13

Application of Agrobacterium *Rol* Genes in Plant Biotechnology: A Natural Phenomenon of Secondary Metabolism Regulation

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1. Introduction

The *rolA*, *rolB* and *rolC* genes are plant oncogenes that are carried in plasmids of the plant pathogen *Agrobacterium rhizogenes*. Following agrobacterial infection, these genes are transferred into the plant genome and cause tumor formation and hairy root disease. The *rolB* and *rolC* genes of *Agrobacterium rhizogenes* were studied extensively for the past two decades as regulators of cell growth and differentiation. A new function for the *rol* genes in plant-Agrobacterium interactions became apparent with the discovery that these genes are also potential activators of secondary metabolism in transformed cells in different plant families (reviewed by Bulgakov, 2008).

Classically, *rolB* and *rolC* have been considered closely related genes, possessing similar biological functions. However, they demonstrated different, or even opposite, effects on cell death processes (Schmulling et al., 1988), calcium balance in transformed cells (Bulgakov et al., 2003), sensitivity to auxin (Maurel et al., 1991), growth of transformed tissues (Capone et al., 1989) and secondary metabolism (Shkryl et al., 2008).

Plant-microbe interactions often lead to the development of defense mechanisms in plant cells. Since reactive oxygen species (ROS) play a pivotal role in the regulation of plant defense mechanisms, extensive experiments were performed to study the relationship between secondary metabolism (phytoalexin production) and the production of ROS in cells transformed with *rol* genes. Here, we summarize these results. Surprisingly, the *rolB* and *rolC* genes not only activated phytoalexin production but also suppressed intracellular ROS levels. This combination of defense responses, coupled with the effect of ROS suppression, represents a unique case in plant-microbe interactions. These findings suggest that bypassing upstream cell control mechanisms may be useful in the construction of plant cells possessing stable production of secondary metabolites. This chapter describes the new findings relating to secondary metabolism and ROS production under the individual and combined expression of the *rol* genes in plant cells.

2. *Agrobacterium rhizogenes rol* genes as activators of secondary metabolism

The interest in *rol* genes stems from the well-known fact that hairy-root cultures, derived from various plants species, stably produce high amounts of secondary metabolites. Among T-DNA genes, three *rol* genes (*rolA*, *rolB* and *rolC*) expressed individually or in a combination (*rolABC*) seem to be most efficient at inducing the production of secondary metabolites (Shkryl et al., 2008). Although it is known that the *rol* genes activate the transcription of defense genes, the mechanism of activation is unclear. Evidence indicates that the *rol* genes mediate uncommon signal transduction pathways in plants. They act on phytoalexin production independently of plant defense hormones and the calcium-dependent NADPH oxidase pathway (Bulgakov, 2008). The extent of secondary metabolism activation varies between plant species, from 2- to 300-fold depending on the group of secondary metabolites and the plant species (Bulgakov, 2008). Transformation with the *rol* genes is especially useful in those cases where different methods commonly used to increase secondary metabolite production (cell selection, elicitor treatments and addition of a biosynthetic precursor) only slightly enhance cell productivity. In some cases, transformation with the *rol* genes provokes a biphasic effect with an initial suppression and the subsequent activation of biosynthesis for particular groups of secondary metabolites (Bulgakov et al., 2005; Bulgakov, 2008; Inyushkina et al., 2009). The information about the effect of *rol* genes on secondary metabolism is still limiting and transformation in some cases causes unpredictable results. For example, *rolB* has been shown to cause a significant stimulatory effect on resveratrol production by cultured cells of *Vitis amurensis* (Kiselev et al., 2007). However, the resveratrol content was lowered after a 2-year cultivation of transformed cells. The reason for this is unknown because only one *rolB*-transformed cell line was analyzed.

The *rolB* and *rolC* genes are the most interesting candidates for plant biochemical engineering. High expression of the *rolB* gene in transformed plant cells dramatically increased the biosynthesis of secondary metabolites (Shkryl et al., 2008); however, excessive expression of the gene inhibited cell growth. Compared to the *rolB* gene, the *rolC* gene activated the biosynthesis of secondary metabolites to a lesser extent. However, the *rolC* gene possesses an important and interesting ability to increase cell growth. Evidence indicates that each of the *rol* genes has its own role in plant metabolic processes (Bulgakov, 2008).

3. Agrobacterium and ROS

Reactive oxygen species play an important role during plant-pathogen interactions. Avirulent and virulent pathogens elicit ROS accumulation in plant cells with different dynamics, and elicitors of defense responses, often referred to as microbe-associated molecular patterns (MAMPs), also trigger oxidative bursts (Torres et al., 2006). ROS act as executioners of pathogens and host cells by causing a hypersensitive response; they also act as signaling molecules that activate defense mechanisms. To ensure their own survival, pathogens commonly inactivate ROS produced during plant-pathogen interactions. The plant pathogen *Pseudomonas syringae* is a well-studied pathogen model that has been used to demonstrate this effect. The effector HopAO1 (HopPtoD2) protein of the *P. syringae* pv. *tomato* strain DC300 is injected from the bacterial cell into the plant cell to promote bacterial growth by suppressing the innate immunity of the host cell. It was shown that HopAO1

suppresses ROS induction in plants (Bretz et al., 2003) as well as several defense mechanisms associated with MAMP-triggered innate immunity (Underwood et al., 2007).
Agrobacterium tumefaciens also employs this strategy to combat the plant cells' defense mechanisms (reviewed by Escobar & Dandekar 2003). This pathogen can detoxify hydrogen peroxide, a primary component of plant ROS, using an agrobacterial catalase KatA (Xu & Pan 2000). *A. tumefaciens* can also suppress the induction of the hypersensitive response in plants elicited by *P. syringae* pv. *phaseolicola* (Robinette & Matthysse 1990). *A. rhizogenes* is a plant pathogen closely related to *A. tumefaciens.* Many laboratories have studied this pathogen extensively, so it is surprising that the effects of *A. rhizogenes* on ROS metabolism in host cells have never been investigated.

4. ROS and secondary metabolism

In some plant cell cultures, ROS are shown to be sufficient for the induction of plant secondary metabolite accumulation, whereas they are not involved in regulation of secondary metabolism in some other plants (reviewed by Zhao et al., 2005). It has been shown that ROS mediate the elicitor-induced accumulation of isoflavonoids in soybean and alfalfa, indole alkaloids in *Catharanthus roseus*, ginsenosides in ginseng, thujaplicin in Mexican cypress, momilactones in rice cell cultures, furanocoumarin in parsley cell cultures, diterpene rishitin and acridone alkaloid *p*-coumaroyloctopamine in potato and capsidiol in tobacco (Zhao et al., 2005).
The involvement of the oxidative burst generated by NADPH oxidase in the process of phytoalexin stimulation is well known (Guo et al., 1998; Jabs et al., 1997). There are, however, several examples of Ca^{2+}-dependent regulation of defense genes, where the NADPH oxidase pathway is not involved (Romeis et al., 2000; Sasabe et al., 2000). It is clear that several different mechanisms regulate secondary metabolism in plants. Although the details of these mechanisms in different regulatory situations are poorly investigated, the general rule postulates that ROS are important inductors of secondary metabolism.

5. Unexpected complicity of related genes: *rolC* inhibits ROS production and *rolB* activates ROS degradation

5.1 ROS levels in transformed cells

Fig. 1 presents results indicating inverse relationship between the production of secondary metabolites (anthraquinones) and ROS levels in callus cultures of *R. cordifolia* transformed with *rol* genes (Bulgakov et al., 2008; Bulgakov et al., submitted). The use of confocal microscopy and fluorogenic dyes revealed the strong inhibitory effect of *rolC* on ROS levels in transformed plant cells. The constitutive expression of the gene led to decreased steady-state levels of ROS in the cells and prevented the ROS accumulation that was induced by different treatments. The ROS inhibition was dose-dependent: the highest levels of the *rolC* expression caused maximal ROS suppression. The maximal inhibition was 46% of the basal level of ROS. This result was confirmed with an independent method employing luminol-based fluorimetric detection of ROS (Bulgakov et al., 2008).
The effect of the *rolB* gene on ROS metabolism in transformed cells has also been studied. One might expect that the gene would act in concert with the *rolC* gene to decrease ROS levels. However, the involvement of *rolB* in the induction of cellular death (necrosis) in callus and leaves of transformed plants (Schmülling et al., 1988) and in the activation of

secondary metabolism (Bulgakov 2008), i.e., in the processes which are often associated with increased production of ROS, would indicate that *rolB* expression is associated with increased ROS levels in transformed tissues. The investigation performed to discriminate between these possibilities showed a high variation of ROS levels in *rolB*-transformed cells. Extensive studies revealed that *rolB* decreased steady-state ROS levels in transformed cells up to 81-85% of control cells. It should be noted that the *rolC* gene was much more active as a ROS suppressor. However, *rolB* was more active in suppressing induced levels of ROS. Expression of *rolB* was sufficient to inhibit excessive elevations of ROS induced by paraquat, menadione and light stress and prevented cell death induced by chronic oxidative stress.

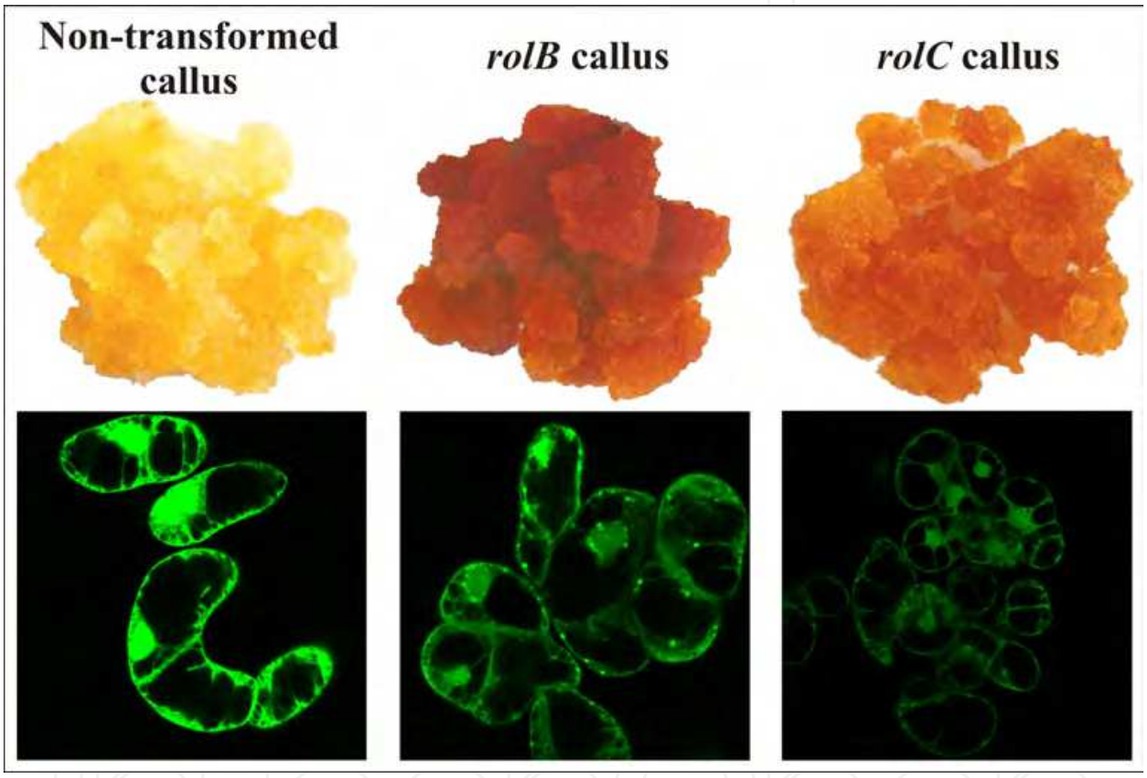

Fig. 1. Relation between secondary metabolism and intracellular ROS level in *R. cordifolia* cells. The upper panel presents phenotypes of *R. cordifolia* calli transformed with the *rol* genes. The *rolB*-calli and *rolC*-calli contained ten times and six times more anthraquinones, respectively, compared to the non-transformed calli. At the same time, ROS levels in cells of these transformed calli were low (see the bottom panel). Green fluorescence inside cells reflects summarized ROS (such as hydrogen peroxide, peroxyl radicals and peroxynitrite) levels measured by laser-scanning confocal microscopy and visualized by dichlorofluorescein diacetate.

5.2 A model in which *rolC* inhibits NADPH oxidase via CDPK

It is known that particular calcium-dependent protein kinase (CDPK) isoforms could activate stress-induced NADPH oxidases of plants by phosphorylation. For example, potato StCDPK5 induces the phosphorylation of StRBOHB (*Solanum tuberosum* NADPH oxidase)

and regulates the oxidative burst (Kobayashi et al., 2007). An investigation of *R. cordifolia* NADPH oxidase genes led to the identification of the *RcRboh1*, *RcRboh2* and *RcRboh3* genes. The alignment of deduced amino acid sequences and comparison with known RBOH proteins showed that RcRBOH1 is most homologous to NADPH oxidases that are responsible for stress-induced oxidative burst, whereas RcRBOH3 is a constitutively active oxidase, supporting ROS homeostasis under normal conditions. Real-time PCR measurements showed dose-dependent inhibition of *RcRboh3* in *rolC*-transformed cultured cells.

By investigating CDPK genes of *R. cordifolia*, 20 CDPK gene isoforms were identified. The closest analog of *StCDPK5* was found to be the *RcCDPK3* gene. We expected that *RcCDPK3* expression in *rolC*-transformed cells is inhibited. In this scenario, the deficient NADPH oxidase phosphorylation should prevent the generation of ROS. This hypothesis was confirmed by experimental data, which showed that *RcCDPK3* expression was inhibited in *rolC*-transformed cells in a dose-dependent manner (the extent of repression was dependent on the strength of *rolC* expression). Interestingly, the expression of most of the other 19 CDPK genes was unchanged in *rolC*-transformed cells, or even upregulated (Shkryl & Veremeichik, unpublished result).

Additional experiments showed that *rolC* does not affect the cellular ROS-detoxifying system. Expression of genes encoding extracellular class III peroxidases, ascorbate peroxidases, catalases and superoxide dismutases was not changed in *rolC*-cells compared to normal cells (Veremeichik et al., submitted; our unpublished result).

These results led us to postulate that *rolC* is involved in the regulation of plant NADPH oxidases. It is likely that *rolC* inhibits the CDPK-mediated NADPH oxidase pathway. This leads to lowered ROS production and ultimately decreases basal intracellular ROS levels. Because ROS levels in transformed cells are low, antioxidant genes are not activated.

In a new research, an Arabidopsis CDPK gene was expressed in *R. cordifolia* cells. Constitutive expression of the gene caused significant activation of anthraquinone biosynthesis (Shkryl et al., 2011), thus confirming our expectation that CDPKs are directly involved in secondary metabolism regulation.

5.3 A model for *rolB*: NADPH oxidase activation leads to the induction of antioxidant defense system

In contrast to *rolC*, the *rolB* gene caused significant activation of both the inducible and constitutive forms of *R. cordifolia* NADPH oxidase genes, *RcRboh1* and *RcRboh3*. This effect is reproducible and does not depend on different external stimuli, such as cold, heat and high salt conditions (Shkryl & Veremeichik, unpublished observation). Thus, *rolB* constitutively activates ROS generation via the activation of the NADPH oxidase ROS-generating system. In this scenario, the cells should defend themselves against the toxicity of excessive ROS. The experimental data showed a massive induction of ROS-detoxifying systems, such as extracellular class III peroxidases (Veremeichik et al., submitted), ascorbate peroxidases and some isoforms of catalases and superoxide dismutases in transformed cells (Shkryl et al., 2010). Thus, *rolB*-transformed cells generate ROS and simultaneously destroy excessive ROS. Equilibrium between these processes determines the resulting ROS level in *rolB*-transformed cells. One can speculate that the slow growth of *rolB*-transformed cells is a consequence of high consumption of cell metabolites and energy in these opposed processes.

5.4 How do the *rol* genes stabilize the biosynthesis of secondary metabolites?

In some cases, the effect of secondary metabolism activation mediated by the *rolB* and *rolC* genes is remarkably stable over time. For example, *R. cordifolia* transformed cells produce large amounts of anthraquinones over a long period of time (over 10 years) without any selection.

It is evident that the *rol* genes somehow avoid the regulatory controls of host cells. The data indicating suppression of basic and induced levels of ROS, together with earlier reported results indicating that the *rol* genes modulate phytoalexin production independently of ethylene, salicylic acid and methyl jasmonate-mediated pathways as well as the NADPH oxidase pathway (Bulgakov et al., 2002, 2003, 2004), point toward a signaling sequence through which the *rol*-gene signals bypass the control mechanisms of host cells. The resulting output is the development of defense reactions that are independent of cellular control mechanisms. In this scenario, the *rol* genes directly activate key genes of secondary metabolism, probably via transcription factors.

Investigation of such complex processes is a subject of systems biology. Comprehensive study of the regulatory networks involved in the biosynthesis of secondary metabolites by proteomics methods is an exciting and new field of knowledge (Bulgakov et al., 2011). This methodology will be used to unravel the complex mechanisms of the *rol* genes.

6. Combined effect of the *rolA*, *B* and *C* genes

The combined action of the *rol* genes on intracellular ROS levels was studied using pRiA4 and *rolABC* constructs. Because the *rolA* gene only slightly affected ROS levels, the main players affecting ROS metabolism were found to be the *rolB* and *rolC* genes. When expressed together, *rolC* and *rolB* balanced the effects of each other. Consequently, ROS levels in pRiA4- and *rolABC*-transformed cells were 83% and 90% of the basal levels, respectively.

It is clear that the combined actions of the *rolA*, *B* and *C* genes do not cause significant ROS suppression. If it were otherwise, the combined effect of the *rol* genes could cause a disruption in ROS homeostasis and cell death. However, the strategy of the phytopathogen *A. rhizogenes* is not to kill cells. Instead, the bacteria, acting via the transferred genes, render cells more tolerant of environmental stresses and increase their defense potential. In many cases, the *rol* genes ensure a high growth rate of transformed cells and their hormonal independence. In this context, the *rol* genes appear to be in tune with each other, providing physiological conditions for better cell fitness in the face of changing environmental conditions (Fig. 2). Perhaps, this is the main effect of the *rol* genes as members of the RolB (*plast*) gene family.

Let's consider the situations in which the *rol* genes would have a favorable effect on cell survival. If *A. rhizogenes*-transformed plant cells were subjected to signals causing ROS elevation (cold, heat, high-intensity light stress, necrotrophic pathogens, etc.), *rolC* would prevent excessive ROS by the suppression of the NADPH oxidase gene. This effect would be strengthened by *rolB*, which activates the antioxidant system. If plant cells were subjected to a signal causing ROS suppression (this is a strategy utilized by many plant pathogens), *rolB* would prevent the decrease of ROS levels by the activation of NADPH oxidase genes. Currently, it is known that *rolC* increases the cold, heat, salt and light resistance of transformed cells. The *rolB* gene exerts the same effects. Additionally, *rolB*-transformed cells are resistant to the ROS-generating herbicide paraquat and external hydrogen peroxide. In contrast, *rolC*-transformed cells are not resistant to these treatments because they do not

possess sustained antioxidant defenses. Simultaneous expression of *rolC* and *rolB* in transformed cells confers resistance to all of these treatments.
In addition, these results reveal an interesting analogy between *rol*-transformed plant cells and mammalian tumor cells, which are also highly viable and resistant to different therapeutic treatments.

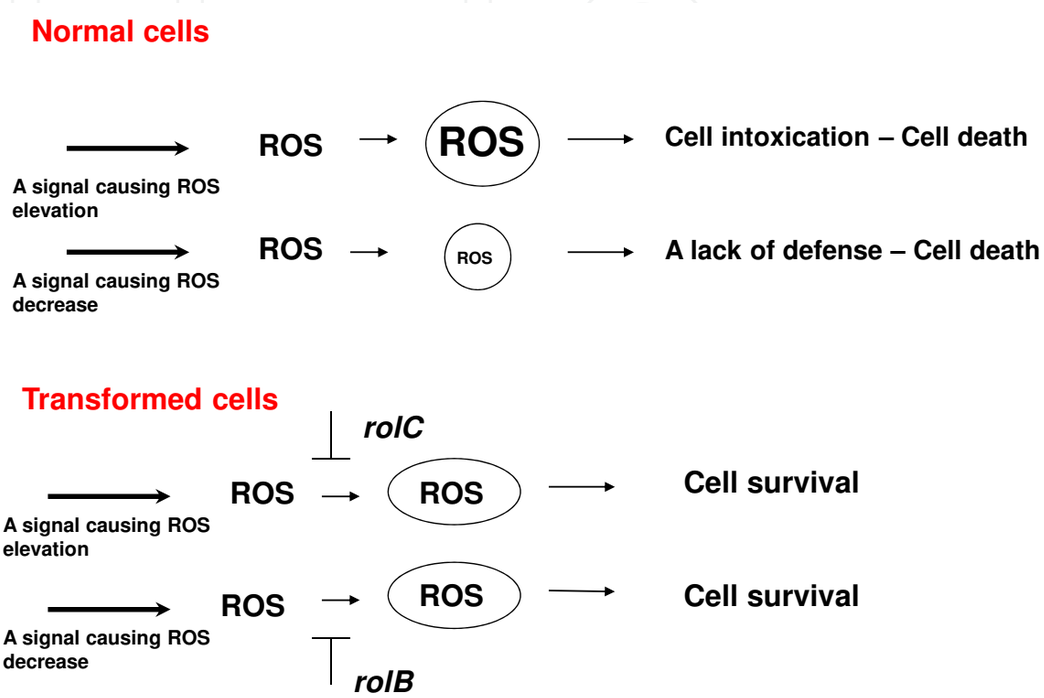

Fig. 2. A scheme illustrating the possible role of *rol* genes in cell survival. The *rolC* and *rolB* genes mitigate ROS changes caused by environmental stimuli. The signals causing acute ROS elevations are high temperature, cold, high salt conditions, excessive light and others. Signals causing decreased intracellular ROS levels are provoked by many pathogens.

7. Conclusion

The combination of defense mechanisms, coupled with the effect of ROS suppression described in this chapter, represents a unique case of plant-microbe interactions. *A. rhizogenes* is closely related to *A. tumefaciens,* which also suppresses the host immune response by ROS inhibition. There is, however, a fundamental difference between these pathogens. *A. tumefaciens* expresses a chromosome catalase gene and suppresses ROS during contact with plant cells. *A. rhizogenes*, acting via T-DNA genes, suppresses ROS in many generations of transformed cells, thereby ensuring a long-term effect on ROS homeostasis. In the studies discussed here, many questions dealing with the relationship between secondary metabolism and ROS metabolism remain to be answered. Nevertheless, the mechanism underlying the action of the *rol* genes is currently emerging. Unraveling this mechanism would allow the engineering of plant cells with improved characteristics and free of shortcomings inherent to the *rol* genes.

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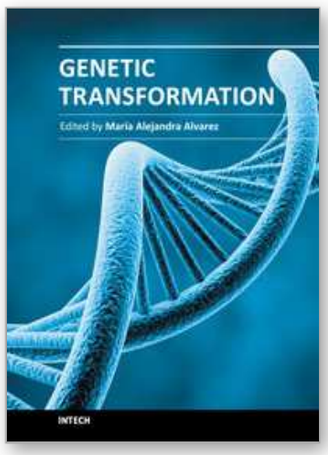

Genetic Transformation

Edited by Prof. MarÃa Alvarez

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Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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