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Faculty of Science

Structure-function analysis of selected hop (*Humulus lupulus* L.) regulatory factors

Ph.D. Thesis

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Annotation

This work concentrated on isolation of novel hop transcription factors from bHLH, bZIP, MYB, and WRKY families involved in the regulation of lupulin flavonoid pathways, followed by their structural and functional analysis. Structural analyses included bioinformatic approaches to elucidate gene organization, domain structure of the putative protein products, and potential post-translational modifications. I performed site-directed mutagenesis to disclose the role of phosphorylation sites in HlbZIP1A stability. Further, this work determined protein-DNA interactions for obtained TFs, giving support to the binding of MYB-bHLH-WDR complexes to the promoter of *chalcone synthase* H1, a key enzyme of the lupulin flavonoid pathways. Employing bioinformatic approaches, quantitative RT-PCR and transient co-expression, I pointed out *chalcone synthase* H1 as a regulatory crossroads in the metabolic (flavonoid) responses during hop stunt viroid pathogenesis.

KEY WORDS: trancription factors, *Humulus lupulus*, flavonoid, hop stunt viroid

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Mgr. Zoltán Füssy

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Last but not least, I am grateful to my partner, family, and friends for their support.

List of papers and author's contributions

The thesis is based on the following papers (listed chronologically):

I. Matoušek J, Kocábek T, Patzak J, Stehlík J, **Füssy Z**, Krofta K, Heyerick A, Roldán-Ruiz I, Maloukh L , De Keukeleire D. Cloning and molecular analysis of *HlbZip1* and *HlbZip2* transcription factors putatively involved in the regulation of the lupulin metabolome in hops (*Humulus lupulus* L.). J Agric Food Chem 2010;58:902-12.

Zoltán Füssy participated in TF sequence cloning and carried out preliminary *HlbZIP1* and *-2* sequence variability determinations.

II. Matoušek J, Kocábek T, Patzak J, **Füssy Z**, Procházková J, Heyerick A. Combinatorial analysis of lupulin gland transcription factors from R2R3Myb, bHLH and WDR families indicates a complex regulation of *chs*_H1 genes essential for prenylflavonoid biosynthesis in hop (*Humulus lupulus* L.). BMC Plant Biol 2012;12:27.

Zoltán Füssy prepared the cDNA library.

III. Matoušek J, Stehlík J, Procházková J, Orctová L, Wullenweber J, **Füssy Z**, Kováčik J, Duraisamy GS, Ziegler A, Schubert J, Steger G. Biological and molecular analysis of the pathogenic variant C3 of potato spindle tuber viroid (PSTVd) evolved during adaptation to chamomile (*Matricaria chamomilla*). Biol Chem 2012;393:605-15.

Zoltán Füssy prepared and purified healthy and PSTVd-infected tomato small RNA species for the deep-sequencing.

IV. **Füssy Z**, Patzak J, Stehlík J, Matoušek J. Imbalance in expression of hop (*Humulus lupulus*) *chalcone synthase* H1 and its regulators during hop stunt viroid pathogenesis. J Plant Physiol 2013; http://dx.doi.org/10.1016/j.jplph.2012.12.006.

Zoltán Füssy participated in the experimental design and metabolite profiling, prepared the HSVd constructs, performed the bioinformatic and statistical analyses, and carried out the transient expression in *Nicotiana benthamiana* and *Galinsoga ciliata*, including part of the transcript quantifications.

Contents

1. PREFACE

For the Czechs, beer is a part of their culture; a social bond between people; a national gold. At the same time, beer contains human health-beneficial compounds, imparted into this drink by the main aromatic ingredient in the process of brewing – hop cones. In the last decades, a branch of the hop research focused on characterising the pathways of secondary metabolites contained in cones and preparing future's hop varieties with enhanced metabolite production via both traditional breeding and genetic engineering.

Plant secondary pathways are regulated in a complex manner to produce a plethora of metabolites in response to developmental, physiological, and pathogen-related signals. Besides determining biological effects of hop secondary metabolites, there is also demand on knowledge of their production mechanisms and regulation of producing enzymes. This dissertation summarises the current knowledge on flavonoid pathways and proposes future perspectives of hop research.

2. INTRODUCTION / THEORETICAL BACKGROUND

2.1. Biology and uses of hops

Hop (*Humulus*) is a genus of herbaceous plants belonging to the group *Cannabaceae*, which includes also genera *Cannabis* (hemp) and *Celtis* (hackberries). Twining shoots of *H. lupulus* typically reach the height of 2 to 15 metres and die back to cold-hardy rhizomes in autumn. Its bines climb by growing clockwise around anything within reach, aided by downward-pointing bristles developed along supporting strings.

The genus *Humulus* includes three species: annual *H. japonicus* (syn. *H. scandens*), and perennials *H. yunnanensis* and *H. lupulus*. Based on morphological characteristics (such as number of lobes on the leaves and hairs on the bine) and geographical distribution, there are five varieties of the species *H. lupulus*: var. *lupulus* from Europe and Western Asia; var. *cordifolius* from Eastern Asia; var. *lupuloides* (syn. *H. americanus*), var. *neomexicanus*, and var. *pubescens* from East, West and midwest North America, respectively (Small 1978). North American and Japanese wild hops resemble each other morphologically and genetically, suggesting a close relationship, while they differ widely from European hops (Patzak and Matoušek 2011).

H. lupulus is native to temperate regions of the Northern Hemisphere; for use in brewing, however, hop plants are grown in many parts of the world. Hop growing is limited by strict day length and temperature (required for flowering and cone production) to regions around 35° latitudes in both hemispheres. In 2011, the most significant regions of hop production (countries with >1,000 ha) were (with decreasing acreage) Germany, the United States, Czechia, China, Poland, Slovenia, and the United Kingdom. The largest area of production is in Germany (18,228 ha producing 38,110 metric tons), while Czechia with approximately one-quarter acreage specialises in aroma hops production (98.4 % of the total area), mostly noble cultivar Saaz (Barth 2012). Besides beer, hops are also used for flavour in some blended teas and carbonated soft drinks. To a much lower extent, hops are also grown as seasonal delicacies, especially young shoots and leaves, as ornamental plants, and for their medicinal and sleep-inducing effects. Hops are also used in herbal medicine as a treatment for anxiety, restlessness, and insomnia.

The species are dioecious, although fertile monoecious individuals appear occasionally. Female plants produce cone-like inflorescences with large number of highly metabolically active glandular trichomes on the inner side of bracteoles, bracts, and strig, while masculine flowers are arranged in panicles and develop fewer glands. Despite structural similarities of the cones of individual *Humulus* species, only *H. lupulus* develops high quantity of lupulin glands (Neve 1991). Small number of lupulin glands also develops on vegetative organs, such as leaves (Fig. 1B) (Wang et al. 2008).

H. lupulus var. *lupulus* found their way into vast European areas after the last glaciations (Murakami et al. 2006), and subsequently, owing to diverse desirable characteristics imparted from cones to beer, gave rise to most cultivars of hops. Varieties "Brewers Gold" and "Northern Brewer" were the first hop cultivars, bred around 1907 by Prof. E.F. Salmon (Lemmens 1998). To date, there is commercial use of about 80 varieties in the world, and many more varieties are in development. Flavour qualities, such as hoppy aroma (Goiris et al. 2002) and bitterness to balance the sweetness of malt (Haseleu et al. 2010), bacteriostatic properties favouring the

brewer's yeast over less desirable microorganisms, and foam-stabilising effects (Hughes 2000), all confer to the complex features of beer. Only female plants are grown and used extensively in brewery; male hops are kept separated from female plants to prevent pollination, because seeds developed in cones may cause undesirable off-flavour.

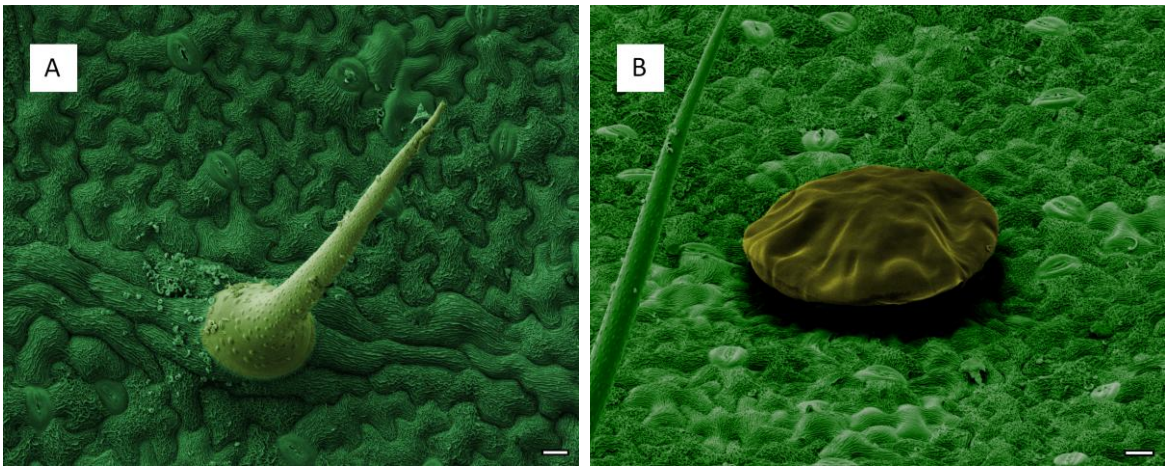

Fig. 1: Colourised electron microscan of hop leaves, lower sides. A hair trichome (A) and a lupulin gland (peltate trichome) (B) on the surface are surrounded by epidermal cells and stomata. The bars represent 10 µm. Courtesy of Tomáš Kocábek.

Hop-specific metabolites produced in cones include the terpenophenolic resin, comprised of bitter acids and prenylflavonoids, and essential oils, a complex mixture of compounds produced in the latter stages of the hop cone ripening, mostly mono- and sesquiterpenes (50-80 % of the whole oil compounds; myrcene, farnesene, humulene, caryophyllene, and selinenes), terpene alcohols (linalool and geraniol), esters, and carbonyl components (Lemmens 1998; Roberts et al. 2004). Terpenophenolics and essential oils of hops accumulate in two types of glandular trichomes: large peltate cup-like glands composed of 100-200 cells (Fig. 1B); and much smaller bulbous glands, containing eight cells at maturity (Sugiyama et al. 2006). Coinciding with the growth and development of the peltate trichomes, hop secondary metabolites accumulate within an intrawall cavity (De Keukeleire et al. 2003; 2007). The resulting mature gland is a biconal structure, filled with secretions (Oliveira and Pais 1990; Kim and Mahlberg 2000; Sugiyama et al. 2006). In contrast to terpenophenolic-rich glandular trichomes, the green tissues of the bracts and bracteoles contain a diverse set of polyphenolic constituents, including catechins, phenolic acids, flavonol (quercetin and kaempferol) glycosides, and proanthocyanidins (PAs) (Kavalier et al. 2011). The terpenophenolics determined by De Keukeleire et al. (2003) are present not only in cones, but also in male inflorescences, albeit in low concentrations (Likens et al. 1978; Haunold et al. 1993; De Keukeleire et al. 2003). Also, leaves of fully grown hops contain detectable levels of hop acids (De Keukeleire et al. 2003), terpenes (Wang et al. 2008), and flavonols (Sägesser and Deinzer 1996).

2.2. Medicinal hops and metabolic engineering

Besides agronomy and folk medicine, hops were proven valuable also in biotechnology. It follows from biological activities attributed to several identified beer constituents, as

summarised by Gerhäuser (2005). Cancer-preventive activities of hops include modulation of carcinogen metabolism assayed *in vitro* as cytochrome P450 1A (Cyp1A) inhibition and NAD(P)H:quinone reductase (QR) activation (Henderson et al. 2000; Gerhäuser et al. 2002a). Induction of apoptosis, cell differentiation (Gerhäuser et al. 2002b), and reduced angiogenesis (Bertl et al. 2004) also contribute to anti-cancerogenic effects of beer compounds, based on *in vivo* tests. Further, anti-inflammatory activities were reported (Gerhäuser et al. 2002a), assayed as the rate of inducible nitric oxide synthase (iNOS) inhibition and cyclooxygenase 1 (Cox-1) induction as a relevant *in vitro* marker (Gerhäuser et al. 2003). Catechins/flavanols were identified as good Cox-1 inhibitors, but only weakly interfering with Cyp1A and QR involved in carcinogen metabolism, both indicators of elevated detoxification. In contrast, beer flavanones were identified as effective inhibitors of Cyp1A and inductors of QR. Some compounds can affect estrogen signalling (Milligan et al. 1999; Gerhäuser et al. 2002b). Finally, antioxidant activities, or reactive species quenching (Ghiselli et al. 2000; Miranda et al. 2000), were reported. Radical-scavenging activity was shown for benzoic and cinnamic acid derivatives, catechins, dimeric and trimeric PAs, and flavones (Gerhäuser 2005). The results obtained suggest that the combination of beer compounds enhances their biological effect because of different respective activity profiles (Gerhäuser 2005). Importantly, Gerhäuser et al. (2002a) pointed out xanthohumol (XN) as a compound demonstrating activity in most bioactivity screening assays.

Lupulin glands produce predominantly flavonoids of the chalcone type, with XN being the most abundant (82–89%) of prenylated flavonoids in European hop varieties (Stevens et al. 1997). It is because of the lack of isomerase activity, necessary for the efficient conversion of chalcones to flavanones (see Chapter 2.3.1), although chalcone isomerase (CHI) transcripts were detected in lupulin glands (Nagel et al. 2008). Still, the total content of prenylflavonoids in hops is low; in most varieties the content of XN and desmethylxanthohumol (DMX) does not exceed 1 % (w/w) of fully grown cone weight (De Keukeleire et al. 2003). Bitter acids accumulate to highest contents in lupulin; particular hop varieties contain up to 19 % (w/w) of α-acids (super-α-hops). The biosynthesis of bitter acids and prenylflavonoids involves common building blocks including malonyl-CoA and dimethylallyl pyrophosphate (DMAPP) (Zuurbier et al. 1998; Okada et al. 2001); hence, the respective pathways may be competitive. Importantly, each hop variety exhibits individual accumulation rate of these metabolites (De Keukeleire et al. 2003). Specific relative levels of major chemicals are rather genetically determined (De Keukeleire et al. 2003) and reasonably constant within a variety, regardless of environment (De Cooman et al. 1998; Heyerick et al. 2002). Therefore, traits may be selected and combined to produce suitable cultivars (e.g. Nesvadba et al. 2011).

Largely independent breeding programmes around the world aspire to develop new and improved cultivars with advantageous traits, such as higher yield, disease resistance, and resin content and chemistry. One direction of hop breeding leads through marker-assisted selection of germplasms, following searches for markers genetically linked with certain traits, e.g. pest and fungus resistance (Weihrauch et al. 2009; Majer et al. 2012), or secondary metabolite content (Patzak 2001; Patzak and Matoušek 2011). Another direction may involve transformation of hop material with either heterologous or homologous sequences, which proved promising for secondary metabolome engineering.

Plant-breeding programs have led to the development of hop varieties that combine unusually high concentrations of α-acids (super-α-hops) with greatly improved resistance against the most relevant diseases (De Keukeleire et al. 2003). Conventional breeding methods however deal with

many constraints, namely long duration, sexual incompatibility of produced lines, and appearance of quality/chemical changes and unintended characteristics. Selection-based breeding is also dependent on the effective utilisation of genetic diversity, which is, unfortunately, limited among the major world's hop cultivars (Jakse et al. 2001). Despite a long cultivation history, current hop cultivars are derived from a narrow genetic source (Murakami et al. 2006). Experimental approaches of material improvement include the enhancement of the genetic potential of traditional cultivars, for instance, via hybridizing of European cultivars with North American wild hops (Stajner et al. 2008).

Genetic transformation has become an established technology to produce single, accurate, and rapid modifications of utilized cultivars, which may be preferred to the breeding of new cultivars (Moir 2000). Very few reports on hop transformation have been published, partly because plant regeneration systems are highly genotype-dependent (Batista et al. 2008). Transgenic hop plants 'Osvald's clone 72' (Oriniakova and Matoušek 1996; Okada et al. 2003), 'Tettnanger' (Horlemann et al. 2003), and 'Aurora' (Škof and Luthar 2005) containing *gus* reporter and/or *nptII* selection genes were produced with the use of *Agrobacterium tumefaciens*-mediated system. After that, transformation of hop 'Tettnanger' with a grapevine stilbene synthase (STS) gene (Schwekendiek et al. 2007) and two genes encoding transcription factors (TFs) (Gatica-Arias et al. 2012b; 2012a) was reported, using the system developed by Horlemann et al. (2003). Several TF-transgenic lines have been produced by Matoušek and colleagues (unpublished). In addition, biolistic transformation has proven to be a powerful and versatile technique with a successful application to hops (Batista et al. 2008).

For the purpose of both marker-assisted selection and genetic modification, the understanding of the variation in the genes of the biosynthetic pathways is instrumental. There is a lack of information on hop flavonoid pathway network, but parallels may be deduced from other models. The content and quality of secondary metabolites in the lupulin glands certainly undergoes complex regulation; however, the similarity in regulation of plant models shows that comparisons can be made across very divergent plant species in looking for common mechanisms of regulation (Durbin 2003).

2.3. Flavonoid pathways and their regulation in plants

Phenylpropanoids are synthesised during the normal development of plant tissues or in response to stress, often compartmented to strategically important sites where they play a signalling role and/or a direct role in defence (Wink 1997; Zhao and Dixon 2010): Their presence not only accompanies plant stress responses upon variation of light or mineral treatment, but also mediate resistance towards pathogens (La Camera et al. 2004). They promote invasion of new habitats (Bais et al. 2003) and provide the biochemical aspects for successful reproduction (Dudareva et al. 2004). Phenylpropanoid-based polymers, like lignin, suberin, or condensed tannins, considerably contribute to the stability and robustness of plants towards wounding or drought. Despite their classification as secondary metabolites, phenylpropanoids turn out to be equivalently relevant to plant survival as photosynthesis or the citrate cycle (Vogt 2010).

Flavonoids represent one of the largest classes of phenylpropanoids with approximately 10,000 structurally different members including flavonols, flavanols, stilbenes, and anthocyanins

(Tahara 2007) (see Chapter 2.3.1.), synthesized from phenylpropanoid and acetate-derived precursors into a common C6-C3-C6 scaffold (Fig. 2), except for the aurones and stilbenes. Flavonoids are classified based upon the oxidation level of the central C heterocycle, the substitution status of the cycles A and B, and additional modifications such as glycosylation, acylation, and polymerisation.

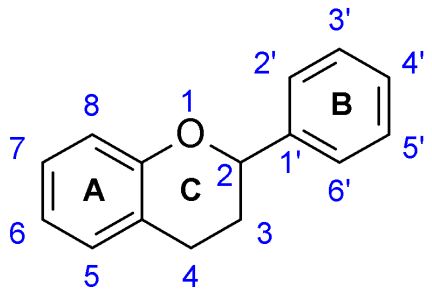

Fig. 2: A general structure and numbering system for the flavonoids.

The largest class of flavonoids, flavanols exist as monomers, dimers, and polymers of eight monomers per molecule on average, proanthocyanidins (PAs) (Abrahams et al. 2003). The PAs accumulate in the seed coat and are thought to function in controlling seed longevity and dormancy, and in protection from pathogens (Debeaujon et al. 2003). The concentration and nature of PAs in leaves is also important to deter herbivores (Aron and Kennedy 2008).

Another group of at least 400 flavonoid compounds are the anthocyanins, the major red, purple and blue pigments, depending on pH, co-pigmentation, metal cations, and covalent modifications (Grotewold 2006; Tanaka et al. 2009). Together with aurones and terpenes, anthocyanins colour flowers, fruits, and pollen in order to attract pollinators and seed dispersers (Winkel-Shirley 2001; Lepiniec et al. 2006).

The functions of developmental regulation and signalling to stress agents are largely restricted to flavonols (Pollastri and Tattini 2011), notably quercetin and kaempferol. The flavonol pathway has remained intact for millions of years as it yields metabolites with varied functional roles to protect plants from diverse unpredictable injuries (Izhaki 2002). In development, flavonoids serve as signalling molecules via modulation of auxin retention or transport (Murphy et al. 2000; Brown et al. 2001; Buer and Muday 2004; Peer et al. 2004). In addition, flavonoid aglycones have the capacity to regulate the activity of different protein kinases in animals (Formica and Regelson 1995).

Flavonoids are synthesised in the cytosol and then mainly transported to the vacuole for storage. They can also be found in cell walls, the nucleus, chloroplasts, and even in the extracellular space, depending on the plant species, the tissue, or the stage of development (Hutzler et al. 1998; Kuras et al. 1999; Feucht et al. 2004), which may mirror their biological functions.

Flavones, flavonols, and anthocyanins accumulate as stress protectives in their glycosylated form after an inductive light treatment in the vacuoles of epidermal cells, waxes, trichomes (Graham 1998; Weisshaar and Jenkins 1998; Veit and Pauli 1999), and also in roots after exposure to light (Hemm et al. 2004). Rather than absorbing UV-B light (Ormrod et al. 1995; Olsson et al. 1998; Solovchenko and Schmitz-Eiberger 2003), flavonoids reduce reactive oxygen species (ROS) formed as a consequence of UV-B penetration in ROS-generating cells (Pollastri and Tattini 2011). Likewise, anthocyanins accumulate upon cold stress in seedlings of maize and *Arabidopsis thaliana* (Christie et al. 1994; Leyva et al. 1995) and berries of grape (Mori et al. 2005).

In plant-microorganism interactions, many flavonoids exhibit antimicrobial and pesticide properties, by acting as a repellent, and inhibiting growth and development of pests (Dixon et al. 2002; Chong et al. 2009). Stilbenes have been shown *in vitro* to have antifungal activity and

overexpression of STS in different species led in most cases to an increased disease resistance against pathogenic fungi (Thomzik et al. 1997; Leckband and Lorz 1998; Hipskind and Paiva 2000; Schwekendiek et al. 2007). (Iso)Flavonoids may, however, promote positive signals of rhizobial symbiosis (Bhattacharya et al. 2010).

2.3.1. The flavonoid biochemical pathway

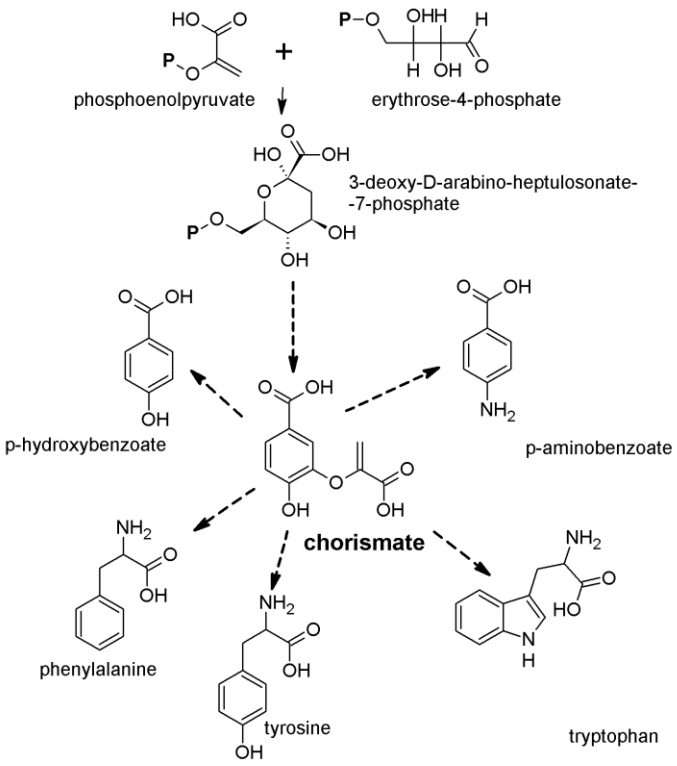

Fig. 3: The main compounds of the plastid shikimate pathway.

The plant shikimate pathway is the source of flavonoid and other phenylpropanoid precursors. Its plastidial location and complex regulation have been investigated for decades (Schmid and Amrhein 1995) (Fig. 3). Regulation of the pathway is accomplished at multiple levels: transcriptional control was shown for 3-deoxy-D-arabinose-heptulosonate synthase (Herrmann and Weaver 1999), while arogenate and prephenate dehydratase are inhibited by phenylalanine, one of the end-products of the pathway (Yamada et al. 2008). The individual shikimate pathway genes respond to changes in light or nutrient content in a tightly regulated manner and more complexly than the transcriptional responses of the genes of phenylpropanoid and flavonoid pathway, encoding phenylalanine ammonia lyase (PAL) or chalcone synthase (CHS) (Lillo et al. 2008). The regulation of this pathway will not be discussed in detail in this work.

The general phenylpropanoid pathway directs the carbon flow from the shikimate pathway to result in coumaroyl-CoA, the substrate for all subsequent phenylpropanoid branches and resulting metabolites (Fig. 4). The initial three steps of the pathway are catalyzed by PAL, cinnamate 4-hydroxylase (C4H), and 4-coumaroyl CoA-Ligase (4-CL). Tyrosine ammonia lyase (TAL) provides an efficient shortcut by circumventing the problematic cytochrome P450 hydroxylase C4H. PAL and TAL catalyze the non-oxidative deamination of phenylalanine and tyrosine to yield trans-cinnamate and 4-coumarate, respectively. Several copies of the PAL-genes are found in all plant species, comprising four genes in *Arabidopsis*, five in poplar and nine in rice (according to UniprotKB database) (UniProt Consortium 2012). TAL genes have been identified in strawberry only, while monocots take advantage of PAL/TAL that is able to utilize both phenylalanine and tyrosine as substrate (Rösler et al. 1997). 4-hydroxylation of *trans*-cinnamate to 4-coumarate is encoded by a single gene encoding *Arabidopsis* C4H/CYP73A5. The subsequent step, encoded by the small gene family of four 4-CLs in *Arabidopsis* (five members in rice),

channels the resulting aromatic CoA-esters to different biosynthetic pathways. 4-coumaroyl CoA probably represents an important branchpoint within the general phenylpropanoid biosynthesis in plants, as it is the direct precursor for flavonoid and lignin branches.

The structural diversity of flavonoids is afterwards achieved by action of enzymes and enzyme complexes that bring about regio-specific condensation, cyclisation, aromatisation, hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation reactions on many positions along their backbone molecule (Fig. 2). The enzymes that catalyse these reactions often belong to large gene families, which can be recognized in expressed sequence tag (EST) and genome datasets through family-specific conserved sequence motifs (Naoumkina et al. 2010).

CHS is the key enzyme synthesizing naringenin chalcone, the flavonoid backbone, by condensation of 4-coumaroyl-CoA and malonyl-CoA moieties. CHS enzymes belong to the polyketide synthase type III superfamily (PKSIII), share high homology with enzymes found in several bacterial genomes (Moore et al. 2002; Austin and Noel 2003), and are distant relatives to the ß-ketoacyl synthases and thiolase enzymes of fatty acid metabolism (Schröder 1997). PKSIII enzymes differ by substrate specificities and the number of condensation repetitions, while the reaction mechanisms are essentially alike. In CHS as an example, the architecture of the active site determines how substrate preference is determined (by residues in the coumaroyl binding pocket), and the cyclisation pocket limits the number of acetate additions and controls the stereochemistry of the endproduct (Ferrer et al. 1999). Site-directed mutagenesis of CHS and structural data support these models (Jez et al. 2000; Suh et al. 2000; Lukacin et al. 2001). STS performs the reaction similarly to CHS, but folds the polyketide intermediate in a different way to release CO_2 and yield a stilbene backbone (Fig. 4) (Tropf et al. 1995).

Chalcone isomerases (CHIs) catalyse the stereospecific isomerisation of chalcones into their corresponding flavanones (Shimada et al. 2003) via an intramolecular lyase reaction resulting in the formation of ring C (Jez and Noel 2002), though this reaction occurs spontaneously in a lower rate. *A. thaliana* and petunia contain 2 and 3 genes encoding CHI, respectively (UniProt Consortium 2012).

Hydroxylation of flavonoid skeletons is important in the biosynthesis of complex flavonoids, accomplished by cytochrome P450 hydroxylases (flavonoid 3' hydroxylase F3'H, flavonoid 3'5' hydroxylase F3'5'H, flavone synthase II FSII, isoflavone synthase IFS, and isoflavone 2' hydroxylase I2'H) and oxoglutarate-dependent dioxygenases (flavanone 3-dioxygenase F3H, flavonol synthase FLS, flavone synthase I FSI, and leucoanthocyanidin dioxygenase LDOX). Addition of hydroxyl groups determines anthocyanin colour, enhances flavonoid stability and solubility, and increases metal-binding (and thus ROS scavenging) and UV-absorbing properties (reviewed in Heim et al. 2002). F3H, F3'H, FLS, and LDOX are encoded by a single gene in *Arabidopsis*, maize, and petunia. Flavonoid skeletons are also modified by several NADPH-dependent reductases (anthocyanidin reductase ANR, dihydroflavonol 4-reductase DFR, leucoanthocyanidin reductase LAR, and isoflavone reductase IFR).

Most acyl-, glycosyl-, and methyltransferases identified up to now exhibit overlapping or promiscuous substrate specificities *in vitro* (Bowles et al. 2006; Vogt 2010). Sugar transfer to flavonoid skeleton lessens the toxic effects of aglycones to host plant. However, flavonoid aglycones (often those involved in defence responses) may possess biological function, and their

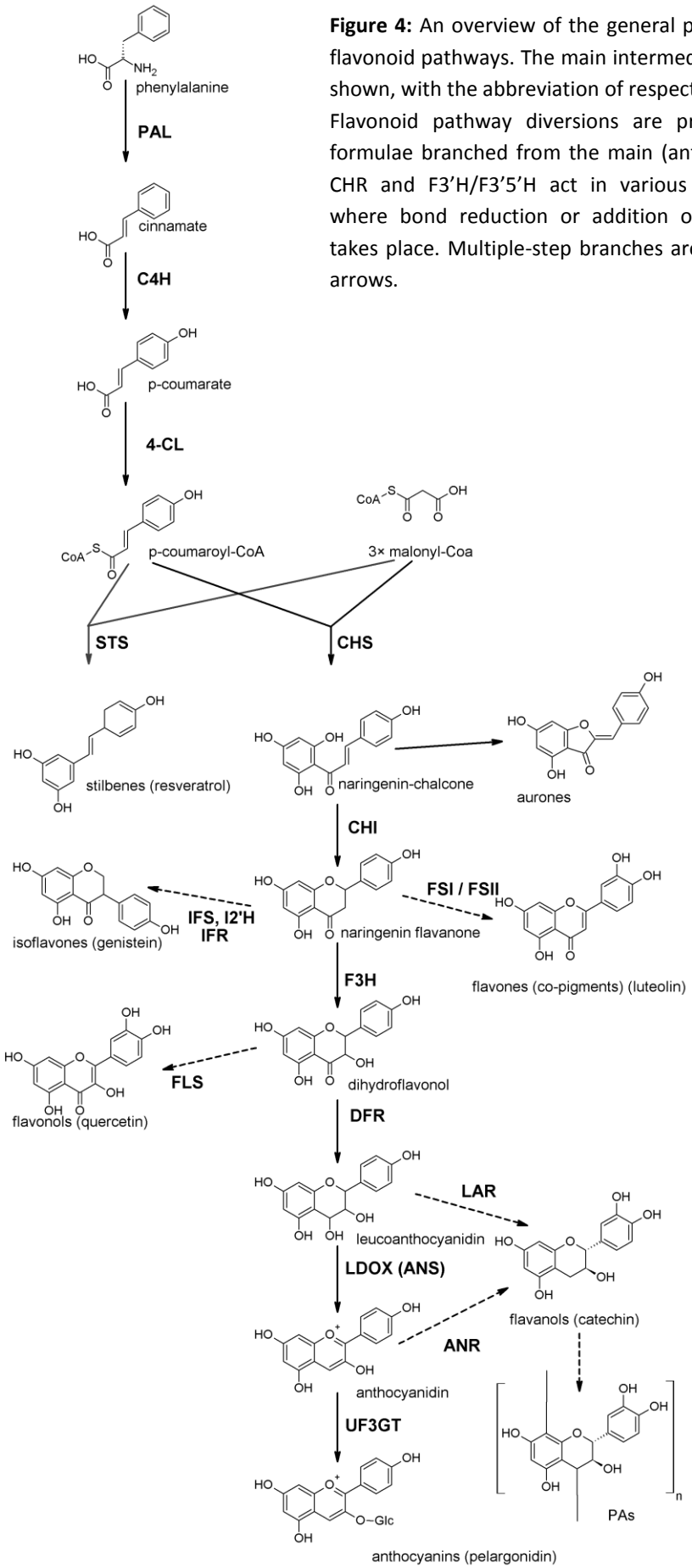

Figure 4: An overview of the general phenylpropanoid and flavonoid pathways. The main intermediate compounds are shown, with the abbreviation of respective enzymatic steps. Flavonoid pathway diversions are presented as named formulae branched from the main (anthocyanin) pathway. CHR and F3'H/F3'5'H act in various steps, respectively, where bond reduction or addition of hydroxyl moieties takes place. Multiple-step branches are marked by dashed arrows.

sugar moiety is removed by highly regulated deglycosylation enzymes once flavonoid glycosides reach their final destination to exert their function (Beckman 2000). Enzymatic *O*-methylation of flavonoids is catalysed by *O*-methyltransferases (OMTs), which transfer a methyl group from *S*-adenosyl-L-methionine to a hydroxyl moiety of the acceptor molecule. The prenylation of flavonoids enhances their antibacterial, antifungal and other biological activities by increasing their lipophilicity and membrane permeability (Sohn et al. 2004).

At least a part – and possibly the entire – flavonoid pathway is likely associated with the cytoplasmic surface of endoplasmic reticulum as a multi-enzyme complex (Winkel 2004). PAL and a flavonoid glucosyltransferase, the first and the last enzyme of the pathway, respectively, are located in the lumen of the endoplasmic reticulum. C4H is membrane-embedded, while other enzyme activities appear to be weakly associated with the cytoplasmic face of endoplasmic reticulum membranes (Hrazdina and Wagner 1985). The channeling of metabolic intermediates through multi-enzyme complexes without their release into general metabolic pools can provide not only the most effective utilisation of (unstable) intermediates in biosynthetic reactions, but also for controlling flux among the multiple pathway branches, often concurrently functional in the same cell. F3'H is proposed to be the membrane anchor for the flavonoid pathway, since CHS and CHI do not co-localise in *f3'h* mutant compared to the wild type (Saslowsky and Winkel-Shirley 2001). Following synthesis, flavonoids are transported to the vacuolar compartment by a combination of transporters (Goodman et al. 2004) and vesicles (Lin et al. 2003) and may be decompartmented upon appropriate signals (Beckman 2000).

2.3.2. Flavonoid pathway regulation

Coordinate transcriptional control of biosynthetic genes emerges as a major mechanism determining the flavonoid metabolic profiles in plant cells. This regulation of biosynthetic pathways is achieved by specific TFs, sequence-specific DNA-binding proteins that interact with the promoter regions of target genes, and modulate the rate of mRNA synthesis. These proteins regulate gene transcription depending on cellular identity, tissue type, in response to internal signals, for example plant hormones, and/or to external signals such as microbial elicitors or UV light, usually in a complex and interconnected manner (Vom Endt et al. 2002). The picture of regulation suggests interplay of various types of regulatory genes acting to regulate not only the flavonoid pathway but sometimes also other seemingly unrelated pathways (Durbin 2003).

Genes of the flavonoid pathway are often referred to as ''early'' or ''late'' genes depending on how the genes are regulated. In *Arabidopsis*, *Antirrhinum*, and petunia, early biosynthesis genes common to different flavonoid branches such as *CHS*, *CHI*, *F3H*, and *F3'H* are induced prior to late biosynthesis genes such as *DFR*, *LDOX*, *ANR*, and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UF3GT) (Pelletier and Shirley 1996). Regulators belonging to different transcription factor families, including R2R3-MYB, bHLH, WD40, WRKY, BZIP, and MADS-box factors, are involved in the transcriptional control of flavonoid biosynthesis genes. Many of these have been identified by genetic studies in *Arabidopsis*, maize, petunia, *Antirrhinum*, and other plants. For clarity reasons, this work concentrates mainly on *Arabidopsis* flavonoid regulation.

MYB

Proteins containing R2R3-MYB domains constitute an important superfamily of transcription factors characteristic by a DNA-binding MYB domain, with a C-terminal region regulating gene expression. MYB TFs bind to different pathway-specific MYB-binding sites (BS), some of them showing certain flexibility (Romero et al. 1998; Jin and Martin 1999). Yanhui et al. (2006) have identified 126 R2R3-MYB genes classified into 25 subgroups in the *Arabidopsis* genome (Kranz et al. 1998; Stracke et al. 2001), where they control one or multiple stimuli: metabolism, cell morphogenesis and cell cycle, development, stress signaling, and disease-resistance (reviewed in Du et al. 2009). Numerous pathway-specific regulators have been identified, as reviewed by Dubos et al. (2010). Most of MYB TFs involved in flavonoid pathway belong to subgroups (SG) 3-7. In *Arabidopsis* for instance, AtMYB11/PFG2 (Production of Flavonol Glycosides2), AtMYB12/PFG1 and AtMYB111/PFG3 (SG7) control flavonol biosynthesis in all tissues (Stracke et al. 2007), AtMYB75/PAP1 (Production of Anthocyanin Pigment1), AtMYB90/PAP2, AtMYB113 and AtMYB114 (SG6) control anthocyanin biosynthesis in vegetative tissues (Gonzalez et al. 2008), and AtMYB123/TT2 (Transparent Testa2) (SG5) control the biosynthesis of PAs in the seed coat of *Arabidopsis* (Baudry et al. 2004). The regulators of the PA and anthocyanin pathways display the $[D/E]Lx_2[R/K]x_3Lx_6Lx_3R$ motif necessary for interaction with bHLH transcription factors in their R3 repeat (Grotewold et al. 2000; Zimmermann et al. 2004), while MYB TFs controlling flavonol biosynthesis share the SG7 $[K/R][R/x][R/K]xGRT[S/x][R/G]x_2[M/x]K$ and the SG7-2 $[W/x][L/x]LS$ motifs in their C-terminal end (Stracke et al. 2001; Czemmel et al. 2009). However, not all flavonoid pathway regulators meet this classification perfectly (Stushnoff et al. 2010).

bHLH

The bHLH TF family has also been associated with a range of functions in plants, frequently in conjunction with MYBs (Ramsay and Glover 2005). The bHLH TFs are named thus regarding their 16-aa basic (b) domain containing basic amino acids followed by two regions of hydrophobic α-Helices separated by a variable stabilising Loop (HLH). The basic domain is essential for DNA binding, recognising the G-box consensus BS (Li et al. 2006) sometimes concurrently with 3' flanking sequences (Shimizu et al. 1997); bHLH factors lacking this domain act as repressors (Toledo-Ortiz et al. 2003). HLH helices are involved in homo- and heterodimerisation; the loop and the second helix may also be involved in DNA binding. They constitute an ancient class of eukaryotic TFs that are found in fungi, plants, and metazoans. In *Arabidopsis*, there are 162 bHLH TF-encoding genes divided into 12 subgroups (Bailey et al. 2003; Heim et al. 2003), while rice encodes 167 bHLH TFs (Li et al. 2006); they regulate many cellular processes such as development of floral organs, photomorphogenesis, fate of epidermal cells such as trichomes, root hair, and stomata, hormonal response, and metal homeostasis (reviewed in Feller et al. 2011). Roles of TFs AtbHLH42/TT8 (Transparent Testa8), AtbHLH1/GL3 (Glabra3), and AtbHLH2/EGL3 (Enhancer of Glabra3) functionally overlap in anthocyanin production and trichome development of *Arabidopsis* (Bernhardt et al. 2003; Zhang et al. 2003). bHLH factors from the same subgroup share a similar sequence length, the position of the bHLH domain, and specific regions outside this domain. Flavonoid pathway regulators from group IIIf (Heim et al. 2003) share several common features: the N-terminal side (200 aa) is involved in the interaction with MYB transcription factors; the following 200 amino acids

often include an acidic region necessary for interaction with WD40 proteins and/or the RNA Pol II complex; and the bHLH domain itself and the C-terminal region are known to participate in homodimer or heterodimer formation (Ferré-d'Amaré et al. 1993; Payne et al. 2000; Zhang et al. 2003; Pattanaik et al. 2008).

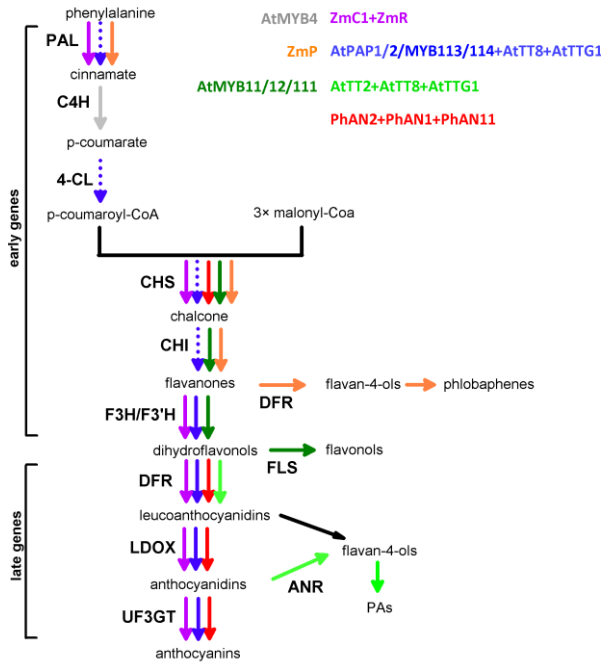

Fig 5. Regulatory factors of early and late genes of flavonoid pathway. Dashed arrows are shown for genes upregulated in plants overexpressing PAP1, unconfirmed by detailed expression studies (Gonzalez el al. 2008).

MYB and bHLH proteins often interact to promote target gene expression (Fig. 5). Examples of functional combinations include AtMYB123/TT2 (Nesi et al. 2001) that interacts with TT8 (Nesi et al. 2000) to activate the PA branch genes *DFR*, *ANR*, and the gene encoding the multidrug and toxic compound extrusion (MATE)-type transporter TT12. Between anthocyanin-related MYBs and bHLHs, interactions were shown for the maize ZmC1 MYB and ZmB/ZmR bHLH TFs, the petunia Anthocyanin2 (AN2) MYB and AN1 and JAF13 bHLHs, and the *Antirrhinum* Ros1, Ros2 and Ve MYBs and the Mut and Del bHLHs (Goff et al. 1992; Goodrich et al. 1992; Mol et al. 1998; Schwinn et al. 2006). The binding characteristics may diverge for TF(s) and target gene combinations. Co-expression of the petunia AN2-JAF13 or *Arabidopsis* TT2-TT8 is necessary to bind and activate *Spinacia oleracea DFR* promoter; however, the JAF13 and TT8 proteins can also individually bind the *SoLDOX* and *AtDFR* promoters (Shimada et al. 2007). Together, these results indicate that the bHLH proteins can bind DNA either alone or as a dimer with MYB, depending on the target promoter.

WDR

Besides the bHLH TFs, R2R3-MYBs often form complexes with a WDR protein, such as Transparent Testa Glabra1 (TTG1). WD40 proteins are characterised by a peptide motif of 44–60 amino acids, typically delimited by the GH dipeptide 11–24 residues from the N-terminus and the WD dipeptide on the C-terminus (Smith et al. 1999). This motif can be tandemly repeated 4–16 times within a given protein forming a higher-order β-propeller structure; a large majority of *Arabidopsis* WDR TFs exhibit 4 or more WD repeats (van Nocker and Ludwig 2003). WDR proteins take part in multitude of cellular processes, including cell division, vesicle formation and trafficking, signal transduction, transcription, and RNA processing (van Nocker and Ludwig 2003). They are not thought to bind DNA, but rather enhance transcription through modifications of the histone proteins and chromatin remodelling (Suganuma et al. 2008; Zhu et al. 2008). In addition, they seem to be a docking platform, as they can interact with several

proteins simultaneously (van Nocker and Ludwig 2003). Only TTG1 was clearly demonstrated to bind the promoter of *AtTTG2* encoding a WRKY TF mainly involved in trichome patterning (Zhao et al. 2008). Their functional versatility is dependent on the available protein partners present in the cells at a given developmental stage (Fig. 6). The fine regulation of flavonoid biosynthesis is hence achieved by combinatorial action(s) of TFs, expressed in a spatially and temporally controlled manner (reviewed in Hichri et al. 2011).

MBW complexes

MYB-bHLH-WDR (MBW) transcription complexes regulate flavonoid biosynthesis as has been clearly demonstrated for *A. thaliana* and petunia complexes TT2-TT8-TTG1 and AN2-AN1-AN11, respectively. The TT2-TT8-TTG1 complex regulates PA accumulation in the seed coat (Debeaujon et al. 2003; Baudry et al. 2004), PAP1-EGL3-TTG1 induces the anthocyanin pathway (Zhang et al. 2003), while the GL1-GL3/EGL3/TT8-TTG1 complex (the bHLH TFs show an overlapping action) controls trichome development (Fig. 6) (Payne et al. 2000; Zhang et al. 2003; Maes et al. 2008). The AN2-AN1-AN11 complex controls the anthocyanin accumulation in petunia (Quattrocchio et al. 1993; deVetten et al. 1997; Spelt et al. 2000), while another MYB, PH4 is recruited by AN1-AN11 to control the vacuolar pH (Quattrocchio et al. 2006). MBW complexes were implicated in anthocyanin pathway regulation also in grapevine, pea, *Lotus japonicus*, and *Pyrus pyrifolia* (Hellens et al. 2010; Matus et al. 2010; Yoshida et al. 2010; Zhang et al. 2011). The flavonol biosynthesis itself, however, is regulated by MYB factors lacking the motif for interaction with bHLH proteins. AtMYB11, AtMYB12, and AtMYB111 activate on their own the early genes *CHS*, *CHI*, *F3H*, and *FLS* expression, but neither *DFR* nor *UFGT* late gene expression (Stracke et al. 2007).

The MBW complex is highly organised and each subunit fulfils a specific function such as recognition of target DNA, activation of expression, or stabilisation of the TF complex. The WDR component stabilises the complex, since it further enhances the *DFR* activation of the TT2-TT8 dyad (Baudry et al. 2004). The subcellular localisation of the complex itself may be determined upon components interaction. For instance, WD40 proteins seem to be translocated into the nucleus upon interaction with a bHLH protein. PFWD and AN11 WDR proteins reside in the cytosol (deVetten et al. 1997; Sompornpailin et al. 2002), while co-expression of MYC-RP bHLH mediates PFWD transport to the nucleus (Sompornpailin et al. 2002). In addition, TTG1 and GL1

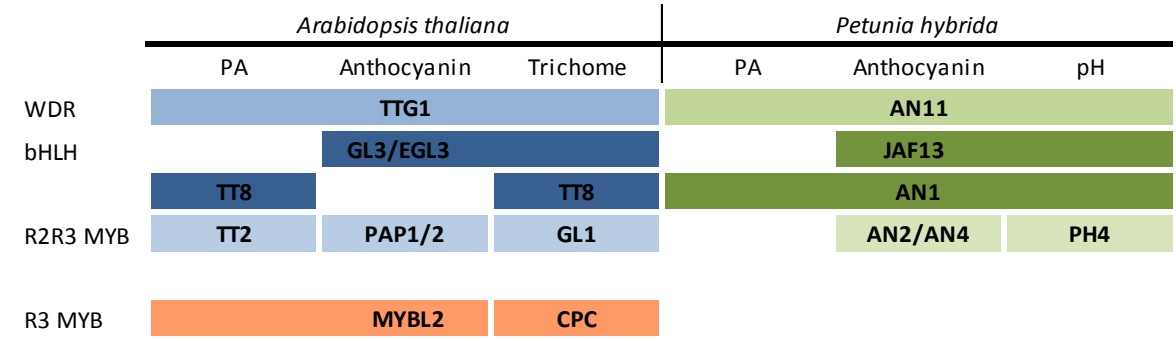

Fig. 6: Regulatory proteins controlling flavonoid accumulation and related signaling in *Arabidopsis* and petunia via MBW complexes. For abbreviations, see text.

TFs are required for the proper subnuclear distribution of GL3 (Zhao et al. 2008). The above mentioned examples suggest that since WD40 and bHLH TFs participate in general and overlapping functions, the target gene specificity seems to be conferred by the MYB component.

Additional regulators

While MYB, bHLH, and WDR proteins play major roles in flavonoid pathway regulation, additional TFs take part in this complex network. A combinatorial interaction towards coordinated expression of flavonoid pathway genes was shown in the work of Hartmann et al. (2005), guided by MYB, bHLH, and bZIP BSs.

Proteins with a basic DNA-binding domain and a leucine zipper dimerisation motif (bZIPs) are present in all eukaryotes analysed to date. *A. thaliana* genome encodes 75 bZIP factors (Jakoby et al. 2002) regulating diverse biological processes such as pathogen defence, light and stress signalling, seed maturation, and flower development. Cooperating with MYB factors, they mediate light-responsiveness of *CHS*, *F3H*, and *FLS* promoters via a light-response unit (LRU) BS (Hartmann et al. 1998; Czemmel et al. 2009), different from the site combination recognised by MBW complexes.

A. thaliana encodes 107 MADS family members, mostly involved in the regulation of flower-related physiological and developmental processes (Parenicova 2003), meristem identity, root development, and fruit dehiscence (Theissen et al. 2000). These proteins share a conserved MADS DNA-binding domain (DBD) binding to the CArG-box (West et al. 1997). An *Arabidopsis* B-sister MADS is required for *DFR* expression in seed PA pathway, acting upstream of TT2 MYB factor (Nesi et al. 2002).

The WRKY superfamily consists of 72 members in *A. thaliana* (http://www.arabidopsis.org/browse/genefamily/WRKY.jsp). Members of this family contain at least one conserved WRKY domain, comprising the highly conserved WRKYGQK peptide sequence, and a zinc finger motif, that generally binds to the W-box DNA element, although alternative BSs have been identified (e.g. van Verk et al. 2008). WRKY factors are crucial in defence response and pathogen resistance (reviewed in Eulgem and Somssich 2007). TTG2 WRKY is involved in PA pathway, downstream of the PAP1/PAP2/TT2-GL3/EGL3-TTG1 complexes (Tohge et al. 2005; Ishida et al. 2007) that directly activate *TTG2* expression.

Regulation of regulators

Plants modulate the expression levels of these regulators to fine-tune flavonoid accumulation. The response cascades of several environmental and developmental cues are briefly summarised in this subchapter.

Light is a key environmental stimulus for flavonoid biosynthesis. *Arabidopsis* utilises more than three independent photoreceptor systems, perceiving the red/far-red (phytochromes, PHYA-E), blue/UV-A (cryptochromes, CRY1 and CRY2) and UV-B (UVB-Resistance 8, UVR8) fractions. Apart from light spectrum sensing through photoreceptors, plants use the photosynthetic electron transfer chain to integrate light information (both the quantity and quality) and

regulate flavonoid pathway genes (Cominelli et al. 2008; Das et al. 2011), together with the plant hormone ethylene and plant hormone-like sugars (Leon and Sheen 2003). Elongated Hypocotyl (HY5) and HY5-Homolog (HYH) serve as points of convergence for UVR8 (Brown and Jenkins 2008; Favory et al. 2009), PHY, and CRY signalling (Gyula et al. 2003). This way, the light-response system can adjust balance of photomorphogenesis and high light-responsive pathways. HY5 and HYH function as positive components controlling several important genes (Tohge et al. 2011), suggesting that the complex structure of light signalling cascades allows adaption to severe changes of light intensity: AtMYB111, AtMYB12, and PAP1 are regulators of flavonol/anthocyanin pathways (Tohge et al. 2005; Stracke et al. 2007); Early Light-Inducible Protein 1 (ELIP1) is a major light-responsive protein mediating tolerance to photoinhibition and photooxidative stress (Rossini et al. 2006); AtMYB4 is a negative regulator of an early phenylpropanoid step, *C4H* (Jin et al. 2000; Hemm et al. 2001). In addition, HY5 and Phytochrome-Interacting Factor3 (PIF3) bHLH positively regulate anthocyanin biosynthesis, binding directly to the promoters of anthocyanin structural genes such as *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, and *LDOX* (Lee et al. 2007; Shin et al. 2007).

Sugar is a common regulator for the expression of genes encoding metabolic enzymes and proteins involved in photosynthesis, carbohydrate metabolism, pathogenesis (Rolland et al. 2002), and anthocyanin biosynthesis (Mita et al. 1997; Baier et al. 2004). Sucrose induced *CHS* (Tsukaya et al. 1991; Takeuchi et al. 1994), *DFR*, and *LDOX* (Gollop et al. 2001; Gollop et al. 2002) expression, possibly via *PAP1* upregulation (Lloyd and Zakhleniuk 2004; Teng et al. 2005; Solfanelli et al. 2006). In contrast, sucrose inhibited flavonol accumulation in hypocotyl and cotyledons. Solfanelli et al. (2006) also indicated that sucrose-induced anthocyanin accumulation is sensed either by sucrose transporters (SUCs) or proteins closely associated with SUCs (Lalonde et al. 1999) and the signal is transferred via a sucrose-specific pathway (reviewed in Rolland et al. 2002). The regulatory roles of other genes cannot be ruled out (Solfanelli et al. 2006).

Another important player in the flavonoid induction is the HY5-independent negative regulation by ethylene (Jeong et al. 2010). This might be a mechanism to balance between carbon assimilation and anthocyanin accumulation in target tissues via the suppression of light- and sugar-induced anthocyanin pigmentation (Das et al. 2011). Ethylene was shown to modulate anthocyanin accumulation in response to phosphate starvation (Lei et al. 2011), interplaying with DELLA growth repressors and gibberelic acid (Jiang et al. 2007). These suppressions were shown to be mediated through regulation of the transcription factors of the MBW complex components such as PAP1, GL3, and EGL3. Ethylene maintains anthocyanin pigmentation in *Arabidopsis* leaves through the upregulation of MYBL2 at the transcriptional level (Jeong et al. 2010), rendering the MBW complex inactive (see below).

Cold stress induces flavonoid pathway genes, as observed for instance in maize, *A. thaliana*, grapevine, purple kale, and strawberry (Christie et al. 1994; Leyva et al. 1995; Mori et al. 2005; Feng et al. 2011; Koehler et al. 2012; Zhang et al. 2012). In grape, the individual enzyme-encoding genes respond differentially to the cold stress during berry development/veraison (Mori et al. 2005). A key role of light in cold-induced flavonoid accumulation is plausible, since *PAL* and *CHS* expression is light-dependent (Leyva et al. 1995). However, the exact signalling pathway is not known to date, except the involvement of PAP1 and TT8 homologs from apple and *Brassica oleracea* (Ban et al. 2007; Zhang et al. 2012).

Besides abiotic stresses, flavonoid biosynthesis genes are responsive to elicitation by microbial signal molecules (Bhattacharya et al. 2010) and pathogen-responsive genes (Lozoya et al. 1991). *PAL* and *4CL* are induced, but *CHS* is silenced rapidly upon elicitor signal (Lozoya et al. 1991). A number of common *cis*-acting elements have been identified in the promoters of PR genes; for instance, the GCC box function as an ethylene-responsive element that is specifically bound by AP2/ERF (Apetala2/Ethylene Responsive Factors)-domain TFs from *Arabidopsis* (Stepanova and Ecker 2000). Ethylene seems to integrate these elicitor-responsive processes, including pathways leading to phenolics accumulation (Beckman 2000; Lee et al. 2009).

As discussed above, the deposition of PA oligomers in the seed coat of *Arabidopsis* is developmentally regulated upon a MADS signal by MBW complexes (Nesi et al. 2002; Baudry et al. 2004). ABI3 (ABA-Insensitive3) is also involved in the expressional activation of downstream TFs (such as AN2 MYB) in seed and flower development and flavonoid accumulation (Kardailsky et al. 1999; Kurup et al. 2000; Suzuki et al. 2003).

Small RNAs are also important regulators of TF gene expression. MYB genes are targets to both microRNAs (miRNAs) and trans-acting, silencing RNAs (ta-siRNAs). miR828 down-regulates *AtMYB113*, involved in anthocyanin biosynthesis. This MYB is also targeted by TAS4-siR81, a dominant ta-siRNA, along with *PAP1* and *PAP2* (Rajagopalan et al. 2006). miR858 targets *AtMYB13*, *-20*, and *-111*, regulating stress responses, secondary cell wall biogenesis, and flavonol biosynthesis, respectively (Addo-Quaye et al. 2008). Furthermore, miR156-targeted *SPL9* (*Squamosa Promoter-Binding-Like Protein9*) gene activity has recently been coupled to MBW complex destabilisation within the developing *Arabidopsis* shoot (Gou et al. 2011).

After an initial signal from signal-transducing cascades, the expression level of TF genes is often controlled by complex interactions. For instance, TT8 forms complex with TTG1 and MYB TFs (TT2 or PAP1) to enhance its own transcription (Tohge et al. 2005; Baudry et al. 2006), in addition to the PAP1-GL3 dimer that also regulates TT8 expression (Baudry et al. 2006). In petunia, the MYB TFs AN2 and AN4 regulate AN1 expression, without affecting JAF13 (Quattrocchio et al. 1998; Spelt et al. 2000). A repressive loop has been described in grape. Overexpression of the specific both anthocyanin and PA regulators, *VlMYBA1* and *VvMYBPA2* respectively (Cutanda-Perez et al. 2009; Terrier et al. 2009), induces the expression of a C2 MYB repressor.

These regulatory interactions may as well include auto-regulation. PcWRKY1 binds the W box elements within the promoters of parsley PR1 genes *in vitro*. However, it also binds a specific arrangement of W boxes in its own promoter augmenting its own expression in response to elicitation, which is necessary and sufficient for early pathogen-responsive activation (Eulgem et al. 1999). In red-fleshed apples, MdMYB10 also binds to and trans-activates its own promoter, where a minisatellite forms an autoregulatory element, comprising five direct repeats of a 23-base element, each one predicted to contain a MYB-BS (Espley et al. 2009).

Despite the activities of most TFs are controlled at the transcriptional level, the activity of some can be regulated by post-translational modifications and/or interactions with various proteins. Post-translational modifications of proteins include phosphorylation, acetylation, hydroxylation, nitrosylation, gluthathiolation, intra- and intermolecular S–S bridge formation, myristoylation, farnesylation, ubiquitination, or glycosylation. TF modifications may alter protein conformation, allow interaction with other regulatory proteins, or affect subcellular localisation. These changes

in turn can affect DNA binding affinity, activation potential, nuclear localisation and/or protein stability. Post-translational modifications in plants are mostly occurring upon TFs acting early in regulatory cascades of light- (Hardtke and Deng 2000), cell cycle-, and stress-response signalling (Schutze et al. 2008; Ishihama and Yoshioka 2012) with consequences for the flavonoid pathway. Indeed, the DNA-binding affinity of an *A. majus* flavonoid regulator AmMYB340 was shown to be negatively affected by phosphorylation (Moyano et al. 1996) via a unknown mechanism. In addition, the transcriptional activity of several MYB proteins related to cell cycle regulation is positively affected by phosphorylation (Dubos et al. 2010 and references therein). Intramolecular S-S bond formation under oxidizing conditions prevents DNA binding of maize P1 (Heine et al. 2004) and is predicted for *Arabidopsis* MYB11, MYB12, and MYB111.

Protein-protein interactions may also regulate TF activity. An example already discussed here is the interaction between MYB, bHLH and WDR TFs. Interactions between TFs may stabilise their interaction with target promoters and/or may have synergistic effects on transcription rate. Negative regulation may occur as nuclear exclusion upon interaction with a 14-3-3 regulatory protein partner (Igarashi et al. 2001). The single-repeat R3-MYB proteins MYBL2 and Caprice are negative regulators of anthocyanin biosynthesis. In addition to being regulated developmentally, their expression also depends upon environmental cues such as high light levels and, presumably, nitrogen deficiency (Dubos et al. 2008; Matsui et al. 2008; Zhu et al. 2009). MYBL2 and Caprice are thought to inhibit anthocyanin biosynthesis by outcompeting positive R2R3-Myb regulators to form an inactive complex, MYBL2/CPC-bHLH-TTG1 (L2BW). Hence, the anthocyanin content in a specific cell type is proposed to be regulated by a balance between MBW and L2BW complexes (Dubos et al. 2008; Zhang et al. 2009; Zhu et al. 2009).

TF abundance can also be regulated by adjustment of protein turnover rate. Protein stability is often regulated via covalent modifications such as phosphorylation and/or ubiquitination, and/or via interaction with other proteins (Vom Endt et al. 2002). An example of regulation by specific proteolysis combined with phosphorylation is given by HY5. Constitutive Photomorphogenesis1 (COP1) is an E3-ubiquitin ligase that is localised to the nucleus under dark conditions and marks unphosphorylated HY5 for degradation (Hardtke and Deng 2000). The activity of casein kinase II maintains a small pool of phosphorylated HY5 that is less susceptible to degradation but physiologically inactive. Upon light stimulus, COP1 is excluded from the nucleus and the HY5-related casein kinase II activity is reduced (Hardtke and Deng 2000), resulting in an increase of active unphosphorylated HY5 level and the activation of its target genes.

2.4. Secondary pathways of hops

The biosynthesis of prenylchalcones such as XN represents a diverged branch from the general flavonoid pathway of plants. As described above, naringenin chalcone represents the first intermediate of the pathway, often rapidly cyclised to naringenin by CHI. This standard route is functional in hop tissues, such as leaves, stems, and cone bracts, as shown by their content of flavonols, PAs, and anthocyanins (Sägesser and Deinzer 1996), but it appears repressed in lupulin glands (Stevens et al. 1997). Despite advances in hop-specific metabolite research, the knowledge about metabolic activities in lupulin glands remains incomplete.

By initiative of several laboratories worldwide (Fortes, Jeltsch, Page, Jakše, Xuechun), the hop EST database has broadened considerably to contain valuable information. Nagel et al. (2008) extracted the counts of ESTs corresponding to identifiable enzymes for the three major lupulin-specific pathways (terpenoid, bitter acid, and XN) and managed, with a few exceptions, to link the transcriptome of lupulin glands with the biosynthetic activities thereof.

Isopentenyl diphosphate (IPP) and DMAPP function both as precursors for terpenoid biosynthesis and as the source of the prenyl side chains of terpenophenolics. In lupulin glands, the plastidic methylerythritol 4-phosphate pathway dominates over cytoplasmic mevalonate pathway in the synthesis of IPP and DMAPP in EST counts (Nagel et al. 2008), and is supported by other evidence (Goese et al. 1999; Dudareva et al. 2005). The entire terpenoid enzymatic machinery was identified in the EST library of Nagel et al. (2008).

XN originates in flavonoid pathway, formed in hops from the CHS_H1 product (Okada et al. 2004), naringenin chalcone, before intramolecular isomerisation takes place. Prenylation of naringenin chalcone (see below) yields DMX, which is methylated to form XN. The 6'-O-methylation of DMX by OMT1 serves to inhibit isomerisation; XN is much more stable than its precursor (Nagel et al. 2008). The trichome-specifically expressed CHS_H1 is encoded by an oligofamily of *chs*_H1 genes (Novák et al. 2003; Matoušek et al. 2006). Humulone and lupulone are derived from primary metabolism by the two-step degradation of Leu and Val to form isovaleryl-CoA and isobutyryl-CoA (Goese et al. 1999) used as substrates by valerophenone synthase (VPS) to form acylphloroglucinols phlorisovalerophenone (PIVP) and PIBP, respectively (Paniego et al. 1999). The resulting acylphloroglucinols then undergo two or three transfers of prenyl moieties catalysed by an aromatic substrate prenyltransferase.

The VPS protein shares a high degree of homology with plant CHSs as well as other hop CHSs; amino acid differences result in a slight, but significant, change in substrate specificity (Paniego et al. 1999; Matoušek et al. 2002; Novák et al. 2006) achieved through variations in the number of rounds of condensation, differences in starter molecule specificity, and the folding pattern of the reaction intermediate (Schröder 1997; Austin and Noel 2003). Naringenin chalcone is also formed by VPS, albeit at a much lower rate, and conversely, CHS_H1 inefficiently accepts isovaleryl-CoA to yield phlorisovalerophenone (Okada et al. 2004). VPS is expected to accumulate substantially and specifically in lupulin glands (Okada et al. 2003); its transcript was the second most abundant in the EST data of Nagel et al. (2008).

Seven expressed OMTs predicted to methylate low molecular weight substrates were identified by Nagel et al. (2008). *OMT4* resembled salicylic acid carboxyl methyltransferase, while *OMT5* and *OMT6* were similar to methyltransferases of caffeoyl-CoA and other phenylpropanoids of lignin biosynthesis. *OMT1*, *OMT2*, *OMT3*, and *OMT7* showed homology to enzymes catalysing methylation of flavonoids and were further characterised as to their substrate specificity. Notably, OMT1 methylated chalcones DMX and xanthogalenol, while OMT2 showed catalytic activity over a broader range of substrates including DMX (on a different hydroxyl group) and XN (Nagel et al. 2008).

Prenyltransferases catalysing aromatic substances prenylation are divalent cation-requiring membrane-bound proteins, and those characterised to date are localised to plastids (Sasaki et al. 2008; Akashi et al. 2009; Sasaki et al. 2011; Tsurumaru et al. 2012). HlPT-1 exhibited a unique broad substrate specificity, catalysing the first transfer of dimethylallyl moiety to PIVP and PIBP

(Tsurumaru et al. 2012), as well as naringenin chalcone to yield XN. Besides HlPT-1, however, there should be other prenyltransferase members showing different substrate specificity (Tsurumaru et al. 2012). Their candidates may be within the 23 ESTs (4 contigs and 3 singletons) identified by Nagel et al. (2008), annotated as small-molecule prenyltransferases, excluding sequences encoding protein prenyltransferases and short-chain terpenoid prenyltransferases.

From other enzymes, CHS2 or CHS4 accept isovaleryl-CoA as substrate, but the reaction stops prematurely (Okada et al. 2004). Despite undisclosed functions, CHS2 and CHS4 ESTs were rather numerous, while CHS3, which appears non-functional, is not expressed (Nagel et al. 2008). A high number of ESTs for CHI–like proteins were found, forming three large contigs and two singletons (Nagel et al. 2008). These proteins may not function as true CHIs, since only trace amounts of isoxanthohumol and other flavanones, products of CHI activity, were detected in lupulin glands. Rather, CHI-like proteins are thought to have enzymatic activity beyond chalcone isomerisation or to possess non-catalytic functions as flavonoid carriers or stabilisers (Gensheimer and Mushegian 2004; Ralston et al. 2005). Several ESTs were found that correspond to FLS, UFGT, and LDOX (Nagel et al. 2008).

Metabolic channeling of intermediates is hypothesised in lupulin glands via a multi-enzyme complex that would prevent chalcone cyclisation (Winkel 2004; Nagel et al. 2008). As mentioned in Chapter 2.3.1., the advantages of such regulation would be the quick conversion of the labile chalcone into stable compounds and the control of metabolic crosstalk, such as the availability of DMAPP for both the humulone and XN pathways.

Despite efforts to screen cDNA libraries of hop for regulatory factors, only two MYB TFs were described (Matoušek et al. 2005; 2007a) until the beginning of this Ph.D. thesis. Based on genomic sequence data, Matoušek et al. (2006) predicted a set of *cis*-regulatory sites in the *chs*_H1 promoter (*Pchs*_H1), with a putative involvement of TFs from MYB, bHLH, and bZIP families. Consistently with this, the *Arabidopsis* PAP1 MYB proved to be a potent *chs*_H1 activator (Matoušek et al. 2006). Therefore, we set out to a journey of discovery and characterisation of other regulators, with a battery of methods to analyse TFs discussed in the following chapter.

2.5. Methods and approaches of TFs research

To achieve a detailed knowledge of the regulatory circuits in plants, a comprehensive set of methods and approaches must be used. The functional analysis of TFs is increasingly important as huge quantities of high-throughput sequence and expression data are generated over the last years, often without adequate experimental support. Approaches of functional analysis may be divided into following categories: bioinformatic methods; molecular function analyses; expression profiling; and phenotype determination (reviewed in Mitsuda and Ohme-Takagi 2009). These approaches complement each other in the characterisation of entire transcriptional regulatory networks.

2.5.1. Sequence analyses

Bioinformatic techniques may be implemented in several steps of TF analysis, based on DNA or protein sequence characteristics and comparison with the published data. Bioinformatics may provide information on evolutionarily conserved domains of pathway regulators that may be screened within sequence databases or cDNA/genomic libraries, or, conversely, identify conserved domains in unknown sequences obtained by cloning and/or sequencing. For instance, it is frequently observed that TFs with high homology in the DBD, such as MYB proteins, function redundantly. Homology searches, using for instance BLAST, may identify functions that are shared in a group of highly homologous proteins. Most described *Arabidopsis* and rice genes are assigned a Gene Ontology (GO) term that characterises their function in the biological system (AmiGO) (Ashburner et al. 2000)). Additionally, many web-based programs can be used to perform conserved domain searches within queried sequences, including InterProScan (Quevillon et al. 2005), while MEME (Bailey et al. 2006) and SALAD (http://salad.dna.affrc.go.jp/salad/en/) perform searches for common motifs within a queried sequence set, which alleviates putative function assignments. Finally, proteins larger than 60 kDa require the presence of a nuclear localisation signal (NLS) for a selective nuclear import (Raikhel 1992) that is vital for proper TF function. The subcellular localisation of TFs may be predicted by routinely used TargetP (Emanuelsson et al. 2000) and WoLF PSORT (Horton et al. 2007).

2.5.2. Expression profiling

The expression of a gene in time, space, and response to varying conditions is vital to its biological function. Expression analysis using quantitative reverse transcription-PCR (qRT-PCR) is routinely employed to assay individual TF transcript abundance throughout the plant body, development, or upon stress. Particular attention must be drawn to the selection of robust reference gene(s) and normalisation (Czechowski et al. 2005). To obtain detailed expression profiles, promoter-reporter experiments may be implemented using *E. coli* β-glucuronidase (GUS) or green fluorescent protein (GFP) coupled to the assayed gene's promoter. This simplified approach has been applied to the hop *VPS* gene (Okada et al. 2003). However, such constructs disregard the possibility of additional regulatory elements, including miRNA targeting sites, elsewhere in the gene's sequence, e.g. in introns (Deyholos and Sieburth 2000), 5' and 3' untranslated regions, or sequences distant from the coding region. For an accurate profiling, it is therefore advisable to use a largest possible genomic fragment, in which the reference is inserted to generate a fusion protein (Wu and Poethig 2006).

Large-scale expression profiles may be obtained using microarray or massively parallel sequencing technologies. In fact, huge amount of microarray data were already accumulated over last years and are accessible via NCBI Gene Expression Omnibus (GEO) and EBI ArrayExpress. Co-expression, i.e. positive or negative correlation of gene expression, with other *Arabidopsis* genes may be analysed using ATTED-II (Obayashi et al. 2009) and BAR eFP (Winter et al. 2007) web sites. Correlated expression of genes may infer functional relation, downstream/upstream position within a transcriptional cascade, and/or putative interaction.

2.5.3. Networking of genes

The comprehensive understanding of the entire regulatory network is an ultimate challenge for TF research. Besides bioinformatics, genetic and non-genetic approaches are employed to achieve this.

Genes that resemble by expression profiles often share regulatory motifs within their sequences. These regulatory motifs may be identified in genomic sequences using several online tools such as PLACE (Higo et al. 1999) and Match based on TRANSFAC (BioBase GmbH, Wolfenbüttel, Germany). Updated cis-regulatory sites may be explored using the software employing MatBase (Genomatix, München, Germany) and TRANSFAC knowledge bases. Though hop genomic sequence data are rather scarce, we can take advantage of the *Cannabis* genome from two cultivars Finola and Purple Kush, available via Cannabis Genome Browser (http://genome.ccbr.utoronto.ca/).

Analysis of mutant lines provides robust data, if suppressors that reduce the phenotypic abnormality of mutants are found. Restored DFR activity in *an11* mutants with induced ectopic expression of *AN2* may serve as a proof that AN11 acts upstream of the MYB TF, i.e. enhances its activation potential (deVetten et al. 1997). Complementation experiments may be carried out to restore wild type phenotype in *A. thaliana* T-DNA insertion mutants with a heterologous gene, if suspected to share function with the insertion mutant.

Non-genetic approaches include high-throughput experiments, such as yeast one-hybrid screening (Luo et al. 1996) to identify upstream-acting TFs. This system uses a tandem repeat of a putative short cis-element as bait to screen a cDNA library prepared for Y1H. An improved protocol employing directly a <500 bp promoter fragment has been successfully applied (Deplancke et al. 2006; Pruneda-Paz et al. 2009). To identify downstream genes of a TF, microarray approaches are most suitable. Morohashi and Grotewold (2009) proposed distinct roles for GL1 and GL3 that form an MBW complex to regulate trichome initiation using these methods.

For identification of direct targets, TF BSs need to be revealed. The consensus BS may be determined via selection of a purified protein's target site from a pool of random oligonucleotides (Wright et al. 1991). Alternatively, electrophoretic mobility shift assay (EMSA) is used to monitor the interaction of a TF with its BS, typically a labelled double-stranded oligonucleotide of 20–25 bp containing a known *cis*-element (Garner and Revzin 1981). The mobility of a TF–BS complex during non-denaturing PAGE is determined by both size and charge: the TF–BS complex will migrate more slowly than free DNA molecules. When unlabelled wild-type BS is added in excess over the labelled probe, the band representing TF-BS complex diminishes by competition. Mutation of the BS prevents competition. Purification of TFs is challenging because of their low abundance and post-translational modifications, so specialised purification and analysis methods have been developed (Jiang et al. 2009). However, EMSA is accomplishable with crude proteins, such as nuclear extract.

Chromatin immunoprecipitation (ChIP) is an approach to identify genomic fragments that are bound by a TF (O'Neill and Turner 1996). It is considered as strong evidence that the assayed TF regulates a putative target gene if its promoter sequence is enriched in the pool of TF-bound fragments. If combined with high-throughput array and sequencing technologies, ChIP-chip and

ChIP-seq, respectively, allow genome-wide BS identification and comprehensive networking (Morohashi and Grotewold 2009).

2.5.4. Molecular function analyses

To characterise activation or repression properties of a TF, an effector-reporter assay is often employed (Mitsuda and Ohme-Takagi 2009). The effector is often an in frame fusion of a TF with the GAL4-DBD, driven by a strong constitutive promoter, such as cauliflower mosaic virus 35S promoter (CaMV-P35S). The reporter consists of a minimal promoter containing tandemly repeated GAL4-BSs that drives the expression of GUS or firefly luciferase. As an internal control or reference, another reporter gene driven by a constitutive promoter is used. Upon transient co-expression of the effector, reporter, and reference constructs, the activity of the reporter is a measure of the assayed TF activation potential. The repressive activity may be examined in a similar way, only with the reporter driven by an additional enhancer in its promoter (such as CaMV-P35S). The decrease of the reporter activity is therefore a measure of the assayed TF repressive potential.

Site-directed mutagenesis further dissects TF function from a mechanistic point of view. It is pointed mainly on known post-translational regulatory sites, e.g. phosphorylation, glycosylation and ligand-binding sites, and NLS. The analysis can be accomplished via random mutagenesis using DNA shuffling systems to a measurable trait, e.g. higher *cis*-element affinity or improved trans-activational properties (so-called *in vitro*, or directed, evolution). For instance, Pattanaik et al. (2006) identified variants with significant increase in transcriptional activities through two rounds of DNA shuffling, with the majority of resulting mutants in the activation domains of the improved variants.

To function properly, TFs may require protein-protein interactions, as discussed in Chapter 2.3.2. For protein-protein interactions prediction, information may be retrieved from databases. EBI stores and updates literature data in the IntAct database (Kerrien et al. 2007), while the *A. thaliana* Protein Interactome Database (AtPID) presents a searching tool with a graphical output of interactions, including pathway depictions (Cui et al. 2008). Protein-protein interactions may be also assayed *in vitro* and *in vivo* by yeast and bacteria two-hybrid systems (Y2H and B2H, respectively). Compared to effector-reporter assay, there are two effectors: GAL4 sequence is split to parts encoding the activation domain (AD) and the DBD. While the DBD sequence is fused to a known protein (referred to as bait) screened for interaction partners, the AD sequence is fused in a separate vector to a different protein (referred to as prey) represented by a single known coding sequence or a library of sequences. The reporter construct consists of a promoter containing GAL4-BS(s) driving the expression of a reporter gene. If bait and prey interact upon co-transfer into the cell, the AD and DBD reconstitute to a functional TF, which recruits RNA Pol II and leads to reporter expression (Fields and Song 1989; reviewed in Bruckner et al. 2009). A modified procedure for *E. coli* was presented by Joung et al. (2000).

The *Agrobacterium tumefaciens*-mediated transient expression *in planta* has several advantages. It allows protein-protein interactions with virtually no limitation for the number of co-expressed TF effectors. Promoter regions of putative downstream genes are coupled to a reporter (typically GUS or GFP), whose activity (Jefferson et al. 1987) is a measure of combinatorial interactions of TFs on this promoter sequence.

2.5.5. Phenotype analyses

Identification of an informative phenotype associated with the assayed TF is instrumental in order to verify its biological function. Flavonoid pathway mutant phenotypes encompass affected metabolite accumulation, morphological changes, and altered stress tolerance. The mutant phenotype may be "hidden", only visible under certain conditions. To induce phenotypic changes by manipulating TFs, two strategies, "gain of function" and "loss of function", are usually applied in either homologous or heterologous plant systems. The "gain of function" approach induces a mutant phenotype by ectopic TF gene expression driven by a CaMV-P35S promoter. Inducible promoter or a hormone-receptor system is useful to prevent bias phenotypes, co-suppression, or lethality (Severin and Schoffl 1990; Aoyama and Chua 1997; Caddick et al. 1998; Zuo et al. 2000). This system may not always reflect the native function of assayed TFs since the expression of a single TF might be insufficient to activate the expression of target genes; a cooperation with other factors may be missing.

Phenotypes induced by "loss of function" analysis should more directly mirror native gene function. Inactivation of genes or of a gene's activity may be accomplished through the expression of complementary RNA, namely antisense RNA, RNA interference (Fire et al. 1998), and hairpin RNA strategies (Wesley et al. 2001). The functional redundancy of TFs sharing similar DBDs is however a major obstacle for "loss of function" approaches. The CRES-T system (Hiratsu et al. 2003) uses a repressor domain fused to the assayed TF to overcome this problem, producing a dominant suppressor of all downstream genes. The native promoter of the TF is preferred to drive the chimeric protein expression to correct tissues.

3. AIMS

▪ Screening for additional transcription factors involved in the regulation of lupulin flavonoid pathways.

▪ Sequence analyses and elucidation of gene organisation – description of promoter region, intron(s), and gene family arrangement, if applicable.

▪ Characterisation of domain structure of putative protein products; phylogenetic analyses; prediction of post-translational modification sites.

▪ Functional analyses in connection to viroid pathogenesis, phenological examination of heterologous transformants.

4. RESULTS

4.1. Identification of bZIP factors involved in secondary pathways of hops

Owing to the roles of flavonoids in UV protection, the light-activation of CHS as the first committed step of their production was described quite early (Duell-Pfaff and Wellmann 1982; Chappell and Hahlbrock 1984; Bruns et al. 1986). The sequences involved in the light-responsiveness of CHS were identified soon after (Schulze-Lefert et al. 1989; Fritze et al. 1991), comprising MYB and bZIP BSs. Also hop *chs*_H1 oligofamily promoter regions was found to contain MYB BSs as well as G-box BSs (Matoušek et al. 2006), generally recognised by the stress-related TFs from bZIP family (Jakoby et al. 2002). Consistently, the PAP1 proved to be a potent *chs*_H1 activator (Matoušek et al. 2006). Interplay between MYB, bZIP and bHLH BSs was shown instrumental for activation of phenylpropanoids in response to light and developmental signals (Hartmann et al. 2005). This directed our research to screen cDNA libraries for bZIP sequences, which resulted in identification of two genes encoding *HlbZIP1* and *-2*, plus a truncated version of the former, described in the following Paper I. Their respective gene products mediated the activation of *chs*_H1 promoter (*Pchs*_H1) and O-methyltransferase 1 promoter (*Pomt1*) in an independent manner as well as in combination with HlMYB3, but did not activate *Pvps*. Their role in flavonoid regulation is underlined by their lupulin gland-specific expression and metabolic changes observed in *P. hybrida HlbZIP* transgenotes.

The following passage (11 pages) is already published in a scientific journal and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

I. Matoušek J, Kocábek T, Patzak J, Stehlík J, **Füssy Z**, Krofta K, Heyerick A, Roldán-Ruiz I, Maloukh L , De Keukeleire D. Cloning and molecular analysis of *HlbZip1* and *HlbZip2* transcription factors putatively involved in the regulation of the lupulin metabolome in hops (*Humulus lupulus* L.). J Agric Food Chem 2010;58:902-12.

Abstract

Hop (*Humulus lupulus* L.), the essential source of beer flavor is of interest from a medicinal perspective in view of its high content in health-beneficial terpenophenolics including prenylflavonoids. The dissection of biosynthetic pathway(s) of these compounds in lupulin glands, as well as its regulation by transcription factors (TFs), is important for efficient biotechnological manipulation of the hop metabolome. TFs of the bZIP class were preselected from the hop transcriptome using a cDNA-AFLP approach and cloned from a cDNA library based on glandular tissue-enriched hop cones. The cloned TFs HlbZIP1A and HlbZIP2 have predicted molecular masses of 27.4 and 34.2 kDa, respectively, and both are similar to the group A3 bZIP TFs according to the composition of characteristic domains. While HlbZIP1A is rather neutral (pI 6.42), HlbZIP2 is strongly basic (pI 8.51). A truncated variant of HlbZIP1 (HlbZIP1B), which is strongly basic but lacks the leucine zipper domain, has also been cloned from hop. Similar to the previously cloned *HlMyb3* from hop, both bZIP TFs show a highly specific expression in lupulin glands, although low expression was observed also in other tissues including roots and immature pollen. Comparative functional analyses of *HlbZip1A*, *HlbZip2*, and subvariants of *HlMyb3* were performed in a transient expression system using *Nicotiana benthamiana* leaf coinfiltration with *Agrobacterium tumefaciens* strains bearing hop TFs and selected promoters fused to the GUS reference gene. Both hop bZIP TFs and *HlMyb3* mainly activated the promoters of chalcone synthase *chs*_H1 and the newly cloned *O*-methyl transferase 1 genes, while the response of the valerophenone synthase promoter to the cloned hop TFs was very low. These analyses also showed that the cloned bZIP TFs are not strictly G-box-specific. HPLC analysis of secondary metabolites in infiltrated *Petunia hybrida* showed that both hop bZIP TFs interfere with the accumulation and the composition of flavonol glycosides, phenolic acids, and anthocyanins, suggesting the possibility of coregulating flavonoid biosynthetic pathways in hop glandular tissue.

4.1.1. Unpublished results – Functional analysis of HlbZIP1A

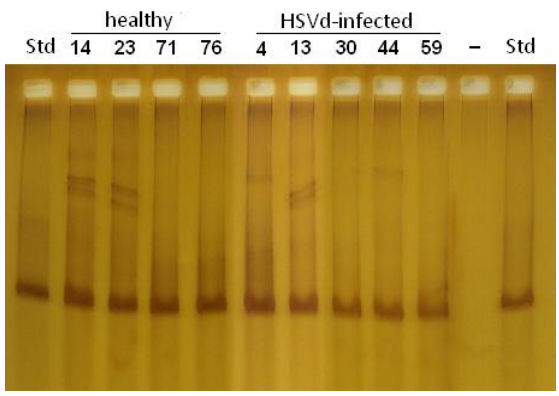

Fig. 7: Example of heteroduplex analysis by PAGE. Sample DNA fragments (numbered) from two *HlbZIP2* cDNA libraries are allowed to form heteroduplex with a standard (Std) DNA fragment and electrophoresed at a temperature favouring resolution of homo- and heteroduplexes (Füssy 2008). Several samples exhibit additional bands as proof of sequence difference(s) from the Std.

My work produced additional data related to hop bZIP TFs. In preliminary studies, I elucidated the expression and sequence variability of hop bZIP TFs during viroid pathogenesis. I discovered some changes in *HlbZIP1* and *-2* expression upon viroid infection (to be expanded in my later work), and sequence variability in case of *HlbZIP2*. Several clones contained nucleotide changes compared to the published *HlbHLH2* sequence, as determined by temperature-gradient gel electrophoresis (Fig. 7) (Matoušek et al. 2001) and sequencing (Füssy 2008), consistent with the observed variability between hop cultivars in terms of *HlbZIP1* and *-2* genomic arrangements (Matoušek et al. 2010).

The following functional experiment was inspired by a bioinformatic prediction. Sharing sequence identity with the Group A of plant bZIP proteins (Choi et al. 2000; Jakoby et al. 2002), HlbZIP1A exhibits motifs 1 and 2 near its N-terminal end. The roles of these phosphorylation sites have not been unequivocally determined (Meggio and Pinna 2003). A casein kinase II (CK2) phosphorylation site deletion diminished light-mediated degradation of PIF1 bHLH (Bu et al. 2011), but another CK2 site is assumed in ABA-related activation of AREB1/2 (Uno et al. 2000; Jakoby et al. 2002).

To shed light on this ambiguity, I performed site-directed mutagenesis to remove two CK2 phosphorylation sites via replacing Ser41 (motif 1) and Thr102 (motif 2) by Ala. The site-directed mutagenesis was carried out on the original HlbZIP1A clone 2547 in pCR-Script, using KOD polymerase (Merck, Darmstadt, Germany) and primers covering the mutated site (Zip1S41Af 5'-GATTCTAGAAACAT-TGCTGCCATGGATGATTTGCTCAAG-3'; Zip1S41Ar 5'-CTTGAGCAAATCATCC-ATGGCAGCAATGTTTCTAGAATC-3'; sites of mutation underlined), designed to replace Ser41 with Ala. After the PCR synthesis of the whole plasmid, the wild type HlbZIP1A-bearing template was degraded with a

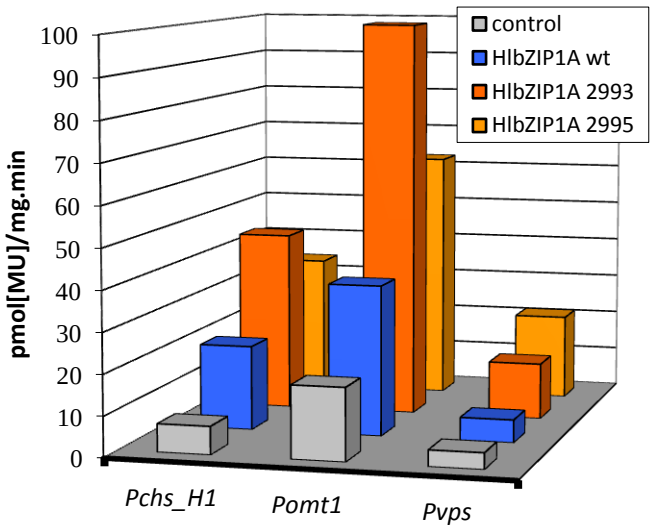

Fig. 8: Activation potential of wild-type and mutated HlbZIP2 proteins, assayed on promoter::GUS reportem *Pchs*_H1, *Pomt1*, and *Pvps* in an *A. tumefaciens*-mediated transient expression assay.

methylation-specific endonuclease *Dpn*I. The procedure was repeated to introduce the second mutation, Thr102Ala (using primers Z1T102Af 5'-GAGATAGGCAGCGCGATGGCCTTGGAGGATTA-CTTGACG-3'; Z1T102Ar 5'-CGTCAAGTAATCCTCCAAGGCCATCGCGCTGCCTATCTC-3'). The coding region was then excised and cloned to an expression vector, as described in Matoušek et al. (2010). The activation potential of the mutated HlbZIP1A proteins was assayed using an *in planta* transient expression system followed by GUS assay (Jefferson et al. 1987; Matoušek et al. 2010).

As seen in Fig. 8, both mutated variants of HlbZIP2 had higher activation potential than the wild type protein, possibly due to higher stability. This, however, has not been analysed into detail.

4.2. An MBW complex is involved in *chs*_H1 activation

As MBW complexes became well-established in the flavonoid pathway regulation (Ramsay and Glover 2005), a question arose whether the characterised hop TFs fulfil their function via a ternary complex as well. In the meantime, we isolated two genes encoding bHLH TFs, *HlbHLH1* and *HlbHLH2*, several MYB TFs, and a WDR protein. Also, despite their description earlier (Matoušek et al. 2005; 2007a), the influence of HlMYB1 and HlMYB3 on the activation of *chs* genes has not been investigated in detail. Intriguingly in the case of HlMYB3, alternative start codon variants s-HlMYB3 and l-HlMYB3 were identified, causing divergent phenotypic and metabolic responses in transgenotes (Matoušek et al. 2007a) and transient expression assays (Matoušek et al. 2010). However, these variants exhibit similar activation capacity with respect to *Pchs*_H1 and *Pomt1* (Matoušek et al. 2010), leaving the function of the N-terminus unspecified.

By means of functional analyses, we validated the model of MBW complexes as potent activators of the *chs*_H1 promoter. *Pchs*_H1 showed the strongest response to the ternary complexes of HlMYB2, HlMYB3, and AtPAP1, combined with HlbHLH2 and HlWDR1, confirmed using GUS reporter construct, as well as "native" *chs*_H1 gene (Matoušek et al. 2006). In contrast, the activation of *Pchs4* is mainly achieved by independent MYB TFs, such as AtMYB12, or their binary MYB-WDR combinations; HlMYB2 and AtPAP1 showed low capability to activate *Pchs4*. This may be due to different promoter architecture of the two *chs* genes, possibly reflecting their diverged functions, as CHS4 lacks the ability to produce naringenin chalcone. In addition, metabolic assays in petunia leaves transiently expressing TFs demonstrate the inability of described hop TFs to activate anthocyanin pathway genes, possibly due to a regulatory role in another flavonoid branch.

In Paper II, we also identified the first hop MYB TF with inhibitory effect on the activation potential of other MYBs on *Pchs*_H1 and *Pchs4*. Despite HlMYB7 shares high sequence similarity with HlMYB2 and AtMYB12, a suppressor motif in its C-terminal region confers suppressive activity.

The two HlMYB3 variants showed an interesting diversion. While l-HlMyb3 shows a maximum of activation for *Pchs4* in the MYB-bHLH combination, s-HlMyb3 is most potent in the activation of *Pchs*_H1 in an MBW complex. The difference at the N-terminus therefore account for some protein–protein interactions, consistently with previous reports (Matoušek et al. 2010).

The following passage (20 pages) is already published in a scientific journal and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

II. Matoušek J, Kocábek T, Patzak J, **Füssy Z**, Procházková J, Heyerick A. Combinatorial analysis of lupulin gland transcription factors from R2R3Myb, bHLH and WDR families indicates a complex regulation of *chs*_H1 genes essential for prenylflavonoid biosynthesis in hop (*Humulus lupulus* L.). BMC Plant Biol 2012;12:27.

Background
Lupulin glands of hop produce a specific metabolome including hop bitter acids valuable for the brewing process and prenylflavonoids with promising health-beneficial activities. The detailed analysis of the transcription factor (TF)-mediated regulation of the oligofamily of one of the key enzymes, i.e., chalcone synthase CHS_H1 that efficiently catalyzes the production of naringenin chalcone, a direct precursor of prenylflavonoids in hop, constitutes an important part of the dissection of the biosynthetic pathways leading to the accumulation of these compounds.

Results
Homologues of flavonoid-regulating TFs *HlMyb2* (M2), *HlbHLH2* (B2) and *HlWDR1* (W1) from hop were cloned using a lupulin gland-specific cDNA library from the hop variety Osvald's 72. Using a "combinatorial" transient GUS expression system it was shown that these unique lupulin-gland-associated TFs significantly activated the promoter (P) of *chs*_H1 in ternary combinations of B2, W1 and either M2 or the previously characterized *Hl*Myb3 (M3). The promoter activation was strongly dependent on the Myb-P binding box TCCTACC having a core sequence CCWACC positioned on its 5' end region and it seems that the complexity of the promoter plays an important role. M2B2W1-mediated activation significantly exceeded the strength of expression of native*chs*_H1 gene driven by the 35S promoter of CaMV, while M3B2W1 resulted in 30% of the 35S:*chs*_H1 expression level, as quantified by real-time PCR. Another newly cloned hop TF,*Hl*Myb7, containing a transcriptional repressor-like motif pdLNLD/ELxiG/S (PDLNLELRIS), was identified as an efficient inhibitor of *chs*_H1-activating TFs. Comparative analyses of hop and *A. thaliana* TFs revealed a complex activation of P*chs*_H1 and P*chs*4 in combinatorial or independent manners.

Conclusions
This study on the sequences and functions of various lupulin gland-specific transcription factors provides insight into the complex character of the regulation of the *chs*_H1 gene that depends on variable activation by combinations of R2R3Myb, bHLH and WDR TF homologues and inhibition by a Myb repressor.

4.2.1. Unpublished results – Functional analyses of the MBW complex

To assay protein-DNA and protein-protein interactions, I implemented EMSA and B2H systems, respectively. Several optimisations were carried out to remove background DNA-binding activity, including optimisation of the extraction procedure and the binding buffer ion content. The final procedure takes into account a major role of unknown light-induced factors in generating background *Pchs*_H1 DNA-binding activity (see below). In Fig. 9, specific protein-DNA interaction can be seen in MBW complex-containing sample (lanes 3) and a partial inhibition of this interaction if mutated *Pchs*_H1 DNA is used as probe. Additional experiments using partially purified protein or supershift assays are however needed to provide conclusive results.

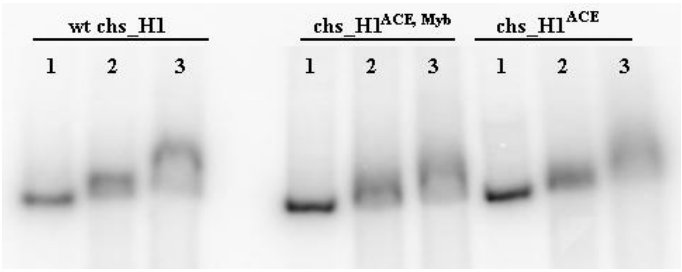

Fig. 9: EMSA of crude protein extracts from plants transiently expressing TFs HlWDR1, HlbHLH2, and s-HlMyb3. As a probe, we used variants of *Pchs*_H1, as indicated above the lanes. (1) blank sample without protein; (2) negative control extract; (3) extract from plant expressing P19, s-*HlMyb3*, *HlbHLH2*, and *HlWDR1*. Mutation of ACE and MYB elements in *Pchs*_H1 did not clearly inhibit the M3B2W1-DNA complex formation.

For B2H assay, I used the BacterioMatch II Two-Hybrid System (Stratagene, La Jolla, CA, USA) according to manufacturer's instructions. The following combinations of TFs were assayed for interaction (bait × target): HlWDR1 × HlbHLH2; l-HlMYB × HlbHLH2; HlWDR1 × s-MYB3; and s-MYB3 × HlbHLH2. Very weak or no interaction was shown for all combinations (not shown), despite clear evidence of co-operation is provided by the transient expression experiments (see Paper II).

As an attempt to determine TF trans-activation, we screened for promoter regions of TF-encoding genes in hop genomic and BAC libraries. I isolated the HlMYB1 promoter and, using MatInspector (Genomatix), identified high-probability BSs for several TF families, suggesting HlMYB1 responsiveness to various stimuli (Fig. 10): light- and stress induction mediated by bZIP BSs and light-responsive GAP-boxes; MYB and bHLH trans-activation; pathogen elicitation via WRKY BSs; floral development-related expression via binding of MADS and homeobox TFs (Matoušek et al. 2005). *In planta* activation of HlMYB1 promoter remains to be established. Cloning and characterisation of several other TF-encoding genes' promoter sequences is in

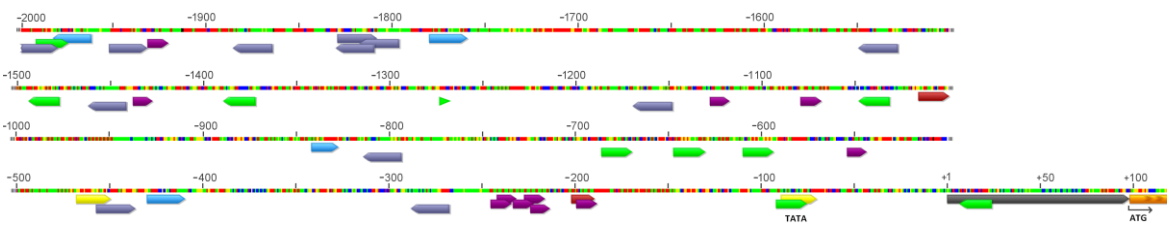

Fig. 10: Organisation of the HlMYB1 promoter region 2000 bp upstream of presumed transcription start (+1). Binding sites of various TFs predicted by MatInspector (Genomatix) are shown: MYB and MYB-like (green), bHLH (yellow), bZIP and light-responsive boxes (light-blue), WRKY (red), MADS (mauve), and homeobox TFs (violet). Note the accumulation of MADS BSs around position -1800 and homeobox protein BSs around position -200.

progress (HlbHLH2, HlMYB3, HlWDR1, and HlWRKY1).

Method: Crude protein extraction and EMSA procedure

Crude protein extracts were prepared using an *A. tumefaciens*-mediated transient expression system in *N. benthamiana* leaves. TFs were co-expressed with a silencing inhibitor (Voinnet et al. 2003), P19, coupled to CaMV 35S promoters, while *A. tumefaciens* expressing only P19 was used as negative control, as in (Matoušek et al. 2012). Following infiltration, plant were kept in a shade for four days, then 4 hours prior to protein extraction transferred to darkness to eliminate background generated by unknown light-activated factors. Following extraction (3 v/w leaf material 83mM Tris-HCl pH=7.5, 66mM KCl, 100mM NaCl, 0,8mM $MgCl_2$, 2mM β-mercaptoethanol, 1mM PMSF), the samples were clarified by centrifugation for 15 minutes at 18,000 rcf and 4 °C, filtered through sterile glass wool and snap frozen to -80 °C until EMSA analysis was carried out.

The probes for EMSA were radiolabelled using modified PCR. Reactions of 50 µl contained 1× PCR buffer (10× = 100mM Tris-HCl pH=8.3, 500mM KCl, 25mM MgCl2, 0.5% Nonidet P40), dATP+dTTP+dGTP 0.2mM each, 0.02mM dCTP, 1mM each primer, 100 ng *Pchs*_H1 DNA fragment as template, 1 U *Taq* polymerase, and 1 pmol α-^{32}P dCTP (0.12 MBq). Subsequently, the PCR product was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Using a scintillation counter, we evaluated the probe activities.

The EMSA procedure was adapted from Maliga et al. (1995). Samples, composed of 75 µg crude extract, 15,000 cpm of probe, 1 µg dsDNA as competitor, 1× binding buffer (10mM Tris-HCl pH=7.5, 40mM NaCl, 4% glycerol, 1mM EDTA, 0.1mM β-mercaptoethanol), and water added to 30 µl, were mixed and kept on ice for 30 min. After that, loading buffer was added (10× = 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol blue) and the samples were run in 4.5% acrylamide gel (19:1) containing 1× TGE buffer (25mM Tris, 0.19M glycine, 1mM EDTA, pH=8.3) at 6 V/cm for 4 hours. The gels were dried and exposed over-night in a PhosphoImager cassette; signal was detected by a Typhoon 9200 Imager (Amersham Pharmacia, Amersham, UK).

4.3. Viroids interfere with hop secondary metabolite composition

Hops are natural host of three viroids genera. During 1952, hop stunt disease emerged in Japan to infect hops, causing stunting, along with abnormal plant growth and a significant decrease in cone yields. The causal agent was later determined to be the hop stunt viroid (HSVd), 297 nucleotides in length (Ohno et al. 1983). Hop latent viroid (HLVd) was first identified as a 256-nt viroid simultaneously occurring with HSVd in hop plants (Pallás et al. 1987). HLVd apparently lacks the detrimental effects of HSVd on hop and is therefore undiscernibly distributed worldwide (Puchta et al. 1988). Still, HLVd is reported to cause bitter acid level changes in infected plants (Barbara et al. 1990). Apple fruit crinkle viroid (AFCVd) has been detected recently in Japan as the third viroid of hops, occasionally causing dwarfing and severe leaf curling in infected plants (Sano et al. 2004).

Viroids comprise of a self-complementary circular single-stranded RNA molecule. Viroids evolved to perform all the processes necessary for their replication and translocation via exploiting the host's molecular machinery that remains largely uncovered (reviewed in Ding 2009). The symptoms, ranging from morphological to metabolic changes, seem entirely dependent on the interaction of viroid and host RNA species. For several reasons, the

involvement of viroid-derived small RNA species (vd-sRNAs) in pathogenesis is plausible (Wang et al. 2004).

Various plant-pathogen interactions also include the biosynthesis of phenylpropanoid compounds. Metabolic changes were reported upon infection by several genera of *Pospiviroidae*, the group HSVd and HLVd belong to (see references in Paper III and IV). The pathogenesis resulting from vd-sRNA is attractive to investigate, because the presumed direct interaction with host RNA world may help reveal regulatory points of symptom-related pathways.

In an attempt to describe the pool of vd-sRNAs generated during a pospiviroid disease, we carried out small RNA sequencing of viroid-infected and viroid-free tomato plants. Using bioinformatic approaches, we subsequently mapped these small RNA sequences along the mature viroid molecule to identify "hot spots", where the majority of small RNAs are generated. This was still a matter of debate for both PSTVd (Itaya et al. 2007; Machida et al. 2007; Diermann et al. 2010) and HSVd (Navarro et al. 2009; Martinez et al. 2010) and appears to be determined by viroid vs. host genetic interaction (Matoušek et al. 2007b), as well as environmental factors, such as temperature (Harris and Browning 1980; Matoušek et al. 2001; Gomez et al. 2008). In addition, we mapped the vd-sRNA pool to the recently sequenced genome of tomato to identify genes potentially affected by small RNA targeting. Four of them, namely TCP3 and VSF growth- and development-related TF, CIPK kinase involved in the cell physiology, and VPE protease as a signalling metacaspase are discussed as potential targets of viroid in Paper III, as they are differentially expressed in healthy and mild strain-infected tomatoes on one side and in severe strain-infected symptomatic plants on the other side.

The following passage (11 pages) is already published in a scientific journal and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

III. Matoušek J, Stehlík J, Procházková J, Orctová L, Wullenweber J, **Füssy Z**, Kováčik J, Duraisamy GS, Ziegler A, Schubert J, Steger G. Biological and molecular analysis of the pathogenic variant C3 of potato spindle tuber viroid (PSTVd) evolved during adaptation to chamomile (*Matricaria chamomilla*). Biol Chem 2012;393:605-15.

Abstract

Viroid-caused pathogenesis is a specific process dependent on viroid and host genotype(s), and may involve viroid-specific small RNAs (vsRNAs). We describe a new PSTVd variant C3, evolved through sequence adaptation to the host chamomile (*Matricaria chamomilla*) after biolistic inoculation with PSTVd-KF440-2, which causes extraordinary strong ('lethal') symptoms. The deletion of a single adenine A in the oligoA stretch of the pathogenicity (P) domain appears characteristic of PSTVd-C3. The pathogenicity and the vsRNA pool of PSTVd-C3 were compared to those of lethal variant PSTVd-AS1, from which PSTVd-C3 differs by five mutations located in the P domain. Both lethal viroid variants showed higher stability and lower variation in analyzed vsRNA pools than the mild PSTVd-QFA. PSTVd-C3 and -AS1 caused similar symptoms on chamomile, tomato, and *Nicotiana benthamiana*, and exhibited similar but species-specific distributions of selected vsRNAs as quantified using TaqMan probes. Both lethal PSTVd variants block biosynthesis of lignin in roots of cultured chamomile and tomato. Four 'expression markers' (TCP3, CIPK, VSF-1, and VPE) were selected from a tomato EST library to quantify their expression upon viroid infection; these markers were strongly downregulated in tomato leaf blades infected by PSTVd-C3- and -AS1 but not by PSTVd-QFA.

Lignin synthesis, branching off the main flavonoid pathway after p-coumarate, is suppressed in PSTVd-infected chamomile (see Paper III). HSVd and HLVd interfere with the biosynthesis of bitter acids (Momma and Takahashi 1984; Barbara et al. 1990; Kawaguchi-Ito et al. 2009), as well. Pospiviroid diseases may be therefore relevant to investigate phenylpropanoid regulation. We chose the interaction model of HSVd × hop 'Admiral' to study the regulation of bitter acids and prenylflavonoid pathways. This is partly due to the presence of lupulin glands on 'Admiral' leaves, accounting for detectable levels of terpenophenolics (De Keukeleire et al. 2003). In addition, despite substantial lupulin gland specificity, *chs*_H1 is significantly expressed in other tissues such as coloured petioles (Matoušek et al. 2002). Changes in the regulatory networks described in Paper II may account for metabolic shifts upon HSVd infection.

In Paper IV, we observed changes in expression of several TF-encoding genes, most notably *HlbHLH2* and *HlMYB3*, and also a marked decrease of *chs*_H1 expression. According to transient co-expression experiments, the latter may be a combined result of TF imbalance and vd-sRNA targeting, both upon HSVd infection. This hypothesis is supported by strain-specific responses of hop 'Admiral' to HSVd, i.e. less imbalanced response to the mild strain compared with the severe strain. We also observed changes in metabolite content, consistent with the downregulation of *chs*_H1.

The following passage (8 pages) is a manuscript recently accepted for publication and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

IV. **Füssy Z**, Patzak J, Stehlík J, Matoušek J. Imbalance in expression of hop (*Humulus lupulus*) *chalcone synthase* H1 and its regulators during hop stunt viroid pathogenesis. J Plant Physiol 2013; http://dx.doi.org/10.1016/j.jplph.2012.12.006.

Abstract

Viroid-derived small RNAs generated during hop stunt viroid (HSVd) pathogenesis may induce the symptoms found in hop cultivar 'Admiral', including observed shifts in phenylpropanoid metabolites and changes in petiole coloration. Using quantitative RT-PCR, we examined hop lupulin gland-specific genes which have been shown to be involved in phenylpropanoid metabolism, for altered expression in response to infection with two HSVd isolates, HSVd-g and CPFVd. Most notably, the expression of a gene encoding a key enzyme for phenylpropanoid synthesis, naringenin-chalcone synthase H1 (*chs*_H1), decreased up to 40-fold in infected samples. In addition, marked decrease in expression of *HlbHLH2* and increase in expression of *HlMyb3* were observed. These two genes encode transcription factors that form a ternary complex with HlWDR1 for *chs*_H1 promoter activation. In a transient expression assay, a decrease in the ternary complex potential to activate the *chs*_H1 promoter was observed upon the decrease of *HlbHLH2* expression. In addition, targeting of the *chs*_H1 transcript by vd-sRNAs may contribute to these expression changes. Our data show that HSVd infection causes a significant imbalance in the expression of phenylpropanoid metabolite-affecting genes via complex mechanism, possibly involving regulatory disorders and direct targeting by vd-sRNA.

4.3.1. Unpublished results– targets of HSVd-derived sRNAs

To clarify HSVd pathogenesis in hop, we searched sequence databases for putative vd-sRNA targets, and assayed whether these hop genes alter expression in symptomatic leaves.

A list of all possible HSVd-derived 25-bp sequences was screened against the *H. lupulus* sequence data (both nr and EST accessions from GenBank) publicly available in September 2011 using psRNATarget (Dai and Zhao 2011). The list of 564 accessions with higher than medium probability expectation (≥2.5), was submitted to BLAST 2.0 (Altschul et al. 1997) and AmiGO (Ashburner et al. 2000) to assign putative functions, resulting in 46 sequences similar to proteins with known functions. These functions can be grouped to kinases and TFs regulating gene expression (3 accessions), proteins involved in nutrition, stress, and developmental signaling (19), RNA processing (2), enzymes (17) including proteases (5) and transporter proteins (8), resistance-related proteins (3) and/or photosynthesis (4). Nine candidates for mRNA quantification were selected according to possible link with observed HSVd symptoms. Importantly, *chs*_H1 proved as a good target using this bioinformatic prediction (see Paper IV).

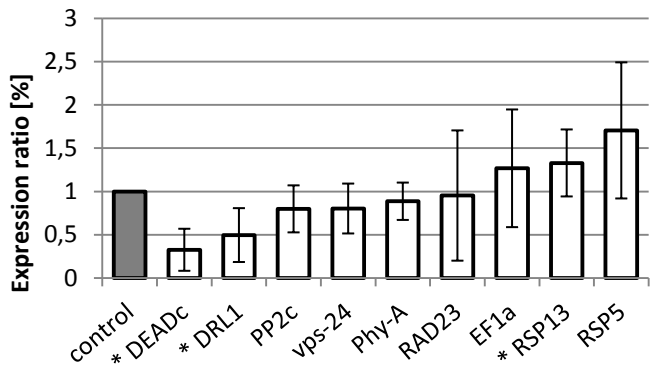

Fig. 11: Changes in candidate target levels upon HSVd-g infection in leaves. The data points show the means of all obtained samples for each variant, bars represent S.E. of these data sets, and asterisks indicate significantly altered genes.

Using the material and methods described in Paper IV, we observed that transcript levels of *DEADc*, putatively encoding a stress-suppressing RNA helicase, and *DRL1*, possibly involved in meristem activity and organ growth, were significantly lower in diseased material (Fig. 11). Increased levels were observed for a transcript showing similarity to ribosomal protein S13-encoding genes (Fig. 11). Further analyses are needed to be carried out to confirm our data and elucidate the roles of these putative targets.

5. CONCLUSION AND FUTURE PROSPECTS

Undoubtedly, transcription factors play important roles in cellular and systemic responses to variety of external and internal signals via target gene regulation. Their concerted interactions enable changes in cells' metabolism and development relevant to the actual set of environmental conditions. Production of 'secondary metabolites' such as flavonoids is regarded as an essential and complex response to developmental, pathogen- and stress-related cues. For this purpose, large families of transcriptional regulators evolved, including Myb, bHLH, bZIP, MADS and WRKY TFs. Their involvement in model plants was reviewed in this work, inspiring research in a non-model crop, *H. lupulus*.

Two main methodical approaches are used to investigate the functions of TFs: on the gene and the protein level. The genetic level includes searching genomic and cDNA libraries for TF genes and analysing their coding and surrounding regulatory sequences using bioinformatics. Within this framework, I identified regulatory sequences of the recently cloned HlMYB1 promoter that are to be functionally characterised in a future work. Bioinformatic analyses were also helpful in identifying HSVd-derived small RNA targets in hops.

Protein investigation includes purification and methods of DNA-binding analysis, e.g. EMSA and ChIP. Protein-protein interaction and transactivation properties are analysed *in vitro* and *in vivo* by one- and two-hybrid systems, as well as similar procedures carried out *in planta* based on transient expression. I carried out EMSA analyses to prove the interaction of HlMYB3-HlbHLH2-HlWDR1 with a promoter fragment of *chs*_H1. Unsatisfactory results were obtained using B2H system to underline protein-protein interactions of MBW components as determined by transient expression system with a *Pchs*_H1:GUS reporter.

For further understanding of protein functions, mutational analyses are frequently carried out. Basic information is obtained by observing phenotype in "loss of function" and "gain of function" mutants, while site-directed mutagenesis might be pointed at protein activity connected with DNA binding or post-transcriptional regulatory motifs including phosphorylation, dimerisation and trans-activation domains. Random mutagenesis or DNA-shuffling methods may help improving TF activity (DNA-binding or transactivation) or clarify the involvement of particular aminoacids. To clarify the roles of putative casein kinase II phosphorylation sites characteristic of Group A bZIP factors, I used site-directed mutagenesis of HlbZIP1A, resulting in a protein of higher activation potential and/or molecular stability.

During the past years, there has been a major increase in genomic and expression data, largely produced by high throughput sequencing technologies. It remains a huge challenge, however, to understand the flavonoid metabolism in detail, with the entirety of structural genes involved and the complexity of transcriptional and hormonal regulation. Non-targeted approaches correlating co-expression data and metabolomics at the organ or tissue level can provide solutions to unravel parts of this intriguing puzzle (Tohge et al. 2005). Still, detailed analyses of single tiles of this puzzle may provide valuable information in non-model plants such as hop. This field of study, thus, remains challenging and might offer many interesting theoretical and practical outcomes.

6. CURRICULUM VITAE

Citizenship: Slovak Republic; **date of birth:** 29 January 1986, Dunajská Streda, Slovak Republic.

Research interests: transcriptional regulation, small RNAs, molecular genetics

Education and Employment:

2013- : Post-doctoral fellow at Laboratory of Evolutionary Protistology, Biology Centre CAS, v.v.i., Institute of Parasitology, České Budějovice.

2008-2013: Ph.D. student (expected finish 03/2013) in Molecular and Cell Biology and Genetics, University of South Bohemia, České Budějovice, Czech Republic. Employed at Laboratory of Molecular Genetics, Biology Centre CAS, v.v.i., Institute of Plant Molecular Biology, České Budějovice. Ph.D. Thesis named „Structural and functional analysis of selected hop (Humulus lupulus) regulatory factors".

2003-2008: Student (Mgr. equiv. MSc.) in Experimental Biology from Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Master Thesis named „Expression analysis of selected regulation factors in hop with relation to symptoms of viroid pathogenesis."

Scientific experiences:

9/2012 Genomics Workshop, České Budějovice.

9-11/2010 Bioinformatics, small RNA library construction experiments. Short stay at the Institute of Physical Biology, Heinrich-Heine University Düsseldorf, Germany.

2006-2007 High-performance liquid chromatography experiments. Short stays at the Faculty of Pharmaceutical Sciences, Ghent University, Belgium.

3/2004 Work with radioactive isotopes, České Budějovice.

Teaching activities:

Genetics I – (laboratory courses; in Czech) – co-lecturer

Basic methods of genetic engineering – (laboratory courses; in Czech) – co-lecturer

7. ABBREVIATIONS

4-CL	– 4-coumaroyl CoA-Ligase
AD	– activation domain
AN1/2/11	– Anthocyanin phenotype-related proteins
ANR	– anthocyanin reductase
B2H	– bacterial two-hybrid system
BS	– binding site
bHLH	– basic Helix-Loop-Helix
C4H	– cinnamate 4-hydroxylase
CHI	– chalcone isomerise
ChIP	– chromatin immunoprecipitation
CHS	– chalcone synthase
CoA	– coenzyme A
COP1	– Constitutive Photomorphogenesis1 protein
Cox-1	– cyclooxygenase 1
CRY	– cryptochrome
Cyp1A	– cytochrome P450 1A
DBD	– DNA-binding domain
DFR	– dihydroflavonol 4-reductase
DMAPP	– dimethylallyl pyrophosphate
EGL	– Enhancer of Glabra3 bHLH protein
EMSA	– electrophoretic mobility shift assay
EST	– expressed sequence tag
F3'H	– flavonoid 3' hydroxylase
F3'5'H	– flavonoid 3'5' hydroxylase
F3H	– flavanone 3-dioxygenase
FLS	– flavonol synthase
FSI	– flavone synthase I
FSII	– flavone synthase II
GFP	– green fluorescent protein
GL1/3	– GLABRA phenotype-related proteins
GUS	– β-glucuronidase
HY5	– Elongated Hypocotyl5
HYH	– HY5-homolog
I2'H	– isoflavone 2' hydroxylase
IFR	– isoflavone reductase
IFS	– isoflavone synthase
iNOS	– inducible nitric oxide synthase
IPP	– isopentenyl diphosphate
L2BW	– MYBL2/CPC-bHLH-TTG1 transcription factor complex
LAR	– leucoanthocyanidin reductase
LDOX	– leucoanthocyanidin dioxygenase/anthocyanin synthase
LRU	– light-response unit, a *cis*-regulatory site
MBW	– a general MYB-bHLH-WDR transcription factor complex
miRNA	– microRNA
OMT	– O-methyltransferase
QR	– NAD(P)H:quinone reductase

qRT-PCR	– quantitative reverse transcription-PCR
PA	– proanthocyanidin
PAL	– phenylalanine ammonia lyase
PAP	– Production of Anthocyanin Pigment MYB protein
PFG	– Production of Flavonol Glycosides MYB protein
PHY	– phytochrome
PIBP, PIVP	– phlorisobutyrophenone, phlorisovalerophenone
PKSIII	– polyketide synthase type III superfamily
ROS	– reactive oxygen species
SG	– subgroup
STS	– stilbene synthase
TAL	– tyrosine ammonia lyase
ta-siRNA	– trans-acting silencing RNA
TT2/8/12	– Transparent Testa phenotype-related proteins
TTG1/2	– Transparent Testa Glabra phenotype-related proteins
TF	– transcription factor
UF3GT	– UDP-glucose:flavonoid 3-O-glucosyltransferase
UVR8	– UVB-Resistance 8 protein
vd-sRNA	– viroid-derived small RNA
Y2H	– yeast two-hybrid system
XN	– xanthohumol

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