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Transcriptional regulation of biosynthetic genes of the plant MEP-derived pathway to boost the metabolic flux towards bioactive diterpenes

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A Michele e ai miei figli

« Da' al mondo il meglio di te, e ti prendereranno a calci.
Non importa, da' il meglio di te. »

Madre Teresa di Calcutta

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Abstract

This project was aimed at enhancing the synthesis of tri-cyclic bioactive abietane diterpenes (*e.g.* aethiopinone, 1-oxoaethiopinone, salvipisone, and ferruginol), synthesized in the roots of *Salvia sclarea* and other *Salvia* species, with known anti-inflammatory and antitumoral activities. There is a great demand of novel molecules to treat melanoma, the most aggressive form of skin cancer, since advanced stages are inevitably resistant to conventional therapeutic agents.We have recently shown that aethiopinone is cytotoxic against the human melanoma A357 cell line at a concentration not toxic to normal cells. In addition, by using the web server *IdTarget* a number of putative proteins overexpressed in melanoma were identified as potential cellular target of aethiopinone. Despite this interesting evidence, this compound can not be easily synthesized by chemical means, and it is only produced in the roots of *Salvia* species in minute amounts (less than 0.5% DW) wich are not sufficient to yield reliable amounts for a deeper understanding of their molecular targets and potential future commercialization.

In order to produce sufficient quantity of this interesting class of compounds, we targeted the plastidial terpenoid MEP-dependent pathway, from which they derive, by two different metabolic engineering strategies in *S. sclarea* hairy roots.

The first approach was based on the coordinated activation of MEP-pathway biosynthetic genes by elicitation or by overexpression of transcription factors. An enhanced content (about a 20-fold increase) of abietane diterpenes in *S. sclarea* hairy roots was induced by elicitation with Methyl-Jasmonate (MJ), due to the increased expression levels of the several MEP-pathway biosynthetic genes, indicating a possible coordinate gene regulation by transcription factors. Four transcription factors (WRKYs and Myc2) of *A. thaliana* were selected on the basis of the presence of MJRE-box in their promoter region. Overexpression of *AtWRKY* and *AtMyc2* genes in *S. sclarea* hairy roots positively regulated transcription of several genes of the terpenoid MEP-pathway. High-level induced-expression of genes acting up-stream [1-Deoxy-D-Xylulose-5-Phosphate Synthase (*DXS*) and 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase (*DXR*)] or downstream [geranylgeranyl-diphosphate synthase (*GGPPS*) and copalyl-diphosphate synthase (*CPPS*)] of this pathway, correlated with high-level of abietane-type diterpenes (3-5 fold increase). To our knowledge, this is the first evidence of TFs activating this specific diterpene pathway. One drawback of this strategy was the impaired growth, at varying level, of transgenic *S. sclarea* hairy roots. However, it was possible to select the best performing over-expressing hairy root lines in which high final biomass was coupled to high content of abietane diterpenes.

The second strategy was aimed at blocking the Ent-copalyl-diphosphate synthase (Ent-CPPS), the first enzyme acting at the lateral competing route from GGPP to gibberellins. Either chemical inhibition of the enzymatic activity of Ent-CPPS with CCC (chlorocholine chloride), a known plant growth retardant, or RNAi-mediated silencing of this gene in *S. sclarea* hairy roots enhanced significantly (>4-fold) the total abietane diterpenes content, without causing any growth impairment compared to control hairy roots.

Overall, these complementary approaches were successful in increasing the content of aethiopinone and other tricyclic abietane diterpenes (from a 3-fold up to a 5-fold increase compared to the content in the control line) in engineered *S. sclarea* hairy roots and might be extended to different plant species

synthesizing other bioactive specialized terpenes. Moreover, the combination of these two approaches are expected to further enhance the accumulation of abietane diterpenes, as for chemical elicitation (with MJ, coronatine etc) coupled with metabolic engineering approaches, currently in progress in our laboratory, are also expected to increase the efficiency of the synthesis of this interesting class of compounds.

Finally, the promising results presented in this study pave the way to a rational design of a hairy root-based production platform to yield reliable amounts of tricyclic abietane diterpenes towards a deeper understanding of their molecular targets and the potential future exploitation as novel plant-derived anti-tumor molecules.

Introduction

1. Introduction

1.1 Medicinal plants as source of bioactive molecules

Plants represent a very rich source of different bioactive molecules, so that consumption of herbal medicines and medicinal plants is widespread and increasing. Medicinal plants play an important role in all cultures as an exclusive source of life-saving drugs maintaining health and combating diseases for majority of the world's population, (Canter *et al*., 2005). The identification of active principles and their molecular targets from traditional medicine provides an enormous opportunity for drug development (Chandra *et al*., 2012). Well over 50,000 plant species are used for medicinal purposes, currently; over 60% of anticancer drugs and 75% of drugs for infectious disease are either natural products or analogues of natural products (Wilson *et al.,* 2012). However, there is a growing concern on loss of genetic diversity since about 75% of the 50,000 different medicinal plant species in use are collected from the wild, with the consequent effects of habitat destruction and loss of genetic diversity. Cultivated material is more suitable for large-scale uses, such as the production of drugs by pharmaceutical companies, which require standardized products of guaranteed or known content and quality. These quality requirements are becoming increasingly important as drug regulations become more stringent in many countries (Canter *et al*., 2005) Domestic cultivation is a valuable alternative and offers the opportunity to overcome the problems that are inherent in herbal extracts, such as misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants. However, the costs of cultivated medicinal plants are frequently prohibitive because of their slow growth rate and the fact that many tropical plants are very difficult to cultivate in a commercial setting. Moreover, yield instability of cultivated medicinal plants due to unpredictable environmental constraints might cause serious problems in the biomass necessary for the extraction of adequate quantities of the purified plant compounds for the market demand, as occurred for worldwide shortage anti-malarial artemisinin in 2007 (Cohen *et al*., 2008). Another main problem associated with the commercial supply of plant secondary metabolites is the limited compound availability (Bouvier *et al*., 2005) Secondary metabolites typically represent <1% dry weight of the plant (Georgiev *et al*., 2009; Wilson *et al*., 2012). Altogether these considerations have opened the way to a new renaissance in the field of genetic and metabolic engineering studies to modify biosynthetic pathways in medicinal plants and to enhance the production of bioactive phytopharmaceuticals. Using modern biotechnology, plants with specific chemical compositions can be mass-propagated and genetically improved for the extraction of bulk active pharmaceuticals (Chandra *et al*., 2012).The possibility of extraction of plant bioactive secondary metabolites from massive plant cell and tissue culture is an attractive alternative (Leone *et al*., 2007; Weather *et al.*, 2010; Hussain *et al*., 2012). Hairy root cultures have been also proven to be an efficient tool for producing secondary metabolites that are normally biosynthesized in roots of differentiated plants (Georgiev *et al*, 2012; Talano *et al*, 2012).

1.2 Hairy roots for the production of medicinal compounds

Hairy roots are differentiated cultures of transformed roots generated by the infection of wounded higher plants with a Gram-negative soil bacterium *Agrobacterium rhizogenes*. This pathogen causes the hairy root disease leading to the neoplastic growth of roots that are characterized by high growth rate in hormone free media and genetic stability (Pistelli *et al.,* 2010). Hairy roots are highly differentiated and provide a reliable and stable system for extensive production of secondary metabolites, whereas other plant cells cultures have a strong tendency to be generally and biochemically unstable and often synthesize very low levels of useful secondary metabolites (Hu *et al*., 2006). The genetic transformation involved in the formation of transformed roots (widely called "hairy roots"; HRs) can be divided into the following steps: 1) chemio-tactism induced movement of agrobacteria towards the plant cells; 2) binding of the bacteria to the surface components of the cell wall; 3) activation of the virulence (*vir*) genes, 4) transfer and integration of the transfer-DNA (T-DNA) into the plant genome, and 5) HR formation (Bensaddek *et al.,* 2008; Georgiev *et al*., 2012). The genetic information allowing this infection process is mainly contained in the Ri plasmid (pRi) carried by the bacteria. In the pRi, the *vir* region contains 6 to 8 genes involved in the DNA transfer (T-DNA) (Bensaddek *et al*., 2008). The T-DNA has two independent sequences, denoted left (T_L) and right (T_R) borders. T_L-DNA is essential to induce HRs. Sequence analysis of T_L –DNA has revealed four open reading frames (among others) that are essential for HR induction (*rol* A, B, C and D). The products of the *rol A,B,C,D* genes all play key roles in HR formation. But the *rol*B gene appears to be the most important in HR induction, because loss-of-function mutation at this locus renders the plasmid avirulent (Georgiev *et al.,* 2011). The wild *A. rhizogenes* strains, many of which have been used to produce hairy roots from medicinal plants, can be classified by their opine type. Agropine strains induce agropine. Mannopine and agropinic acid production while the mannopine strains induce the production of one single opine. Agropine strains pRi transfer independently both the T_L-DNA and T_R-DNA to the plant genome, while mannopine strains only transfer the T_L-DNA. The choice of a bacterial strain is very important, since some plants are very resistant to infection. For example monocotyledonous are harder to transform with *Agrobacterium* than dicotyledonous plants. Moreover, bacterial strains are more or less virulent according to the plant species (Bensaddek *et al.,* 2008). The hairy root system is stable and highly productive under hormone-free culture conditions. The fast growth, low doubling time, ease of maintenance, and ability to synthesize a range of chemical compounds of hairy roots cultures offer additional advantages as continuous sources for the production of valuable secondary metabolites can also synthesize more than a single metabolite and, therefore, prove economical for commercial production purposes. Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced shown a relatively high productivity of secondary metabolites, which are potentially important pharmaceutical products (Oksman-Caldentey *et al*., 2004; Ono *et al*., 2011). However, sometimes the efficiency of secondary metabolic production is not so desirable. Metabolic engineering offers new perspectives for improving the production of secondary metabolites in medicinal and may lead to the accumulation of the target products (Hu *et al*., 2006; Leone *et al*., 2007).

1.3 Plant isoprenoids

Plant terpenoids are the most functionally and structurally diverse group of plant metabolites reported to date (Phillips *et al*., 2008) and are the most diverse class of natural products consisting of over 40,000 structurally different compounds, which have many essentially biological functions in the plant kingdom (Ajikumar *et al*., 2008) and in human health, have extensively been applied to pharmaceuticals (*e.g.*, artemisinin sesquiterpene; taxol; diterpene), herbal medicines (*e.g*., glycyrrhizin and ginsenoides; triterpenes), nutraceuticals (*e.g*., astaxanthin and lycopene; carotenoids), flavors (*e.g.,* limonene and linalool; monoterpenes), fragrances (*e.g.,* citronellol and geraniol; monoterpenes), cosmetics (*e.g*., astaxanthin), colorants (*e.g*., β-carotene; carotenoid), or agrichemicals (*e.g*., gibberellins, diterpenes) (Roberts, et al*.,* 2007). Currently there are very active studies on isoprenoids biosynthesis worldwide, and the knowledge and interests in isoprenoid biosynthesis pathways and bioengineering have been recently increased tremendously (Liu *et al*., 2005; Liao *et al.,* 2006; Verpoorte *et al*, 2007; Roberts, 2007). All isoprenoids are biosynthesized from just two universal C_5 precursors IPP and its isomer DMAPP. Sesquiterpenes have three C_5 units. Triterpenes are C_{30} terpenes utilizing six C_5 units and polyterpenes contain more than eight C_5 units. Although IPP and DMAPP are the universal precursors for isoprenoid biosynthesis there are two distinct pathways to biosynthesize isoprenoid in plant kingdom: the well- studied mevalonate (MVA) pathway and the recently unveiled deoxyxylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DXP/MEP) mevalonate-independent pathway (Fig. 1.1) Furthermore, the two distinct pathways are localized in different compartments at the subcellular level: the MVA pathway predominates in cytosol and the DXP pathway in plastid. Terpenoids constitute a large and diverse class of natural products that serve many functions in nature. Most of the tens of thousands of the discovered terpenoids are synthesized by plants, where they function as primary metabolites involved in growth and development, or as secondary metabolites that optimize the interaction between the plant and its environment. Through MEP-pathway are also synthesized gibberellins (GAs), a class of phytohormones that impact various aspects of plant growth and development (Fleet and Sun, 2005). For more than 50 years, Gas have been known for their dramatic impact on plant stature. Inhibition of GA biosynthesis results in dwarfism (Ninnemann et al., 1964), whereas exogenously applied gibberellic acid promotes internodal stem growth (Brian et al., 1954). Recent evidence suggests that Gas also play an important role in lateral root development. Mutants defective in GA biosynthesis (Berova and Zlatev, 2000) or signalling (Busov et al., 2006) were found to have enhanced lateral root formation. Several plant terpenoids are economically important molecules that serve many applications as pharmaceuticals, pesticides, etc. Major challenges for the commercialization of plant-derived terpenoids include their low production levels in *planta* and the continuous demand of industry for novel molecules with new or superior biological activities (Moses et al., 2013).

1.3.1 Isoprenoid biosynthesis in plastid through DXP pathway

Pyruvate and glyceraldehyde 3-phosphate (G3P) are the precursors for synthesizing isoprenoids on the MEP pathway. Pyruvate and glyceraldehydes are condensed to 1-deoxy-D-xylulose 5-phosphate synthase (DXP) by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and this is the first committed step on the plastid isoprenoid biosynthesis. DXP is subsequently rearranged and reduced to 2-C-methyl-D-erythritol 4-phosphate (MEP) catalyzed by 1-deoxy-D-xylulose 5- phosphate reductoisomerase (DXR). Then, MEP is conjugated with CDP by MEP cytidyltransferase (MCT) to form 4-(cytidine 5-diphospho)-2-C-methylerythritol kinase (CMK) catalyzed the phosphorylation of CDP-ME to form CDP-MEP is subsequently converted to 2-C-methylerythritol 2,4-cyclodiphosphate (ME-cPP) by 2-C-methylerythritol 2,4-cyclodiphosphate synthase (MECPS). The final two steps of DXP pathway include the formation of hydroxymethylbutenyl 4-diphosphate (HMBPP) from MecPP catalyzed by hydroxymethylbutenyl 4-diphosphate synthase (HDS), and the direct conversion of HMBPP into 5:1 mixture of IPP and DMAPP by IPP and DMAPP synthase (IDS). The isoprenoids can be classified into different groups according to the number of C_5 units that are used to build the skeletons of isoprenoids. The known simplest isoprenoid, isoprene, contains only a single C_5 units. Monoterpenes consists of two C_5 units.

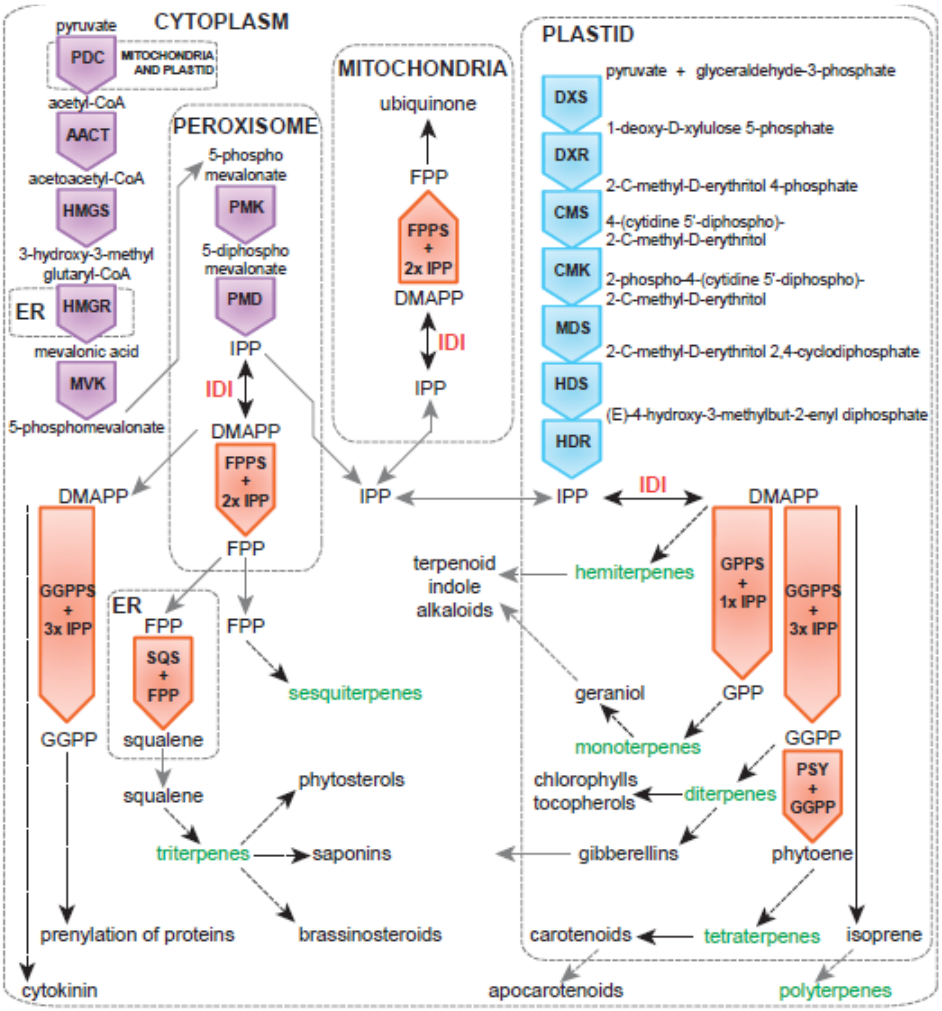


Figure 1.1 - The cytoplasmic mevalonic acid pathway (MVA) (purple) and the plastid MEP Pathway (blue). for the synthesis of isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The prenyltransferases (orange) generate the immediate precursors for the different terpenoid classes (green). Dotted arrows indicate multiple reactions. Dotted grey boxes indicate the subcellular localization of the pathway. Grey arrows indicate metabolites that are transported between subcellular compartments. **AACT**, acetoacetyl-CoA thiolase; **CMK**, 4-diphosphocytidyl-methylerythritol kinase; **CMS**, 4-diphosphocytidyl-methylerythritol synthase; **DMAPP**, dimethylallyl pyrophosphate; **DXR**, deoxyxylulose 5-phosphate reductoisomerase; **DXS**, deoxyxylulose 5-phosphate synthase; FPP, farnesyl pyrophosphate; **FPPS**, FPP synthase; **GGPP**, geranylgeranyl pyrophosphate; **GGPPS**, GGPP synthase; GPP, geranyl pyrophosphate; **GPPS**, GPP synthase; **HDR**, hydroxymethylbutenyl 4-diphosphate reductase; **HDS**, hydroxymethylbutenyl 4-diphosphate synthase; **HMGR**, 3-hydroxy-3-methylglutaryl-CoA reductase; **HMGS**, 3-hydroxy-3-methylglutaryl-CoA synthase; **IDI**, isopentenyl diphosphate isomerase; **IPP**, isopentenyl pyrophosphate; **MDS**, methylerythritol 2,4-cyclodiphosphate synthase; **MVK**, mevalonate kinase; **PDC**, pyruvate dehydrogenase complex; **PMD**, 5-diphosphomevalonate decarboxylase; **PMK,** 5-phosphomevalonate kinase; **PSY**, phytoene synthase; **SQS**, squalene synthase. (from Moses et al., 2013).

1.3.2 Plant diterpenoids

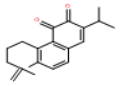
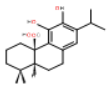
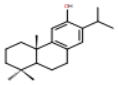
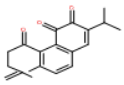
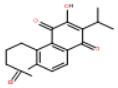
Diterpenoids constitute a large class of chemically diverse metabolites, derived from geranylgeranyl diphosphate (GGPP), which form a large group of >12,000 natural products that are widely distributed throughout the plant kingdom. Because of their various biological activities in humans, diterpenoids of plant origins have substantial economical relevance as bioproducts for a variety of applications (Caniard *et al.,* 2012). The plastid is the main if not the unique site for the synthesis of geranylgeranyl diphosphate (GGPP) that constitutes the backbone for the synthesis of diverse diterpenes (Bouvier *et al.*, 2005). Geranylgeranyl diphosphate synthase (GGPPS) catalyzes the consecutive condensation of the dimethylallyl diphosphate (DMAPP) with tree molecules of IPP to produce geranylgeranyl diphosphate (GGPP), the universal precursor for biosynthesis of diterpenes. While all the enzymatic steps and the encoding genes involved in the synthesis of GGPP have been completely elucidated in different plant species, unfortunately, very little is known about the subsequent enzymatic steps that from GGPP lead to biosynthesis of diterpenes in plants. Cyclic diterpenoids are commonly biosynthesized from geranylgeranyl diphosphate (GGPP) through the formation of carbon skeletons by specific cyclases or diterpene cyclases and subsequent chemical modifications, such as oxidation, reduction, methylation, and glucosidation (Toyomasu *et al.,* 2008, 2009). Different classes of diterpene synthases (or cyclases), have been cloned and their functional proteins sequenced (Toyomasu *et al.,* 2009). However, besides the well known biosynthetic pathway of gibberellins (Yagamuchi *et al.,* 2008), the two most studied and advanced examples are the synthesis of the anti-tumoral diterpene taxol (Kirby and Keasling, 2009), and abietane diterpenoids which are the major constituents of conifer resins (Keeling *et al.,* 2011).

1.3.3 Abietane diterpenes synthesized in *Salvia* spp

The genus *Salvia* constitutes the largest of the plant family Lamiaceae with 900 species widespread throughout the world. The genus has yielded various classes of natural products, including the major class of terpenoids, particularly the diterpenoids. Diterpenoids constitute a large class of chemically diverse metabolites, widely distributed throughout the plant kingdom with more than 12,000 known examples (Hanson *et al*., 2009). Most of the diterpenoids has diverse biological properties, such as antitumor (Devappa *et al.,* 2010) cytotoxic antibacterial (Aiyelaagbe *et al.,* 2007), antiplasmodial (Clarkson *et al.,* 2003; Sutthivaiyakit *et al*., 2003), leishmanicidal, gastroprotection, molluscicidal (Schmeda-Hirschmann *et al.,* 1996), antifungal (Schmeda *et al*., 1992), insecticidal, and antiproliferative activities. Some of them have effects on cardiovascular and central nervous systems (Bonito *et al.,* 2011) exhibit *in vitro* antiproliferative activity against the human cervical cancer cell line (HeLa) (Choudhary *et al.,* 2012). All the diterpene compounds are derived from geranylgeranyl pyrophosphate (GGPP), but the structural changes involved in the last stages of the biosynthetic pathway lead to a wide variety of complex chemical structures, including acyclic compounds, bi-tri-and tetra-cyclic. The main terpenic constituents of the *Salvia* species are represented by tricyclic diterpenoids, which can be classified in abietane and neo-clerodane diterpenoids In particular, more than 400 diterpenoids with different abietane skeletons have been isolated from *Salvia* plants (Zhou *et al.,* 2009).

The roots of *S. sclarea* are rich in abietane diterpenoids (*e.g.* carnosic acid, aethiopinone, 1-oxoaethiopinone, salvipisone, and ferruginol), with known antibacterial, antifungal, and sedative pharmacological properties. Examination of the *Salvia* species has afforded a series of abietane-quinone type diterpenoids, the secondary metabolites that are widely known and studied. Aethiopinone, salvipisone, 1-oxoaethiopinone and ferruginol, abietane-phenolic type, obtained from *Salvia sclarea*, proved to be bacteriostatic as well as bactericidal for the cultures of *Staphylococcus aureus* and *Staphylococcus epidermidis* strain (Kuzma *et al.,* 2007). It has been reported by Walencka *et al*. (2007) that salvipisone and aethiopinone from *Salvia sclarea* showed synergy activity with ß-lactam antibiotics through alterations of cell surface hydrophobicity and permeability, but not by changing penicillin binding protein. This class of tricyclic diterpenoids has raised also much attention for its cytotoxic and antitumoral activity against human tumor cell lines (Hernández-Pérez *et al*., 1995; Rozalski *et al*., 2006; Vaccaro et al., 2014) (Table 1.1). Abietane diterpenes, especially those containing quinone, are often reported to have cytotoxic effects on cancer cell lines and deserve greater attention because several cancer chemotherapeutic agents also possess the quinone structural feature. Salvipisone and aethiopinone showed relatively high cytotoxicity against HL-60 and NALM-6 leukemia cells (IC_{50} range 0.6-7.7 µg ml^{-1} which is equal to 2.0-2.47 µM), whereas 1-oxoaethiopinone and ferruginol were less active in this regard. Caspase-3 activity determinations showed that salvipisone and aethiopinone were able to induce apoptosis in a time- and concentration-dependent manner (Rozalski *et al.* 2006). Recently, aethiopinone was proved to be cytotoxic also to different solid tumor cell lines, i.e. MCF7 (breast adenocarcinoma), HeLa (epithelial carcinoma), PC3 (prostate adenocarcinoma), and A375 (human melanoma) (Vaccaro et al., 2014) These results point at *S. sclarea* aethiopinone as useful plant-derived new compound for the treatment of human cancers, especially in the case of drug resistance.

Table 1.1 Biological activity from different abietane diterpenes synthesized in the roots of *S. sclarea*

Diterpene	Biological Activity	References
Aethiopinone 	Antiinflamatory Cytotoxic and Proapoptotic Bactericidal	Vaccaro et al., 2014 Walencka *et al., 2007* Rózalski *et al*., 2006 Benrezzouk *et al.,* 2001
Carnosic acid 	Apoptosis. Antioxidant Anti-angiogenic	Kar *et al*., 2012 Erkan, 2008 Tsai CW *et al.,* 2011 López-Jimenez *et al.,* 2011
Ferruginol 	Cardioactive. Antiplasmodial. Cytotoxic	Ullubelen *et al*., 2002 Clarkson *et al*., 2003 Rózalski *et al*., 2006
1-Oxoaethiopinone 	Antimicrobial	Kuzma *et al*., 2007
Salvipisone 	Antibiofilm Antimicrobial Cytotoxic	Walencka *et al*., 2007 Rózalski *et al*., 2006

1.4 Metabolic engineering in plants

In 1991, Bailey defined metabolic engineering as ‘‘the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technologies” (Bailey., 1991). Today, the availability of the complete genome sequence for several plants, together with the development of powerful techniques for the transformation and stable or transient expression of genes in plants brings plant metabolic engineering as a strong alternative to classical chemical synthesis for the production of pharmaceuticals and other important industrial compounds. Plant metabolic engineering involves the manipulation of existing metabolic pathways by either increasing or diverting flux to desired or from undesired products, respectively, or the generation of chemical entities not normally found in the plant production system through the introduction of genes from other organisms. In general terms, development of metabolic engineering strategies to boost the production of bioactive secondary metabolites in plants or in plant tissue cultures requires:

- **i)** a systematic expansion of the available molecular toolboxes (*i.e.* cloned genes for enzymes involved in a specific pathway and regulatory genes);
- **ii)** detailed and accurate knowledge on the biochemistry of the metabolic pathways under study, including rate limiting enzymatic reactions, on

the cellular compartmentation of the specific compounds and their catabolism;

iii) appropriate and efficient systems for genetic transformation of the plant of interest.

Pioneer work in engineering metabolically medicinal plants was based on overexpressing one or few genes thereby overcoming specific rate limiting steps in the pathway, or to shut down competitive pathways or to decrease catabolism of the target product (Fig. 1.2). However, identifying rate limiting reactions in one specific pathway is often difficult and, besides, the level of the end-product accumulation can be controlled by more than one enzymatic activity. Secondly, attempts have been made to change the expression of regulatory genes that control multiple biosynthetic genes. Pioneer work in engineering metabolically medicinal plants was based on over-expressing one or few genes thereby overcoming specific rate limiting steps in the pathway, or to shut down competitive pathways or to decrease catabolism of the target product. However, identifying rate limiting reactions in one specific pathway is often difficult and, besides, the level of the end-product accumulation can be controlled by more than one enzymatic activity. Secondly, attempts have been made to change the expression of regulatory genes that control multiple biosynthetic genes (Fig. 1.1). This has been, in many cases, the most successful strategy in boosting the synthesis of bioactive secondary metabolites in plants. Because of the ability of controlling multiple, if not all steps in a particular metabolic pathway, transcription factors (TFs) provide attractive tools for overcoming flux bottlenecks involving multiple enzymatic steps, or for deploying pathway genes in specific organs, cell types or even plants where they normally do not express. The potential of a TF for the predictive manipulation of plant metabolism is intimately linked to understanding how it fits in the gene regulatory organization. The knowledge gained over the past decade on how plant pathways are controlled together with increasing efforts aimed at deciphering the overall architecture of plant gene regulatory networks are starting to realize the potential of TFs for predictive plant metabolic engineering (Jirschitzka et al., 2013).

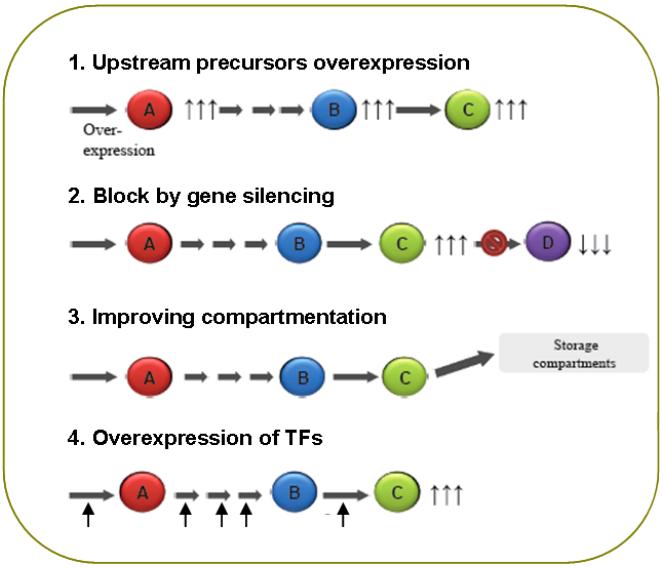


Fig. 1.2 **Strategies to modulate organic compound levels in plants.** A and B are the precursors of C; C is the target product; D is the result of the target product conversion. **(1)** Upstream precursors enhancement by increasing flux through the pathway by overexpressing the enzyme(s) that catalyze(s) the first committed step of the pathway. **(2)** Blocked pathway branch points by RNA interference or antisense. **(3)** Enhanced accumulation of target metabolite by increasing sink compartments. **(4)** Enhanced accumulation of target metabolite by overexpressing transcription factors. (Modified from Zorrilla-Lopez et al., 2013).

1.5 Targeting entire pathways with transcription factors

Metabolic pathways are often controlled by regulatory proteins that bind to the promoters of the genes encoding the enzymes and activate their transcription. Thus, transcription factors provide an attractive alternative to the manipulation of single or multiple enzymes for increasing flux (Table 1.2). Because of the conspicuous pigmentation provided by anthocyanin pigments, the flavonoid pathway provides one of the best-studied cases of control of a plant metabolic pathway (Mol et al., 1998). Anthocyanin accumulation is regulated in many plant species by the concerted action of transcription factors corresponding to the MYB and bHLH families (Irani et al., 2003). Not surprisingly, the regulators of anthocyanin biosynthesis have been among the first ones used to demonstrate the potential of transcription factors to activate entire pathways (Goff et al.,1990). Such transcription factors are known for defence pathways, and are drawn mainly from the bZIP, MYB, MYC, WRKY and ERF families (Singh et al., 2002). For example, the ORCA3 transcription factor from Catharanthus roseus is a classic example of a defence pathway regulator. It controls a major portion of the terpenoid indole alkaloid pathway, and like various other defence transcription factors, its own expression is induced by jasmonic acid signaling (van der Fits et al., 2000) and by other transcription factors, including a basic helix loop-helix protein known as CrMyc2 (Zhang et al., 2011). In the case of *C. roseus*, the expression of tryptophan decarboxylase (TDC) and of other pathway genes was upregulated by the overexpression of CrWRKY1. This transformation resulted in the overproduction of the alkaloid serpentine. The jasmonate-induced NbbHLB1 and NbbHLH2 transcription factors are both

involved in nicotine biosynthesis in *Nicotiana benthamiana*. The overexpression of one or the other factor singly produced higher levels of nicotine, but expression of both together provided even more nicotine (Todd et al., 2010). The value of transcription factors in plant pathway engineering is usually not limited to the original source species. In recent years several factors have been shown to be active in other species that are not necessarily of close taxonomic affinity to the source species (Akagi et al., 2010; Zhou et al., 2011; Dal Cin et al., 2011). For example, the transcription factor AtMYB12, which partially regulates the phenylpropanoid pathway in *A. thaliana*, also increases the concentration of phenylpropanoids and flavonoids in *N. tabacum* when heterologously expressed in that species. The resulting plants had increased resistance to larvae of *Spodoptera litura* and *Helicoverpa armigera* in comparison to wild-type tobacco (Misra et al., 2010). The identification of novel plant repressor domains, such as the EAR motif, expands the use of transcription factors to not only activate entire metabolic pathways, but also to inhibit them (Hiratsu et al., 2003). In addition to using natural transcription factors, an emerging approach is to artificially create transcription factors, primarily from the Zn-finger family, with known DNA-binding specificities (Jantz et al., 2004).Thus, the use of artificial DNA-binding domains fused to either transcriptional activation or repression motifs is likely to provide a powerful future tool for altering flux through entire metabolic pathways using a single transgene.

1.6 Transcriptional regulation of terpenoids

The synthesis and proper accumulation of secondary metabolites are strictly controlled in a spatial and temporal manner and influenced by a number of biotic and abiotic factors. The spatio-temporal transcriptional regulation of metabolic pathways is controlled by a complex network involving many regulatory proteins known as transcription factors (TFs). TFs are sequence specific DNA binding proteins that recognize specific cis-regulatory sequences in the promoters of target genes and activate or repress their expression in response to developmental and/or other environmental cues. Some TFs do not bind DNA but interact with other co-factors to form complexes that regulate the expression of the target genes (Yang et al., 2012). Terpenes comprise the largest class of plant secondary metabolites, containing in excess of 50,000 chemicals identified (Vranová et al., 2012), and many bio-synthetic enzymes in the pathway have been characterized. Despite the wealth of knowledge regarding the synthesis of terpenes, transcriptional regulation of these compounds is the least well studied, partially due to alternative methods available to increase chemical production (Wu et al., 2006). As with alkaloids, the AP2-ERF and WRKY transcription factor families are quickly emerging as important regulators of terpene biosynthesis. Recent advances in *Artemisia annua* have uncovered several TFs leading to the biosynthesis of artemisinin, a sequiterpene lactone widely utilized in the treatment of malaria. Two jasmonate responsive AP2-ERF TFs from *A. annua*, AaERF1 and AaERF2, regulate the transcription of *AMORPHA-4,11-DIENE SYNTHASE (ADS)* and *CYP SEQUITERPENE OXIDASE (CYP71AV1)* (Yu et al., 2012). Both AaERF1 and AaERF2 belong to the same B3 subfamily of AP2-ERF TFs as *Catharanthus* ORCA2 and ORCA3. *ADS* and *CYP71AV1* are also regulated by the WRKY TF, AaWRKY1, that also regulates *3-HYDROXY 3-METHYLGLUTARYL-COA*

REDUCTASE (HMGR) and *ARTEMISINIC ALDEHYDE Δ11(13) REDUCTASE (DBR2).* Recently, the AP2/ERF TF AaORA1, has been shown to regulate accumulation of artemisinin, and contribute to *A. annua* defence against the necrotrophic plant pathogen, *Botrytis cinerea* (Lu et al., 2013). With the exception of *HMGR*, AaORA1 regulates the same enzymes as AaWRKY1. AaORA1 and AaWRKY1 also regulate DBR2, which catalyzes the conversion of artemisinic aldehyde into dihydroartemisinic acid, directing the pathway towards artemisinin formation. In *Artemisia*, increased production of either artemisinin or artemisinic acid is desirable as a semisynthetic conversion of artemisinic acid to artemisinin is possible (Roth et al., 1989). WRKY TFs have also been cloned and characterized from several other terpene producing plants. *Taxus* species provide the valuable anti-cancer drug paclitaxel. The enzyme 10-DEACETYLBACCATIN III-10 β-O-ACETYL TRANSFERASE (DBAT) is a key rate limiting step in the synthesis of paclitaxel (Walker et al., 2000). Overexpression of DBAT was previously shown to increase the accumulation of paclitaxel in cell suspension (Zhang et al., 2011). Li et al. (2013) recently identified aWRKY TF, TaWRKY1, from *Taxus chinensis* cells, as a regulator of DBAT. GaWRKY1, from cotton (*Gossypium arboreum*), regulates the expression of (+)-δ-CADINENE SYNTHASE (CAD1), a branch point in the synthesis of sesquiterpenes leading to gossypol (Xu et al., 2004). GaWRKY1 not only regulates CAD1 developmentally in a temporal and spatial manner, but also in response to fungal and jasmonate elicitor treatment in cell suspension cultures (Xu et al., 2004). Recently, HbEREBP1 and HbWRKY1, from rubber tree (*Hevea brasiliensis*), have been implicated in regulation of latex production (Chen et al., 2012; Zhang et al., 2012). HbWRKY1 was found to be strongly induced by abscisic acid, ethylene, jasmonate, osmotic stress, *Oidium heveae* infection, and wounding (Zhang et al., 2012). HbEREBP1 may be a negative regulator of early jasmonate and wounding induction of latex biosynthesis (Chen et al., 2012). Together, these examples demonstrate that the WRKY transcription factor family clearly plays a prominent role in the regulation of terpenoid compounds.

Table 1.2. Transcription factors employed in the engineering of plant metabolic pathways (from Jirschitzka et al., 2013)

Family	Transcription factor	Plant species	Defense metabolites	GenBank
bHLH	CrMYC2	*Catharanthus roseus*	Terpenoid indole alkaloids	AF283507
	MYC2	*Arabidopsis thaliana*	Sesquiterpenes	At1g32640
	NbbHLH1	*Nicotiana benthamiana*	Nicotine	GQ859152
	NbbHLH2	*Nicotiana benthamiana*	Nicotine	GQ859153
MYB	MYB134	*Populus tremuloides*	Proanthocyanidins	ACR83705
	MYB28	*Arabidopsis thaliana*	Glucosinolates	Q9SPG2
	MYB29	*Arabidopsis thaliana*	Glucosinolates	Q9FLR1
	MYB76	*Arabidopsis thaliana*	Glucosinolates	Q9SPG5
	DkMyb2	*Diospyros kaki*	Proanthocyanidins	BAI49719
	GmMYBZ2	*Glycine max*	Terpenoid indole alkaloids	DQ902861
	AtMYB12	*Arabidopsis thaliana*	Flavonoids	NM_130314
	ODORANT1	*Petunia hybrida*	Phenylpropanoids	AAV98200
WRKY	AaWRKY1	*Artemisia annua*	Artemisinin	FJ390842
	CrWRKY1	*Catharanthus roseus*	Terpenoid indole alkaloids	HQ646368
	SoWRKY1	*Spinacia oleracea*	Compounds responding to wounding and salicylic acid	N/A
ERF	ORC1	*Nicotiana tabacum*	Nicotine	CQ808982
	AaERF1	*Artemisia annua*	Artemisinin	JN162091
	AaERF2	*Artemisia annua*	Artemisinin	JN162092

Abbreviations: bHLH, basic helix-loop-helix; ERF, ethylene response factor.

1.7 Transcription factors regulated by Methyl-jasmonate

The biosynthesis of secondary metabolites in plants is widely believed to be part of response to biotic or abiotic stress (Hahlbrock et al., 2003). It has been well-documented that endogenous signal compounds, such as JAs, are involved in elicitor-induced secondary metabolite biosynthesis. JAs are oxylipin-derived phytohormones that regulate a wide variety of physiological plant processes ranging from growth and development to reproduction and defence. Originally, JAs were labelled as secondary metabolites present in the scent of jasmine flowers (*Jasminum* spp.). Now, it has become clear that they themselves act as elicitors of the production of secondary metabolites across the plant kingdom, from angiosperms to gymnosperms (Wasternack., 2007; Pauwels et al., 2009). JAs can induce the synthesis of molecules in all three major classes of plant secondary metabolites (terpenoids, alkaloids and phenylpropanoids) (Zhao et al., 2005). JA, itself, increases secondary metabolite production and inhibition of JA biosynthesis abolishes elicitor-induced metabolite accumulation and the expression of secondary metabolite synthesis genes (Zhao et al., 2005; Wasternack, 2007). In addition, JAs can modulate particular primary metabolic pathways to supply connected secondary metabolite pathways with the necessary substrates. Hence, downstream of a conserved elicitation mechanism, species-specific secondary metabolic pathways have evolved under JA control (Memelink, 2009). Several genome-wide transcript profiling studies have demonstrated that JA treatment triggers an extensive transcriptional reprogramming of metabolism (De Geyter et al., 2012). The expression of genes encoding enzymes involved in one particular secondary metabolic pathway often displayed a marked concerted upregulation after JA elicitation, leading to the recognition of so-called 'transcriptional regulons' (Pauwels et al., 2009). Transcriptional regulators of three other TF families have been reported to be involved in transcriptional reprogramming of secondary metabolite pathways in a JA-inducible manner, but their exact position in the JA signalling cascades and/or their interaction with the JA core

module remains unclear. A number of TFs, MJ activated, have been identified (Table 1.3). Among these, the OCTADECANOID-DERIVATIVE RESPONSIVE CATHARANTHUS AP2-DOMAIN 2 and 3 (ORCA2 and ORCA3), driving terpenoid indole alkaloid (TIA) synthesis in *C. roseus* (Memelink et al., 2001) or PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) and C1/R, steering anthocyanin biosynthesis in Arabidopsis and maize (*Zea mays*), respectively (Dubos et al., 2010; Petroni et al., 2011). Myc2 has been shown to be both directly and indirectly involved in regulating secondary metabolite induction. It positively regulates TFs and biosynthetic enzymes of flavonoid biosynthesis but negatively controls tryptophan (Trp)-derived indole glucosinolate synthesis (Dombrecht et al., 2007). The *C. roseus* Myc2 homologue regulates the expression of the ORCA TFs by direct binding to the 'on/off switch' in the promoter of the ORCA3 gene, thereby controlling expression of several TIA biosynthesis genes (Zhang et al., 2011). In common tobacco (*N. tabacum*), Myc2 upregulates the ORCA-related NIC2 locus APETALA2/ETHYLENE Response Factor (AP2/ERF) TFs that regulate nicotine biosynthesis. In parallel, they also directly bind the target promoters of several nicotine biosynthesis genes (Shoji et al., 2011; Zhang et al., 2012). Accordingly, co-expression with the Myc2 TF stimulated the functionality of at least one of these NIC2 locus AP2/ERFs, whereas co-expression with the JAZ proteins reduced it (De Boer et al., 2011) In the related species *N. benthamiana*, the Myc2 homologues NbbHLH1 and NbbHLH2 also function as positive regulators in the JA mediated activation of nicotine biosynthesis (Todd et al., 2010). The WRKY TFs have been implicated in a diverse range of stress tolerance and development programmes (Agarwal et al., 2011; Rushton et al., 2010). Several WRKY TFs may regulate secondary metabolism biosynthesis in response to JA elicitation, as suggested by their (fast) upregulation by JA treatment. For instances, the induction of *Medicago truncatula* cell suspension culture with MJ lead to the up-regulation of several *WRKY* factors and overexpression of four *WRKY* genes in tabacco demonstrated their regulatory roles in lignin deposition, *PR* gene expression, and systemic defence responses against Tobacco mosaic virus (Naoumkina et al., 2007). Moreover, in recent years, many WRKY genes have been isolated from medicinal plants. The *Cj*WRKY1 from *Coptis japonica* represents the first discovery of transcriptional regulator of benzyl isoquinoline alkaloid biosynthesis (BIA). Silencing of Cj*WRKY1* transcripts led to a substantial reduction in the expression of several gene transcripts involved in berberine biosynthesis, whereas expression of Cj*WRKY1* in protoplasts of a berberine-producing *C. japonica* cell line increased expression of berberine biosynthetic genes (Kato et al., 2007). A full-length cDNA *AaWRKY1* was isolated from a cDNA library of the glandular secretory trichomes of *A. annua*, in which artemisinin is synthesized and sequestered. Transient expression of *AaWRKY*1 cDNA in *A. annua* leaves clearly activated the expression of the majority of artemisinin biosynthetic genes, suggesting the involvement of the AaWRKY1 transcription factor in the regulation of artemisinin biosynthesis and indicating that *Amorpha-4,11-diene synthase* (*ADS*) is a target gene of AaWRKY1 in *A. annua* (Ma et al., 2009). The cotton (+)-δ-Cadinine synthase (CAD1), a sesquiterpene cyclase, catalyzes the first committed step in the formation of the phytoalexin gossypol. Its promoter contains a W-box palindrome with two reversely oriented TGAC repeats, which are proposed binding sites of WRKY transcription factors. Thus, GaWRKY1, an orthologous of AtWRKY18, has been isolated and participated in regulation of sesquiterpene biosynthesis in cotton. Coordinated expression of *GaWRKY1* and *CAD1-A* were

observed in floral organs and in response to fungal elicitor treatment (Xu et al., 2004). Recently, an high throughput screening performed both in *A. thaliana* and *C. roseus* identificated new WRKY TFs jasmonate responsive. The seventy-five percent of the jasmonate esponsive CrWRKY's were orthologs of AtWRKYs known to be regulated by jasmonate. (Schluttenhofer et al., 2014) These new identified TFs represent potential candidates for regulation of TIA accumulation in *C. roseus*. In general, identification of jasmonate responsive WRKY provide useful information on plant defense and natural product regulatory networks.

Table 1.3 Overview of TFs recruited by JA signalling to steer secondary metabolites biosynthesis (from De Geyter et al., 2012)

TF name	Accession no.	Plant species	Target secondary metabolite pathway
AP2/ERF			
ORCA2	AJ238740	*Catharanthus roseus*	Terpenoid indole alkaloids
ORCA3	EU072424	*C. roseus*	Terpenoid indole alkaloids
ERF189	–	*Nicotiana tabacum*	Nicotine
ERF221/ORC1	CQ808982	*N. tabacum*	Nicotine
ERF1	JN162091	*Artemisia annua*	Artemisinin
ERF2	JN162092	*A. annua*	Artemisinin
bHLH			
MYC2/At1g32640	NM_102998	*Arabidopsis thaliana*	Indole glucosinolates and anthocyanins
MYC2	AF283507	*C. roseus*	Terpenoid indole alkaloids
MYC2a	HM466974	*N. tabacum*	Nicotine
MYC2b	HM466975	*N. tabacum*	Nicotine
NbbHLH1	GQ859152	*Nicotiana benthamiana*	Nicotine
NbbHLH2	GQ859153	*N. benthamiana*	Nicotine
GL3/At5g41315	NM_148067	*A. thaliana*	Anthocyanins
EGL3/At1g63650	NM_105042	*A. thaliana*	Anthocyanins
TT8/At4g09820	NM_117050	*A. thaliana*	Anthocyanins
R2R3-MYB			
PAP1/At1g56650	NM_104541	*A. thaliana*	Anthocyanins
MYB14	DQ399056	*Pinus taeda*	Flavonoids and isoprenoids
MYB29/At5g07690	NM_120851	*A. thaliana*	Aliphatic glucosinolates
MYBJS1	AB236951	*N. tabacum*	Phenylpropanoids
MYB8	GU451752	*N. attenuata*	Phenylpropanoids
WRKY			
WRKY1	AY507929	*Gossypium arboretum*	Gossypol
WRKY1	FJ390842	*A. annua*	Artemisinin
WRKY1	HQ646368	*C. roseus*	Terpenoid indole alkaloids
WRKY3	AY456271	*N. attenuata*	Volatile terpenes
WRKY6	AY456272	*N. attenuata*	Volatile terpenes
WRKY33/At2g38470	NM_129404	*A. thaliana*	Camalexin
NAC			
ANAC042	NM_129861	*A. thaliana*	Camalexin
DOF			
OBP2/Dof1.1/At1g07640	NM_001035911	*A. thaliana*	Indole glucosinolates
DOF4;2/At4g21030	NM_118221	*A. thaliana*	Flavonoids
HD-ZIP			
HAHB4	AF339748	*H. annuus*	Green leaf volatiles
TFIIIA zinc finger			
ZCT1	AJ632082	*C. roseus*	Terpenoid indole alkaloids
ZCT2	AJ632083	*C. roseus*	Terpenoid indole alkaloids
ZCT3	AJ632084	*C. roseus*	Terpenoid indole alkaloids

1.8 The WRKY transcription factors

The WRKY TF family is primarily found in the plant kingdom and belongs also to the ten largest families of TFs in higher plants. The superfamily of WRKY transcription factors consists of 74 members in *Arabidopsis*, 109 in rice, 66 in papaya (*Carica papaya*) and 104 in poplar (*Populus* spp.), and represents one of the ten largest families of transcription factors in higher plants (Rushton et al., 2010). Almost two decades have already passed since their discovery (Rushton et al., 1995, 1996) and by now a lot of different functions have been attributed to the WRKY TFs. They participate in the regulation of many plant processes including the responses to pathogen infestation (Chujo et al., 2013), abiotic stresses (Scarpeci et al., 2013) and senescence (Besseau et al., 2012). A more recently described physiological activity of WRKY factors is their participation in the biosynthesis of alkaloids (Suttipanta et al., 2011).
All WRKY factors share their characteristic DNA binding domain, called the WRKY domain. The WRKY domain is about 60 amino acid residues in length. It contains at the N-terminus the almost invariant eponymous peptide signature WRKYGQK, whereas the C-terminal part harbours an atypical zinc finger motif (either Cx4-5Cx22-23HxH or Cx7Cx23HxC), in which the cysteine and histidine residues bind one zinc atom and generate a finger like structure. Both, the WRKYGQK motif and the zinc-finger structure are necessary for the DNA-binding activity of WRKY TFs. Mutations in the invariable WRKYGQK motif significantly reduced the DNA-binding activity and substitutions of the conserved C and H residues of the zinc-finger even abolished the DNA-binding (Maeo et al., 2001). All WRKY proteins contain one or two of these DNA-binding WRKY domains and are categorized into three subgroups dependent on their number of WRKY domains and the zinc-finger structure. Group I WRKY proteins are marked by two WRKY domains with a C2H2 zinc-finger structure. Group II and III WRKY proteins consist of only one WRKY domain with a C2H2 and a C2HC zinc-finger structure, respectively. The group II WRKY proteins were originally further divided into IIa, IIb, IIc, IId, and IIe based on their primary amino acid sequence (Eulgem et al., 2000). The WRKY domain binds to a so called W-box (TTGACC/T) in the promoters of target genes. This sequence is the minimal core element necessary for binding of a WRKY protein to DNA (Rushton et al., 1996). W-boxes can be found in the promoters systemic acquired resistance related (SAR) genes, including *isochorismate synthase 1*, *non-expressor of PR genes 1*, and *pathogenesis related 1* (Fu and Dong, 2013); or ABA signaling-related genes such as *ABI4*, *ABI5*, and *ABA responsive element binding factor 4* (Rushton et al., 2012). Often there are several W-boxes in one promoter, and even motif clusters can be found. Remarkably, W-boxes are also found in the promoter of *WRKY* genes, suggesting a potentially strong transcriptional networking between WRKY proteins. WRKY TFs bind W-boxes in the promoters of target genes to regulate their expression. But almost all WRKY factors bind W-boxes raising the question, how specificity is achieved between certain promoters and different WRKY TFs. Binding studies revealed that only the presence of W-boxes is not sufficient for a DNA–protein interaction but most likely the surrounding sequences and the overall structures are important. It has been demonstrated for five different WRKY TFs (WRKY6, WRKY11, WRKY26, WRKY38, and WRKY43) that the DNA-binding selectivity depends on neighboring sequences (Ciolkowski et al., 2008). An interesting point that has emerged in promoter analysis of WRKY TFs is the enrichment of W-boxes in their own promoters. The analysis of promoter sequence upstream

of 72 WRKY genes in *Arabidopsis*, revealed that 83% of the WRKY genes contain at least two perfect W-boxes (TTGACC/T) and 58% contain even four or more TTGAC core elements suggesting a regulatory network between the WRKY factors (Dong et al., 2003). Further detailed studies of several WRKY promoters also confirmed the presence of multiple W-boxes. In agreement with the W-box enrichment in their own promoters, it has been demonstrated that the WRKY proteins act on the promoters of their own genes and on other WRKY genes (Potschin et al., 2013). In addition to transcriptional networking, WRKY proteins can also form dimers and are also capable to form heterodimers. Furthermore, many other proteins have been characterized to form protein complexes with WRKY proteins thereby regulating their function. The growing number of discovered interaction partners reveals that there is also a networking between the WRKY factors on the protein level. Moreover, there is some evidence that these WRKY heterodimers act in a different way on transcriptional regulation than homodimers or monomers. Recently, a participation of *At*WRKY18 in the senescence process was discovered (Potschin et al., 2013). *At*WRKY18 can physically interact with *At*WRKY53, an important regulator of early senescence, leading to different transcriptional activation in a reporter gene assay of the heterodimer in comparison to the single proteins. A well-investigated network exists between the three *Arabidopsis* WRKY factors WRKY18, WRKY40, and WRKY60. It was shown by that these three WRKYs interact with each other in a yeast two-hybrid assay and form homo- and heterodimers (Xu et al., 2006). In gel shift assays, WRKY18 and WRKY40 heterodimers bind much stronger to different W-box carrying sequences than the respective homodimers. In contrast, if WRKY40 is mixed with WRKY60 proteins the binding affinity declines. Since WRKY60 alone shows almost no binding activity for the used DNA sequences the effect has to be due to heterodimer formation. An example for the regulation activity of WRKY18/WRKY40 heterodimers is given by Chen et al., 2010. *WRKY60* is expressed after ABA treatment and this induction is almost lost in the *wrky18* and *wrky40* mutants, suggesting that *WRKY60* is regulated by WRKY18/WRKY40 in the ABA signaling pathway. In addition, they could show activation of the *WRKY60* promoter by WRKY18/WRKY40 heterodimers in a reporter gene assay, whereas the homodimers had no effect (Chen et al., 2010). These three WRKY proteins participate in the ABA signaling pathway through direct regulation of ABI4 and ABI5. Interestingly, not only different binding effects to these two genes were observed for the heterodimers, by using fragments of the ABI4 and ABI5 promoters in gel shift assays, binding activity of a combination of all three WRKYs together was sometimes completely abolished binding, although all possible heterodimers could bind to the same fragment. This indicates that an interaction between all three WRKY proteins takes place and that this higher order complex has again a distinct functionality (Liu et al., 2012). W-boxes in the promoters of target genes are often clustered. Since one WRKY domain is thought to bind one W-box, such W-box clusters in the DNA can mediate a complex formation of higher order protein complexes between different WRKY proteins. Depending on the orientation and the number of nucleotides between the W-boxes, the WRKY DNA-binding protein complex is composed of WRKY proteins with specific conformations. WRKY TFs activity can also be modulated by phosphorylation. In the case of WRKYs, phosphorylation can be mediated through the mitogen-activated protein kinase (MAPK) pathway (Asai et al., 2002). Most WRKY TFs are located in the nucleus for direct transcriptional regulation (Zheng et al., 2006). However, an interesting

example for WRKY TFs that regulate gene expression by changing their subcellular localization is given by Shang et al., 2010. Usually, WRKY40 inhibits expression of ABA-responsive genes in the nucleus. Triggered by high concentrations of ABA, *At*WRKY40 interacts strongly with magnesium-protoporphyrin IX chelatase H subunit [CHLH]/putative ABA receptor (ABAR) inhibiting further regulatory function of *At*WRKY40 in the nucleus. ABAR is localized predominantly in the outer chloroplast membrane, with its N- and C-terminus exposed to the cytosol. ABAR binds ABA and appears to be an ABA receptor.*At*WRKY40 interaction with the C-terminus of ABAR in the cytosol releases inhibition of ABA response genes in the nucleus and ABA response can occur. Furthermore, the expression of *AtWRKY40* is repressed after ABA treatment (Shang et al., 2010).

1.9 The transcription factor Myc2

Myc2, a basic helix-loop-helix (bHLH) domain–containing TF, acts as both activator and repressor of distinct JA-responsive gene expression in Arabidopsis (Lorenzo et al., 2004). Myc2 is allelic to the JAI1/JIN1 (for JASMONATE-INSENSITIVE1) locus, which was first identified in a mutant screen for reduced sensitivity of its roots to exogenous JA (Berger et al., 1996). Myc2 is also known as RD22BP1, RAP-1, or ZBF1 (Yadav et al., 2005). Despite the potential significance of Myc2 as a major player in the JA signalling pathway (Lorenzo and Solano, 2005), only a few Myc2-regulated genes have been identified to date. These genes include the JA-responsive pathogen defence genes PDF1.2, CHIB/PR3, and HEL/PR4 and are negatively regulated by Myc2 (Lorenzo et al., 2004). Consequently, myc2/jin1 mutant plants show increased resistance to fungal pathogens such as *Plectosphaerella cucumerina*, *Botrytis cinerea*, and *Fusarium oxysporum* (Lorenzo et al., 2004) and the bacterial pathogen *Pseudomonas syringae* (Laurie-Berry et al., 2006). In addition, Myc2 positively regulates the JA- and wound/insect-responsive genes VSP, LOX, and TAT (Lorenzo et al., 2004). Also unknown is whether Myc2 has additional roles in modulating other JA-regulated genes and plant functions. The JA signaling pathway interacts extensively with other hormonal and developmental signalling pathways, and emerging evidence suggests that Myc2 plays a pivotal role in modulating some of these interactions. For instance, Myc2 also acts as a positive regulator of abscisic acid–dependent drought responses (Abe et al., 2003) and is required for the suppression of salicylic acid–dependent defences during infection by *P. syringae* (Laurie-Berry et al., 2006). Interactions between JA and ET as well as JA and auxin signalling are also known (Woodward and Bartel, 2005), but it is not known whether Myc2 has a role in regulating such interactions. Myc2 is required for the inhibition of root elongation by auxin transport inhibitors. Finally, differential expression of diverse TF genes during JA signaling in the myc2/jin1 mutant along with DNA binding and expression studies of T-DNA lines of Myc2-modulated TFs have led to the proposal that Myc2 probably acts through the transcriptional orchestration of other TFs, which in turn regulate downstream JA response genes involved in diverse JA-dependent plant processes. Recent investigations have defined COI1/JAZs/Myc2 as a core JA signalling pathway module. CORONATINE INSENSITIVE1 (COI1), an F-box protein, is a key component of the jasmonate receptor complex (Yan et al., 2009). Similar to the GA/DELLA signalling system, JASMONATEZIM-DOMAIN (JAZ) proteins are ubiquitinated via SCF^{COI1} in

response to JA. The JAZ family proteins function as repressors of the JA signalling pathway, and a recent structural and pharmacological study showed that COI1 and JAZ form a coreceptor complex (Sheard et al., 2010). JAZ proteins have been shown to directly bind to Myc2 and its close homologs MYC3 and MYC4 to block their function. Jasmonoyl Ile, an active form of JA, promotes the degradation of JAZ proteins and, in turn, frees the transcriptional regulation activity of MYC2, the major transcription factor of jasmonate-mediated gene expression (Kazan and Manners 2013).

1.9 Diverting flux using loss of function approaches

While the overexpression of metabolic enzymes provides a powerful tool in metabolic engineering, it is often as important to minimize flux through a pathway that results in an undesired product. Several approaches are currently available for the downregulation or knockout of a specific enzymatic step:

1. Interfering with protein function using specific inhibitors. There are many protein inhibitors of metabolic enzymes that, when overexpressed, could have the potential to inhibit specific enzymatic steps (Rausch and Greiner, 2004). Non-protein inhibitors of metabolic enzymes are also extensively used to inhibit non essential pathways (Forkmannand Martens, 2001).
2. Knocking out gene function by targeted RNA degradation. Although there are several ways to induce gene silencing in plants, *i.e* the antisense or co-suppression technologies (Bourque, 1995), the use of ribozymes (Puerta-Fernandez et al., 203) and the more recent application of artificial microRNA (Schwab et al., 2010), the double-stranded RNA interference (dsRNAi) represent the preferred method to knock out gene function (Baulcombe, 2004).

RNA interference (RNAi) is considered to be the most effective strategy for the suppression of gene expression (silencing) at the posttranscriptional level in plants and other organisms (Tang and Galili, 2004). However, often, the most impressive pathway manipulation results are obtained when gain and loss-of-function approaches are combined to increase flux through one pathway while decreasing flux through a competing pathway. This combinatorial approach has proven to be very successful in modifying multiple lignin traits in aspen trees (Li et al., 2003). RNA interference can be used in order to obtain a new pharmaceutical plant. Opiate poppy is a well known source of morphine and codeine formed during the majority of enzymatic reactions from L-tyrosine. The codeinone reductase enzyme (COR) is responsible for codeinone transformation into codeine followed by its demethylation into morphine. RNA interference allowed for the suppression of all seven enzymes from the COR family, which resulted in the accumulation in the poppy of reticulin and its methylated derivatives, components of the drug used for malaria treatment. The same research group conducted a study involving the suppression of the synthesis of other alkaloids located in different poppy parts via antisense RNA (Champa et al., 2012) and RNA interference (Allen et al., 2008) strategies, due to the inhibition of other enzymes involved in morphine biosynthesis. The use of RNA interference allowed for the construction of a tobacco plant characterized by an increased nicotine content and an absence of carcinogens (Gavilano et al., 2006). Tobacco's nicotine has the ability for demethylation, leading to the formation of nornicotine, a precursor of the carcinogen compound nitrozonornicotine. The silencing of CYP82E4, encoding nicotine demethylase,

via RNAi, resulted in the almost complete absence of nicotine transformation into nornicotine in leaves. The inhibition of another enzyme, putrescine-N-methyltransferase, via RNAi caused a decrease in the nicotine content in leaves of different lines of the tobacco plant by up to 9.1–96.7% compared to the control (Wang et al., 2008). Japanese researchers have been trying to obtain transgenic coffee plants with caffeine free seeds. Caffeine is synthesized from adenine during nine reactions catalyzed by various methyltransferases. The theobromine synthase gene (CaMXMT1) encoding one of the methyltransferases involved in caffeine synthesis has been suppressed via both antisense RNA and RNAi. The caffeine and theobromine content in transgenic plants was shown to diminish rapidly and no harmful side substances were detected in the coffee seeds (Ogita et al., 2003).

1.11 Starting point of the present project

Extracts of *Salvia* species are used in traditional medicine to treat various human diseases. The economic importance of this genus has increased in recent years due to evidence that some of its secondary metabolites have valuable pharmaceutical and nutraceutical properties (Marchev et al., 2014). In particular, the roots of *Salvia sclarea* accumulate bioactive abietanic diterpenes (e.g. aethiopinone, 1-oxo-aethiopinone, salvipisone, and ferruginol) with interesting pharmacological properties (Topcu and Goren 2007). We have demonstrated that it is possible to increase the production of these compounds in *S. sclarea* hairy roots (up to 3-4 fold) by overexpressing the genes *DXS* or *DXR*, encoding the enzymes acting more upstream to the MEP-derived metabolic pathway, from which abietane diterpenes derive (Vaccaro et al., 2014). Diterpenes, isolated in *S. sclarea* roots (Ulubelen et al. 1997) and hairy roots (Kuzma et al. 2006), belong to the class of tri-cyclic diterpenes, either of the abietane-phenolic or abietane-quinone-type. Since several plant-derived compounds with the quinone moiety present some anti-proliferation and anti-metastasis effects in various cancer types both *in vitro* and *in vivo* (Fronza et al. 2012), abietane-quinone diterpenes would appear to deserve greater attention. In particular, aethiopinone has recently attracted attention due to its high cytotoxic activity against HL-60 and NALM-6 leukemia cells (Rozalski et al. 2006). In a recent study conducted in the *Planta*LAB, aethiopinone was proved to be cytotoxic also to different solid tumor cell lines, i.e. MCF7 (breast adenocarcinoma, IC50 30 lM), HeLa (epithelial carcinoma, IC50 33 lM), PC3 (prostate adenocarcinoma, IC50 48 lM), and A375 (human melanoma, IC50 11.4 lM). Preliminary results have also shown that aethiopinone treatment induced apoptosis of A375 cells with 28.5 and 42.5 % cell death at 5 and 10 lM respectively (Vaccaro et al., 2014). The highest cytotoxicity was found in human melanoma cells. The incidence of malignant melanoma is increasing faster than any other cancer, and successful systemic chemotherapy is rare (Rigel and Carucci 2000). For a deeper understanding of the molecular mechanism underlying the more pronounced cytotoxic effect of the aethiopinone against melanoma cells, a research of putative molecular targets of this compound was carried out by using the free available web server IdTarget (http://idtarget.rcas.sinica.edu.tw). The deposited structure of the aethiopinone, downloaded from Pubchem database (https://pubchem.ncbi.nlm.nih.gov/), was submitted to the algorithm of IdTarget and a number of putative targets, screened among all protein structures deposited in the Protein Data Bank

(PDB), were listed in the output. For each protein the parameters ΔG^{pred} (kcal/mol) and the Ki^{pred} , indicative of the strength of the predicted interaction, were reported. As shown in table 1, this screening identified, as putative targets of aethiopinone, several proteins whose expression is impaired in melanoma. Among these, the proteins MMP-9 (Matrix metallopeptidase 9) and MMP-13 (Matrix metallopeptidase 13) showed the lowest ΔG^{pred} and the Ki^{pred}, making them potential candidates as molecular targets of aethiopinone in melanoma cells.

Table 1.4 Identification of potential protein targets of aethiopinone

Protein name	ΔG^{pred} (kcal/mol)	Ki^{pred}
MMP-9	-9.75	71.2 nM
MMP-13	-9.67	81.5 nM
Gelsolin	-8.72	405 nM
Wee1-like protein kinase	-8.72	405 nM
Cathepsin S	-8.41	683 nM
NEDD9	-8.38	719 nM
erbB-4	-8.30	823 nM
erbB-3	-8.01	1.3 µM
S100-A4	-7.99	1.4 µM
S100-B	-7.41	1.4 µM
Mdm2	-7.91	1.6 µM

These preliminary results need to be confirmed by *in vivo* analysis in order to verify the effective interaction between the purified compound aethiopinone and each of these purified proteins. However, Despite this interesting evidence, this compound can not be easily synthesized by chemical means, and it is only produced in the roots of *Salvia* species in minute amounts (less than 0.5% DW) wich are not sufficient to yield reliable amounts for a deeper understanding of their molecular targets and potential future commercialization.

1.12 Aims of the project

The main objective of this project was to increase the production of the abietane diterpenes in *S. sclarea* hairy roots by targeting MEP-derived terpenoid biosynthetic pathway, from which they derive, through metabolic engineering approaches. This goal was achieved by the following strategies:

- **activation of the entire metabolic pathway** by elicitation with Methyl-jasmonate (MJ) and by the overexpression of transcription factors upregulating MEP-pathway biosynthetic genes;

- **RNAi-mediated silencing** of the gene encoding the Ent-copalyl-diphosphate synthase (Ent-CPPS), the first enzyme of the competitive gibberellin synthesis route.

Materials and Methods

2. Material and methods

2.1 Plant material and growth conditions

Salvia sclarea plants (B&T World Seeds, France) were surface sterilized with 70% (v/v) ethanol for 1 min and then in 2% (v/v) sodium hypochlorite solution for 10 min, thoroughly washed and sown in Murashige & Skoog (MS) (Murashige and Skoog, 1962) pH 5.8 medium containing 30 g l^{-1} sucrose and 9 g l^{-1} agar. The plants were grown at 23°C under a photoperiod of 8 hour dark and 16 hour light (110 µmol m^{-2} s^{-1}) in a controlled growth chamber.

2.2 Treatments

Equal amounts of *S. sclarea* hairy roots, sub-cultured in MS hormone-free medium for three weeks, were treated for seven days with 150 µM Methyl-jasmonate (MJ) (or an equal volume of DMSO as mock control) or for thirty days with 100 µM Chlorocholine chloride (CCC) (or an equal volume of H_2O as mock control).
A. thaliana two-week-old seedlings (normally with six leaves) were transferred in liquid medium. Fresh MS medium was added every 3 days. Seven days after transfer to liquid medium, the seedlings were treated with 150 µM MJ.
S. sclarea hairy roots and *A. thaliana* seedlings were harvested, quickly washed and then frozen in liquid nitrogen.

2.3 Bioinformatic analysis

Promoter regions (1000 bp upstream the TSS) of *A. thaliana WRKY18, WRKY40, WRKY60 e Myc2* and of MEP-pathway biosynthetic genes (*DXS, DXR, CMK, MCS, HDS, GGPPS*) were obtained by the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nih.nim.gov/) and scanned by the softwares Transfac (http://www.biobase-international.com/product/transcription-factor-binding-sites), PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) e Genomatix Matinspector professional (http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.p1).

2.4 Plasmid construction

Plasmids containing the full-length cDNA of *A. thaliana WRKY60* (Accession n. U23247), *WRKY18* (Accession n. U14890), *WRKY40* (Accession n. C105126) e *Myc2* (Accession n. U12679) were obtained from the *Arabidopsis Biological Resource Center*. The coding sequence of the genes was amplified by polymerase chain reaction (PCR) using a *High Fidelity* DNA Polymerase (*Pfx*, Invitrogen, Carlsbad, CA, USA) with specific primers (Table 2.1) characterized by the presence of the short sequence *CACC* at 5' for the cloning in pENTR / kit D-TOPO ® (Invitrogen) to generate an *Entry-Clone.* The correct insertion and the absence of mutations were verified by sequencing (Primm Biotech, Milan, Italy). Subsequently, TF coding sequences were subcloned through the LR reaction (*The Gateway® LR Clonase™ enzyme mix kit, Invitrogen*) in the gateway *Destination vector* pGBW17, driven by the constitutive strong viral 35SCaMV promoter, and containing the c-terminal 4xmyc-tag and kanamycin resistance selectable marker (Schardl et al. 1987), through a site-specific recombination (Xu and Li, 2008). The resulting binary vectors were shuttled into the *A. rhizogenes* RI 15834, by the standard freeze thaw and $CaCl_2$ method (Weigel and Glazebrook 2006). For the cloning of the partial sequence (450 bp) of the *SsEntCPPS* gene into pHELLSGATE12 RNAi binary vector (characterized by the presence of the constitutive strong viral 35SCaMV and kanamycin resistance) was used the same procedure (gateway technology based) described above for the cloning of TFs.

2.5 *S. sclarea* hairy root cultures

The *TF (WRKY60*, *WRKY18*, *WRKY40*,*Myc2)* recombinant *A. rhizogenes* strain RI 15834 were grown (OD_{600} = 0.3-0.5) in Yeast Extract Broth liquid medium (0.1% Yeast extract, 0.5% Beefextract, 0.5% Peptone, 0.5% Sucrose, 0.04% $MgSO_4$), containing 50 mg l^{-1} kanamycin, at 28 °C . *S. sclarea* sterile leaf sections, from 20 day-old plants, were submerged in the bacterial suspension for 30 min, co-cultivated at 28 °C for 3 days in MS medium solidified with 9 g l^{-1} and then transferred to fresh MS medium with 50 mg

l^{-1} kanamycin and cefotaxime 100 mg l^{-1}. After three weeks of several transfers to medium containing 50 mg l^{-1} kanamycin and decreasing concentrations of cefotaxime (100 down to 50 mg l^{-1}), hairy roots developing from the infected areas were individually excised, sub-cultured several times on hormone-free medium and maintained at 23°C in the dark. Kanamycin-resistant hairy root independent lines, with no bacterial contamination, were selected, and sub-cultured into 250 ml flasks, containing a hormone-free liquid MS medium supplemented with 50 mg l^{-1} kanamycin and kept on a gyratory shaker at 120 rpm at 23°C in the dark. The cultures were sub-cultured every week.

2.6 DNA isolation and PCR analysis

Genomic DNA was extracted from hairy roots by the cetryl trimethyl ammonium bromide (CTAB) method as described in Doyle and Doyle (1990). The DNA was used as the template in PCR analysis to detecting the presence of exogenous genes in transgenic hairy roots using a forward primer located at the promoter of the PGBW17:35S[2] or the PHELLSGATE12 vector and a reverse primer located at the 3' end of the gene. The reactions were performed using 2.5 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA), the conditions for amplification were as follows: 94°C for 5 min, followed by 30 cycles at 94°C 30 sec, annealing 30 sec at the different $T_{m,}$ according to the primers melting temperatures, 72°C 1 min, and at final extension 72°C for 5 min. To detect the absence of contaminating agrobacteria, the hairy root genomic DNA was also amplified with *virD2* specific primers (Haas *et al.* 1995). (Oligonucleotide sequences are reported in table 2.1).

2.7 Isolation of *Salvia sclarea EntCopalyl diphosphate synthase* coding sequence

Partially degenerate PCR primers (Table 2.1) were designed on the highly conserved EntCPPS protein domain. A 518-bp product was PCR amplified by the degenerate primers using first strand cDNA synthesized from RNA isolated from *S. sclarea* stem, and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) followed by sequencing. Elongation of the sequence of *S. sclarea EntCPPS* gene was obtained by 3'-RACE, using the *SMART RACE cDNA Amplification Kit* (Clontech, Takara Bio Company) according to the manufacturer's instructions. The predicted amino acid sequence of the *S. sclarea* EntCPPS and other available EntCPPS proteins were compared using the Basic Local Alignment Search Tool (BLAST) service (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of nucleotide and deduced amino acid sequences were performed using the CLUSTAL W program (Larkin et al., 2007) with the default parameters through the service of European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw).

2.8 RNA extraction and RT-PCR analysis

Total RNA was extracted using the plant RNA/DNA Purification kit (Norgen Biotek Corporation Ontario, Canada), according to the manufacturer's protocol. For semi-quantitative RT-PCR, complementary DNA was synthesized from 1 µg total RNA, previously treated with RNase-free DNAse I (Invitrogen, Carlsbad, CA, USA), using random hexamers and the Superscript III RT (Invitrogen, Carlsbad, CA, USA) at 50°C for 50 min. In the PCR reactions one microliter of cDNA was used as template with specific primers (Table 2.1) and 2.5 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA), using the following condition: initial denaturation at 94°C 1 min, followed by 30 cycles denaturation at 94°C 30 sec, annealing 1 min at the different T_m, according to the primers melting temperatures, extension at 72°C 1 min and final extension at 72°C for 5 min. The *S. sclarea 18S* gene-specific primers were used to normalize equal amounts of RNA for each samples as a internal standard.

2.9 Quantitative real-time reverse transcription-PCR

Complementary DNA was synthesized from 1 µg total RNA and reverse-transcribed by Superscript III Reverse transcriptase (Invitrogen Carlsbad, CA, USA). Quantitative RT-PCR was performed using a Light Cycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions. Reactions

were performed in a 20 µl volume with 0.5 µM primers and Light Cycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK). The sequences of specific primers are listed in Table 2.1. Transcript levels of genes in independent transgenic hairy roots were quantified using SYBR green detection and the CT value of *S. sclarea 18S* rRNA was subtracted from that of the gene of interest to obtain the ΔCT value. Standard deviations were calculated from three different replica for each independent hairy root lines.

2.10 Production of the recombinat *AtWRKY18* protein

The coding sequence of the *AtWRKY18* gene was amplified using specific primers (Table 2.1). The resulting amplicons were cloned behind an N-terminal 6X-His-Tag into the *KpnI*/*SalI* sites of the pQE30 expression vector and the resulting vector transformed into the *E. coli* M15 pREP4 strain. The recombinant protein was extracted and purified from 500 ml of bacterial culture induced with 1mM IPTG at 30 °C for 3 h. The bacterial pellet was dissolved in GTE buffer (Glucose 50 mM; TrisHCl 25 mM, pH 8; EDTA 10 mM pH 8) supplemented with lysozyme (1mg ml^{-1}), and sonicated for 5 min at 10 sec. After centrifugation, the pellet was resuspended in buffer (TrisHCl 1.5 M, pH8; NaCl 5 M; EDTA 0.5 M) and the recombinant protein purified on a Ni-Sepharose High Performance agarose column, according to the manufacture's instructions (GE Healthcare Bio-Science, Uppsala, Sweden). Western blot analysis with an anti-His antibody was performed during amplification and purification of His-fusion protein, following standard procedures (Maniatis et al., 1991).

2.11 Electrophoretic mobility shift assay (EMSA)

For EMSA experiments DNA probes, with or without biotin labeling, were synthesized (Primm Biotech, Milan, Italy) based on the W-box elements of the *AtDXS or AtDXR* promoters. As negative control were synthesized identical probes in which the W-box core sequence was destroyed by mutation (Table 2.1). The double-strand probes and competitor fragments were generated from annealing of separately synthesized strands, with or without one strand being 5′ biotin-labeled. The biotin-labeled and competitor DNA fragments were incubated for 20 min with 100 ng of purified recombinant protein AtWRKY18 in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 5% glycerol and 100 ng polydI:dC, in a final volume of 20 µl) according to the EMSA kit (Pierce, Rockford, IL). The DNA-protein complexes were resolved by electrophoresis on 6% non-denaturing polyacrylamide gels and then transferred to Biodyne®B modified membrane (0.45 µm; Pierce, Rockford, IL). The band-shifts were detected by a chemiluminescent nucleic acid detection module (Pierce, Rockford, IL), according to the manufacture's instructions.

2.12 Hairy roots growth

Equal amounts (0.5 g) of control line or TF overexpressing hairy root lines were inoculated into MS hormone-free liquid medium and dry weight was monitored for one month at 1-week intervals.

2.13 Qualitative and quantitative determination of abietane diterpenes

Lyophilized and powdered hairy roots (0,5 g) were extracted with acetone for 72 h at room temperature. The extract was filtered through a Millipore filter (0.45 µm) and evaporated under reduced pressure. The residue was dissolved in methanol and subjected to HPLC-DAD analysis (Agilent1200 Series, G1312A binary pump, G1329A automatic sample injector, G1315D diode array detector). The HPLC fingerprint was carried out on a C_8 column (Agilent, Zorbax eclips C_8 250 X 4.6 mm) with a sample injection volume of 50 µl. The mobile phase was a gradient elution of water acidified with 0.1% formic acid (solvent A) and acetonitrile (solvent B), starting with 35 % B and rising to 100 % B after 30 min, at a flow rate of 1.0 ml min^{-1}.

The different diterpenes were detected at 280 nm and concentration calculated by the interpolation of the peak areas with calibration curves, constructed with standard purified compounds over the range 10-200 µg ml^{-1}. Content of diterpenoids in roots was expressed as mg g^{-1} of root dry weight. These analysis were performed in collaboration

with the Prof. Nunziatina De Tommasi (Department of Pharmacy, University of Salerno, Italy)

2.14 Statistical analysis

All reported data represent the mean ± SD of at least three independent experiments performed in triplicate. The statistical significance of transcript and protein level was analyzed by one-way analysis of variance (ANOVA), with Tukey's post-test, using GraphPad Prism 5 software. The statistical significance of hairy root growth rate and diterpene content was examined in the two-way analysis of variance (ANOVA) with Bonferroni post-test analysis using GraphPad Prism 5 software.

Table 2.1 List of oligonucleotides used in this study

Cod.	Name	Sequence 5'- 3'	Use
1	WRKY60-F	**CACC**ATGGACTATGATCCCAACAC	Cloning into pEntr-D-TOPO
2	WRKY60-R	TGTTCTTGAATGCTCTATCAA	Cloning into pEntr-D-TOPO
3	WRKY18-F	**CACC**ATGGACGGTTCTTCGTTTCT	Cloning into pEntr-D-TOPO
5	WRKY18-R	TGTTCTAGATTGCTCCATTA	Cloning into pEntr-D-TOPO
6	WRKY40-F	**CACC**ATGGATCAGTACTCATCCTC	Cloning into pEntr-D-TOPO
7	WRKY40-R	AAAAACTCTAACGGAATTTGAA	Cloning into pEntr-D-TOPO
8	MYC2-F	**CACC**ATGACTGATTACCGGCTACA	Cloning into pEntr-D-TOPO
9	MYC2-R	ACCGATTTTTGAAATCAAACTT	Cloning into pEntr-D-TOPO
10	GUSpEntr-F	**CACC**TGTTACGTCCTGTAGAAACC	Cloning into pEntr-D-TOPO
11	GUS174bpR	TGCATCGGCGAACTGATCGT	Cloning into pEntr-D-TOPO
12	RT-WRKY18-F	TACTTGTAGCGACATACGAAG	qRT-PCR
13	RT-WRKY18-R	CAGCAGCAAGAGCAGCTGTA	qRT-PCR
14	RT-WRKY40-F	GTGGAGGATCAGTCCGTGTT	qRT-PCR
15	RT-WRKY40-R	TGAAGCTGAACCACCATGAG	qRT-PCR
16	RT-WRKY60-F	GGCTTGAACCAGTTGAGGAA	qRT-PCR
17	RT-WRKY60-R	CAATCTCCCGGAAATAGCAG	qRT-PCR
18	RT-MYC2-F	GCGTTGATGGATTTGGAGTT	qRT-PCR
19	RT-MYC2-R	TTGCTCTGAGCTGTTCTTGC	qRT-PCR
20	RT-SsDXS-F	AGTGGAGGCCAATGGTTCTT	qRT-PCR
21	RT-SsDXS-R	CCACCAATAAGTGACACAAC	qRT-PCR
22	RT-SsDXR-F	GAAACACAGGAT TCATCCGTG	qRT-PCR
23	RT-SsDXR-R	AGCTCGTCCAGCAGCATAAG	qRT-PCR
24	RT-SsHDS-F	AGCACCGATAACCAAGTCGT	qRT-PCR
25	RT-SsHDS-R	CAGCAGAGTTCATGCTGCAA	qRT-PCR
26	RT-SsGPPS-F	GTGGTGGACATCAACTGCAC	qRT-PCR
27	RT-SsGPPS-R	AAAATGGCCCCCAAAACTAC	qRT-PCR
28	RT-SsCPS-F	GCGAAGACCGATTTCAAGAG	qRT-PCR
29	RT-SsCPS-R	CAGTCGCCAGGAAATAGGAA	qRT-PCR
30	RT-SsActina-F	GGTGCCCTGAGGTCCTGTT	qRT-PCR
31	RT-Ss-Actina-R	GAGCCACCACTGAGGACAAT	qRT-PCR
32	35S^2-Fw	GACTCTAGAGTTATCAACAAGT	PCR
33	Gen-WRKY18-R	TGTTGAAGCTATCAGTGACTG	PCR
34	Gen-WRKY40-R	GATGGATTGTCTCTAGTCACT	PCR
35	Gen-WRKY60-R	GATGGATTATCTCTCGTAATCT	PCR
36	Gen-Myc2-R	GAGTTAAGCTCTCTCAACACT	PCR
37	AtWRKY18KpnIF	GGGGTACCGACGGTTCTTCGTTTCTCGAC	Cloning into PQE30 vector
38	AtWRKY18SalIR	ACGCGTCGACTCATGTTCTAGATTGCTCCAT	Cloning into PQE30 vector
39	-928DXS	TTTGTTTATTCATATTTGACTCAATTGAAACCC AATAATCAAGAATGTCAAATATTATATGTTTTG	EMSA
40	-928DXSmut	TTTGTTTATTCATATTTAAGTCAATTGAAACCC AATAATCAAGAATCTTAAATATTATATGTTTTG	EMSA
41	-85DXS	CTTGGATTTCATGGTTGACGTGGCCCAACCAA AA	EMSA
42	-85DXSmut	CTTGGATTTCATGGTTAAGTGTGGCCCAACCA AAA	EMSA
43	-168DXR	TAATAATAATTATATTTGACAAACTATATAATTG A	EMSA
44	-168DXRmut	TAATAATAATTATATTTAAGAAACTATATAATTG A	EMSA
45	degEntCopFw	GARCAYATGCCNATHGGNTTYGARGT	PCR
46	degEntCop319Rev	TCNACNGGRTANACRTTNGGNACNCC	PCR
47	3RaceEntCop	GCAGAGACTGGGAGTTTCGCGTTATT	3' RACE-PCR
48	siEntCopFw	CACCGCACACGGCTGTGACGTTCAT	Cloning into pENTR-D-TOPO
49	siEntCopRev	GAGTACTTTGGCAATATGGAA	Cloning into pENTR-D-TOPO

Results

3. Results

3.1 Enhancing the synthesis of abietane diterpenes through coordinated activation of the MEP-derived metabolic pathway

In this study, the synthesis of bioactive abietane diterpenes in *Salvia sclarea* hairy roots was enhanced by on activating the entire MEP-derived metabolic pathway through elicitation with Methyl-jasmonate (MJ) and overexpression of transcription factors.

3.1.1 Elicitation of *Salvia sclarea* hairy roots with Methyl-jasmonate (MJ) and quantitative analysis of bioactive abietane diterpenes

In order to stimulate the biosynthesis of bioactive diterpenoids, *S. sclarea* hairy roots were elicited with Methyl-jasmonate (MJ), a derivative form of the jasmonic acid involved in the plant defense signaling cascade. *S. sclarea* hairy roots, obtained by transformation with the *A. rhizogenes* strain ATCC 15835, were grown for thirty days in the dark, in a hormone–free medium, and treated for 24 h with 150 µM MJ. Acetonic extracts from MJ-treated and control DMSO-treated hairy roots were compared by HPLC-DAD analysis for targeted abietane diterpenes. The total diterpenoid content of MJ-treated hairy roots was 29.91 mg g^{-1} dry weight, about 20 times higher than the content of control hairy roots (Table 3.1). The contents of all monitored diterpenoids (carnosic acid, ferruginol, 1-oxo-ferruginol, salvipisone, aethiopinone and 1-oxo-aethiopinone) in MJ-treated hairy roots were significantly enhanced (P≤0.001) compared to the control roots. The most relevant increase was achieved for the content of aethiopinone (10-fold increase) (Fig. 3.1).

Table 3.1 Total abietane diterpene content in MJ- treated hairy roots

Treatment	Total content (mg g^{-1} dry weight)	Fold-increase
Mock	1.42 ± 0.1	-
MJ 150 µM	29.97 ± 0.5***	20.94

*** P≤0.001

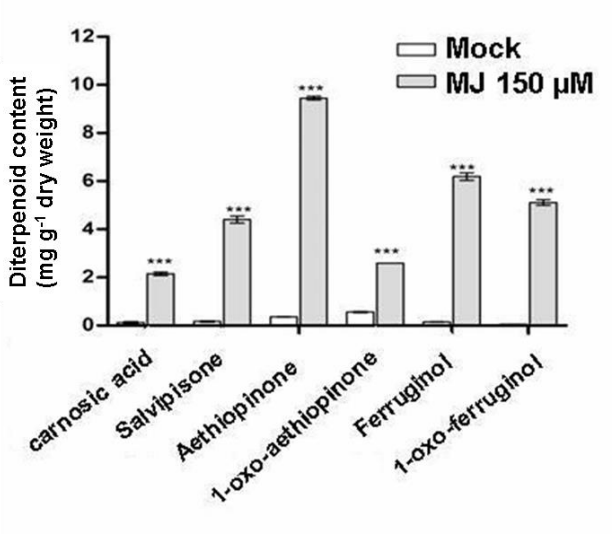


Figure 3.1 - **Content of the principal abietane diterpenes in *S. sclarea* hairy roots**, analyzed by quantitative HPLC-DAD. The amount of each compound in Methyl-jasmonate (MJ) treated roots was compared with the control line (mock). Values are means ± SD of triplicate technical analyses from two biological replicates. *** indicate significant difference at P≤ 0.001.

3.1.2 MJ treatment induces transcriptional activation of MEP-pathway biosynthetic genes

The enhanced content of abietane diterpenes in *S. sclarea* hairy roots elicited by MJ might be a direct consequence of the increased transcription of genes encoding enzymes of the MEP-derived pathway. To verify this, qRT-PCR analyses were performed using RNA isolated from *S. sclarea* hairy roots elicited with 150 µM MJ in a time course study (3h, 6h, 12h, 24h). Oligonucleotides specifically designed on previously isolated partial sequences of *S. sclarea* endogenous genes were used in the amplification reactions (Table 2.1). Compared to untreated control roots, the expression of biosynthetic genes acting upstream of GGPP, the common precursor of diterpenes and other terpenoids, (*SsDXS, SsDXR, SsCMK, SsMCT, SsMDS, SsHDS*) was up-regulated within 3 h after exposure to 150 µM MJ and gradually increased reaching the maximum level at 24h (Fig. 3.2). Also the transcript levels *of SsGGPS,* the enzyme catalyzing the condensation of Isopentenyl pyrophosphate (IPP) and dimetylallil pyrophosphate (DMAPP) into GGPP, increased in response to MJ treatment. Interestingly, *SsCPPS*, encoding the first enzyme that specifically diverts GGPP towards the synthesis of abietane diterpenes, was the most significant upregulated gene induced by MJ (70-fold increase over the level of the control line, P≤0.001). Although to a lesser extent than *SsCPPS*, the expression levels of the biosynthetic genes *SsDXS* (54-fold increase)*, SsDXR* (56-fold increase)*, SsHDS* (40-fold increase)*, SsMDS* (20-fold increase) and *SsGGPS* (25-fold increase) significantly increased in MJ treated hairy roots (P≤0.001), compared to the control roots (Fig. 3.2). No significant change in the transcript levels of *SsCMK* and *SsMCS* was induced by MJ treatment.

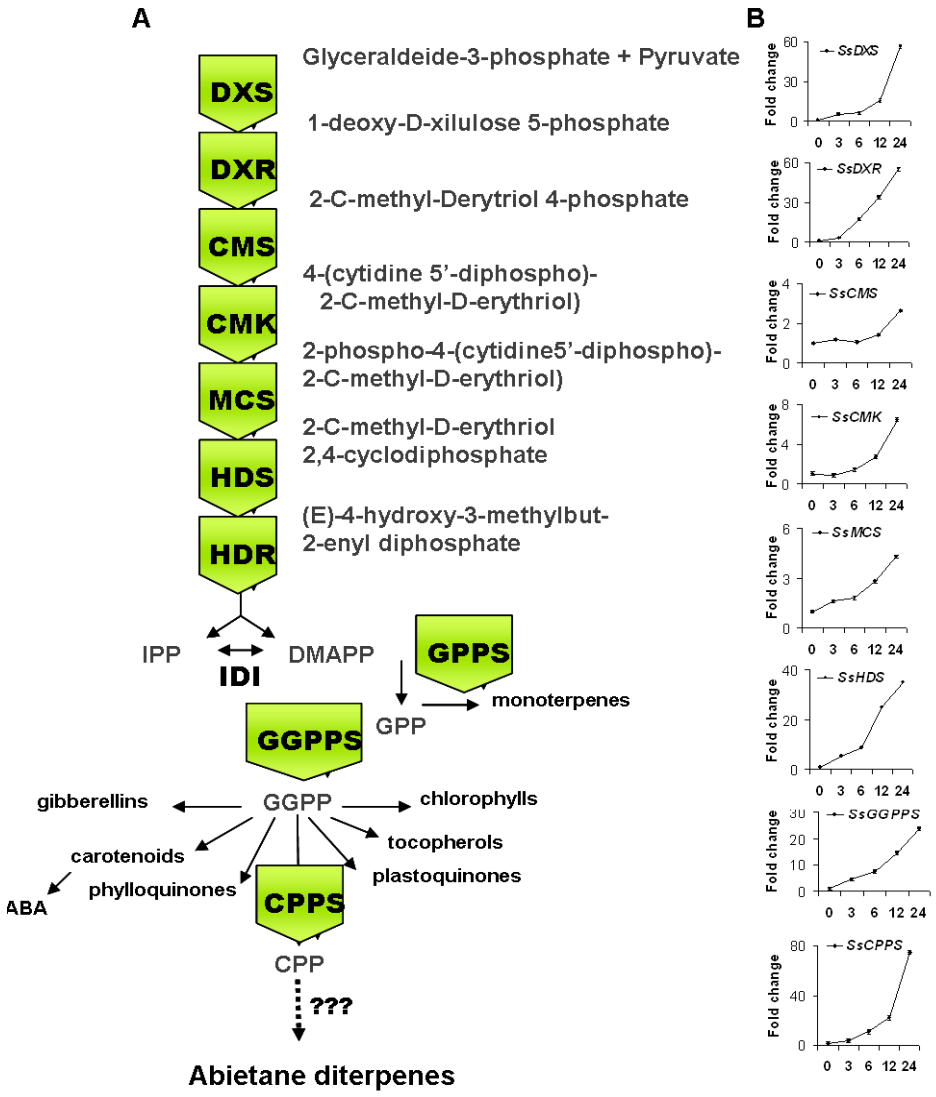


Figure 3.2 - Schematic representation of the plastidial MEP-derived pathway (A) and quantitative expression levels of MEP pathway genes in MJ treated *S. sclarea* hairy roots during 24h of MJ treatment (B). Data represent mean values from three separate replicates ± SD. Enzyme abbreviations are presented in bold capital letters in the green boxes. **DXS**, deoxyxylulose 5-phosphate synthase; **DXR**, deoxyxylulose 5-phosphate reductoisomerase; **CMS**, 4-diphosphocytidyl-methylerythritol synthase; **CMK,** 4-diphosphocytidyl-methylerythritol kinase; **MCS**, methylerythritol 2,4-cyclodiphosphate synthase; **HDS**, hydroxymethylbutenyl 4-diphosphate synthase; **HDR**, hydroxymethylbutenyl 4 diphosphate reductase; **GPPS,** GPP synthase; **GGPPS**, GGPP synthase; **IDI**, isopentenyl diphosphate isomerase; **CPPS,** CPP synthase; **IPP**, isopentenyl pyrophosphate; **DMAPP**, dimethylallyl pyrophosphate; **GPP**, geranyl pyrophosphate; **GGPP**, geranylgeranyl pyrophosphate; **CPP**, copalyl pyrophosphate.

3.1.3 Searching for MJ-inducible transcription factors regulating the genes belonging to the plastidial MEP-derived pathway

Specific TFs are often capable of coordinating the transcription of multiple biosynthetic pathway genes, making them particularly effective in metabolic pathway engineering. Although recently information on transcriptome of *S. sclarea* has been resealed (Legrand et al., 2010), no information is available on the genome of *S. sclarea*. For this reason, it was decided to mine microarray data of *A. thaliana*, to identify putative transcription factors of *A. thaliana* up-regulated by MJ, that might be overexpressed in *S. sclarea* hairy roots in an attempt to boost the synthesis of abietane diterpenes. Among several TFs identified, we focused our attention on *At*WRKY18, *At*WRKY40, and *At*Myc2, reported to be MJ-inducible (Wang et al., 2008). In addition, *At*WRKY60 was also selected considering its physical and functional interaction with *At*WRKY18 and *At*WRKY40 in plant defense responses (Xu et al., 2006.). The choice of these TFs was further strengthened by a promoter analysis, revealing the presence of the consensus sequence *TGACT*, known as **M**ethyl-**J**asmonate **R**esponsive **E**lements (MJRE), in the 1000 bp genomic region upstream the transcription start site of *A. thaliana AtWRKY18, AtWRKY40, AtWRKY60*, and *AtMyc2* genes (Fig. 3.3).

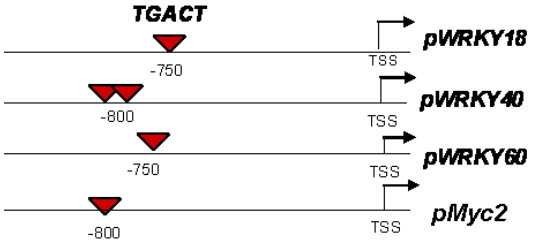


Figure 3.3 - Identification of **M**ethyl-**J**asmonate **R**esponsive **E**lements (MJRE) in the promoters of the identified *A. thaliana* MJ-inducible transcription factors, showing the presence of MJRE (*TGACT*) in the 1000 bp region upstream the transcription start site (TSS).

In addition, the expression pattern of these TFs in response to MJ was characterized in a time-course experiment by qRT-PCR. Two-week-old *A. thaliana* ecotype Columbia (*Col-0)* seedlings were exposed to 150 µM MJ or an equal volume of DMSO (as mock control). In agreement with findings reported by Wang et al (2008), the expression of *AtWRKY18, AtWRKY40*, and *AtMyc2* was rapidly and transiently induced by MJ treatment, reaching the peak after 0.5 h (17-, 60- and 6-fold increase, respectively, $P \leq 0.001$). By contrast, the expression of *AtWRKY60* appeared to be unaffected by MJ treatment (Fig. 3.4). Quantitative expression analysis of *A. thaliana* MEP-pathway biosynthetic genes (*DXS*, *DXR*, *CMK*, *MCT*, *HDS*, *GGPPS*) revealed that this set of genes is indeed up-regulated in *A. thaliana* seedlings by a treatment of 150 µM MJ, although in a transient manner. Transcript levels of all genes increased after 4 hours of treatment, reaching the peak after 6 hours (Fig. 3.5).

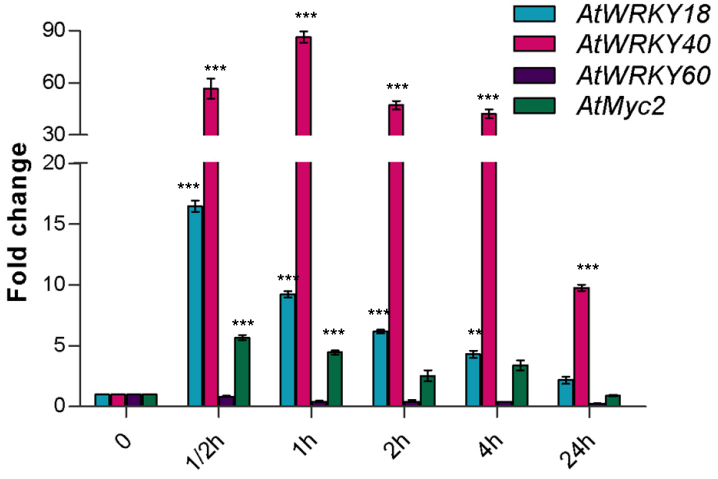


Figure 3.4 - Transcriptional activation of *AtWRKY18, AtWRKY40, AtWRKK60* and *AtMyc2* TFs in *A. thaliana* seedlings in response to elicitation with MJ 150 µM measures by qRT-PCR**.** Values are means ± SD of triplicate technical analyses from three biological replicates *** P≤0.001; ** P≤0.01

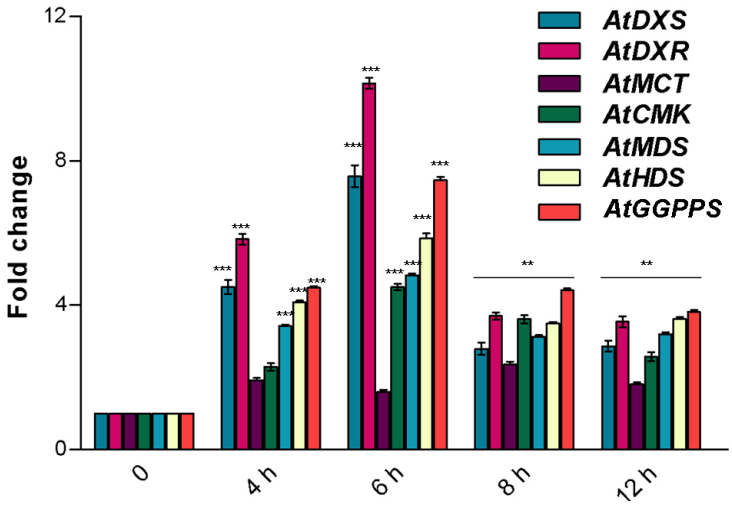


Figure 3.5- Time-course transcriptional activation of *A. thaliana* MEP-pathway biosynthetic genes (At*DXS*, At*DXR*, At*CMK*, At*MCT*, At*HDS*, At*GGPPS*), in response to MJ treatment, measured by qRT-PCR. Values are means ± SD of triplicate technical analyses from three biological replicates *** P≤0.001; ** P≤0.01

A scanning of a region of 1000 bp upstream the transcription start site (TSS) of several MEP-pathway biosynthetic genes revealed also the presence of W-box (consensus binding sequence WRKY TFs) and G-box (consensus binding sequence of Myc2). As shown in figure 3.6, several cis-acting elements, W-box and G-box, were found in the promoter regions of the analyzed biosynthetic genes (*DXS*, *DXR*, *CMS*, *CMK*, *MCS*, *HDS*, *HDR*, *GGPPS*), thus suggesting the possible involvement of WRKYs and Myc2 in the transcriptional regulation of this set of genes.

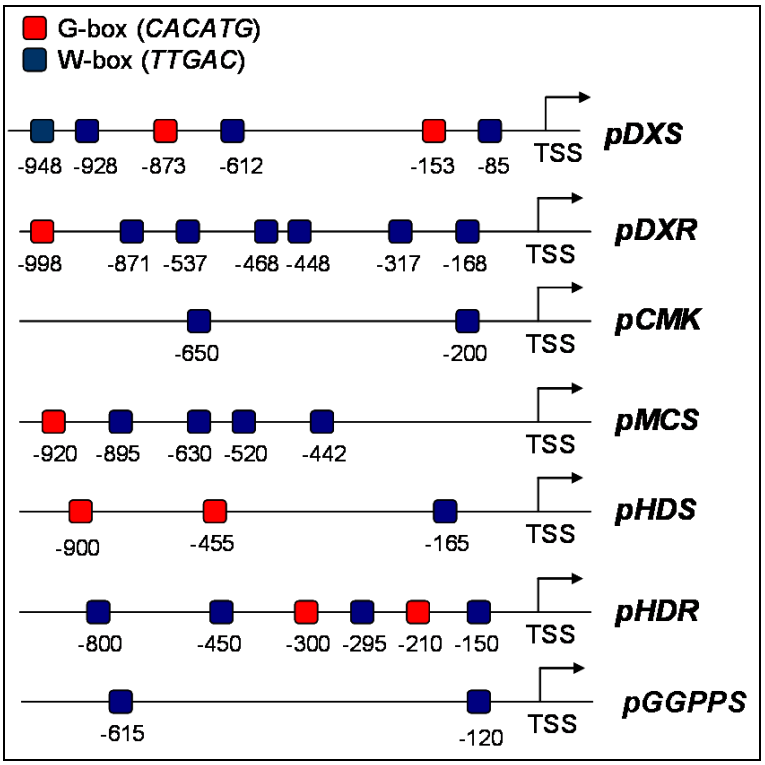


Figure 3.6 – Localization of W-box and G-box, binding sites of WRKY and Myc2 in the promoters of *A. thaliana* MEP-pathway biosynthetic genes.

3.1.4 Generation of *S. sclarea* hairy roots overexpressing *At*WRKY and *At*Myc2 TFs

On the basis of the previous results, *AtWRKY18, AtWRKY40*, *AtWRKY60* and *AtMyc2 TFs* were selected as potential positive transcriptional regulators of the genes belonging to the plastidial MEP-pathway. In order to evaluate whether the overexpression of these TFs could be a valid strategy to boost the synthesis of abietane diterpenes in *S. sclarea* hairy roots, plant binary vectors containing the respective full-length cDNAs were generated. In detail, coding sequences of *A. thaliana* TF ***WRKY60*** (Accession n. U23247), ***WRKY18*** (Accession n. U14890), ***WRKY40*** (Accession n. C105126) e ***Myc2*** (Accession n. U12679) and a 173 bp fragment of the *A. thaliana* gene *GUS* (used as control) were amplified and introduced in the pENTR/D-TOPO vector (Invitrogen) to generate an *Entry-Clone.* The correct insertion and the absence of mutations were verified by sequencing. Subsequently, TF coding sequences were subcloned in the gateway *Destination vector* pGBW17, driven by the constitutive strong viral 35SCaMV promoter, and containing the c-terminal 4xmyc-tag and kanamycin resistance, through a site-specific recombination (Fig. 3.7).

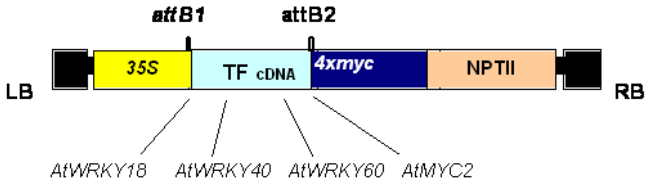


Figure 3.7 - **Schematic representation of the plasmid constructs** used for the constitutive overexpression of *AtWRKY18, AtWRKY40, AtWRKY60* and *AtMyc2* genes. LB, left border; RB, right border; attb1 and attb2, homologous recombination sites.

The binary vectors were then shuttled into the *A. rhizogenes* strain ATCC 15834 and used for transformation of leaf sections taken from axenic *S. sclarea* plantlets. Resistance to kanamycin was used for a primary selection of putatively transformed *S. sclarea* hairy roots lines. Hairy roots differentiating after 20-30 days from transformation were excised from the necrotic explants tissues and subcultured on fresh agar solidified MS30 kanamycin (kan) selective medium. Several independent *kan*$^{+}$ hairy root lines for each construct were subcultured for about 8 weeks in solid medium with cefotaxime, to eliminate bacteria, and then transferred to free-hormone liquid medium (Figure 3.8). Ten *kan*$^{+}$ hairy root lines for each construct, able to grow without adding phytohormones in the medium, indicating the insertion of the *rol* genes of the *A. rhizogenes* T-DNA in the host plant genome, were further characterized for stable insertion of the expression cassette into *S. sclarea* genome and the expression of the heterologous genes. Among them, three independent transgenic hairy root lines for each transcription factor were selected for further analysis. As shown in figure 3.8, the overexpression of TF in hairy roots did not result in phenotypic changes except for a visible reddish color, which indicate indirectly the accumulation of abietane diterpenes.

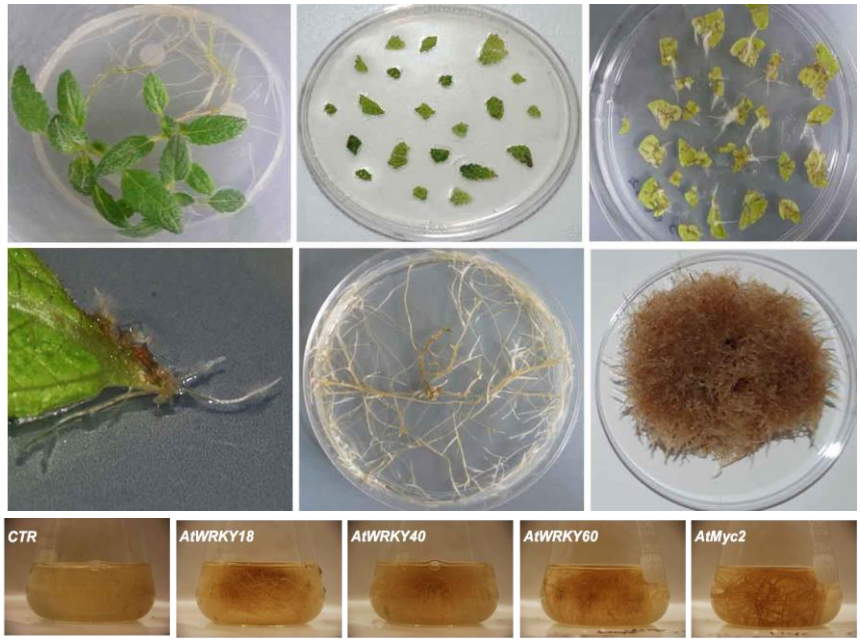


Figure 3.8- Different stages of *A. rhizogenes* mediated transformation of *S. sclarea* leaf sections with vectors shuttling different *A. thaliana* transcription factor genes and hairy roots differentiation. In the bottom panel, TF overexpressing hairy roots, grown in hormone-free liquid medium, are reported.

3.1.5 *AtWRKY18* overexpressing hairy roots

3.1.5.1 Molecular characterization of *AtWRKY18* hairy root lines

Ten *kan*$^{+}$ hairy root lines overexpressing the TF *AtWRKY18* were screened by PCR for the presence of *neomycin phosphotransferase II* (*NPTII*) gene, conferring kanamycin resistance, the presence of *rolB* gene, and for the stable genomic insertion of the heterologous gene *AtWRKY18.* By using primers specially designed to overlap part of the heterologous *AtWRKY18* gene and the CaMV35S promoter sequence (Table 2.1), a single amplicon with the expected size was amplified in all the tested transgenic hairy root lines, while no bands was detected in the control vector transformed lines (C^{-}). Absence of contaminant bacteria was confirmed by missing amplification of the bacterial *virD2* gene. *A. rhizogenes* transformed with the construct overexpressing *AtWRKY18* was used as PCR positive control (C^{+}) (Fig 3.9 A). Since T-DNA mediated insertion of exogenous genes into the plant genome occurs randomly, different independent transgenic lines were analyzed by RT-PCR, to select transgenic root lines expressing the exogenous TF. As shown in Fig. 3.9 B detectable levels of *AtWRKY18* transcripts were present at varying degree in all the ten independent transgenic hairy root lines. As expected, in the control line (CV), transformed with a vector harboring a fragment of the *A. thaliana* gene *GUS*, the specific primers for *AtWRKY18* did not amplify any band. For a more accurate measurement of transcript levels, three different lines were analyzed by qRT-PCR. Transcript level was calculated as 2^(-ddCt) and the root line with the higher ΔCT was arbitrarily considered equal to 1. The *S. sclarea* ribosomal

18S endogenous transcript was used as internal reference gene. As shown in figure 3.9 C, the transcript level *AtWRKY18* in the root line #6 was taken to be equal to 1, while lines #9 and #10 showed respectively transcript levels equal to 1.47- and 1.64-fold higher than the lowest expressing line.

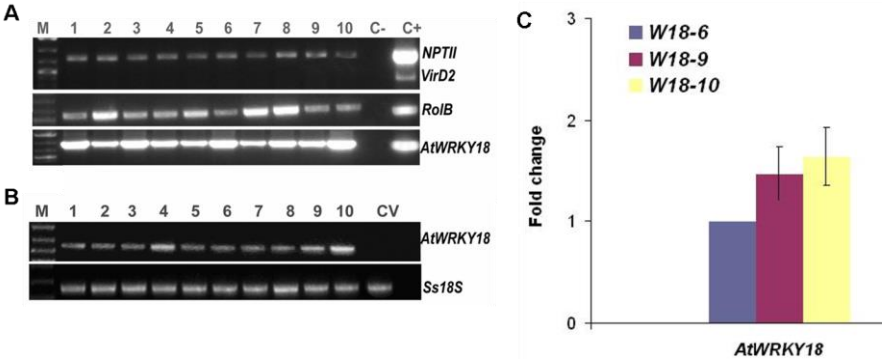


Figure 3.9 - **Molecular characterization of *AtWRKY18* overexpressing hairy root lines. A)** PCR amplification of genomic DNA using specific primers for *NPTII*, *rolB* and *AtWRKY18* genes. Genomic DNA was also used to amplify the *VirD2* gene to confirm the absence of contaminant bacteria. M, marker; C-, negative control without DNA sample; C+, *A. rhizogenes* plasmid carrying the exogenous gene. **B)** Semi-quantitative RT-PCR showing the expression of the exogenous *AtWRKY18* transcript in all transgenic hairy root lines. CV, hairy root line transformed with control vector **C)** qRT-PCR measured the expression levels of *AtWRKY18* in the three transgenic hairy root lines selected for further analyses. Data represent mean values from three separate biological replicates ± SD.

3.1.5.2 Growth of *AtWRKY18* overexpressing hairy roots

In order to ascertain potential detrimental effects of the overexpression of the heterologous gene *AtWRKY18* on biomass growth, the growth rate of the AtWRK18 overexpressing roots was compared to that of the control transformed hairy roots, by monitoring the dry weight at one week interval during a period of 4 weeks (Fig 3.10). The line #9 and #10 showed a longer lag-phase and the final dry weight was lower compared to that of the control root line. However, overexpression of *At*WRKY18 did not cause any significant negative effect on growth of the line #6, characterized by the lowest transcript level of the exogenous TF.

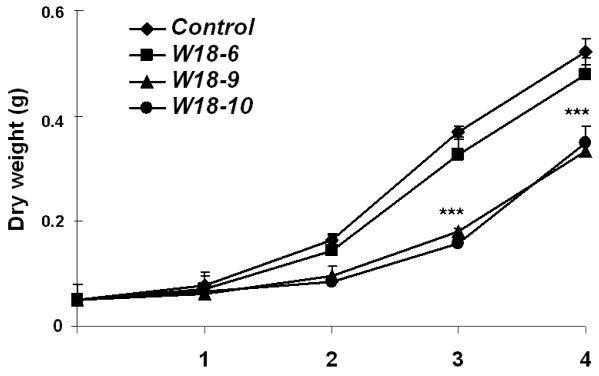


Figure 3.10 - **Biomass production** (mg g^{-1} hairy root dry weight) of three different *AtWRKY18* overexpressing hairy root lines compared to control root line during 4 weeks of culture. Data represent mean values from three separate replicates ± SD. *** P≤0.001.

3.1.5.3 Transcriptional levels of MEP-pathway biosynthetic genes in *AtWRKY18* hairy roots

Transcriptional levels of different MEP-pathway genes of *S. sclarea* were measured by qRT-PCR in *AtWRKY18* overexpressing hairy root lines. As shown in figure 3.11, the expression levels of biosynthetic endogenous genes *SsDXS, SsDXR, SsHDS, SsGGPPS* and *SsCPPS* were upregulated (3-5- fold increase) in all three transgenic root lines. The gene showing the highest induction was the *SsCPPS* gene (5-fold increase), encoding the first synthase responsible of conversion of GGPP into CPP. No transcriptional activation was observed for the transcript *SsCMK*. These data confirmed that WRKY18 is a positive transcriptional regulator of several genes encoding enzymes acting up-stream of GGPP and, interestingly, of CPPS, the first committed enzyme acting at the lateral branch of diterpenoid biosynthetic pathway.

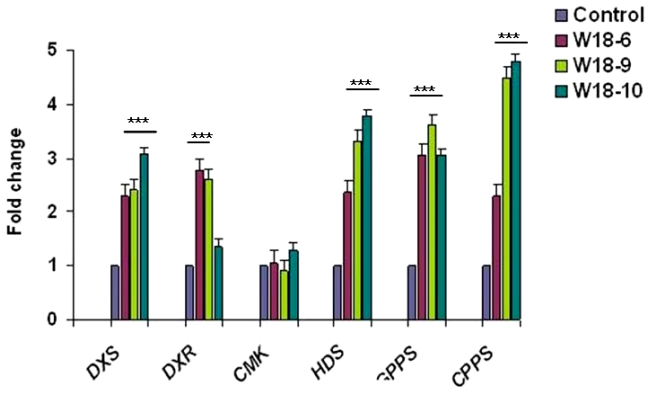


Figure 3.11 - **Expression profile of *S. sclarea* endogenous MEP-pathway biosynthetic genes** in *AtWRKY18* overexpressing hairy root lines, by qRT-PCR. Data represent mean values from three separate replicates ± SD. *** P≤0.001

3.1.5.4 Metabolic profile of *AtWRKY18* overexpressing hairy roots

To verify the effect of the overexpression of the TF *AtWRKY18* on the production of abietane diterpenes, acetonic extracts from three independent transgenic overexpressing hairy root lines were analyzed by targeted HPLC-DAD and compared to the control line. The total abietane diterpene content was different in the three hairy root lines (Table 3.3). The line #10 showed the highest abietane diterpene content (4.23 mg g^{-1}) with a 2.94 fold increase (P≤0.001), the line #6 produced 3.17 mg g^{-1} with a fold increase of 2.2; the line # 9 did not show significant increase in abietane diterpene production compared to the control line. The content of the analyzed diterpenes was significantly enhanced in overexpressing hairy roots. The most relevant effect was the increase in the production of the aethiopinone, whose content ranged from 0.75 mg g^{-1} DW of the line #6 up to 1.42 mg g^{-1} in the line #10, compared to the control line in which the amount of aethiopinone was 0.28 mg g^{-1} (Fig. 3.12). These results strongly supported the role of the AtWRKY18 as a positive regulator of MEP-pathway biosynthetic genes.

Table 3.3 Total abietane diterpene content in At*WRKY18* hairy roots compared to control roots

Hairy root line	Total content (mg g^{-1} dry weight)	Fold- increase
Control	1.44 ± 0.1	-
WRKY18-6	3.17 ± 0.3***	2.20
WRKY18-9	1.86 ± 0.1	1.29
WRKY18-10	4.23 ± 0.3***	2.94

*** P<0.001

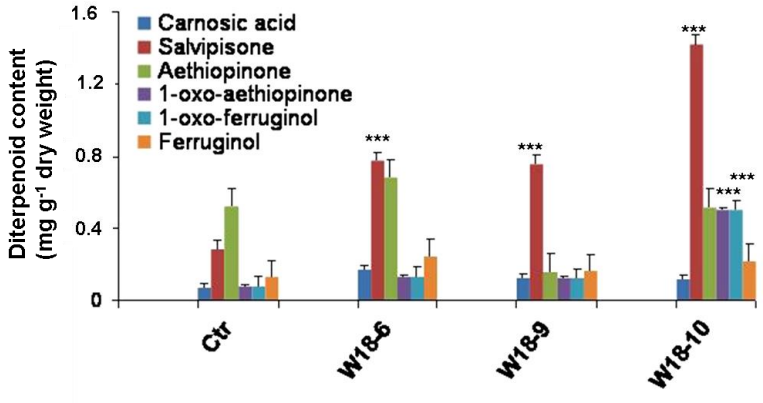


Figure 3.12 - Content of the principal abietane diterpenes synthesized in *S. sclarea*, by HPLC-DAD analysis, in different *WRKY18* overexpressing hairy root lines. Values are means ± SD from two biological replicates. *** P≤0.001

3.1.5.4 Binding of AtWRKY18 to the *AtDXS* and *AtDXR* gene promoters

A number of WRKY proteins have been shown to bind the *TTGACC/T* sequence (W-box) (Eulgem et al., 2000; Llorca et al., 2014). Our data have revealed a strong upregulation of MEP-pathway biosynthetic genes in *AtWRKY18* overexpressing hairy roots. In order to verify the direct involvement of AtWRKY18 in MEP-pathway transcriptional regulation, the recombinant protein HIS-AtWRKY18 was expressed and purified (Fig. 3.13) and tested for its *in vitro* binding to the promoter regions of *AtDXS* and *AtDXR* genes, which contain W-box sequences. Since transcription factors are likely to form homo-complexes, we reasoned that they might also recognize DNA sequences containing more than one W-box. To investigate this possibility, we designed DNA oligonucleotides containing two consecutive W-box separated by a number of nucleotides (Fig. 3.14 A). The correct folding of the purified recombinant protein HIS-AtWRKY18 was preliminarily tested with an oligonucleotide probe specifically designed on *NPR1* gene promoter containing a W-box, for which it has been previously proved to be recognized by WRKY18 (Yu et al., 2001) (lane 1, Fig, 3.14 B). However, at least with the oligonucleotides we have tested, the recombinant AtWRKY18 protein did not seem to bind to the W-box elements of the *AtDXS* gene promoter in the 1000 bp upstream transcription start site (Fig. 3.14 B). Same results were obtained using oligonucleotides designed on the promoter region of the *AtDXR* gene (data not shown).

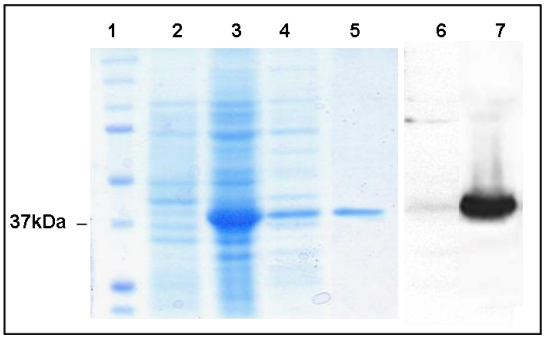


Figure 3.13 - **Expression and purification of recombinant AtWRKY18 native protein in *E. coli M15* strain**. Protein MW marker in kDa (1), bacterial extract before induction by IPTG (2); crude bacterial lysate 3 h after induction (3); unbound fraction (4); recombinant proteins purified from bacterial lysate by affinity chromatography using Ni-NTA resin (5); anti-His immunobloting on bacterial extract before induction by IPTG (6); crude bacterial lysate 3 h after induction (7).

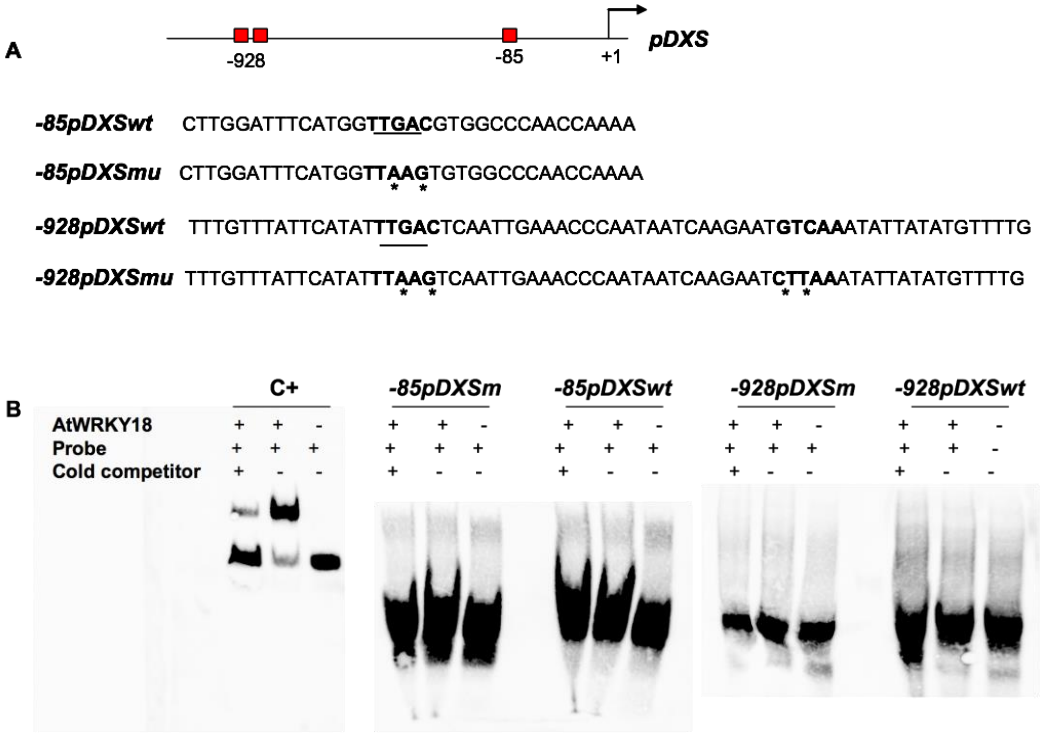


Figure 3.14 - Elecrophoretic Moblity Shift Analysis - A) Sequences of the *AtDXSwt* probes and the *AtDXSmut* probes with the *TTGAC* mutated in *TTAAG*. Underlining indicate W-box sequences. Asterisks represent the mutated bases in the W-box elements. **B)** The recombinant AtWRKY18 protein was incubated with biotin-labeled probes in the presence of cold competor (unlabeled probes) (lane 1), or in absence of cold competitor (lane 2); in the lane 3 only biotin-labeled probes have been loaded .

3.1.6 *AtWRKY40* overexpressing hairy roots

3.1.6.1 Molecular characterization of *AtWRKY40* hairy root lines

Ten *kan*$^{+}$ hairy root lines overexpressing the TF *AtWRKY40*, were screened by PCR for the presence of *NPTII* gene, *rolB* gene, and for the stable insertion of the heterologous *AtWRKY40* gene. By using specific primers (Table 2.1), a single amplicon with the expected size was amplified in the tested transgenic hairy root lines, while no bands were detected in the control vector transformed lines (C-). Absence of contaminant bacteria was confirmed by missing amplification of the bacterial *virD2* gene. A template *A. rhizogenes* transformed with the construct overexpressing *AtWRKY40* was used as PCR positive control (C+) (Fig 3.15 A). Correct expression of the exogenous gene the heterologous *AtWRKY40* gene was verified by RT-PCR. As shown in Fig. 3.15 B, detectable levels of *AtWRKY18* transcripts were present at varying degree in all the ten independent transgenic hairy root lines. In the control line (C-), transformed with a vector harboring a fragment of the *A. thaliana* gene *GUS*, as expected, the specific primers for *AtWRKY18* did not amplify any band. For a more accurate measurement of transcript levels, three different lines were selected for qRT-

PCR analysis. Transcript level was calculated as 2^(-ddCt) and the root line with the higher ΔCT was arbitrarily considered equal to unity. The *S. sclarea ribosomal 18S* endogenous transcript was used as internal reference gene. As shown in figure 3.15 C, the transcript level *AtWRKY40* in the root line #10 was taken to be equal to 1, while lines #9 and #7 showed respectively transcript levels equal to 1.32 and 1.77 higher than the lowest expressing transgenic hairy root line**.**

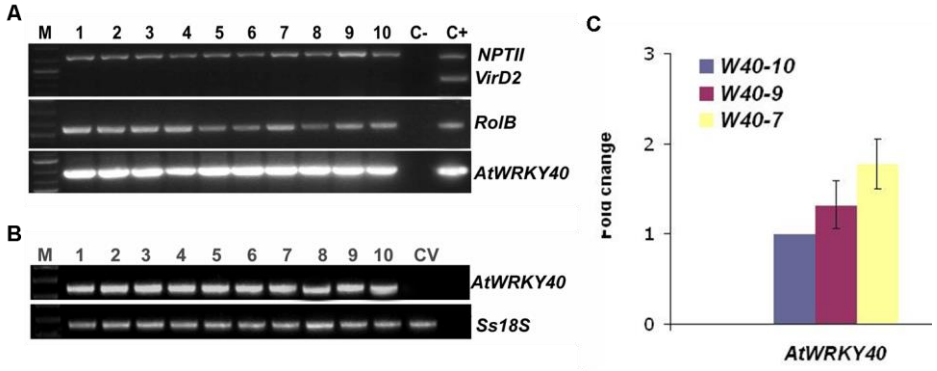


Figure 3.15 - Molecular characterization of *AtWRKY40* overexpressing hairy root lines. **A)** PCR amplification of genomic DNA using specific primers for *NPTII*, *rolB* and *AtWRKY40* genes. Genomic DNA was also used to amplify the *virD2* gene to confirm the absence of contaminant bacteria. M, marker; C-, negative control without DNA sample; C+, *A. rhizogenes* plasmid carrying the exogenous gene. **B)** Semiquantitative RT-PCR showing the expression of the exogenous *AtWRKY40* transcript in all transgenic hairy root lines. CV, hairy root line transformed with control vector. **C)** qRT-PCR on three different root lines with different levels of transgene expression. Data represent mean values from three separate biological replicates ± SD.

3.1.6.2 Growth of *AtWRKY40* overexpressing hairy roots

Biomass, as dry weight, of the *AtWRK40* overexpressing hairy roots was compared to the control roots to check for any detrimental effects on biomass growth due to the overexpression of the heterologous gene. *S*tarting from 0.5 g of hairy roots, the growth was monitored during four weeks of inoculation into fresh hormone-free medium. Although the overexpression of *AtWRK40* caused a longer lag phase in all three overexpressing root lines, after four weeks the final dry weight of the lower overexpressing line #10 (0.43± 0.025 mg g^{-1}) was not significantly different from that of the control line (0.52± 0.028 mg g^{-1}) (Fig. 3.16).

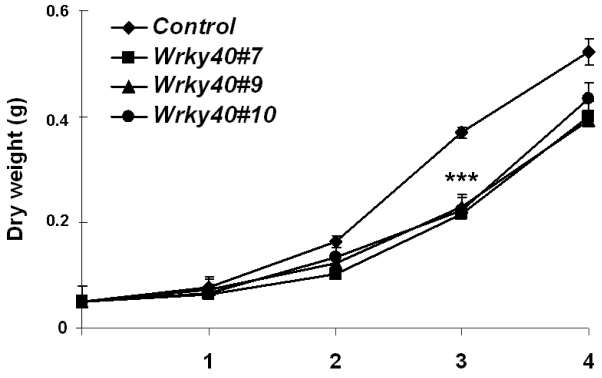


Figure 3.16 - **Biomass production** (mg g^{-1} hairy root dry weight) of three different *AtWRKY40* overexpressing hairy root lines compared to control root line during a period of 4 weeks. Data represent mean values from three separate replicates ± SD. *** P≤0.001

3.1.6.3 Trascriptional levels of MEP-pathway biosynthetic genes in *AtWRKY40* overexpressing hairy roots

Transcriptional levels of MEP-pathway genes of *Salvia sclarea SsDXS, SsDXR, SsCMK, SsHDS, SsGGPPS* and *SsCPPS* were measured to test whether the overexpression of the TF *AtWRKY40* was able to positively stimulate their expression. As shown in figure 3.17, in contrast with what found in the AtWRKY18 overexpressing line, overexpression of this WRKY TF was able to regulate preferentially the expression of *SsDXS,* the first committed enzyme of the MEP-derived pathway, and of *SsCPPS*, acting at the lateral route leading to abietane diterpene synthesis.

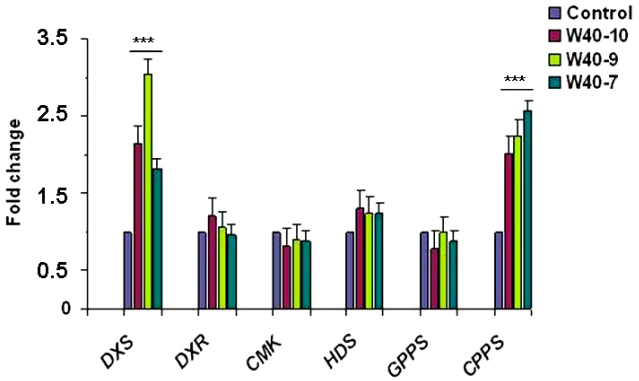


Figure 3.17 - **Expression profile of *S. sclarea* MEP-pathway biosynthetic genes** in *AtWRKY40* overexpressing hairy root lines, by qRT-PCR. Data represent mean values from three biological separate replicates ± SD. *** P≤0.001

3.1.6.4 Metabolic profile of *AtWRKY40* hairy roots

In order to evaluate the effect of the overexpression of the TF *AtWRKY40* on the production of abietane diterpenes in *S. sclarea* hairy roots, acetonic extracts from three independent samples of each transgenic hairy root line were analyzed by HPLC-DAD and compared to the control line. Total abietane diterpene content was increased in overexpressing hairy roots (Table 3.5). As shown in figure 3.18, the most relevant effect of the AtWRKY40 overexpression was the increase in the production of the salvipisone, whose content ranged from 0.754 mg g^{-1} DW of the line #-9 up to 1.67 mg g^{-1} in the line #7. A consistent accumulation of the abietane-quinone type diterpene aethiopinone was also detected in the line #7 (1.03 mg g^{-1}) and in the line #9 (2.25 mg g^{-1}) compared to the control line, in which aethiopinone production was 0.52 mg g^{-1}. The line #9 showed the highest abietane diterpene content (5.03 mg g^{-1}) with a 3.49 fold increase (P≤0.001); the line **#7** produced 4.49 mg g^{-1} with a fold increase of 3.12; the line #9 produced 2.97 mg g^{-1} of total abietane diterpene with 2-fold increase compared to the control line. These data pointed at AtWRKY40 as another positive regulator of the expression of biosynthetic genes encoding enzymes of the plastidial MEP-derived isoprenoid pathway.

Table 3.5 Total abietane diterpene content of *WRKY40* overexpressing hairy roots compared to control roots

Transgenic root line	Total content (mg g^{-1} dry weight)	Fold- increase
Control	1.44 ± 0.1	-
WRKY40-7	4.49 ± 0.4***	3.11
WRKY40-9	5.03 ± 0.3***	3.44
WRKY40-10	2.97 ± 0.2***	2.06

*** P≤0.001

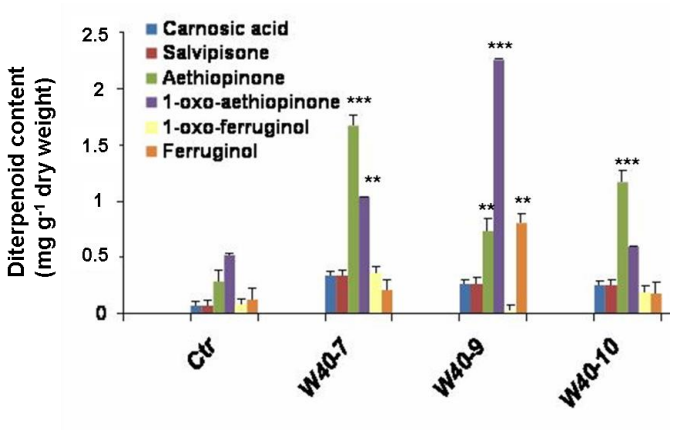


Figure 3.18 - Content of the principal abietane diterpenes synthesized in *S. sclare*, by HPLC-DAD analysis in different *WRKY40* overexpressing hairy root lines. Values are means ± SD from two biological replicates. *** P≤0.001

3.1.7 *AtWRKY60* overexpressing hairy roots

3.1.7.1 Molecular characterization *AtWRKY60* overexpressing hairy root lines

Ten *kan*$^{+}$ hairy root lines transformed overexpressing the TF *AtWRKY60* were screened by PCR for the presence of *NPTII* and *rolB* genes, and for the stable insertion of the heterologous gene *AtWRKY60.* Single amplicon with the expected size was amplified in the tested transgenic hairy root lines, while no bands was detected in the control vector transformed lines (C^{-}). Absence of contaminant bacteria was confirmed by missing amplification of the bacterial *virD2* gene. As PCR positive control (C^{+}) was used as template *A. rhizogenes* transformed with the construct overexpressing *AtWRKY60* (Fig. 3.19 A). As shown in Fig 3.19 B, detectable levels of *AtWRKY60* transcripts were present at varying degree in all the ten independent transgenic hairy root lines. In the control line (CV), transformed with a binary vector containing a fragment of the *A. thaliana* gene *GUS*, as expected, the specific primers for *AtWRKY60* did not amplify any band. Three different lines were selected for a more accurate measurement of transcript levels by qRT-PCR. As shown in figure 3.19 C, the transcript level *AtWRKY60* in the root line #3 was taken to be equal to 1, while lines #5 and #9 showed respectively transcript levels equal to 1.49 and 2.13.

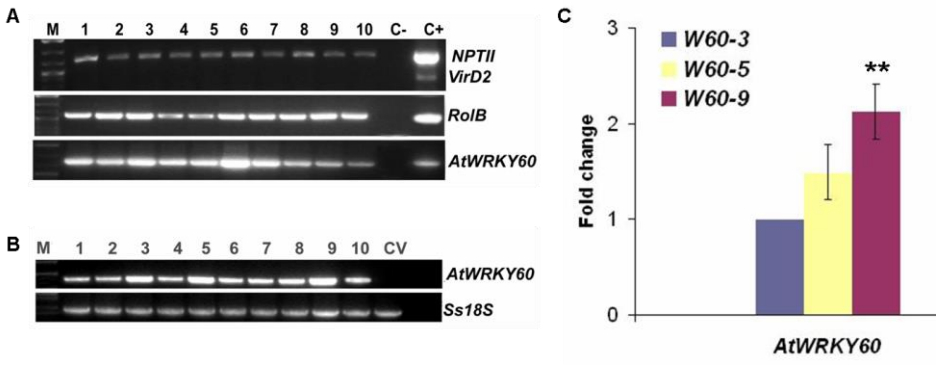


Figure 3.19 - Molecular characterization of *AtWRKY60* overexpressing hairy roots. **A)** PCR amplification of genomic DNA using specific primers for *NPTII*, *rolB* and *AtWRKY60* genes. Genomic DNA was also used to amplify the *VirD2* gene to confirm the absence of contaminant bacteria. M, marker; C-, negative control without DNA sample; C+, *A. rhizogenes* plasmid carrying the exogenous gene. **B)** Semi-quantitative RT-PCR showing the expression of the exogenous *AtWRKY60* transcript in all transgenic hairy root lines. CV, hairy root line transformed with control vector. **C)** qRT-PCR on tree different root lines with different levels of transgene expression. Data represent mean values from three separate biological replicates ± SD. ** P≤0.01

3.1.7.2 Growth of *AtWRKY60* hairy roots

Equal amounts (0.5 g) of each roots overexpressing AtWRKY60 were inoculated into MS hormone-free liquid medium and their growth was monitored at 1-week intervals during a period of one month. As shown in figure 3.20, after two weeks the final dry weight of overexpressing hairy roots was lower than that of the control.

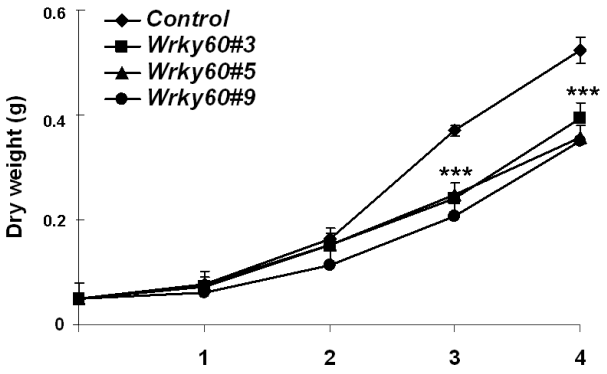


Figure 3.20 - **Biomass production** (mg g^{-1} hairy root dry weight) of three different *AtWRKY60* overexpressing hairy root lines compared to control root line during 4 weeks of culture. Data represent mean values from three separate biological replicates ± SD. *** P≤0.001

3.1.7.3 Transcriptional levels of MEP-pathway biosynthetic genes in *AtWRKY60* overexpressing hairy roots

Transcriptional levels of MEP-pathway endogenous genes of *S. sclarea SsDXS, SsDXR, SsCMK, SsHDS, SsGGPPS* and *SsCPPS* were measured to evaluate whether the overexpression of the TF *AtWRKY60* was able to activate their transcription. As shown in figure 3.21, the transcript levels of biosynthetic genes in overexpressing roots are only slightly up-regulated by overxpression of this WRKY TF, compared to the other two we have analyzed.

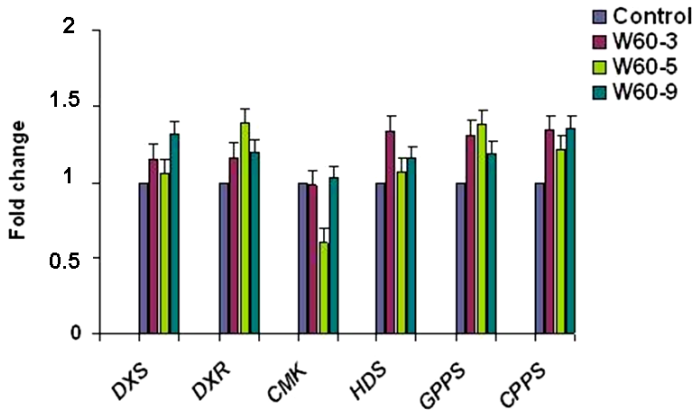


Figure 3.21 - Expression profile of *S. sclarea* MEP-pathway biosynthetic genes in *AtWRKY60* overexpressing hairy root lines, by qRT-PCR. Data represent mean values from three separate biological replicates ± SD.

3.1.7.4 Metabolic profile of *AtWRKY60* overexpressing hairy roots

To assess whether overexpression of the TF *AtWRKY60* was able to boost the production of abietane diterpenes in *S. sclarea* hairy roots, acetonic extracts from three independent samples of each transgenic hairy root line were analyzed by HPLC-DAD. In table 3.6 is shown the total abietane diterpene content. The line #9 showed the highest diterpenoid content (3.13 mg g^{-1}) with a 2.17 fold increase (P≤0.001); the line #3 produced 2.97 mg g^{-1} of total abietane diterpene with a 2-fold increase; the line #5 produced 2.5 mg g^{-1} with a fold increase of 1.74 compared to the control line. The most prominent effect of the *AtWRKY60* overexpression was the increase in the production of the salvipisone, whose content ranged from 0.92 mg g^{-1} DW of the line #-5 up to 1.28 mg g^{-1} in the line #3 (Fig. 3.22).

Table 3.6 Total diterpene content of *WRKY60* overexpressing hairy roots compared to control roots

Transgenic root line	Total content (mg g^{-1} dry weight)	Fold- increase
Control	1.44 ± 0.1	-
WRKY60-3	2.97 ± 0.2***	2.06
WRKY60-5	2.52 ± 0.3***	1.75
WRKY60-9	3.13 ± 0.4***	2.17

*** P<0.001

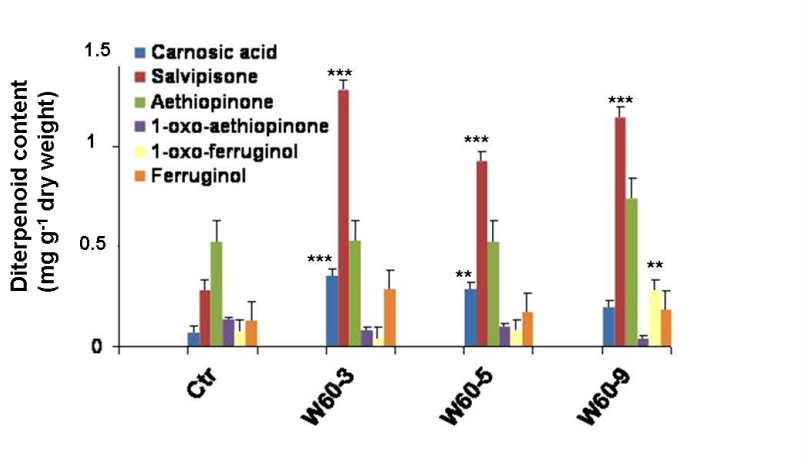


Figure 3.22 - **Content of the principal abietane diterpenes synthesized in *S. sclarea,*** by HPLC-DAD analysis, in different *WRKY60* overexpressing hairy root lines. Values are means ± SD from two biological replicates.*** P≤0.001

3.1.8 *AtMyc2* overexpressing hairy roots

3.1.8.1 Molecular characterization of *AtMyc2* overexpressing hairy roots

The last TF we have tested for its potential ability to enhance the synthesis of abietane diterpenes belongs to well-studied TF Myc family, able to bind to the consensus sequence G-box (*CACATG*), that we have identified in promoters of several biosynthetic genes of the MEP-derived pathway. Ten *kan*$^{+}$ hairy root lines transformed overexpressing the TF *AtMyc2* were screened by PCR for the presence of *NPTII* and *rolB* gene, and for the stable insertion of the heterologous *AtMyc2* gene. A single amplicon with the expected size was amplified in the tested transgenic hairy root lines, while no bands was detected in the control vector transformed lines (C-). Absence of contaminant bacteria was confirmed by missing amplification of the bacterial *virD2* gene. As PCR positive control (C+) was used as template *A. rhizogenes* transformed with the construct containing the coding region of *AtMyc2* (Fig. 3.23 A). The tested hairy root lines expressed varying levels of *AtMyc2* transcripts (Fig. 3.23 B) and three different lines were further examined by qRT-PCR (Fig, 3.23 C). The transcript level *AtMyc2* in the root line #6 was taken to be equal to 1, while lines #9 and #10 showed respectively transcript levels equal to 1.63 and 3.23.

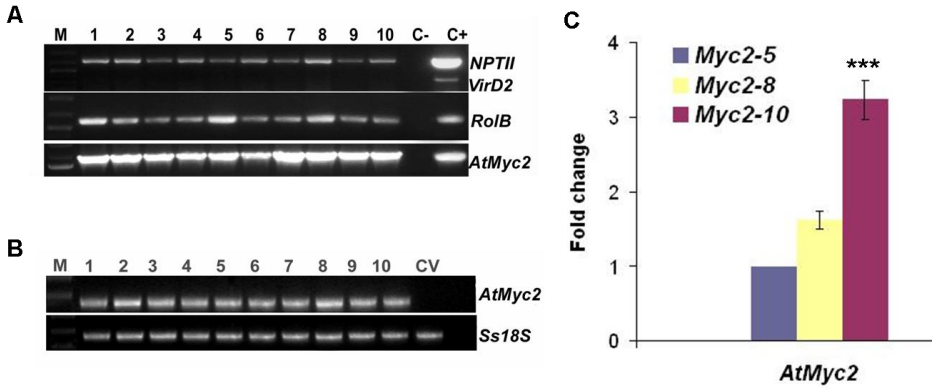


Figure 3.23 - Molecular characterization of *AtMyc2* overexpressing hairy roots. **A)** PCR amplification of genomic DNA using specific primers for *NPTII*, *rolB* and *AtMyc2* genes. Genomic DNA was also used to amplify the *virD2* gene to confirm the absence of contaminant bacteria. M, marker; C-, negative control without DNA sample; C+, *A. rhizogenes* plasmid carrying the exogenous gene. **B)** Semi-quantitative RT-PCR showing the expression of the exogenous *AtMyc2* transcript in all transgenic hairy root lines. CV control hairy root line transformed with control vector. **C)** qRT-PCR on tree different root lines with different levels of transgene expression. Values are means ± SD from three biological replicates. *** P≤0.001

3.1.8.2 Growth of *AtMyc2* overexpressing hairy root lines

Compared to what found in *S. sclarea* hairy roots lines overexpressing the WRKY TFs, hairy root growth was severely affected by overexpression of *AtMyc2* After four weeks, the final dry weight barely increased in the three analyzed overexpressing lines, indicating that even a low expression is associated with detrimental growth defects (Fig. 3.24).

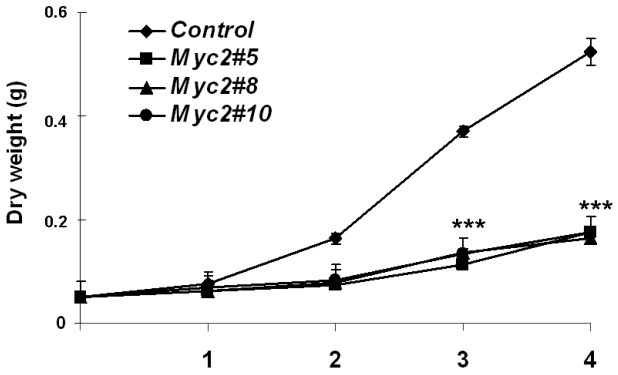


Figure 3.24 - Biomass production (mg g^{-1} hairy root dry weight) of three different *AtMyc2* overexpressing hairy root lines compared to control root line during 4 weeks of culture. Data represent mean values from three biological separate replicates ± SD. *** P≤0.001

3.1.8.3 Transcriptional levels of MEP-pathway biosynthetic genes in *AtMyc2* overexpressing hairy root lines

Transcriptional levels of MEP-pathway endogenous genes of *Salvia sclarea SsDXS, SsDXR, SsCMK, SsHDS, SsGGPGS* and *SsCPPS* were measured to examine whether the overexpression of the TF *AtMyc2* was able to positively stimulate their expression. As shown in figure 25, the expression levels of biosynthetic endogenous genes *SsDXS, SsDXR, SsHDS, SsGGPPS* and *SsCPPS* was upregulated in all three transgenic root lines. The genes showing the highest expression were *SsDXS* (5-fold increase) and *SsCPPS* (more than 4-fold increase in the line #10), while no activation was observed for the transcript *SsCMK*. These data strongly suggest a direct involvement of this TF in the transcriptional regulation of biosynthetic genes of the MEP-dependent isoprenoid pathway, albeit its strong negative effects on root biomass.

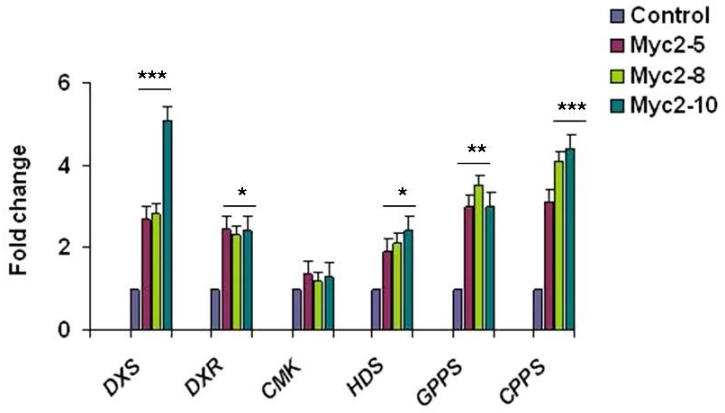


Figure 3.25 - Expression profile of *S. sclarea* endogenous MEP-pathway biosynthetic genes in *AtMyc2* overexpressing hairy root lines, by qRT-PCR. Data represent mean values from three biological separate replicates ± SD. *** P≤0.001; ** P≤0.01; * P≤0.05

3.1.8.4 Metabolic profile of *AtMyc2* overexpressing hairy root lines

Metabolic analyses of *AtMyc2* overexpressing hairy roots were not performed due to their very low biomass although this TF, along with AtWRK18, was one of the most effective in enhancing transcription of several biosynthetic genes of the MEP-derived terpene pathway. To fully exploit the potential of this TF in triggering biosynthesis of bioactive abietane diterpenes, it would be useful to select hairy roots with lower expression of the *AtMyc2* gene or, alternatively, to use inducible promoters (e.g. ethanol-, dexamethasone-, beta-estradiol-inducible), in order to avoid/reduce the negative impact on the final biomass associated to an excessive overxpression,

3.2 Increasing the production of abietane diterpenes by blocking a competitive lateral route of the MEP-derived pathway

The second strategy used to enhance the synthesis of bioactive abietane diterpenes in *S. sclarea* hairy roots was to direct GGPP (geranylgeranyl diphosphate) preferentially towards abietane diterpenes by silencing the gene encoding the EntCopalyl diphosphate synthase (EntCPPS), the enzyme catalyzing the first committed step in the gibberellin biosynthetic pathway. The geranylgeranil- diphosphate (GGPP) is, in fact, the common substrate for both the enzyme EntCPPS and the enzyme CPPS, the first enzyme leading to the biosynthesis of abietane diterpenes. Inhibition of this enzyme might make the GGPP substrate more available for the enzyme CPPS and push the metabolic flux to abietane diterpenes biosynthesis. This competitive lateral route was targeted by using the Chlorocholine chloride (CCC), a known chemical growth retardant, which inhibits the catalytic activity of EntCPPS, or by using double stranded RNA targeting *EntCPPS* mRNA (RNAi) (Fig. 3.26).

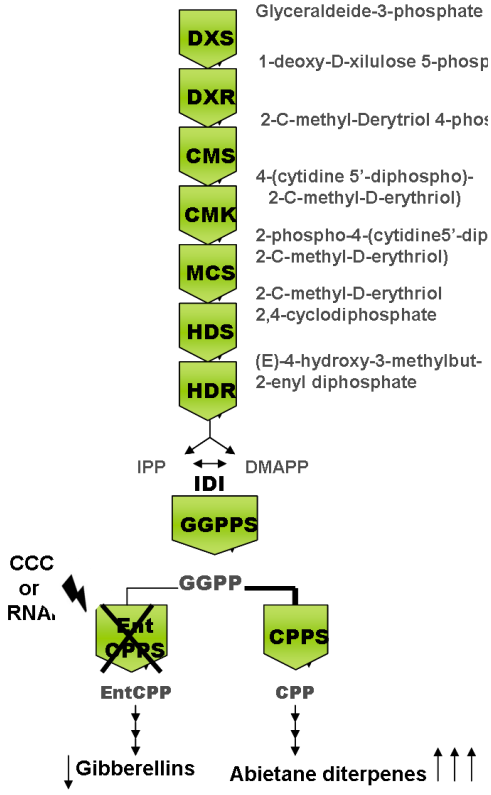


Figure 3.26 - Schematic representation of the block of the competitive gibberellin biosynthetic pathway to divert GGPP towards a higher synthesis of abietane diterpenes. (For abbreviations see figure 3.2)

3.2.1 Treatment of *S. sclarea* hairy roots with Chlorocholine chloride (CCC) and quantitative analysis of bioactive diterpenes in CCC-treated *Salvia sclarea* hairy roots

S. sclarea hairy roots, obtained by transformation with the *A. rhizogenes* strain ATCC 15835, were grown for thirty days in the dark, in a hormone-free medium, and treated for thirty days with 100 µM Chlorocholine chloride (CCC). Acetonic extracts from CCC-treated and control, water treated, hairy roots were compared for targeted abietane diterpenes. Total abietane diterpene content of CCC-treated hairy roots was 4.34 mg g^{-1} dry weight, about 3.5 times higher than the content of control hairy roots (Table 3.8). The content of all monitored diterpenes (carnosic acid, ferruginol, 1-oxo-ferruginol, salvipisone, aethiopinone and 1-oxo-aethiopinone) in CCC-treated hairy roots were significantly enhanced (P≤0.001) compared to the control roots, with the most relevant increase in the content of aethiopinone (7-fold increase) (Fig. 3.27).

Table 3.8 Total abietane diterpenes in CCC- treated *S. sclarea* hairy roots

Treatment	Total content (mg g^{-1} dry weight)	Fold- increase
Mock	1.23 ± 0.03	-
CCC 100 µM	4.34 ± 0.08***	3.5

*** P≤0.001

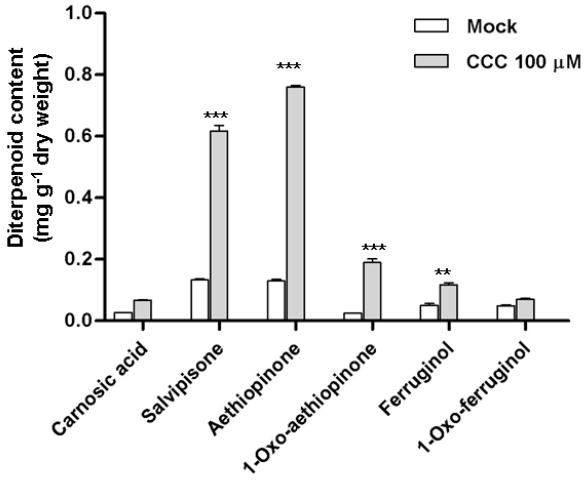


Figure 3.27 - **Content of the principal abietane diterpenes in *S. sclarea* hairy roots**, analyzed by HPLC-DAD analysis. The amount of each compound in CCC-treated roots was compared with the control line (mock). Values are means ± SD of triplicate technical analyses from two biological replicates. *** indicate significant difference at P≤ 0.001. ** P≤0.01

3.2.2 Growth of CCC-treated hairy roots

In order to ascertain any potential detrimental effects of the CCC treatment on biomass growth, the growth rate of CCC-treated roots was compared to the control hairy roots, by monitoring the dry weight at one week interval during a period of 4 weeks. As shown in figure 28, the treatment with CCC compound did not cause any negative effects on growth of the hairy root line. The growth rate of treated roots resulted even improved, making the CCC treatment an advantageous strategy to increase the content of abietane diterpenes in *S. sclarea* hairy roots.

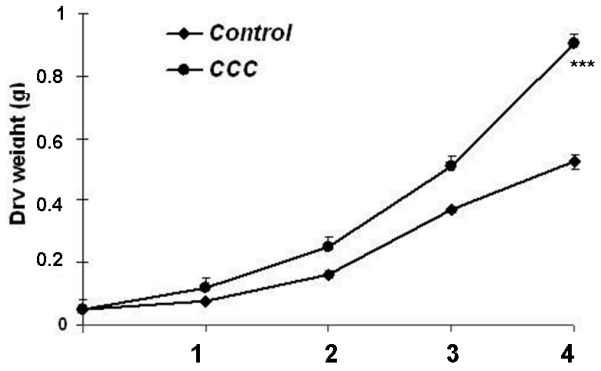


Figure 3.28 - **Biomass production (mg g^{-1} hairy root dry weight) of CCC-treated hairy root lines** compared to control root line during 4 weeks of culture. Data represent mean values from three biological separate replicates ± SD. *** P≤0.001.

3.2.3 RNAi-mediated silencing of the gene *EntCopalyl diphosphate synthase*

3.2.3.1 Identification and characterization of the *S. sclarea EntCPPS* gene

The competitive gibberellin biosynthetic route was also inhibited by RNA interference of the gene *EntCopalyl diphosphate synthase* (*EntCPPS*), encoding the enzyme catalyzing the first committed step in the gibberellin biosynthesis. Since, the coding sequence of the *S. sclarea EntCPPS* gene was not known, a set of degenerate primers designed on conserved regions of EntCPPS proteins of different plant species were used to amplify a partial sequence fragment of this gene starting from a *S. sclarea* stem cDNA pool. As shown in figure 3.29 A, an amplicon with the expected size (518 bp) was obtained (lane 5), using the combination of primers *(Fw GARCAYATGCCNATHGGNTTYGARGT; Rev. TCNACNGGRTANACRTTNGGNACNCC).* This PCR product was purified and cloned into pCR2.1 vector and three random clones were sequenced. The three amplified cDNAs were identical to each other and similar (>70% identity), at the amino acid level, to the coding sequence of the known *A. thaliana* and *Zea mays EntCPPS* hortologues genes. On the basis of this partial sequence, a specific primer was designed for extending the coding sequence by 3'-RACE-PCR. An amplicon of expected size (1400 bp) was obtained (Fig. 3.29 B),

cloned and sequenced. Overall, a fragment of 1821 bp of the gene *EntCPPS* was obtained, corresponding to the portion of deduced protein from the amino acid in position 200 to the stop codon (Fig 3.29 C). The *S. sclarea* sequence, showing a 53% identity to the *A. thaliana* hortologue, was compared to the EntCPPS amino acid sequences available for a number of plant species, to verify the presence of functional domains, such as the aspartate-rich “DIDD” box, responsible of the synthase activity (Fig. 3.30). Expression analysis by qRT-PCR revealed that the gene is ubiquitously expressed in plant tissues (stem, root, leaves and hairy roots) although at higher levels in stem (Fig. 3.31), as reported in other plant species.

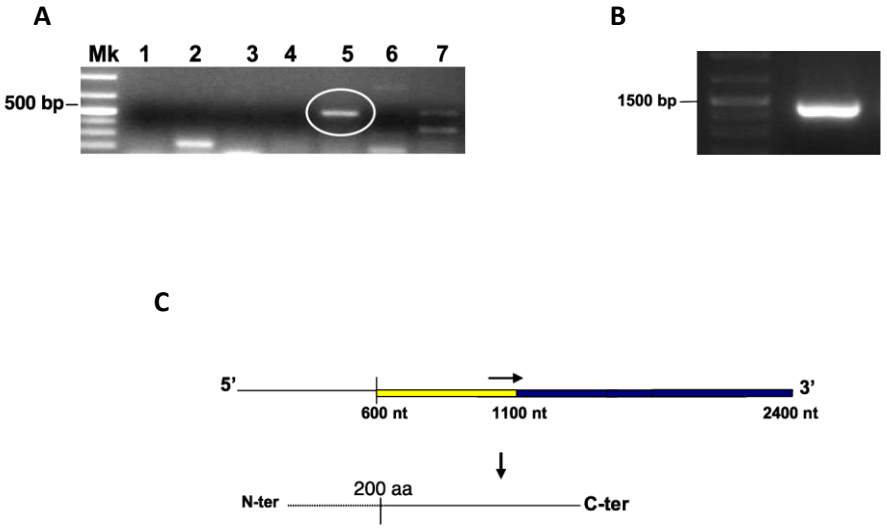


Figure 3.29 - **Amplification of a cDNA fragment relative to the partial sequence of the gene *SsEnt-CPPS*** by RT-PCR, using seven different combinations of degenerated primers (lane 1-7). The band in lane 5 corresponds to the expected molecular weight of 518 bp **(A)** and elongation of the *SsEnt-CPPS* coding sequence using specific primers by 3’-RACE-PCR **(B). C)** Schematic representation of the gene elongation: in yellow, the fragment isolated by amplification with degenerate primers; in blue, the 3’ sequence elongated by RACE-PCR Bottom, the graph indicating the amino acid sequence corresponding to the isolated cDNA.

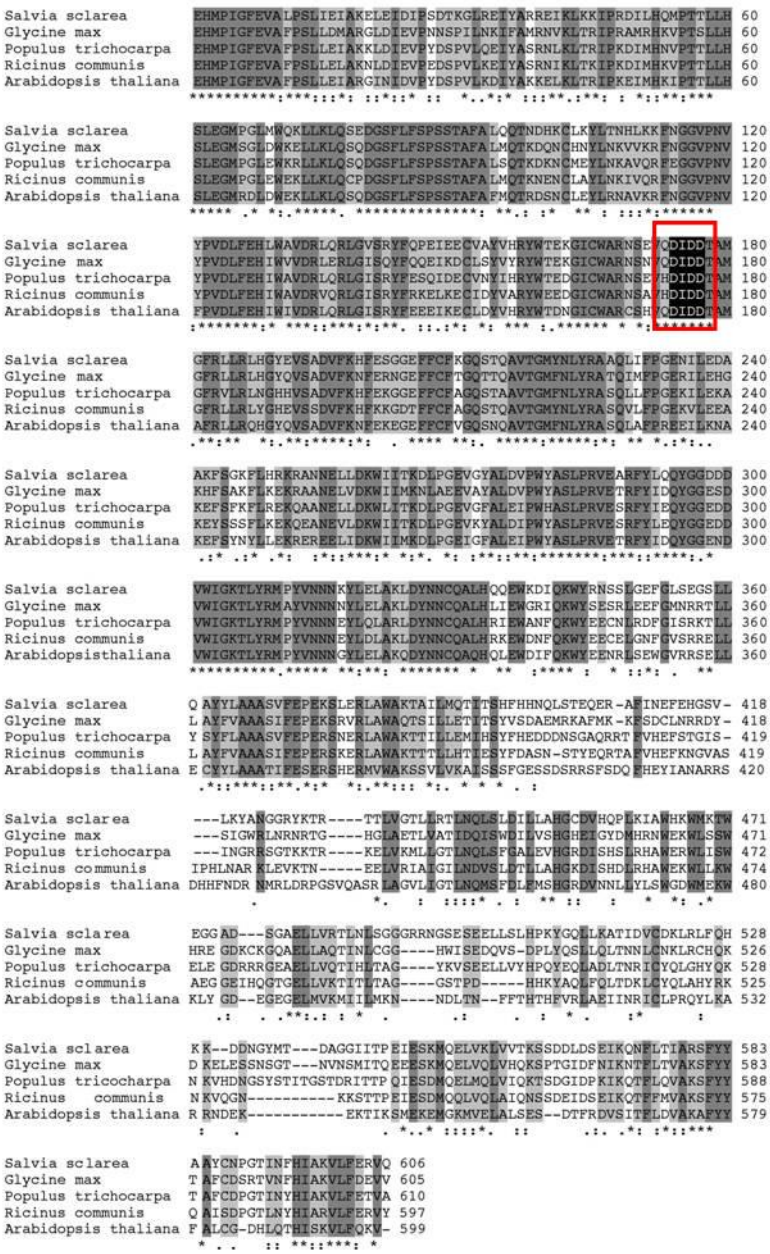


Figure 3.30 - Multiple Alignment of deduced amino acid sequences of SsEntCPPS. Dark shading and gray shading indicate identical and similar amino acid residues, respectively. Conserved DIDD motifis, corresponding to the enzymatic synthase activity, reported in black and evidenced by a red box. Accession numbers: *Glycine max* (XP_003520571); *Populus trichocarpa* (XP_002302110); *Ricinus communis* (XP_002520733); *Arabidopsis thaliana* (At4g02780).

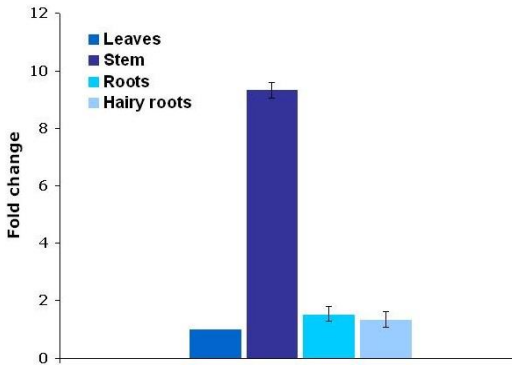


Figure 3.31 - Expression levels of *SsEntCPPS* in different plant tissues. Transcript levels were measured by qRT-PCR using total RNA from *S.sclarea* leaves, stem, root and hairy roots. Transcript level was calculated as 2^(-ddCt). The tissue with the higher ΔCT (lower transcript level) was arbitrarily considered equal to unity. The *S. sclarea ribosomal 18S* endogenous transcript was used as reference. Data represent mean values from three separate biological replicates ± SD.

3.2.3.2 Generation of *EntCPPS* silenced *S. sclarea* hairy roots

Once isolated, the cDNA of the *S. sclarea* gene *EntCPPS* was used as template to amplify a short fragment (450 bp) of the 3' coding sequence, a region with low similarity to other sequences. As shown in figure 3.32, this fragment was inserted by recombination between *att*B1/*att*B2 and *att*B2/*att*B1 into the pHELLSGATE12 binary vector. When the vector is expressed in the plant, a hairpin RNA (hpRNA) with the intron spliced out is produced, targeting the endogenous *Ent-CPPS* mRNA.

Figure 3.32 - Schematic representation of the plasmid constructs used for the silencing RNAi-mediated of *S. sclarea* gene *EntCPPS* gene. The transcription is driven by the constitutive strong viral 35SCaMV promoter. *NPTII* (resistance to kanamycine); LB, left border; RB, right border; attb1 and attb2, homologous recombination sites.

Ten *kan*$^{+}$ hairy root lines putatively silenced were characterized for the presence of the *neomycin phosphotransferase II* (*NPTII*) and *rolB* gene. Absence of contaminant bacteria was confirmed by missing amplification of the bacterial *virD2* gene. *A. rhizogenes* transformed with the RNAi vector was used as PCR positive control (C^{+}) (Fig 3.33 A). RT-PCR analysis revealed that the endogenous *EntCPPS* gene was down-regulated at varying degree in all the ten independent transgenic hairy root lines (Fig. 3.33 B). A quantitative measurement of *SsEntCPPS* transcript levels (qRT-PCR) in the three hairy root lines revealed that gene silencing ranged from 60% in the line #2 to 90% in the line #7 (Fig. 3.33 C).

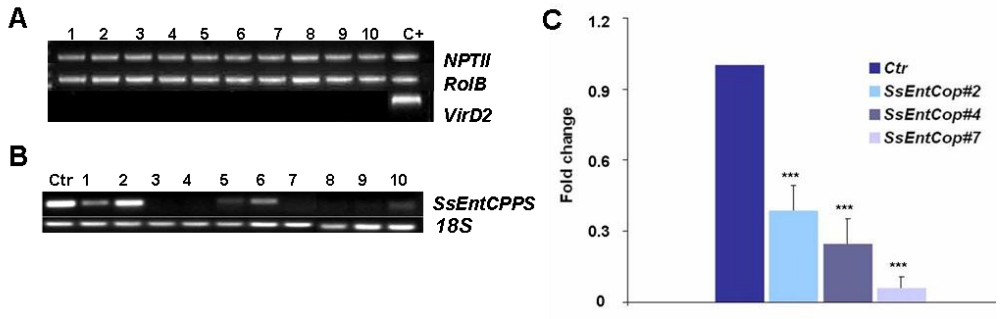


Figure 3.33 - **Molecular characterization of *EntCPPS* silenced hairy root lines**. **A)** PCR amplification of genomic DNA using specific primers for *NPTII* and *rolB* genes. Genomic DNA was also used to amplify the *virD2* gene to confirm the absence of contaminant bacteria. C^+, *A. rhizogenes* plasmid carrying the RNAi construct. **B)** Semi-quantitative RT-PCR showing the expression of the endogenous transcript in independent silenced hairy root lines. **C)** Expression levels of the endogenous *EntCPPS* gene in three silenced hairy roots, by qRT-PCR analysis, calculated as 2^(-ddCt) compared to those of control line. *S. sclarea ribosomal 18S* endogenous transcript was used as reference. Data represent mean values from three separate biological replicates ± SD. *** P≤0.001

3.2.3.3 Growth and morphology of *EntCPPS* silenced *S. sclarea* hairy roots

The growth rate of the transgenic roots was compared to the control roots, starting from 0.5 g of tissue and monitoring the growth by measuring the weight each week. The silenced lines grew very similarly to the control line (Fig. 3.34 A). Moreover, the silencing of *EntCPPS* did not cause any appreciable phenotypic changes, except for the typical reddish colour, indicative of an accumulation of abietane diterpenes (Fig. 3.34 B).

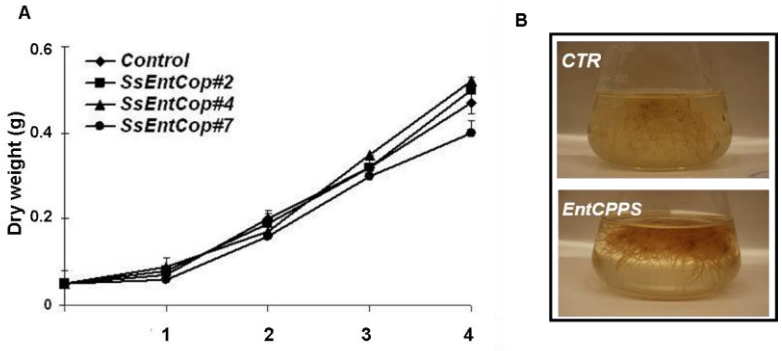


Figure 3.34 - **Biomass growth (mg g^{-1} hairy root dry weight) of three different *EntCPPS*** silenced *S. sclarea* hairy root lines compared to control root line over a period of 4 weeks. Data represent mean values from three separate biological replicates ± SD.

3.3 Metabolic profile of transgenic silenced root lines

To verify the effect of the silencing of the gene *EntCPPS* on the production of abietane diterpenes, acetonic extracts from three independent transgenic hairy root lines were analyzed by targeted HPLC-DAD and compared to the control line. The increase in total abietane diterpene content was quite similar in the three silenced hairy root lines (Fig. 3.35 A). The line #7 showed the highest abietane diterpene content (6.06 mg g^{-1}) with a 4.52 fold increase (P≤0.001), the line #4 produced 5.99 mg g^{-1} with a fold increase of 4.43; the line #2 produced 4.89 mg g^{-1} with a fold increase of 3.62. The content of the single abietane diterpenes was significantly enhanced in silenced hairy roots, as shown in the figure 3.34 B. The most relevant change was the increase in the production of the aethiopinone, whose content ranged from 1.97 mg g^{-1} DW of the line #4 up to 2.64 mg g^{-1} in the line #2, compared to the control line in which the amount of aethiopinone was 0.52 mg g^{-1} (Fig. 3.35 B). These results strongly supported the efficacy of blocking the gibberellin biosynthetic lateral route to boost the production of this class of compounds in *S. sclarea* hairy roots.

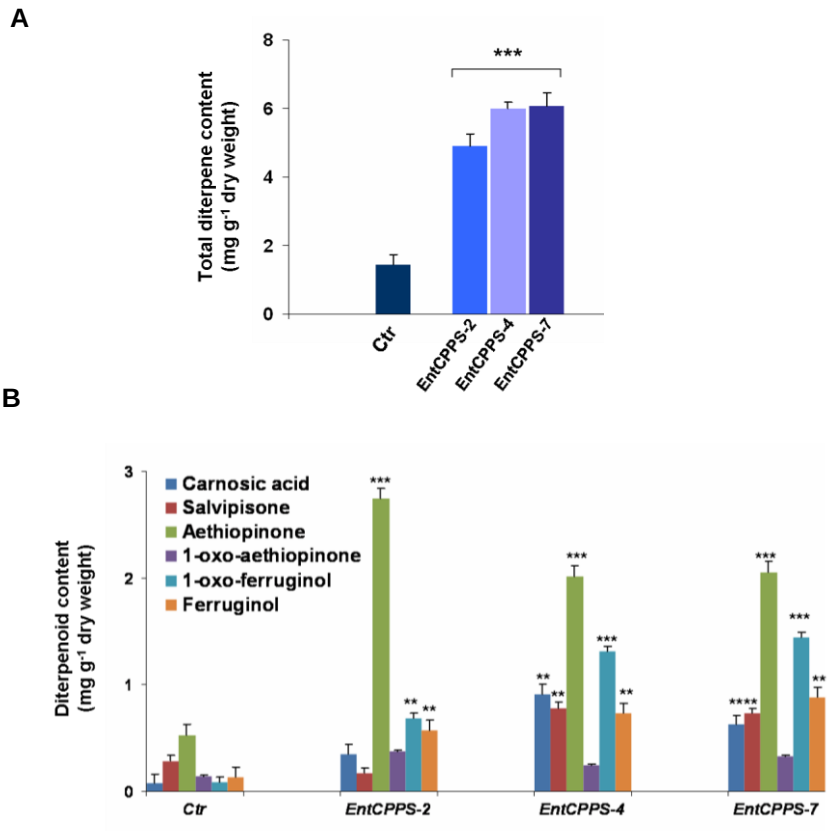


Figure 3.35- Total abietane diterpene content in *S. sclarea* hairy roots, analyzed by quantitative HPLC-DAD analysis (**A)** and content of the principal abietane diterpenes (**B**). The amount of each compound of silenced hairy roots was compared to the control line. Values are means ± SD of triplicate technical analyses from two biological replicates. *** P≤ 0.001; ** P≤0.01

Discussion

4. Discussion

4.1 Enhancing the synthesis of abietane diterpenes through coordinated activation of the MEP-derived metabolic pathway

Diterpenes of *S. sclarea* roots belong to the class of tri-cyclic diterpenes either of the abietane-phenolic type (e.g. carnosic acid and ferruginol) or abietane-quinone-type (aethiopinone, salvipisone and 1-oxoaethiopinone) (Ulubelen et al, 1994; Kuźma et al., 2005). It has been demonstrated that aethiopinone has a relatively high cytotoxicity against HL-60 and NALM-6 leukemia cells (Rozalski et al., 2006). In addition, we have recently shown that aethiopinone showed cytotoxic activity against human melanoma A357 cell line (IC_{50}=11.5 µM) at a concentration which was not toxic for not tumor cells. Aethiopinone treatment induced apoptosis in A375 cells with 37% and 50.5% cell death at 5 µM and 10 µM respectively (Vaccaro et al., 2014). Melanoma is a malignant tumor, which primarily involves the skin and is potentially the most dangerous form of skin tumor with a very high mortality (Garbe *et al*., 2010).The incidence of malignant melanoma is increasing faster than any other cancer, and successful systemic chemotherapy is rare (Rigel and Carucci, 2000). Preliminary results from docking analysis by using the web server *IdTarget* (http://idtarget.rcas.sinica.edu.tw/) identified a list of putative protein targets of aethiopinone and, among these, many proteins overexpressed in melanoma such as NEDD9, a melanoma metastasis gene (Kim et al., 2006), MMP9 and MMP13, matrix metalloproteases (MMPs), with the ability to degrade the extracellular matrix, playing an important role in tissue invasion by cutaneous malignant melanoma (Nagase et al., 1999). Despite their potential pharmacological relevance, abietane diterpenes are synthesized in the roots of *S. sclarea*, but also in other *Salvia spp* (e.g. *S. aethiopis* and others) at very low level (<0.5% dry weight), as already reported for other interesting plant bioactive compounds. Consistent amount of the purified compounds are needed to uncover the molecular mechanisms underlying the cytotoxicity that we found primarily in human melanoma cells and for further studies towards the development of an anti-proliferative agent to treat tumors, such as melanomas. To enhance the synthesis of this class of metabolites, we have developed complementary approaches of elicitation and metabolic engineering strategies in hairy roots of *S. sclarea*. (Vaccaro et al., 2010; Vaccaro et al., 2014). By overexpression of *AtDXS* and *AtDXR* genes, acting up-stream of the MEP-derived terpene pathway, we were able to enhance the content of abietane diterpens by 2-3 times (Vaccaro et al, 2014). Parallel elicitation experiments with MJ triggered a much more increase in the content of aethiopinone, in *S. sclarea* hairy roots, pointing at the possibility of a coordinated up-regulation of the biosynthetic genes of this pathway by regulatory proteins.

4.1.1 MJ-induced abietane diterpene production in *S. sclarea* hairy roots is due to coordinated up-regulation of biosynthetic genes of the MEP-dependent pathway

MJ is a potent regulator of genes involved in cell growth and biotic and abiotic stress responses (Santino, 2013). It is well known that jasmonate and its methyl-ester methyl-jasmonate (MJ) act as elicitors, boosting the synthesis of different secondary metabolites. Following synthesis, MJ is perceived by a receptor protein, and this activates a signal transduction pathway that

culminates in the transcriptional activation or repression of a large number of JA-responsive genes (Memelink, 2009). In the present study, we have demonstrated a significant increase of abietane diterpenes in MJ-elicited *S. sclarea* hairy roots, and, in particular, of aethiopinone, the most interesting compound having cytotoxic activity on different tumor cell line (Rozalsky et al., 2006; Vaccaro et al., 2014). We demonstrated that MJ-elicitation triggered the transcriptional activation of several biosynthetic genes of the plastidial MEP-dependent pathway. *S. sclarea* genes *SsDXS, SsDXR, SsCMK, SsMCS, SsHDS, SsHPR, SsGGPPS* and *SsCPPS* were rapidly upregulated in MJ-elicited hairy roots. The transcription of three genes were the most MJ-induced: *SsDXS*, the first committed enzyme of the MEP-derived pathway, reported to be limiting of terpene synthesis in many plant species (Harker and Bramley, 1999; Kuzuyama et al., 2000), *SsGGPPS*, a synthase producing GGPPS, the common precursor of many terpene of plastidial origin (Beck et al., 2013) and *SsCPPS*, the synthase controlling the first step of conversion of GGPP into copalyl-diphosphate (CPP), from which abietane diterpenes originate (Flory et al., 2000). Similar results have been reported in *Salvia milthiorriza*, in which MJ elicitation resulted in increased production of tanshinone (Ma et al., 2012) and upregulation of biosynthetic genes (Gao et al., 2014). MJ has been proved to induce simultaneously all biosynthetic genes in specific pathways of other plant species, as reported for the TIA (terpenoid-indole-alkaloid in *C. roseus (*van der Fits and Memelik., 2000), for nicotine in, *N. tabacum* (Shoji et al., 2010) and for artemisinin in *A. annua* (Maes et al., 2011). These data indicate that the coordinated overexpression of more genes is an efficient strategy to boost the production of secondary metabolites (Naqvi et al., 2010), as experimentally reported in different plant species. For example, the introduction of three genes encoding the last three reaction of the benzyl-glucosinolates, natural pesticide typical of Brassicacae, under the control of a single promoter CaMV35S, resulted in the synthesis of these metabolites usually not synthesized in tobacco plants (Moldrup et al., 2012). A consistent increase in artemisinin content has been obtained in transgenic *A. annua* plants, by introducing five biosynthetic genes under the control of different promoters contained in the same vectors (Zhang et al., 2011). Beside the “multi staking” strategy, the overexpression of transcription factors is a very promising approach to obtain the coordinate activation of more genes (Gantet and Memelink 2002; Broun et al., 2006; Grotewold et al., 2008, Jirschitzka et al., 2013). TFs are essential components of biosynthetic pathways as master regulatory proteins controlling the transcriptional cascade. Because transcription factors, differently to most structural genes, tend to control multiple pathway steps, they represent a powerful tool for the manipulation of complex metabolic pathways in plants. In *A. annua*, a consistent increase of artemisinin has been obtained by overexpressing the endogenous TF WRKY1 (Ma et al., 2009). Significant increase of ajmalicine production in *C. roseus* cells has been achieved by overexpression of an *Arabidopsis* TF, Agamous-like 12 (Montiel et al., 2007). Increased catharanthine accumulation has been detected in *C. roseus* hairy roots upon co-expression of the TF ORCA3 and the enzyme geraniol 10-hydroxylase (G10H) (Wang et al., 2010). As discussed above, multipoint metabolic engineering can supersede single-point engineering as the best way to manipulate metabolic flux in transgenic plants. Several points in a given metabolic pathway can be controlled simultaneously either by overexpressing and/or suppressing several enzymes or through the use of transcriptional regulators to control several endogenous genes. MJ perception triggers

extensive transcriptional reprogramming leading to the concerted activation of entire metabolic pathways. This observation inspired numerous quests for “master” regulators capable at enhancing the production of specific sets of valuable plant metabolites (Kazan and Manners, 2008). Many MJ-activated transcription factors were discovered in the last few years (De Geyter et al., 2012). However, so far, only few TFs regulating MEP-derived terpene pathway have been identified. On the basis of these considerations, the principal aim of this work was to identify putative transcription factors able to activate the MEP-pathway in order to push metabolic flux to the synthesis of abietane diterpenes in *S. sclarea* hairy roots.

4.1.2 Overexpression of AtWRKY and AtMYC2 TFs activates transcription of biosynthetic genes belonging to the MEP-derived pathway

As far as diterpenes, beside the well known biosynthetic pathway of gibberellins (Yagamuchi et al., 2008), the two most studied and advanced examples are the synthesis of the anti-tumoral diterpene taxol (Kirbly and Keasling, 2009), and of the diterpenes of the conifer resins (Keeling et al., 2010, 2011). Only in the last years, transcriptomic resources for *S. sclarea*, by RNA sequencing of flower calyx (Caniard et al., 2012), for *S. miltiorrizha* AFLP-cDNAs from MJ-treated hairy roots (Dongfeng et al., 2012) and RNA-sequencing (Cui et al., 2011; Wenping et al., 2011; Ma et al., 2012) are contributing to elucidate partially the biosynthetic pathway of abietane diterpenes in *Salvia* spp and providing availability of genes encoding diterpene synthases involved in the synthesis of these classes of diterpenoids. Moreover, to date, transcription factors regulating in a coordinated fashion the expression of genes involved in the isoprenoid biosynthesis have not been identified, except some evidence on the putative role of the plant WRKY transcription factors, for which it has been recently shown their function in regulating the terpenoid pathway (Yang et al., 2012) and *Arabidopsis* Myc2, a basic helix-loop-helix transcription factor, directly binding to promoters of the sesquiterpene synthase genes TPS21 and TPS11 to activate their expression (Hong et al., 2012). *S. sclarea*, as many medicinal plants, is very poorly characterized genetically. Although recently information on transcriptome of *S. sclarea* has been resealed (Legrand et al., 2010), no valuable information is available on the genome of *S. sclarea*. For this reason, it was decided to mine microarray data of *A. thaliana*, to identify putative transcription factors upregulated by MJ, which might be overexpressed in *S. sclarea* hairy roots in an attempt to boost the synthesis of abietane diterpenes. Published microarray data (Wang et al., 2008), showed the upregulation of AtWRKY18, AtWRKY40, and Myc2 in MJ elicited *A. thaliana* seedlings. In addition, AtWRKY60 was also selected considering its physical and functional interaction with AtWRKY18 and AtWRKY40 in plant defense responses (Xu et al., 2006). Myc2, a basic helix-loop-helix (bHLH) domain containing TF, acts as both activator and repressor of JA-responsive gene expression in *Arabidopsis*, preferentially binding G-box motifs (Dombrecht et al., 2007). WRKY TFs belong to a plant specific family and are characterized by a conserved WRKY domain that consists of the peptide motif WRKYGQK and a zinc finger (Yamasaki et al., 2005). Among other biological processes, they play dynamic roles in biotic and abiotic stress responses (Rushton et al., 2010). WRKY TFs function alone or in combination with other regulators to activate, repress or de-repress transcription (Agarwal et al., 2011). The choice of these TFs was further strengthened by a bioinformatic analysis revealing the presence of Methyl-jasmonate responsive

elements (MJRE) in their promoter regions. Interestingly, a MJRE site localized at -800 bp from the TSS, raised attention for its conserved position in all analyzed promoters. This site could be further studied, by site-specific mutagenesis, to shed light on the mechanism underlying the regulation of these TF by MJ. Our analyses, accordingly with results of Wang et al (2008), demonstrated that the TF WRKY40 was the most upregulated by MJ elicitation probably due to the presence in its promoter of two adjacent MJRE cis-acting elements, while, the expression level of WRKY60 was not changed. MEP-pathway biosynthetic genes were also upregulated in MJ-elicited *A. thaliana* seedlings, although the activation was less pronounced compared to *S. sclarea* hairy roots, probably because in *A. thaliana*, MJ trigger preferentially other pathways, such as the biosynthesis of glucosinolates, characteristic defense compounds of the order Brassicales (Brader et al., 2001). In addition, the analysis of the promoter region of *A. thaliana* MEP-dependent terpenoid pathway genes revealed the presence of W-box (*TTGAC*), binding sites of WRKY TFs, and G-box (*CACGT*), binding sites of Myc2, strongly supporting the involvement of these TFs in the transcriptional regulation of these genes. In Hua et al. (2011), the promoter of the *S. milthiorriza* gene *GGPPS,* encoding the enzyme catalyzing the condensation of Isopentenyl-pyrophosphate (IPP) and Dimetylallil-pyrophosphate (DMAPP) into GGPP, was isolated and characterized revealing the presence of W-box and G-box, thus suggesting a role of WRKY and Myc2 in the transcription of this gene, encoding an important branch point enzyme in terpenoid biosynthesis. On the basis of MJ-inducibility and these predictive analyses, the *A. thaliana* TF WRKY18, WRKY40, WRKY60 and Myc2 were used for their ectopic expression in *S. sclarea* hairy roots with the final aim of increasing the production of abietane diterpenes. A number of independent hairy root lines overexpressing each TF were obtained and characterized. Molecular analyses revealed that these heterologous genes, under the control of the strong viral 35S-CaMV constitutive promoter, were correctly transcribed and translated in *S. sclarea* hairy roots, although at variable level in the different lines, due to random non-homologous insertion of the transgene in the plant genome. This variability in the transgene expression in plants emphasizes the need to evaluate different independent transgenic lines to have a thorough analysis on the final metabolic effect of such overexpression. The overexpression of *WRKY18*, *WRKY40* and *Myc2* genes induced the upregulation of *S. sclarea* biosynthetic genes belonging to the plastidial MEP-derived pathway. The TFs WRKY18 and MYC2 positively regulated *S. sclarea* genes *DXS, DXR, HDS, GPPS* and *CPPS.* WRKY40 was able to activate preferentially the transcription of *DXS* and *CPPS* genes. By contrast, the overexpression of WRKY60 activated only slightly the transcription of MEP-pathway biosynthetic genes, consistently with previous expression studies showing unchanged transcription levels of WRKY60 upon elicitation with MJ. However, we can not completely exclude the involvement of WRKY60 in MEP-pathway regulation, since it could regulate other genes leading to the synthesis of abietane diterpenes not yet identified. In fact, genes encoding enzymes catalyzing the reactions downstream CPP (copalyl-disphoshate) in *S. sclarea* are still unknown. Since the over-expression of WRKY18 was the most effective in up-regulating transcriptional levels of MEP-pathway genes, EMSA assay were performed to verify a direct binding of this TF to the W-box in the promoter region of the genes *DXS* and *DXR*, encoding for the first enzymes of this metabolic way. However, a first set of EMSA experiments using oligonucleotides designed on a short promoter region containing the W-box of

the *AtDXS* and *AtDXR* did not reveal any interaction with the recombinant protein WRKY18. It has to point that the absence of interaction did not exclude a role of WRKY18 in the transcriptional activation of MEP-pathway genes. Transcriptional regulation is a very fine and sophisticated mechanism and it has to point that, the absence of interaction did not exclude a role of WRKY18 in the transcriptional activation of MEP-pathway genes. It is well known that members of WRKY family bind preferentially to W box but, although the W-box core is required, adjacent sequences also play a role in determining binding-site preference (Ciolkowski *et al.,* 2008). For this reason, a more thorough scanning of regulatory regions more distant from TSS is needed, since we focused only on the first 1000 bp upstream the TSS. Another important consideration is that the binding activity of the TF WRKY18 on gene promoters is significantly enhanced in the presence of WRKY40 and WRKY60 (Xu *et al.,* 2006). In addition, it has been demonstrated that WRKY18, WRKY40 and WRKY60 interact physically and functionally (Chen et al., 2010).Further studies are required to elucidate the functional role of the TF WRKY18 in transcriptional regulation of MEP-biosynthetic pathway, includinthe the possibility to co-express these three TFs in different combinations in trying to further optimize the synthesis of abietane diterpenes in *S. sclarea* hairy roots.

4.1.3 Using transcription factors to boost production of abietane diterpenes in *S. sclarea* hairy roots

Target metabolic profiling of all WRKY overexpressing hairy root lines revealed increased accumulation of abietane diterpenes compared to control line. However, there was no obvious correlation between expression levels of transgenes, transcriptional activation of MEP-pathway biosynthetic genes, growth rate and abietane diterpene accumulation. The overexpression of the WRKY40 TF was the most effective in triggering the accumulation of abietane diterpenes in *S. sclarea* hairy roots, especially in the production of the diterpenes salvipisone and aethiopinone. Salvipisone, like the aethiopinone, has been also proved to have cytotoxic activity against leukemia cells inducing apoptosis in a time- and concentration-dependent manner (Rozalsky et al., 2006). AtWRKY18 TF was the most efficient to transactivate MEP-pathway biosynthetic genes we have analyzed, but its overexpression was not associated to the highest accumulation of abietane diterpenes in *S. sclarea* hairy roots. This might be due to a major effect on the transactivation of genes of lateral competitive routes, as previously discussed, diverting precursors towards the synthesis of MEP-derived terpenes other than abietane diterpenes. Moreover, it has been demonstrated that AtWRKY18 is able to repress its own transcription when expressed at high levels in *A. thaliana* seedlings (Chen and Chen, 2002), and, therefore, the WRK18 protein level might be accurately tuned to avoid this. By contrast, the overexpression of WRKY60, that upregulates only slightly the expression levels of the MEP-pathway biosynthetic genes, induced a significant accumulation of abietane diterpenes in transgenic hairy roots, compared to control line. This could be probably due to a regulation by WRKY60 of other genes encoding enzymes of this pathway not yet identified, and not analyzed in this study, or also, to a possible concomitant negative regulation of genes of lateral competitive routes. High-throughput transcriptome approaches (microarray, RNA-seq) in *S. sclarea* TF overexpressing hairy roots might contribute to better understand the interplay among these WRKY TFs and to have a complete picture of genes activated and/or repressed by them. In

general, our results confirm that the overexpression of TFs is a successful strategy to enhance the production of valuable secondary metabolites in plants, as already reported for other plant species. The overexpression of the CrWRKY1 in hairy roots of *Catharantus roseus* resulted in increased expression levels of *CrDXS* and *CrSLS* genes, followed by a three fold-increase accumulation of the Indole-alcaloid serpentine (Suttipanta et al., 2011). In cell cultures of *Coptis japonica*, the overexpression of CjWRKY1induced increased levels of the alkaloid berberin through the activation of biosynthetic genes (Kato et al. 2007). The overexpression of ORCA3 in culture cells of *C. roseus* induced increased levels of TIA, precursor of the antitumoral compounds vinblastine and vincristine by upregulating many biosynthetic genes, including also *CrDXS* (Van derFits and Memelink 2000).

4.1.4 Trade-off between growth and accumulation of secondary metabolites

One caveat of the ectopic overexpression of TFs in plants is the often-reported negative pleiotropic effect on plant growth. Active growth and final biomass are important parameters for implementing industrial platform based on hairy roots as source of extraction of purified bioactive secondary metabolites. It has been reported that transgenic *A. thaliana* plants overexpressing WRKY18 or WRKY40 are significantly smaller in size (Chen and Chen, 2002; Xu et al., 2006), while WRKY60 overexpression did not exhibited different growth compared to wild type *A. thaliana* seedlings. The constitutive expression of Myc2 in *A. thaliana* plants resulted in an enhanced inhibition in growth, not only of the root but also of the aerial parts (Lorenzo et al., 2004). In fact, these TFs are active in plants during stress responses, usually resulting in growth retardation, as responding to stress can be costly and reduce fitness in terms of growth and yield (Herms and Mattson, 1992; Tian et al., 2003; Smith and Stitt, 2007; Bechtold et al., 2010). Moreover, the overexpression of TFs could impair the tight and coordinated regulation of signaling pathways in which TFs are the main players. It is known that the WRKY proteins control the complex mechanisms of transcriptional reprogramming via protein–protein interaction and auto-regulation or cross-regulation (Xu et al., 2006; Rushton et al., 2010, 2012). In this way, they may function as negative or positive regulators of cell signaling in different physiological processes (Eulgem et al., 2000; Eulgem and Somssich, 2007; Pandey and Somssich, 2009). Auto- and cross-repression of WRKY18, WRKY40, and WRKY60 suggest a sophisticated mechanism to maintain homeostasis of the WRKY transcription. In addition, it has been shown that WRKY18, WRKY40, and WRKY60 are negative regulators of ABA signaling (Chen et al., 2010; Shang et al., 2010), by inhibiting the expression of a set of ABA-responsive genes (Shang et al., 2010), necessary for plants to avoid growth arrest induced by expression of these ABA-responsive genes and thus keep up their vigorous development under environmental conditions favorable to growth. Moreover WRKY18, WRKY40, and WRKY60 proteins directly bind the W-box motifs in various regions of the promoters of all three WRKY genes showing auto- and cross-regulation. The homeostasis of such transcription factors in plant cells may be of particular importance to balance growth promotion and arrest of growth. In general, we also found that constitutive high expression level of the TFs was associated to growth inhibition of transgenic *S. sclarea* hairy roots. AtMYC2 was one of the most effective TFs in activating transcription of the biosynthetic genes of the MEP-derived

pathway, but it caused dramatic growth impairment in *AtMYC2* over-expressing hairy roots. This is not so surprisingly given the multiplicity of roles of this transcription factor, considered a “master player” in JA signal transduction. Myc2 coordinates JA-mediated defense responses, regulate crosstalk between the signaling pathways of JA and those of other phytohormones such as abscisic acid (ABA), salicylic acid (SA), gibberellins (GAs) and auxin (IAA); regulates interactions between JA signaling and light, phytochrome signaling and the circadian clock; is involved in JA-regulated plant development, lateral and adventitious root formation, flowering time and shade avoidance syndrome (Kazan and Manners, 2013). In addition, since MEP-derived precursors are used as building blocks for other isoprenoids involved in plant growth and development, such as phytohormones (cytokines, gibberellins or ABA) causing undesirable side-effects on overexpressing hairy root growth, or variation in chlorophyll and carotenoids, that, might interfere with other plastidial metabolic processes, resulting ultimately in a growth penalty. However, low amounts of TFs are generally sufficient to activate gene transcription and, in this study, it was evident that there was a trade-off between growth and accumulation of abietane diterpenes in low-expressing WRKY hairy root lines, such as WRKY40 line#10, WRKY18 line#6 and WRK60 line #3. These results point to the possibility of selecting the best performing over-expressing hairy root lines in which high final biomass is coupled to high content of abietane diterpenes. The characterization of these transcription factors will aid in finding activator proteins that regulate diterpenoid biosynthesis pathway. In turn, further elucidation of the regulatory machinery controlling diterpenoids biosynthetic genes will allow greater advancement of diterpenoids biotechnology.

4.2 Increasing the production of abietane diterpenes by blocking a competitive lateral pathway

Genetic engineering of a secondary metabolic pathway aims at either increasing or decreasing the quantity of a certain compound or group of compounds (Staniek et al., 2013). While the overexpression of metabolic enzymes provides a powerful tool in metabolic engineering, it is often as important to minimize flux through a pathway that results in an undesired product. To decrease the production of a certain unwanted (group of) compound(s) several approaches are possible. The production of a certain compound can be reduced by decreasing the flux towards that product by bring down the level of enzyme in the pathway, increasing its catabolism, and increasing flux into competitive pathways (Gómez-Galera et al., 2007). An enzymatic step in the pathway can be knocked out, for example, by reducing the level of the corresponding mRNA via anti-sense, co-suppression or RNA interference technologies, or by using chemical compounds that inhibit the enzymatic activity. In the present study, the block of a competitive metabolic pathway was used as strategy to enhance the synthesis of bioactive abietane diterpenes in *S. sclarea* hairy roots. In particular, a higher availability of GGPP (geranylgeranyl-diphosphate) was directed preferentially towards abietane diterpenes by silencing the gene encoding the ent-copalyl-diphosphate synthase (EntCPPS), the enzyme catalyzing the first committed step in the gibberellin biosynthetic pathway. GGPP is, in fact, the common substrate for both the enzyme EntCPPS and the enzyme CPPS, the first enzyme leading to the biosynthesis of abietane diterpenes. In order to make available the whole GGPP substrate for the enzyme CPPS and push the metabolic flux towards

abietane diterpene biosynthesis, the competitive lateral route, leading to the biosynthesis of gibberellins, was blocked by using the chlorocholine chloride (CCC), a chemical compound able to inhibit the catalytic activity of the EntCPPS (Lone et al., 2010) or by using double stranded RNA targeting the *EntCPPS* mRNA (RNAi). CCC is a quaternary ammonium compound type, commonly used in agriculture as plant growth retardant. It has been reported that the treatment with CCC increased metabolite content by inhibiting gibberellin biosynthesis also in other plant species. In hydroponically grown taxus plants treated with 100 µM CCC, roots showed increased growth by 22%, and exhibited high amount of taxol, an anti tumor diterpene, compared to control plants (Enaksha et al, 1996). Increased levels of the alkaloid serpentine were obtained by treating *C. roseus* cell cultures with CCC and other growth retardants, without adverse effects on biomass accumulation (El-Sayed and Veerporte, 2007). Treatment of potato (*Solanum tuberosum* L.) with CCC, applied twice as a foliar spray 25 and 30 days after planting, significantly increased tuber dry mass and increased contents of chlorophyll and carotenoids in leaves (Wang et al., 2009). CCC also stimulated the production of total phenols and flavonoids in calli and leaves in *in vitro* grown *Stevia rebaudiana* plants (Dey et al., 2013). The ginkgo seedlings, grown in the greenhouse conditions with foliar applications of CCC, showed significantly increased contents of total polyphenols, flavonoids, anthocyanins, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI) activities, suggesting that the foliar treatment with CCC might be a useful means for improving pharmacological properties of *G. biloba* leaves (Zhang et al., 2013). The total abietane diterpene content of CCC-treated *S. sclarea* hairy roots was significantly enhanced, about 3.5 times higher than the content of control hairy roots and the most relevant increase was achieved for the content of aethiopinone (7-fold increase).The growth rate of CCC-treated roots resulted even improved, making the CCC treatment an advantageous strategy for the purpose of a large scale production of abietane diterpenes from *S. sclarea* hairy roots. GAs are phytohormones that regulate a wide range of developmental processes, including seed germination, leaf expansion, stem elongation, flowering, and fruit and seed development (Sun and Gubler, 2004; Swain and Singh, 2005). However, GA's role in root development is poorly understood although several studies have suggested a role for GAs in primary root elongation. GA appears to affect cell expansion in the root elongation zone via destabilizing DELLA proteins like Repressor of GA1 (RGA) (Fu and Harberd, 2003). GA1 (other name of EntCPPS) was found to play a role in endodermal cell expansion in the elongation zone of *A. thaliana* primary roots (Ubeda-Tomas et al., 2008). Recent evidence suggests that GAs also play an important role in lateral root development. Transgenic poplar plants exhibited increased lateral root proliferation and elongation under *in vitro* and greenhouse conditions, and these effects were reversed by exogenous GA treatment (Gou et al., 2010). In the light of these findings, it is possible to explain the increased biomass production of *S. sclarea* hairy roots treated with CCC. The positive and promising results obtained by treating *S. sclarea* hairy roots with the CCC encouraged the design of a RNAi strategy to silence the expression of the gene *EntCPPS.* A fragment of the coding sequence of this gene, obtained by RT-PCR using degenerate primers, was then extended at 3' by RACE-PCR. A fragment of the isolated sequence was cloned into pHELLSGATE12 vector, which has been proved to be very efficient for silencing plant genes (Heliwell and Waterhouse 2003), by producing a hairpin RNA (hpRNA), consisting of an

inverted repeat of a gene fragment separated by an intron, targeting the endogenous gene. The proportion of transgenic plants showing silencing and the degree of silencing with these constructs are much greater than that obtained using either co-suppression or antisense constructs (Wesley et al., 2001). By transforming *S. sclarea* hairy roots with the pHELLSGATE12-*EntCPPS* construct, several independent transgenic lines, showing differing degrees of silencing, were obtained. Among these, three root lines, showing the 60%, 75% and 90% of gene silencing, respectively, were further analyzed for the content of abietane diterpenes. Similarly to what obtained with CCC treatment, the growth of silenced roots was not impaired and the total abietane diterpene content significantly increased up to five-times compared to the amount in control roots, with a major effects on the content of aethiopinone (up to 5-fold increase). These analysis also revealed that there is a correlation between metabolite accumulation and the degree of *EntCPPS* silencing. In fact, the silenced hairy root line #7, characterized by a 90% of *EntCPPS* down-regulation, showed the highest content of abietane diterpenes (4.5 fold increase) and any significant growth retard compared to the control line. By contrast the line #2, showing the 60% of gene reduction, exhibited the lowest content of abietane diterpenes (3.5-fold increase). This means that a major accumulation of abietane diterpenes is associated to a stronger *EntCPPS* silencing, with the final result to have a higher availability of the GGPP substrate for the synthesis of abietane diterpenes. RNAi technology is emerged as an efficient metabolic engineering tool to improve pharmacological, but also nutritional properties of several plant species. An example is given by *Papaver somniferum*, that remains the sole source of morphine. The commercial chemical synthesis of morphine, codeine, and other benzylisoquinoline alkaloids is not economically feasible due to the complexity of the molecule and multiple chiral centers (McCoy et al., 2008). The enzyme salutaridinol 7- *O* –acetyltransferase (SaIAT) that catalyzes the transfer of the acetyl group from acetyl coenzyme A to the 7-OH group of salutaridinol yielding salutaridinol-7-O-acetate, which is an intermediate in morphine biosynthesis. Morphine, codeine, and thebaine levels were increased in SaIAT RNAi plants (Allen et al., 2008). RNAi-mediated silencing on δ-cadinene synthase in cotton (*Gossypium hirsutum* L.) triggered a significant and selective reduction of gossypol content from cottonseed (Sunikumar et al., 2006). The δ–cadinene synthase is a key enzyme in the biosynthesis of gossypol and other sesquiterpenoids. Gossypol is a cardiotoxic and hepatotoxic terpenoid, unsafe for human and animal consumption, thus avoiding the use of cottonseed as a source of proteins and calories, after fiber extraction. In this way, by reducing the gossypol content, cotton could become a nutritionally important crop, not only in developed countries but also in many developing countries where malnutrition and starvation are widespread and it is mainly used for fiber production. Our results in *S. sclarea* hairy roots demonstrated that the block of the competitive lateral route for gibberellin synthesis by the chemical compound CCC or by RNAi were valid strategy to boost the synthesis of abietane ditepenes. Both strategies proved to be successful in the terms of biomass production and of abietane diterpene accumulation and offer many advantages. The CCC is inexpensive and water soluble but, of course, hairy roots need to be treated for one month to accumulate metabolites, while, on the other hand, RNAi silenced hairy root lines, once obtained, represent a constant and stable source from which to extract bioactive diterpenes. In addition, the RNAi approach allows also to obtain a greater production of abietane diterpenes by selecting transgenic lines

with the highest degree of gene silencing and the best growth. Moreover, another ongoing strategy in the *Planta*LAB, is the combined use of CCC or silencing of *EntCPPS* gene in hairy root lines co-overexpressing *GGPPS* and *CPPS* genes. Preliminary results have shown that co-expression of these two genes enhanced the content of abietane diterpenes more than what we have shown for overxpression of WRKY TFs (Vaccaro, personal communication). In fact, the most impressive pathway modification are expected when gain and loss-of-function approaches are combined to increase flux through one while decreasing flux through a competing pathway.

Conclusions and perspectives

5. Conclusions and perspectives

In the present study, a molecular toolbox was successfully developed to engineer the metabolic flux in *Salvia sclarea* hairy roots towards an enhanced accumulation of tryciclic abietane diterpenes.
The main results achieved are here summarized:

- identification of *A. thaliana At*WRK18, AtWRK40, and AtMYC2 TFs, containing MJRE boxes in their promoter and whose transcription is strongly inducible by MJ;
- overexpression of these heterologous TFs in *S. sclarea* hairy roots positively regulating the transcription of several genes of the terpenoid MEP-pathway (especially *Ss*DXS and *Ss*DXR, acting in the up-stream reactions of the MEP-dependent terpene pathway, *Ss*GGPPS, a synthase yielding the common precursor of most of the plastidial terpenes, included also diterpenes, and *Ss*CPPS, the first committed enzyme for abietane diterpene biosynthesis;
- transcriptional up-regulation of this set of genes highly correlated with an enhanced content (3-5 times higher than control hairy roots) of tryciclic abietane diterpenes, especially salvipisone and aethiopinone, which we have previously demonstrated to be cytotoxic against human melanoma tumor cells;
- selection of high performing AtWRKY overexpressing hairy roots lines, with a trade off between high final biomass and increased abietane diterpene content;
- identification and RNAi-mediated silencing of the *SsEntCCPS* gene in *S. sclarea* hairy roots, encoding the first enzyme acting at the lateral competing route from GGPP to gibberellins, which enhanced significantly (>4-fold) the total abietane diterpenes content, without causing any growth impairment compared to control hairy roots.

Overall, these complementary approaches were successful in increasing the content of aethiopinone and other tricyclic abietane diterpenes (from a 3-fold up to a 5-fold increase compared to the content in the control line) in engineered *S. sclarea* hairy roots and might be applied to other plant species synthesizing different bioactive specialized terpenes.
Moreover, we are currently investigating the possibility to enhance further the accumulation of abietane diterpenes in *S. sclarea* hairy roots by combining TF overepression and RNAi gene silencing/chemical inhibition of the EntCPPS to drive the metabolic flux toward higher level of this class of compounds.Another possibility to boost further the synthesis of these compounds under investigation is the co-expression of the different AtWRKY TFs, since, as we have discussed, they act in combination.
Finally, the promising results presented in this study pave the way to a rational design of a hairy root-based production platform to yield reliable amounts of tricyclic abietane diterpenes towards a deeper understanding of their molecular targets and the potential future exploitation as novel plant-derived anti-tumor molecules.

References

6. References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki, T, Hosokawa D, and Shinozaki K. **1997**. Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell*. **9**: 1859-868.

Agarwal P, Reddy MP, Chikara J. **2011**. WRKY: its structure, evolutionary relationship, DNA-binding selectivity, role in stress tolerance and development of plants. *Mol. Biol. Rep*. **38:** 3883-896.

Aiyelaagbe OO, Adesogan K, Ekundayo O, Gloer JB: **2007**. Antibacterial diterpenoids from *Jatropha podagrica* Hook. *Phytochemistry* **68**: 2420-425.

Ajikumar PK, Tyo K, Carlsen S, Mucha O, PhonTH, Stephanopoulous G. **2008**. Biosynthesis of Natural product drugs using engineered microorganisms. *Mol Pharm* **5:** 167-190.

Akagi T, Ikegami A, Yonemori K: **2010**. DkMyb2 wound-induced transcription factor of persimmon (Diospyros kaki Thunb.), contributes to proanthocyanidin regulation. *Planta*, **232**:1045-1059.

Allen RS, Miller JAC, Chitty JA, Fist AJ, Gerlach WL and Larkin PJ. **2008**. Metabolic engineering of morphinan alkaloids by over-expression and RNAi suppression of salutaridinol 7-O-acetyltransferase in opium poppy. *Plant Biotechnol*. *J*. **6**: 2-6.

Bailey, J.E. Toward a science of metabolic engineering. **1991**. *Science*, **252**: 1668-675.

Baulcombe D. **2004**. RNA silencing in plants. *Nature* **431**: 356-363.

Bechtold U, Lawson T, Mejia-Carranza J, Meyer RC, Brown IR, Altmann T, Ton J, Mullineaux PM. **2010**. Constitutive salicylic acid defences do not compromise seed yield, drought tolerance and water productivity in the *Arabidopsis* accession C24. *Plant, Cell and Environment.* **33**:1959-1973.

Beck G, Coman D, Herren E, Ruiz-Sola MA, Rodríguez-Concepción M, Gruissem W, Vranová E. **2013**. Characterization of the GGPP synthase gene family in *Arabidopsis thaliana. Plant Mol Biol.* **82**: 393-416.

Bensaddek L, Villarreal ML, Fliniaux MA. **2008**. Induction and growth of hairy roots for the production of medicinal compounds. *J Integ Biosc* **3**: 2-9.

Benrezzouk R, Terencio MC, Ferrandiz ML, Hernandez-Perez M. Rabanl R. Alcaraz MJ. **2001**. Inhibition of 5-lipoxygenase activity by the natural antiinflammatory compound aethiopinone. *Inflamm Res* **50**: 96-101.

Berger S, Bell E, and Mullet JE. **1996**. Two methyl jasmonateinsensitive mutants show altered expression of AtVSP in response to methyl jasmonate and wounding. *Plant Physiol*. **111**: 525–531.

Berova, M., and Zlatev, Z. **2000**. Physiological response and yield of paclobutrazol treated tomato plants (Lycopersicon esculentum Mill.). *Plant Growth Regul*. **30**: 117–123.

Bonito MC, Cicala C, Marcotullio MC, Maione F, Mascolo N. 2011. Biological activity of bicyclic and tricyclic diterpenoids from *Salvia* species of immediate pharmacological and pharmaceutical interest. *Nat Prod Commun* **6**: 1205–1215.

Bourque JE. **1995**. Antisense strategies for genetic manipulations in plants. *Plant Science*, **105**: 125–149.

Bouvier F, Rahier A, Camara B. **2005**. Biogenesis, molecular regulation and function of plant isoprenoids. *Prog Lip Res* **44**: 357-429.

Brader G, Tas E , and Tapio-Palva E. **2001**. Jasmonate-Dependent Induction of Indole Glucosinolates in Arabidopsis by Culture Filtrates of the Nonspecific Pathogen *Erwinia carotovora*. *Plant Physiology*. **126**: 849-860.

Broun P, Liu Y, Queen E, Schwarz Y, Abenes ML, Leibman M. **2006**. Importance of transcription factors in the regulation of plant secondary metabolism and their relevance to the control of the terpenoid accumulation. *Phytochemistry Review* **5**: 27-38.

Busov, V., Meilan, R., Pearce, D.W., Rood, S.B., Ma, C., Tschaplinski, T.J., and Strauss, S.H. **2006**. Transgenic modification of gai or rgl1 causes dwarfing and alters gibberellins, root growth, and metabolite profiles in Populus. *Planta*. **224**: 288–299.

Canter PH, Thomas H, Ernst E. **2005**. Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends Biotechnol* **23**: 180-185.

Caniard A, Zerbe P, Legrand S, Cohade A, Valot N, Magnard JL, Bohlmann J, Legendre L. **2012**. Discovery and functional characterization of two diterpene synthase for sclareol biosynthesis in *Salvia sclarea* (L.) and their relevance for perfume manufacture. *BMC Plant Biol* **12**: 119-132.

Champa P. Wijekoon and Peter J. Facchini. **2012**. Systematic knockdown of morphine pathway enzymes in opium poppy using virus-induced gene silencing. *The Plant Journal* **69**: 1052–1063.

Chandra S. **2012**. Natural plant genetic engineer *Agrobacterium rhizogenes:* role of T-DNA in plant secondary metabolism. *Biotech Lett* **34**: 407-415.

Chen C, Chen Z. **2002**. Potentiation of developmentally regulated plant defense response by atwrky18, a pathogen-induced arabidopsis transcription factor. *Plant Physiol* **129**: 706-716.

Chen H, Lai Z, Shi J, Xiao Y, Chen Z, and Xu X. **2010**. Roles of *Arabidopsis* WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. *BMC Plant Biol* . **10**:281.

Chen YY, Wang LF, Dai LJ, Yang SG, Tian WM. **2012**. Characterization of HbEREBP1, a wound-responsive transcription factor gene in laticifers of Hevea brasiliensis Muell. *Arg. Mol. Biol. Rep*. **39**: 3713–3719.

Choudhary MI, Hussain A, Ali Z, Adhikari A, Sattar SA, Ayatollahi SAM, Al-Majid AMA. **2012**. Atta-ur-Rahman: Diterpenoids including a novel dimeric conjugate from *Salvia lieriaefolia*. *Planta Medica* **78**: 269–275.

Ciolkowski I, Wanke D, Birkenbihl RP, and Somssich IE. **2008**. Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. *Plant Mol. Biol.* **68**: 81–92.

Clarkson C, Musonda CC, Chibale K, Campbell WE, Smith P. **2003**. Synthesis of totarol amino alcohol derivatives and their antiplasmodial activity and cytotoxicity. *Bioorg Med Chem* **11**: 4417-22.

Cohen JM, Singh I, O'Brien ME. **2008**. Predicting Global Fund grant disbursements for procurement of artemisinin-based combination therapies. *Malar J* **7:** 200-215.

Cui G, Huang L, Tang X, Zhao J. **2011**. Candidate genes involved in tanshinone biosynthesis in hairy roots of Salvia miltiorrhiza revealed by cDNA microarray. *Mol Biol Rep* **38**: 2471–2478.

Dal Cin V, Tieman DM, Tohge T, McQuinn R, de Vos RCH, Osorio S, Schmelz EA, Taylor MG, Smits-Kroon MT, Schuurink RC et al. **2011**: Identification of genes in the phenylalanine metabolic pathway by ectopic expression of a MYB transcription factor in tomato fruit. *Plant Cell*, **23**:2738-2753.

De Boer K, Tilleman K, Pawels L, Bossche RV, De Sutter V, Hilson P, Hamill JD and Goosens A. **2011** APETALA2/ETHYLENE RESPONSE FACTOR and basic helix–loop–helix tobacco transcription factors cooperatively mediate jasmonate-elicited nicotine biosynthesis. *Plant J*. **66**, 1053–1065.

De Geyter N, Gholami A, Goormachtig S and Goossens A. **2012**. Transcriptional machineries in jasmonate-elicited plant secondary metabolism. *Trends in Plant Science*. **17**: 349-359

Devappa RK, Makkar HPS, Becker K. **2010**. *Jatropha* diterpenes: a review. *J Am Oil Chem* **88**:301–322.

Dey A, Paul S, Kundu S, Bandyopadhyay A, Bhattacharjee A. **2013**. Elevated antioxidant potential of chlorocholine chloride-treated in vitro grown Stevia rebaudiana Bertoni. *Acta physiologiae plantarum* **35**: 1775-1783.

Dong J, Chen C and Chen Z. **2003**. Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. *Plant Mol. Biol.* **51**, 21–37.
Dongfeng Y, Ma P, Liang X, Liang Z, Zhang M, et al. **2012**. Metabolic Profiles and cDNA-AFLP Analysis of *Salvia miltiorrhiza* and *Salvia castanea* Diel f. *tomentosa. Stib. Plos One* **7**: 378-385.

Doyle, JJ, Doyle JL. **1990**. A rapid total DNA preparation procedure for fresh plant tissue. *Focus* **12**: 13-15.

Dombrecht, B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JA, Reid JB, Fitt GP, Sewelam, Schenk PM, Manners JM, Kazan K. **2007**. MYC2 differentially modulates diverse jasmonate-dependent functions in Arabidopsis. *Plant Cell* **19**: 2225-2245.

Dubos, C. **2010**. MYB transcription factors in Arabidopsis. *Trends Plant Sci*. **15**: 573–581.

El-Sayed M and Veerporte R. **2007**. Influence of growth retardants on serpentine accumulation in *Catharanthus roseus* cell suspension cultures. *American journal of Plant Phyiology*. **2**: 373-377.

Enaksha RM, Wickremesinhe R, Arteca N. **1996**. Effects of plant growth regulators applied to the roots of hydroponically grown *Taxus x media* plants on the production of taxol and related taxanes. *Plant science*, **121**: 29-38.

Erkan N, Ayranci G, Ayranci E. **2008**. Antioxidant activities of rosemary (*Rosmarinus Officinalis L.)* extract blackseed (*Nigella sativa L.)* essential oil carnosic acid rosmarinic acid and sesamol. *Food Chem* **110**: 76-82.

Eulgem T, Rushton PJ, Robatzek S, Somssich IE. **2000**. The WRKY superfamily of plant transcription factors. *Trends Plant Sci*. **5**:199-206.

Eulgem T, Somssich IE. **2007**. Networks of WRKY transcription factors in defense signaling. *Current Opinion in Plant Biol.* **10**(4):366-71.

Fleet, C.M., and Sun, T.P. **2005**. A DELLAcate balance: The role of gibberellin in plant morphogenesis. *Curr. Opin. Plant Biol*. **8**: 77–85.

Flory RB, Jetter JE, Ravn R, Lee MM, Coates HJ, Croteau RB. **2000**. Abietadiene synthase from grand fir (*Abies grandis*): characterization and mechanism of action of the "pseudomature" recombinant enzyme. *Biochemistry.* **39**: 15592–602.

Forkmann G, Martens S. **2001**. Metabolic engineering and applications of flavonoids. *Curr. Opin.Biotechnol*., **12**: 155–160.

Fronza M, Lamy E, Günther S, Heinzmann B, Laufer Stefan, Merfort Irmgard. **2012**. Abietane diterpenes induce cytotoxic effects in human pancreatic cancer cell line MIA PaCa-2 through different modes of action. *Phytochemistry* **78**: 107-119.

Fu ZQ and Dong X. **2013**. Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* **64**, 839–863.

Fu X, and Harberd NP. **2003**. Auxin promotes Arabidopsis root growth by modulating gibberellin response. *Nature* **421**: 740–743.

Gantet P, Memelink J. **2002**. Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. *Trends in Pharmacological Sciences* **12**: 563-569.

Gao W**,** Sun HX, Xiao H, Cui G, Hillwig ML, Jackson A**,** Wang X**,** Shen Y**,** Zhao N**,** Zhang L**,** Wang XJ, Peters RJ and Huang L. **2014**. Combining metabolomics and transcriptomics to characterize tanshinone biosynthesis in *Salvia miltiorrhiza. BMC Genomics.* **15**:73.

Garbe C, Peris K, Hauschild A, Saiag P, Middleton M, Spatz A, Grob JJ, Malvehy J, Newton-Bishop J, Stratigos A, Pehamberger H, Eggermont A. **2010**. Diagnosis and treatment of melanoma: European consensus-based interdisciplinary guideline. *J Eur Cancer* **46**: 270-283.

Gavilano LB, Coleman NP, Burnley LE, Bowman ML, Kalengamaliro NE, Hayes A, Bush L, Siminszky B. **2006**. Genetic engineering of Nicotiana tabacum for reduced nornicotine content. *J Agric Food Chem*. **54**: 1-8.

.Georgiev M, Weber J, Maciuk A. **2009**. Bioprocessing of plant cell cultures for mass production of targeted compounds. *Appl Microbiol Biotechnol* **83**: 809-823.

Georgiev MI. **2011**. Contemporary approaches towards production of phytopharmaceuticals: plant biotechnology meets medicinal chemistry. *Mini Rev Med Chem* **1**: 822-826.

Georgiev M, Agostini E, Ludwig-Muller J, Xu J. **2012**. Genetically transformed roots: from plant disease to biotechnological re source. *Trends in Biotech* **30**: 528-537.

Goff SA; Klein TM, Roth BA, Fromm ME, Cone KC, Radicella JP, Chandler VL. **1990**. Transactivation of anthocyanin biosynthetic genes following transfer of B regulatory genes into maize tissues. *EMBO J*. **9**: 2517-2522.

Gómez Galera S, Pelacho AM, Gené A, Capell T, Christou P. **2007**. The genetic manipulation of medicinal and aromatic plants. *Plant Cell Rep* **26:**1689-1715.

Gou J, Strauss SH, Tsai CJ, Fang K, Chen Y, Jiang X, Busov VB. **2010**. Gibberellins regulate lateral root formation in Populus through interactions with auxin and other hormones. Plant Cell **22**: 623-39.

Grotewold E., **2008**. Transcription factors for predictive plant metabolic engineering: are we there yet? *Current Opinion in Biotechnology* **19**: 138-144.

Haas JH, Moore LW, Ream W, Manulis S. **1995**. Universal PCR primers for detection of phytopathogenic Agrobacterium strains. *Appl Environ Microbiol* **61**:2879–2884.

Hahlbrock K, Bednarek P, Ciolkowski I, Hamberger B, Heise A, Liedgens H, Logemann E, Nürnberger T, Schmelzer E, Somssich IE, Tan J. **2003**. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *Proc Natl Acad Sci U S A,* **100**: 14569– 14576.

Harker M, Bramley PM. **1999**. Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Lett.* **448**:115-119.

Hanson JR. **2009**. Diterpenoids. *Nat Prod Rep* **26**:1156-1171.

Herms DA, Mattson WJ. **1992**. The dilemma of plants—to grow or defend. *Quarterly Review of Biology*. **67**: 283-335.

Hernández-Pérez M, Rabanal RM, de la Torre MC, Rodríguez B. **1995**. Analgesic, anti-inflammatory, antipyretic and haematological effects of aethiopinone, an o-naphthoquinone diterpenoid from *Salvia aethiopis* roots and two hemisynthetic derivatives. *Planta Medica* **61**: 505-509.

Hiratsu, K.; Matsui, K.; Koyama, T.; Ohme-Takagi, M. **2003**.Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *Plant J.* **34**: 733–739.

Hong GL, Xue XY, Mao YB, Wang LJ, Chen XY. **2012**. *Arabidopsis* MYC_2 Interacts with DELLA Proteins in regulating sesquiterpenes synthase gene expression. *Plant Cell* **24**: 2635-2648.

Hu Z, Du M. **2006**. Hairy Root and its application in plant genetic engineering. *J Int Plant Biol* **48**: 121-127.
Hua W, Song J, Li C, Wang Z. **2011**. Molecular cloning and characterization of the promoter of SmGGPPs and its expression pattern in *Salvia miltiorrhiza. Mol Biol Rep*. **39**(5):5775-83.

Hussain S, Fareed S, Ansari S, Rahman A, Ahamad IZ, Saeed M. **2012**. Current approaches toward production of secondary plant metabolites. *J Pharm Bioallied Sci* **4**: 10-20.

Irani, N.G.; Hernandez, J.M.; Grotewold, E. **2003**. Regulation of anthocyanin pigmentation. *Recent Adv. Phytochem*., **38**: 59–78.

Jantz, D.; Amann, B.T.; Gatto, G.J. Jr.; Berg,J.M. **2004**. The design of functional DNA-binding proteins based on zinc finger domains. *Chem.Rev*., **104**: 789–799.

Jirschitzka J, Mattern DJ, Gershenzon J, D'Auria JC. **2013**. Learning from nature: new approaches to the metabolic engineering of plant defense pathways. *Curr Opin Biotechnol*. **24**: 320-8.

Kar S, Palit S, Basy Ball W, Das P. **2012**. Carnosic acid modulates AkT/IKK/NFkB signaling by PP2A and induces intrinsic and extrinsic pathway mediated apoptosis in human prostate carcinoma PC-3 cells. *Apoptosis* **17**: 735-747.

Kato N, Dubouzet E, Kokabu Y and Yoshida S, Taniguchi Y, Dubouzet JG, Yazaki K, Sato F. **2007**. Identification of a WRKY Protein as a Transcriptional Regulator of Benzylisoquinoline Alkaloid Biosynthesis in *Coptis japonica*. *Plant and Cell Physiology*. **48**: 8-18.
Kazan K and Manners JM. **2013**. MYC2: The master in action. *Molecular plant*. **6**: 686-703

Keeling CI, Dullat HK, Yuen M, Ralph SG, Jancsik S, Bohlmann J. **2010**. Identification and functional characterization of monofunctional entcopalyl diphosphate and ent-kaurene synthases in white spruce reveal different patterns for diterpene synthase evolution for primary and secondary metabolism in gymnosperms. *Plant Physiol* **152**: 1197–1208.

Keeling CI, Weisshaar S, Ralph SG, Jancsik S, Hamberger B, Dullat HK, Bohlmann J. **2011**. Transcriptome mining, functional characterization, and phylogeny of a large terpene synthase gene family in spruce (Picea spp.) *Plant Biol* **11**: 43-58.

Kim M, Gans JD, Nogueira C, Wang A, Paik JH, Feng B, Brennan C, Hahn WC, Cordon-Cardo C, Wagner, SN, Flotte TJ, Duncan LM, Granter SR, Chin L. **2006**. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell.* **30**: 1269-81.

Kirby J, Keasling J. **2009**. Biosynthesis of Plant Isoprenoids: Perspectives for Microbial Engineering. *Annual Rev Plant Biol* **60**: 335-355.

Kuźma Ł, Skrzypek Z, Wysokińska H. **2005**. Diterpenoids and triterpenoids in hairy roots of *Salvia sclarea*. *Plant Cell Tiss Org Cult* **84**: 100152–100160.

Kuzma L, Rozalski M, Walencka E, Rozalska B, Wysokinska H. **2007**. Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L: salvipisone as a potential antibiofilm agent active against antibiotic resistant staphylococci. *Phytomed* **14**: 31-35.

Kuzuyama T, Takagi M, Takahashi S, and Seto H. **2000**. Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from *Streptomyces* sp. strain CL190, which uses both the mavalonate and nonmevalonate pathways for isopentenyl dophosphate biosynthesis. *J Bacteriol* **182**: 891-897.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG**. 2007**. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Laurie-Berry N, Joardar V, Street IH, and Kunkel BN. **2006**. The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid–dependent defenses during infection by *Pseudomonas syringae. Mol. Plant Microbe Interact*. **19**: 789–800.

Legrand S, Valot N, Nicolé F, Moja S, Baudino S, Jullien F, Magnard JL, Caissard JC, Legendre L., **2009**. One-step identification of conserved miRNAs, their targets, potential transcription factors and

effector genes of complete secondary metabolism pathways after 454 pyrosequencing of calyx cDNAs from the Labiate *Salvia scl*area L. *Gene*.**15**: 55-62.

Leone A, Grillo S, Monti L, Cardi T. **2007**. Molecular tailoring and boosting of bioactive secondary metabolites in medicinal plant. *Improvement of crop plants for industrial end uses.* Ranalli, P (Ed) **IX**: 471-507.

Li L., Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL. **2003**. Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proc. Natl. Acad. Sci. USA*, **100**: 4939–4944.

Li S, Zhang P, Zhang M, Fu C, Yu L. **2013**. Functional analysis of a WRKY transcription factor involved in transcriptional activation of the DBAT gene in Taxus chinensis. *Plant Biol*. **15**: 19–26.

Liao ZH, Chen M, Gong YF, Miao ZQ, Sun XF, Tang K. **2006**. Isoprenoid Biosynthesis in Plants: Pathways, Genes, Regulation and Metabolic Engineering. *J Bio Sci* **6**: 209-219.

Liu Y, Wang H, Ye H-C. **2005**. Advances in the plant isoprenoid biosynthesis Pathway and its metabolic engineering. *J Int Plant Biol* **47**:769-782.

Liu ZQ, Yan L, Wu Z, Mei C, Lu K, Yu YT. **2012**. Cooperation of three WRKY-domain transcription factors WRKY18, WRKY40, and WRKY60 in repressing two ABA-responsive genes ABI4 and ABI5 in Arabidopsis.*J. Exp. Bot.* **63**: 6371–6392.

Lone, E. **2010.** Effect of chlorocholine chloride (CCC) on plant growth and development. *International Journal of Current Research* **6**: 001-007.

López-Jimenez A, García-Caballero M, Medina MA, Quesada A. **2011**. Anti-angiogenic properties of carnosol and carnosic acid, two major dietary compounds from rosemary. *Eu J Nutrit* **10**: 1432-1440.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. **2004**. JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* **16**: 1938–1950.

Lorenzo, O., and Solano, R. **2005**. Molecular players regulating the jasmonate signalling network. *Curr. Opin. Plant Biol*. **8**: 532–540.

Lu X, Zhang L, Zhang F, Jiang W, Shen Q, Zhang L, Lv Z, Wang G, Tang K. **2013**. AaORA, a trichome-specific AP2/ERF transcription factor of Artemisia annua, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to Botrytis cinerea. *New Phytologist* **198**:1191-1202.

Ma D, Pu G, Lei C, Ma L, Wang H, Guo Y, Chen J, Du Z, Wang H, Li G, Ye H, Liu B. **2009**. Isolation and characterization of AaWRKY1, an *Artemisia annua* transcription factor that regulates the amorpha-4,11-diene synthase gene, a key gene of artemisinin biosynthesis. *Plant Cell Physiol.* **50**(12): 2146-61.

Ma Y, Lchaj Y, Wu B,Li X, Chen S, Lu S. **2012**. Genome-wide identification and characterization of novel genes involved in terpenoid biosynthesis in *Salvia miltiorrhiza. J Exp Bot* **63**: 2809-2823.

Maeo K, Hayashi S, Kojima-Suzuki H, Morikami A, and Nakamura K. **2001**. Role of conserved residues of the WRKY domain in the DNA-binding of tobacco WRKY family proteins. *Biosci. Biotechnol. Biochem.* **65**: 2428–2436.

Maes L, Van Nieuwerburgh FC, Zhang Y, Reed DW. **2011**. Dissection of the phytohormonal regulation of trichome formation and biosynthesis of the antimalarial compound artemisinin in Artemisia annua plants. *New Phytol*. **189**: 176-189.

Maniatis T, Fritsch EF, Sambrook J. **1991.** Molecular cloning: a laboratory manual. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y*.

Marchev A, Haas C, Shultz S, Georgiev V, Bley T, Pavlov A. **2014**. Sage in vitro cultures: a promising tool for the production of bioactive terpenes and phenolic substances. *Biothecnology Letters*. **36**:211-221.

McCoy E and O ' Connor SE. **2008**. Natural products from plant cell cultures . *Prog. Drug Res.* **65:** 331 -370.

Memelink, J. **2001**. ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends Plant Sci*. **6**: 212–219.

Memelink, J. **2009**. Regulation of gene expression by jasmonate hormones. *Phytochemistry* **70**: 1560–1570.

Misra P, Pandey A, Tiwari M, Chandrashekar K, Sidhu OP, Asif MH, Chakrabarty D, Singh PK, Trivedi PK, Nath P. **2010**. Modulation of transcriptome and metabolome of tobacco by Arabidopsis transcription factor, AtMYB12, leads to insect resistance. *Plant Physiol*, **152**: 2258-2268.

Mol J, Grotewold E Koes R. **1998**. How genes paint flowers and seeds. *Trends Plant Sci*. **3**: 212–217.

Moldrup ME, Geu-Flores F, de Vos M, Olsen CE, Sun J, Jander G, Halkier BA. **2012**. Engineering of benzylglucosinolate in tobacco provides proof-of-concept for dead-end trap crops genetically modified to attract *Plutella xylostella* (diamondback moth). *Plant Biotechnol* **10**:435-442.

Montiel G, Breton C, Thiersault M, Burlat V, Jay-Allemand C, Gantet P. **2007**. Transcription factor Agamous-like 12 from *Arabidopsis* promotes tissue-like organization and alkaloid biosynthesis in *Catharanthus roseus* suspension cells. *Metabolic engineering*. **9**: 125-132.

Moses T, Pollier J, Thevelein JM, Goossens A. **2013**. Bioengineering of plant (tri)terpenoids: from metabolic engineering of plants to synthetic biology *in vivo* and *in vitro*. *New Phytologist* **200**: 27-43.

Murashige T, Skoog F. **1962.** A revised Medium for rapid Growth and Bio assays with Tobacco tissue cultures. *Plant Physiol* **15**: 473-497.

Nagase H, Woessner JF (1999). "Matrix metalloproteinases". *The Journal of Biological Chemistry* **274**: 21491–4.

Naoumkina M, Mohamed AF, Sumner LW, Tang Y, Liu CJ, Dixon RA. **2007**. Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula.* *PNAS* **104**: 17909-17915.

Naqvi S, Farre G, Sanahuja G, Capell T, Zhu CF, Christou P. **2010**. When more is better: multigene engineering in plants. *Trends Plant*. **15**:48-56.

Ninnemann, H., Zeevaart, J.A.D, Kende, H., and Lang, A. **1964**. The plant growth retardant CCC as inhibitor of gibberellin biosynthesis in *Fusarium moniliforme*. *Planta*. **61**: 229–235.

Ninomiya K, Matsuda H, Shimoda H, Nishida N, Kasajima N, Yoshimo T, Morikawa T, Yoshikawa M. **2004**. Carnosic acid a new class of lipid absorption inhibitor from sage. *Bioorg & Med Chem Lett* **14**: 1943-1946.

Noori S, Hassan ZM, Mohammad M Habibi Z, Sohrabi N, Bavanolhagh S. **2010**. Sclareol modulates the Treg intra-tumoral infiltrated cell and inhibits tumor growth in vivo. *Cell Immunol* **263**: 148-153.

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N and Sano Y. **2003**. RNA interference: Producing decaffeinated coffee plants. *Nature* **423**, 823.

Oksman-Caldentey KM, Inzé D. **2004**. Plant cell factories in the post genomic era: new ways to produce designer secondary metabolites. *Trends Plant Sci* **9**: 433-440.

Ono N, Tian L. **2011**. The multiplicity of hairy root cultures: Prolific possibilities. *Plant Sci* **180**: 439-446.

Pandey SP, Somssich IE. **2009**. The role of WRKY transcription factors in plant immunity. *Plant Physiology* **150**:1648–1655.

Pauwels, L. **2009**. Jasmonate-inducible gene: what does it mean? *Trends Plant Sci*. **14**: 87–91

Petroni, K. and Tonelli, C. **2011**. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Sci.* **181**, 219–229.

Pistelli L, Giovannini A, Ruffoni B, Bertoli A, Pistelli L. **2010**. Hairy root cultures for secondary metabolites production. *Bio-Farms Nutraceu* **698**: 167-184.

Poeckel D, Greiner C, Verhoff M, Rau O, Taush L, Hornig C, Steinhilber D, Schubert-Zsilavecz M, Werz O. **2008.** Carnosic acid and carnosol, potently inhibit human 5-lipoxygenase and suppress pro-inflammatory responses of stimulated human polymorphonuclear leukocytes. *Biochem Pharmacol* **76:** 91-97.

Phillips M, León P, Boronat A, Rodríguez-Concepción M. **2008**. The plastidial MEP pathway unified nomenclature and resources. *Trend Plant Sci* **13**: 619-623.

Potschin M, Schlienger S, Bieker S, and Zentgraf U. **2013**. Senescence networking: WRKY18 is an upstream regulator, a downstream target gene, and a protein interaction partner of WRKY53. *J. Plant Growth Regul.* **33**: 106–118.

Puerta-Fernández E, Romero-López C, Barroso-delJesus A, Berzal-Herranz A. **2003**. Ribozymes: recent advances in the development of RNA tools. *FEMS Microbiol Rev*. Apr;**27**(1): 75-97.

Rausch T, Greiner S. **2004**. Plant protein inhibitors of invertases. *Biochim. Biophys. Acta*, **1696**: 253–261.

Rigel DS, Carucci JA. **2000**. Malignant melanoma: prevention, early detection, and treatment in the 21st century. *J CA Cancer Clin* **50**: 215-236.

Roberts SC. **2007**. Production and engineering of terpenoids in plant cell culture. *Nature Chem Biol* **3:** 387-395.

Roth RJ, Acton N. **1989**. A simple conversion of artemisinic acid into artemisinin, *J. Nat.Prod*. **52**: 1183–1185.

Rózalski M, Kuzma L, Krajewska U, Wysokinska H. **2006**. Cytotoxic and proapoptotic activity of diterpenoids from in vitro cultivated *Salvia sclarea* roots. Studies on the Leukemia Cell Lines. *Naturforsch* **61**: 483-488.

Rushton PJ, Somssich IE, Ringler P, and Shen QJ. **2010**. WRKY transcription factors. *Trends Plant Sci.* **15**: 247–258.

Rushton PJ, Macdonald H, Huttly AK, Lazarus CM, and Hooley R. **1995**. Members of a new family of DNA-binding proteins bind to a conserved *cis*-element in the promoters of alpha-Amy2 genes. *Plant Mol. Biol.* **29**: 691–702.

Rushton PJ, Torres JT, Parniske M, Wernert P, Hahlbrock K, and Somssich IE. **1996**. Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes.*EMBO J.* **15**: 5690–5700.

Santino A, Taurino M, • De Domenico S, •Bonsegna S, • Poltronieri P, •Pastor V, • Flors V. **2013**. Jasmonate signaling in plant development and defense responseto multiple (a)biotic stresses. *Plant Cell Rep* **32**:1085–1098.

Schardl CL, Byrd AD, Bezion G, Altschuler MA. **1987.** Hildebrand DF, Hunt AG. Design and construction of a versatile system for the expression of foreign genes in plants. *Gene* **61:** 1-11.

Scarpeci TE, Zanor MI, Mueller-Roeber B, and Valle EM **2013**. Overexpression of AtWRKY30 enhances abiotic stress tolerance during early growth stages in *Arabidopsis thaliana*. *Plant Mol. Biol.* **83**, 265–277.

Shang Y, Yan L, Liu ZQ, Cao Z, Mei C, Xin Q. **2010**. The Mg-chelatase H subunit of *Arabidopsis*antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* **22**: 1909–1935.

Schmeda-Hirschmann G, Tsichritzis F, Jakupovic J. **1992**. Diterpenes and a lignan from *Jatropha grossidentata*. *Phytochemistry* **31**: 1731–1735.

Schmeda-Hirschmann G, Razmilic I, Sauvain M, Moretti C, Munoz V, Ruiz E, Balanza E, Fournet A. **1996.** Antiprotozoal activity of jatrogrossidione from *Jatropha grossidentata* and jatrophone from *Jatropha isabelli*. *Phytotherapy Res* **10:** 375–378.

Sheard LB. **2010**. Jasmonate perception by inositol-phosphatepotentiated COI1-JAZ co-receptor. *Nature* **468**: 400–405.

Shoji T. and Hashimoto T. **2011**. Tobacco MYC2 regulates jasmonateinducible nicotine biosynthesis genes directly and by way of the NIC2- locus ERF genes. *Plant Cell Physiol*. **52**: 1117–1130.

Schluttenhofer C, Pattanaik S, Patra B, Yuan L**. 2014**. Analyses of *Catharanthus roseus* and *Arabidopsis thaliana* WRKY transcription factors reveal involvement in jasmonate signalling. *BMC Genomics*. **15**: 502.

Schwab R, Ossowski S, Warthmann N, Weigel D. **2010**. Directed gene silencing with artificial microRNAs. *Methods Mol Biol.* **592**:71-88.

Singh KB, Foley RC, Onate-Sanchez L: **2002**. Transcription factors in plant defense and stress responses. *Curr Opin Plant Biol*, **5**:430-436.

Smith AM, Stitt M. **2007**. Coordination of carbon supply and plant growth. *Plant, Cell and Environment* . **30**:1126-1149.

Staniek A, Bouwmeester H, Fraser PD, Kayser O, Martens S, Tissier A, van der Krol S, Wessjohann L, Warzecha H. **2013**. Natural products - modifying metabolite pathways in plants. *Biotechnol J* **8**: 1159-71.

Sun TP, and Gubler F. **2004**. Molecular mechanism of gibberellin signaling in plants. *Annu. Rev. Plant Biol*. **55**: 197–223.

Sunilkumar G, Campbell LM, Puckhaber L, Stipanovic RD and Rathore KS. **2006**. Engineering cottonseed for use in human nutrition by tissue - specific reduction of toxic gossypol. *Proc. Natl. Acad. Sci. U.S.A.* **103**: 18054-18059.

Sutthivaiyakit S, Mongkolvisut W, Ponsitipiboon P, Prabpai S, Kongsaeree P, Ruchirawat S, Mahidol C. 2003. A novel 8, 9-secorhamnofolane and a new rhamnofolane endoperoxide from *Jatropha integerrima* roots. *Tetrahedron Lett.* **44**: 3637–3640.

Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, and Yuan L. **2011**. The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *Plant Physiol.***157**: 2081–2093.

Sung HJ, Choi SM, Yoon Y, An KS. **1999.**. Tanshinome IIA, an ingredient of *Salvia miltiorrhiza* BUNGE, induces apoptosis in human leukemia cell lines through the activation of caspase-3. *Exp Mol Med* **31**: 174-178.

Swain SM, and Singh DP. **2005**. Tall tales from sly dwarves: Novel functions of gibberellins in plant development. *Trends Plant Sci*. **10**: 123–129.

Talano M, Oller A, González P, Agostini E. **2012**. Hairy roots their multiple applications and recents patents. *Recent Plant Biotech* **6**: 115-133.

Tang G, Galili G. **2004**. Using RNAi to improve plant nutritional value: from mechanism to application. *Trends Biotechnol*., **22**: 463–469.

Tian D, Traw MB, Chen JQ, Kreitman M, Bergelson J. **2003**. Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana*. *Nature*. **423**:74-77

Todd AT, Liu EW, Polvi SL, Pammett RT, Page JE: **2010**. A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in Nicotiana benthamiana. *Plant J*, **62**: 589-600.

Topcu G, Goren AC. **2007**. Biological activity of diterpenoids isolated from Anatolian laminaceae plants. *Rec Nat Prod* **1**:1–16.

Toyomasu T. **2008.** Recent Advances Regarding Diterpene Cyclase Genes in Higher Plants and Fungi. *Biosci Biotech Biochem* **72**: 1168–1175.

Toyomasu T, Kagahara T, Hirose Y, Usuj M, Abe S, Okada K, Koga J, Mitsuhashi W, Yamane H. **2009**. Cloning and characterization of cDNAs encoding ent-copalyl diphosphate synthase in wheat: insight into the evlotion of rica phytoalexin biosynthetic genes. *Biosci Biotech Biochem* **23**: 772-775.

Tsai CW, Lin CY, Lin HH, Chen JH. **2011**. Carnosic acid, a rosemary phenolic compounds, induces apoptosis through reactive oxygen species-mediated p38 activation in human neuroplastoma IMR-32cells. *Neurochem Res* **36**: 2442-2451.

Ubeda-Tomás S. **2008**. Root growth in Arabidopsis requires gibberellin/DELLA signalling in the endodermis. *Nat Cell Biol* **10**: 625–628.

Ulubelen A, Topcu G, Eriş C, Sönmez U, Kartal M, Kurucu S, Bozok-Johansson C. **1994**. Terpenoids from *Salvia sclarea*. *Phytochemistry* **36**: 971–974.

Ulubelen A. **2003.** Cardioactive and antibacterial terpenoids from some Salvia species. *Phytochemistry* **64:** 395-399.

Vaccaro, M.C., Ocampo, V.E, Fasano, R., De Tommasi, N., Leone, A., Ectopic over-expression of up-stream DXS and DXR biosyntheticgenes in *S.sclarea* hairy roots to enhance synthesis of antitumoral diterpenoids, *LIV SIGA Annual Congress* September 27-30, **2010** Matera (IT).

Vaccaro MC, Malafronte N, Alfieri M, De Tommasi N, Leone A, **2014** - Enhanced biosynthesis of bioactive abietane diterpenes by overexprtessing *AtDXS* or *AtDXR* genes in *Salvia sclarea* hairy roots. *Plant Tissue and Organ Culture* **119**: 65-77.

Van der Fits L, Zhang H, Menke FL, Deneka M, Memelink J. **2000.** A Catharanthus roseus BPF-1 homologue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene Str and is induced by elicitor via a JA-independent signal transduction pathway. *Plant Mol Biol* **44:** 675-685.

Verpoorte R, Choi YH, Mustafa NR, Kim HK. **2007.** Metabolomics: back to basics. *Phytochem Rev* **7:** 525-537.

Vranová E, Coman D, Gruissem W. **2012**. Structure and dynamics of the isoprenoid pathway network, *Mol. Plant* **5**: 318–333.

Walencka E, Rozalska S, Wysokinska H, Rozalski M, Kuzma L, Rozalka B. **2007.** Salvipisone and aethiopinone from *Salvia sclarea* hairy roots modulate Staphylococcal antibiotic resistance and express anti-biofilm activity. *Planta Medica* **73:** 545-551.

Walker K, Croteau R. **2000**. Molecular cloning of a 10-deacetylbaccatin III-10-O-acetyl transferase cDNA from taxus and functional expression in Escherichia coli., *Proc. Natl. Acad. Sci. U.S.A.* **97**: 583–587.

Wang P, Liang Z, Zeng J, Li W, Sun X, Miao Z, Tang K. **2008**. Generation of tobacco lines with widely different reduction in nicotine levels via RNA silencing approaches. *J Biosci*. **33**: 177-84.

Wang H, Li H, Liu F, Xiao L. **2009**. Chlorocholine chloride application effects on photosynthetic capacity and photoassimilates partitioning in potato (*Solanum tuberosum* L.). *Scientia Horticulturae* **119**:113-116.

Wang H, Ma CZ., Ma L, Wang H, Ye H, Xu G, Liu B. **2010**. Effects of exogenous methyl jasmonate on artemisinin biosynthesis and secondary metabolites in *Artemisia annua* L. *Industrial crops and products.* **31**: 214–218.

Wasternack, C. **2007**. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot*. **100**: 681–697.
Weigel D, Glazebrook J.**2006**. Transformation of Agrobacterium using the freeze-thaw method. *CSH Protoc* **7:** 110-115.

Weather PJ. Towler MJ, Xu J. **2010.** Bench to batch: Advances in plant cell culture for producing useful products. *Appl Microbiol Biotech* **85:** 1339-1351.

Wenping H, Yuan Z, Jie S, Lijun Z, Zhesnj W. **2011**. De novo transcriptome sequencing in *Salvia miltiorrhiza* to identify genes involved in the biosynthesis of active ingredients. *Genomics* **98**: 272-279.

Waterhouse PM and Helliwell CA **2003**. Exploring plant genomes by RNA-induced gene silencing. *Nat*. *Rev. Genet*. **4**: 29-8.

Wesley SV, Helliwell A, Smith NA. **2001**. Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J*. **27**: 581-90.

Wilson S, Roberts SC. **2012**. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotech J* **10**: 249-268.

Woodward AW, and Bartel B. **2005**. Auxin: Regulation, action, and interaction. *Ann. Bot. (Lond.)* **95**: 707–735.

Wu S, Shalk M, Clark A, Brandon Miles R, Coates R, Chappell J. **2006**. Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nature Biotech* **24**: 1141-1147.

Xu YH, Wang JW, Wang S, Wang JY, Chen XY. **2004**. Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-a. *Plant Physiol.* **135**: 507–515.

Xu X, Chen C, Fan B, and Chen Z. **2006**. Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* **18**: 1310–1326.

Xu R and Li QQ. **2008**. Protocol: Streamline cloning of genes into binary vectors in *Agrobacterium* via the Gateway® TOPO vector system. *Methodology.***4**:4.

Yamaguchi S. **2008**. Gibberellin Metabolism and its Regulation. *Annu Rev Plant Biol* **59**: 225-251.

Yamasaki K, Kigawa T, Inoue M, Tateno M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda M, Tomo Y, Hayam N, Terada T, Shirouzu M, Tanaka A, Seki M, Shinozaki M, and Yokoyama S. **2005**. Solution Structure of an Arabidopsis WRKY DNA Binding Domain. *The Plant Cell*. **17**: 944–956.

Yang D, Ma P, Liang X, Wei Z, Liang Z, Liu Y, Liu F. **2012**. PEG and ABA trigger methyl jasmonate accumulation to induce the MEP pathway and increase tanshinone production in *Salvia miltiorrhiza* hairy roots. *Physiol Plant* **146**: 173-183.

Yang CQ, Fang X, Wu XM, Mao Y.B., Wang LJ, Chen XY. **2012**. Transcriptional regulation of plant secondary metabolism, *J. Integr. Plant Biol*. **54**: 703–712.

Yu D, Chen C, and Chen Z. **2001**. Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* **13**: 1527–1540.

Yu ZX, Li JX, Yang CQ, Hu WL, Wang LJ, Chen XY. **2012**.The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in Artemisia annua L., *Mol. Plant* **5**: 353–365.

Zhang HT, Hedhili S, Montiel G, Zhang YX, Chatel G, Pre M, Gantet P, Memelink J: **2011**. The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the ORCA genes that regulate alkaloid biosynthesis in *Catharanthus roseus. Plant J*, **67**: 61-71.

Zhao, J. **2005**. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv*. **23**: 283–333.

Zhang YS, Nowak G, Reed DW, Covello PS. **2011**. The production of artemisinin precursors in tobacco. *Plant Biotechnol* **9**:445-454.

Zhang P, Li ST, Liu TT, Fu CH, Zhou PP, Zhao CF, Yu LJ. **2011**. Overexpression of a 10 deacetylbaccatin III-10-O-acetyltransferase gene leads to increased taxol yield in cells of *Taxus chinensis*, *Plant Cell Tissue Organ Cult*. **106**: 63–70.

Zhang Q., Zhu J., Ni Y., Cai Y., Zhang Z. **2012**. Expression profiling of HbWRKY1, an ethephon-induced WRKY gene in latex from *Hevea brasiliensis* in responding to wounding and drought, *Trees* **26**: 587–595.

Zhang W, Xu F, Cheng H, Li L, Cao F, Cheng F. **2013**. Effect of Chlorocholine Chloride on Chlorophyll, Photosynthesis, Soluble Sugar and Flavonoids of *Ginkgo biloba. Not Bot Horti Agrobo* **41**: 97-103.

Zheng Z, Qamar SA, Chen Z, Mengiste T **2006**. *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant J.* **48**: 592–605.

Zhou ML, Hou HL, Zhu XM, Shao JR, Wu YM, Tang YX: **2011**. Soybean transcription factor GmMYBZ2 represses catharanthine biosynthesis in hairy roots of *Catharanthus roseus*. *Appl Microbiol Biotechnol*, **91**:1095-1105.

Zhou Y, Xua G, Choia FFK, Dingc LS, Hana QB, Songa JZ, Qiaoa CF, Zhaob QS, Xua. **2009**. HX: Qualitative and quantitative analysis of diterpenoids in *Salvia* species by liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *J Chromatogr A*. **1216**: 4847–4858.

Zorrilla-López U, Masip G, Arjó G, Bai C, Banakar R, Bassie L, Berman J, Farré G, Miralpeix B, Pérez-Massot E, Sabalza M, Sanahuja G, Vamvaka E, Twyman R. M, Christou P, Zhu C, Capell T. **2013**. Engineering metabolic pathways in plants by multigene transformation. *Int. J. Dev. Biol.* **57**:565-576.

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