Agrobacterial Plant-transforming Oncogenes: Emerging Complexity of Their Action and a Functional Parallelism with Some Animal Protooncogenes

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Abstract—**Following agrobacterial infection, the *Agrobacterium rhizogenes rolA*, *rolB* and *rolC* genes are transferred to plant genome, causing tumor formation and hairy root disease. The emerging complexity of the *rol*-gene triggered effects and the involvement of signals generated by these genes in basic processes of cell biology such as calcium and ROS signaling and modulation of expression of calcium-dependent protein kinase genes indicate that the plant oncogenes, like some animal protooncogenes, use sophisticated strategies to affect cell growth and differentiation. Recent data raise the intriguing possibility that some components of plant and animal oncogene signaling pathways share common features. The *rol* oncogenes are involved in regulation of secondary metabolism performing a function not existing in animals. Studying of processes representing hot spots in animal cell biology, such as the triangle combining the interplay between oncogene expression, ROS production and cell senescence/apoptosis, calcium signaling and processes of protein phosphorylation/dephosphorylation will help in understanding functions of the plant oncogenes. In this respect, new directions of *rol*-gene studies are highlighted in this article underlying the necessity to use metabolomic, genomic and proteomic approaches in further investigations.**

***Index Terms*—calcium-dependent protein kinase, plant oncogenes, reactive oxygen species, *rol* genes, transformation.**

I. INTRODUCTION

The *Agrobacterium*-plant cell interaction is the only known natural example of DNA transport between kingdoms. In this process, the plant pathogens *Agrobacterium tumefaciens* and *A. rhizogenes* transfer a part of their plasmid DNA (T-DNA) to plant genome. Twenty years ago, *rolA*, *rolC* and *rolB* of *Agrobacterium rhizogenes* were shown to induce root formation in transformed plant cells [1, 2]. This discovery stimulated investigations aimed at understanding how the genes affected plant development. Studies of *rol* genes are abounding in detective findings which, however, are somewhat mosaic and therefore cannot form complete picture of oncogenesis. This is not surprising, because the oncogenes cause multiple biochemical and physiological alterations in transformed plants and appear to act in a complex manner, and in this aspect they are similar to animal oncogenes. "The *rol* genes saga" written by Costantino et al. [3] and continued in other review articles [4-6] summarized different aspects of growth, development and hormone metabolism in transformed plants. A new function of the *rol* genes in plant-Agrobacterium interaction became apparent with the discovery that these genes are potential activators of secondary metabolism in transformed cells of different plant families [7]. In some cases, the activator effect of individual *rol* genes was sufficient to overcome inability of cultured plant cells to produce large amounts of secondary metabolites [7].

This review highlights the necessity to use different "omics" methods to provide new insights in further understanding how the plant oncogenes affect different cellular functions. While at least partial "interactome" networks of several model plants are already available, progress in the plant field is less prominent comparing with animal studies. However, peculiarities of network organization (the modules, motifs and cliques) are increasingly recognized as the operational units of plant biological functions. Analysis of the protein interaction networks in plant cells transformed with the *rol* genes is a valuable tool for a molecular understanding functional specificities and regulatory components and pathways mediated by these genes.

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II. HOW THE *ROL* GENES CAN BE INTEGRATED IN PLANT SIGNALING PATHWAYS

Biochemical function of rol genes and their effects on secondary metabolism

The RolA protein probably belongs to the group of DNA-binding proteins, sharing a structural similarity with the papillomavirus E2 DNA-binding domain [8]. The biochemical function of RolA is generally unknown. A stimulatory effect of the *rolA* gene on nicotine production in tobacco transformed cells has been demonstrated [9]. The *rolA*-expressing calli of *Rubia cordifolia* produced a 2.8-fold higher level of anthraquinones (AQs) when compared with the control calli [10]. An interesting biotechnological peculiarity of *rolA* is that the gene, being expressed in *R. cordifolia* calli, ensured remarkably invariable levels of AQs and simultaneously provided conditions for vigorous callus

growth. This effect was stable over a 7 year period of observation of a *rolA*-transformed callus line [10].

Early studies suggested that the RolB and RolC proteins are glucosidases, liberating auxin and cytokinins, respectively, from their bound forms [11, 12]. Other investigations have not supported this proposition, arguing that the changes in levels of free hormones observed in some *rolC* and *rolB*-transformed plants reflect non-specific effects of the oncogenes [13-15]. More recently, Faiss et al. [16] proposed that RolC could act as a non-specific glucosidase that hydrolyzes the plant cell wall, thereby interfering with plant development via the release of oligosaccharides. To date, there have been no additional studies to elucidate the biochemical function of RolC. In transformed plants and plant cell cultures, the *rolC* gene alone is capable of stimulating production of tropane alkaloids [17], pyridine alkaloids [18], indole alkaloids [19], ginsenosides [20] and anthraquinones [21, 22, 10].

By contrast, two breakthroughs have revealed important new insights into the mechanism of RolB action. RolB was shown to exhibit tyrosine phosphatase activity [23] and to interact with 14-3-3 proteins [24]. These studies in turn highlight an interesting parallel between plant and animal oncogenes, given that both Tyr phosphatases and 14-3-3 proteins are known to play a pivotal role in mammalian oncogenesis, participating in complex processes of cell growth, differentiation and death [25, 26]. The tyrosine phosphatase function of RolB needs further examination, because the CX_5R motif of the pRiA4 RolB protein, which is characteristic for the tyrosine phosphatase family, is lacking in most other RolB proteins [27]. Among *rol* genes, this gene is apparently the most powerful inductor of secondary metabolism and, at the same time, the most powerful suppressor of cell growth. In *R. cordifolia* transformed calli, *rolB* expression positively correlated with increase of expression of the *ICS* gene (a key gene for AQ biosynthesis) and anthraquinone production [10]. Investigation of growth parameters of these calli showed that high expression of *rolB* inhibited callus growth. The *rolA* and *rolC* genes did not show negative effect on callus growth. When combined with the *rolA* and *C* genes in the *rolABC* construct, *rolB* no longer exhibited the adverse effect on cell growth [10]. The *rolB* gene had the maximal effect on AQ accumulation when compared with *rolA* and *rolC*. In the high-*rolB*-expressing culture, a 15-fold increase in AQs was detected. However, the stimulatory effect of the *rolB* gene on AQ formation was weaker when the gene was combined with the *rolC* and *rolA* genes in the *rolABC* construct. The most prominent example of the effectiveness of the *rolB*-transformation have been recently demonstrated in the case of *Vitis amurensis* cells where the gene ensured more than a 100-fold increase in resveratrol production [28]. Resveratrol is a stilbene, which prevents carcinogenesis at stages of tumor initiation, promotion and progression. In the investigation, normal cell cultures of wild-growing grape (*Vitis amurensis*) were developed. The cultures produced low levels of resveratrol, up to 0.026% dry wt., i.e., comparable to levels reported for other grape cell cultures. Different methods commonly used to increase secondary metabolite production (cell selection, elicitor treatments and addition of a biosynthetic precursor) only slightly enhanced cell productivity. The *rolB*-transformed calli were capable of producing up to 3.15% dry wt. of resveratrol. The capability to resveratrol biosynthesis was correlated with the abundance of *rolB* mRNA transcripts [28].

Growth suppression is a limiting factor of usage of *rolB*-transformed cells in practical aspects. As *rolB* encodes a protein possessing tyrosine phosphatase activity [23], the authors of both studies attempted to block the effects of *rolB* by using tyrosine phosphatase inhibitors. The inhibitors partially prevented toxic effect of *rolB* on cell growth and lowered the production of secondary metabolites [10, 28].

Nothing is known about involvement of processes of tyrosine phosphorylation and processes mediated by 14-3-3 proteins in plant secondary metabolism. Recent studies suggest that protein Tyr phosphorylation performs critical functions in plants, regulating activity of MAP kinases, transcription factors and ROS signaling [29]. The existence of Tyr phosphatases in plants has been controversial until recently when several members of the protein Tyr phosphatase family were characterized from Arabidopsis [30]. The inability of plant cells to produce high resveratrol levels and a low effectiveness of standard biotechnological methods is the major problem of resveratrol production in vitro. The facility with which *rolB* transformation overcomes this problem [28] demonstrates high potential of engineering of the plant Tyr phosporylation-mediated pathways and potentially may be exploited to produce high-yielding cell lines for particular groups of secondary metabolites.

A role of ROS in rolC-mediated effects

Processes of reactive oxygen species (ROS) generation by mammalian oncogenes, as well as the role of oxidative stress in many aspects of oncology, are subjects of unfailing interest of numerous investigations. However, such a theme for plant oncogenes has never been considered. Involvement of important animal regulators of oncogenesis, such as Rac1, v-Ha-ras, c-Myc and NF-κB in intracellular ROS production is well documented [31-34]. Increased expression of these animal protooncogenes causes ROS elevation, except NF-κB, which prevents accumulation of ROS [34]. The investigation aimed on understanding whether tumor formation mediated by the *rolC* gene is associated with increased production of ROS was performed with *R. cordifolia* cells. Single-cell assays based on confocal microscopy and fluorogenic dyes showed reduced steady-state levels of ROS in *rolC*-expressing madder cells compared with normal cells [35]. ROS inducers caused significant ROS elevation in normal cells, but had little effect on *rolC*-transformed cells. The results indicated that the *rolC* gene acts as a powerful ROS suppressor. In contrast to several animal protooncogenes which accelerate tumor progression via enhanced ROS production, *rolC* probably does not act in a similar way because expression of the gene does not trigger oxidative burst. It is interesting that despite the active influence of *rolC* on many cellular processes, its expression does not deteriorate growth of transformed cells. The ROS-suppressing function of *rolC* requires further careful examination to evaluate whether or not RolC exerts functionality similar to those animal protooncoproteins (such as NF-κB) which repress apoptosis by ROS suppression, thereby ensuring survival of tumors.

After elicitation of plants, the amplification of ROS production is dependent on Ca^{2+} influx. Data from our group indicate a striking difference in responses of normal and

rolC-transformed cells of *R. cordifolia* to H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevations. In *rolC*-transformed cells, almost complete blockade of H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevations was detected, showing a positive correlation between levels of ROS and H_2O_2-induced $[Ca^{2+}]_{cyt}$ fluxes [36]. If ROS and $[Ca^{2+}]_{cyt}$ signaling is impaired in *rolC*-transformed cells, one can suppose that the oncogene bypasses upstream plant control mechanisms regulated by these important signaling molecules.

Modulation of expression of calcium-dependent protein kinase genes

Constitutive expression of *rolC* in cultured plant cells leads to altering hormone (auxin) sensitivity [37], causes somatic-to-embryo transition [38] and calcium disbalance [22], activates production of PR-2 proteins [39] and production of secondary metabolites [7]. The complex picture revealed in the *rolC*-gene studies raises the question as to how *rolC* could affect such different processes. In recent years, several publications have shown an important regulatory role for Ca^{2+}/calmodulin-dependent protein kinases and calcium-dependent protein kinases (CDPK) in nodule formation during plant-rhizobium interaction [40-42]. As *Agrobacterium rhizogenes* is related to rhizobia microorganisms, one could hypothesize that the *A. rhizogenes* could develop a similar strategy to affect signaling components of host plant cells. Because CDPKs are commonly accepted as molecules that mediate cross-talk between signaling pathways [43], modulation of their expression and/or activity could explain numerous unrelated and largely unexplained effects seen in *rolC*-transformed cultures. Indeed, it was shown recently that expression of *rolC* in cultured cells of *Panax ginseng*, *Vitis amurensis* and *Eritrichium sericeum* resulted in changes of expression of different CDPK genes and provoked generation of new transcripts with modified sequences corresponding to catalytic Ser/Thr kinase subdomains [44, 45]. The function of each CDPK gene whose expression is modified in *rolC*-expressing cell cultures needs further examination. The observation that *rolC* expression induces the appearance of truncated or long CDPK transcripts (*PgCDPK1a-s*, *VaCPK1a-s*, *VaCDPK1-L*$_1$ and *EsCDPK1-s*) in *P. ginseng*, *V. amurensis* and *E. sericeum* cells is particularly interesting. It is likely that *rolC* affects multiple biochemical processes in transformed cells through changing expression of various CDPK genes and by generation of new CDPK transcripts.

III. LONG-LASTING EFFECTS IN *ROLC*-TRANSFORMED CELLS

There is evidence indicating that processes of growth, differentiation and secondary metabolism in *rol*-transformed cells could be changed during prolonged periods of cultivation. For example, five independently transformed primary *rolC*-tumors of *P. ginseng* were subcultured for a long time to study their root-forming capability [38]. The appearance of roots in these cultures was registered after 3-15-month cultivation. After 1.5-5 years in culture, all independently established primary tumor cultures ceased to form roots and began instead to form somatic embryos and shoot primordia [38]. Growth of the *rolC*-transformed cell cultures can be also changed during a long-term cultivation. A study performed with seven-year-old calli of *R. cordifolia* indicated a conservation of *rolC*-transformed callus phenotypes, stable expression of *rolC* and high AQ levels during the long-term cultivation [10]. A contrasting difference was revealed in respect of growth parameters: during the long-term cultivation, the *rolC*-transformed calli progressively increased the intensity of growth, whereas growth of the control calli remained stable. The highest growth rate was observed in a low-*rolC*-expressing callus line. Similar peculiarity was also observed for the low-*rolC*-expressing calli (2c2 line) of *P. ginseng*. These calli are characterized by remarkably stable and vigorous growth for a long time (over 10 years) without any selection [38].

An unexpected result has been reported for *rolC*-transformed cell cultures of *Eritrichium sericeum* and *Lithospermum erythrorhizon* where a clear negative effect on secondary metabolism was revealed [46]. Callus cultures of these plants contain large amounts of caffeic acid metabolites rabdosiin and rosmarinic acid, which possess a profound anti-nephritic activity [47, 48]. The *rolC*-transformed callus cultures of both plants yielded 2-3-fold less levels of caffeic acid metabolites than respective control cultures. The conclusion about inhibitor action of the *rolC* gene on secondary metabolism was made basing on chemical analyses performed over a 2-year period of observations [46]. However, further investigation performed during next 2-3 years revealed progressive increase of production of rabdosiin and rosmarinic acid in *E. sericeum* *rolC*-transformed cells. Likewise, a similar effect was observed for the *rolC*-transformed roots of *P. ginseng* where a 6-month lag phase preceded the activation of ginsenoside production [49].

It is evident that after integration of the *rolC* gene into the plant genome, the competence to growth, differentiation and secondary metabolism was changed on the time-dependent manner indicating that dynamic processes proceeded after transformation. These effects have proven difficult to unravel largely because it is unclear to what extent the oncogene rearranges the genetic apparatus of cells and to what extent cells undergo somatic mutations independently on the oncogene. Studying of such properties of *rol*-induced tumors may have a broad biological significance by analogy with recent studies dealing with processes of somatic mutations in human cancer genomes [50]. In human cancers, some mutations, so called 'passenger', are fortuitous. Whereas these mutations do not contribute to oncogenesis, a substantial part of mutations are 'driver' ones conferring growth advantage on the cell in which they occur [50]. Intriguingly, many of the driver mutations occurred within sequences corresponding kinase domains of different protein kinase genes [50]. The discussed above modifications of kinase domains of CDPK genes in *rolC*-transformed plant cells are interesting in this respect.

IV. UNCOMMON SIGNAL TRANSDUCTION

The possibility that the *rolB* and *rolC* genes might function through mechanisms alternative to the conventional mechanisms described for plants is intriguing. Indeed, pharmacological experiments suggested that the *rolC* gene acted on phytoalexin production independently of defense hormone-mediated pathways and the calcium-dependent NADPH oxidase pathway [21, 22, 51, 46]. Likewise,

production of phytoalexins in *rolC*-transformed cells of *R. cordifolia* is not dependent on oxidative burst [35]. One can conceive a situation in which the *rolC* and *rolB* genes performs its own critical function in regulation of secondary metabolism by bypassing upstream plant control mechanisms and directing defense reactions via a "short cut". Considering natural history of *A. rhizogenes*-plant interaction, when ancestral T-DNA genes participated in ancient infection processes and played a role in early events of plant species differentiation [52, 53], one can speculate that *rolC* influences primitive plant regulatory networks and fails to affect flexible instruments developed by plants more recently through ROS and defense hormone signaling.

V. SIMILARITY BETWEEN SOME FUNCTIONS OF PLANT AND ANIMAL ONCOGENES

Protooncogenes, originally discovered as the cellular cognates of retroviral growth-promoting transforming genes, have been highly conserved during evolution, a fact that indicates an essential function of their protein products in the basic regulation of cellular metabolism. Plants contain several animal protooncogene homologues, such as *c-myb*, *c-myc*, *c-fos*, *c-jun* and *ras* [54]. Some of the protooncogenes are involved in regulation of secondary metabolism performing a function not existing in animals [54]. After the publication by the V. Citovsky group [55] showing that Agrobacterium could transform HeLa cells, it was hypothesized that Agrobacterium could transform any eukaryotic organisms [56]. Recently, agrobacterial transformation of sea urchin embryos was performed and the *rolC* and *rolB* oncogenes have been shown to promote formation of teratoma-like structures in the animal embryos [57]. The ability of plant oncogenes to function in animals [57] and the ability of some animal protooncogenes (such as members of the Bcl-2 family) to function in plants [58] suggests that processes of oncogenesis in plant and animals may have some common features. The ROS suppressor function of *rolC* discussed above is similar to that demonstrated by NF-κB [34]; some functional analogy with the *rolC* gene shows animal protooncogene *Bcl-2*, whose expression in transgenic mice correlated with the inhibition of H_2O_2-induced intracellular Ca^{+2} fluxes [59].

VI. PERSPECTIVES

It is becoming clear that *rol* genes are interesting candidates in terms of their biotechnological application. The *rolA* gene emerges as a stimulator of growth and secondary metabolism in cultured plant cells. The *rolB* gene seems to be the most powerful activator, but its practical usage is presently questionable because of its growth-suppressing effect. The *rolC* gene is most studied in this respect and usage of the gene is promising with respect to activation of secondary metabolism. Stimulation of secondary metabolism mediated by the *rol* genes is remarkably stable over long-term cultivation. Further investigation of the activator functions of these genes is highly dependent upon investigation of their general biochemical functions. Such investigation would discover new signaling pathways in cultured plant cells, thus expanding a base for genetic engineering of secondary metabolism.

It is evident that *A. rhizogenes* has a mechanism to manipulate defense pathways in transformed plant cells via expression of T-DNA oncogenes. Are the alterations in secondary metabolism beneficial for *A. rhizogenes* or for plants? In other words, whether the bacteria evolved to cause such type of plants defense reaction or plants developed the defense mechanism as a response to the pathogen intervention (realizing a specific type of systemic acquired resistance). Both variants are possible. Investigation of the *rol* genes may help us to understand why closely related microorganisms such as *A. rhizogenes*, *A. tumefaciens* and Rhizobium species have evolved to develop such different interactions with plants as pathogenic or symbiotic.

Further investigation of regulatory components and pathways mediated by the plant oncogenes and comparison of protein networks in tumors caused by plant and animal oncogenes is interesting with respect to evaluating earliest events in oncogenes evolution.

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