PROJECT PERIODIC REPORT

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Periodic report:	1st ☐ 2nd ☐ 3rd ☐ 4th X
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[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.
[2] The home page of the website should contain the generic European flag and the FP7 logo which are available in electronic format at the Europa website (logo of the European flag: http://europa.eu/abc/symbols/emblem/index_en.htm logo of the 7th FP: http://ec.europa.eu/research/fp7/index_en.cfm?pg=logos). The area of activity of the project should also be mentioned.

1 PUBLISHABLE SUMMARY

1.1 A summary description of project context and objectives

The overall scientific objective of SmartCell was to gain a comprehensive understanding of the extraordinarily complex biochemical capacity of plants, leading to the development of enabling technologies that will facilitate rational pathway engineering in plants and plant cells so that valuable secondary metabolites can be produced systematically in a predictable and reproducible manner. These enabling technologies include platforms for multigene transformation and metabolomics, as well as bioinformatics tools that can handle the large data sets generated during the project. We also aimed to establish and optimize large-scale production platforms for plants and plant cell cultures prior to demonstration activities that were carried out in this last period of the project. The specific aims for the fourth reporting period were:

- to perform the last experiments to validate the best subset(s) of genes and subcellular localization strategies for the optimised production of 10-hydroxygeraniol (fast track)
- to validate new genes encoding enzymes, transporters and transcription factors in the iridoid and TIA pathways (advanced track) up to secologanin
- to optimize the final production platforms based on tobacco and *C. roseus*
- to manufacture a small amount of geraniol in the optimized culture system (demonstration activity)
- to disseminate the results in terms of scientific publications and also general articles

1.2 A description of the work during the fourth reporting period

The SmartCell project has progressed according to the work plan during the fourth reporting period, with some minor deviations explained and justified in Section 2 of the current periodic report. The collaboration between partners has been active and fruitful. The reporting period included final project meeting and a joint conference with TERPNET 2013 and COST FA 1006 PlantEngine in Crete, Greece.

The terpenoid pathway engineering component of the project was divided in two themes: a fast track leading to 8-hydroxygeraniol and an advanced track leading to secologanin. In this fourth reporting period we focused on validating the function of the selected candidate genes. The validation was performed in yeast (membrane-anchored cytochrome P450 enzymes) and in *Escherichia coli* (soluble proteins). A set of 15 enzymes were expressed and screened for activity against predicted substrates. We also reconstructed transiently the pathway in *Nicotiana benthamiana* using the agro-inflitration method and transformed the genes stably both in tobacco and *C. roseus* plants and/or hairy roots. All pathway enzymes were successfully characterized in the SmartCell screen and the complete pathway is now validated. We also succeeded in studying the subcellular localization of the secologanin pathway enzymes and identified transporter genes which are involved in loganin and secologanin transport. Our major achievement was the discovery of the last four missing steps of the (seco)iridoid biosynthesis pathway leading to secologanin and to strictosidine. RNA sequencing and co-expression analysis also led to a set of 20 candidate transcription factors from which three have shown very promising results. Their final role in the terpenoid indole alkaloid pathway is currently under investigation.

For the tests of the biological activities the comprehensive solvent extraction including three temperatures and three pressures was optimized for obtaining a wide range of metabolites. The NMR spectra of all samples were obtained, and the results were subject of multivariate data analysis. There are clear differences in the metabolite profiles of the the various samples. Altogether 162 tobacco hairy root extracts were tested in three different concentrations on colorectal cancer cell line for the possible cytotoxicity effect and the proliferation of the cells were measured. The genetic modification of the tobacco hairy roots did not modify the cytotoxic activity. Various *C. roseus* hairy root lines carring different gene constructs are now also processed and ready for biological activity tests.These experiments will include more complex and diverse models for anti-cancer activity (e.g. 3D cell model systems) and they will be carried out after SmartCell project has ended.

Different tobacco production platforms including whole plants grown in greenhouses, *in vitro* hairy roots and suspension cell cultures have been evaluated to optimize geraniol production. We found that hairy root and suspension cell cultures provide the highest product yields in the shortest time. Furthermore, both systems can be cultivated under controlled conditions in bioreactors enabling the reproducible and homogenous production of geraniol. We have optimized biomass accumulation and product yields in whole plants, hairy roots and suspension cells even further by improving the nutritional and physical culture conditions in factorial design experiments. Fermentation processes using plant tissue and cell cultures have also been carried out using disposable bioreactors to allow larger-scale production. The wave-mixed single-use CultiBag system was optimized.

Our final aim was to demonstrate that our optimized plant systems and procedures can be used to manufacture geraniol. This experiment was successfully conducted using cell suspension cultures carrying geraniol synthase gene (*VoGes*) in 20 liter volume both in CultiBag and ATMI integrity WandMixer bioreactors. A maximum fresh weight up 300 g/L was achieved after 16 days of cultivation in CultiBag and at the same time highest geraniol content of 12 µg/g fw was detected.

After a thorough analysis no exploitable results in terms of patents have been identified by the Exploitation Committee. SmartCell has actively been involved in dissemination of the results of the project. A large number of original research articles, reviews, popular articles, interviews and other dissemination activities have been published, and SmartCell researchers have been presented many posters and talks at scientific congresses and educational events.

1.3 The expected final results and their potential impact and use

The SmartCell project addressed the use of plants as green factories for sustainable non-food products. It focused on the development of enabling technologies allowing the manufacture of plant-based products in rationally-engineered plants and plant cells, especially pharmaceuticals and speciality chemicals. This has a positive impact on the environment and will promote sustainable economic improvement and international competitiveness. The inclusion of plant genomics, proteomics and metabolomics as used in SmartCell will significantly improve the productivity and composition of plant raw materials in terms of known and new high-value added products. The expected impact in Europe is the significant gain of knowledge concerning the molecular, biochemical, genetic and physiological aspects of plant metabolic pathways at the systems level allowing the rational, efficient and sustainable production of important industrial compounds.

The cutting edge of basic plant research is rapidly evolving from understanding the function of individual genes to studying networks of genes that control complex systems-level biological processes such as the production of metabolites. The SmartCell demonstration component (**WP7**) which was performed in this last period of the project involved the successful manufacture of geraniol in an optimized large-scale system. This has provided SmartCell with a unique opportunity to embark on the transition from fundamental science to applications, thus validating (within the lifetime of the project) concepts, tools, tangible materials, resources, intellectual property and regulatory/biosafety issues that affect the commercial manufacture of value-added products from plants. Now when the project is complete, the gene bank, metabolomics/pathway database, compound library and cell culture collection developed during the project will be made available to the wider academic and industrial communities. The direct involvement of the companies in SmartCell supports the competitiveness of European industries, specifically those dealing with the industrial application of new technologies, processes and products.

1.4 The address of the project public website

http://www.smart-cell.org