium and an adjustment phase for a few days, the cells passed into a strong growth phase. The general tendency shows a time-delayed increase of protein content in the medium in the course of time. After a week of growth most essential nutrients were consumed. This lack of resources led to the start of upcoming cell lysis, resulting in continuously decreasing cell weights and protein content.

In the course of this work, the suspension cells were able to accustom themselves more and more to the medium and weekly inoculation. Later on ($\geq 50^{th}$ subculture), up to 20 g cells (fresh weight) could be obtained from a suspension culture at day 7 at its maximum. This, unfortunately, was accompanied by a considerable loss of secondary metabolites like silymarin. Therefore the culture characterisation was carried out as early as possible.

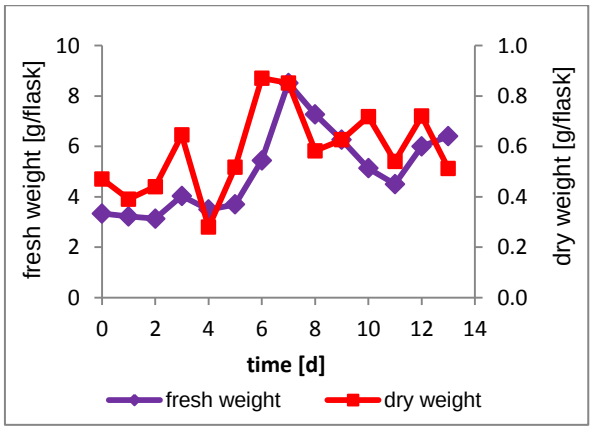


Fig. 14: Growth parameters (fresh and dry weight per flask) of *Silybum marianum* suspension cultures.

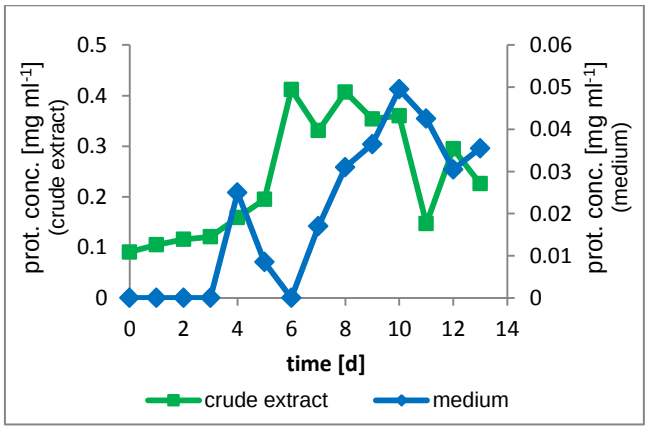


Fig. 15: Growth parameters (protein content in crude extracts and the corresponding medium) of *Silybum marianum* suspension cultures.

1.3 Flavonolignan content

The calculated silymarin content during the observation period approximately follows the progress of the cells' dry weight and thus its growth curve. During the first few days and at the end of the cultivation period the content of silymarin fluctuates around an average of 60 µg per flask. The huge drop in silymarin content at day thirteen cannot be really explained, at most with far progressed cell lysis. Despite renewed reprocessing of the third sample and repeated measuring by HPLC no result could be achieved. The fifth sample got lost because of boiling retardation in the ultrasonic bath. Between day 7 and 9 the highest yield in cell dry weight was reached (Fig. 16). This correlates to the maximum of silymarin content on day eight (~14 µg in 0.06 g dried cells ≙ 0.023% dry weight). This is equivalent to 235 µg silymarin in 1.0 g dried cells (between 100 and 140 µg per flask). The two diastereomeric pairs of isosilybin and silybin make up the largest part of the silymarin content (> 95%). The two positional isomers silydianin and silychristin were only present in very low amounts, thus hard to measure precisely by HPLC. Hasanloo et al. (2008) made similar observations during their evaluations of flavonolignan content in *Silybum marianum* suspensions cells. The first to deal with this problem were Becker and Schrall (1977). At that time, neither silymarin nor taxifolin could be detected in callus or suspensions cultures by means of thin-layer chromatography. The silymarin content measured in untransformed root cultures was about one-tenth lower compared to suspension cells (Cacho et al., 1999; Alikaridis et al., 2000). The former group, however, came to 10-fold the amount of silymarin in suspensions cells of the 8th subculture (~0.3% dry weight). Yet, this value is certainly difficult to ascertain after all these years, even though herbal *in vitro* cells can vary widely with regard to quantities of specific metabolites.

Based on this very small yield a commercial utilisation is not recommended. In general, it can be specified that the flavonolignan content extracted from cell suspension cultures is much lower compared to the amounts detectable in fruit coats of milk thistle. Interestingly, even the developed root cultures, grown on MS-Li agar plates, contained small amounts of silymarin. With only about 0.0004% dry weight, however, this is hardly worth mentioning. Therefore new elicitors for higher production and accumulation of these specialised metabolites by *in vitro* cultures are being constantly evaluated.

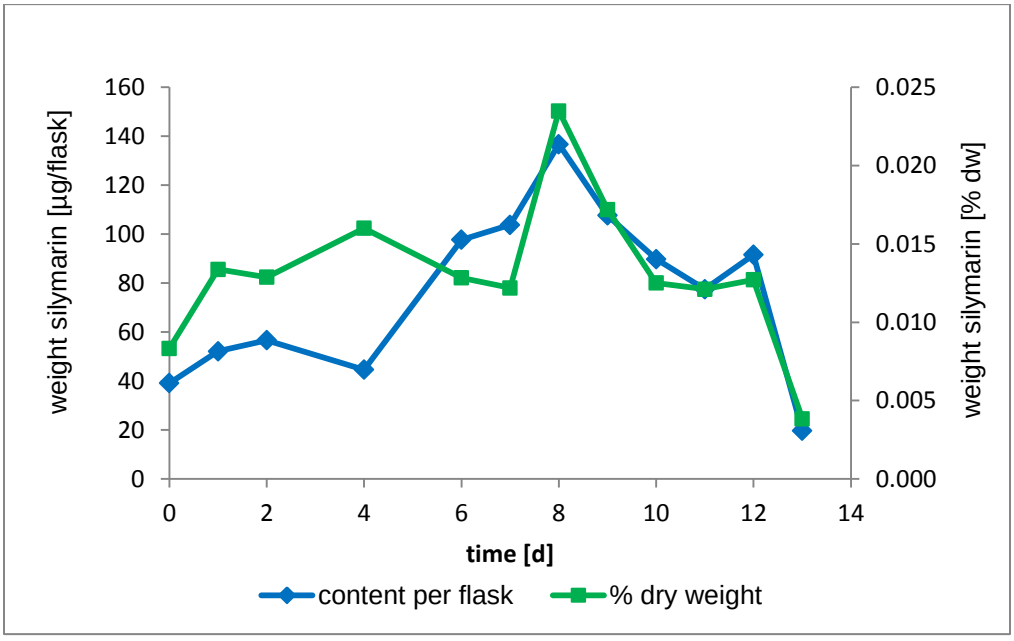


Fig. 16: Flavonolignan content (per flask and in % dry weight) of suspension cells of *Silybum marianum*.

1.4 Enzyme activities

For the enzyme assays performed with the individual protein samples prepared from suspension cells or the corresponding medium several things are striking. First, the formation of the two regioisomers silybin and isosilybin takes place preferably. Again, they represent the largest share of the formed silymarin mixture (> 99%). Furthermore, the distribution of the individual regioisomers formed during enzyme assays was almost uniform over the course of the characterisation. Therefore only the total amounts of silymarin are compared further on.

On closer examination of the curve with respect to the activity of the crude protein extract the highest activity has been measured on day one. Between days three and four the lowest activity could be recorded. Probably the newly transferred suspension cells react to their new environment with biochemical stress reactions. This could lead to a higher enzyme expression rate and an increase in secondary metabolites, possibly explaining the higher silymarin content in dried cells on day two.

Previous observations (V.1.2) have shown that the highest cell growth of suspension cultures occured between days six and eight. This reveals no correlation between the quantity of cells and the activity of enzymes catalysing silymarin formation. Enzyme activity in crude extracts seems to stay at a constant level from day 5 on.

In contrast, the activity of proteins released to the medium of cell suspension cultures somewhat seems to correlate with increasing cell growth but rather with beginning cell lysis. Higher extracellular protein concentrations would be the consequence. A big increase in activity could be observed on day nine and ten, followed by a big drop on day eleven. This could be an outlier. The last few days of cultivation again revealed a small rise of products, thus activity. However the highest activity did not correlate with the time of highest cell growth, but rather with beginning lysis and dying of plant cells. This seems reasonable, since more proteins were released into the medium. Furthermore, this agrees with the observations on the measured protein concentration in the medium, which also had its maximum peak on those days.

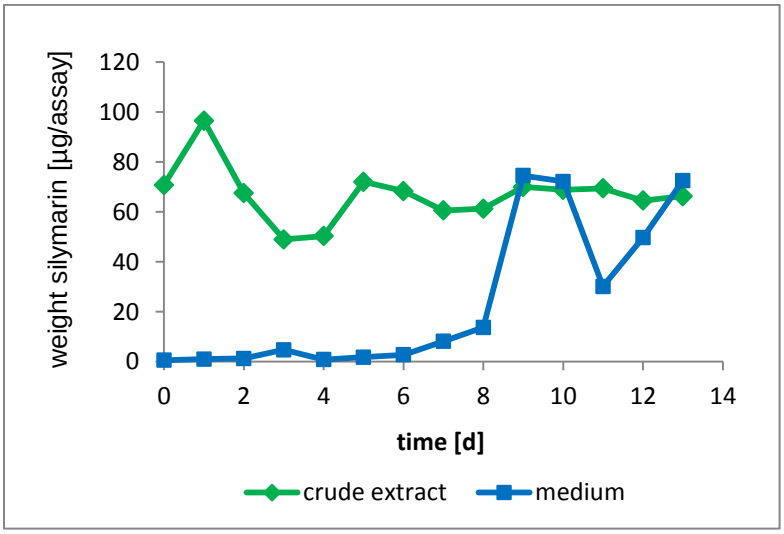


Fig. 17: Silymarin content formed in standard enzyme assays with crude protein extracts or the corresponding medium of suspension cultures of *Silybum marianum*.

2. Culture elicitation

In previous studies it was reported that methyl jasmonate (MeJA) or cyclodextrins can induce the release of taxifolin, coniferyl alcohol and all of the isomers of the silymarin mixture to the extracellular medium (Sánchez-Sampedro et al., 2005; Belchi-Navarro et al., 2011). Many other substances have also proven their effectiveness as elicitors in various studies. These include, for example, picloram (Hasanloo et al., 2008), salicylic acid (Khalili et al., 2009), chitosan (Hasanloo et al., 2014), synthetic pyrazinecarboxamide derivatives (Tůmová et al., 2011) or silver ions (Vildová et al., 2014). In the course of this work the choice yet fell on MeJA, a known elicitor for secondary metabolites in plants, because other groups achieved good results with this elicitor.

2.1 Flavonolignan content

In order to increase the silymarin formation and study possible effects on its release into the outer compartments *in vitro* cells of *Silybum marianum* were treated with either MeJA or EtOH as control. The addition was made on day three of freshly inoculated suspensions cells in B5 medium. Every 12 h, starting with 0 h, a flask was taken and analysed for its flavonolignan content.

Comparing the silymarin content in the suspension cells, no big differences could be detected between control and samples. The trend of the curves is very similar. The flavonolignan content increases slowly until reaching its maximum with about 0.028% dry weight after three days of incubation, and then decreases steadily in the following days. Merely cell samples obtained after 12-24 h of elicitation showed a small increase of silymarin content (~0.005% dry weight) (Fig. 18). This effect seems to correlate with the amount of silymarin released into the medium, shifted for about 12 h. A higher biosynthesis of flavonolignans in suspension cells also could have an enhancing effect on transport systems.

Flavonolignans were released into the medium in a similar amount in the control group and in elicited samples. Only marginal differences could be detected during the first 12 h of elicitation. After 24 h the effect of the elicitor showed its full impact on putative transport systems. Elicited samples showed about 70% more silymarin in the medium at that time. The control group with EtOH nearly catches up with a delay of 12 h showing a parallel curve from that time onwards (Fig. 19). Yet the amount stays about 15-20% lower.

In general, increased flavonolignan formation started after 12 h, peaked between 24 and 48 h and slightly decreased afterwards. These observations are in agreement to other studies (Sánchez-Sampedro et al., 2005). Conversely, Belchi-Navarro et al. (2011) found that MeJA only markedly influenced silymarin production if combined with cyclodextrins and only after delayed addition. It is also reported that flavonolignan accumulation in the medium of suspension cells treated with MeJA is closely related to an increased activity of the enzyme phospholipase D (Madrid and Corchete, 2010). In 2013, Prieto and Corchete demonstrated that extracellular accumulation of flavonolignans in *Silybum marianum* cultures is dominated by ATP-dependent primary transport that involves ATP-binding cassette-like transporters. Their results suggest that the elicitor-induced secondary metabolite release from cells into the medium could also be regarded as an induced excretion via activation of specific transport systems. They postulated that a Ras-related protein of the Rab family, usually involved in transmitting signals within cells, as well as an ATP-binding cassette transporter (ABC transporter) is upregulated in cultures elicited with MeJA. Both enzymes could play an important role in secretion and/or recycling of cell wall components (Prieto and Corchete, 2014).

Nevertheless, even after successful elicitation of milk thistle suspension cells, flavonolignan quantities are still far too low for an economic benefit. Extraction from mature achenes is still significantly more profitable. A far more interesting topic would be to clarify how silymarin is released to the medium, which mechanisms are involved and if so, as a result, whether this could affect the flavonolignan regioisomer distributions. This in turn could be a possible indication of similar processes for silymarin accumulation in the mature fruits of the plants. Even though this has been investigated a lot in recent years it is certainly an interesting topic for new insights into plant processes in the future.

Recent studies predict vesicles or specialised organelles transporting newly synthesised compounds to other storage compartments or to the plasma membrane for efflux (Weston et al., 2012), thus vesicle-associated pathways for the delivery of silymarin and/or their precursors to the extracellular medium are conceivable. “Taken into account that the extracellular medium of suspension cultures could be considered as an apoplastic space, thus resembling the fruit wall, it could be postulated that coniferyl alcohol and taxifolin could be exported independently and radical coupling of these precursors would take part extracellularly by apoplastic proteins. Concerning flavonoids, the enzymes for their biosynthesis are believed to be localised on the cytosolic side of the endoplasmic reticulum (ER), and efficient flavonoid transport systems

deliver these metabolites to their destination (Agati et al., 2012). There are even studies about flavonoids that are secreted from the cells (Kitamaru, 2006).”

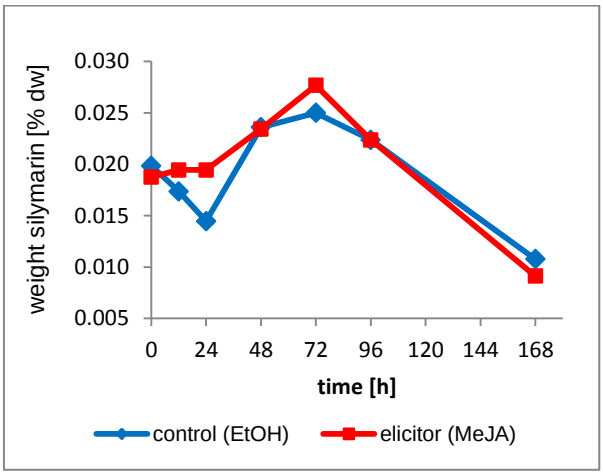


Fig. 18: Silymarin content in suspension cells of *Silybum marianum* treated with either EtOH or MeJA for 168 h. The values come from a single series of measurements, since the first attempt got destroyed in the process.

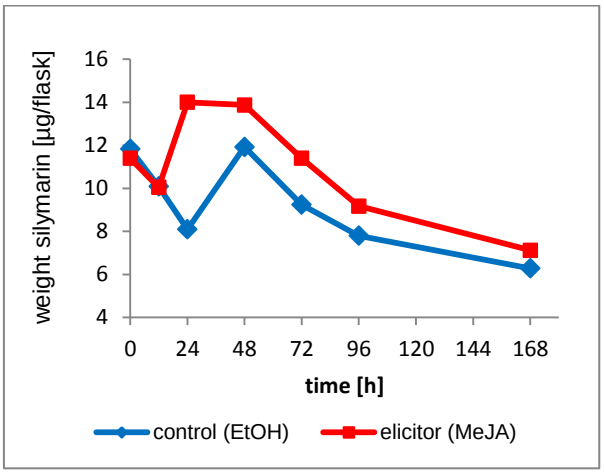


Fig. 19: Silymarin content extracted from the medium of *Silybum marianum* suspension cells treated with either EtOH or MeJA over 168 h. Each value represents the mean of two independent measurements.

2.2 Silymarin degradation

Since the suspension cells of *Silybum marianum* also release flavonolignans and precursors to the medium, it is important to investigate possible reasons and mechanisms, but also whether the released compounds have sufficient stability in the medium or are degraded by plant enzymes. In the plant itself, silymarin is almost exclusively stored in the fruit coat of the achenes. This was tested by careful separation of pericarp and testa from the embryo, followed by individual flavonolignan extraction and determination (V.8). Similar results were reported by AbouZid et al. (2016). It has yet to be explored if silymarin is synthesised in the fruit coat or if the individual flavonolignan compounds and precursors are formed somewhere else and are transported into this compartment. Furthermore, Greimel and Koch (1977) stated that the silymarin components interfere with or even inhibit enzymes of oxidative metabolism, like peroxidases. This would indicate a clear separation. The possibility of relocation to specific parts of the fruit is not possible within the *in vitro* culture or replaced by targeted transport into the medium. Since this is rather a compensation process, the stability of silymarin in the medium has to be investigated.

The stability of silymarin and its possible degradation was tested by incubating a specific amount of silymarin standard (~240 µg) in 50 µl purified medium for 24 h under normal culture conditions (IV.3.4). Different pH conditions, as well as simple TRIS-HCl buffer as a reference, were tested.

In the evaluation of the collected data it is striking, that there was about 12% less silymarin after shaking it in habituated medium in the dark at 26 °C for 24 h. Compared to simple TRIS-HCl buffer of the same pH this was even 7% more degradation. The effect increased with rising pH values particularly for the positional isomer silydianin. About 50% less silydianin and 20% less of the other regioisomers could be detected after incubation at pH 10.0 for 24 h.

The results show that released flavonolignans are rather unstable in the culture medium. Temperature and pH probably play the major role, but degradation by enzymes cannot be excluded. In 2007, Sánchez-Sampedro et al. reported that even though peroxidases from cell extracts and, to a higher degree from the spent medium, promote flavonolignan formation, silymarin compounds were also degraded by suspension culture peroxidases. Although a higher pH (~9.5) for the formation of silymarin in enzyme assays is very favourable, a rising pH in the culture medium, as observed at the end of the culture period, shows rather opposite effects. This is reflected particularly in the stability of the positional isomer silydianin. This shows that an *in vitro* culture is only an attempted imitation and is inferior to the plant in many ways. This is especially true for storage of specialised metabolites in special compartments of the plant.

3. Flavonolignan composition from different milk thistle chemotypes

Since the middle of the 1990s, Hetz et al. (1995) found that there are different lines of *Silybum marianum* plants with a varying silymarin composition. A decade later, more research groups took up this topic and discovered distinct chemotypic variations, particularly in the fruits, from plants grown and harvested in different areas (Hasanloo et al., 1995; Martin et al., 2006; Shokrpour et al., 2007). However, very little is known about why these differences occur and how the plant discriminates between the individual silymarin regioisomers.

Simple seasonal differences due to environmental factors, harvesting times or also further processing methods, for example the drying processes could affect the silymarin composition. Much more interesting, however, could also be factors on the enzymatic and genomic level of the

plant which are largely independent from external influences. Initial conditions such as substrate concentrations or transport systems can also be discussed at this point.

In order to obtain further information on processes within the plant regarding variations of the silymarin components, milk thistle fruits of various European origins were examined for their flavonolignan content and composition (Poppe and Petersen, 2016). Of the variants with clearly different proportions of its silymarin regioisomers, callus and suspensions cultures were established. After further analyses and determinations of the flavonolignan composition, these allow conclusions about possible causes and involved systems in the cells or the corresponding medium. In addition, proteins involved in silymarin biosynthesis and present in enzyme preparations could differ. Fractionation and isolation of participating enzymes could establish a direct link between the varieties and the biosynthesis of the individual positional isomers. Thus, in addition to possible differences in the crucial oxidising enzyme(s), involved dirigent proteins would be detectable and could as well be isolated by means of fractionation. Enzyme assays with these individual fractions then could further provide information about the presence of such proteins in *Silybum marianum* and clarify a possible direct influence on the diastereomer formation of silybin and/or isosilybin or even affect the distribution of the individual positional isomers.

3.1 Extraction method

The validation of the extraction method for fruits of *Silybum marianum* revealed unequivocal results. A thin-layer chromatography first provided a rough overview. Even though silydianin, silybin and taxifolin could be detected as separated spots (V.8) this method did not allow any statements about exact amounts. Thus, the more accurate evaluation by HPLC was carried out.

Best results could be achieved with MeOH as extraction solvent. The single extraction step of the fruits with MeOH led to a 60% higher yield of flavonolignans than extraction with EtOAc, while the extraction with acetone led to a 13% reduction in yield (Fig. 20). Very similar results were also observed by others groups (AbouZid et al., 2016).

“Unfortunately, the single extraction method is not suited for quantitative determination of the flavonolignan content in milk thistle fruits. Only about 50% of the total flavonolignan content could be extracted by a single extraction step. This could be increased by repetitive extractions.

Each additional step with fresh solvent resulted in about half of the yield achieved before. Thus, about five repetitions would be necessary to get a > 95% yield of silymarin. However, the number of extraction steps did not affect the ratios of flavonolignan regioisomer distribution (Fig. 21). In conclusion, the single extraction method is suitable to determine variations in the composition of the flavonolignan mixture and the chemotypic variations of *Silybum marianum*" (Poppe and Petersen, 2016).

About 25 mg silymarin could have been be extracted from 1 g fruits (≙ 2.5%) using repetitive extractions and MeOH as extractant. On the other hand, the extractions with acetone and EtOAc would have yielded in only about 14 mg and 3 mg, respectively. In order to get equal amounts, much more repetitions would have been necessary.

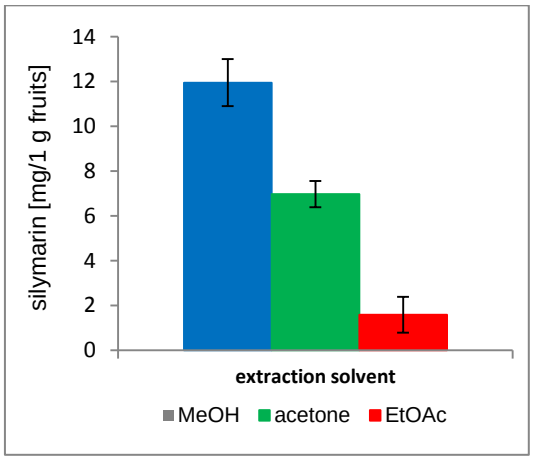


Fig. 20: Silymarin content extracted from 1 g milk thistle fruits using a single extraction method with 10 ml of the indicated solvents. The data represent mean values of three replicate extractions (±s.d.).

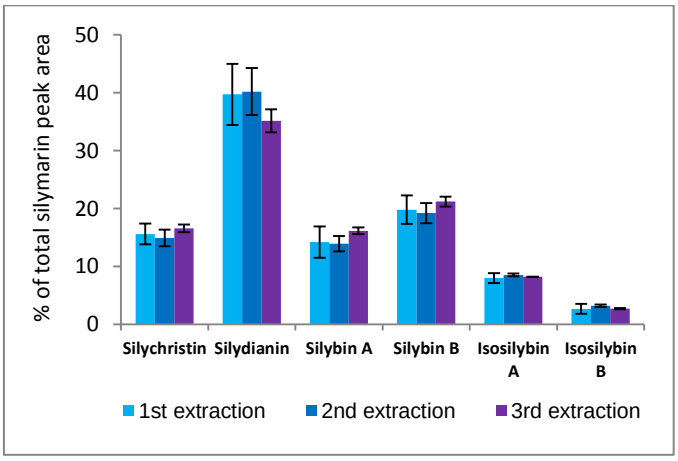


Fig. 21: Percentage share of the total peak area of the individual regioisomers after 1-3 repetitive extractions of 1 g milk thistle fruits with 10 ml MeOH. The data represent mean values of three replicate experimental procedures (±s.d.).

3.2 Silymarin content and composition in fruits

To obtain a rough overview of the quality and the silymarin content of the fruits obtained from different habitats in Europe these were first extracted and compared. The flavonolignan content in fruits varied from appr. 0.8% (Bombastus/Bulgaria) to 2.7% (Klenk/Poland) using a single MeOH extraction step (Fig. 22). The yield could be increased up to a maximum of approximately

6% depending on the number of extractions and additional discrimination of damaged fruits (Fig. 23). This is comparable to other studies (Cacho et al., 1999; Greenlee et al., 2007).

The colour of the fruits ranged from a pale greyish white tone (Bombastus/Bulgaria) to a darker grey (Klenk/Poland; Caelo/Poland) up to brown (Galke/Hungary; Marburg) and almost shiny black (Frankfurt). However, there was no specific connection between outer appearance and silymarin content; also the distinct distribution of regioisomers was in no context. Only the fruits of the pharmaceutical wholesaler Bombastus seemed to be not fully mature, which was reflected in colour and silymarin content. Normally the colour of milk thistle fruits changes during maturation from creamy white to light/dark brown to almost shiny black when they are ripe and fully mature. Accordingly, the early developmental stages also contain substantially less total flavonolignans. A thin-layer chromatography of extracted unripe fruits revealed no detectable flavonolignans at all. A few years ago, Elwekeel et al. (2013) could demonstrate a correlation between fruit colour, maturity stage and silymarin content as well.

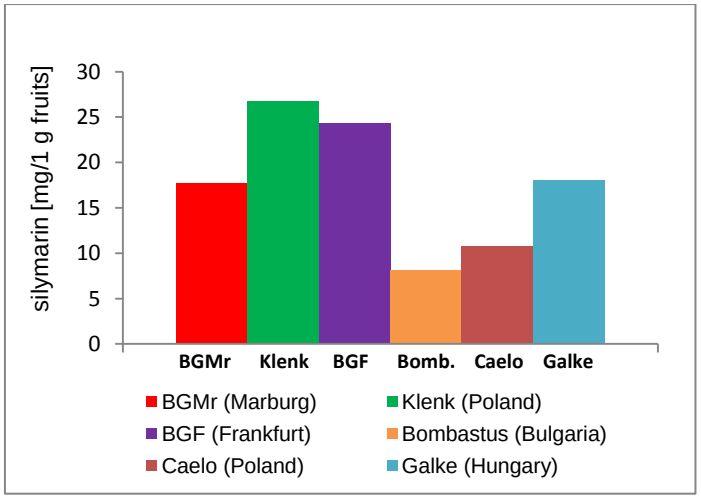


Fig. 22: Silymarin content in milk thistle fruits from different origins after a single extraction step with MeOH as extraction solvent.

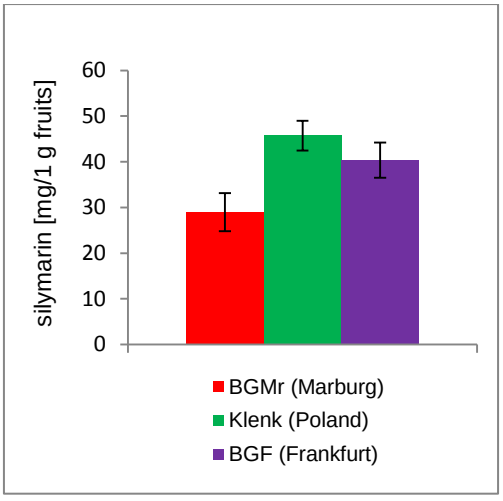


Fig. 23: Silymarin content in fully mature undamaged milk thistle fruits from different origins after three replicate extraction steps. The data represent mean values (±s.d.).

Closer examination of the distribution of the individual components of the silymarin mixture extracted from the fruits of *Silybum marianum* cultivars led to interesting results. Of the achene extracts from the 6 different origins two showed a completely different pattern [type I + II]. The remaining 4 shared a very similar silymarin composition [type III]. This includes the fruit extracts

from Frankfurt, Hungary, Poland (Caelo) and Bulgaria. In this milk thistle chemotype the amounts of silychristin, silybin A and B and isosilybin A each were between 10 and 20%, calculated as the share of the total silymarin peak area, while isosilybin B amounts only to about 5%. The dominant positional isomer was silydianin with about 40%. The achenes from Poland (Klenk) contained between 25-35% each of the total silymarin peak area as silychristin, silybin A and silybin B, while the silydianin content was extremely low (< 10%) [type II]. Compared to these, the Marburg line (BGMr) revealed the highest amount of the regioisomer silydianin with about 70% of the total silymarin peak area, but on the other hand, the lowest silychristin and silybin amounts [type I]. The percentage share of the positional isomer isosilybin was roughly comparable in all samples (Fig. 24).

Representatives of the three lines with the most striking differences in their regioisomeric pattern of silymarin were chosen for a second comparison to validate the results. The Frankfurt line was chosen for the type III cultivar for further experiments. Additionally, only fully mature and undamaged fruits were selected. The above-mentioned results could be reproduced. This was also true when comparing absolute quantities of the individual flavonolignans. The characteristic patterns of the regioisomer distribution of the three types are easily recognisable. Again, the silychristin (0.4 mg/1 g fruits) and silybin (0.9 mg/1 g fruits) quantities of the Marburg cultivar [type I] were very low compared to the other two. The value of silydianin is the highest (2.65 mg/1 g fruits), whereas the Klenk line revealed very low silydianin amounts (0.5 mg/1 g fruits). In turn, this is characterised by a high proportion of silychristin (4.2 mg/1 g fruits) and silybin (10 mg/1 g fruits) [type II]. To some extent, one could label these two cultivars as lines with opposite distribution of the proportions of regioisomers in the total silymarin mixture. Solely, the share of isosilybin is very similar for all three cultivars with values between 3-4 mg/1 g fruits. Compared with type I+II, the proportions of the positional isomers of the Frankfurt line almost have an average character [type III] (Fig. 25).

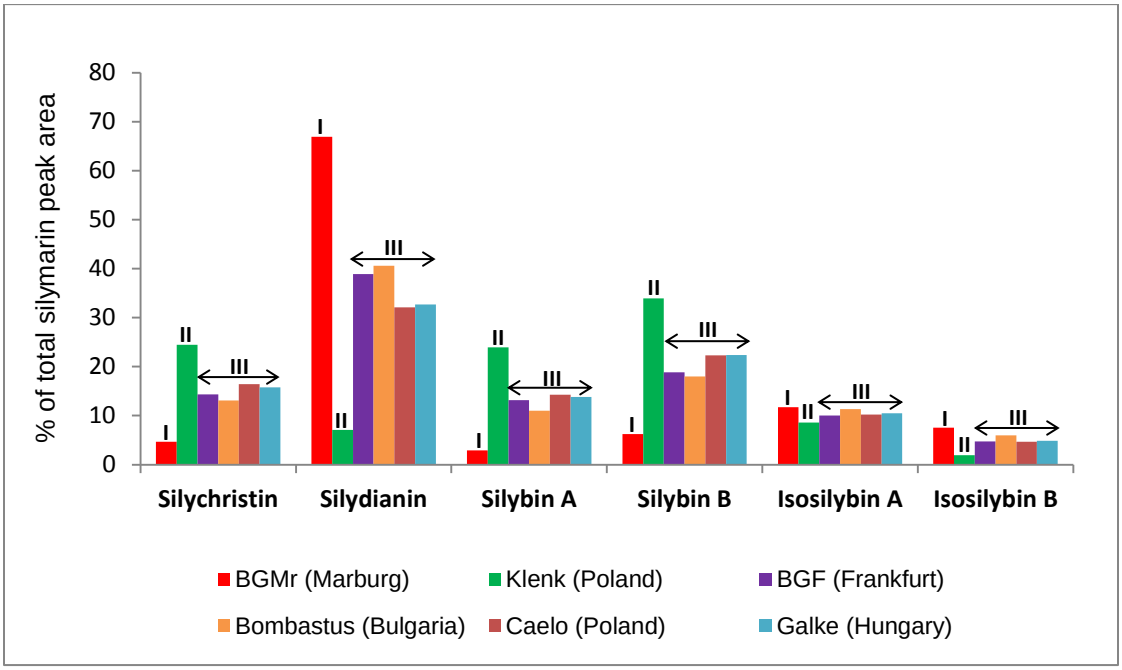


Fig. 24: Distribution of flavonolignan components in *Silybum marianum* fruits from different cultivation areas. The abbreviations indicate the origin of the fruits: Botanical Garden Frankfurt (BGF), Botanical Garden Marburg (BGMr), Poland (Klenk), Hungary (Galke), Poland (Caelo) and Bulgaria (Bombastus). Three different types of regioisomer distribution (chemotypes) are marked with I, II, III. Silymarin components were compared based on the percentage of the total silymarin peak area. The data represent mean values of three replicate extractions (±s.d.).

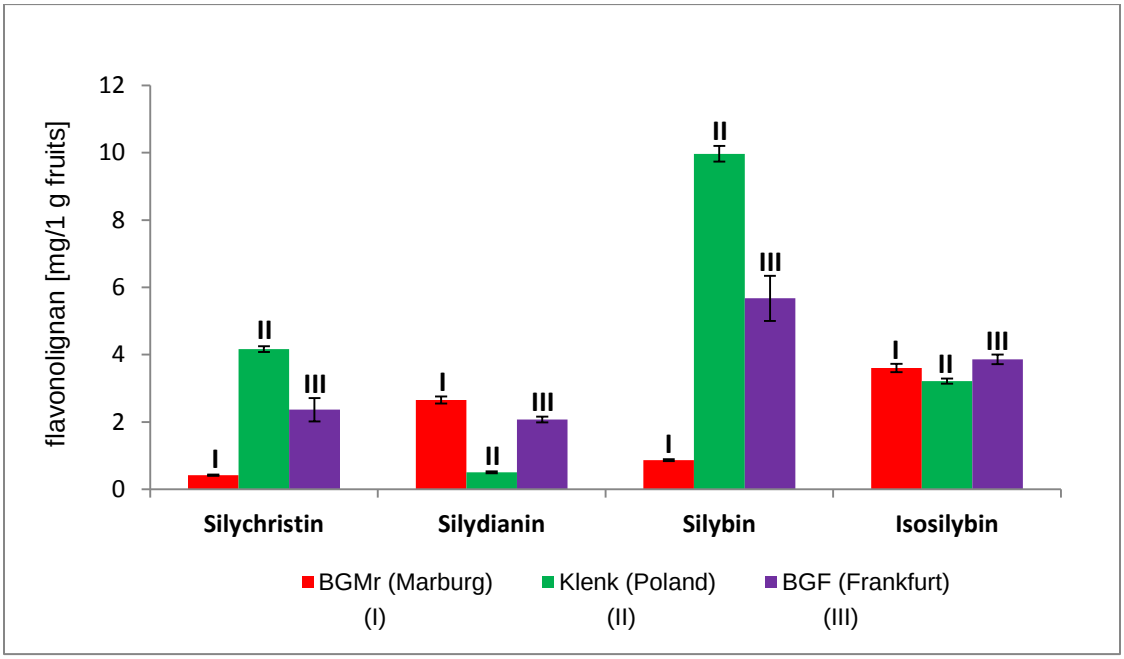


Fig. 25: Distribution of flavonolignan components from selected fully mature, undamaged fruits of *Silybum marianum* from the three chemotypes BGMr (I), Klenk/Poland (II) and BGF (III). The data represent mean values of three replicate extractions (±s.d.).

Taking these findings into account we can assume that there are at least three different chemotypic variations of *Silybum marianum* with distinct properties. Somehow the plant discriminates between the regioisomers of silymarin. The variation in total amounts of silymarin has on the one hand been assigned to the maturity of the fruits and on the other hand to the genotype (Martin et al., 2006). Whether this is caused due to active processes, to what extent external factors play a role or if there is some kind of special advantage to the plant yet has to be clarified. During the investigation of milk thistle cultivars from different regions of Iran and Hungary, in addition to a strong diversification of the individual silymarin components, even variants with completely missing regioisomers were found (Hasanloo et al., 2005).

In this context, of course, seasonal variations must be discussed. Flavonolignan biosynthesis, thereby also the ratio of its constituents, might vary with the season due to environmental influences. In addition to the stage of fruit development, biotic and abiotic stress factors might play an important role as well (Sahoo et al., 2012). For example, sun exposure, drought or precursor concentrations could influence the individual regioisomer formation of silymarin. In 2015, Khan et al. demonstrated a direct link between growth conditions and the concentrations of various silymarin components. Additionally, *in vitro* stress conditions revealed a strong impact on the respective biosynthetic pathways. As pointed out by Abbasi et al. (2016), this suggests that wild grown milk thistle plants are susceptible to seasonal variations and environmental pollutants.

On closer inspection of the milk thistle fruit material used in our experiments (Table 1) the varying drying processes and harvesting times have to be mentioned. These could have an impact on the amounts of positional isomers of the silymarin mixture. However, the three presented chemotypes revealed no correlation with respect to harvest year, drying process or cultivation area. For example, the samples of Bombastus/Bulgaria, Caelo/Poland and Galke/Hungary shared their chemotypic properties with fruits from the Botanical Garden in Frankfurt although drying process and region of cultivation clearly differed. Apart from that, the achenes from Frankfurt and the Old Botanical Garden in Marburg were dried similarly, harvested in the same year and cultivated in the same region but showed obvious differences in their flavonolignan regioisomer distribution. Although it can be assumed that the above-mentioned factors may influence the total silymarin accumulation in the fruits, the three chemotypic variations discovered in this study certainly cannot be related to extrinsic factors only but could rather be caused by the genotype of the plant (Poppe and Petersen, 2016).

3.3 Silymarin composition in suspension-cultured cells and medium

The *in vitro*-cultivated *Silybum marianum* cells of the three different chemotypes already revealed visible differences in their phenotype. Suspension cells from the chemotype Poland (Klenk; II) grew in bigger flake-like aggregates and were cream-coloured, whereas the BGF chemotype (III) was much finer and light brown. This way of surface enlargement even increased with the total number of subcultures. Cells of the Marburg variety (I) had small dark brown, spherical cell aggregates (Fig. 26).

Fig. 26: Phenotype of suspension cells after 7 days of growing in B5 medium, incubated in the dark at 26 °C and shaken at 120 rpm. The abbreviations represent the respective origin of the achenes: Botanical Garden Marburg (BGMr), Botanical Garden Frankfurt (BGF) and Poland (Klenk).

The individual regioisomeric patterns of silymarin in the suspension cells of the three cell lines seem familiar. In fact, the *in vitro* cultured suspension cells resembled the fruit chemotype. The highest silydianin amount again could be detected in the Marburg variety [type I]. The same applies for the Klenk cells [type II], which on the one hand had the lowest amount of silydianin, but then again revealed high quantities of the positional isomers silychristin and especially silybin. Cells from the Frankfurt chemotype [type III] took a similar position in its distribution of flavonolignans as discovered in fruits of the same type. Its silydianin and silybin amounts ranged between the two other varieties, only silychristin amounts were higher than expected. With respect to the isosilybin proportions no major differences could be detected between the three cell lines (Fig. 27).

These results support the hypothesis, that there could be some kind of active or passive mechanism (e.g. the involvement of specialised proteins) that discriminates between the formation of the single regioisomers and underline the existence of high genotypic variations (Martin et al., 2006; Poppe and Petersen, 2016).

The analysis of the cell culture media revealed clear distinctions in the isomeric flavonolignan ratios. Especially the very low amount of silydianin and silychristin are striking. Both compounds made up for the dominant silymarin components in cell extracts. The remarkable divergence between the diastereomers of silybin and isosilybin even within a cell line is particularly interesting as well. These were always very uniformly spread in the fruits, suspension cells or even enzyme assays. Although the cells of the Marburg variety [type I] revealed the lowest amount of silybin in the cells, more was released to the medium compared to the other two types. The isosilybin B value of the Klenk line [type II] seems peculiarly high. In summary, it can be said that the respective medium does not resemble the fruit chemotype and no real correlation is recognisable (Fig. 28).

Certainly, a large influence on the distribution of the individual silymarin components in the medium could be its stability as mentioned in V.2.2. Degradation due to rising pH values or extracellular enzymes can be expected but cannot solely explain this diverse distribution. In case of the regioisomers silychristin and silydianin these could either be retained inside the cells or may be tightly linked to the cell well. Selective transport mechanisms for silymarin components and/or its precursors have been proposed by Prieto and Corchete (2014) who, however, did not analyse a possible variation in the ratio of different silymarin components.

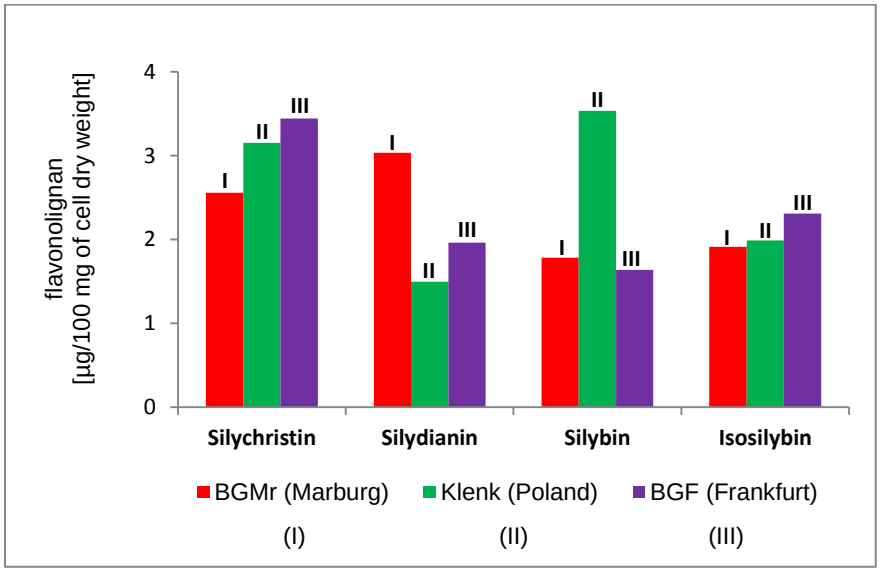


Fig. 27: Distribution of flavonolignan components extracted after 5, 20 and 50 subcultures of suspension cell lines of three different chemotypes. The data represent mean values of three extractions. Standard deviations were omitted due to the fact that the individual flavonolignan content decreases with the number of subcultures.

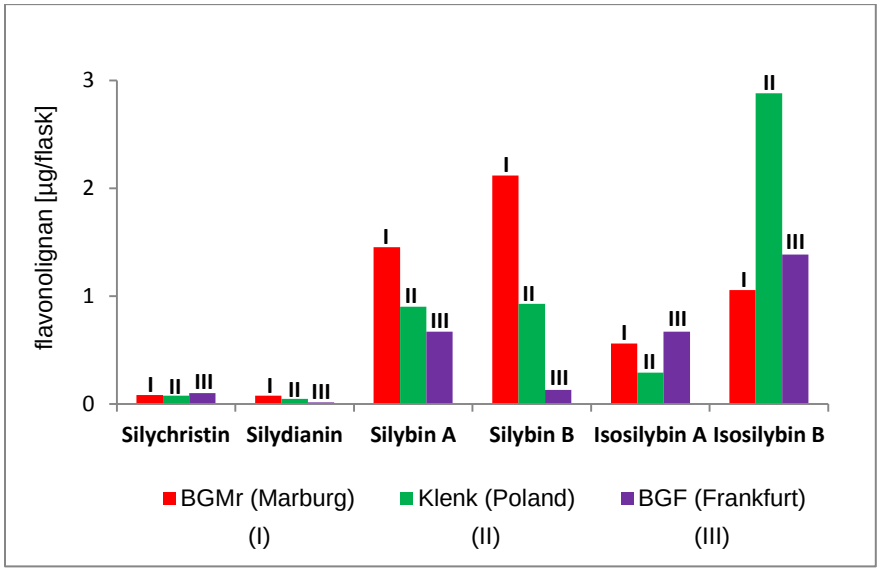


Fig. 28: Flavonolignan content of regioisomers and diastereomers extracted from media of suspension cultures of *Silybum marianum* after 5, 20 and 50 subcultures. The data represent mean values of three extractions (high outliers have been omitted). Standard deviations were omitted due to the fact that the individual flavonolignan content decreases with the number of subcultures.

The opposite proportions of the regioisomer distribution and location between the flavonolignan content extracted from cells and its corresponding medium is best shown in an "anti-correlation" diagram (Fig. 29). Whereas silychristin and silydianin represent the greater part of the total silymarin in the cells, it is opposite for silybin and isosilybin. These positional isomers mostly make up for the greater part in the respective medium. The low amounts of the diastereomer silybin B in the medium of the Frankfurt variety as well as the isosilybin B value for the Marburg cell line are the only exceptions.

If one considers the cell medium ("apoplastic space") and the cells themselves as a large unit and sums up the amounts of the individual silymarin components, changes in the ratio of the isomeric flavonolignans would be plausible. However, compared to the amounts of flavonolignans extracted from the cells, the silymarin amounts released to the medium are minute, thus do not have a great impact on the whole composition.

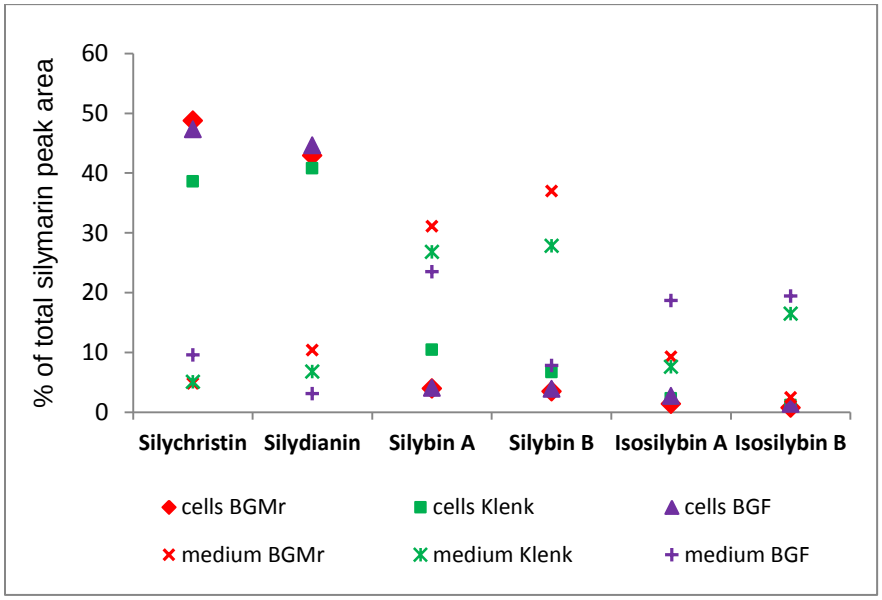


Fig. 29: "Anti-correlation" of the content of different flavonolignans extracted from cells and the corresponding medium. Silymarin components were compared based on the percentage of the total silymarin peak area.

4. Enzymatic formation of silymarin components

Surprisingly, crude protein extracts prepared from suspension cells of the three different varieties barely showed any difference in flavonolignan composition in *in vitro* assays. The analysis of flavonolignans did not resemble the characteristic patterns as observed for the fruits or the suspension cells. Only the total silymarin amount varied slightly. Likewise experiments with chemical reagents (Ag_2O) or horseradish peroxidase or a laccase from *Rhus verniciflua* led to similar ratios. This also applied for assays with filtered or concentrated medium (Fig. 30).

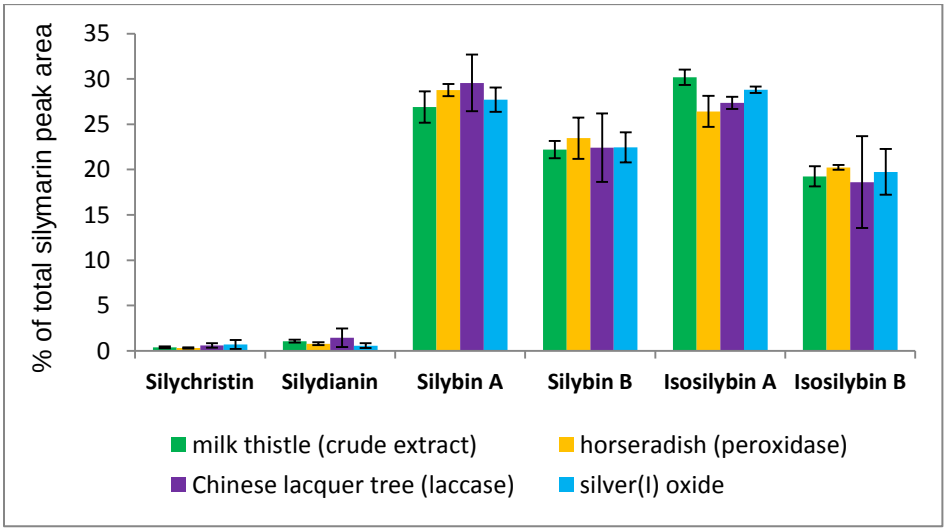


Fig. 30: Regioisomer and diastereomer ratio of flavonolignans analysed from assays performed with different enzymes/reagents. A crude protein extract from suspension cells of *Silybum marianum,* a peroxidase (*Armoracia rusticana)*, a laccase (*Rhus verniciflua)* and silver(I)oxide (Ag_2O) were tested. Silymarin components were compared based on the percentage of the total peak area.

For a more accurate investigation crude extracts as well as concentrated medium were further fractionated and chromatographically purified by FPLC (IV.5.2). Typical elution diagrams are shown in chapter V.10. The screening for peroxidase-active fractions via colour assays with ABTS (V.4.1) is explained below. After size exclusion chromatography the fraction with high peroxidase activity (fraction 5) from all three cultivars was tested in standard enzyme assays. Furthermore, every 2nd fraction from the anion exchange chromatography was tested for the presence of dirigent proteins (IV.5.10.c).

4.1 Qualitative peroxidase activity assays

By fractionation with help of an FPLC system, a peroxidase from crude extracts and concentrated medium of suspension cells of *Silybum marianum* could be separated and purified. After anion exchange chromatography (AEC), the collected fractions 10-14 showed most peroxidase activity (Fig. 31). After further size exclusion chromatography (SEC) of these pooled, concentrated fractions only the 5th revealed high peroxidase activity (Fig. 32). The respective chromatograms can be seen in V.10. The same applies for the determination of the approximate molecular weight.

With the help of the adjusted Bradford assay (IV.5.6) for very small amounts of proteins protein concentrations in fraction 5 could be determined to be around 20-50 µg ml^{-1}. 20 µl of this fraction were sufficient to oxidise ABTS within a few seconds and form the typical deep green radical cation. The coupling reaction between the two substrates taxifolin and coniferyl alcohol also proceeded very quickly. 10 µl of fraction 5 were sufficient to use up the applied substrate(s) and form the specific silymarin products within 5-10 min in a standard enzyme assay. 10 µl of applied HRP (1 mg ml^{-1}) from *Armoracia rusticana* (EC 1.11.1.7) resulted in less then half the amount of silymarin in the same time. Compared to unpurified medium the specific activity could be doubled. In relation to enzyme assays performed with crude extracts from suspension cells it even increased 50-fold. This indicates a successful enzyme concentration and purification.

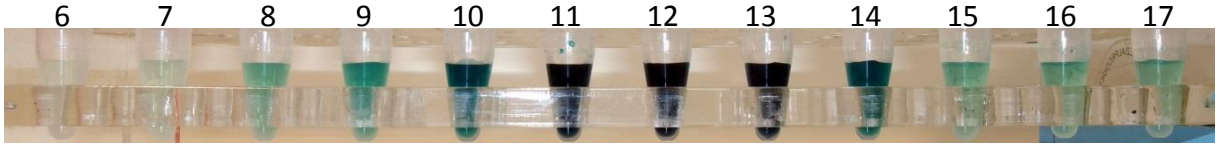


Fig. 31: ABTS assays with 20 µl of each individual fraction after AEC via FPLC and 10 min reaction time. About 8 ml crude extract of suspensions cells from the Frankfurt cell line were used in one chromatography run. Fraction 10-14 were pooled and concentrated before further purification by SEC.

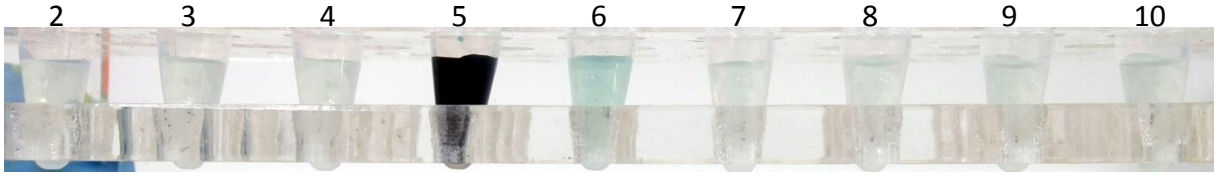


Fig. 32: ABTS assays with 20 µl of the collected fractions after SEC via FPLC and 10 min reaction time. Only fraction 5 revealed high peroxidase activity. This applied for all runs with either crude extract or medium.

4.2 Enzymatic silymarin formation

Despite showing high specific activity in catalysing the coupling reaction between taxifolin and coniferyl alcohol, again no differences in the regioisomer distribution occurred (Fig. 33). Neither the purified peroxidase from the crude extracts nor the corresponding medium preparation could reproduce the characteristic chemotypic patterns observed in the fruits before. Interestingly, the formation of silybin and isosilybin always seems to take place preferably although the two regioisomers silychristin and silydianin made up for a big part in the fruits. This could be a

question of stability of the oxidised radical form of coniferyl alcohol and/or taxifolin or the resulting flavonolignan product. It has already been shown that silydianin is more susceptible towards higher pH (V.2.2). Also steric interferences during the coupling reaction can be discussed. The reaction conditions could determine the final composition of the product when competing reaction pathways lead to different products. In this case, the reaction would be subjected to thermodynamic or kinetic control.

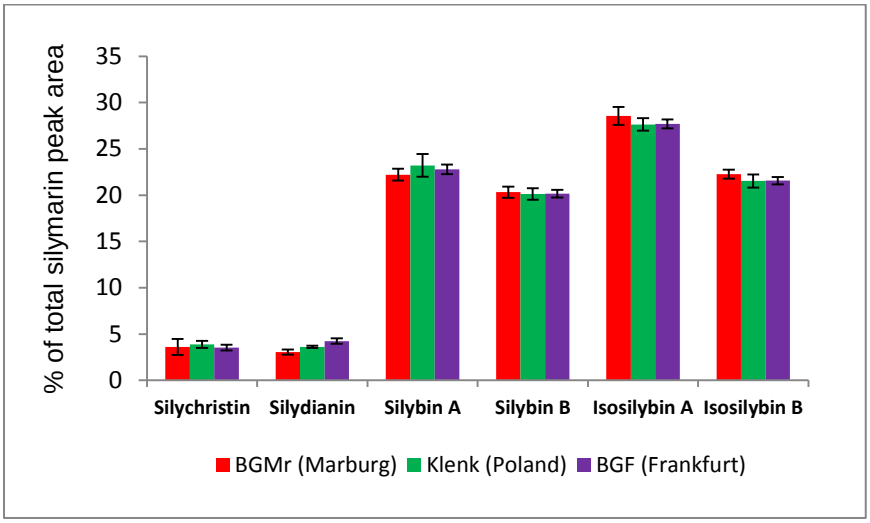


Fig. 33: Regioisomer and diastereomer ratio of flavonolignans analysed from assays performed with purified peroxidase fractionated from crude protein extracts of suspension cells from BGMr, Klenk/Poland and BGF. Silymarin components are compared based on the percentage of the total peak area. The data represent mean values of three replicate assays (±s.d.).

Also the test for dirigent proteins (IV.5.10.c) was negative. All enzyme assays with additionally added protein fractions after size exclusion chromatography resulted in the same distribution of silymarin components. Thus, a participation of dirigent proteins in flavonolignan biosynthesis regulating the formation of diastereomers or even positional isomers, at this time, seems rather unlikely. Even if such proteins may have been found in the plant genome (V.11), their expression might be plant organ- (seed/fruit coat) and/or time-specific (maturity phase) or their function must probably be sought elsewhere in the plant.

4.3 Modified assays for silymarin formation

In the search for parameters which could influence the formation of the individual regioisomers of silymarin enzyme tests with varying concentrations of taxifolin and coniferyl alcohol revealed interesting new insights. Modified substrate concentrations, especially in unequal ratios, greatly affected the formation of the two positional isomers silychristin and silydianin. A fourfold taxifolin concentration (2.5 mM) at an unchanged concentration of coniferyl alcohol (0.625 mM) stimulated the formation of silychristin 234-fold and silydianin 34-fold while the formation of the other silymarin components remained largely unchanged. Vice versa, the same increase in coniferyl alcohol concentration (2.5 mM) only led to a slight increase in silydianin amounts (13-fold). The total silymarin content mostly remained constant, since silydianin and silychristin usually are minor components of the *in vitro* formed flavonolignan mixture (Fig. 34). In conclusion, we can say that the formation of these two components is highly dependent on the available taxifolin concentration.

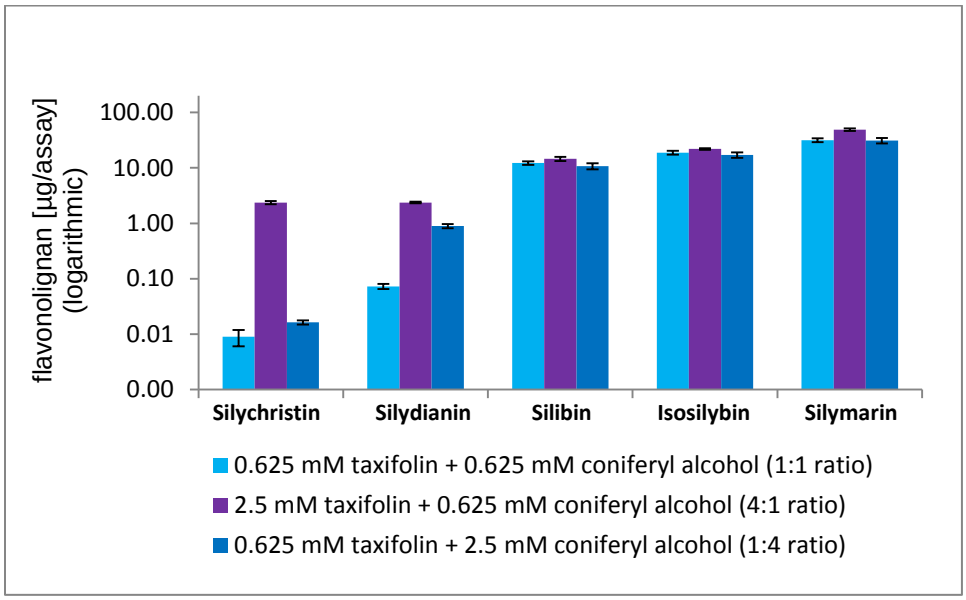


Fig. 34: Formation of flavonolignan components in enzyme assays with varying concentrations and ratios of taxifolin and coniferyl alcohol. Flavonolignan formation is given in µg per assay in a logarithmic scale for better visualisation.

4.4 Optimal buffer and pH for reaction

For an adequate buffer system as the basis for enzyme assays, standard buffers like potassium phosphate buffer (KPi; 0.1 mol l^{-1} pH 7.0) and TRIS-HCl (0.05 mol l^{-1} pH 7.3) were tested against dist. H_2O as reference. It became quite clear that the reaction is relatively susceptible to ionic buffer systems. Only half the amount of silymarin was formed in assays with KPi as buffer. Even the organic TRIS-HCl buffer resulted in 20% less flavonolignan products compared to distilled water as reaction medium (Fig. 35). Certainly, the pH also greatly influences the coupling reaction between taxifolin and coniferyl alcohol. To determine the exact impact of the pH during the reaction, a series of assays with different starting pH values was tested. In addition, possible inhibitors of the enzyme reaction were investigated more closely.

The starting pH values of the 0.05 mM TRIS-HCl buffer systems ranged between 7.0 and 11.0, in steps of 0.5 pH units. In addition, the pH was measured right before and after the enzyme assay with a small electrode to record the exact value and possible pH shifts.

Beginning with a lower pH like 7.0 resulted in a slightly higher pH (7.2) at the end of reaction. Starting with 11.0 led to a lower pH (10.3). Addition of enzyme (dissolved in pH 10.0 buffer) and conversion of substrates could be responsible for this. Furthermore, a 10 mM silymarin solution in dist. H_2O already results in a slightly alkaline pH (8.45). The most effective buffer pH before starting the reaction is about 10.0. After addition of all assay components the reaction starts with a pH at about 9.5 and ends at 9.3 (Fig. 36). Hence, this method led to the best yield in flavonolignan products.

A possible explanation could be that the responsible enzyme(s) generally are more stable at alkaline pH than at neutral/acidic pH. For example, Stoilova et al. (2010) observed this property for laccases probably due to the OH^- inhibition of autoxidation. This could extend to peroxidases as well. However, if the pH is too high (> 9.5) in the beginning, there is a huge decrease in silymarin formation. This is probably due to the fact that there are not enough protons in the surrounding medium of the catalytic center of the enzyme. Thus, previously positively charged molecules, like amino groups ($-NH_3^+$), would discharge a proton and become neutral. Conversely, this also naturally applies to a lower pH, resulting in uncharged carboxyl groups (-COOH). Altogether the number of electrostatic bonds, important for spatial distribution and structure of the catalytic center, would drop significantly. As a consequence the enzyme activity decreases because substrates can not fit into the modified active sites of the protein anymore.

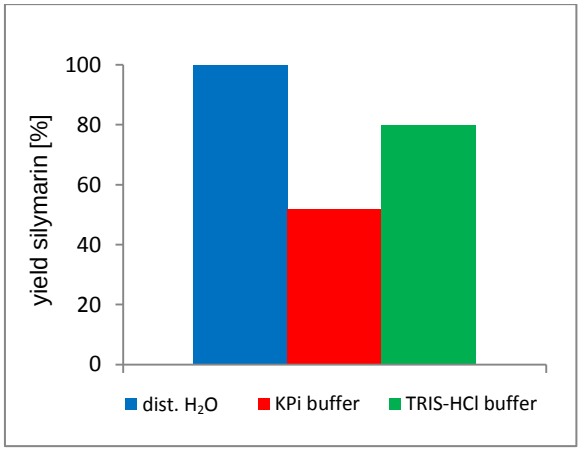


Fig. 35: Silymarin yield in % extracted from standard enzyme assays with crude extract from milk thistle suspension cells. Dist. H_2O was used as reference (100%).

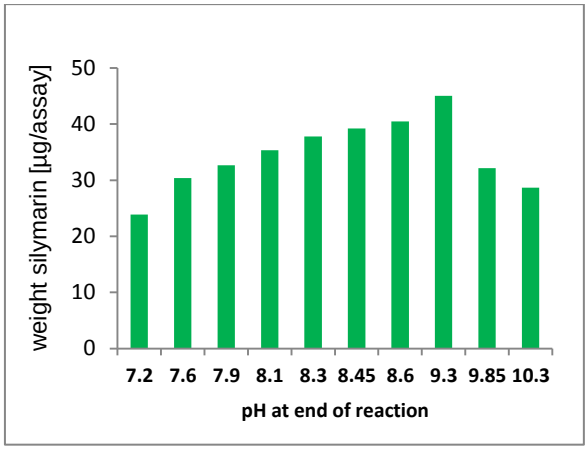


Fig. 36: Silymarin content formed in standard enzyme assays with different pH values of the applied TRIS-HCl buffer (7.0-11.0). Every column is a 0.5 step increase in pH value. Calculation was done by evaluating the induvial regioisomer amounts and adding up for total flavonolignan content.

4.5 Inhibitor studies

The use of dithiothreitol (DTT) and the chelating agent EDTA in enzyme assays performed with crude extracts almost led to total inhibition of the responsible enzyme(s). Inhibitors including metal ions (Co^{2+}, Sn^{2+}, Zn^{2+}) also revealed strong inhibition by 25-40% (Fig. 37) though the resulting pH shift (decreased to 7.5-8.0) caused by these substances probably was jointly responsible for the decreased silymarin formation. As noted above, the investigated reaction is pH sensitive. Metal ions like K^+ or Na^+ resulted in only about 5% less flavonolignan formation. Here, however, the pH shift was also not as drastic. Known inhibitors of peroxidases, like fluoride (F^-), cyanide (CN^-) or quaternary ammonium compounds (NH_4^+), revealed inhibitory effects between 10-15% (Fig. 38).

It is known that laccases and peroxidases are highly dependent on the state of oxidation of the copper/heme atoms. This is one of the reasons for high sensitivity towards chelating agents. The selective removal of Cu/Fe by chelating agents (EDTA, dimethyl glyoxime, diethyldithiocarbamic acid or nitrilotriacetic acid) leads to a loss of catalytic activity (Abdullah et al., 2012). In turn, the strong reducing agent DTT leads to effective cleavage of disulphide bonds playing an important role in the three-dimensional structure, thus the stability and activity of

proteins, especially those secreted into the extracellular medium (Sevier and Kaiser, 2002). Small anions such as azide (N_3^-), CN^-/SCN^-, halides (e.g. F^-) and hydroxide bind to the type 2 and type 3 Cu/Fe, resulting in the interruption of internal electron transfer and inhibition of activity. Other inhibitors including metal ions, sulfhydryl reagents, hydroxyglycine, kojic acid, deferoxamin or cationic quaternary ammonium detergents (NH_4^+) could interfere in the reaction which may cause amino acid residue modifications, conformational changes or Cu/Fe chelation (Call and Mücke, 1997; Gianfreda et al., 1999). Vanadate (VO_4^{3-}) can lead to inhibition due to interference with the peroxidase-H_2O_2 system (Serra et al., 1989).

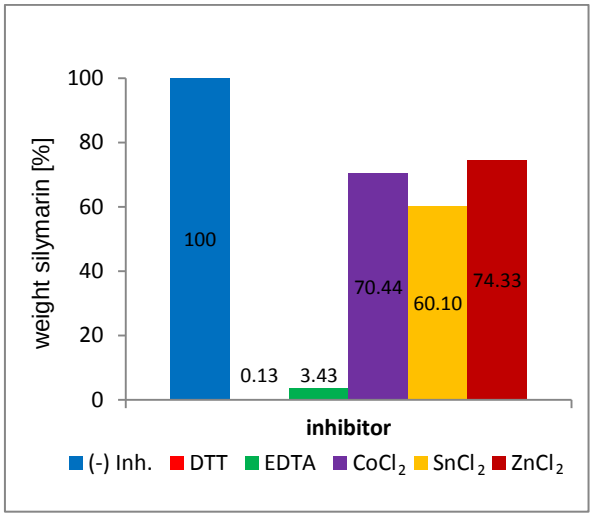


Fig. 37: Silymarin yield in % extracted from enzyme assays with additionally applied inhibitors (1.25 mM). A standard enzyme assay was used as reference (100%).

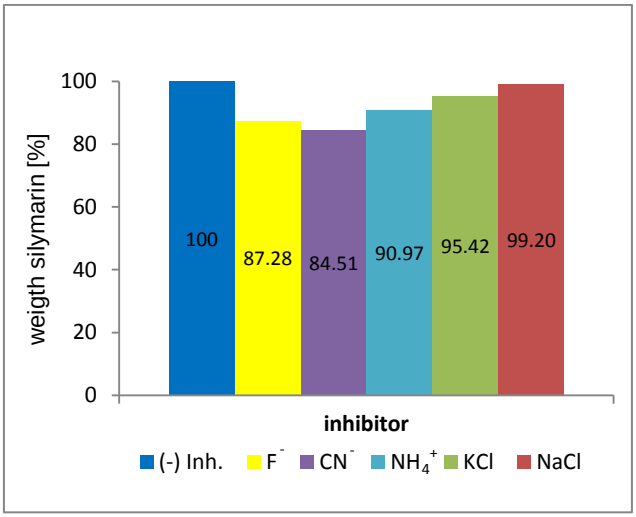


Fig. 38: Silymarin yield in % extracted from enzyme assays with additionally applied inhibitors (1.25 mM). A standard enzyme assay was used as reference (100%).

According to literature and data bases kojic acid (a chelating agent produced by several species of fungi; Yabuta, 1924) is a more or less selective inhibitor for laccases, whereas 4-aminobenzoic acid (an intermediate in the synthesis of folate in bacteria, plants and fungi; Green et al., 1996; Cossins and Chen, 1997) should only have a negative effect on the function of peroxidases. A comparison between these two inhibitors and the effect on the silymarin-forming enzyme(s) in the crude extract tested in enzyme assays should reveal further indications to the specific type of enzyme responsible for the coupling reaction. As references a peroxidase from horse radish and a laccase from Chinese lacquer tree were treated in a similar way.

The experiments showed that kojic acid is not a selective inhibitor for laccases. Enzyme assays with both, laccase from Chinese lacquer tree and horse radish peroxidase, resulted in a 70-85% less silymarin yield. This also applied for the silymarin-forming enzymes present in the crude extract from milk thistle suspension cells. 4-Aminobenzoic acid (PABA), on the other hand, showed unexpected results. Instead of blocking the peroxidase it enhanced the coupling reaction between taxifolin and coniferyl alcohol. About 170% more silymarin was formed. Also the laccase was rather supported in its function (30% more). Only the enzyme assay with crude cell extracts was slightly inhibited with PABA by about 15% (Fig. 39-41).

These controversial results unfortunately give little insight into the possible type of enzyme(s) involved. Again, it only shows how important the pH is during silymarin formation. The pH optimum of horse radish peroxidase is in the range of 6.0-6.5 (Sigma-Aldrich database). Laccase from *Rhus verniciflua* displayed maximal activity in neutral and weakly alkaline solutions (Morozova et al., 2007). Since the addition of PABA shifts the pH to a more neutral pH (~7.5) activity of horse radish peroxidase is increased, thus much more flavonolignans are formed. The same applies to the small increase in activity of the laccase.

Due to the chelating properties of kojic acid both enzyme classes were largely inhibited. Concerning the enzymes present in the crude extract, no statements regarding the specific type can be made, at least at the present pH conditions used for optimal silymarin formation.

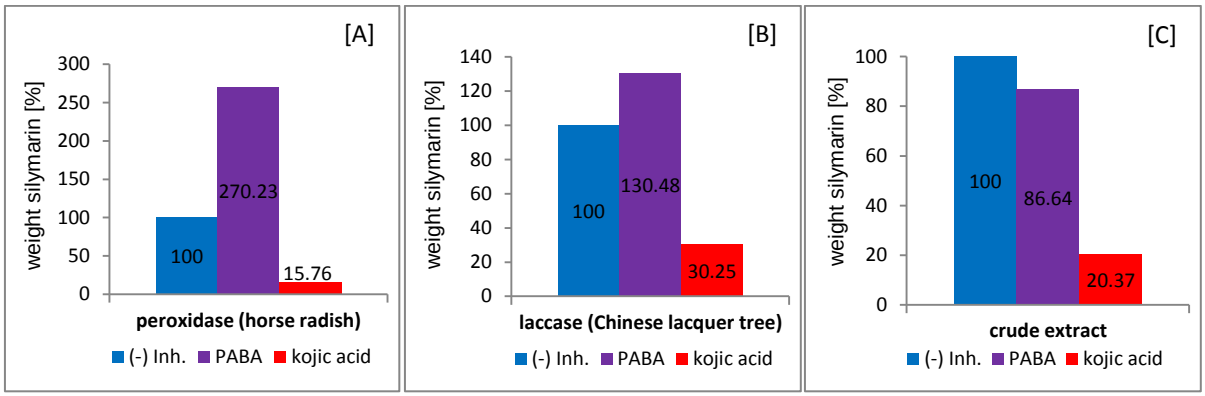


Fig. 39-41: Silymarin yield in % extracted from enzyme assays with additionally applied inhibitors (1.25 mM). A standard enzyme assay was used as reference (100%). Either peroxidase (horse radish) [A], laccase (Chinese lacquer tree) [B] or milk thistle crude extract [C] were used as enzyme source.

4.6 Electron donor

Enzyme assays with laccase from *Rhus verniciflua* or milk thistle crude extracts with previously removed O_2 unfortunately did not give new insights. Samples treated with glucose oxidase only showed slightly less enzyme activity. The difference in flavonolignan content extracted from assays was small. Especially because the amount of formed silymarin by laccase is very low it is difficult to estimate the effectivity. In addition, it is difficult to remove all O_2 from the system.

Omitting H_2O_2 in enzyme assays with horse radish peroxidase or crude extracts from *Silybum marianum* suspension cells provided much more significant results. The pure horse radish peroxidase protein from *Armoracia rusticana* lost about 90% of its activity in that case. Similar effects were observed for the flavonolignan content formed by the species-specific proteins present in the crude extract. By omitting H_2O_2 about 80% enzyme activity got lost (Fig. 42). This is further evidence for the importance of the presence of an electron donor system. Furthermore, this experiment confirms the assumption, that a peroxidase-similar protein is responsible for linking taxifolin and coniferyl alcohol in *Silybum marianum* to form silymarin. Substitution experiments with NADPH did not affect these ratios thus could not replace the missing electron donor H_2O_2.

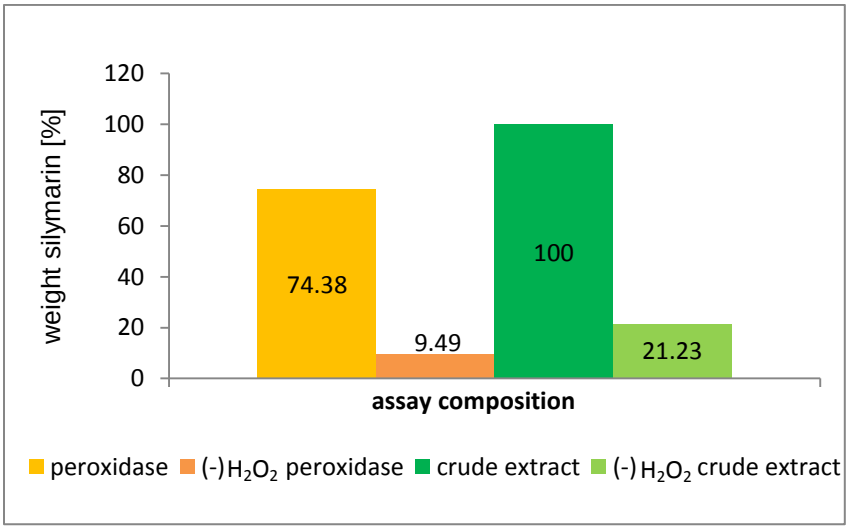


Fig. 42: Silymarin yield in % extracted from enzyme assays with and without electron donor (H_2O_2). A standard enzyme assay was used as reference (100%). Either peroxidase (horse radish) or milk thistle crude extract were used as enzyme source.

5. Cell wall protein extraction (CWPE)

At an early stage of this work during the treatment of suspensions cells with TRIS-HCl buffer it was observed that even after several washing steps by vigorous shaking, the respective washing fractions showed enzyme activity in assays. In conclusion, the typically secreted enzymes responsible for the coupling reaction to form silymarin can be easily extracted from the cell wall of *Silybum marianum* suspension cells.

After repetitive extraction (IV.5.1.c) of cells from the three chemotypic varieties (BGMr, BGF and Poland/Klenk) and reduction of the volume to 10% the obtained samples were tested for activity. All three revealed high activities for the formation of silymarin, especially samples from the Marburg and Klenk varieties. About 80 µg total silymarin could be extracted from the best *in vitro* assay (Fig. 43).

The concentrated samples then were precipitated with TCA and subjected to SDS gel electrophoresis. This revealed promising bands at 34 kDa for both highly active protein samples. For the sample with less activity (BGF) no specific band could be detected, probably because the protein concentration was too low (Fig. 44). This is in agreement with the calculation of the molecular weight of the peroxidase found in the genome of *Silybum marianum* discovered by different PCR methods (V.11). However, this applies to the denatured proteins without probable glycosylations. Unfortunately, at that time protein identification after tryptic digestion via mass spectroscopy was not undertaken.

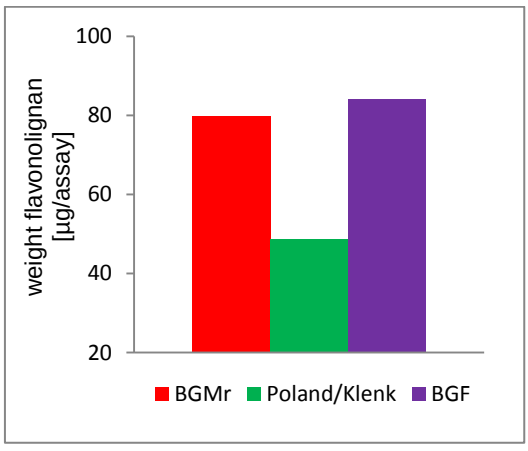


Fig. 43: Flavonolignans extracted from standard enzyme assays with cell wall protein extracts from suspension cells of the three chemotypic varieties (BGMr, BGF and Poland/Klenk).

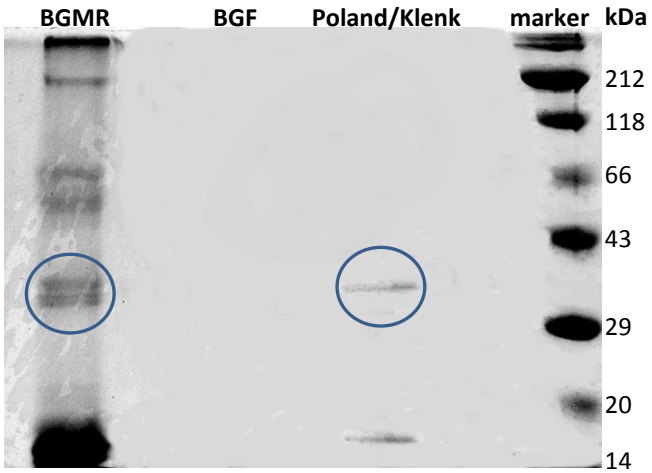


Fig. 44: SDS-PAGE of TCA-precipitated, concentrated cell wall protein extracts from suspension cells of the three chemotypic varieties (BGMr, BGF and Poland/Klenk). The encircled bands probably represent a denatured peroxidase from *Silybum marianum* at ~34 kDa.

Later, a similar attempt for visualisation by SDS gel electrophoresis and additional silver staining (IV.5.8.b) was carried out for TCA-precipitated samples (1.5 ml) of the collected fraction 5 (V.4.1) after separation and purification by FPLC (IV.5.2).

Again, specific bands could be detected in the range of 30-37 kDa. Unfortunately, the staining was too intense, resulting in large and difficult to distinguish bands (Fig. 45). Due to various impurities this still was not usable for a mass-spectrometrical analysis. However, the presence of one or more denatured isoenzymes of a peroxidase in this area could be assumed.

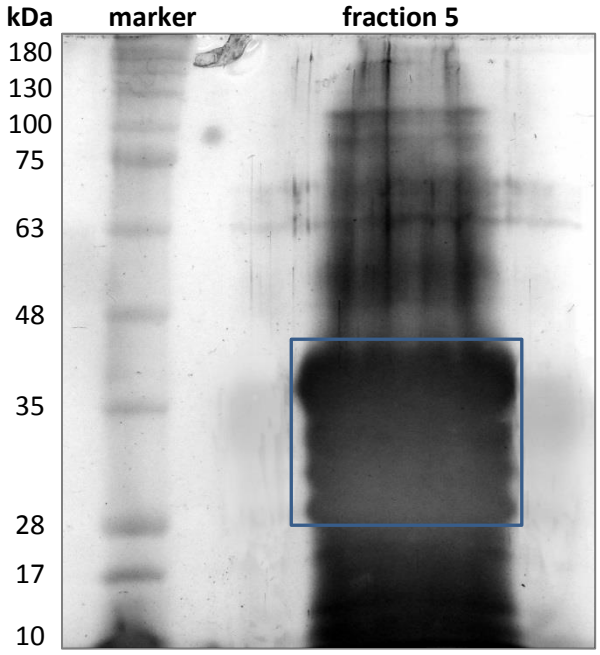


Fig. 45: TCA-precipitated fraction 5 (1.5 ml) after SDS-PAGE and silver staining. The bands in the black box could represent denatured peroxidase isoenzymes from *Silybum marianum* at ~30-37 kDa.

6. Protoplast isolation

With the help of protoplast isolation from *Silybum marianum* suspension cells it was be tested, whether the flavonolignans are stored in the cell walls or rather in the protoplast respectively the vacuole. This could provide useful information about similar *in planta* conditions. For this purpose, the cell walls of suspension cultures of *Silybum marianum* were digested with cellulase and pectinase under normal culture conditions for 24 h (IV.6). Afterwards, protoplasts and medium were separated.

However, after extraction, evaporation and analysis by HPLC, the medium did not contain substantially higher quantities of flavonolignans. In fact, the silymarin yield in the outer compartments remained constant. As a consequence it can be expected that most of the flavonolignans are not stored in the cell wall, but instead either in the interior of cells. They could be driven out through transport systems into outer layers/medium from there. Since this method was very susceptible to interference like contamination and we generally had to deal with very low silymarin amounts in the medium the results should be interpreted with caution. A repetition of this experiment under sterile conditions and additional investigation of the protoplasts for their silymarin content is recommended and necessary for conformation.

7. Spectrophotometrical detection of silymarin in enzyme assays

In the reaction between taxifolin and coniferyl alcohol in an alkaline buffer, a strong yellow colouration occurs. This can be photometrically determined at an absorption maximum at 325 nm. However, the silymarin formation is not responsible for this change in colour. Even though the flavonolignan mixture itself has a slight yellowish-translucent colour in TRIS-HCl buffer at pH 10.0 its absorption maximum is at 290 nm. This could be demonstrated in approaches with either silymarin alone, or the individual substrates with and without peroxidase. The result shows that a photometric test for the determination of the silymarin concentration is not possible.

The strong yellow colouration with the absorption maximum at 325 nm only occurred after addition of a radical-forming enzyme or protein preparations with peroxidase activity (Fig. 47). However, it is not the formed silymarin or coniferyl alcohol, but an intermediate of taxifolin, which leads to this deep change in colour. It could be caused by the taxifolin phenoxy radical formation in the presence of OH^- ions. Addition of 75 µl 6 N HCl instantly quenched the specific colour (Fig. 48).

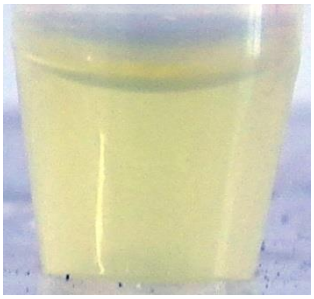


Fig. 46: Typical colour of a standard assay before enzymatic conversion at pH 9.5.

Fig. 47: Typical colour of a standard assay after enzymatic conversion for 15 min at 30 °C and pH 9.3.

Fig. 48: Typical colour of a standard assay after enzymatic conversion and addition of 6 N HCl (pH < 1).

8. Analysis of plant samples by thin-layer chromatography (TLC)

Leaves, roots and fruits of *Silybum marianum* were investigated for their silymarin content using thin-layer chromatography (IV.5.12). Additionally, fruits were separated into embryo and fruit wall to obtain further information on the exact silymarin storage location. Several reference substances, mostly flavonolignans, were applied as well for comparison.

After spraying with "Naturstoffreagenz" the plates showed the yellow-green fluorescing silybin in the middle and the caffeic acid reference with a light blue fluorescent at the same height. Below there are several obvious visible fluorescent areas, among these taxifolin with a brown colour and silydianin and silychristin with a yellow-green fluorescence right next to it (Fig. 49). Under UV light (312 nm), these specific bands become even more apparent (Fig. 50). However, the retention factor of silydianin and silychristin is very similar using this eluent (R_f = 0.29). The same applies to the regioisomers silybin and isosilybin (R_f = 0.54). Since these substances are very similar in structure, other separation techniques would be necessary.

There were no detectable silymarin compounds in the leaves or the embryos of *Silybum marianum*. Only the fruit coat showed high quantities of flavonolignans. Most of the silymarin compounds are only produced during seed maturation and probably stored in the thick-walled cells of the seed and/or fruit coat. Preliminary tests with unripe fruits showed no measurable flavonolignan content. Silymarin accumulation in the fruit coat could, therefore, increase the efficiency of its role as a barrier to protect the embryo against pathogens due to its antioxidant and antimicrobial effects.

In 1977, Greimel and Koch showed that some silymarin regioisomers could inhibit horseradish peroxidase *in vitro*. Furthermore, they investigated the activity of peroxidase isoenzymes in cress seedlings (*Lepidium sativum* L.) and possible inhibitory effects by silymarin. The results led to the conclusion that silymarin even could negatively affect the *de novo* synthesis of these peroxidases. Similar conditions could also apply to peroxidase activity in *Silybum marianum*. This would further support the hypothesis of a strict separation between storage site and place of formation of flavonolignans.

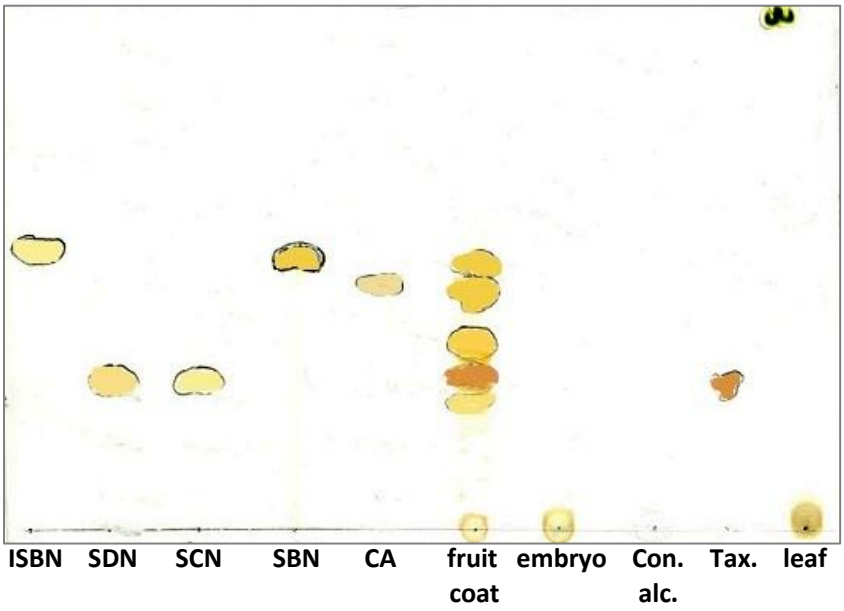


Fig. 49: TLC of extracts from *Silybum marianum* plant parts and reference compounds in daylight. Mobile phase: $CHCl_3$-acetone-formic acid (37.5:8.25:4.25, v/v). Detection: 1% "Naturstoffreagenz A" and 5 % PEG 4000 in MeOH. Abbreviations: isosilybin (ISBN), silydianin (SDN), silychristin (SCN), silybin (SBN), caffeic acid (CA), coniferyl alcohol (Con. alc.) and taxifolin (Tax.). In each case, 10 µl of references (10 mmol l^{-1}) and 30 µl of the sample solutions were applied.

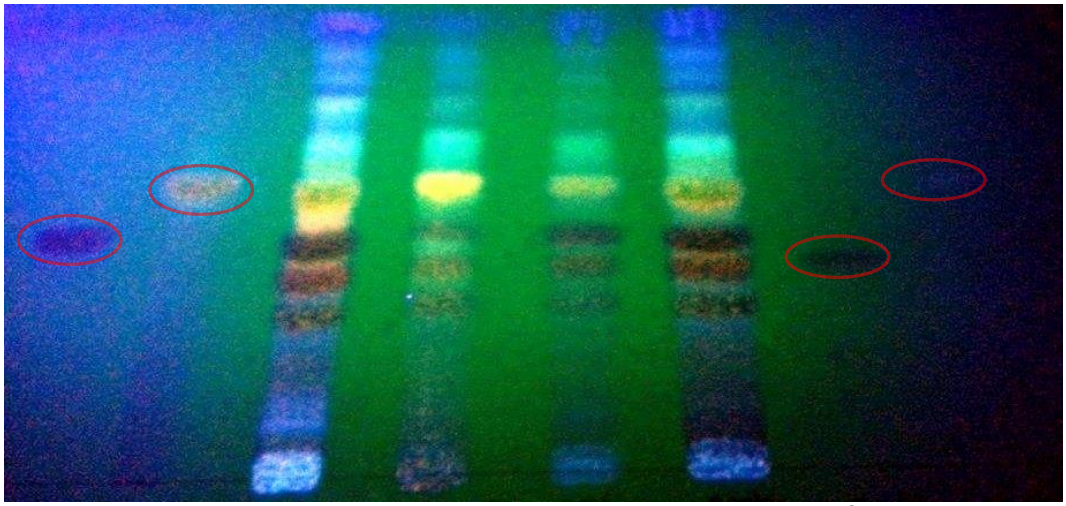


Fig. 50: TLC of milk thistle fruit extracts and individual flavonolignan references under ultraviolet light (312 nm). Mobile phase: $CHCl_3$-acetone-formic acid (37.5:8.25:4.25, v/v). Detection: 1% "Naturstoffreagenz A" and 5% PEG 4000 in MeOH. The red circles mark otherwise difficultly visible bands. Abbreviations: silydianin (SDN), silybin (SBN) and isosilybin (ISBN). 100 µl of the extracted and previously diluted (1:5) fruit samples were applied.

Meanwhile, some seed coat specific genes have been identified in various plants. For example, a cysteine proteinase from *Brassica napus* (rape) associated with programmed cell death of the inner integument (Wan et al., 2002) or a subtilisin-like gene from *Glycine max* (soybean) being associated with differentiation processes of the thick-walled parenchyma cells (Batchelor et al., 2000). Several genes expressed specifically in the seed coat of the model organism *Arabidopsis thaliana* have been described as well. Esfandiari et al. (2013) identified a promoter regulatory region from a dirigent protein gene that drives expression specifically in the outer layers of the seed coat epidermis from *Arabidopsis thaliana*. A corresponding mutant lacking this dirigent protein was longer able to synthesise seed specific phenylpropanoid compounds (Matsuda et al., 2010).

The same could apply for flavonolignan biosynthesis in *Silybum marianum*. The expression of dirigent proteins could occur only during a specific developmental phase, to be precise, during the maturing process of the achenes. This could also explain why crude enzyme extracts of milk thistle suspension cells were not able to regulate the preferential formation of specific positional isomers of silymarin, although several dirigent protein-like genes could be identified using PCR methods. This also applies for the fractions separated by anion exchange chromatography tested for the presence of dirigent proteins in enzyme assays (V.4.2). Differences in the function and

expression of seed or fruit coat specific genes in *Silybum marianum* could explain the presence of milk thistle cultivars with distinct chemotypic properties regarding the silymarin regioisomer distribution. The heterologous expression of such protein(s) or mutation studies of corresponding genes in the plant would provide clarity.

9. Separation and identification of silymarin compounds by high-performance-liquid chromatography (HPLC)

Using isocratic elution as described in IV.5.13 the positional isomers silychristin and silydianin could be detected as separate peaks. Additionally, the HPLC conditions allowed a partial resolution of the diastereomers silybin A and B and isosilybin A and B as also shown by other groups (Fig. 51). Assignment of the respective diastereomers was done according to published data acquired under similar HPLC conditions (Cai et al., 2009; Kuki et al., 2012; Radjabian et al., 2008). Peak areas of the respective diastereomers were calculated by the percentage of the total peak area of a known concentration of the isomeric flavonolignan mixture. Additionally, standard calibration curves of silymarin and the diastereomer mixtures of silybin and isosilybin were evaluated. As a result, calculating the specific flavonolignan weights, by showing the proportional relations between the individual peak areas and its corresponding applied quantity, is appropriate (Poppe and Petersen, 2016). Switching to gradient elution enabled faster run times with simultaneous better separation of the individual flavonolignan peaks (Fig. 52).

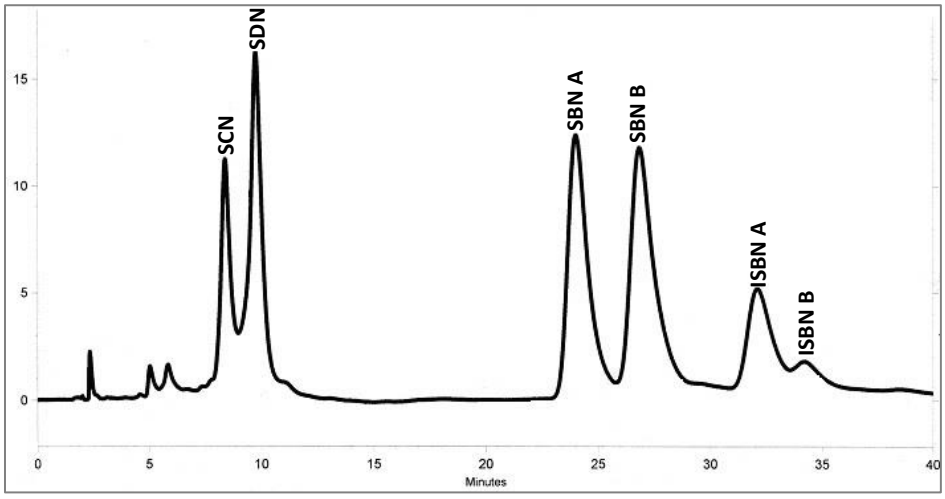


Fig. 51: Chromatogram of a mixed flavonolignan standard [9.65 µg ml^{-1} (SCN+SDN) and 48.25 µg ml^{-1} (SBN+ISBN)]. HPLC analysis was carried out on an Equisil ODS-column (4 mm ø, 250 mm length; with precolumn) using a mixture of 85% H_3PO_4–MeOH–H_2O (0.01:46.5:53.5, v/v) as mobile phase (**isocratic** elution) at a flow-rate of 1 ml min^{-1}. Flavonolignans were detected at 288 nm.

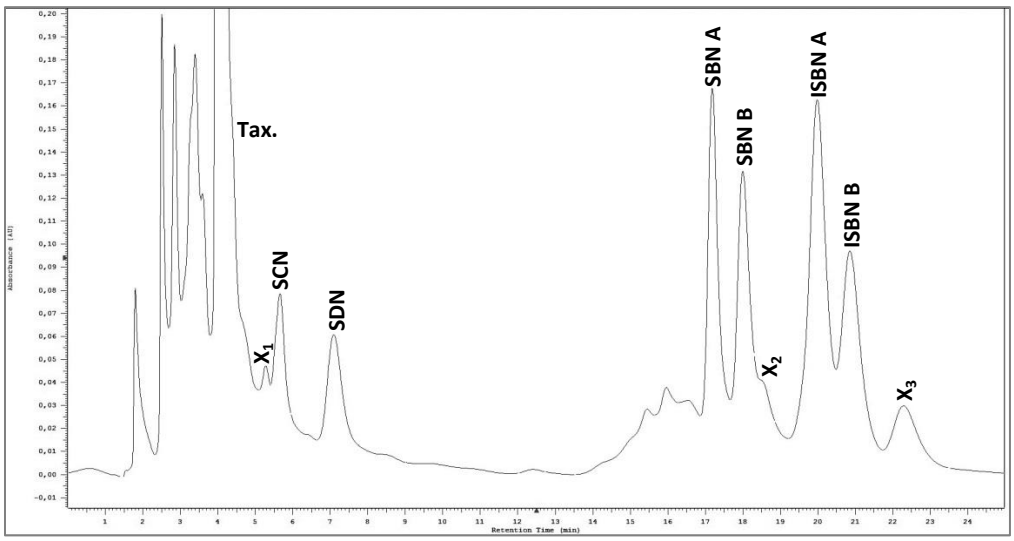


Fig. 52: Chromatogram of an enzyme assay with taxifolin and coniferyl alcohol as substrates at a ratio of 3:1 (IV.5.10.b). Enzyme source: 10 µl fraction 5 (V.4.1). HPLC analysis was carried out on an Equisil ODS-column (4 mm ø, 250 mm length; with precolumn) using **gradient** elution (IV.5.13) at a flow-rate of 1 ml min^{-1}. Flavonolignans were detected at 288 nm.

The peaks marked with X_{1-3} could be flavonolignans also formed during enzyme assays (Fig. 52). Based on their retention time and after comparison with scientific literature these could be isosilychristin (X_1) and 2,3-*cis*-isomers of silybin ($X_{2;}$ shoulder) and isosilybin (X_3) (Kuki et al., 2012; Novotná et al., 2014). Unfortunately, no standards were available for these substances. In addition, mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR) would be necessary for structural elucidation. Furthermore, the possible formation of pinoresinol by the coupling of two coniferyl alcohol units could never be unequivocally proven.

10. Purification of peroxidase by fast protein liquid chromatography (FPLC)

Crude extracts and medium of suspension cells of *Silybum marianum* were fractionated and enriched by anion exchange chromatography (AEC). Run times and conditions as well as buffers are explained in IV.5.2.a.

The chromatogram was obtained with help of the UNICORN™ Control Software of the ÄKTA pure system. It shows all important parameters (Fig. 53). Whereas the precolumn pressure should always remain constant, conductivity increases with rising concentration of the elution buffer. In case of purified medium NaCl concentrations between 0.2-0.3 mol l^{-1} were sufficient to elute most protein(s) from the column resulting in a clear peak with an UV maximum at about 125 mAU (Fig. 53). These fractions (10-14) also showed the highest peroxidase activity (V.4.1; Fig. 31). Accordingly, fractionated crude extracts resulted in more peaks with higher absorptions, since these also contained significantly higher amounts of diverse cellular proteins (Fig. 54). Furthermore, fractions with highest peroxidase activity could slightly shift.

The pooled enzyme fractions positive for high peroxidase activity were concentrated with help of centrifugal ultrafiltration and fractionated by means of size exclusion chromatography (SEC). Details on the running conditions are explained in IV.5.2.b. The highest peak (absorption) in the UV chromatogram of the fractionated medium can be seen in fraction 5 (Fig. 55), only this fraction showed peroxidase activity (V.4.1). This was also true for fractionated enzyme crude extracts but with lower absorption values, thus peroxidase yields (Fig. 56). Therefore it can be assumed that the purification of the peroxidase from milk thistle suspension cells was successful. Yet, purification and fractionation of the medium seems more profitable, since yields are higher and far less side products/proteins have to be removed. These results further confirm that high amounts of peroxidase are secreted to the outer compartments.

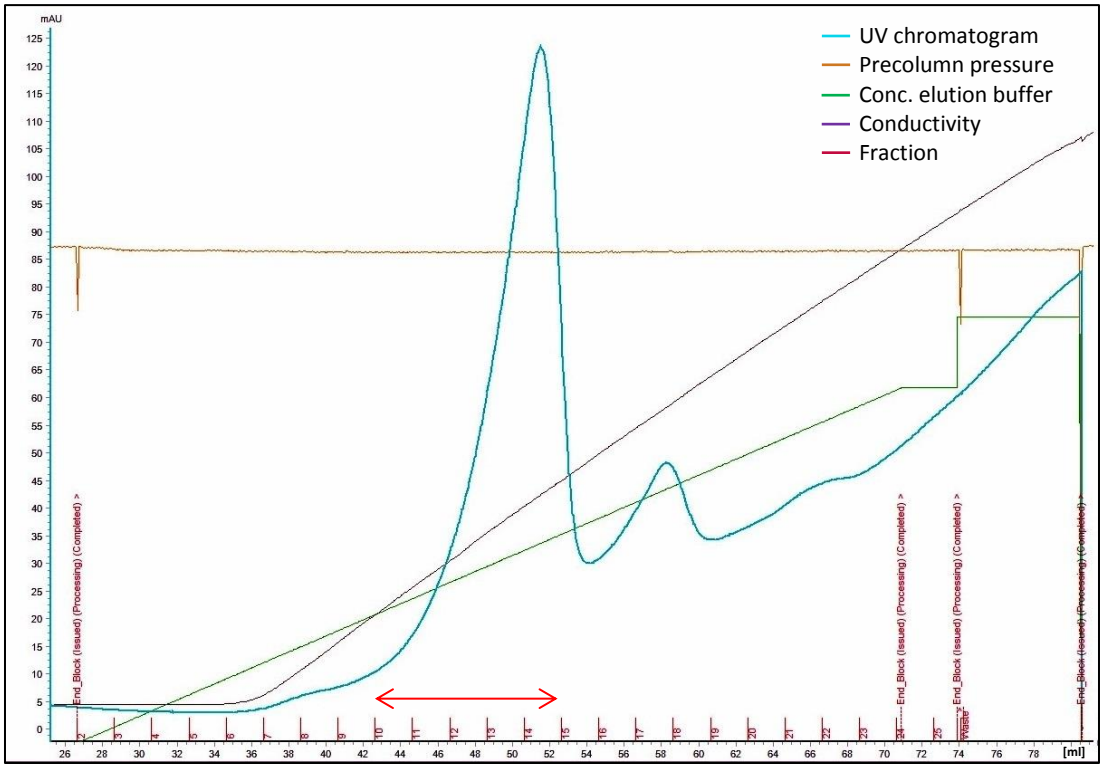


Fig. 53: Anion exchange chromatography of concentrated medium (Frankfurt variety) (IV.5.2.a). Fractions 10-14 (↔) revealed high peroxidase activity correlating with the highest peak (absorption) in the UV chromatogram (~125 mAU).

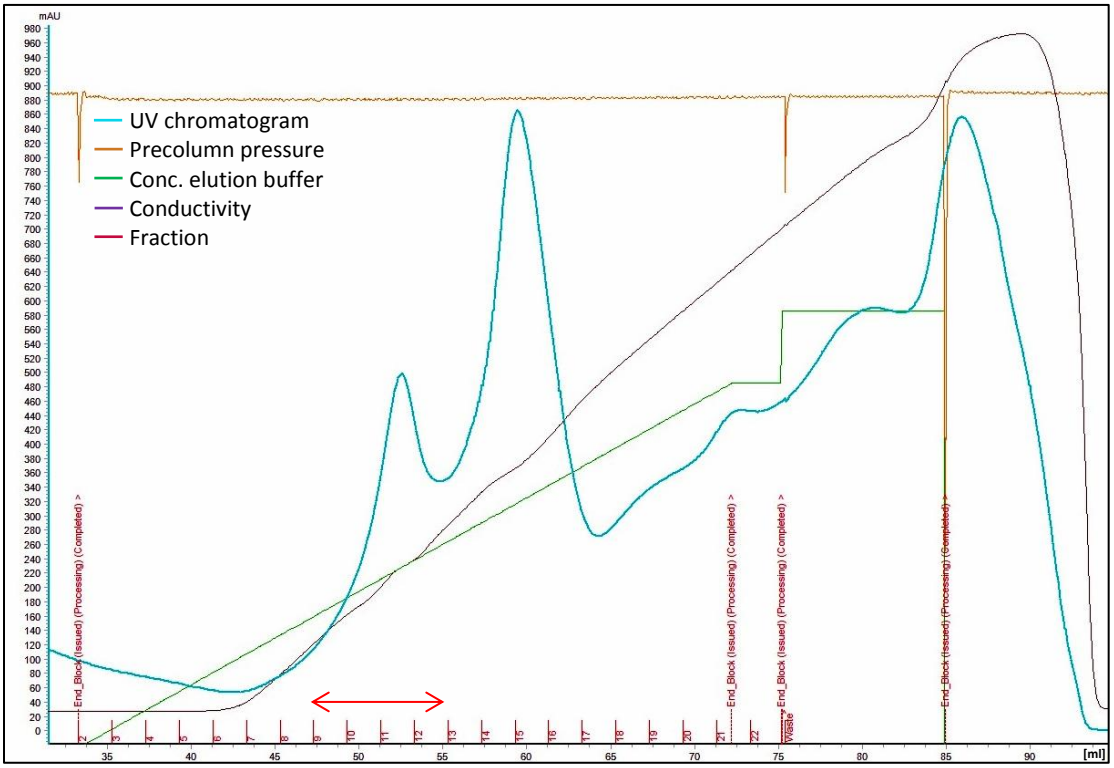


Fig. 54: Anion exchange chromatography of crude enzyme extracts (Marburg variety) (IV.5.2.a). Fractions 9-13 (↔) revealed high peroxidase activity correlating with the first big peak in the UV chromatogram (~500 mAU).

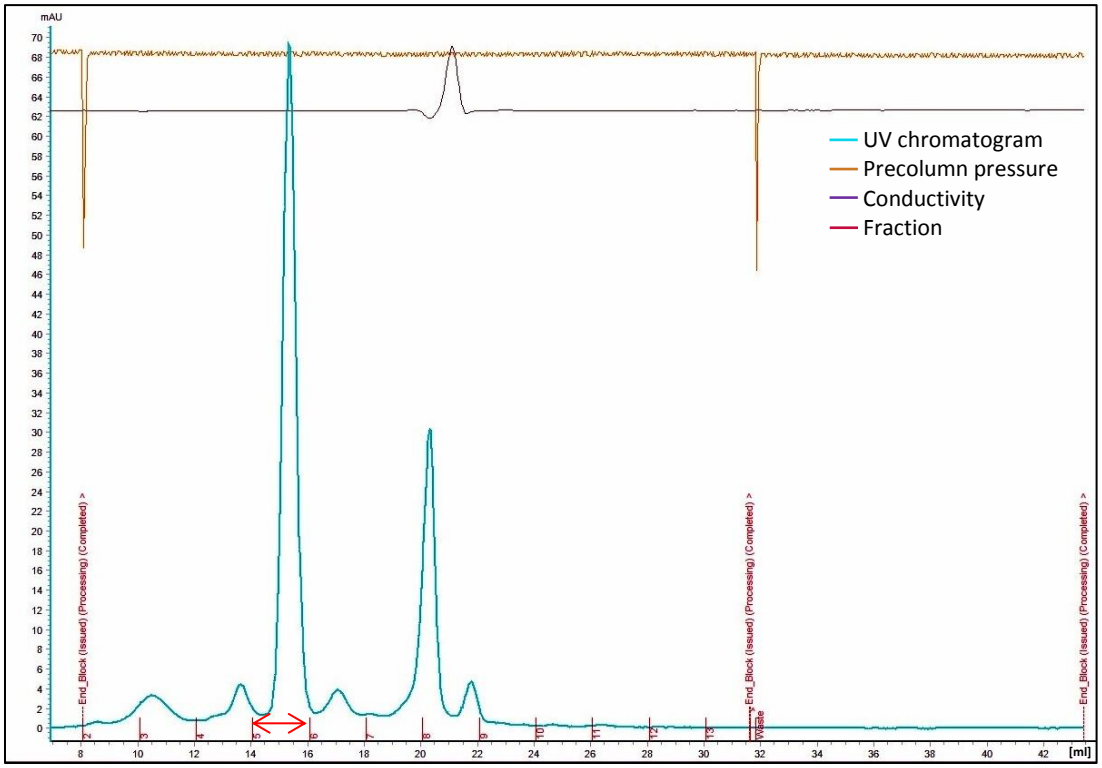


Fig. 55: Size exclusion chromatography of the pooled and concentrated fractions (10-14 from Fig. 53) (IV.5.2.b). Only fraction 5 (↔) revealed high peroxidase activity correlating with the highest peak (absorption) in the UV chromatogram (~70 mAU).

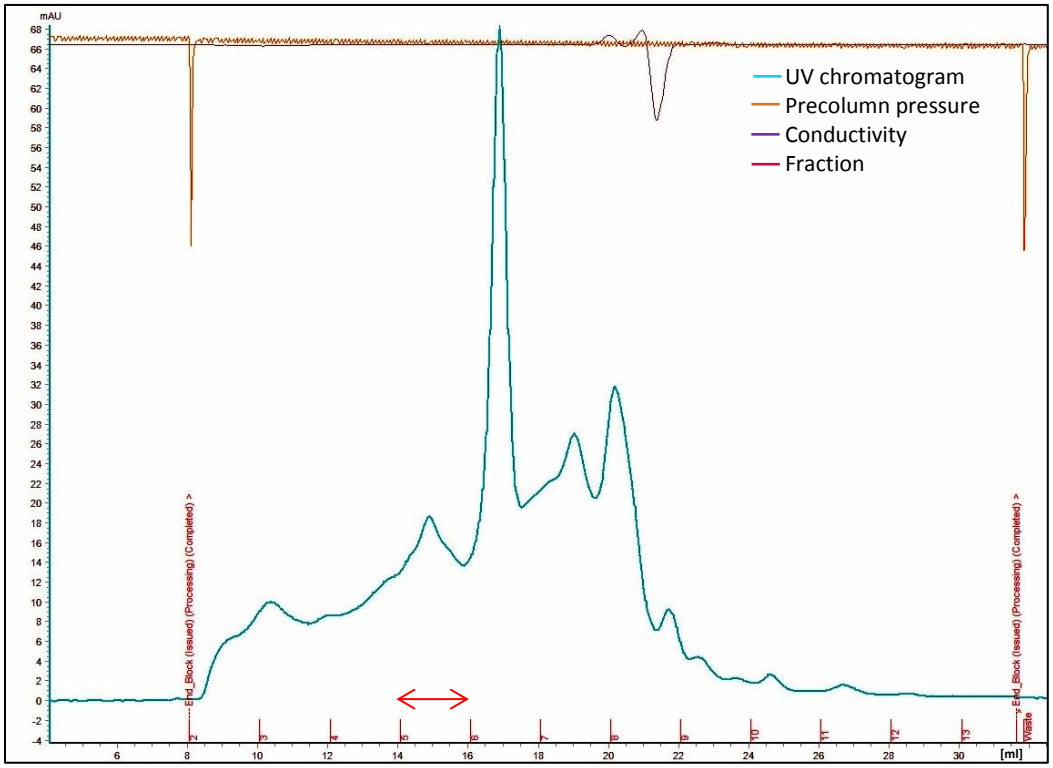


Fig. 56: Size exclusion chromatography of the pooled and concentrated fractions (9-13 from Fig. 54) (IV.5.2.b). Only fraction 5 (↔) revealed high peroxidase activity.

Using calibration proteins which were separated under the same conditions, the approximate molecular weight of the protein of interest was determined. The individual substances were assigned to a respective fraction by UV-absorption (Fig. 57). Since cytochrome c showed a red coloration in the elution buffer it could be easily assigned to fraction 7. The fraction with high peroxidase activity always was fraction 5 which corresponds to the molecular weight of egg albumin. Accordingly, the molecular weight of the active peroxidase, responsible for coupling the precursors taxifolin and coniferyl alcohol in *Silybum marianum*, is about 45 kDa. This is very close to the molecular weight of HRP (44 kDa) (Welinder, 1979).

Since the peroxidase from *Silybum marianum* found by PCR methods only had a calculated molecular weight of about 34 kDa further modifications such as glycosylations, similar to those determined for HRP, can be hypothesised. Assuming an average carbohydrate side chain molecular weight of 2-2.5 kDa about 4-5 glycosylations can be expected.

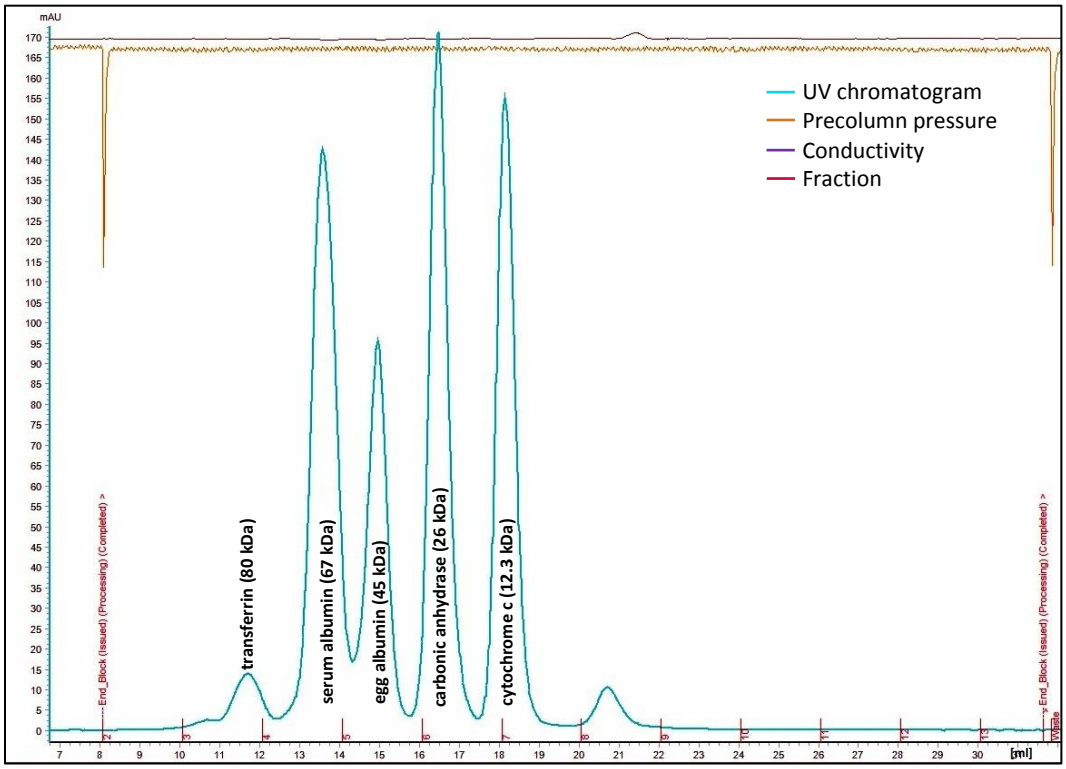


Fig. 57: Size exclusion chromatography of calibration proteins (IV.5.2.b). The proteins were adjusted to 1 mg ml^{-1} each and 200 µl of the mixed solution was loaded to the gel filtration column via capillary loop. Running conditions were the same as in Fig. 55 and Fig. 56.

11. Molecular cloning of genes putatively involved in silymarin formation

11.1 PCR with degenerate primers

Using degenerate primers and standard PCR methods (IV.6.3.a) different partial genes, putatively encoding a peroxidase (POD), two laccases (LACs) and several possible dirigent proteins (DIRs) could be found. The focus for the DIRs, however, was on the two most apparent PCR products.

The *Silybum marianum* partial POD sequence had a length of 701 bp (Fig. 58). The first partial DIR sequence contained 316 bp and the second 223 bp (Fig. 60). Investigations for laccases resulted in two different partial isoforms of 1067 and 1065 bp (Fig. 59). The individual numbered lanes represent PCR products which are formed under the same conditions.

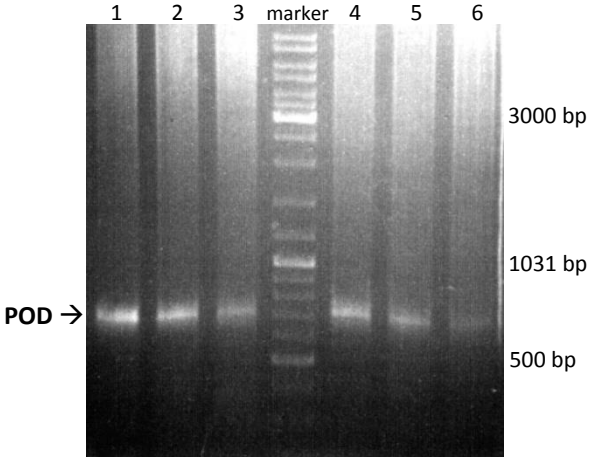


Fig. 58: POD PCR, product at ~700 bp.

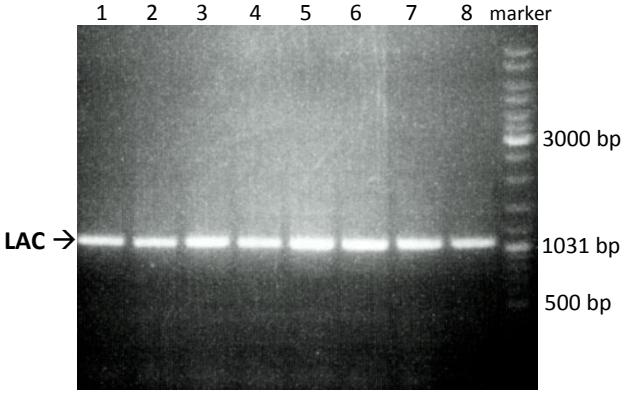


Fig. 59: LAC PCR, product at ~1050 bp.

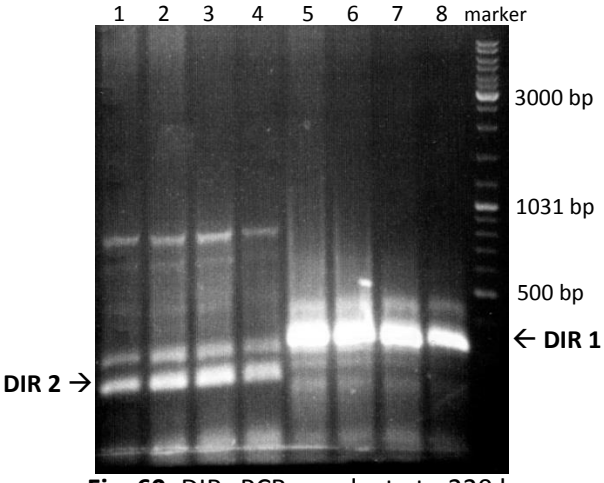


Fig. 60: DIRs PCR, product at ~220 bp (lanes 1-4) and ~315 bp (lanes 5-8).

11.2 RACE PCR

Following the protocol of the SMARTer® RACE Kit enabled the amplification of the 5' and 3' ends of the sequences of interest. In case of the DIRs, nested PCR was performed due to very high sequence similarity. The focus for the laccase was on the longer sequence (1067 bp).

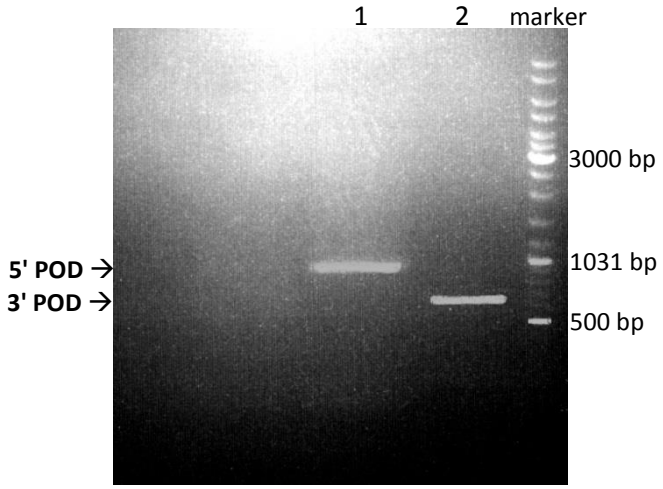


Fig. 61: POD RACE PCR, products at ~1000 bp (5') and ~650 bp (3').

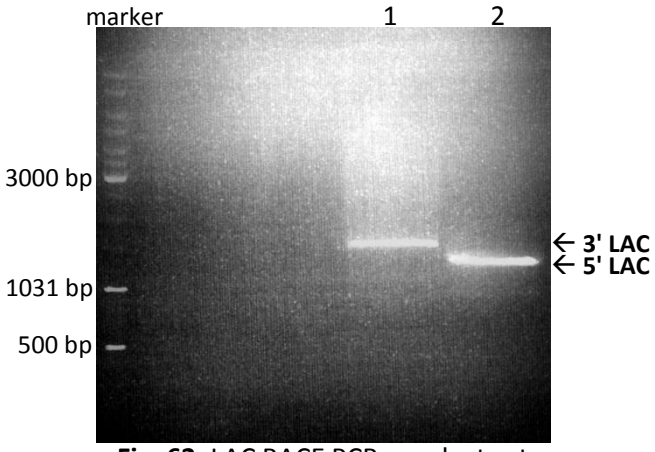


Fig. 62: LAC RACE PCR, products at ~1300 bp (5') and ~1600 bp (3').

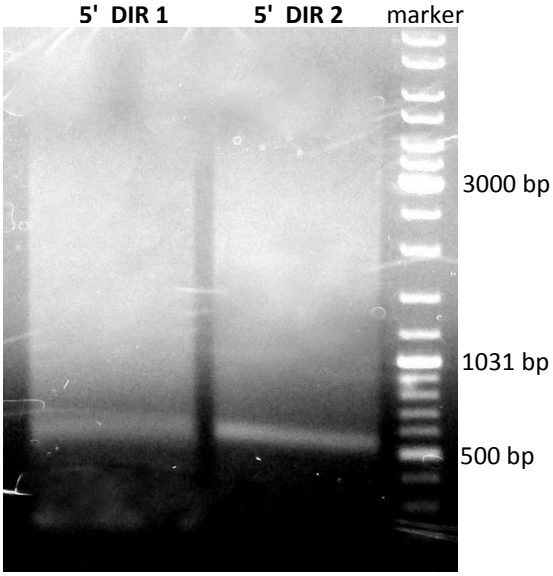


Fig. 63: 5' DIRs nested PCR (after RACE) with short primer (products at ~550 bp).

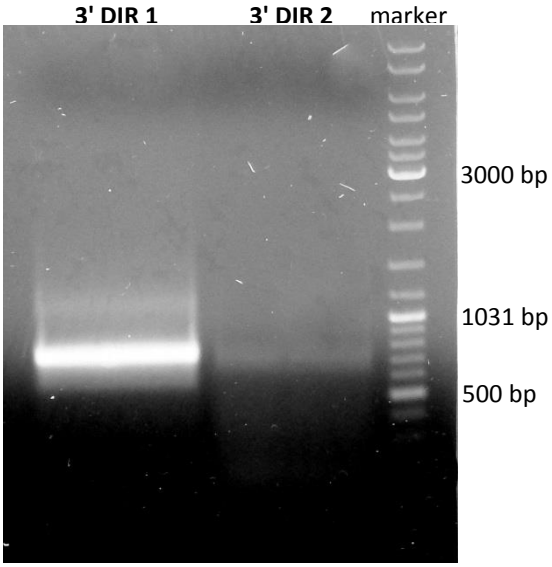


Fig. 64: 3' DIRs nested PCR (after RACE) with short primer (products at ~700 bp).

11.3 Full length sequences

After assembling of the complete coding sequences full length primers were designed for PCR-amplification with a proofreading polymerase, ligation in pDrive, verification, multiplication and transformation of competent *E. coli* cells (EZ from Qiagen) for long-term storage. Consequently, genes highly likely coding for a class III secretory peroxidase, a laccase and two different dirigent proteins could be identified to be a part of the gene pool of *Silybum marianum* (Fig. 65).

These were further compared with already published, known sequences of similar proteins with help of an alignment tool (EMBOSS Needle) from the European Bioinformatics Institute (EBI). In addition, the translated amino acid sequences were tested for the presence and location of potential signal peptide cleavage sites via SignalP 4.1 (Petersen et al., 2011) and for possible glycosylation sites with help of GlykoEP (Chauhan et al., 2013). Signature and binding sites were assigned according to InterProScan (Jones et al., 2014).

All four identified proteins are very likely to have signal sequences, a short peptide present at the *N*-terminus of many *de novo* synthesised proteins that are destined towards the secretory pathway (von Heijne, 1990). Usually, these signal peptides determine the destination to which that protein is delivered. In many instances the signal peptide (~16-30 amino acid residues) is cleaved off the protein once its final destination has been reached (Kapp et al., 2009).

In the identified proteins from *Silybum marianum* it is noticeable that the most obvious differences in the primary structure compared with similar proteins from other plants occur in the signal sequence. This is also true for the otherwise very similar dirigent proteins, even from the same organism. This could be seen as an indication for the correct and specific target destination of the enzymes. For example, processes that could be associated with the maturation of the fruits and thus also the silymarin formation and/or storage in the fruit coats of the achenes. The secretion of the peroxidase, responsible for the coupling reaction between taxifolin and coniferyl alcohol, into the apoplastic space (medium) could be demonstrated in several experiments (V.1.4; V.4.1; V.10; Sánchez-Sampedro et al., 2007).

Furthermore, all identified proteins contain several putative *N*-glycosylation patterns. Their synthesis starts in the endoplasmic reticulum, continues in the Golgi apparatus and ends at the plasma membrane, where these glycoproteins either become embedded in the plasma membrane or are secreted (Cooper, 2000). In fact, a large number of proteins are *N*-glycosylated in the secretory pathway and it has become the most common protein modification in eukaryotes (Aebi,

2013). “These glycosylatory processes are important for the extensive range of structures and allows any one glycoprotein to be tailored for a precise role in the organism” (Cole and Smith, 1989).

The peroxidase from *Silybum marianum* should be similar in structure to other class III plant secretory peroxidases. The presence of an iron(III) atom associated with a protoporphyrin IX as well as two calcium ions, which are essential for both the structure and function of the enzyme, can be supposed. Likewise, four conserved disulphide bridges should have an important role in terms of activity and stability. But especially differences in the glycosylation patterns and the signal peptide could determine substrate specificity and target localisation. However, nano-LC-MS with repeated ion-isolation/fragmentation cycles would be necessary for precise structure elucidation. Crystallisation studies or X-ray structure analyses would also be good options. The three-dimensional structures of several other plant peroxidases have already been determined by X-ray crystallography. This also applies for relevant catalytic intermediates and substrate complexes. In addition, specific roles for particular amino acid residues and conserved regions have been pointed out as well (Veitch, 2004; Protein Data Base). Meanwhile, over 6000 class III peroxidase entries are listed in PeroxiBase, a database for peroxidases (Fawal et al., 2013).

In 2012, Kazenwadel et al. could demonstrate a clear correlation between the loss of dirigent protein activity and the disappearance of fully glycosylated protein as well. Furthermore, they proposed that “proper glycosylation may be necessary for the binding and orientation of coniferyl alcohol to maximise the probability for the specific coupling event yielding the respective product and that glycosylation may affect tertiary structure with an indirect effect on dirigent activity”.

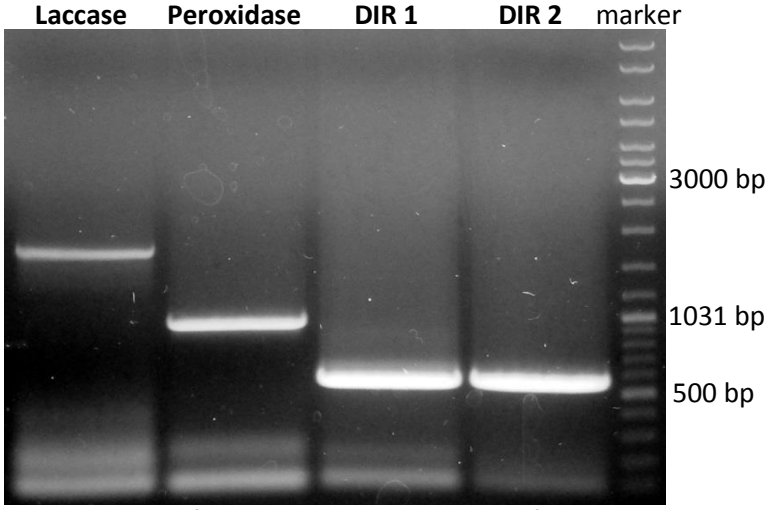


Fig. 65: PCR with full length primers, Phusion® polymerase (V.6.3.c) and restriction sites for expression in pYES2 NT/ C (HindIII + XhoI). Products at 1740 bp (LAC), 984 bp (POD) and 585/582 bp (DIRs).

Peroxidase cDNA (969 bp), nucleotide sequence

ATGGCTCGTTCGTCGTCGTCTTACTCGTATATCGTACTAACATGTATATTATGTTTATGGGTTTTGCTCGGGACGGCTTCAGGTC
AACTTTCGGCAAACTTTTACGCGACCTCATGCCCTAATTTCAGCTCTATTATATCAAACGCTGTGAGCTCTGCCGTTTCAAATGAA
GCTCGAATGGGTGCTTCGCTACTTCGACTTCACTTTCATGATTGTTTTGTTAATGGATGTGATGCGTCGGTTTTATTGGATGATA
CTACAAATTTCGCTGGAGAAAAAACAGCCAGACCTAATAATAATTCGTTGAGAGGATTTGACGTTATCGATACTATTAAAAGTC
AATTAGAGAGTTCATGCCCTGGTGTTGTCTCTTGTGCAGATTTATTAACAACCGCTTCTCGTGATTCGGTTGTAGCACTTGGTGG
CCCGAGTTGGAACCTTGTGTTCGGTAGGAGGGACTCGACCACAGCTAGTTTGAGTGCGGCTAATTCCAACATCCCCGCACCTAC
TTTGAACCTTAGTGGTCTCATATCCTCCTTTTCAAACCAAGGGTTTAATGCGAATGAAATGGTTGCTTTATCAGGATCTCATACA
ATTGGGCAAGCAATGTGTACAGTATTTCGAGCTCGACTCTATAATGAGAACAACATAAACTCGTCGTTTGCTACATCGCTAAGA
GCAAATTGTCCTTCAAGTGGAGGCGACAATAACTTGTCGCCATTAGACGTTGTTTCTCCCACGTCATTCGACAATGCATACTTCA
GTAATTTGATAAGTCAAAGTGGTGTTTTGCATTCGGATCAAGAACTATTTAACGGTGGTTCAACCGATGCCCAAGTGAGAACTT
ACGGTTCAAATTCCGCAACCTTTTCGACAGACTTTGCAAACGCAATGGTTAAGATGAGTAACCTCAACCCGTTGACTGGATCAA
GTGGTGAGGTTCGGACGAATTGCAGAAGAACTAATTAA

Peroxidase, translated amino acid sequence (322 aa)

MARSSSSYSYIVLTCILCLWVLLGTASGQLSANFYATSCP**N**FSSIISNAVSSAVSNEARMGASLLRLHFHDCFVNGCDASVL
LDDTT**N**FTGEKTARPN**N**NSLRGFDVIDTIKSQLESSCPGVVSCADLLTTASRDSVVALGGPSWNLVFGRRDSTTASLSAA
NSNIPAPTL**N**LSGLISSFSNQGFNANEMVALSGSHTIGQAMCTVFRARLYNENNI**N**SSFATSLRANCPSSGGDN**N**LSPLD
VVSPTSFDNAYFSNLISQSGVLHSDQELFNGGSTDAQVRTYGSNSATFSTDFANAMVKMSNLNPLTGSSGEVRTNCRR
TN-

Potential *N*-glycosylation sites are written in **bold**.

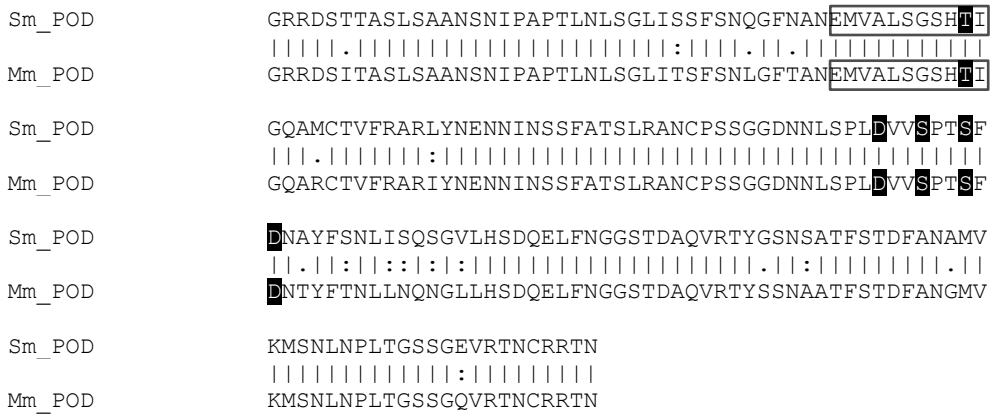
The presumed signal peptide is underlined. The predicted cleavage site shifted four amino acids further downstream in the sequence with recent program updates.

The obtained peroxidase cDNA from *Silybum marianum* shows many conserved motifs of a secretory peroxidase belonging to the plant peroxidase-like superfamily. In particular, this includes the active site signature 61-72 and the proximal heme-ligand signature 189-199. The FHDCFV and VSCAD motifs are conserved in class III peroxidases across plant species (González et al., 2010). In addition, several Ca^{2+} binding sites are recognisable. An alignment with a protein sequence of a known peroxidase from *Micania micrantha* (FJ872511; bitter vine; Asteraceae) shows high similarity (93.2%) and identity (89.2%).

```
Sm_POD          MARS-SSSYSYIVLTCILCLWVLLGTASGQLSANFYATSCPNFSSIISNA
                ||.| ||.||..:.  ||||.||..||.|||||||||||||||||:|
Mm_POD          MALSFSSMYSLPIY--ILCLCVLSDTALGQLSANFYATSCPNFSSIISSA

Sm_POD          VSSAVSNEARMGASLLRLHFHDCFVNGCDASVLLDDTTNFTGEKTARPNN
                |:|||||||||||||||||||||||||||||||||||||||||.|||
Mm_POD          VNSAVSNEARMGASLLRLHFHDCFVNGCDASVLLDDTTNFTGEKTAGPNN

Sm_POD          NSLRGFDVIDTIKSQLESSCPGVVSCADLLTTASRDSVVALGGPSWNLVF
                |||||||||||||||||||||||||||||.||:|||||||||||||.|
Mm_POD          NSLRGFDVIDTIKSQLESSCPGVVSCADLLATAARDSVVALGGPSWNLAF
```



Laccase cDNA (1725 bp), nucleotide sequence

ATGGATCGTGTGTTCACCACCATTGCATTGTTTCTATTGGGCTTGTTGCTTCTCTTTTCAAGTGTGGCCTCTATGCCAAATGCAAA
GACTCACTACCATGACTTCGTCGTTCAAGCAACAAAAGTTAAGAGGCTGTGCAAAACCCACAATTCTATCACGGTGAATGGCCA
GTTCCCAGGTCCGACTTTGGAAGTGAACAATGACGACACTTTAGTCATACATGTTGTCAACAAAGCTAGATATAATGTCACCATT
CACTGGCATGGTGCGAGACAAATGAGGACAGCATGGGCAGATGGACCAGAGTTCATTACGCAATGTCCAATTAGACCAGGAG
GAAGTTACACCTACAGGTTTACGATTTCAGGACAAGAAGGAACACTTTGGTGGCACGCACATAGTTCATGGCTTAGAGCCACT
GTCTATGGTGCTATTATTATTCACCCTAAACAAGGATCTTCCTACCCGTTCCCTAAGCCCAACCGTGATTCACTCATTCTTCTCGG
TGAATGGTGGGATGCAAACCCAATAGATGTTATAAGAGAAGCCACAAGAACAGGAGCTGCTCCAAATGTTTCTGATGCATATA
CCATCAATGGTCAACCCGGTGATCTTTATAACTGCTCTAGCAAAGATACTGTCATAGTACCAGTTGATTCAGGAGAGACAAACC
TCATTCGGGTGATCAATGCAGCTTTAAACCAACAGCTTTTTGTAACAATCGCTAACCACAAACTTACTGTGGTAGGAGCTGATGC
TTCCTATGTAAAACCCTTCACCACCTCGGTCCTAATGCTTGGACCAGGCCAAACCACTGATGTACTCATCAAGGCTGACCAGCCA
CCGGCTAGATACTACATAGCTGCACGTGCTTATGCCAGTGCACAAGGTGCTCCTTTTGACAACACCACCACTACCGCCATTTTGG
AGTACAAAACCGCCCCTTGTACTACAGCCAAATGCACCACTTCAAAACCCATTATGCCACGTCTACCAGCATATAACGACACCAC
AACCGCCACTGCCTTCACAACCAGCTTCAGGAGCCCCAGAAAGGTCTATGTGCCCACTGAAATCGATGAAAATCTGTTCATAAC
CGCGGGTCTTGGAATCAACCAATGCCCACCGAAAACCAGAGCCAGAAACTGTCAGGGACCCAATGGGACCCGCTTCACCGCCA
GCATGAACAACGTATCTTTTGTACTCCCATCCAATTTCTCCTTGCTTCAAGCACATCACCAAGGCGTACGTGGAGTTTTCACCACT
GACTTCCCCGTGAAACCTCCAGTGCCGTTTGATTATACTGGTAATGTGAGCCGGTCTTTATGGCAGCCAACTCGTGGAACAAAA
GTCTACAGGTTAAAGTATGGATCAAGGGTGCAGATTGTGTTGCAAGGAACAAACATCTTCACAGCTGAGAACCATCCAATCCAT
CTTCATGGATATGATTTCTATATACTTGCAGAAGGATTTGGAAACTTCAACACAAAAACAGACACCGCTAAATTCAACCTTGTGG
ATCCACCTCTCAGAAATACAGTTAGTTTGCCTGTAAAAGGGTGGGCAGTCATTAGATTTGTTGCAGATAATCCAGGTGTTTGGA
TTATGCATTGCCACTTAGACGTTCACATTGGCTGGGGTTTGGCTACAGTATTTCTTGTGGACAACGGAGTTGGACCATTGCAGC
AATTGGAGCAACCACCACTAGACTTACCCGTGTGTTGA

Laccase, translated amino acid sequence (574 aa)

MDRVFTTIALFLLGLLLLFSSVASMPNAKTHYHDFVVQATKVKRLCKTHNSITVNGQFPGPTLEVNNDDTLVIHVVNKAR
Y**N**VTIHWHGARQMRTAWADGPEFITQCPIRPGGSYTYRFTISGQEGTLWWHAHSSWLRATVYGAIIIHPKQGSSYPFP
KPNRDSLILLGEWWDANPIDVIREATRTGAAP**N**VSDAYTINGQPGDLY**N**CSSKDTVIVPVDSGETNLIRVINAALNQQLF
VTIANHKLTVVGADASYVKPFTTSVLMLGPGQTTDVLIKADQPPARYYIAARAYASAQGAPFD**N**TTTTAILEYKTAPCTTA
KCTTSKPIMPRLPAY**N**DTTTATAFTTSFRSPRKVYVPTEIDENLFITAGLGINQCPPKTRARNCQGP**N**GTRFTASMN**N**VS
FVLPS**N**FSLLQAHHQGVRGVFTTDFPVKPPVPFDYTG**N**VSRSLWQPTRGTKVYRLKYGSRVQIVLQGTNIFTAENHPIHL
HGYDFYILAEGFGNFNTKTDTAKFNLVDPPLRNTVSLPVKGWAVIRFVADNPGVWIMHCHLDVHIGWGLATVFLVDN
GVGPLQQLEQPPLDLPVC-

Potential *N*-glycosylation sites are written in **bold**.

The presumed signal peptide is <u>underlined</u>.

The obtained laccase cDNA from *Silybum marianum* shows many conserved motifs of a copper oxidase belonging to the cupredoxin superfamily. This is especially true for the multicopper oxidase signature region towards the end of the protein sequence 537-548. Furthermore, several trinuclear Cu and four type 1 Cu binding sites can be found. These as well are highly conserved regions among multicopper oxidases, especially laccases (Giardina et al., 2010; Reiss et al., 2013). An alignment with a protein sequence of a known laccase from *Solanum lycopersicum* (XM_004239911; tomato; Solanaceae) shows high similarity (87.6%) and identity (80.1%).

```
Sm_LAC          MDRVFTTIA-----LFLLGLLLLFSSVASMPNAKTHYHDFVVQATKVKRL
                 .....:||     |..|.:.|||:..||   |||||||||:|||.||||
Sl_LAC          -MEALKSIAKPLCYLLFLCIFLLFADAAS---AKTHYHDFVIQATPVKRL

Sm_LAC          CKTHNSITVNGQFPGPTLEVNNDDTLVIHVVNKARYNVTIHWHGARQMRT
                |||||:|||||||||||||.||||:.|||:||||||||||.|||||:
Sl_LAC          CKTHNTITVNGQFPGPTLEVNNGDTLVVKVVNRARYNVTIHWHGVRQMRS

Sm_LAC          AWADGPEFITQCPIRPGGSYTYRFTISGQEGTLWWHAHSSWLRATVYGAI
                ||||||||||||||||.||||||||.|||||||||||||||||||||:
Sl_LAC          AWADGPEFITQCPIRPGKSYTYRFTIQGQEGTLWWHAHSSWLRATVYGAL

Sm_LAC          IIHPKQGSSYPFPKPNRDSLILLGEWWDANPIDVIREATRTGAAPNVSDA
                |||||:|.:|||||||.|::.|||||||||||||:|||||||||||||||
Sl_LAC          IIHPKEGGNYPFPKPRRETPILLGEWWDANPIDVVREATRTGAAPNVSDA

Sm_LAC          YTINGQPGDLYNCSSKDTVIVPVDSGETNLIRVINAALNQQLFVTIANHK
                ||||||||||.|||:||.||.:|||||||:|||||.:|||:||.|:||||
Sl_LAC          YTINGQPGDLYKCSSQDTTIVHMDSGETNLLRVINAGMNQELFFTVANHK

Sm_LAC          LTVVGADASYVKPFTTSVLMLGPGQTTDVLIKADQPPARYYIAARAYASA
                .|||||||||||||||||||||||||||||||||||:|||:||||||||
Sl_LAC          FTVVGADASYVKPFTTSVLMLGPGQTTDVLIKADQPPSRYYMAARAYASA

Sm_LAC          QGAPFDNTTTTAILEYKTAPCTTAKCTTSKPIMPRLPAYNDTTTATAFTT
                |||||||||||||||||:.| ::.|..:.|:.|.|||||||.|||||||
Sl_LAC          QGAPFDNTTTTAILEYKTSSC-SSNCVKTNPVFPSLPAYNDTATATAFTT

Sm_LAC          SFRSPRKVYVPTEIDENLFITAGLGINQCPPKTRARNCQGPNGTRFTASM
                .|||||:|.||.|||||||.|.|||:|.||...|:|||||||||||||||
Sl_LAC          KFRSPRRVEVPKEIDENLFFTVGLGLNNCPRGARSRNCQGPNGTRFTASM

Sm_LAC          NNVSFVLPSNFSLLQAHHQGVRGVFTTDFPVKPPVPFDYTGNVSRSLWQP
                ||||||||||||||||||.|:.|||:||||..|||.||||||||||||||.
Sl_LAC          NNVSFVLPSNFSLLQAHHNGIPGVFSTDFPAVPPVKFDYTGNVSRSLWQL

Sm_LAC          TRGTKVYRLKYGSRVQIVLQGTNIFTAENHPIHLHGYDFYILAEGFGNFN
                .||||:|:||||:|||:|||||||.|||||||||||||||:||||||||
Sl_LAC          IRGTKLYKLKYGARVQVVLQGTNISTAENHPIHLHGYDFYIIAEGFGNFN

Sm_LAC          TKTDTAKFNLVDPPLRNTVSLPVKGWAVIRFVADNPGVWIMHCHLDVHIG
                .|.||:||||||||||||.|:||.|||||||||||||:|||||||||||.
Sl_LAC          PKRDTSKFNLVDPPLRNTASVPVNGWAVIRFVADNPGVWLMHCHLDVHIT
```

```
Sm_LAC          WGLATVFLVDNGVGPLQQLEQPPLDLPVC
                ||||..|||:|||..|:.||.||:|||||
Sl_LAC          WGLAMAFLVENGVSQLESLEAPPVDLPVC
```

Dirigent protein No. **1** (570 bp), cDNA sequence

ATGAGTTCTAAGTATCAAAACTCAACACTTCTCTTAACAATCTTTTTCTTGCTTATTTTAGGATGTTCTGCTTCACCTAGAAAGAA
CATTCGCTCTCGACGTCCTTGTAAAGAAATGGTGTTTTATTTTCACGATATTCTTTATAATGGCAAGAACTTCAAGAATGCAACT
GCAGCCATTGTAGGTGCTCCAGCTTGGGGTAATAACACAATTTTGGCGAATAATAATCATTTTGGGAACATAGTAGTATTCGAT
GATCCTATCACATTGGACAACAACTTGCACTCACCCCCTGTTGGGCGAGCACAAGGGTTTTATATCTATGACAAAAAAGAAATT
TTTACAGCCTGGCTTGGATTCTCGTTTGTCTTTAATTCAACTGCACATAAGGGTAGCATTAACTTTGCAGGGGCCGATCCTTTGA
TGAACAAGACAAGAGACATTTCTGTGATTGGTGGAACTGGTGATTTCTTCATGACCAGAGGCGTGGCTACCATTATGACAGACT
CGTTTGAAGGGGAGGTTTATTTTAGGCTTCGTGTTGACATTAAGTTCTATGAATGTTGGTGA

Dirigent protein No. **1**, translated amino acid sequence (189 aa)

MSSKYQ**N**STLLLTIFFLLILGCSASPRKNIRSRRPCKEMVFYFHDILYNGKNFK**N**ATAAIVGAPAWG**N**NTILANNNHFGNI
VVFDDPITLDNNLHSPPVGRAQGFYIYDKKEIFTAWLGFSFVF**N**STAHKGSINFAGADPLM**N**KTRDISVIGGTGDFFMTR
GVATIMTDSFEGEVYFRLRVDIKFYECW-

Potential *N*-glycosylation sites are written in **bold**.

The presumed signal peptide cleavage site is underlined.

The obtained cDNA sequence of dirigent protein No. 1 shows high similarities to known sequences from, for example, *Thuja plicata*, *Linum usitatissimum*, *Glycine max* and other disease resistance response proteins. An alignment with a protein sequence of a known dirigent protein from *Forsythia x intermedia* (AAF25357; border forsythia; Oleaceae) shows high similarity (79.4%) and identity (67.2%). This dirigent protein is known to be active in the stereoselective biosynthesis of (+)-pinoresinol from coniferyl alcohol monomers (Davin et al., 1997).

```
Sm_DIR1         MSSKYQNSTLLLTIFFLLILGCSASPRKNIRSRRPCKEMVFYFHDILYNG
                |.||.|...|.|.   .|....||:..:..|.||||||:||||||:|:.|
Fi_DIR1         MVSKTQIVALFLC---FLTSTSSATYGRKPRPRRPCKELVFYFHDVLFKG

Sm_DIR1         KNFKNATAAIVGAPAWGNNTILANNNHFGNIVVFDDPITLDNNLHSPPVG
                .|:.|||:||||:|.|||.|.:|...::|::||||||||||||||||||
Fi_DIR1         NNYHNATSAIVGSPQWGNKTAMAVPFNYGDLVVFDDPITLDNNLHSPPVG

Sm_DIR1         RAQGFYIYDKKEIFTAWLGFSFVFNSTAHKGSINFAGADPLMNKTRDISV
                ||||.|.||:|..:.|||||||:||||.:.|::|||||||||:||||||||
Fi_DIR1         RAQGMYFYDQKNTYNAWLGFSFLFNSTKYVGTLNFAGADPLLNKTRDISV

Sm_DIR1         IGGTGDFFMTRGVATIMTDSFEGEVYFRLRVDIKFYECW
                |||||||||.|||||:|||:|||:||||||||..||||
Fi_DIR1         IGGTGDFFMARGVATLMTDAFEGDVYFRLRVDINLYECW
```

Dirigent protein No. **2** (567 bp), cDNA sequence

ATGAGTGTAAAAACAAGTTCAAATATAGTTGTTCTTTTGTTTATGTTTCTTACAGTTTCTTCATCATACAATCATGAAGGAAACAA
TAAACATTACAAACCATGCAAACGTCTAGTTTTGTTTTTCCATAATATTCTCTACAATGGCAATAATAAAGCCAATTCGACAGCA
GCTATTGTGGCTGCACCCGAATGGGGCAACTTGACAATCTTGACTAGCGAGTTTCATTTTGGGGATCTCGTGGTTTTCGATGAC
CCTATTACTTTAGACAACAATTTGCATTCCTCTCCGGTTGGTCGGGCTCGGGGTCAATACATCTATGACGCCAAGAACTCCTCCT
CATCATGGTTAGGGTTCTCGTTTGTTCTTAATAGTACGGATTATCATGGAAGCATCAATTTCATTGGGGCTAATCCACATCCACT
CAAGTTTAGGGATATATCAGTTGTTGGTGGGACTGGAGACTTCTTTATGCATAGAGGAATTGCAACTCTTATGAGTGATGCAAC
TGAAGGCCAAGTGTATTTTAGACTCCGCGTTGACATAAAGTTCTATGAGTGTTGGTAA

Dirigent protein No. **2**, translated amino acid sequence (188 aa)

MSVKTSSNIVVLLFMFLTVSSSYNHEGNNKHYKPCKRLVLFFHNILYNGNNKA**N**STAAIVAAPEWG**N**LTILTSEFHFGDL
VVFDDPITLDNNLHSSPVGRARGQYIYDAK**N**SSSSWLGFSFVL**N**STDYHGSINFIGANPHPLKFRDISVVGGTGDFFMHR
GIATLMSDATEGQVYFRLRVDIKFYECW-

Potential *N*-glycosylation sites are written in **bold**.

The presumed signal peptide cleavage site is underlined.

The obtained cDNA sequence of dirigent protein No. 2 shows high similarities to known sequences of, for example, *Thuja plicata*, *Forsythia x intermedia*, *Glycine max* and other disease resistance response proteins as well. An alignment with a protein sequence of a known dirigent protein from *Linum usitatissimum* (KM433755; flax; Linaceae) shows high similarity (79.3%) and identity (62.8%). This protein is also known for its involvement in the formation of (+)- or (−)-pinoresinol by stereoselective coupling of coniferyl alcohol monomers (Dalisay et al., 2015).

```
Sm_DIR2           MSVKTSSNIVVLLFMFLTVSSSYNHEGNNKHYKPCKRLVLFFHNILYNGN
                  |::..|.  :.|.|:|....||.:.....|.:.|||.||||||:|:|||.
Lu_DIR2           MTISRSK--IALFFIFFIYLSSTSSSAKKKQHAPCKELVLFFHDIIYNGQ

Sm_DIR2           NKANSTAAIVAAPEWGNLTILTSEFHFGDLVVFDDPITLDNNLHSSPVGR
                  ||||:|||||||||..|.|||..|.|||::.|||||||||||||.||||
Lu_DIR2           NKANATAAIVAAPEGSNRTILAGESHFGNIAVFDDPITLDNNLHSPPVGR

Sm_DIR2           ARGQYIYDAKNSSSSWLGFSFVLNSTDYHGSINFIGANPHPLKFRDISVV
                  |:|.|:||.||:.::||||:|.||||::.|:|||:||:|...|.||:|:|
Lu_DIR2           AQGMYLYDTKNTFTAWLGFTFCLNSTEHQGTINFMGADPLMNKTRDVSIV

Sm_DIR2           GGTGDFFMHRGIATLMSDATEGQVYFRLRVDIKFYECW
                  ||||||||||:||:|:|:.||:|||||||:|||:||
Lu_DIR2           GGTGDFFMHRGVATIMTDSYEGEVYFRLRVDMKFYDCW
```

A comparison of the two identified dirigent proteins from *Silybum marianum* shows, on the one hand, that these are also quite related (similarity: 76.7%, identity: 62.4%), but nevertheless differ at several positions and amino acids. This could be an indication for similar enzymatic processes within the plant and at the same time possible distinct catalytic activities based on the resulting product.

```
Sm_DIR1           MSSKYQNSTLLLTIFFLLILGCSASPRKNIRSRRPCKEMVFYFHDILYNG
                  ||.| .:|.::::.:|..|.:..|.:...|.:..:|||.:|.:||:|||||
Sm_DIR2           MSVK-TSSNIVVLLFMFLTVSSSYNHEGNNKHYKPCKRLVLFFHNILYNG

Sm_DIR1           KNFKNATAAIVGAPAWGNNTILANNNHFGNIVVFDDPITLDNNLHSPPVG
                  .|..|:|||||.||.|||.|||.:..|||::|||||||||||||||.|||
Sm_DIR2           NNKANSTAAIVAAPEWGNLTILTSEFHFGDLVVFDDPITLDNNLHSSPVG

Sm_DIR1           RAQGFYIYDKKEIFTAWLGFSFVFNSTAHKGSINFAGADPLMNKTRDISV
                  ||:|.||||.|...::|||||||.|||.:.|||||.||:|...|.|||||
Sm_DIR2           RARGQYIYDAKNSSSSWLGFSFVLNSTDYHGSINFIGANPHPLKFRDISV

Sm_DIR1           IGGTGDFFMTRGVATIMTDSFEGEVYFRLRVDIKFYECW
                  :||||||||.||:||:|:|:.||:|||||||||||||||
Sm_DIR2           VGGTGDFFMHRGIATLMSDATEGQVYFRLRVDIKFYECW
```

12. Expression studies for peroxidases, laccases and dirigent proteins

12.1 Prokaryotic cell lines (*E. coli*)

Initially, the discovered sequences were expressed in *E. coli* cell lines in order to obtain large protein quantities in short time. For this purpose, they were ligated into the expression vector pET-15b and introduced in either BL21(DE3)pLysS or BL21-CodonPlus (DE3)-RIPL cells. A polyhistidine-tag for purification, already present on the plasmid, was attached to the *N*-terminus. Expression was done as described in IV.7.7.a.

The formation of the desired proteins, however, could not be detected. SDS-PAGE after affinity chromatography for proteins containing a polyhistidine tag showed no gene-specific bands in the expected size ranges. Likewise, enzyme activity tests with insoluble and soluble protein fractions were negative. Therefore, the structure of the proteins could be too complex for expression in prokaryotic systems.

A possible approach to this problem would be the removal or replacement of the signal sequences since prokaryotes do not have a membrane-enclosed secretion system analogous to the eukaryotes. Even then, problems such as insoluble protein inclusion bodies could be expected. Extensive refolding techniques of intracellularly produced proteins would be the consequence. In 2011, Pickel came to a similar result and conclusion during his PhD thesis. An approach would be the co-expression of chaperones possibly resulting in a higher expression level of the soluble protein complex which, for example, was the case for a dirigent protein from *Arabidopsis thaliana* (Kazenwadel 2013).

Furthermore, the expected glycosylations, probably responsible for the correct structure and function of these proteins, tend to be major problems for *E. coli*-based expression systems because of its limited post-translational machinery function (Khow and Suntrarachun, 2012). Meanwhile, problem-solving approaches by a succesful transfer of a gene cluster encoding a complete *N*-linked protein glycosylation pathway from subdivisions of Proteobacteria into *E. coli* were presented (Fisher et al., 2011; Nothaft and Szymanski, 2013).

According to Krainer and Glieder (2015) it has to be pointed out that the production of, for example, secreted class III peroxidases in *E. coli* is still by far not suitable for biotechnological applications due to the extremely low yields. The maximum achievable yields from *E. coli* for HRP did not surpass 10 mg l^{-1} so far (Grigorenko et al., 1999).

12.2 Eukaryotic cell lines (yeast)

a. *Pichia pastoris* X-33 strain

In the further course of this work the decision for the next expression attempt fell to a methanol-inducible system known for the high-level secretion of recombinant proteins (Ahmad et al., 2014). For example, the group of Kazenwadel et al. (2012) could achieve good results for high glycosylated proteins containing signal peptides. A fed batch fermentation process yielded about 47 mg l^{-1} of a dirigent protein.

This time the cDNA sequences of the proteins were inserted into the expression vector once with signal sequence and once without signal peptides in order to cover both possibilities for expression of active enzyme. After ligating into the expression vector pPICZα A and conformation of the correct insert within the reading frame the constructs were linearised and *P. pastoris* cells transformed by electroporation (IV.7.2.b). In each case, the His-Tag present on the plasmid was attached to the C-terminal end of the sequence for easier detection and purification.

The ability to utilise MeOH (Mut phenotype) was tested using Zeocin™-resistant transformants on MD and MM plates (IV.8.7). Mut^+ colonies with possible multiple gene insertions were used for further isolation of pure clonal isolates. Putative multi-copy recombinants were selected by picking well growing clones from YPD plates with adjusted high Zeocin™ concentration (1 mg ml^{-1}). The presence of the insert was confirmed via colony PCR (IV.6.3.d). Expression experiments were undertaken as described in IV.7.7.b.

The supernatants and cell pellets were analysed for protein expression by SDS-PAGE and the more sensitive Western blot or enzyme assays. Unfortunately, neither the inserts with nor those without signal peptide resulted in any detectable expressed protein. No recombinant protein could be detected at any time between 0 and 72 h of expression, neither secreted nor on the intracellular level. Concentrating the supernatant by ammonium sulphate precipitation (IV.5.3.a) did not result in the desired success either. Variation of the temperature (28-30 °C) during expression or the regularly added MeOH concentration (0.5-1%) also led to no improvement. Since no recombinant protein could be found within the cells as well it can be assumed that the expression in *P. pastoris* did not work properly and would have to be optimised on many levels.

Following *Pichia* protocols, the biggest hurdle seems to be generating initial success, meaning expression of recombinant protein at any level (Higgins and Cregg, 1998). The secreted protein might also be susceptible to neutral pH proteases which could be circumvented by expressing in unbuffered medium. The addition of 1% casamino acids with buffered medium could inhibit extracellular proteases (Werten et al., 1999). In addition, heavily glycosylated proteins with possible interchain disulphide bonds would probably not go through the secretion pathway and therefore not be modified (Gasser et al., 2006). Then again, intended or casual deglycosylation could likely lead to loss of activity. A class III peroxidase mutant for HRP that contained mutations in all *N*-glycosylation sites showed a 300-fold reduction in catalytic activity (Capone et al., 2014). Even the number of glycosylations per isoenzyme could affect both purification factor and recovery yield as reported for isoenzymes of HRP (Krainer et al., 2014).

Another very important aspect could be the α-mating factor secretion signal sequence of the *P. pastoris* strains used for expression. Extensive site-directed mutagenesis of the respective prepro region led to interesting results. Though some mutations clearly dampened protein expression, deletion of specific amino acids led to about 50% increased secretion of reporter proteins (Lin-Cereghino et al., 2013).

b. *Saccharomyces cerevisiae* CB018 and InvSc1 strains

As an alternative to the *P. pastoris* system optimised for the secretion of recombinant proteins a simpler strategic approach was undertaken. The full length sequences were ligated into the vector pYES2/NT C and again a His-Tag was attached to the *C*-terminal end of the proteins. After verification of the inserts within the correct reading frame transformation of the *Saccharomyces cerevisiae* strains CB018 and InvSc1 followed by using the lithium acetate method (IV.7.2.c). InvSc1 is a common fast-growing *S. cerevisiae* strain; CB018 was established in the W303 strain background with deficiencies for major vacuolar proteases (Pep^-). Again, successful transformation was confirmed by colony PCR (IV.6.3.d). Both strains should theoretically be able to produce the proteins intracellularly. For expression conditions see IV.7.7.c.

The only visible results, thus possibly expressed recombinant protein, could be detected after 24 h of expression mainly in the insoluble and soluble fractions of the cells of the InvSc1 strain, however, only after separation by SDS-PAGE and subsequent Western blot analysis (Fig. 64-65). This was also only true for two of the four genes, the peroxidase and dirigent protein No. 2. The two others showed no gene-specific bands at all. Transformed control samples with empty vectors were negative as well.

The Western blot of the expressed dirigent protein No. 2 (Fig. 67) showed two visible and clearly separated bands between 24 and 26 kDa in the insoluble and the soluble fraction after His-Tag purification (IV.5.2.c). This would be in accordance with the expected length. The expressed peroxidase (Fig. 66) appears as a smear on the membrane. Hyperglycosylation might be responsible for this phenomenon, since *S. cerevisiae* usually adds longer *N*-linked sugar chains to proteins than *P. pastoris* (Romanos *et al.*, 1992). In case of expressed HRP in *P. pastoris* heavy modifications in the glycosylation sites led to a smear on a SDS-polyacrylamide gel at a size of approximately 65 kDa instead of its non-glycosylated size of 35 kDa (Morawski et al., 2000; Dietzsch et al., 2011). Furthermore, the amount and type of *N*-glycosylations could differ. The most obvious band is at about 42.5 kDa which was also detectable in the flow through. This would correspond to the peroxidase gene (34 kDa) with attached V5 Epitope plus *C*-terminal His-Tag (~3.5 kDa) and about two glycosylations (~5 kDa).

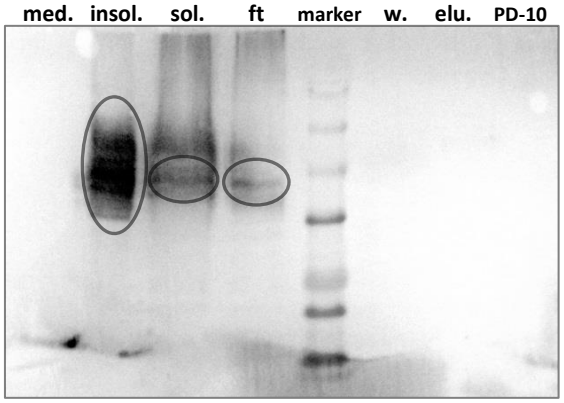


Fig. 66: Western blot of a denatured peroxidase from *Silybum marianum* possibly expressed in InvSc1 cells. Detection was done with anti-His-Tag antibody. Abbreviations: medium (med.), insoluble fraction (insol.), soluble fraction (sol.), flow through (ft), washing fraction (w.), elution (elu.) and the respective purified elution fraction after desalting with PD-10 columns.

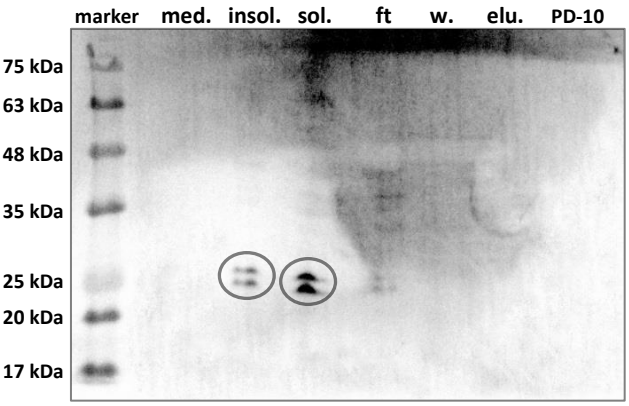


Fig. 67: Western blot of a denatured dirigent protein from *Silybum marianum* possibly expressed in InvSc1 cells. Detection was done with anti-His-Tag antibody. Abbreviations: medium (med.), insoluble fraction (insol.), soluble fraction (sol.), flow through (ft), washing fraction (w.), elution (elu.) and the respective purified elution fraction after desalting with PD-10 columns.

Unfortunately, the failed purification rather indicates an incorrect formation or inside flap of the polyhistidine-tag. Purification under denaturing conditions with refolding possibilities led to no further improvement. The same applied for shorter or longer expression times. Possible secretion of proteins could not be detected, whereby the system is not designed for that purpose in the first place. Enzyme activity tests were all negative. The yield simply could have been too low. Falsely synthesised or non-present glycosylation may have contributed to that fact. For further use of this system optimisations at some points are possible and apparently necessary in order to get higher amounts of active protein.

At first, a secretory expression system similar to the *P. pastoris* project is also possible for *S. cerevisiae*. However, one would have to consider here again if it would be best to omit the signal sequence at the *N*-terminus of the proteins. Morawski et al. (2000) could express heterologous recombinant HRP protein in the pYEX-S1 vector system in the BJ5465 *S. cerevisiae* strain and in *P. pastoris* with pPIZα B as vector as well. All in all, systems optimised for the secretion of recombinant proteins in yeast currently seem to be the most promising production systems based on total yields and expenditure of time.

Another important and easy to accomplish factor would be the addition of molecules that could support the native protoporphyrin IX formation or rather the heme-biosynthesis pathway in yeast or even *E. coli* which is crucial for the structure and function of class III peroxidases. In many cases the addition of δ-aminolevulinic acid, a precursor of the porphyrin synthesis pathway, and/or iron compounds like iron(III) citrate or sulphate led to higher yields of active recombinant HRP (Antipov et al., 2009; Dietzsch et al., 2011). In case of laccases the addition of copper compounds to the expression medium also led to significantly increased protein yields (Gomaa and Momtaz, 2015).

Finally, it can be said that there are several possible approaches which could lead to better yields of recombinant protein. According to Idiris et al. (2000) common strategies for strain engineering of secreted proteins is focused mainly on four topics: "engineering of protein folding and quality control system in the endoplasmic reticulum, engineering of the intracellular protein trafficking pathway, minimisation of post-secretory proteolytic degradation, and engineering of post-translational glycosylations".

If all these attempts fail there are still ways like codon optimisation, transient or stable transformation of plant systems like *Nicotiana tabacum* or, ultimately, custom peptide synthesis.

VI. Closing Discussion and Outlook

In the course of this work eligible enzymes and several factors possibly involved in flavonolignan biosynthesis in *Silybum marianum* have been investigted. A secretory class III heme peroxidase could be identified and isolated from established cell suspension cultures. This enzyme and propable isoforms may be responsible for the radical formation and coupling reaction between taxifolin and coniferyl alcohol. However, peroxidases and other oxidative enzymes or substances alone could not regulate the formation of flavonolignan diastereomers or even regioisomers present in the silymarin mixture. In fruits of distinct milk thistle ecotypes/cultivars the distribution of these flavonolignans can clearly differ. Interestingly, the regioisomer composition in suspension cells resembled the chemotype of these plant origins.

Since solely extrinsic factors, like seasonal variations, could be excluded to be responsible for this phenomenon high genotypic variations with distinct chemotypic properties can be assumed. The plant must have ways to regulate the formation of its individual flavonolignan structures. It could be shown, that changing concentrations of the substrates could contribute to this to a small extent. Yet, additional active mechanisms seem probable. Precursors and/or flavonolignans could be transported individually to their destination in the testa/pericarp and also discriminated in the same process. The search for transport proteins possibly involved and responsible for translocation of flavonolignans and accumulation of these substances in specific plant parts seems an interesting approach. Extensive characterisation by functional genomic efforts, like metabolic profiling followed by reverse genetic experimentation, would be necessary for further information.

Another possibility would be that silymarin is mostly only formed at its storage location. This would indicate a participation of further enzymes involved in flavonolignan biosynthesis. At this point, dirigent proteins have been hypothesised. An involvement in regulating the individual flavonolignan structures in *Silybum marianum* seems possible. Two different dirigent proteins could be identified to be part of the gene pool. Since these share high similarity with proteins from non related plant families also regulating the formation of diastereomeric structures a similar function in milk thistle seems plausible. Even new types of dirigent proteins, playing an active role in coupling the phenoxy radicals and thus possibly regulate the biosynthesis of specific positional isomers of silymarin, are possible and would certainly be a major discovery.

Enzyme preparations from *Silybum marianum* cells provided no further information regarding the presence and involvement of dirigent proteins. The expression of these proteins might be plant organ and/or time specific. With help of quantitiative real-time PCR (qPCR) with isolated RNA from distinct plant parts at different stages of plant development it could be tested, whether involved proteins are active only during the fruit development and maturation phase. This could further indicate fruit coat specific dirigent proteins possibly involved in flavonolignan biosynthesis.

The main focus for further clarification of this topic should be the expression and characterisation of the proteins found by means of PCR methods from *Silybum marianum* plant material. Since the laccase, peroxidase and the two dirigent proteins all contain a signal peptide and show many glycosylation patterns, most likely crucial for structure and activity, this has to be considered when choosing a suitable expression system. For this purpose, yeast systems optimised for the secretion of recombinant proteins could be used first. The signal sequence should be omitted. Furthermore, molecules supporting the native protoporphyrin IX formation should be added. δ-aminolevulinic acid is particularly well suited for that purpose. Depending on the ion contained in the centre of the protoporphyrin, addition of Fe^{2+}/Cu^{2+} to the medium could also have a positive impact on expression.

Even better would be the expression in High Five™ insect cell lines specialised for secreted expression of recombinant proteins. Also stable transformation of plant-based systems could be considered. An expression of active protein by modified Gateway cloning strategies for tagged proteins should certainly be possible. Especially with regard to heavily glycosylated proteins with signal peptides these represent promising alternatives. The expression rate as well could be increased by performing codon optimisation. Only those amino acids which are most strongly expressed in the respective species would be used for the synthesis of a recombinant protein.

Ultimately, the expressed peroxidase and laccase should be tested in enzyme assays for their radical formation potential and their ability to form silymarin. Especially the possible involvement of the two dirigent proteins should be clarified, but also whether they differ in their function. In case the laccase and both dirigent proteins are not involved in silymarin biogenesis the participation in other enzymatic reactions can not be excluded. Lignification processes, like the formation of monolignol radicals and the stereoselective coupling of monolignol units should be considered and investigated. X-ray christallography would clarify post-translational modifications and extended glycosylation patterns.

VII. Summary

Flavonolignan Biosynthesis in *Silybum marianum* – Potential Regulatory Mechanisms and Candidate Genes

Lennart Poppe

Silymarin, a flavonolignan mixture from milk thistle (*Silybum marianum*, Asteraceae), is mainly used for the supportive therapy of chronic liver diseases or to prevent toxic liver damage. In addition, beneficial effects for human health like tumor inhibition and immunomodulatory mechanisms have been reported. The plant produces and accumulates silymarin mainly in the testa and pericarp for protection of its sporophytic embryo against plant herbivores and to limit or prevent bacterial, fungal or viral infections.

The final step in the biosynthetic pathway of silymarin is not yet fully elucidated. Starting point is the amino acid L-phenylalanine which is converted to 4-coumaroyl-CoA which is the essential precursor for flavonoids and monolignols. A flavonoid (taxifolin) and a monolignol (coniferyl alcohol) then are transformed to silymarin by radical formation (oxidation), coupling, rearrangement and subsequent cyclisation. Several positional isomers (mainly silychristin, silydianin, silybin and isosilybin) occur, the latter two being present as diastereomeric pairs. The distribution of these regioisomers differs in ecotypes/cultivars and genotypes. Whereas a peroxidase (POD) is probably responsible for the radical formation, very little is known about how the plant discriminates between its regioisomers and diastereomers. A potential involvement of dirigent proteins (DIRs), controlling the formation, has been investigated.

In vitro cultures have been established from seedlings of three *Silybum marianum* varieties for further insights into flavonolignan content and composition in suspension cultures and the release of flavonolignans to the outer compartments. Additionally, over a period of two weeks, one culture line was characterised based on various medium parameters, silymarin formation and corresponding enzyme activities. The same culture line was also subjected to an elicitation attempt. However, despite a slight increase in the quantities of the specialised metabolites of the silymarin mixture, the amounts of flavonolignans produced in suspension cells of *Silybum marianum* are very low.

Interestingly, a connection between the individual positional isomer amounts of silymarin extracted from the mature fruit skins and the respective *in vitro* cells could be determined. The regioisomer composition in suspension cells resembled the chemotype of the plant origin. This underlined the assumption of the existence of high genotypic variations and the possible presence of involved regulatory mechanisms. Higher taxifolin concentrations increased the formation of two specific regioisomers in enzyme assays but no further direct mechanism for discrimination could be found. Since enzyme activity and flavonolignans could also be detected in the medium of suspension cells, transport systems might play a role as well.

A class III secretory peroxidase, a laccase of the cupredoxin superfamily and two different dirigent proteins could be identified to be a part of the gene pool of *Silybum marianum*. Unfortunately, the expression of these genes in different *E. coli* and yeast strains turned out to be a big hurdle. Signal peptides and several glycosylation sites, crucial for structure and activity, greatly contribute to this. Optimisation at some points would be necessary in order to yield recombinant proteins.

On the other hand, the enzyme(s) involved in the coupling reaction between taxifolin and coniferyl alcohol could be extracted from *in vitro* cells and the respective medium. The radical-forming protein could successfully be identified as a peroxidase with a molecular weight of about 45 kDa using chromatographical methods. In general, structure and function should be similar to the versatile used horseradish peroxidase (HRP). However, this protein alone could not specifically regulate the formation of individual silymarin regioisomers. Even though dirigent proteins could be identified on the genomic level their presence in enzyme preparations or an actual involvement could never be proven. Their expression and utilisation could be plant organ- and/or time-specific, namely active only during the fruit development and maturation phase.

In summary, in the scope of this thesis, enzymes probably involved in the final step of the silymarin biosynthesis and numerous factors possibly regulating the positional isomer ratios were discussed and highlighted. While issues concerning the expression of recombinant proteins remained to be challenging, reasonable solutions were presented. A continuation of this project seems promising and very interesting for further clarification of this subject.

VIII. Zusammenfassung

Flavonolignan Biosynthese in *Silybum marianum* – Mögliche Regulationsmechanismen und Kandidatengene

Lennart Poppe

Silymarin, ein Flavonolignangemisch aus der Mariendistel (*Silybum marianum*, Asteraceae), wird überwiegend zur unterstützenden Therapie von chronischen Lebererkrankungen sowie zur Prävention toxischer Leberschäden genutzt. Zusätzlich werden positive Effekte wie Tumorhemmung und immunmodulatorische Mechanismen beschrieben. Die Pflanze reichert Silymarin hauptsächlich in der Samenschale und Fruchtwand an. Dies dient zum Schutz des Keimlings gegen Pflanzenfresser und um Bakterien-, Pilz- oder Virusinfektionen einzudämmen oder zu verhindern.

Der letzte Schritt im Biosyntheseweg von Silymarin ist noch nicht vollständig aufgeklärt. Ausgangssubstrat ist die Aminosäure L-Phenylalanin, die zu 4-Cumaroyl-CoA umgewandelt wird, welches eine essentielle Vorstufe für die Synthese von Flavonoiden und Monolignolen ist. Ein Flavonoid (Taxifolin) und ein Monolignol (Coniferylalkohol) reagieren dann über Radikalbildung, Verknüpfung, Umlagerung und anschließendem Ringschluss zu Silymarin. Dabei entstehen mehrere Positionsisomere (hauptsächlich Silychristin, Silydianin, Silybin und Isosilybin), wobei die beiden zuletzt genannten als Diastereomerenpaare vorliegen. Die Verteilung der Regioisomere unterscheidet sich in Ökotypen/Kultursorten und Genotypen. Für die Radikalbildung ist sehr wahrscheinlich eine Peroxidase (POD) verantwortlich. Es ist nur sehr wenig darüber bekannt, wie die Differenzierung zwischen den Regioisomeren und Diastereomeren in der Pflanze abläuft, deshalb wurde eine mögliche Beteiligung von dirigierenden Proteinen (DIRs) untersucht.

Aus Keimlingen dreier *Silybum marianum* Varianten wurden *in-vitro* Kulturen angelegt, um zusätzliche Erkenntnisse über Gehalt und Zusammensetzung der Flavonolignane zu erhalten. Weiterhin wurde die Sekretion der Sekundärstoffe in Zellwand und Medium von Suspensionskulturen untersucht. Eine Kulturlinie wurde über einen zweiwöchigen Zeitraum in Bezug auf verschiedene Mediumskenngrößen, Silymarinbildung und entsprechende Enzymaktivitäten charakterisiert. Mit der gleichen Kulturlinie wurde auch eine Elicitierung durchgeführt. Obwohl eine geringe Zunahme an Silymarinkomponenten beobachtet werden

konnte, sind die in Suspensionszellen von *Silybum marianum* produzierten Mengen an Flavonolignanen leider sehr gering.

Bemerkenswert dabei war ein Zusammenhang zwischen den einzelnen Positionsisomeren des Silymarins, extrahiert aus den reifen Fruchtschalen, und den entsprechenden *in-vitro* Kulturen. Die Zusammensetzung der Regioisomere in den Suspensionszellen hat Ähnlichkeit mit dem Chemotyp der Pflanzenherkunft. Dies untermauert die Annahme von genotypischen Variationen und eine mögliche Beteiligung regulatorischer Mechanismen. Erhöhte Taxifolinkonzentrationen in Enzymassays steigerten zwar die Bildung von zwei spezifischen Regioisomeren, weitere direkte Wege der Differenzierung konnten jedoch nicht gefunden werden. Da auch das Medium der Suspensionszellen Enzymaktivität und Flavonolignane aufwies, spielen vermutlich auch Transportsysteme eine Rolle.

Durch molekularbiologische Methoden konnten eine sekretorische Peroxidase (Klasse III), eine Laccase der Cupredoxin-Familie und zwei dirigierende Proteine im Genpool von *Silybum marianum* identifiziert werden. Die Expression dieser Gene in verschiedenen *E. coli* und Hefestämmen erwies sich leider als sehr schwierig. Diese Problematik wurde verstärkt durch die Anwesenheit von Signalpeptiden und mehreren Glykosylierungsstellen, welche wichtig für Aufbau und Funktion der Proteine sind. Für die Gewinnung rekombinanter Proteine wäre eine weitere Optimierung notwendig.

Die an der Kopplungsreaktion zwischen Taxifolin und Coniferylalkohol beteiligen Enzyme konnten auch aus *in-vitro* Zellen und dem Medium gewonnen werden. Das verantwortliche Enzym konnte durch chromatographische Trennverfahren erfolgreich als Peroxidase mit einem Molekulargewicht von etwa 45 kDa bestimmt werden. Im Allgemeinen sollte sie in Struktur und Funktion der Meerrettich-Peroxidase (HRP) ähneln. Allerdings konnte dieses Protein allein nicht die Bildung einzelner Regioisomere des Silymarins gezielt steuern. Obwohl dirigierende Proteine auf genomischer Ebene nachgewiesen wurden, konnte ihre Gegenwart in Enzymextrakten oder ihre tatsächliche Beteiligung an der Bildung unterschiedlicher Silymarinkomponenten nicht belegt werden. Sie könnten organspezifisch oder zu verschiedenen Entwicklungstadien der Pflanze exprimiert werden. Eine Beteiligung nur während der Fruchtentwicklung und Reifungsphase wäre denkbar.

Zusammenfassend lässt sich sagen, dass im Rahmen dieser Arbeit Enzyme untersucht wurden, die vermutlich den letzten Schritt der Silymarin-Biosynthese katalysieren. Zahlreiche Faktoren wurden angesprochen, die möglicherweise die Zusammensetzung der Positionsisomere

regulieren. Während die Expression der rekombinanten Proteine eine Herausforderung bleibt, wurden weitere Lösungsvorschläge vorgestellt. Eine Fortsetzung dieses Projektes scheint vielversprechend und hinsichtlich einer weiteren Aufklärung dieses Themas sehr interessant.

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