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.