UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI BIOTECNOLOGIE

SCUOLA DI DOTTORATO DI SCIENZE INGEGNERIA MEDICINA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE APPLICATE

CICLO XXV

THE MODIFICATION OF METABOLIC PROFILE IN PLANT CELL CULTURES AS A STRATEGY TO INVESTIGATE THE BIOLOGICAL ROLE OF SECONDARY METABOLITES

Coordinatore: Prof.ssa Paola Dominici

Tutori: Dott.ssa Flavia Guzzo
Prof.ssa Marisa Levi

Dottoranda: Pamela Strazzer

THE MODIFICATION OF METABOLIC PROFILE IN PLANT CELL CULTURES AS A STRATEGY TO INVESTIGATE THE BIOLOGICAL ROLE OF SECONDARY METABOLITES

Pamela Strazzer

CONTENTS

Chapter 1

General introduction

Secondary metabolites are a way by which plants communicate or respond to external stimuli (Bouwmeester et al., 2007; Maffei, 2010; Rasmann and Turlings, 2008; Frost et al., 2008). Their apparent lack of primary function in the plant, combined with the observation that many secondary metabolites have specific negative impacts on other organisms such as herbivores and pathogens, led to the hypothesis that they have evolved because of their protective role.

Each plant species has its own ability to synthesize and accumulate a very specific set of secondary metabolites, so that they can sometimes be used as taxonomic characters in classifying plants. Such remarkable variety and diversity of wild type secondary metabolite profiles depends, at least partially, on the ability of plant cells to chemically link secondary metabolites each other and with other molecules such as organic acids, amino acids and sugars. Furthermore, the secondary metabolism of plants, and the expressed metabolite levels, may further change considerably due to the influence of several biotic and abiotic stress signals (Pavarini et al., 2012).

The possible physiological role of the presence and relative abundance of different secondary metabolites and the interactions between these different compounds have not been explored in details for most of them. One way to investigate the biological role is to modify the secondary metabolome and investigate the impact of such modifications on the phenotype. However, the complexity of plant metabolome makes difficult to recognise the role of the various secondary metabolites during a specific stress response.

In vitro cell cultures as a model to study the role of secondary metabolites

Unlike field-grown plants, *in vitro* plants and *in vitro* cultures (e.g., calluses, cell suspensions, tissues, organs) are cultivated independently from climate, soil, season, day length and weather conditions. Beside their use in the large-scale production of

biochemicals, they may be an useful tool for basic studies on plant biochemistry and molecular biology.

In vitro organ cultures or intact plants can be suitable for studying tissue-specific biosynthetic pathways, which are not always expressed in cell suspension cultures. On the other hand, *in vitro* plant cell suspension cultures are more convenient to investigate the cellular and molecular processes as they offer the advantage of a simplified model system. Indeed, cell suspension cultures allow rapid and uniform access to nutrition, precursors, growth hormones and signal compounds for the cells (Mustafa et al., 2011) and a better control of external factors. Cell cultures have an higher rate of metabolism than intact plants and biosynthetic cycles compressed into shorter time periods (Zenk, 1991).

Although usually they are defined as “undifferentiated”, because not organized into tissues and organs, in plant cell cultures co-exist different sub-populations of cells, differing in morphology, gene expression, epitopes, morphogenetic capacity and ability to produce different chemicals (Guzzo et al., 2002). Due to this phenotypic difference in cell mixture, the cell lines can be guided to reach a desired phenotype in terms of accumulation of specific metabolites, through visual selection processes (e.g. the selection of red cells accumulating anthocyanin from non-pigmented cell lines). This long and often unpredictable selection activity can finally yield a stable cell line, which maintains relatively constant metabolic traits.

Furthermore, *in vitro* cultivated plant cells, that retain many biosynthetic properties of the plant tissues, are still well able to accumulate different classes of phenylpropanoids, such as hydroxycinnamic acids, hydroxybenzoic acids, coumarins, stilbenes, as well as flavonoids, including anthocyanins. The accumulation of biochemically related molecules depends on highly complex networks of production and consumption. As consequence*, in vitro* plant cell cultures, selected for desired features, have been used as a simplified model system in which to perform alternative

approaches to modify the secondary metabolome in order to clarify the biological role of different classes of secondary metabolites.

Biosynthesis of phenylpropanoids

The upstream part of the phenylpropanoid metabolism consists of three enzymatic steps leading to 4-coumaroyl CoA (Fig. 1.1). The first step is the deamination of the amino acid phenylalanine, derived from the shikimate pathway, to generate cinnamic acid. The phenylalanine ammonia lyase (PAL) enzyme is a major switch to channel organic carbon from primary to secondary metabolism. The subsequent reaction is the hydroxylation of the aromatic ring of cinnamic acid in *para* position (carbon 4) by cinammate-4-hydroxylase (C4H), which generates *p*-coumaric acid. C4H enzyme is a member of the structural family of cytochrome P450 proteins, which catalyse monooxygenation of a broad range of substrates within all organisms (Werck-Reichhart and Feyereisen, 2000).

p-coumaric acid represents a key molecule in the phenylpropanoid pathway, leading to the formation of different groups of metabolites, including: 1) metabolites sharing the C6-C3 carbon framework (hydroxycinnamic acids and their alcoholic derivatives, monolignols, which are precursors of lignans and lignin and some coumarins); 2) metabolites deriving from the extension of the C6-C3 framework (stilbenes and flavonoids, including anthocyanins).

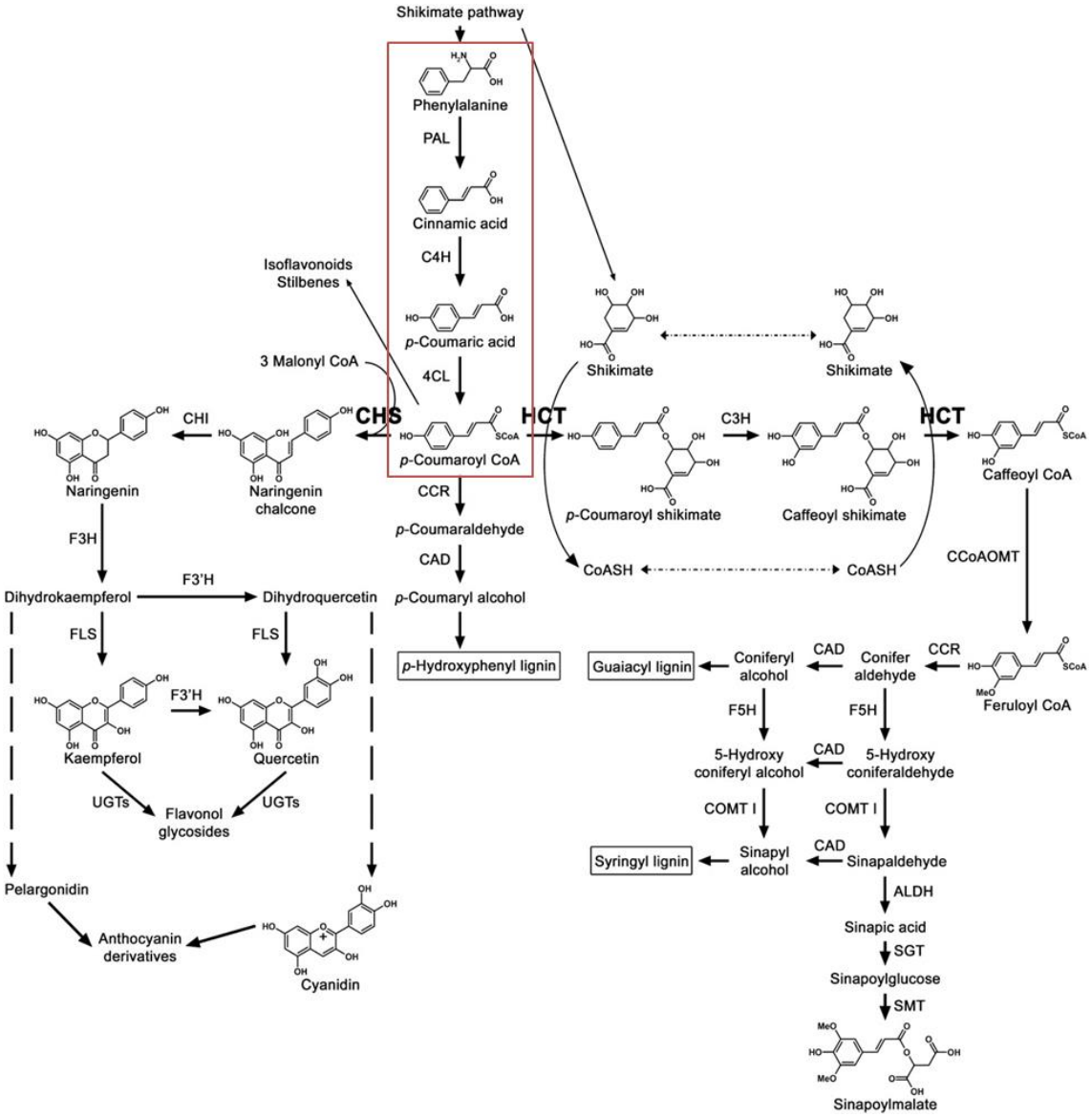


Fig. 1.1 The phenylpropanoid pathway (Besseau et al., 2007).

Among the hydroxycinnamic acids (C6–C3 carbon framework), *p*-coumaric, caffeic, ferulic, and sinapic acids, shown in the Fig. 1.2, are the most common (Bravo, 1998). The generation of caffeic acid, which differs from *p*-coumaric acid for an additional hydroxylation of the aromatic ring, is still under debate. This molecule could be directly produced by the *meta*-hydroxylation of the aromatic ring (carbon 3) of the *p*-coumaric acid by a cytochrome P450 hydroxylase (C3H). However, in *Arabidopsis thaliana,* sweet basil and coffee trees, the C3H enzymes, do not use free *p*-coumaric acid as

substrate, but its quinate and shikimate ester to produce caffeoyl quinic and caffeoyl shikimic acid (Mahesh et al., 2007; Schoch et al., 2001; Gang et al., 2002). The biosynthesis of the *p*-coumaroyl quinic or shikimic acids, which are the substrates of the hydroxylation reaction catalysed by C3H, requires the previous activation of the *p*-coumaric acid as *p*-coumaroyl-CoA thioester by the *p*-coumaroyl-CoA ligase enzyme (4CL). The *p*-coumaroyl-CoA is in turn used as substrate for coumaroyl quinic or shikimic acid production by the transfer of an acylic group from one molecule to another. The enzymes that direct this transesterification reaction are the hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), (Hoffmann et al., 2003), and the hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT), (Sonnante et al., 2004; Niggeweg et al., 2010).

The other hydroxycinnamic acids (HCAs) are synthetized from caffeic acid and/or caffeic acid derivatives by an O-metylation reaction that leads to the generation of ferulic acid. A further hydroxylation produces hydroxyferulic acid and a second O-methylation generates sinapic acid. These reactions are directed by O-methyltransferase (OMT) and cytochrome P450 hydroxylases.

The HCAs are usually further modified. The conjugation with mono and oligosaccharides to generate glycosilated derivatives appears to use free HCAs and are catalysed by UDP-glucose:glucosyltransferase (UGT) enzymes (Lim et al., 2003), while the conjugation with other molecules (organic acids, amino acids and polyammines) seems to depend mainly on acyl-transfer reactions using HC-O-glucose esters or HC-CoA as acyl donors (Stehle et al., 2009). The formation of more complex structures with anthocyanins or other flavonoids, requires other transferases that catalyse the transfer of HCAs on the phenylpropanoid acceptor molecules using the same acyl donor molecules (Stehle et al., 2009). Furthermore HCAs are precursors of monolignols, such as *p*-coumaryl, coniferyl and sinapyl alcohol, that are required for lignan and lignin biosynthesis.

In response to wounding numerous plant species accumulate HCAs esters, that can act directly as defense compounds or can strengthen the cell wall (Hahlbrock and Scheel, 1989; Bernards and Lewis, 1992). Moreover, epidermal located HCAs are considered, as flavonoids, ultraviolet (UV)-absorbing phenolic compounds, that can shield the underlying tissues in plants against harmful UV-radiation (Burchard et al., 2000).

HCAs exhibit high antioxidant activity *in vitro* (Kikuzami et al, 2002). Compared to the corresponding hydroxybenzoic acids (compound with C6–C1 structure, such as gallic, *p*- hydroxybenzoic, protocatechuic, vanillic and syringic acids), the HCAs show an higher activity, that could be due to the CH=CH–COOH group, which ensures greater H-donating ability and radical stabilization than the –COOH group in the hydroxybenzoic acids (Rice-Evans et al., 1996).

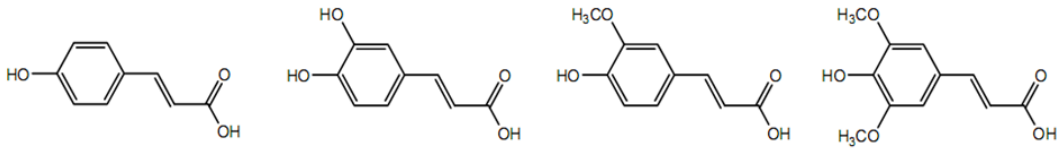


Fig. 1.2 Chemical structure of the most common hydroxycinnamic acids.

Flavonoids belong to the large family of phenolic compounds, including condensed tannins, aurones, isoflavonoids, flavones, flavonols, flavanols, and anthocyanins (Fig. 1.3). Except for the aurones (C6–C2–C6), all flavonoids display a C6–C3–C6 skeleton structure (Harborne and Williams, 2000; Marais et al., 2006).

Plant species have exploited the flavonoid chemical diversity in unique ways, and flavonoids that have important functions in one plant might not serve a similar function in another.

The chalcone synthase (CHS), a polyketide synthase, is the first enzyme in the flavonoid biosynthetic pathway, and catalyzes the condensation of three acetate units from *p*-malonyl-CoA with *p*-coumaroyl-CoA. This reaction, that generates a molecule of tetrahydroxychalcone (THC), builds the second aromatic ring (B) of flavonoids. THC is rapidly isomerized to the colorless naringenin (flavanone) by chalcone isomerase (CHI), which closes the central heterocyclic ring (C) producing the typical flavonoid structure.

The classification of different flavonoid classes depends on the oxidation level of the central C heterocycle: the formation of a double bond between C2 and C3 generates the flavones; the migration of the ring B from C2 to C3 and the concomitant formation of the C2-C3 double bond produces the isoflavones; the hydroxylation of C3 results in the production of flavonols (with C2-C3 double bond) and flavandiols (without the C2-C3 double bond and without the oxygen bound to C4), which in turn can be used to generate procyanidins and anthocyanins.

The individual flavonoids differ also for the presence of hydroxyl and methyl substitutions on the A and B rings, and also on supplemental modifications such as glycosylation, acylation, and polymerization (Kong et al., 2003; Aron and Kennedy, 2008).

A wide array of biological roles have been attributed to flavonoids. Flavonoids attract pollinators in flowers and serve as deterrent against herbivores, but also screen photosynthetically-active radiation to protect plant cells from excess of light and scavenge reactive oxygen species, although this role *in vivo* is a matter of debate (Hernández et al., 2010). Hernández et al. (2010) proposed, in addition to well-known functions, that flavonoids may play a role as energy escape valves. The synthesis of flavonoids consumes more energy and photoassimilates than that of other simple phenylpropanoids, without sequestering any nitrogen or phosphorous, which may make flavonoids a more efficient excess energy outlet compared to simple

phenylopropanoids. On the other hand, there is growing evidence that flavonoids also act as signalling molecules within plant cells. The best described example is the regulation of polar auxin transport of flavonoids, particularly flavonols like quercetin (Peer and Murphy, 2008).

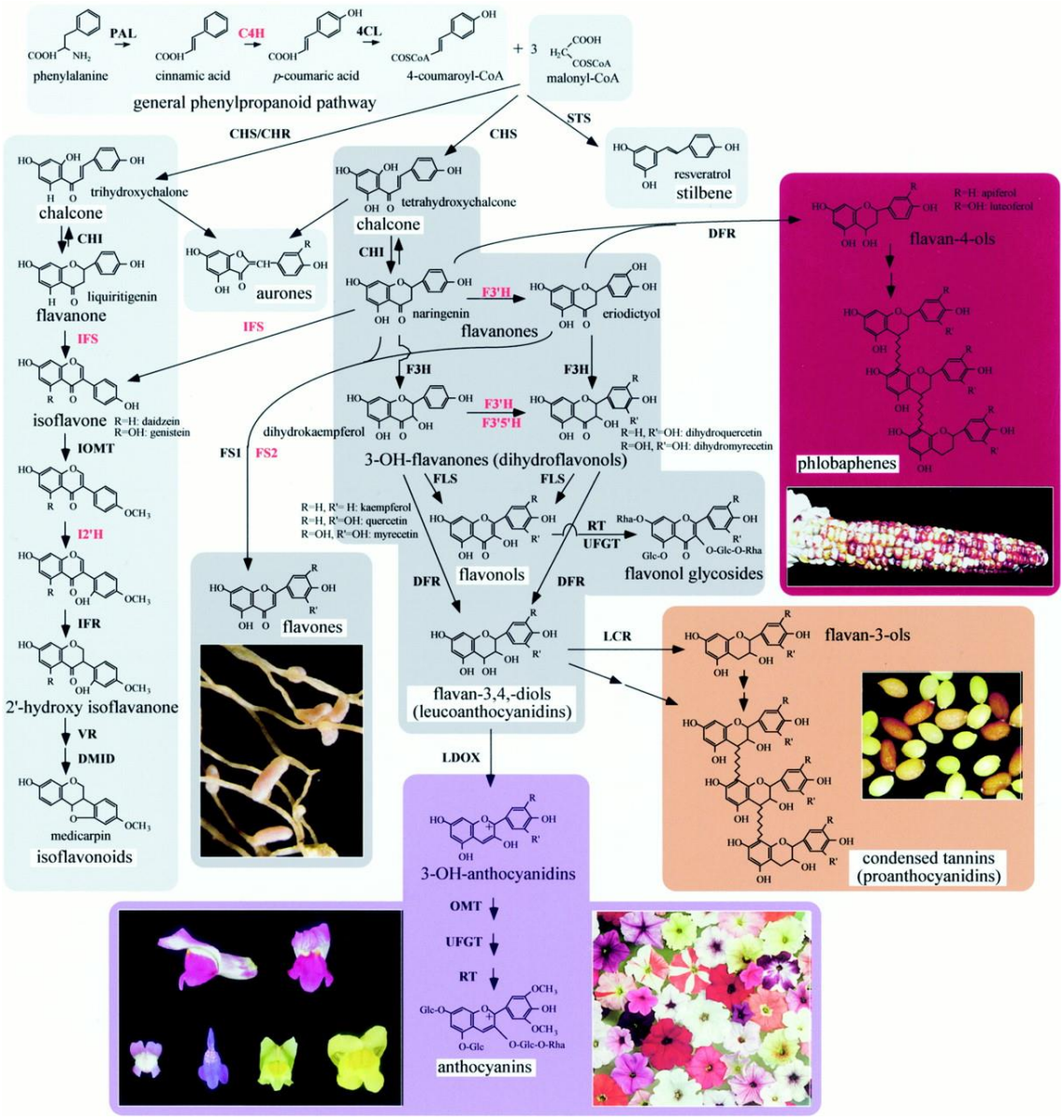
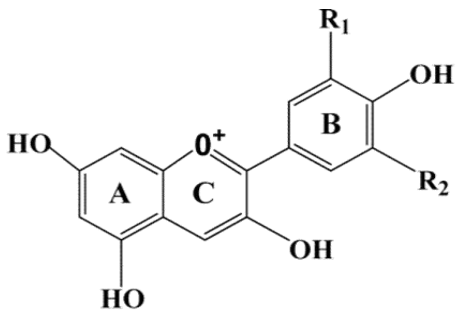


Fig. 1.3 Schematic of the major branch pathways of flavonoid biosynthesis, starting with the general phenylpropanoid metabolism and leading to the major subgroups: the colorless chalcones, aurones, isoflavonoids, flavones, flavonold, and flavandiols (grey boxes) and anthocyanins, condensed tannins and, phlobaphene pigments (colored boxes) (Winkel-Shirley, 2001).

Although the presence of flavonols is well documented in a number of subcellular localizations (chloroplasts, nuclei, cytosol, etc.), when in massive amounts, they usually accumulate in the vacuole or the apoplast.

Anthocyanins, one of the most ubiquitous classes of flavonoids, were described as 'Nature's Swiss army knife' by Gould (2004). The basic structure of anthocyanins, called "flavylium ion", can show a different degree of hydroxylation and methoxylation, giving rise to different types of molecules including the most common cyanidin, delphinidin, peonidin, petunidin, malvidin and pelargonidin, shown in Fig.1.4. The degree and position of hydroxylation and methoxylation in the B ring affects their stability, reactivity and also the antioxidant property (Pereira et al., 1997; Rice-Evans et al., 1996). Indeed, besides the obvious functions, such as in attracting pollinators and seed dispersers, anthocyanins are believed to be involved in protection from oxidative damage by scavenging reactive oxygen species.



AGLYCONE	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Fig. 1.4 General structural formula of anthocyanin and substitution patterns of some common anthocyanins.

Further modifications such as glycosylation and acylation lead to the formation of a wide range of complex structures (Fig. 1.5). The primary role of glycosylation seems to be the stabilization of the molecule against nucleophilic attack or enzymatic degradation (Ford et al., 1998). Sugar groups attached to anthocyanins can be

acylated with residues from aromatic (hydroxybenzoic and hydroxycinnamic acids) or aliphatic (malonic acid, acetic acid) acids. It has been proposed that aromatic acylation makes anthocyanins more stable by copigmentation with polyphenols and that the aliphatic acylation is important for enhancing pigment solubility in water, protecting glycosides from enzymatic degradation, stabilizing anthocyanin structures, and promoting the uptake of anthocyanins into vacuole (Nakayama et al., 2003).

The relationship between the chemical structure and physical-chemical properties of anthocyanins, such as light absorption, stability, reactivity and interactions with other molecules, has been elucidated by several investigators (Kähkönen and Heinonen, 2003; Giusti and Wrolstad, 2003; de Freitas and Mateus, 2006). However, little is known about the relationship between the biological function of these pigments and their chemical structure. Moreover the existence of such a diverse range of types of anthocyanins, in different species but also in the same species and even in the same type of cells, raises the question whether these different compounds have different physiological functions.

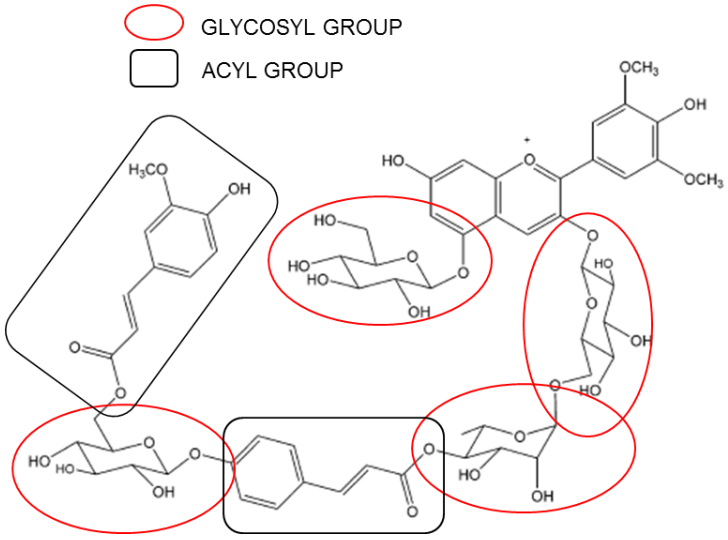


Fig. 1.5 Anthocyanin found in *Petunia hybrida* cv 'Festival' (Gonzalez et al, 2001). The malvidin derivative is characterized by a 3-syde-chain featuring alternating glucosyl, rhamnosyl, coumaroyl, glucosyl and feruloyl groups. Glycosyl groups are indicated by red circles and acyl groups by black rectangles.

Research aims

Secondary metabolites are present in all higher plants, in a high structural diversity, giving rise to complex patterns. They are considered necessary for plant adaptation to the variable environment; however, only for few secondary metabolites the specific functions are known in detail.

One possible approach to investigate the roles of secondary metabolites in plants is to modify the secondary metabolic profile and study the impact of such modifications on the phenotype.

The general experimental approach in use in the research group, in which the experimental work for this project have been performed, consists in different steps. The first step is to perform a metabolic analysis of the methanolic cell extract to identify the specific metabolic profile of the cell culture under investigation. In the second step short stresses (e.g. heat shock) are applied to cells and stressed cells are characterized from a cytological point of view in order to identify specific phenotypic traits caused by stress application. Finally, the metabolic profile of the cells is modified by precursor and inhibitor administration strategies, that are the main object of this research project, and the effects of the modification of metabolic profile on stress responses are evaluated. The main goal of the present research is to develop experimental strategies to modify the secondary metabolome in *in vitro* cell cultures by 1) the use of specific inhibitors of some of the early enzymes of phenylpropanoid metabolism and 2) feeding cells with biosynthetic precursors of phenylpropanoids.

In vitro cell cultures under investigation

Three different cell lines derived from three different species, T2b (*Ocimum basilicum*), Sw4i (*Petunia hybrida*), R3M (*Daucus carota*) (Fig. 1.6), have been chosen since they showed three different type of metabolic profiles in terms of secondary metabolites,

especially in phenylpropanoid composition. All these lines have been selected for the stable accumulation of anthocyanins, in the light or in the dark, and they accumulated also other flavonoids, hydroxycinnamic acid and hydroxybenzoic acid derivatives. The diversity in metabolic profile of these cell cultures allowed to develop different strategies in order to modify the ratios of accumulation of different secondary metabolites. Each of this cell lines potentially showed advantages and disadvantages for the purposes of this project.

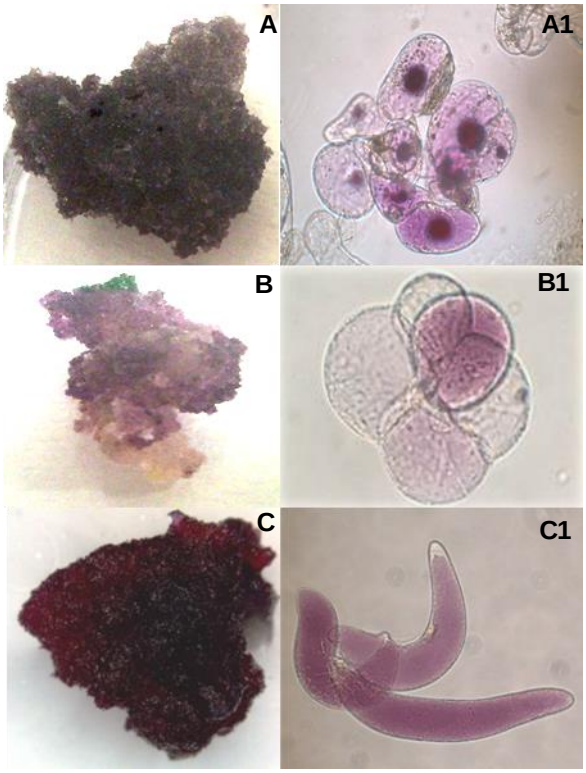


Fig. 1.6 Callus cultures (A, B, C) and cells in suspension (A1, B1, C1) derived from T2b line of *Ocimum basilicum*, Sw4i line of *Petunia hybrida*, R3M line of *Daucus carota*, respectively. Some different features can be observed: basil callus is the most dark, probably due to the different accumulation of anthocyanins in T2b cells, packed in anthocyanin vacuolar inclusions (AVIs); petunia callus shows heterogeneity in the pigmentation as well as cells in suspension that are not completely pigmented, whereas carrot callus is the most friable and, as in petunia cells, anthocyanins are accumulated only as soluble material in the vacuole.

Basil cell lines

Ocimum basilicum, as *Rosmarinus officinalis, Salvia officinalis,* and *Perilla frutescens*, belongs to Lamiaceae species (subfamily Nepetoideae). For these species it has been reported the capability to accumulate high basal levels of rosmarinic acid, a hydroxycinnamic ester, deriving from caffeic acid (Zgórka and Glowniak, 2001). The rosmarinic acid levels remain high even in the red varieties, that accumulate also anthocyanins.

The biosynthesis of rosmarinic acid, including the several enzymes involved, has been elucidated in suspension cultures of *Anchusa officinalis* (Boraginaceae) and *Coleus blumei* (Lamiaceae) (Fig. 1.7; Petersen et al., 1993; Petersen, 1997).

The first step in the rosmarinic acid biosynthesis involves the deamination of L-phenylalanine to *t*-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL). The following reaction is the hydroxylation of *t*-cinnamic acid in position 4 for the generation of 4-coumaric acid by the cytochrome P450 monooxygenase cinnamate 4-hydroxylase (CAH) which has been characterized from suspension cells of *Coleus blumei* (Petersen, 1997). It is generally accepted that hydroxycinnamic acids have to be activated before further reactions can take place (Petersen and Simmonds, 2003). In most cases a coenzyme A thioester is formed, but also glucose esters or other cinnamic acid esters, e.g. chlorogenic acid have been shown to be able to serve as donors of hydroxycinnamic acid moieties (Petersen and Simmonds, 2003).

The other amino acid precursor for the rosmarinic acid formation is the tyrosine. The first reaction involves the transamination of L-tyrosine with 2-oxoglutarate to the formation of 4-hydroxyphenylpyruvate by the pyridoxalphosphate-dependent transaminase tyrosine aminotransferase (TAT). In the following step 4-hydroxyphenylpyruvate is reduced to the corresponding 4-hydroxyphenyllactate by hydroxyphenylpyruvate reductase (HPPR). The resulting hydroxyphenyllactate is

accepted by the hydroxycinnamoyl transferase, (rosmarinic acid synthase, RAS) to generate the ester. Under release of coenzyme A, the ester linkage is formed between the carboxyl group of 4-coumaric acid and the aliphatic hydroxyl group of 4-hydroxyphenyllactate. The resulting ester is 4-coumaroyl-4'-hydroxyphenyllactate which is hydroxylated in positions 3 and 3' of the aromatic rings by two cytochrome P450 monooxygenases for the generation of rosmarinic acid (Petersen, 1997).

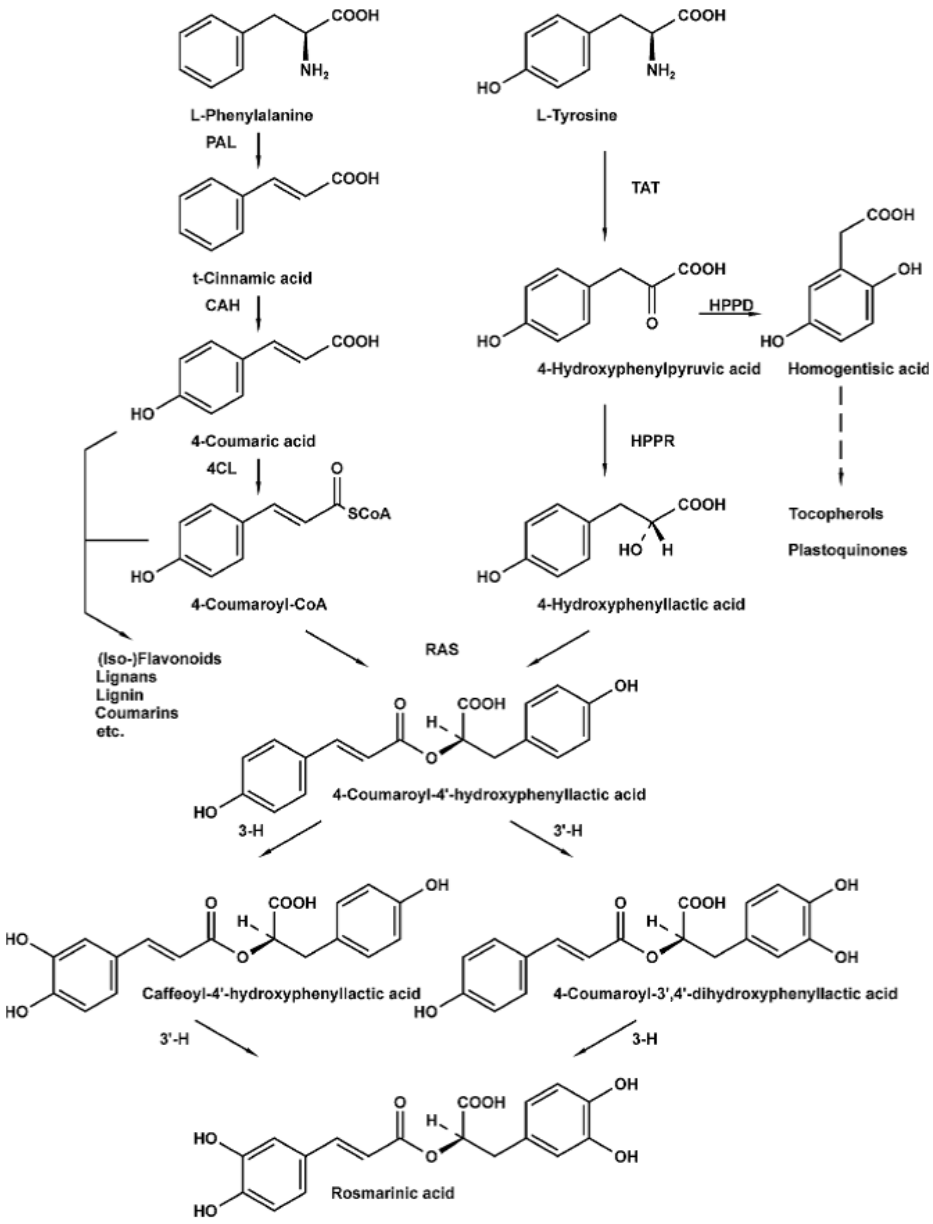


Fig. 1.7 Biosynthetic pathway for rosmarinic acid as found in suspension cultures of *Coleus blumei* (Petersen and Simmonds, 2003).

cDNAs encoding cytochrome P450s, belonging to the CYP98 family, with the capability of hydroxylation to generate esters (e.g. 4-coumaroyl-shikimate) from 4-coumaric acid or 4-coumaroyl moiety, have been unraveled recently (Schoch et al., 2001; Franke et al., 2002; Anterola et al., 2002). Furthermore it has been proposed an alternative biosynthetic pathway for rosmarinic acid in *Menta arvense* and *Mentha piperita*, in which not only RAS, but also HPPR is able to use as substrate a 3,4-dihydroxylated form, the 3-idroxy-tyrosine DOPA (Ellis and Towers, 1970).

Rosmarinic acid and anthocyanins share an initial common biosynthetic pathway from phenylalanine to 4-coumaroyl CoA. Indeed, 4-coumaroyl CoA is the substrate for rosmarinic acid synthase (RAS) in the biosynthesis of rosmarinic acid and for chalcone synthase (CHS) for the formation of flavonoids, including anthocyanins. Furthermore, they show similar or overlapping biological functions. Indeed, as reported also for anthocyanins, rosmarinic acid is a well-known antioxidant and scavenger of reactive oxygen species (ROS), and is implicated in the prevention of lipid peroxidation, photoprotection and protection from ionizing radiation (Sánchez-Campillo et al., 2009). Its biological activity has been deeply investigated from a pharmacological perspective for the interesting health-promoting effects (reviewed by Petersen and Simmonds 2003; Jiang et al., 2005).

Cell cultures producing both anthocyanins and rosmarinic acid represent an interesting experimental system to study the modulation and interactions of these metabolic pathways. The red suspension culture of *Ocimum basilicum*, T2b, showed anthocyanins accumulation in the dark, even in the presence of high levels of rosmarinic acid. However, T2b cells allowed only limited cytological analysis because of the cell clustering.

Petunia cell lines

Petunia species represents one of the best models for the study of flavonoid biosynthesis. Indeed, the use of genetic and molecular approaches have allowed the isolation and characterization of a large number of genes affecting flower color, including those encoding biosynthetic enzymes, regulators of their expression, and vacuolar functions (Tornielli et al., 2002).

In the first step of anthocyanin and flavonol biosynthetic pathway (Fig. 1.8), chalcone synthase (CHS) catalyzes the condensation of three molecules of malonyl-CoA and 4-coumaroyl-CoA. In the following step chalcone isomerase (CHI) catalyzes tetrahydroxychalcone to colorless naringenin. The subsequent hydroxylation of flavanone by flavanone 3-hydroxylase (F3H) leads to the formation of dihydrokaempferol (DHK). The DHK can be hydroxylated by flavonoid 3- hydroxylase (F3H) to generate dihydroquercetin (DHQ) or by flavonoid 3,5-hydroxylase (F3,5H) to produce dihydromyricetin (DHM). Afterwards the colorless dihydroflavonols (DHK, DHQ and DHM) could be converted into anthocyanins. However, petunia does not synthetize pelargonidin-type anthocyanins, since the dihydroflavonol reductase (DFR) in this species cannot reduce DHK to leucopelargonidin, while it converts DHM to leucodelphinidin with high efficiency, and DHQ to leucocyanidin with low efficiency (Tornielli et al. 2002). The conversion of leucocyanidin and leucodelphinidin to the corresponding colored anthocyanidins is catalyzed by anthocyanidin synthase (ANS). Modifications of anthocyanidins encompass glycosylation, acylation, and methylation steps. All anthocyanins found in flowers of petunia are glucosylated at the 3-position. Depending on the genetic background, anthocyanin 3-glucosides can be modified by sequential rhamnosylation, acylation (generally with *p*-coumaric acid), glucosylation at the 5-position, and methylation at the 3'- and 3',5'-positions. Methylation at the 3' position in cyanidin- type anthocyanins generates peonidins, whereas delphinidin- type

anthocyanins methylated at the 3' position form petunidins; a second methylation at the 5'-position gives rise to malvidins.

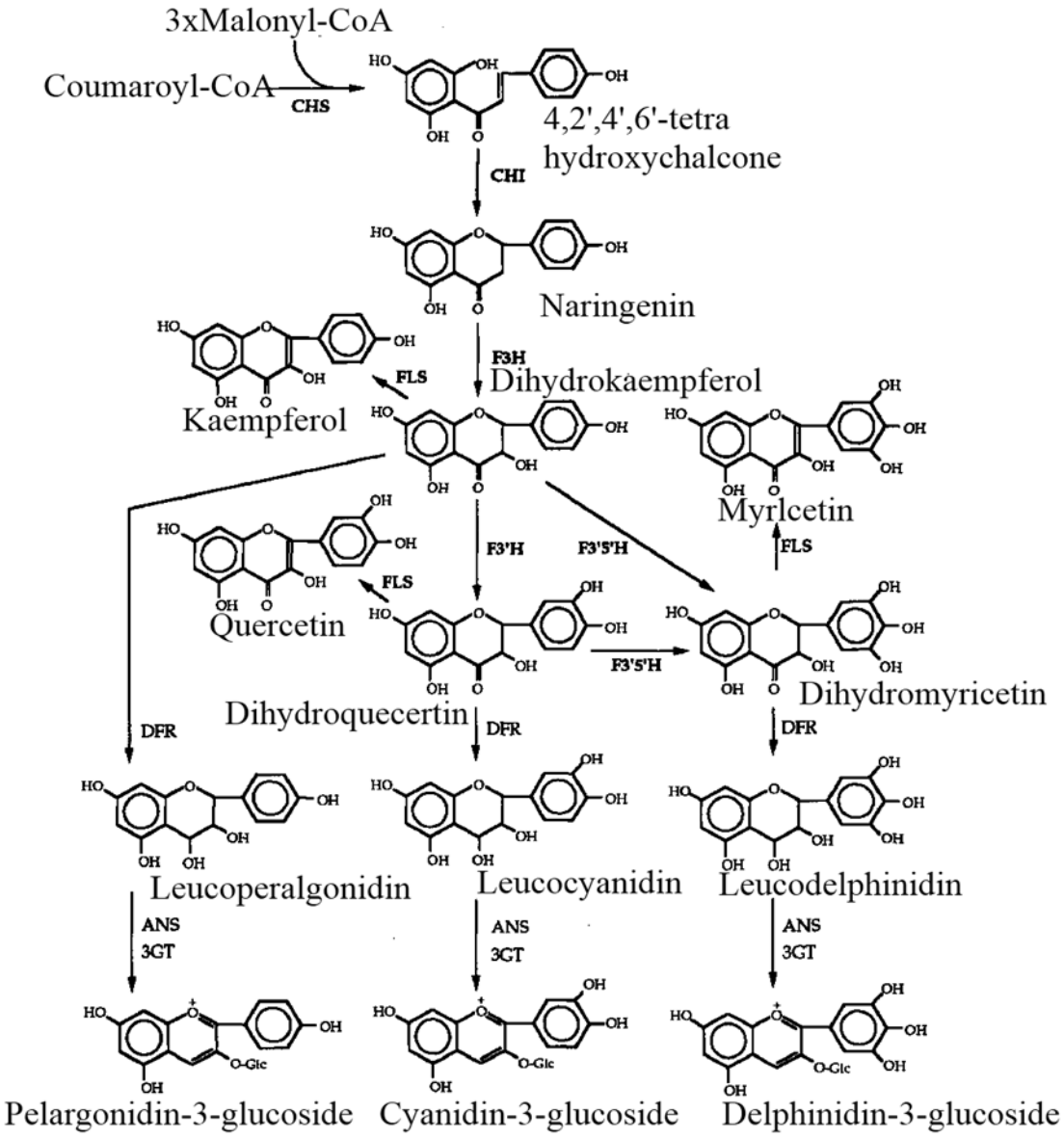


Fig. 1.8 Anthocyanin and Flavonol biosynthetic pathway (Holton and Cornish, 1995).

The cell line of *Petunia hybrida*, Sw4i, accumulated anthocyanins in the light. The most predominant pigments were glycosides of petunidins and malvidins, in some cases acylated with coumaric acid or ferulic acid. In terms of hydroxycinnamic acids, the cell

line accumulated high level of ferulic acid derivatives and a lower level of caffeic acid and coumaric acid derivatives.

Petunia hybrida could offer further investigations from a molecular point of view (a lot of genes are characterized, mutants available), but the cells organized in cluster, as in basil cell line, were not the best option for cytological analysis.

Carrot cell lines

Phenylpropanoid composition, especially anthocyanins and phenolic acids, has been characterized in detail in roots extracts and cell suspensions of various carrot varieties (Ceoldo et al., 2009).

Anthocyanins in carrot are mainly represented by glycosides of cyanidin, often acylated with hydroxycinnamic and hydroxybenzoic acids, although in some black varieties peonidin and pelargonidin glycosides have also been found.

Some studies reported that acylated cyanidin-triglycosides are the dominant pigments in carrot cell cultures (Gläßgen and Seitz, 1992). The aglycone cyanidin shows a galactose moiety attached at the C-3 position, which in turn is linked to one xylose and one glucose residue. The mono-acylation of the anthocyanins can occur at the C-6 of the glucose moiety.

Baker et al. (1994) and Dougall et al. (1998) also showed that cell feeding with different cinnamic and benzoic acid derivatives could alter the acylation pattern of the anthocyanins. The carrot cell suspension culture was able to metabolize non-natural 3,4-dimethoxy- and 3,4,5-trimethoxy-cinnamic acids and in each case a new mono-acylated anthocyanin was formed (Baker et al., 1994). This suggested that in carrot the biosynthesis of anthocyanins is not specific only for sinapic acid, but other naturally occurring cinnamic acids and “non-natural” cinnamic acids can be incorporated into anthocyanins.

The pigmented cell line of *Daucus carota*, R3M, was selected for the ability to accumulate high-yields of anthocyanins in the light, specifically glycosides of cyanidin acylated with coumaric, caffeic, ferulic, sinapic or hydroxybenzoic acid. Furthermore this cell line represented a good candidate for cytological analyses (relatively single cells, without big aggregates or clumps) and was quite stable in liquid medium, allowing not only the modification of the metabolic profile, but also the identification of specific phenotypic traits caused by heat stress and the investigation on the protective role of specific classes of secondary metabolites after this stress application.

Supplementation of inhibitors and precursors to *in vitro* cell cultures

The selective inhibition of plant enzymes *in vivo* is an exploited tool to unravel metabolic routes of plant secondary metabolites. The consequences of inhibition of an enzymatic pathway may contribute to elucidate the regulatory mechanism in the accumulation of specific metabolites (Hartmann et al., 1988). Furthermore inhibition experiments can help to identify steps in a pathway in which the activity of an enzyme or the availability of its substrate may limit the metabolic flux (Han et al., 2002). For instance, lovastatin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the mevalonic acid (MVA) pathway and clomazone, an inhibitor in the methyl-D-erythritol 4-phpsphate (MEP) biosynthesis, were supplied to fungal elicited *Cinchona* “Robusta” cell cultures in order to investigate which pathway was involved in anthraquinone biosynthesis (Han et al., 2002). Furthermore, in order to get an insight on the accumulation of *p*-hydroxybenzoic acid in elicited cell cultures of *Cocos nucifera* (coconut), the effect of supplementation of phenylpropanoid enzyme inhibitors was studied (Chakraborty et al., 2009).

As it has been mentioned above, the upstream part of phenylpropanoid metabolism consists of three enzymatic steps leading to 4-coumaroyl CoA. The cinnamate 4-hydroxylase (C4H) catalyzes the second step, that converts *trans*-cinnamic acid to *p*-

coumaric acid. This enzyme is a member of the structural family of cytochrome P450 heme thiolate proteins, which catalyse critical hydroxylation steps and variety of more complex reactions. The most efficient inhibitors of the C4H have to share the same structural characteristics of the natural substrate. One of the most effective inhibitors reported is piperonylic acid (PIP) (Fig. 1.9). PIP is a natural compound isolated from the bark of Paracoto tree (unknown botanical source, probably belonging to species of *Cryptocarya/ Laurineae/ Aniba coto*) and is a selective and potent mechanism-based quasi irreversible inhibitor of the C4H (Schalk et al.,1998). In elicitor-treated BY2 cells, PIP also behaved as an effective C4H inactivator and inhibited the formation of *p*-coumarate and accumulation of the downstream metabolite scopoletin (Schalk et al.,1998).

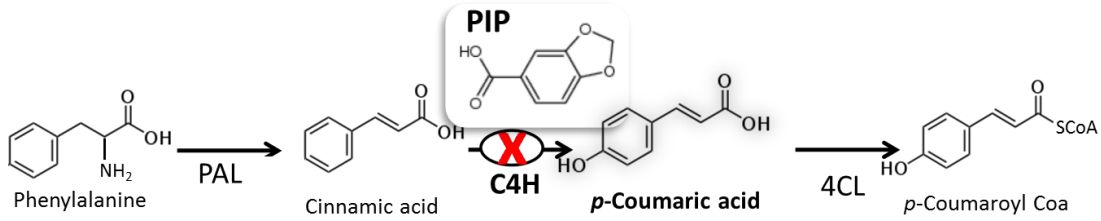


Fig. 1.9 Branching in the upper phenylpropanoid pathway. Piperonylic acid (PIP) is an inhibitor of cinnamate 4-hydroxylase (CH4).

3,4-(Methylenedioxy)cinnamic acid (MDCA) is the competitive inhibitor of hydroxycinnamate CoA-ligase (4CL), enzyme that converts, in the third step of phenylpropanoid biosynthesis, the *p*-coumaric acid to *p*-coumaroyl CoA thioester (Chakraborty et al., 2009) (Fig. 1.10). In some studies to elucidate *p*-hydroxybenzoic acid biosynthetic pathway, supplementation of MDCA did not decrease *p*-hydroxybenzoic acid level but rather decreased lignin and total flavonoid content, suggesting that 4CL activity was not required for *p*-hydroxybenzoic acid accumulation in chitosan-elicited hairy roots of *Daucus carota* (Sircar et al., 2009).

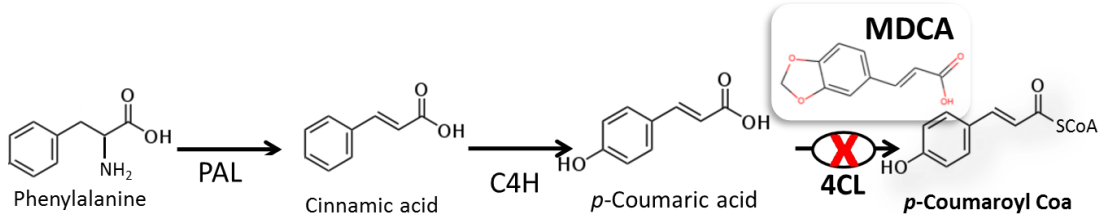


Fig. 1.10 Branching in the upper phenylpropanoid pathway. 3,4-(Methylenedioxy)cinnamic acid (MDCA) is an inhibitor of 4-hydroxycinnamate CoA ligase (4CL).

The inverse strategy to the use of specific inhibitors is the administration of biosynthetic precursors through feeding experiments.

The concept of “feeding precursors” is based upon the idea that any compound, which represents an intermediate in a secondary metabolite biosynthetic route, is a good chance of increasing the yield of the final product (Ramachandra Rao and Ravishankar, 2002).

Han et al. (2002) also suggested that supplementation of the medium with precursors could increase the availability of the substrate which might limit the overall flux through the pathway and eventually enhance the production of secondary metabolites.

Apart from the obvious and popular aim to increase the production of high-value secondary metabolites for pharmaceutical and nutritional purposes through plant cell cultures (Fett-Neto et al., 1993), feeding with precursor or intermediate compounds can be useful to better understand the biosynthetic routes of secondary metabolites. For instance, Silvestrini et al. (2002) investigated the biosynthesis of camptothecin, an alkaloid produced in the Chinese tree *Campotheca acuminata* Decaisne (Nyssaceae) by feeding with potential precursors of biosynthesis.

Often the two approaches are combined. As reported by Sun et al (2009), in order to identify the biosynthetic pathway and improve the production of the anti-tumor compound aspergiolide A, specific inhibitors and precursors of different biosynthetic pathway were fed to the culture of the fungus *Aspergillus glaucus*. Since both

precursors of polyketide pathway and inhibitors of mevalonate pathway increased aspergiolide A production, a novel strategy to control the metabolism was performed based on simultaneous treatment with the precursor acetate and a specific inhibitor of mevalonate pathway; these two treatments behaved in a synergic way, greatly improving the aspergiolide A accumulation.

Inactivation or enhancement of selected steps of a biosynthetic pathway by a chemical approach, may be an alternative tool to metabolic engineering (mutations or genetic transformation techniques) for the investigation on the role of some plant secondary metabolites. One of the main advantages of using inhibitor and precursor administration is that the genetic and epigenetic background of the cell remains unchanged. On the other hand, the overexpression of a transcriptional factor controlling a metabolic pathway can affect other cellular processes, modifying the expression of numerous other genes, not directly involved in the metabolic pathway of interest.

Consequently the development of such chemical strategies is genetic-knowledge independent, and only the knowledge of biosynthetic pathways and related enzymes, often extensively characterised, is required to choose the successful strategy. Indeed, time-consuming transgenic techniques for gene up- and down- regulation can be escaped from easy administrations of chemicals to cell cultures.

References

Anterola AM, Jeon JH, Davin LB, Lewis NG. Transcriptional control of monolignol biosynthesis in *Pinus taeda*. Factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. J Biol Chem 2002;277:18272–18280.

Aron PM, Kennedy JA. Flavan-3-ols: nature, occurrence and biological activity. Mol Nutr and Food Res 2008;52:79–104.

Baker DC, Dougall DK, Glassgen WE, Johnson SC, Metzger JW, Rose A, Seitz HU. Effects of supplied cinnamic acids and biosynthetic intermediates on the anthocyanins accumulated by wild carrot suspension cultures. Plant Cell Tiss Org 1994;39: 79–91.

Bernards MA, Lewls NG. Alkyl ferulates in wound healing potato tubers. Phytochemistry 1992;31:3409–3412.

Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M. Flavonoid Accumulation in *Arabidopsis* Repressed in Lignin Synthesis Affects Auxin Transport and Plant Growth. Plant Cell 2007;19:148–162.

Bouwmeester HJ, Roux C, Lopez-Raez JA, Bécard G. Rhizosphere communication of plants, parasitic plants and AM fungi. Trends Plant Sci 2007;12:224–230.

Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 1998;56:317–333.

Burchard P, Bilger W, Weissenböck G. Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultravioletinduced chlorophyll fluorescence measurements. Plant Cell Environ 2000;23:1373–1380.

Ceoldo S, Toffali K, Mantovani S, Baldan G, Levi M, Guzzo F. Metabolomics of *Daucus carota* cultured cell lines under stressing conditions reveals interactions between phenolic compounds. Plant Sci 2009;176:553–65.

Chakrabortya M, Karunb A, Mitra A. Accumulation of phenylpropanoid derivatives in chitosan-induced cell suspension culture of *Cocos nucifera*. J Plant Physiol 2009;166:63–71.

De Freitas V, Mateus N. Chemical transformations of anthocyanins yielding a variety of colours. Environ Chem Lett 2006;4:175–183.

Dougall DK, Baker DC, Gakh EG, Redus MA, Whittemore NA. Anthocyanins from wild carrot suspension cultures acylated with supplied carboxylic acids. Carbohydr Res 1998;310:177–189.

Dougall DK. Sinapic acid stimulator of anthocyanin accumulation in carrot cell cultures. Plant Sci 1989;60:259–262.

Ellis BE, Towers GHN. Biogenesis of rosmarinic acid in *Mentha*. Biochem J 1970;118: 291–297.

Fett-Neto AG, Melanson SJ, Sakata K, Di Cosmo F. Improved growth and taxol yield in developing callus of *Taxus cuspidata* by medium composition modification. Nat Biotechnol 1993;11:731–4.

Ford CM, Boss PK, Hoj PB. Cloning and characterization of *Vitis vinifera* UDP glucose:flavonoid 3-*O*-glucosyltransferase, a homologue of the enzyme encoded by the maize *bronze-1* locus that may primarily serve to glucosylate anthocyanidins *in vivo.* J Biol Chem 1998;273:9224–33.

Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C. The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J 2002;30:33–45.

Frost CJ, Mescher MC, Carlson JE, de Moraes CM. Plant defense priming against herbivores: getting ready for a different battle. Plant Physiol 2008;146:818–824.

Gang DR, Beuerle T, Ullmann P, Werck-Reichhart D, Pichersky E. Differential production of meta hydroxylater phenylpropanoids in sweet basil peltate glandular trichomes and leaves is controlled by the activities of specific acyltransferase and hydroxylases. Plant Physiol 2002;130:1536–1544.

Giusti MM, Wrolstad RE. Acylated anthocyanins from edible sources and their applications in food systems. Biochem Eng J 2003;14:217–225.

Gläβgen WE & Seitz HU. Acylation of anthocyanins with hydroxycinnamic acids via 1-O-acylglucosides by protein preparations from cell cultures of *Daucus carota* L. Planta 1992;186: 582–585.

Gonzalez E, Fougerousse A, Brouillard R. Two diacylated malvidin glycosides from *Petunia hybrida* flowers. Phytochemistry 2001;58:1257–1262.

Gould SK. Nature's swiss army knife: the diverse protective roles of anthocyanins in leaves. J Biomed Biotechnol 2004;5:314–320.

Guzzo F, Cantamessa K, Portaluppi P, Levi M. Flow cytometry and sorting of protoplasts from carrot cell cultures reveal two cell subpopulations with different morphogenetic potential. Plant Cell Rep 2002;21:214–219.

Hahlbrock KM, Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 1989;40:347–369.

Han YS, Heijden R, Verpoorte R. Improved anthraquinone accumulation in cell cultures of *Cinchona* 'Robusta' by feeding of biosynthetic precursors and inhibitors. Biotechnol Lett 2002;24:705–10.

Harborne JB, Williams CA. Advances in flavonoid research since 1992. Phytochemistry 2000;55:481–504.

Hartmann T, Sander H, Toppel G. Metabolic links between the biosynthesis of pyrrolizidine alkaloids and polyamines in root cultures of *Senecio vulgaris*. Planta 1988;175:82–90.

Hernández I, Van Breusegemb F. Opinion on the possible role of flavonoids as energy escape valves: Novel tools for nature's Swiss army knife? Plant Sci 2010;179: 297–301.

Hoffmann L, Maury S, Martz F, Geoffroy P, Legrand M. Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. J Biol Chem 2003;278:95–103.

Holton T, Cornish EC. Genetics and Biochemistry of Anthocyanin Biosynthesis. Plant Cell 1995;7:1071–1083.

Jiang RW, Laua KM, Hona PM, Makb TCW, Wooc KS, Fung KP. Chemistry and biological activities of caffeic acid derivatives from *Salvia miltiorrhiza*. Curr Med Chem 2005;12:237–246.

Kähkönen MP, Heinonen M. Antioxidant Activity of Anthocyanins and Their Aglycons. J Agric Food Chem 2003;51:628–633.

Kikuzami H, Hisamoto M, Horose K, Akiyama K, Taniguchi H. Antioxidant Properties of Ferulic Acid and Its Related Compounds. J Agric Food Chem 2002;50:2161–2168.

Kong JM, Chia LS, Goh NK, Chia TF, Brouillard R. Analysis and biological activities of anthocyanins. Phytochemistry 2003;64:923–933.

Lim EK, Higgins GS, Li Y, Bowles DJ. Regioselectivity of glucosylation of caffeic acid by a UDP-glucose:glucosyltransferase is maintained in planta. Biochem J 2003;373:987–992.

Maffei ME. Sites of synthesis, biochemistry and functional role of plant volatiles. S Afr J Bot 2010;76:612–631.

Mahesh V, Million-Rousseau R, Ullmann P, Chabrillange N, Bustamante J, Mondolot L, Morant M, Noirot M, Hamon S, de Kochko A. Functional characterization of two p-coumaroyl ester 3-hydroxylase genes from coffee tree: evidence of a candidate for chlorogenic acid biosynthesis. Plant Mol Biol 2007;64:145–159.

Marais JPJ, Deavours B, Dixon RA, Ferreira D. The stereochemistry of flavonoids. The science of flavonoids 2006;1–46.

Mustafa NR, de Winter W, van Iren F, Verpoorte R. Initiation, growth and cryopreservation of plant cell suspension culture. Nat Protoc 2011;6:715–742.

Nakayama T, Suzuki H, Nishino T. Anthocyanins acyltransferase: specificities, mechanism, phylogenetics, and applications. J Mol Catal B-Enzym 2010;23:117–132.

Niggeweg R, Michael AJ, Martin C. Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat Biotechnol 2004;2:746–754.

Pavarini DP, Pavarini SP, Niehues M, Lopes NP. Exogenous influences on plant secondary metabolite levels. Anim Feed Sci Tech 2012;176:5–16.

Peer WA, Murphy AS. Flavonoids as signaling molecules. The Science of Flavonoids 2008;239–268.Pereira GK, Donate PM, Galembeck SE. Effects of substitution for hydroxyl in the B-ring of the flavylium cation. J Mol Struct 1997;392:169–179.

Petersen M. Cytochrome P-450-Dependent hydroxylation in the biosyntheis of rosmarinic acid in *Coleus*. Phytochemistry 1997;45:1165–1172.

Petersen M, Häusler E, Karwatzki B, Meinhard J. Proposed biosynthetic pathway for rosmarinic acid in cell cultures of *Coleus blumei* Benth. Planta 1993;189:10–14.

Petersen M, Simmonds MSJ. Rosmarinic acid. Phytochemistry 2003;62:121–5.

Ramachandra Rao S, Ravishankar GA. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol Adv 2002;20: 101–153.

Rasmann S, Turlings TCJ. First insights into specificity of belowground tritrophic interactions. Oikos 2008;117:362–369.

Rice-Evans CA, Miller NJ, Paganga G. Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol Med 1996;20:933–956.

Sanchez-Campillo M, Gabaldon JA, Castillo J, Benavente-Garcia O, Del Baño MJ, Alcaraz M. Rosmarinic acid, a photo-protective agent against UV and other ionizing radiations. Food Chem Toxicol 2009;47:386–92.

Schalk M. Piperonylic Acid, a Selective, Mechanism-Based Inactivator of the trans-Cinnamate 4-Hydroxylase: A New Tool to Control the Flux of Metabolites in the Phenylpropanoid Pathway. Plant Physiol 1998;118: 209–218.

Schoch G, Goepfert S, Morant M. CYP98A3 from *Arabidopsis thaliana* is a 3¢-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J Biol Chem 2001;276:36566–36574.

Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichhart D. CYP98A3 from *Arabidopsis thaliana* is a 30-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J Biol Chem 2001;276:36566–36574.

Silvestrini A, Pasqua G, Botta B, Monacelli B, van der Heijden R, Verpoorte R. Effects of alkaloid precursor feeding on a *Camptotheca acuminate* cell line. Plant Physiol Biochem 2002;40:749–753.

Sircar D, Mitra A. Accumulation of p-hydroxybenzoic acid in hairy roots of *Daucus carota* 2: Confirming biosynthetic steps through feeding of inhibitors and precursors. J Plant Physiol 2009;166:1370–1380.

Sonnante G, D'Amore R, Blanco E, Pierri CL, De Palma M, Luo J, Tucci M, Martin C. Novel hydroxycinnamoyl-Coenzyme A Quinate Transferase genes from Artichoke involved in the synthesis of chlorogenic acid. Plant Physiol 2010;153:1224–1238.

Stehle F, Brandt W, Stubb MT, Milkowski C, Strack D. Sinapoyltransferases in the ligh of molecular evolution. Phytochemistry 2009;70:1652–1662.

Sun X, Zhou X, Cai M, Tao K, Zhang Y. Identified biosynthetic pathway of aspergiolide A and a novel strategy to increase its production in a marine-derived fungus *Aspergillus glaucus* by feeding of biosynthetic precursors and inhibitors simultaneously. Bioresource Technol 2009;100:4244–4251.

Tornielli G, Koes R, Quattrocchio F. The genetics of flower color. Petunia 2009; 13:269–299.

Werck-Reichhart D, Feyereisen R. Cytochromes P450: a success story. Genome Biol 2000;3003.1–3003.9.

Winkel-Shirley B. Update on Flavonoid Biosynthesis: Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiol 2001;126: 485–493.

Zenk MH. Chasing the enzymes of plant secondary metabolism: plant cell cultures as a pot of gold. Phytochemistry 1991;30:3861–3.

Zgorka G, Glowniak K. Variation of free phenolic acids in medicinal plants belonging to the Lamiaceae family. J Pharm Biomed Anal 2001;26:79–87.

Chapter 2

Modification of metabolic profile in plant cell cultures: a chemical approach

Introduction

A cell line (T2b) of a red variety (Dark Opal) of *Ocimum basilicum*, characterized by the presence of high levels of rosmarinic acid (RA) (800 µg/g fresh weight) has been selected for the stable accumulation of anthocyanins (ACs) in the dark (Strazzer et al., 2011). The HPLC-DAD profile for ACs (520 nm chromatogram) showed sixteen principal peaks. The chemical composition of each individual peak was investigated by tandem mass spectrometry by Strazzer et al. (2011). All ACs were identified as cyanidin-based ACs linked to glycosyl and acyl moieties. Almost all ACs were acylated with coumaric acid and, in some case, the aromatic acylation was supplemented with a further aliphatic acylation with malonic acid.

The HPLC-DAD chromatogram representing methanol:HCl cell extracts at 329 nm showed a single peak, corresponding to a single molecule with a m/z(-) of 359. The comparison of its retention time, absorbance spectrum and fragmentation pattern with those of authentic standards identified it as RA. MS and MS/MS spectra facilitated the putative annotation of RA derivatives, such as RA hexosides and lithospermic acid B, a major component of *Salvia miltiorrhiza* with antioxidant activities (Jiang et al., 2005), previously detected in basil hairy roots (Tada et al., 1996). With regard to other hydroxycinnamic acids, coumaric acid and caffeic acid derivatives have been detected in the cell extracts. Considering flavonoids, beside ACs, in T2b many flavanone derivatives have been found, such as naringenin, eriodictyol, dihydroquercetin, instead of the corresponding flavones (apigenin, luteolin and quercetin), suggesting that the flavonoid synthetic pathway could be arrested at this point.

Since AC accumulation in red cultures of *Daucus carota* increased following enhanced agitation (Ceoldo et al., 2009), in previous investigations in our laboratory, the T2b cell line has been exposed to similar stress conditions in order to investigate the effect on RA and AC accumulation. Mechanical stress increased the total AC and RA contents

but reduced biomass accumulation. Other hydroxycinnamic acids and flavonoids were induced by mechanical stress, whereas the abundance of some RA dimers was reduced (Strazzer et al., 2011). A strong correlation between the increases in AC and RA levels has been observed, both in the light and in the dark, but more RA was accumulated in the light. From these previous results, although ACs and RA share a common early biosynthetic pathway and could have similar or overlapping biological activities, there appears to be no competition between their individual pathways, which are individually regulated by light (Strazzer et al., 2011).

Cell cultures producing both ACs and RA can represent an interesting experimental system in which to better elucidate the modulation and relationship of these two metabolic pathways.

The cell line Sw4i of *Petunia hybrida* was visually selected for the stable production of ACs in the light. The HPLC-DAD analysis of methanolic cell extracts led to a separation of ACs in six main peaks. The fragmentation pattern of single ACs obtained by tandem mass spectrometry and the comparison of such profiles with those of ACs of petunia already reported in literature, allowed to putatively identify the ACs accumulated as derivatives of various aglycones. Petunidins and malvidins were the most predominant, but also a lower accumulation of delphinidins was detected. The AC glycosylation consisted of a rhamnosyl-glucose (rutinose), in some cases with an additional glucose residue. Some ACs were further monoacylated with coumaric acid or ferulic acid. Considering hydroxycinnamic acid levels, Sw4i accumulated also ferulic acid derivatives, that corresponded to the two principal peaks in the HPLC-DAD chromatogram at 320 nm, but also caffeic acid derivatives and a lower content of coumaric acid derivatives were present.

A pigmented cell line, R3M, was selected from the non-pigmented *Daucus carota* L. cv. Flakkese K1 cell line, for the ability to produce high-yields of ACs in the light. As described by Ceoldo et al. (2009), HPLC-DAD analysis at 520 nm revealed the

presence of chromatographic peaks corresponding to ACs, whose chemical composition has been unraveled through HPLC-MS. The eight ACs identified were differently glycosylated with pentose and/or hexose residues and mono-acylated with coumaric, caffeic, ferulic, sinapic or hydroxybenzoic acid. Beside the ACs, within the identified molecules, hydroxycinnamic and hydroxybenzoic acid derivatives were the most represented. Among the hydroxycinnamic acid derivatives accumulated in R3M, caffeic acid derivatives were the most abundant and some of these were putatively identified as caffeic esters linked to quinic acid or daucic acid and caffeic acid glycosides. Glycosides and other sinapic, ferulic and coumaric acid derivatives were also detected. The hydroxybenzoic acid peak detected with HPLC–DA was revealed by HPLC–MS as a mixture of different co-eluting molecules, all derivatives of vanillic and hydroxybenzoic acid (Ceoldo et al., 2009).

A chemical approach using inhibitors and precursors of phenylpropanoid biosynthesis has been developed in order to modify the metabolic profile in the three different cell lines. This represents the first step in order to elucidate possible protective roles of specific secondary metabolites and to unravel specie-specific features of the phenylpropanoid pathway.

Materials and Methods

Generation of plant cell cultures

The red cell line T2b (*Ocimum basilicum* L., cv. Dark Opal) was established from the root–stem transition zone as described by Strazzer et al. (2011). The anthocyanin producing cell line R3M was visually selected from an established non-pigmented cell line called K1 (*Daucus carota* L., cv. Flakkese) as described by Ceoldo et al. (2005). The Sw4i cell line of petunia was generated by a long-term selection of red cells from calli obtained from hypocotyls of *Petunia hybrida* F1 hybrid M1XV30 plantlets.

The anthocyanin productive trait of the cell lines has been maintained through continuous visual selection of highly pigmented cells during the subculture process. All the suspension cultures were generated from calli transferred to the B5 Gamborg's liquid medium supplemented with 2% sucrose and 0.5 mg/L 2,4-dichlorophenoxyacetic (pH 5.9) on a rotary shaker at 90 rpm and then subcultured every 14 d at an initial density of 4% (v/v) cells.

Cell lines of basil were maintained in the dark in a growth chamber at 25±1 °C, whereas petunia and carrot cell lines were maintained with a 16 h light/ 8 h dark photoperiod at 25±1 °C.

Treatment with inhibitors and feeding precursors

3,4-methylenedioxy cinnamic acid (MDCA) and piperonylic acid (PIP) were dissolved in dimethyl sulfoxide (DMSO). Each enzyme inhibitor was prepared as concentrated stock solutions and added at the define final concentration into the liquid medium containing ten-day-old cells.

Basil cells were treated with MDCA at the final concentration of 0,2 mM. PIP was supplied at the final concentration of 0,1 and 0,5 mM to carrot and petunia cells, respectively.

In the feeding experiments dihydroquercetin (DHQ) was dissolved in methanol and fed to ten-day-old petunia cell cultures in a final concentration of 1mM. The treatment with DHQ (1mM) in the presence of the inhibitor MDCA (0,2 mM) was carried out simultaneously in basil cells.

Non-treated cultures were fed with equal volume of solvents and considered as control. Cells were collected after 24 h until 6 d, extracted and analyzed by HPLC-DAD and HPLC-MS.

Cell extracts

After the treatments, cells were washed by mild centrifugation at 220 g for 5 min, resuspended in Gamborg B5+ and washed twice in the same medium, in order to remove possible excesses of chemicals added to the liquid medium during the treatments. Cells were centrifuged at 1200 g for 10 min at 4 °C. Different extraction times and solvent volumes were tested in order to find the optimum condition to simultaneously extract as much phenylpropanoids as possible.

The optimum conditions for extractions were: 1) basil cells: resuspended in ten volumes of 99:1 methanol:HCl (v/v) and incubated for 2 h on ice; 2) petunia cells: resuspended in two volumes of 99:1 methanol:HCl (v/v) and incubated for 2 h on ice; 3) carrot cells: resuspended in four volumes of 99:1 methanol:HCl (v/v) and incubated for 20 min on ice. After the extraction, cells were centrifuged again as above. The resulting supernatants were stored at −20 °C.

HPLC-DAD analysis

HPLC-DAD analysis was performed using a Beckman Coulter Gold 126 Solvent Module equipped with a Gold 168 Diode Array Detector (Beckman Coulter, Fullerton, CA). A C18 guard column (7.5 9 2.1 mm) and an analytical Alltima HP C18 column (150 9 2.1 mm, particle size 3 µm) (Alltech Associates Inc, Derfield, IL) were used. Solvents were (A): 0,5% (v/v) formic acid and 5% (v/v) acetonitrile in water; (B): 100% acetonitrile.

The solvent gradient was: 1) basil cells: 0–10% B in 5 min, 10–20% B in 20min, 20–80% B in 20 min, and 80–0% B in 1 min; 2) petunia and carrot cells: 0–10% B in 5 min, 10–20% B in 20min, 20–25% B in 5 min, 25–70% B in 15 min and 70–0% B in 1 min. Dilution ratio and injection volumes of cell extracts (iv) were: 1) basil cells: 1:5 with water, 50 µl iv; 2) petunia cells: 1:2 with water, 50 µl iv; 3) carrot cells: 1:5 with water, 20 µl iv.

The flow rate was set to 0.2 mL min^{-1}. The wavelength for HPLC-DAD analysis was 190-600 nm. Chromatographic data were collected and processed using 32 Karat Software version 7.0 (Beckman Coulter Inc., Fullerton, CA).

Anthocyanins were monitored by absorbance at 520 nm. Total anthocyanin levels were determined by the sum of the peak areas detected at 520 nm and were expressed as cyanidin equivalent on the basis of a calibration curve prepared with commercial cyanidin (Extrasynthese, France).

In basil, chromatographic peak at 329 nm was used for rosmarinic acid determination. Accumulation of rosmarinic acid was expressed on the basis of a calibration curve obtained with an authentic standard (Extrasynthese, France). Hydroxycinnamic acids were monitored by absorbance at 320 nm.

Absorbance spectra of HPLC-DAD peaks were used to facilitate the putative annotation of the chemical composition based on tandem mass spectrometry.

LC-MS analysis

To gain information about the different HPLC-DAD peaks, cell extracts were analyzed by LC–MS using the same HPLC system coupled to an ion trap mass spectrometer equipped with an ESI (electrospray ionization) source (Esquire 6000, Bruker Daltoniks). The chromatographic and MS data were collected using the Bruker Daltonics Esquire 5.2-Esquire Control 5.2 software, and processed using the Bruker Daltonics Esquire 5.2-Data Analysis 3.2 software (Bruker Daltonik GmbH, Germany).

The HPLC parameters were the same as described above for HPLC-DAD. Nitrogen was used as the nebulizing gas (pressure 50 psi, temperature 350 ◦C) and drying gas (12 L min−1). Helium was used as the collision gas. Negative and positive ion mass spectra were recorded in the range 50–3000 m/z. For tandem mass spectrometry analysis, ms/ms and ms^3 spectra both in negative and positive mode were recorded, in the range of 50–3000 m/z, with a fragmentation amplitude of 1 V. In order to putatively

identify the compounds, the fragmentation patterns (ms/ms and ms^3) of the detected molecules were compared with those of commercial standards. When the direct comparison of the retention time and the fragmentation pattern of the detected compounds with those of known molecules was not possible for the absence of appropriate commercial standards, the fragmentation patterns of the detected molecules were also compared with those reported in the literature.

Since internal standards were not applied, the relative quantitation (i.e. comparison between samples) was based on the area of each of the signals extracted from the chromatograms and expressed as intensity in arbitrary units (a.u).

Data analysis

MS data were transformed in .cdf format with the Bruker Daltonics Esquire 5.2 Data analysis software, and were analyzed, aligned and filtered with MZmine software.

The data matrices were exported from Excel to Simca v. 13 (Umetrics, Sweden) for multivariate statistical analyses, including Principal Component Analysis (PCA), Partial Least Square Discriminant Analysis (PLS-DA) and Orthogonal Bidirectional Projections to Latent Structures Discriminant Analysis (O2PLS-DA), using pareto scaling. O2PLS-DA models were validated with a permutation test using 200 permutations.

Results

The T2b cell line (*Ocimum basilicum*)

The T2b cell line accumulates cyanidin-type anthocyanins (ACs) esterified with coumaric acid and high levels of rosmarinic acid (RA). The ability of this cell line to accumulate high levels of both ACs and hydroxycinnamic acid derivatives, makes it an attractive model to investigate the reciprocal roles of these two classes of molecules. In order to investigate possible non-overlapping biological roles of these two classes of

molecules, it is previously necessary to be able to perturb the system differentially increasing or decreasing only one class of molecule.

Since the external stimuli have been proved to act in the same way on the two classes of molecules, indicating a common regulation of the two pathways, the chemical approach seems especially suitable to modify the ratio between AC and RA accumulation. Thus, a strategy was designed in which the inhibitor 3,4-methylenedioxy cinnamic acid (MDCA) was used to block the synthesis of the common precursor *p*-coumaroyl CoA, while the simultaneous treatment with the precursor of ACs, dihydroquercetin (DHQ), was used to support the AC formation (Fig. 2.1).

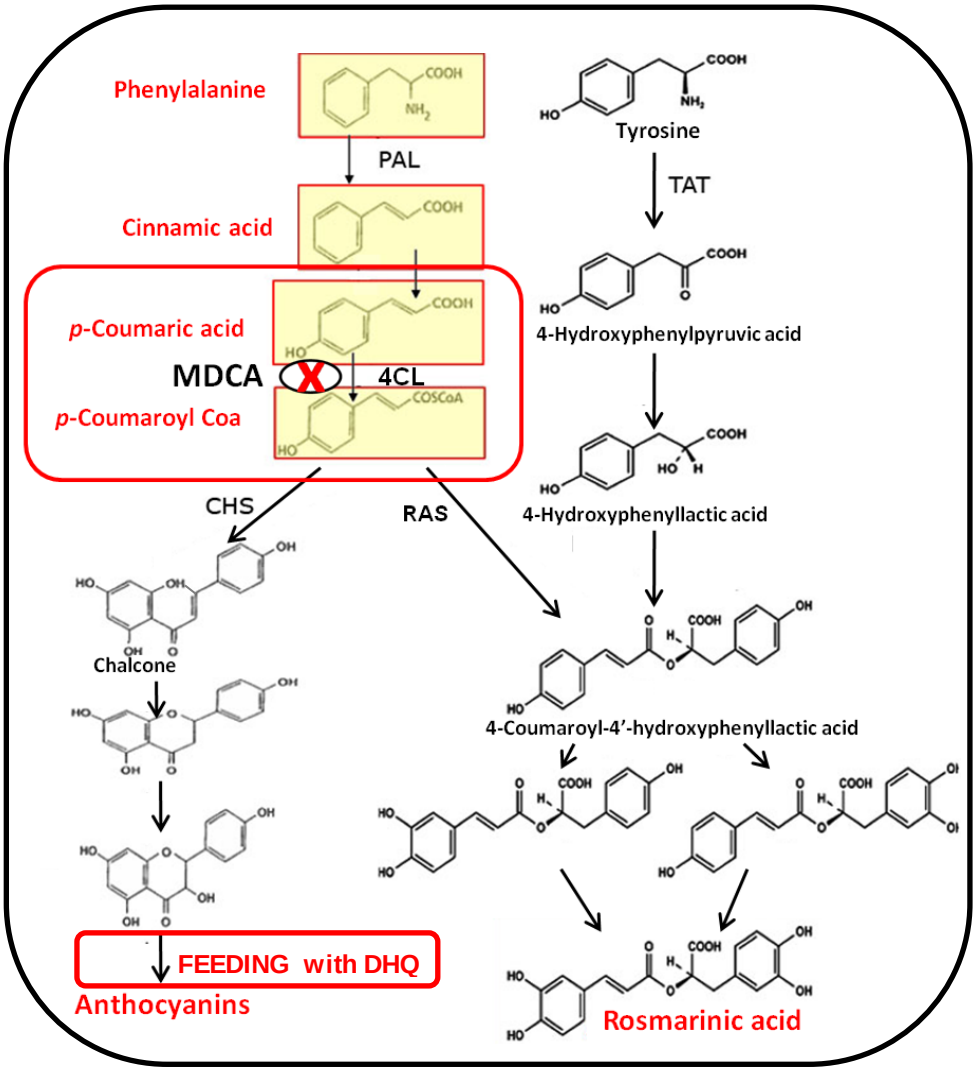


Fig. 2.1 Strategy designed in T2b cell line of *Ocimum basilicum*

Before combining MDCA treatment and DHQ supplementation, the effect of inhibitor on the metabolism of the T2b cell culture was determined. Cells were treated with 0,2 mM MDCA for 24, 48, 72 h and 6 d, after which the levels of ACs and RA were determined.

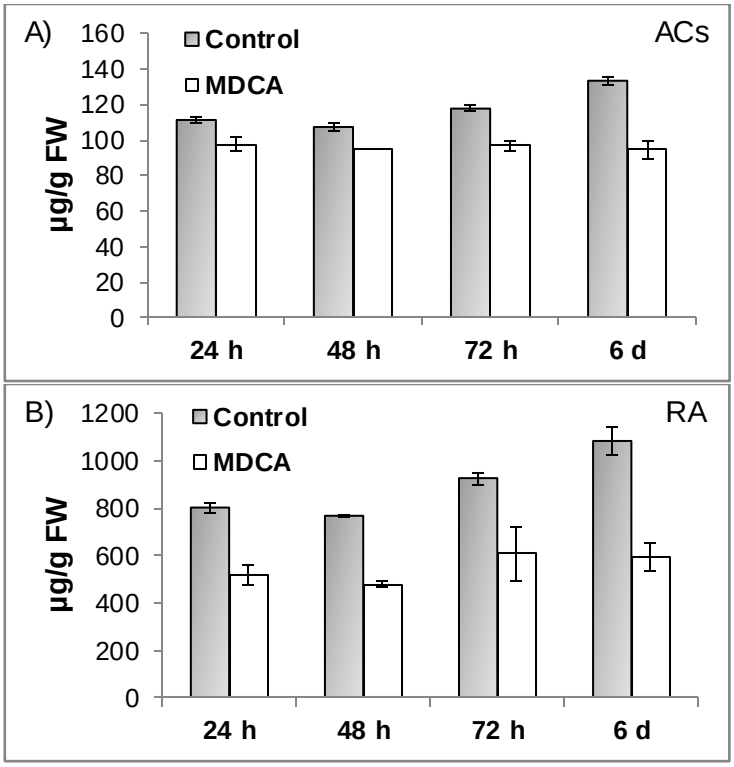


Fig. 2.2 Accumulation of ACs (A) and RA (B) in non-treated cells and cells treated for 24, 48, 72 h and 6 d with 0,2 mM MDCA, as determined by HPLC-DAD analysis.

As shown in Fig. 2.2, all the treatments resulted in the reduction of RA and AC levels. In order to get more information on the metabolic profiles of cells treated with 0,2 mM MDCA for 24, 48, 72 h and 6 d, a semiquantitative untargeted HPLC-ESI-MS analysis was carried out, and the resulting data matrix was evaluated by O2PLS-DA. The *loading plot* reported in Fig. 2.3, shows the correlation between the metabolites (black triangles) and the various treatments (classes, red square) expressed as pq(corr) (= correlation between P, the class of sample, and q, the metabolites). The analysis shows that after the MDCA treatment, a group of metabolites positively characterizes

the control cells (Tab. 2.1), while another group characterizes the MDCA treated cells (Tab. 2.2).

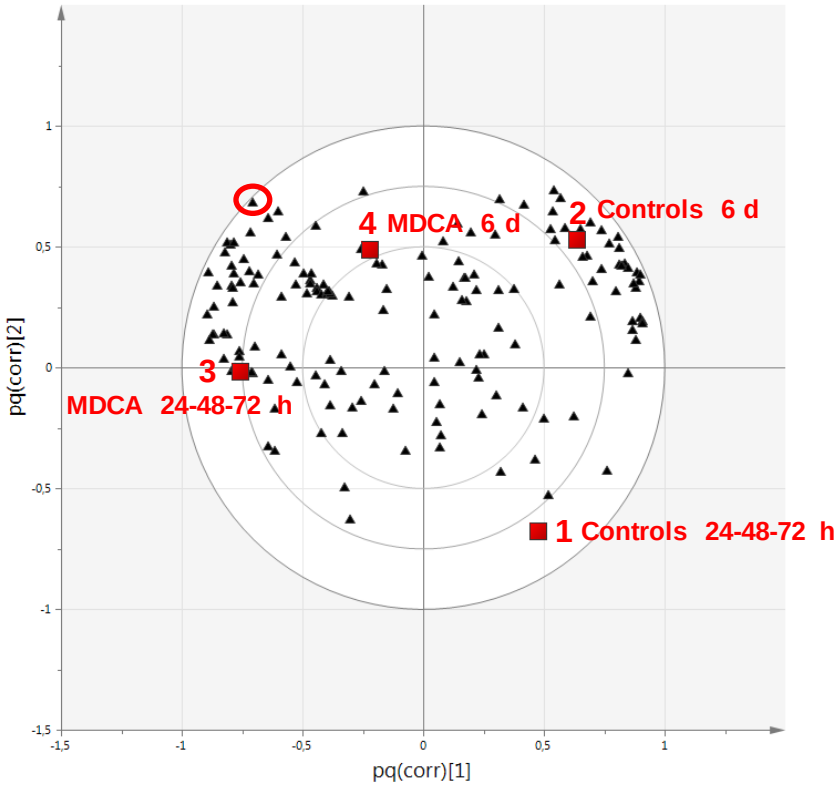


Fig. 2.3 Loading plot of the O2PLS-DA analysis.

ID	pq(corr)[1]	pq(corr)[2]	putative identification	m/z (-)	Rt
1	0,885669	0,392063	Rosmarinic acid	359	24,8
3	0,898533	0,203835	Methyl rosmarinic acid	373	34,4
28	0,882388	0,112568	Coumaric acid C hex	326	5,5
55	0,907768	0,186491	Dihydroxybenzoic acid derivative	492	28,4
58	0,798941	0,312895	Rosmarinic hex II	521	21,4
66	0,864577	0,190015	Coumaroyl hex	325	8,3
77	0,811575	0,424034	Cyanidin 3 coum mal hex hex deoxy	987	32,9
83	0,824786	0,419695	Cyanidin 3 coum methyl mal hex	693	35,3
98	0,769993	0,511653	Rosmarinic acid hex I	521	19,6
108	0,847126	0,410536	Cyanidin 3 coum mal hex	679	30,5
111	0,893384	0,355507	Rosmarinic acid derivative II	667	35,0
154	0,897781	0,381448	Rosmarinic acid derivative	667	32,1
175	0,872078	0,346743	Cyanidin 3 coum mal hex hex	841	23,0

Tab. 2.1 Molecule positively correlated with non-treated samples and negatively with samples treated with MDCA. hex, hexose; coum, coumaric acid; mal, malonic acid.

ID	pq(corr)[1]	pq(corr)[2]	putative identification	m/z (-)	Rt
100	-0,795794	0,334715	Methylmalonyl caffeoyl hex	441	17,4
164	-0,796057	-0,016881	MDCA methylmalonyl hex adduct	489	34,6
169	-0,86939	0,135895	MDCA malonyl hex -COO adduct	443	26,3
173	-0,755242	0,348964	Malonylhexoside caffeic acid -COO	383	8,5
188	-0,892375	0,389078	MDCA derivative	533	36,2
197	-0,852715	0,335343	Malonyl hex caffeic acid	427	8,6
199	-0,812622	0,135457	Caffeoyl gluc derivative	490	37,7
213	-0,827701	0,033225	MDCA malonyl hex	440	26,4

Tab. 2.2 Molecule positively correlated with samples treated with MDCA and negatively with non-treated samples. hex, hexose.

Rosmarinic acid and some its derivatives, coumaric acid derivatives, some anthocyanins and dihydroxybenzoic acid derivatives (Tab. 2.2) are positively correlated with the control samples and negatively with MDCA treated cells. Therefore, the inhibitor MDCA led to a reduction of these classes of metabolites. The metabolites that characterize MDCA treated cells are some derivatives of this molecule, indicating that the inhibitor is stored by the cell, and some caffeoyl derivatives.

The deoxy RA, which is the intermediate in the final steps of rosmarinic acid biosynthesis, (Var ID 5 indicated in red in Fig. 2.3, m/z- 343; pq(corr)[1] 0,705804, pq(corr)[2] 0,678927; Rt 29,8), and is one of the most abundant compound normally accumulated by cells, further strongly increased following MDCA treatment.

Once that the efficacy of MDCA in inhibiting both AC and RA accumulation in T2b cells has been proved, it was administrated together with the AC precursor DHQ (Fig. 2.1). However, this strategy, that combined the use of 0,2 mM MDCA and 1 mM DHQ for 24 h, has led to an unvaried accumulation of ACs and only a slight reduction of RA (Fig. 2.4). The latter unexpected negative result could depend on the higher content of RA accumulated by the cell line compared with that of the previous experiments. Since in the same time the cell line was evaluated also from the cytological point of view, showing massive cell clustering that could seriously prevent the cytological analysis

necessary to go in details on the biological role of the metabolites, the investigation on T2b line was abandoned.

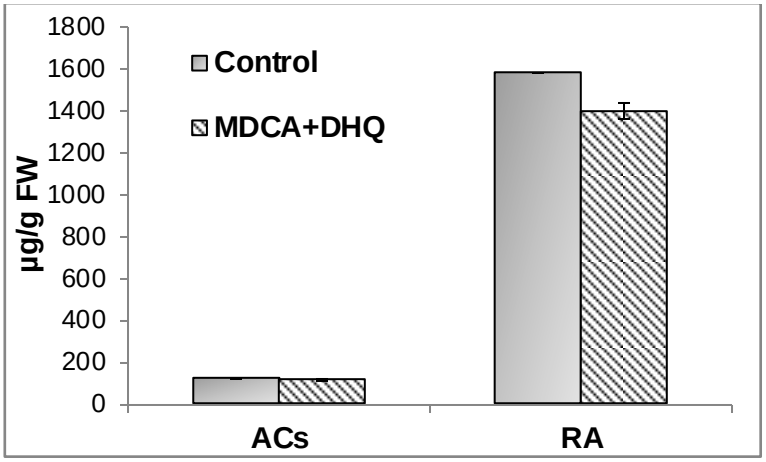


Fig. 2.4 Accumulation of ACs and RA in the samples of control and in the samples treated with MDCA and supplemented with DHQ for 24 h.

The Sw4i cell line (*Petunia hybrida*)

The capability of Sw4i cell line to accumulate different type of AC aglycones (delphinidin, petunidin and malvidin) makes it an interesting system to investigate their possible different biological roles. For this purpose, an experimental approach was developed with the aim of modulating the proportion of different aglycones in the cell line. Sw4i cells were fed with DHQ, precursor upstream the biosynthesis of the non-methylated ACs (i.e. delphinidins), which in turn generate the methylated ones (i.e. petunidins, malvidins), in order to change the ratio between the methylated versus non-methylated ACs.

Since DHQ is also a precursor for the biosynthesis of cyanidins, this approach could also stimulate the accumulation of these ACs, which are non-methylated ACs (Fig. 2.5).

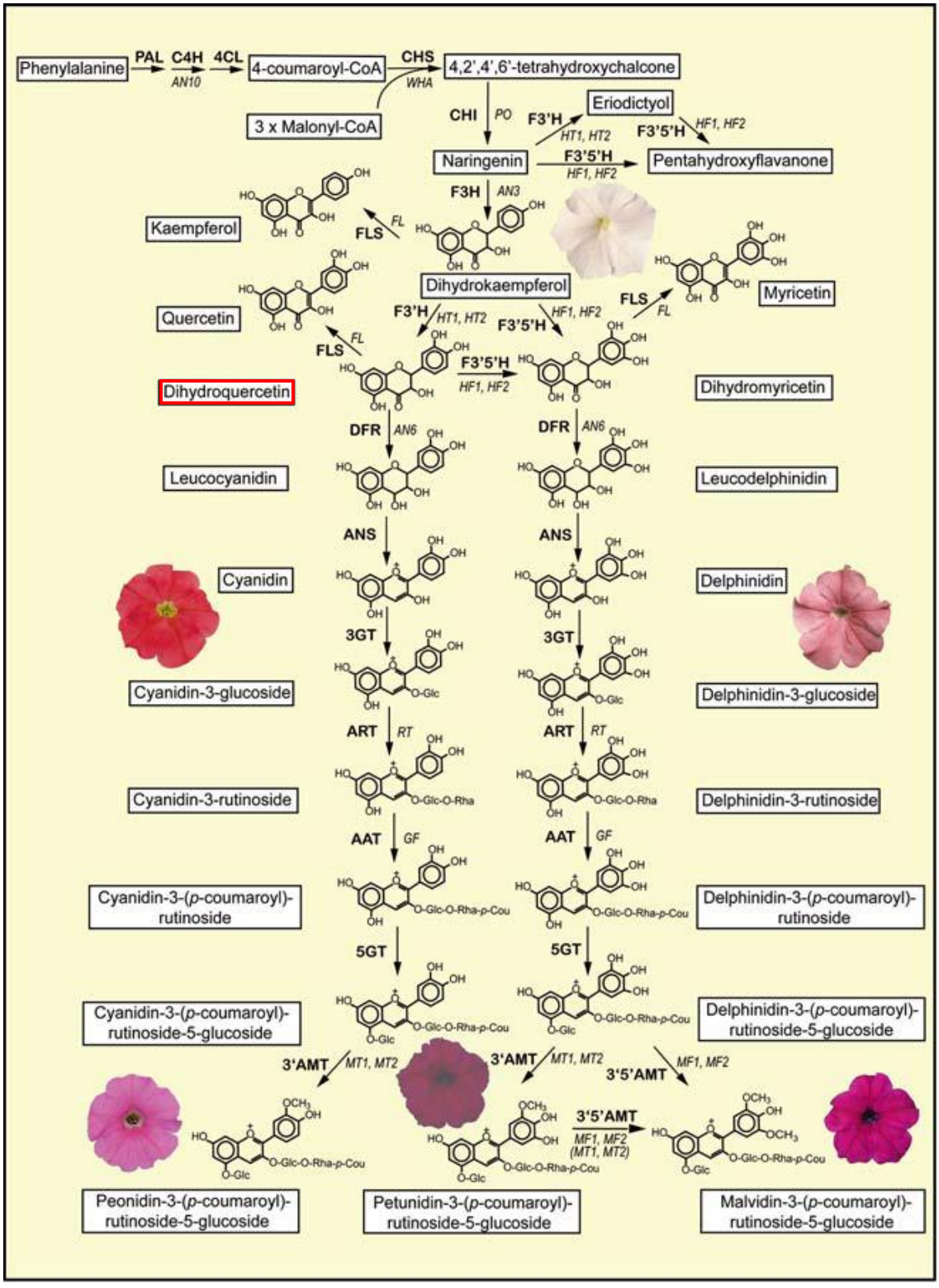


Fig. 2.5 Scheme of the flavonoid pathway in Petunia flower (Tornielli et al., 2002).

Moreover, since this cell line has the ability to accumulate ACs both in non-acylated and acylated form (especially with coumaric acid), it can also be used as a model to investigate the biological importance of the presence of AC acylation. Thus, the inhibitor PIP, which interferes with the synthesis of *p*-coumaric acid, the main substrate for AC acylation, was used in order to reduce the accumulation of acylated ACs (Fig. 2.6).

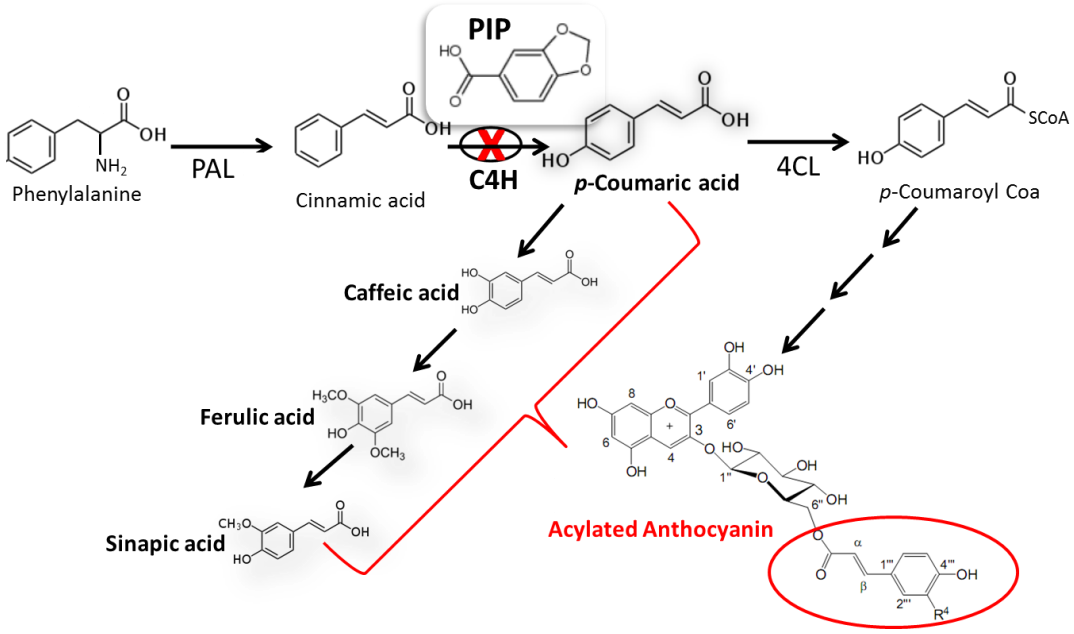


Fig. 2.6 Possible target and outcome of PIP treatment in Sw4i cell line of *Petunia hybrid*a.

The content of ACs in Sw4i suspension line changed after each inoculum during the growth cycle, as shown in Fig. 2.7, with a peak of accumulation during the last days of culture.

Considering the main ACs, non-acylated ACs continue to increase their accumulation until 18th day, while acylated ACs reach their maximum level at 14th day (Fig. 2.8). In order to obtain a stronger modification in the AC profile, the treatments were applied at day 10, just before the expected peak of AC accumulation.

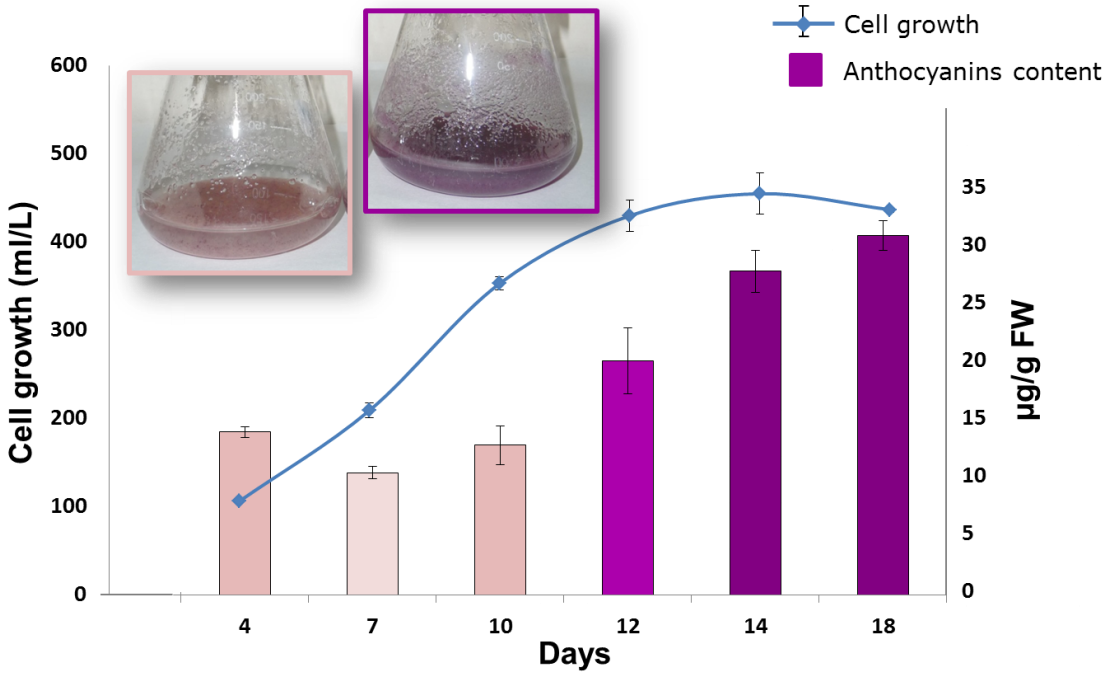


Fig. 2.7 Correlation between AC accumulation and cell growth in Sw4i cell line.

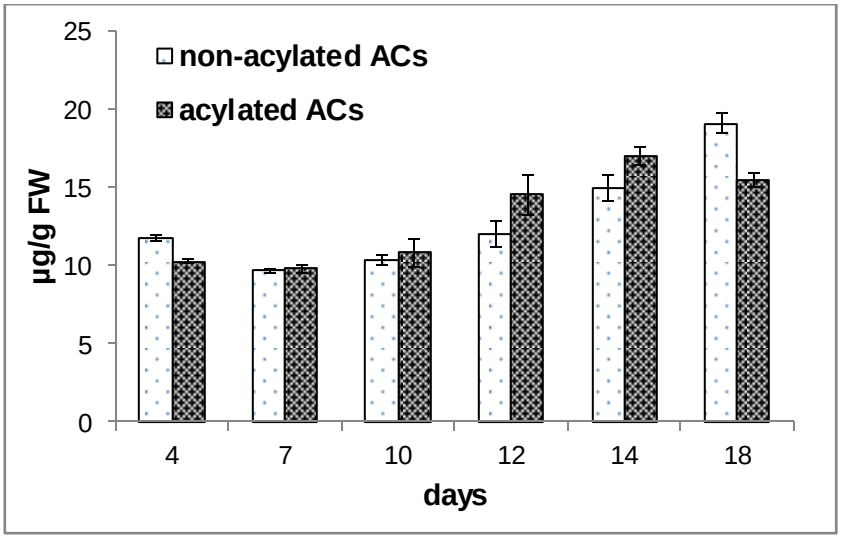


Fig. 2.8 Non-acylated and acylated ACs accumulation during the growth cycle until 18 d (HPLC-DAD analysis).

Treatment with the inhibitor piperonylic acid (PIP)

The inhibitor PIP was used to inhibit coumaric acid biosynthesis and thus to reduce the biosynthesis of ACs and their acylation with coumaric acid. Moreover, since coumaric acid is also the precursor for the biosynthesis of the other hydroxycinnamic acids (HCAs) used for ACs acylation, ferulic acid included, also a specific decrease of this acylation reaction was expected (Fig. 2.6).

0,5 mM PIP was supplied to cells for 24 and 48 h. After the treatment, the inhibitor itself appeared within the cells as PIP hexosides, as indicated by LC-ESI-MS analysis (Fig. 2.10 A).

The main effect of PIP treatment on Sw4i AC profile, as expected, was the strong reduction in the total AC content of cells. Considering the individual ACs, the acylated ACs decreased more than the non acylated ones (Fig. 2.9).

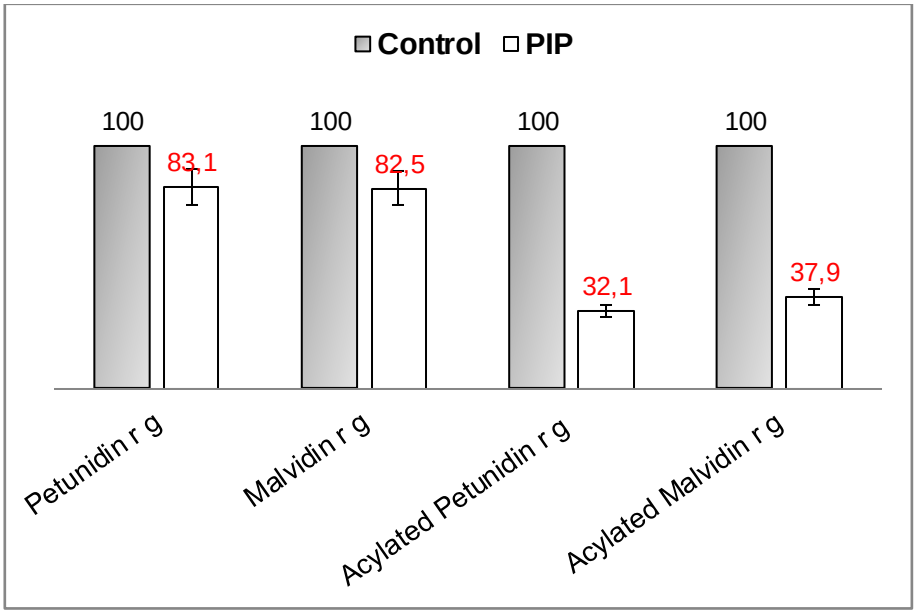
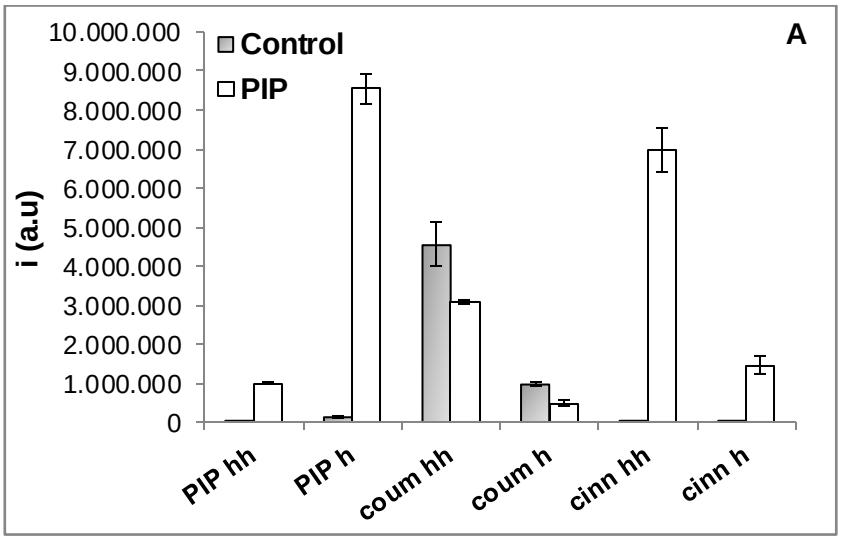
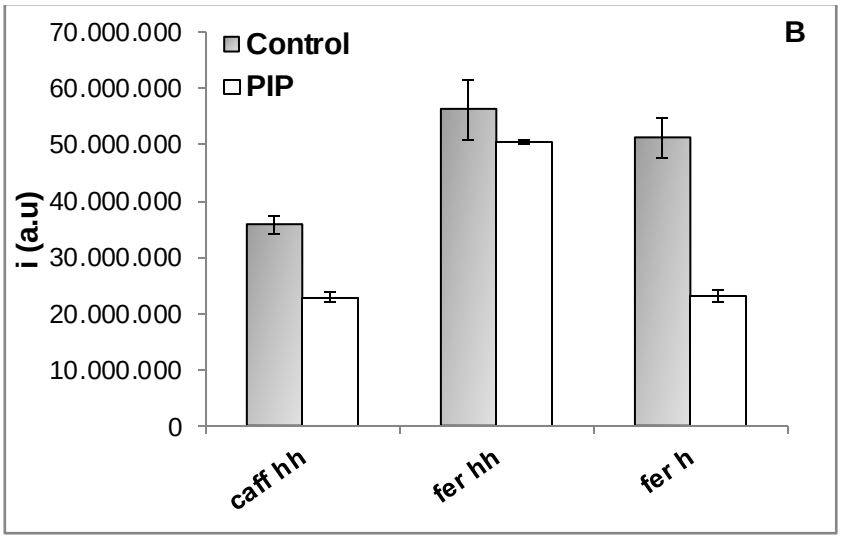


Fig. 2.9 Percentage of reduction of non-acylated and acylated AC after 48h of treatment with PIP. Samples of control were normalized at 100% (HPLC-DAD analysis). r, rutinoside; g, glucoside.

Furthermore, as expected, the treatment with PIP led to a reduction of coumaric acid derivatives and an enhancement of cinnamic acid derivatives, since coumaric acid and cinnamic acid represent respectively the product and the substrate of the reaction inhibited by PIP (Fig. 2.10 A). Some other HCA derivatives, such as caffeic and ferulic acid hexosides and di-hexosides underwent a decrease after PIP inhibition experiments (Fig. 2.10 B). With regard to hydroxybenzoic acids, some of them decreased while other increased after PIP supplementation (Fig. 2.10 C).





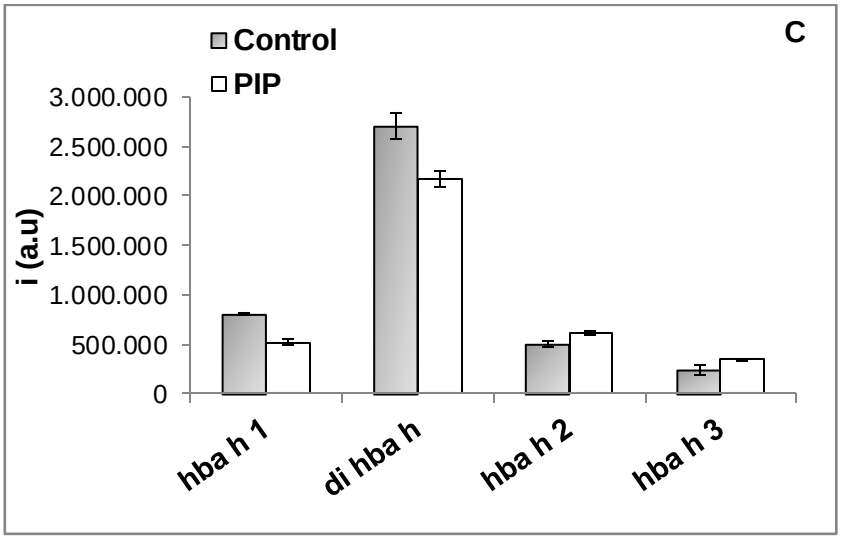


Fig. 2.10 MS intensity signals expressed in arbitrary units (a.u), obtained after the MzMine software analysis of the LC-MS chromatograms, were used for the quantification of different classes of metabolites in non-treated cells and in cells treated with PIP: A) piperonylic acid, coumaric acid and cinnamic acid hexosides; B) other hydroxycinnamic acid hexosides; C) di or mono hydroxybenzoic acid hexosides.
PIP, piperonylic acid, coum, coumaric acid; cinn, cinnamic acid; caff, caffeic acid; fer, ferulic acid; hba, hydroxybenzoic acid; h, hexoside.

Feeding with the AC precursor dihydroquercetin (DHQ)

In order to change the proportion between the various AC aglycones, cells were treated with 1 mM DHQ for 24 h, which is a precursor for the biosynthesis of the delphinidins and cyanidins in petunia species.

This treatment caused an expected increase of total ACs. Considering the individual ACs, delphinidin derivatives, which normally accumulated at very low levels underwent a remarkable increase (BPC 3,4 in Fig. 2.11). Moreover completely new ACs were stimulated after DHQ treatment, which were putatively identified as cyanidin derivatives (BPC 5,6 in Fig. 2.11).

The LC-MS analysis allowed the identification of these accumulated AC, as shown in Tab. 2.3.

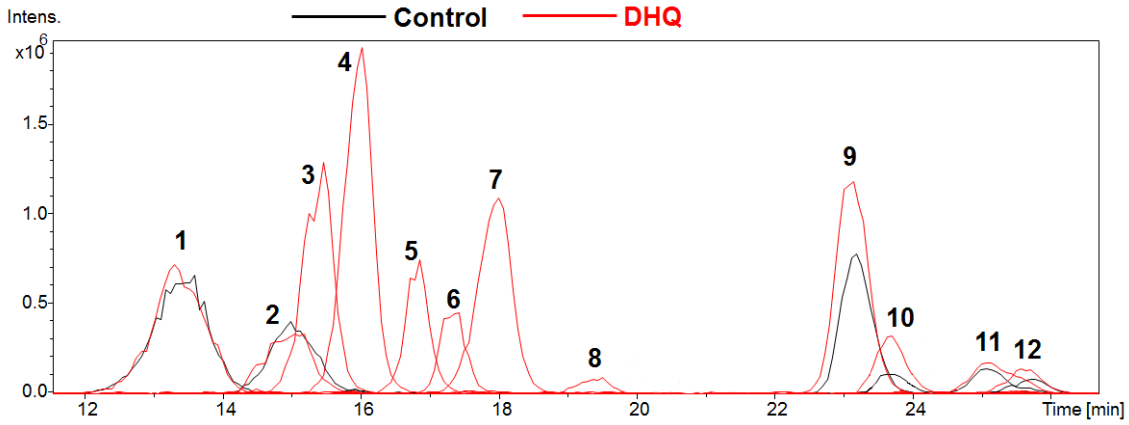


Fig. 2.11 Base peak chromatograms (BPC) of m/z (+) corresponding to mainly ACs in a representative non-treated sample (black) and sample supplemented with DHQ for 24 h (red) (LC-MS/MS analysis).

BPC	m/z (+)	Putative identification
1	787	Petunidin rutinoside glucoside
2	801	Malvidin rutinoside glucoside
3	465	Delphinidin glucoside
4	611	Delphinidin rutinoside
5	449	Cyanidin glucoside
6	595	Cyanidin rutinoside
7	625	Petunidin rutinoside
8	609	Peonidin rutinoside
9	933	Petunidin coumaroyl rutinoside glucoside
10	963	Petunidin feruloyl rutinoside glucoside
11	947	Malvidin coumaroyl rutinoside glucoside
12	977	Malvidin feruloyl rutinoside glucoside

Tab. 2.3 ACs putatively identified by LC-MS/MS analysis corresponding to BPC numbered in figure above.

The newly accumulated ACs included both cyanidin and delphinidin derivatives. Moreover, the profiles showed high variability in different independent experiments, without an overall predominance of cyanidin or delphinidin-type AC accumulation, but the ratio of accumulation between methylated ACs (petunidin, malvidin, peonidin) and non-methylated ACs (delphinidin, cyanidin) highly changed, with a strong increase of non-methylated ones (Fig. 2.12).

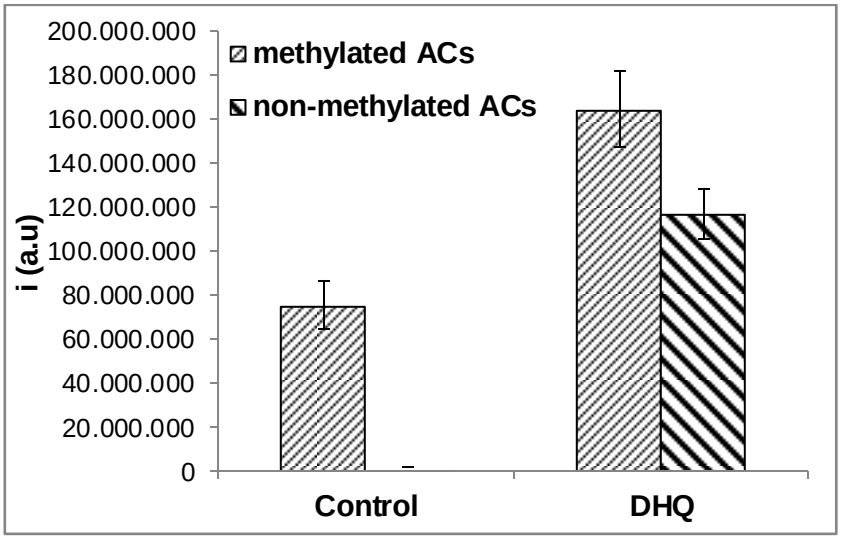
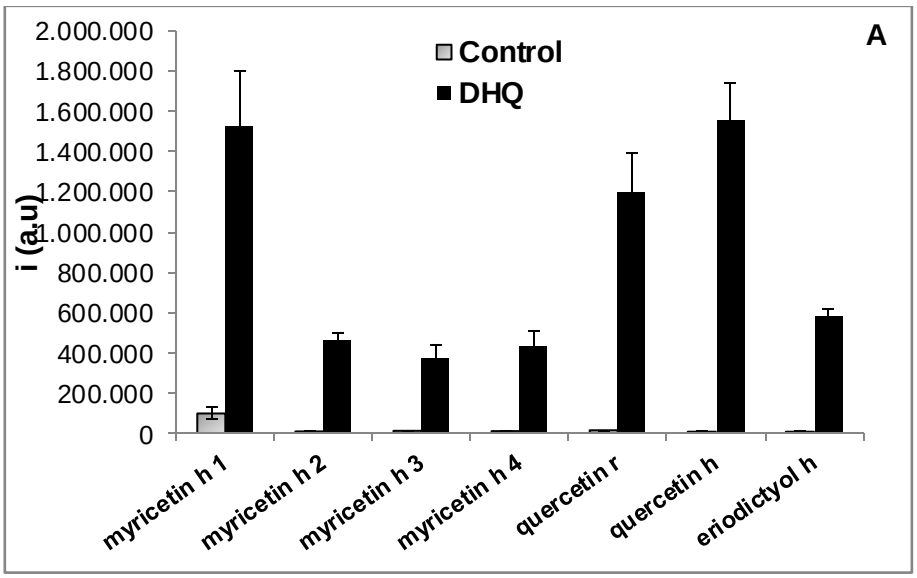


Fig. 2.12 MS intensity signals expressed in arbitrary units (a.u) used for the quantification of methylated ACs (petunidin, malvidin and peonidin derivatives) and non-methylated ACs (delphinidin and cyadinin derivatives) in non-treated samples and samples supplemented with DHQ for 24 h.

Furthermore feeding experiments induced an accumulation of DHQ hexosides and some other flavonoids, which derived from the DHQ biosynthesis, such as quercetin, myricetin and eriodictyol hexosides (Fig. 2.13).



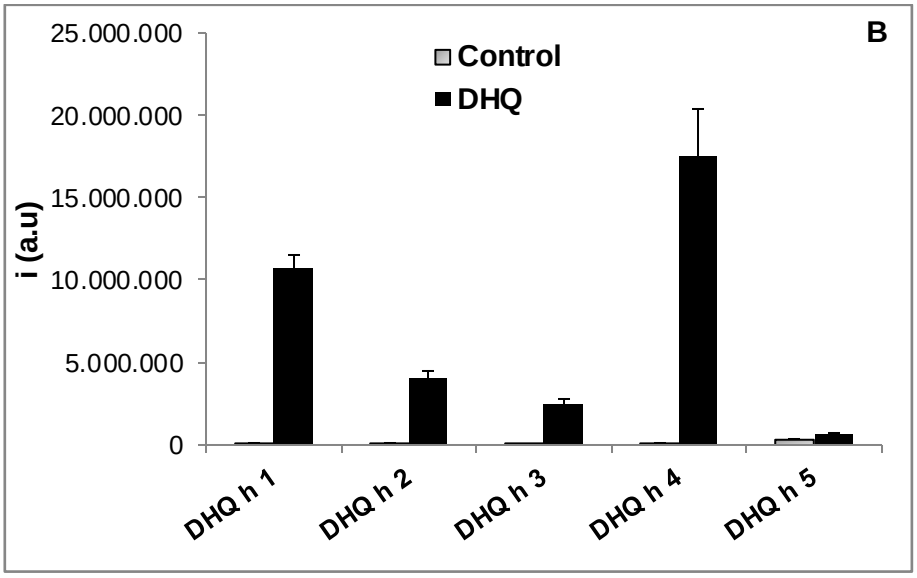


Fig. 2.13 MS intensity signals expressed in arbitrary units (a.u) used for the quantification of different classes of metabolites in non-treated cells and in cells fed with DHQ: A) myricetin, quercetin and eriodictyol derivatives; B) dihydroquercetin derivatives.
h, hexoside; r, rutinoside; DHQ, dihydroquercetin.

The R3M cell line (*Daucus carota*)

The carrot cell line R3M is able to accumulate cyanidin-based ACs, both non-acylated or acylated with coumaric, caffeic, ferulic, sinapic and hydroxybenzoic acid. Thus, it could represent a suitable model in which to investigate the possible different biological roles of acylated versus non acylated cyanidin-based ACs.

Inhibition experiments, with 0,1 mM piperonylic acid (PIP) supplied to ten-day-old cells for 24 and 48 h, were performed in order to specifically change the metabolic profile of R3M.

Treated samples were characterized by the appearance of PIP derivatives and cinnamic acid derivatives that, as expected, accumulated in the cells after treatment (Fig. 2.14).

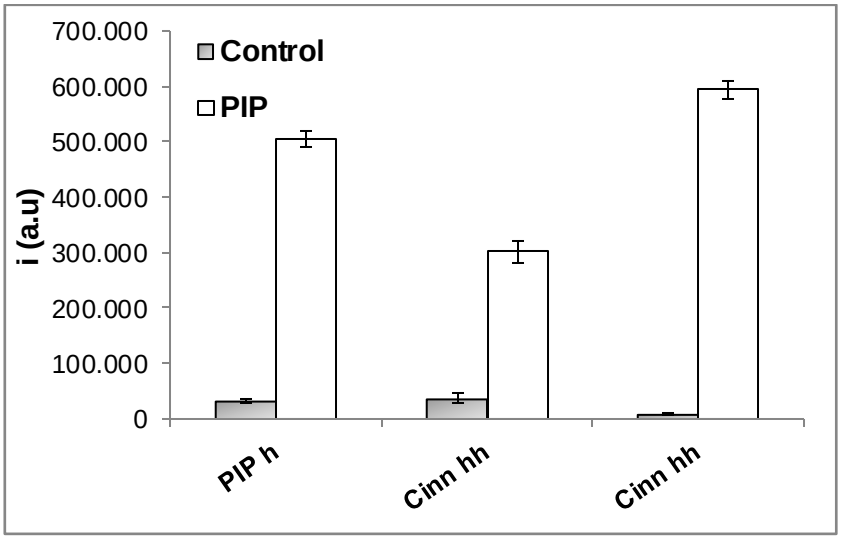


Fig. 2.14 MS intensity signals expressed in arbitrary units (a.u) used for the quantification of piperonylic acid and cinnamic acid hexosides in non-treated cells and in cells treated with PIP. PIP, piperonylic acid; Cinn, cinnamic acid; h, hexoside.

Considering ACs, the treatment with PIP caused the appearance of two new chromatographic peaks (Fig. 2.15) corresponding to the AC conjugated with PIP (peak 6) and the AC acylated with cinnamic acid (peak 7), the substrate of the inhibition reaction of PIP that is normally not present in carrot (Tab. 2.4, in red).

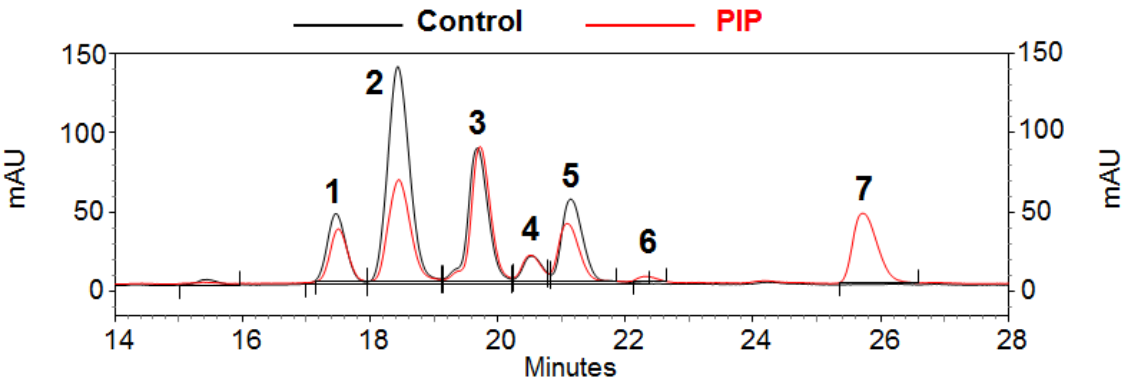


Fig. 2.15 HPLC-DAD chromatograms at 520 nm of two representative samples, treated sample with PIP (red) and non-treated sample (black). AC peaks are numbered.

Peak	m/z (-)	Putative identification
1	741	Cyanidin hexosyl pentosyl hexoside
2	579	Cyanidin hexosyl pentoside
	903	Cyanidin (caffeoyl) hexosyl pentosyl hexoside
	933	Cyanidin (caffeoyl) hexosyl hexosyl hexoside
3	947	Cyanidin (sinapoyl) pentosyl hexosyl hexoside
	861	Cyanidin (hydroxybenzoyl) hexosyl pentosyl hexoside
4	917	Cyanidin (feruloyl) pentosyl hexosyl hexoside
5	887	Cyanidin (coumaroyl) pentosyl hexosyl hexoside
6	889	Cyanidin (PIP) pentosyl hexosyl hexoside
7	871	Cyanidin (cinnamoyl) hexosyl pentosyl hexoside

Tab. 2.4 Putative annotation of AC peaks detected by HPLC-DAD, based on m/z(-) values and fragmentation patterns of LC-MS/MS analysis. HPLC-DAD peak 2-3 correspond to different ACs which elute at the same retention time.

The detected signal in HPLC-MS attributable to single ACs revealed that, unlike the results in petunia cell line, non-acylated ACs, mainly the cyanidin hexosyl pentoside (Cy ph, m/z (-) 579; HPLC-DAD peak 2), underwent a remarkable reduction compared to acylated ACs (Fig. 2.16).

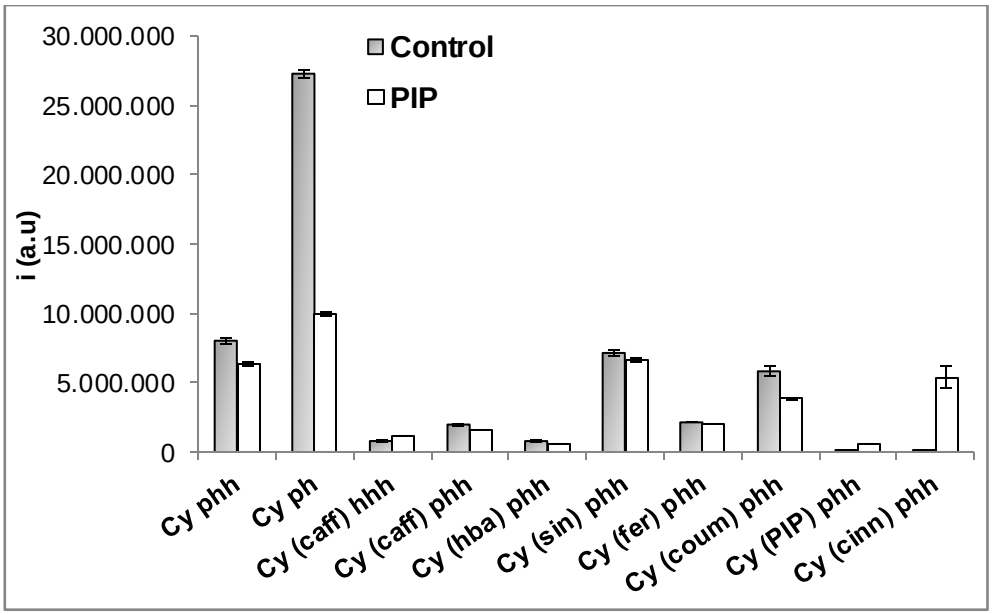
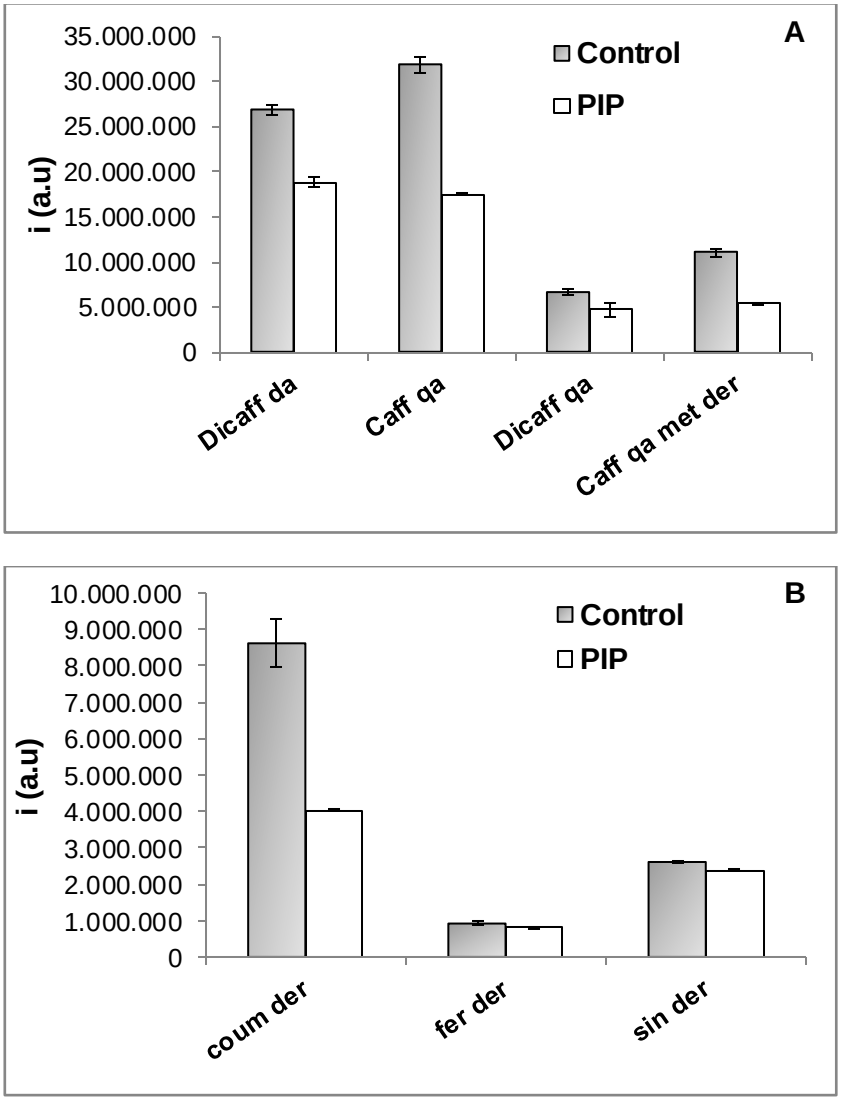


Fig. 2.16 MS intensity signals expressed in arbitrary units (a.u) used for the quantification of different ACs in non-treated cells and in cells treated with PIP.
Cy, cyanidin; p, pentose; h, hexose; caff, caffeic acid; hba, hydroxybenzoic acid; sin, sinapic acid; fer, ferulic acid; coum, coumaric acid; PIP, piperonylic acid; cinn, cinnamic acid.

In term of hydroxycinnamic acid derivatives, the effect of PIP reduced to a great extent the levels of the most abundant caffeic acid derivatives (dicaffeoyl daucic acid, caffeoyl quinic acid, dicaffeoyl quinic acid, caffeoyl quinic acid methyl derivatives) accumulated in the line (Fig. 2.17 A). Furthermore also coumaric acid derivatives were significantly more abundant in the control cells (Fig. 2.17 B).

Some flavonoids, dihydroquercetin (DHQ) and quercetin (Q) derivatives, were less accumulated in the cells treated with PIP, whereas naringenin and dihydrokaempferol derivatives which are more upstream in the biosynthetic pathway for the generation of DHQ and Q, were accumulated in the cells under the effect of PIP (Fig. 2.17 C).



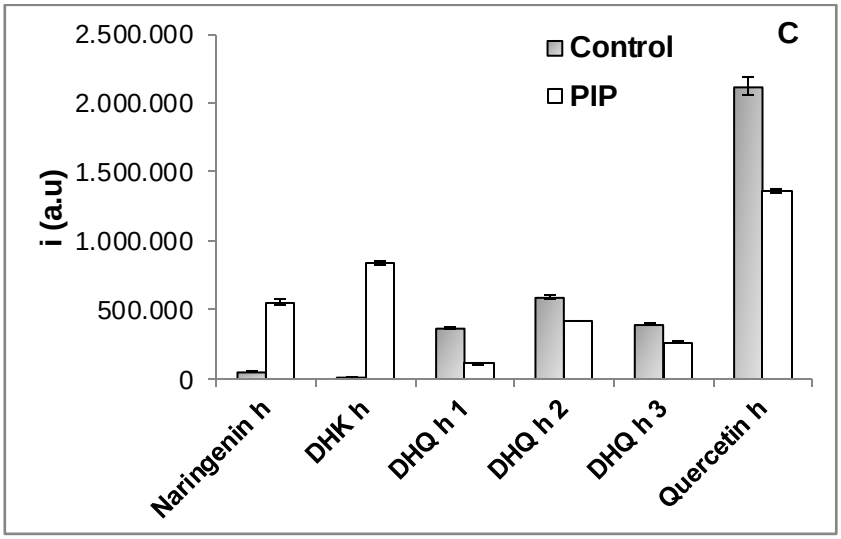


Fig. 2.17 MS intensity signals expressed in arbitrary units (a.u) used for the quantification of different classes of metabolites in non-treated cells and in cells treated with PIP: A) most abundant caffeic acid derivatives; B) other hydroxycinnamic acid derivatives; C) main other flavonoid derivatives.
Dicaff da, dicaffeoyl daucic acid; Caff qa, caffeoyl quinic acid; Dicaff qa, dicaffeoyl quinic acid; Caff qa met der; caffeoyl quinic acid methyl derivative; coum, coumaric acid; fer, ferulic acid; sin, sinapic acid; der, derivatives; h, hexoside; DHK, dihydrokaempferol; DHQ, dihydroquercetin.

Discussion

The plant secondary metabolites have been extensively studied for their possible biological effect on humans when consumed as part of the diet or as medicine, using a plethora of approaches including the administration of the molecules to animal cells systems. Surprisingly, less investigations involved the biological roles of these molecules within the plant cells that produce and accumulate them.

A possible approach to investigate the biological role of secondary metabolites *in vivo* in the cells that produce and accumulate them, is to change the secondary metabolite profile and to investigate in the new phenotypic traits due to these modifications. In turn, various strategies can be used in order to modify the secondary metabolite profile. The use of chemical inhibition of specific enzymes and the precursor administration can be a useful alternative to genetic-molecular approaches.

We used precursor feeding and specific inhibitor treatments in order to develop potential strategies to change the phenylpropanoid profiles in different *in vitro* cell cultures, characterized by different metabolomes.

The T2b cell line of *Ocimum basilicum* was used to investigate the relationship between ACs and RA, molecules that share part of the biosynthetic pathway and for which a protective function as antioxidant agents have been already described. Previous results indicated no competition between ACs and RA biosynthetic pathways, since mechanical stress experiments highlighted an increase of both compounds (Strazzer et al., 2011). The use of the inhibitor MDCA, that inhibits coumaroyl CoA-ligase in the reaction for the production of coumaroyl CoA (common intermediate between ACs and RA), led to the reduction of both ACs and RA, and, on the other hand, to a high accumulation of deoxy rosmarinic acid (4-coumaroyl-3',4'-dihydroxyphenyllactic acid, m/z(-) 343). In the final reaction for the generation of RA, deoxy RA ester is hydroxylated in positions 3 and 3' of the aromatic rings by two cytochrome P450 monooxygenases (Petersen, 1997). Although MDCA has been described as a potent inhibitor of the coumaroyl CoA-ligase (Funk and Brodelius, 1990), it remains also a potential inhibitor of the other P450 enzymes. In fact it is known that MDCA belongs to MDP (methylenedioxy) compounds, that are commonly used to characterize P450-dependent reactions, and are often considered to be broad-specificity inhibitors of P450 enzymes (Schalk et al., 1998). For this reason, MDCA could block the hydroxylation that leads to the formation of RA and promote the accumulation of deoxy RA in the cells.

A manipulation of the metabolome that could lead to a reduction in the levels of RA, maintaining the same levels of ACs, could be a suitable strategy to better elucidate the roles and relationships between the two type of compounds. For this purpose, feeding experiments with the AC precursor, DHQ, in the presence of the inhibitor (MDCA) were subsequently carried out. However, in preliminary experiments an unexpected non-

significant reduction of RA was obtained when MDCA was administered together with DHQ. Moreover, since T2b cells did not allow a complete cytological analysis because of the cell clustering, the strategy for the modification of metabolic profile in this line has not been further developed.

Compared to cyanidin-type AC profile expressed in T2b cell line, Sw4i cells of *Petunia hybrida* accumulated different type of ACs. The most predominant were petunidins and malvidins, but also delphinidins were accumulated at lower levels. By feeding cells with DHQ, which is a precursor in the formation of delphinidin based ACs, a modulation between non-methylated ACs (delphinidins) and methylated ACs (petunidins, malvidins) accumulation was desired. Going into details, DHQ can be converted in dihydromyricetin (DHM), which is in turn converted in colorless leucodelphinidin, the precursor of delphinidins. Further methylations in the B ring give rise to methylated ACs (petunidins, malvins). Furthermore, since in petunia species DHQ is also the direct substrate for the generation of leucocyanidin that generates cyanidin-type ACs, the strategy designed could also promote a further accumulation of cyanidin based ACs. As expected, this approach led to an increase of total anthocyanin level in Sw4i, mainly due to an increase of delphinidin-based ACs, normally present in trace amounts only. The appearance of novel cyanidins and of low levels of peonidins revealed that DHQ was both used for cyanidin-based AC production and converted in dihydromyricetin for delphinidin-based AC synthesis. The total amounts of methylated versus non-methylated ACs detected by LC-MS analysis showed a significant increase of non-methylated ones after DHQ feeding.

DHQ was not normally produced by the cell line, because probably it was completely converted in delphinidin-type ACs. However, when such precursor was administrated to cells, it was not totally consumed for the generation of the ACs commonly accumulated from Sw4i (delphinidin and its derivatives), since other ACs (cyanidin and

its derivatives) and flavonoids (DHQ, quercetin, myricetin and eriodictyol derivatives) have been observed.

Furthermore, while in basil cells all ACs were acylated with coumaric acid, in Sw4i cell line ACs were accumulated both as non-acylated and acylated forms. The inhibitor PIP was used in order to interfere with the biosynthesis of coumaric acid, a key precursor of ACs and at the same time, a substrate for the reaction of acylation, to obtain lower levels of acylated ACs. PIP turned out to be a promising tool to modulate AC profile in cell cultures, since cells treated with PIP showed a decrease in AC content with a more significant reduction of acylated ACs. Furthermore the reduction of coumaric acid derivatives accompanied by an accumulation of cinnamic acid derivatives suggested that PIP is an effective inactivator of coumaric acid synthesis *in vivo*.

A similar experimental approach has been applied to R3M of *Daucus carota*, a cell line already studied in detail in previous recent works (Ceoldo et al., 2005; Ceoldo et al., 2009). The ACs detected in the carrot suspension culture were qualitatively different from those detected in petunia cell line. R3M accumulated only glycosides of cyanidin, both non-acylated and acylated with many different hydroxycinnamic acids and with hydroxybenzoic acid. The inhibition experiments with PIP aimed, as in petunia cell line, to decrease the content of acylated ACs.

When the cells were treated with PIP, two new anthocyanins appeared, the AC conjugated with PIP, at very low levels, and the AC acylated with cinnamic acid, which became one of the major ACs. This new synthesis presumably reflects the limited specificity of the enzyme(s) that activate the acid (Halaweish and Dougall, 1990) or acylate the AC (Gläßgen and Seitz, 1992). In fact the possibility that the carrot suspension culture system can use a number of both natural and 'non-natural' cinnamic acids to acylate ACs has already been reported by Baker et al. (1994) and PIP can be considered as an analogue of a “non-natural” cinnamic acid. Moreover, Halaweish and Dougall (1990) provided evidence that extracts of carrot suspension cultures could

synthesize 1-O-sinapoyl-l]-D-glucose from sinapic acid and UDP-glucose and that similar activity occurred with all the natural 4-hydroxy-cinnamic acids and also with the unnatural 3,4-dimethoxycinnamic acid.

Beside the appearance of two new ACs in the cell line, inhibition experiments with PIP led to alterations in the relative proportions of the ACs accumulated with a strong decrease in the levels of non-acylated ACs. The apparent ineffectiveness of inhibitor in modulating negatively the content of acylated ACs is probably due to the non-specificity of acyltransferase enzymes in carrot cell line. In fact, PIP supplied to cells and cinnamic acid accumulated as substrate of the inhibited reaction, are used to generate acylated ACs at the expense of non-acylated ACs. It is not clear, at the same time, if these two newly accumulated AC can have a real biological activity due to the "non-natural" acyl groups. Since the number of the hydroxyl-groups on the benzene ring is an indication of some activities, such as the scavenging ability of hydroxycinnamic acids (Kikuzaki et al., 2002) and no hydroxyl-groups in the phenolic ring are present in PIP and cinnamic acid, it could be reasonable not to attribute any biological significance to the acylation of the newly synthesized ACs.

Furthermore, also in R3M cell line, PIP acts as a metabolic inhibitor of coumaric acid synthesis under *in vivo* condition, decreasing the levels of coumaric acid derivatives. The reduction in the levels of caffeic acid derivatives could be due to the reduction of coumaric acid that is the intermediate in the generation of caffeic acid derivatives or to the potential inactivation of any P450 enzyme by PIP. As we discussed before for MDCA, PIP is a potent inhibitor of CH4, but it could also inhibit other P450s (Schalk et al., 1998).

In our cell lines, PIP treatment caused also the increase of dihydrokaempferol and naringenin and the decrease of DHQ and quercetin. These compounds belong to the flavonoid pool of the cells, and their transformation (for instance the synthesis of DHQ from dihydrokaempferol) requires hydroxylation steps catalysed by cytochrome P-450

enzyme superfamily. It is reasonable to suppose that the observed perturbation of the flavonoid pool could be due to an inhibitory effect of PIP on the above class of enzymes.

References

Baker DC, Dougall DK, Gläßgen WE, Johnson SC, Metzger JW, Rose A, Seitz HU. Effects of supplied cinnamic acids and biosynthetic intermediates on the anthocyanins accumulated by wild carrot suspension cultures. Plant Cell Tiss and Org 1994;39:79–91.

Ceoldo S, Levi M, Marconi AM, Baldan G, Giarola M, Guzzo F. Image analysis and *in vivo* imaging as tools for investigation of productivity dynamics in anthocyanin producing cell cultures of *Daucus carota*. The New Phytol 2005;166:339–52.

Ceoldo S, Toffali K, Mantovani S, Baldan G, Levi M, Guzzo F. Metabolomics of *Daucus carota* cultured cell lines under stressing conditions reveals interactions between phenolic compounds. Plant Sci 2009;176:553–65.

Funk C, Brodelius PE. Phenylpropanoid metabolism in suspension cultures of *Vanillia planifolia* Andr. II. Effects of precursor feeding and metabolic inhibitors. Plant Physiol 1990;94:95–101.

Gläßgen WE, Seitz HU. Acylation of anthocyanins with hydroxycinnamic acids via 1-O-acylglucosides by protein preparations from cell cultures of *Daucus carota* L. Planta 1992;186:582–585.

Halaweish FT, Dougall DK. Sinapoyl glucose synthesis by extracts of wild carrot cell cultures. Plant Sci 1990;71:179–184.

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu CY, Farcy E, Stevenson TW, Cornish EC. Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 1993;366:276–279.

Jiang R, Lau K, Hon P, Mak TCW, Woo K, Fung K. Chemistry and Biological Activities of Caffeic Acid Derivatives from *Salvia miltiorrhiza*. Curr Med Chem 2005;12:237–246.

Kikuzaki H, Hisamoto M, Horose K, Akiyama K, Taniguchi H. Antioxidant Properties of Ferulic Acid and Its Related Compounds. J Agric Food Chem 2002;50:2161–2168.

Petersen M. Cytochrome P-450-Dependent hydroxylation in the biosynthesis of rosmarinic acid in *Coleus*. Phytochemistry 1997;45:1165–1172.

Schalk M., Cabello-Hurtado F, Pierrel MA, Atanossova R, Saindrenan P, Werck-Reichhart D. Piperonylic Acid, a Selective, Mechanism-Based Inactivator of the trans-Cinnamate 4-Hydroxylase: A New Tool to Control the Flux of Metabolites in the Phenylpropanoid Pathway. Plant Physiol 1998;118: 209–218.

Strazzer P, Guzzo F, Levi M. Correlated accumulation of anthocyanins and rosmarinic acid in mechanically stressed red cell suspensions of basil (*Ocimum basilicum*). J Plant Physiol 2011;168:288–293.

Tada H, Murakami Y, Omoto T, Shimomura K, Ishimaru K. Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum*. Phytochemistry 1996;42:431–4.

Tornielli G, Koes R, Quattrocchio F. The genetics of flower color. Petunia 2009;13:269–299.

Chapter 3

Effect of the metabolic modification on stress response

Introduction

In order to investigate the possible defensive role of secondary metabolites in plant cell cultures, a road map has been proposed in which the first step, already described in the previous chapter, consists in the modification of metabolic profile of cells by inhibitor and precursor administration. Once cells with specific metabolic modifications were obtained, the second step is to evaluate if these changes in the accumulation of specific secondary metabolites affect the cellular response against a stress treatment that causes a cellular damage. However, *Petunia hybrida* cell line was difficult to analyze from the cytological point of view because cells in suspension culture tend to aggregate and form large clumps. The presence of cell aggregates, which typically range in size up to several hundred cells, represented the major obstacle to the study of specific phenotypic traits induced by stress application.

Among the three different cell lines established, only R3M of carrot has proven to be suitable for cytological investigations. For this cell line it was possible to detect specific features in individual cells after heat stress application (44°C for 1h). In this conditions, the heat treatment caused deep modifications of cell organization with the appearance of internal patches instead of cytoplasmic streams typical of healthy cells (Commisso, Toffali and Guzzo, personal communication). It was also demonstrated, through *in vivo* imaging techniques, that the patched cells were committed to a slow cell death anticipated by a vacuole shrinkage. The same authors were also able to find a correlation between the modification of the metabolic profile of R3M cells and a reduction of damaged cells after heat stress; moreover, this protection correlated with a specific enhancement of acylated anthocyanins and hydroxycinnamic acid derivatives. These correlative results suggested but did not demonstrate a role of these compounds in heat stress protection, since minor modification of the metabolome or other not

predicted cell modification induced by feeding could be also responsible for the observed protection from heat stress.

In order to get more clues on the role of the above secondary metabolites on heat stress protection, the reverse approach, i.e. the inhibition of anthocyanin (AC) and hydroxycinnamic acid (HCA) accumulation by piperonylic acid (PIP) was used.

Materials and Methods

Treatment with PIP in cell cultures

PIP was dissolved in dimethyl sulfoxide (DMSO) and added at the final concentration of 0,1 mM into Petri plates containing 6 ml of ten-day-old cells. Non-treated cells were fed with an equal volume of DMSO and considered as control.

After 24 h of treatment, the liquid medium of treated and non-treated cells was substituted with eleven-day-old conditioned medium from another batch of cells, in order to remove the residual PIP from the medium avoiding at the same time any effect due to fresh medium. After every PIP treatment, an aliquot of cells was collected and used for metabolite analysis by HPLC-DAD and HPLC-MS.

Heat treatment

After 24 h of treatment with PIP, sufficient to cause the desired secondary metabolome modification, an heat stress has been applied to PIP-treated and untreated cells, by placing Petri dishes containing 6 ml of culture in an incubator at 44°C for 1 h.

After the heat treatment cells were maintained in the growth chamber at 25°C with a photoperiod of 16 h light / 8 h of darkness. Cells were monitored by microscopic analysis until six days after the stress application.

Cytological analysis

Cellular viability was determined via staining with fluorescein diacetate (FDA). FDA is a non-fluorescent non-polar molecule capable of entering in the intact cells where it is hydrolysed by cytoplasmic esterases producing fluorescein, which is a fluorescent polar molecule. The fluorescein cannot permeate the intact membranes of cells and accumulates in the cytoplasm conferring an intense green fluorescence. Cells were stained with 5 µg/ml FDA solution for 2 minutes and observed using optical fluorescence microscope Leica DMRB equipped with a Leica DC100 camera. Fluorescence emission, recorded through the following filter set: a band pass filter BP 470/90, a dichroic mirror DM 510 and a long pass filter LP 520, was used to distinguish viable (fluorescent) from non-viable (non-fluorescent) cells. Weakly fluorescent cells were not considered to be viable.

The effects of treatments were evaluated monitoring both the viability and the morphology of cells via staining with FDA. A small aliquot of each sample was used for determining the frequency of cells with different morphologies using a Nageotte chamber. From three to six technical replicates were included in the analysis and about 800 cells were observed in each sample.

Results

The inhibitor PIP has been supplied to carrot cells in order to decrease the accumulation of specific phenylpropanoids normally accumulated and to investigate the role of such metabolites after an heat stress (1 h, 44°C). Thus, cells were pretreated with 0,1 mM PIP for a congruous period of time to change their metabolite profiles, and then treated to 44°C for 1 h to test the effects of the profile modifications on heat stress responses. Since the patched cells were proven to be committed to die, the frequency of the patched cells was considered as an index of cell damage. The frequency of cells

displaying normal or damaged phenotype was determined through a Nageotte chamber. The frequency of damaged cells with modified metabolome (=cells pretreated with PIP) was compared with the frequency of untreated damaged control cells (= cells without metabolome modification). Moreover, to have a direct link between cell damage degree and metabolites, the metabolome of the control cells and cells pretreated with PIP were compared in each experiment.

Thus, as shown in Fig. 3.1 and in Tab. 3.1, the pre-treatment of carrot cells with PIP caused the reduction of non-acylated ACs, mainly of the most predominant cyanidin hexosyl pentose (BPC 3). The inhibition with PIP did not affect the accumulation of acylated ACs, beside a slight reduction of the AC acylated with coumaric acid (see Chapter 2).

In terms of HCAs, the inhibition remarkably reduced the content of caffeic acid derivatives, that were the most abundant HCA derivatives accumulated in the cell line (caffeoyl quinic acid, caffeoyl quinic acid methyl derivatives, dicaffeoyl quinic acid, dicaffeoyl daucic acid; BPC 1, 5, 8, 9).

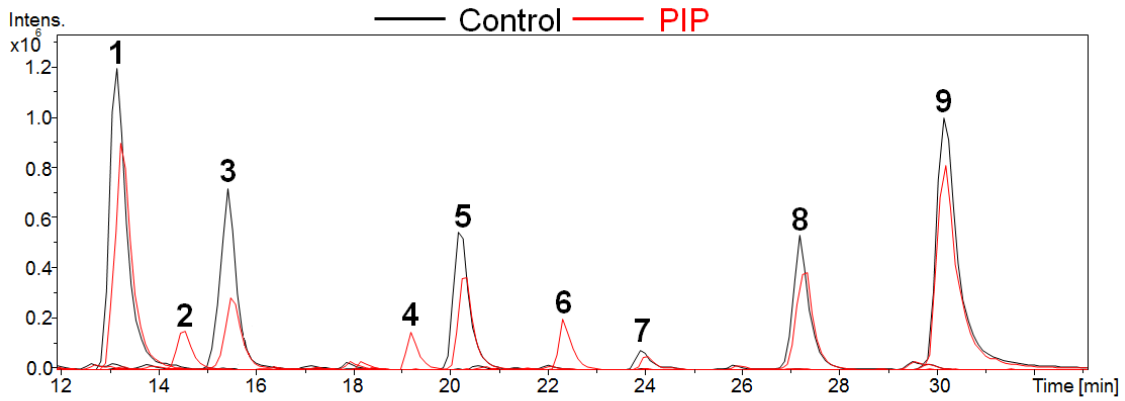


Fig. 3.1 Base peak chromatograms (BPC) of m/z (-) corresponding to the main molecules differently accumulated in cells pretreated with PIP (red) and cells with wild type metabolome (black), before heat stress application.

BPC	m/z (-)	Putative identification	Control	PIP
1	353	Caffeoyl quinic acid	+	
2	373	PIP hexoside formic adduct		+
3	579	Cyanidin hexosyl pentose	+	
4	889	Cyanidin (PIP) pentosyl hexosyl hexoside		+
5	367	Caffeoyl quinic acid methyl derivative	+	
6	871	Cyanidin (cinnamic acid) pentosyl hexosyl hexoside		+
7	463	Quercetin hexoside	+	
8	515	Dicaffeoyl quinic acid	+	
9	527	Dicaffeoyl daucic acid	+	

Tab. 3.1 Putative identification of the molecules corresponding to the base peak chromatograms numbered above.

The treatment with PIP caused an increase of the frequency of damaged cells after the heat treatment, as shown in Fig. 3.2.

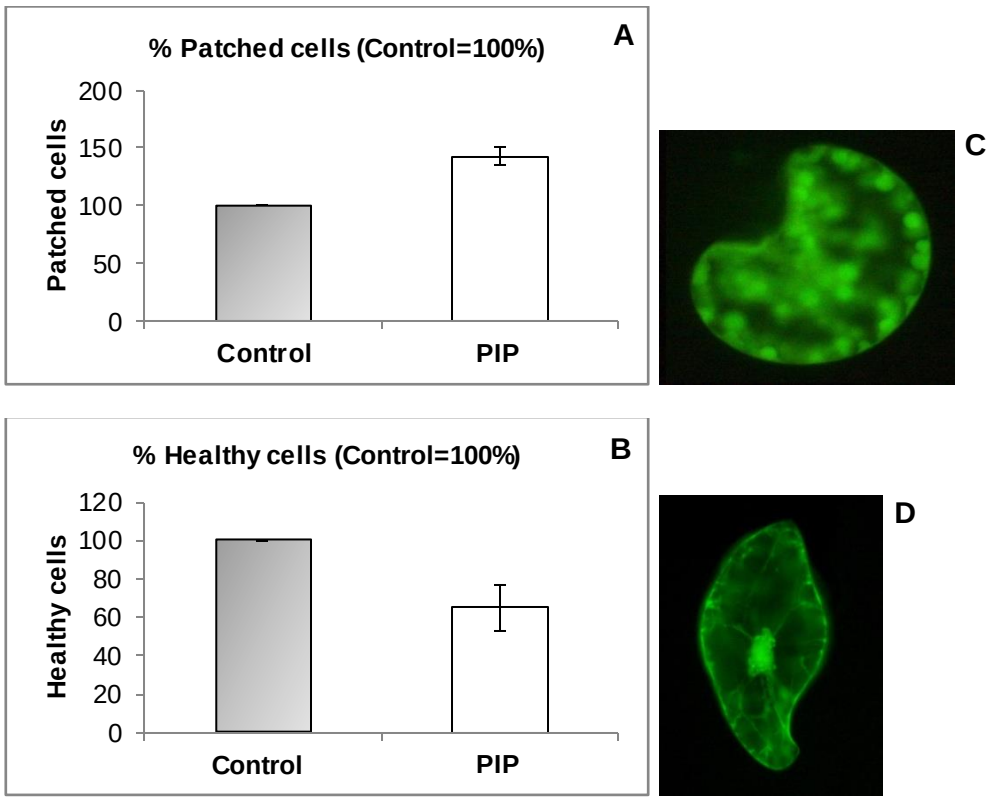


Fig. 3.2 Percentage of patched cells (A) and healthy cells (B) within cells with modified metabolome (=cells pretreated with PIP) and cells with wild type metabolome (control), after heat stress application. Fluorescence microscopy images C) Heat stressed cells with patches; D) Control cells with cytoplasmic strands.

Thus, the strong enhancement of damaged cells in samples pre-treated with the inhibitor PIP, after heat stress, was correlated with the reduction of non-acylated ACs and HCA derivatives, mainly caffeic acid derivatives.

Discussion

In the previous studies, the cytological damages induced after the heat treatment at 44°C for 1 h in R3M carrot cells was characterized, and this allowed to better investigate the possible protective effects of specific secondary metabolites, which levels of accumulation have been increased by feeding with precursors before the heat stress. More in detail, heat treatment of carrot cells resulted in an appearance of cytoplasmic patches surrounded by endoplasmic reticulum; this organization has been associated to the structure of autophagic vacuoles which are often stress-induced (Dunn, 1990; Jia et al., 1997; Mitou et al., 2009); it was demonstrated that the carrot cells showing this morphology were committed to a slow cell death fate.

Feeding R3M cells with HCAs before the heat treatment caused an increase in acylated ACs and HCAs derivatives and the reduction of the number of cells with patches. The opposite effect was obtained, within this research project, by supplying PIP that caused a decrease of the level of certain phenylpropanoids and an increase of the number of cells with damaged phenotype.

The heat-induced damage in the cell culture treated with the PIP inhibitor before the heat treatment, correlated with the reduction of non-acylated ACs and HCA derivatives, mainly caffeic acid derivatives. In contrast with the results obtained by HCAs feeding experiments, in this reverse approach it was not possible to assess the role of acylated ACs since they did not decrease after PIP administration, apart from a slight reduction of cyanidin coumaroyl penthosyl hexoside. Furthermore it has to be clarified the physiological importance of the appearance of unnatural ACs acylated with PIP and

cinnamic acid, as well as the accumulation of high level of other cinnamic acid and PIP derivatives following the administration of the inhibitor. With regards to ACs, it is not clear whether the acylation with these unnatural substrates, which do not show any hydroxyl-groups in the phenolic ring (Kikuzaki et al., 2002), might affect the ACs in terms of physical-chemical properties and might play a physiological role.

In conclusion, previous experiments in the same cell line showed a strong relationship between the enhancement of acylated ACs and HCAs induced by feeding and protection against the heat stress, while the enhancement of non-acylated ACs did not show the same effect. In the present experiments, the reduction of both non-acylated ACs and HCAs resulted in an increase of cell damaged after heat stress. Combining the two series of experiments it can be concluded that HCAs have a role in heat stress protection, while the eventual roles of acylated and non-acylated ACs needs to be further investigated.

References

Dunn WA. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J Cell Biol 1990;110:1923–1933.

Jia L, Dourmashkin RR, Allen PD, Gray AB, Newland AC, Kelsey SM. Inhibition of autophagy abrogates tumor necrosis factor a induced apoptosis in human T-lymphoblastic leukaemic cells. Brit J Haematol 1997;98:673–685.

Kikuzaki H, Hisamoto M, Horose K, Akiyama K, Taniguchi H. Antioxidant Properties of Ferulic Acid and Its Related Compounds. J Agric Food Chem 2002;50:2161–2168.

Mitou G, Budak H, Gozuacik D. Techniques to study autophagy in plants. Int J Plant Genomics 2009;451357.

Chapter 4

Concluding Remarks

Being sessile organisms, plants cannot run away nor can rely on an immune system when they are attacked by enemies. On the basis of the available experimental evidences, it seems they have evolved defence mechanisms based upon chemicals (so-called secondary metabolites) to protect themselves from different biotic and abiotic stresses. Despite several studies have elucidated the function for some of these compounds (Feucht and Treutter, 1999; Hopkins et al., 2009), plant secondary metabolism remains functionally poorly characterised. Furthermore in each plant species, secondary metabolites are present in a high structural diversity (Wink, 2003) that depends, at least partially, on the ability of plant cells to chemically link secondary metabolites each other and with other molecules such as organic acid, amino acids and sugars. The specific functions of these chemical reactions is unknown except for few molecules or classes of molecules, e.g. some non-toxic glycosides that generate toxic aglycones when the plant tissue is damaged by a predators attack (Morant et al., 2008).

Thus, the complexity and the variability of plant metabolome makes difficult to recognise the role of the various secondary metabolites and to define which components are both necessary and sufficient to confer a potential protection during a specific stress response.

In vitro plant cells, retain the same ability of plant tissues to accumulate various secondary metabolites (Gillet et al., 2000; Roat and Ramawat, 2009), such as different classes of phenylpropanoids (e.g. hydroxycinnamic acids, hydroxybenzoic acids, coumarins, stilbenes, flavonoids, anthocyanins). As consequence*, in vitro* plant cell cultures, selected for desired features, can be used as simplified model systems in which to investigate the biological role of different classes of secondary metabolites. The overall goal of the present project is the investigation on the possible biological role of specific secondary metabolites, accumulated in plant cultured cells, mainly belonging to the class of phenylpropanoids, in the protection against different stresses.

Phenylpropanoids are believed to be implicated in primary functions in plant, such as auxin transport regulation (Murphy et al., 2000; Brown et al., 2001; Peer et al., 2004) and in the protection of plant cell against stress, through their well-known activity of reactive oxygen species scavenging (Agati et al., 2012) and UV-absorbing capacity (Chapple et al., 1992; Ryan et al., 2002).

These proposed roles for phenylpropanoid compounds in plant defence have traditionally been based on biological activity *in vitro* (Kähkönen and Heinonen, 2003; Dixon and Paiva, 1995) and on correlations between both biotic and abiotic stress induction and increased accumulation of phenylpropanoids in plant cells and tissues with consequent expression of resistance *in vivo* (Christie et al., 1994; Hernandez et al., 2009). However the precise function of most of these molecules in the plant cell is still unclear.

In this research project an approach to functionally characterise specific phenylpropanoids was proposed; this approach was based on the modification of cell metabolome through administration of inhibitors and/or precursors followed by the investigation of the effect of these modification in the ability to respond to severe stress application.

The present work consists of two distinct parts: in the first (Chapter 2), a chemical approach was developed in order to modify qualitatively and quantitatively the metabolic profile of different cell cultures in terms of phenylpropanoid accumulation; in the second part (Chapter 3), the possible protective role of specific metabolites against stress application has been investigated through cytological analysis.

The investigations were carried out in cell cultures belonging to different plant species, to allow a comparison among different metabolic profiles and biosynthetic pathways, in order to gain a more complete and detailed understanding of how a different metabolome composition could be implicated in stress response. Moreover, as *in vitro* cell cultures are relatively easier to manipulate and constitute a simplified system from

a metabolic point of view, they can provide initial clues to move the investigations on intact plants.

By using chemicals as a strategy to modify the metabolic profile, the genetic background of cell cultures does not change, making this approach a good alternative or complement to metabolic engineering. Specifically, *in vivo* applications of specific enzyme inhibitors might mimic the results of gene silencing experiments, while feeding precursors might be an excellent tool to simulate the possible results of the introduction of a structural gene (i.e. an enzyme encoding gene) or overcome a rate limiting step (Martens et al., 2003). Moreover these strategies might give an insight to specie-specific features of the phenylpropanoid pathway (Chakrabortya et al., 2009; Funk and Brodelius, 1990).

In the inhibition experiments, two different enzyme inhibitors were used, piperonylic acid (PIP) and 3,4- (methylenedioxy)cinnamic acid (MDCA). They are selective inhibitors of two enzymatic reactions that take place in the upstream part of phenylpropanoid metabolism. PIP inhibits the cinnamate 4-hydroxylase (C4H), that converts *trans*-cinnamic acid to *p*-coumaric acid (Schalk. 1998), while MDCA is the inhibitor of hydroxycinnamate CoA-ligase (4CL), enzyme that converts in the following step, the *p*-coumaric acid to *p*-coumaroyl CoA thioester (Chakraborty et al., 2009).

On the other side, feeding experiments were carried out by using dihydroquercetin (DHQ), an anthocyanin precursor, which was already used in a previous work. The DHQ coupled with an acylation substrate have allowed to successfully increase specific anthocyanins in carrot cultured cells (Toffali et al., personal communication).

T2b cell line of *Ocimum basilicum* differs from the other cultured cells because it accumulated very high levels of rosmarinic acid (RA) in respect to the accumulation of other phenylpropanoids, and anthocyanins (ACs), with which it shares antioxidant functions. The relationship between RA and ACs has been investigated in a previous work, in which mechanical stress induced an enhancement in the levels of both

compounds (Strazzer et al., 2011). In the light of the common biosynthetic pathway shared between ACs and RA (from phenylalanine to coumaroyl CoA), a specific strategy was designed in order to better elucidate the relationships between the two compounds. As expected, the inhibitor of coumaroyl CoA-ligase enzyme, MDCA, blocking the biosynthesis of coumaroyl CoA, was been able to decrease the content of both RA and ACs in basil cells. In order to modify the ratio between RA and ACs, the treatment with MDCA was performed in presence of dihydroquercetin (DHQ), the precursor of ACs, in order to support the biosynthesis of ACs and allow a reduction of RA level. This experiment led to some promising clues, since it allowed to decrease the levels of RA, while ACs levels remained almost invariable. However the decrease of RA was not strong enough to allow the use of this treatment to investigate the biological role of this metabolite. This unexpected result and the difficulty to perform cytological analysis induced to abandon the investigations.

The diversity in the AC composition in terms of aglycones in Sw4i of petunia allowed to devise a strategy to change the ratio between different aglycones. The feeding with DHQ, a precursor in the biosynthesis of delphinidin and cyanidin type-ACs in petunia species, induced a modification in the accumulation of methylated ACs (petunidin, malvinid and peonidin) versus non-methylated ACs (cyanidin, delphinidin). Furthermore, since Sw4i cell line accumulated both non-acylated and acylated ACs, a strategy using the inhibitor PIP was set up in order to change the ratio between acylated ACs versus non-acylated ACs. Indeed, PIP, an inhibitor of cinnamate 4-hydroxylase (Schalk et al., 1998), blocks the synthesis of *p*-coumaric acid, a precursor of ACs biosynthesis and a substrate for acylation of ACs. This approach turned out to be effective to modulate AC profile in cell cultures, since cells treated with PIP showed a decrease in AC content with a more significant reduction of acylated ACs.

Since the carrot cell line, R3M, accumulated both non-acylated and acylated cyanidins with many different hydroxycinnamic acids and with hydroxybenzoic acid, the use of

PIP in this line was aimed, as in petunia cell line, to decrease the content of acylated ACs. PIP led to a strong decrease in the levels of non-acylated ACs and to the appearance of the AC conjugated with PIP and with cinnamic acid, the substrate of the reaction inhibited by PIP. This different result obtained in the different cell lines allowed to make some consideration regarding specie-specific features of the phenylpropanoid pathway. Carrot suspension culture system can use a number of both natural and 'non-natural' cinnamic acids (such as PIP) to acylate ACs (Baker et al., 1994), showing that the enzyme(s) that activate the acids (Halaweish and Dougall, 1990) or acylate the AC (Gläßgen and Seitz, 1992) show a poor specificity. This strategy may also reveal non-desired effects, such as the synthesis of novel compounds from intermediates accumulated upstream of the inhibited step of the pathway, such as the accumulation, in our experiments, of derivatives of cinnamic acid, which is the substrate of the reaction inhibited by the inhibitor PIP.

With regard to the possible protective role of the modified metabolome against stress, extensive efforts were spent to try to find specific phenotypic traits to be used as markers of cell damage after stress. Various stressing conditions have been used, such as applications of the glucose-glucose oxidase H_2O_2 generating system, that causes a severe oxidative stress, the direct administration of H_2O_2 (data not shown) and heat stress. However, no characteristic phenotypic traits to be used as cell damage markers were found in petunia suspension culture Sw4i. On the other side, R3M cell line of *Daucus carota* turned out to be a good candidate, not only for the modification of cell metabolome, but also for the investigation on the protective role of specific secondary metabolites, because specific markers of cell damages were found.

From a cytological point of view, R3M cells showed a typical cytoplasm organization, with a high complex network of mobile cytoplasmic streams. The heat treatment at 44°C for 1 h affected the cytoplasmic organization, causing the disappearance of the streams and the formation of very characteristic cytoplasmic patches surrounded by

endoplasmic reticulum. Furthermore *in vivo* imaging techniques allowed, in a previous work, to reveal that the cells with cytoplasmic patches (damaged cells), induced by heat stress, were committed to die, mainly by a slow cell death.

Previous feeding experiments with hydroxycinnamic acids (HCAs) allowed to modify in a predictable way the secondary metabolite profile of the carrot cultured cells (Toffali et al., personal communication). Specifically, the administration of different HCAs caused the increase of specific acylated ACs and HCAs derivatives. In order to evaluate the possible protective roles of acylated ACs and HCAs derivatives, cells fed with HCAs and untreated cells were subjected to the heat treatment, and the percentage of cells with cytoplasmic patches was determined. Cells fed with HCAs showed a lower percentage of damaged cells compared to the control cells.

In this work, the inhibitor PIP was used in order to perform a reverse approach, i.e. to decrease both HCA and AC levels in carrot cells. Indeed, PIP blocks the hydroxylation of cinnamic acid to *p*-coumaric acid, a precursors in the biosynthesis of ACs and HCAs. The treatment with PIP caused an increase of the percentage of heat damaged cells in treated samples compared with the control ones, suggesting a greater sensitivity to heat stress of the cells treated with PIP.

The classes of secondary metabolites which decrease after PIP treatment were mainly non-acylated ACs and HCA derivatives, especially caffeic acid derivatives. In these inhibition experiments these classes of metabolites were those that correlated with the increased sensitivity to heat stress.

Comparing the two complementary strategies, the implication of acylated ACs and non-acylated ACs in the protection to heat stress is still unclear. However, in both approaches, the increase (feeding experiments) or the reduction (inhibition experiments) of HCAs derivatives correlated with the reduction or increase of damaged cells, respectively. Therefore, these investigations suggested that HCAs play a role in the prevention of cellular damages induced by heat stress in R3M carrot cells.

References

Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci 2012;196:67–76.

Baker DC, Dougall DK, Gläßgen WE, Johnson SC, Metzger JW, Rose A, Seitz HU. Effects of supplied cinnamic acids and biosynthetic intermediates on the anthocyanins accumulated by wild carrot suspension cultures. Plant Cell Tiss Org 1994;39:79–91.

Brown DE, Rashotte AM, Murphy AS, Normanly J, Tague BW, Peer WA, Taiz L, Muday GK. Flavonoids act as negative regulators of auxin transport in vivo in *Arabidopsis*. Plant Physiol 2001;126:524–535.

Chakrabortya M, Karunb A, Mitra A. Accumulation of phenylpropanoid derivatives in chitosan-induced cell suspension culture of *Cocos nucifera*. J Plant Physiol 2009;166:63–71.

Chapple CCS, Vogt T, Ellis BE, Somerville CR. An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell 1992;4:1413–1424.

Christie PJ, Alfenito MR, and Walbot V. Impact of low temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta 1994:194;541–549.

Dixon RA, Paiva NL. Stress-Induced Phenylpropanoid Metabolism. Plant Cell 1995;7:1085–1097.

Feucht W, Treutter D. The role of flavan-3-ols and proanthocyanidins in plant defence. In: Inderjit S, Dakshini KMM, Foy CL (eds) Principles and practices in plant ecology 1999;307–338. CRC Press, Boca Raton.

Funk C, Brodelius PE. Phenylpropanoid metabolism in suspension cultures of *Vanillia planifolia* Andr. II. Effects of precursor feeding and metabolic inhibitors. Plant Physiol 1990;94:95–101.

Gillet F, Roisin C, Fliniaux MA, Jacquin–Dubreuil A, Barbotin JN, Nava–Saucedo JE. Immobilization of *Nicotiana tabacum* plant cell suspensions within calcium alginate gel beads for the production of enhanced amounts of scopolin. Enzyme Microb Tech 2000;26: 229–234.

Gläßgen WE, Seitz HU. Acylation of anthocyanins with hydroxycinnamic acids via 1-O-acylglucosides by protein preparations from cell cultures of *Daucus carota* L. Planta 1992;186:582–585.

Halaweish FT, Dougall DK. Sinapoyl glucose synthesis by extracts of wild carrot cell cultures. Plant Sci 1990;71:179–184.

Hernández I, Alegre L, Van Breusegem F, Munné-Bosch S. How relevant are flavonoids as antioxidants in plants?. Trends Plant Sci 2009;14:125–132.

Hopkins RJ, van Dam NM, van Loon JJA. Role of Glucosinolates in Insect-Plant Relationships and Multitrophic Interactions. Annu Rev Entomol 2009;54:57–83.

Kähkönen MP, Heinonen M. Antioxidant Activity of Anthocyanins and Their Aglycons. J Agric Food Chem 2003;51:628–633.

Martens S, Knott J, Seitz CA, Janvari L, Yu S-N, Forkmann G. Impact of biochemical pre-studies on specific metabolic engineering strategies of flavonoid biosynthesis in plant tissues. Biochem Eng J 2003;14:227–235.

Morant AV, Jørgensen C, Paquette SM, Sánchez-Pérez R, Møller BL, Bak S. β-Glucosidases as detonators of plant chemical defense. Phytochem 2008; 69: 1795–1813.

Murphy A, Peer WA, Taiz L. Regulation of auxin transport by aminopeptidases and endogenous flavonoids. Planta 2000;211:315–324.

Peer WA, Bandyopadhyay A, Blakeslee JJ, Makam SN, Chen R, Mason P, Murphy A. Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in *Arabidopsis thaliana*. Plant Cell 2004;6:1898–1911.

Roat C, Ramawat KG. Elicitor-induced accumulation of stilbenes in cell suspension cultures of *Cayratia trifolia* (L.) Domin. Plant Biotechnol Rep 2009;3:135–138.

Ryan KG, Swinny EE, Markham KR, Winefield C. Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. Phytochem 2002;59:23–32.

Schalk M., Cabello-Hurtado F, Pierrel MA, Atanossova R, Saindrenan P, Werck-Reichhart D. Piperonylic Acid, a Selective, Mechanism-Based Inactivator of the trans-Cinnamate 4-Hydroxylase: A New Tool to Control the Flux of Metabolites in the Phenylpropanoid Pathway. Plant Physiol 1998;118: 209–218.

Strazzer P, Guzzo F, Levi M. Correlated accumulation of anthocyanins and rosmarinic acid in mechanically stressed red cell suspensions of basil (*Ocimum basilicum*). J Plant Physiol 2011;168:288–293.

Wink M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochem 2003;64:3–19.

Summary

The precise physiological role of specific plant secondary metabolites has not been explored in details for most of them. One possible approach to elucidate their roles in plant is to modify the secondary metabolite profile of *in vitro* cell cultures and investigate the impact of such modifications on the phenotype.

The general experimental approach followed in this investigation can be summarized by these four different steps: 1) detailed analysis of the secondary metabolite profile of the chosen cell lines; this preliminary knowledge is necessary in order to design the proper strategy to selectively modify the accumulation or depletion of specific secondary metabolites; 2) the metabolite profile of the cells is modified by precursor and inhibitor administration, i.e. the main object of this research project, and the effects of any treatment on the metabolome are monitored though HPLC-DAD and HPLC-ESI-MS; 3) short stresses are applied to cells and the effects of stress are characterized from a cytological point of view in order to identify specific phenotypic traits caused by stress application; 4) the effects of the metabolome modification on cellular response against the stress are evaluated.

Three *in vitro* cell lines derived from different plant species, T2b (*Ocimum basilicum*), Sw4i (*Petunia hybrida*), R3M (*Daucus carota*), have been chosen for their ability to accumulate different set of secondary metabolites, especially in terms of phenylpropanoids. Different strategies have been developed in order to modify the cell metabolome.

In the basil cell line, T2b, a strategy using the inhibitor of the biosynthesis of coumaroyl CoA (3,4- (methylenedioxy) cinnamic acid, MDCA) coupled with the supplementation of an AC precursor (dihydroquercetin, DHQ) was performed, in order to change the ratio between rosmarinic acid (RA) and anthocyanins (ACs).

In the petunia cell line, Sw4i, two different strategies were designed in order to modulate the metabolic profile. A modification of the ratio between methylated ACs

(petunidin, malvidin, peonidin) and non-methylated ACs (delphinidin, cyanidin) was obtained feeding cells with DHQ, a precursor of cyanidin and delphinidin based-ACs in petunia species. In the second strategy, the use of piperonylic acid (PIP), an inhibitor of the biosynthesis of *p*-coumaric acid, allowed to modify the accumulation of acylated ACs versus non-acylated ACs, with a significant decrease of acylated ACs.

In the carrot cell line, R3M, cells were treated with PIP in order to decrease the accumulation of acylated ACs, as obtained in petunia. Surprisingly, after the treatment, non-acylated ACs underwent a strong reduction. This unexpected result might be due to the non-specificity of acyltransferase enzymes in carrot cell line. In fact, PIP supplied to cells and cinnamic acid accumulated as substrate of the inhibited reaction, might be used to generate acylated ACs at the expense of non-acylated ACs.

In R3M cell line specific phenotypic traits caused by heat stress were identified; in this line the protective role of specific classes of secondary metabolites after stress application could be investigated both through precursor and inhibitor administration.

The heat treatment at 44°C for 1 h induced the appearance of cytoplasmic patches surrounded by endoplasmic reticulum; it was also demonstrated that the carrot cells showing this morphology were committed to a slow cell death fate.

Previous experiments of feeding with hydroxycinnamic acids (HCAs) before the heat treatment caused an increase in acylated ACs and HCAs derivatives and the reduction of the number of cells with patches. By supplying PIP to R3M cells we obtained the decrease of the level of non-acylated ACs and HCA derivatives and an increase of the number of cells with stressed phenotype.

Comparing the results obtained by the two complementary approaches, it was not possible to assess the role of ACs. However both strategies suggested a role of HCAs in the prevention of cellular damages induced by heat stress in R3M carrot cells.

A word of gratitude

I am really grateful to Prof. Marisa Levi and Dr. Flavia Guzzo for giving me the opportunity to stay in their laboratory and helping me to realize this thesis. Thank you for your advice, time, input and mentorship.

I would like to thank all my colleagues for your help, support and friendship throughout the duration of this study.