

Agrobacterium rhizogenes *rolB* oncogene: An intriguing player for many roles

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ARTICLE INFO

Keywords:

Agrobacterium rhizogenes
rolB gene
 Plant fitness
 Photosynthesis
 Biotic and abiotic stress
 Secondary metabolites
 Epigenetic regulation

ABSTRACT

The *rolB* oncogene is one of the so-called *rol* genes found in the T-DNA region of the *Agrobacterium rhizogenes* Ri plasmid and involved in the *hairy root* syndrome, a tumour characterized by adventitious root overgrowth on plant stem.

rolB produces in plants a peculiar phenotype that, together with its root-inducing capacity, has been connected to auxin sensitivity. The gene is able to modify the plant genetic programme to induce meristem cells and direct them to differentiate not only roots, but also other cells, tissues or organs.

Besides its essential function in *hairy root* pathogenesis, the *rolB* role has been progressively extended to cover several physiological aspects in the transgenic plants: from secondary metabolites production and ROS inhibition, to abiotic and biotic stress tolerance and photosynthesis improvement. Some of the observed effects could be determined, at least in part, through microRNAs molecules, suggesting an epigenetic control *rolB*-mediated. These multifaceted capacities could allow plants to withstand adverse environmental conditions, enhancing fitness.

In spite of this expanding knowledge, functional analyses did not detect yet any definitive *rolB*-derived biochemical product, even if more than one enzymatic activity has been ascribed to it. Moreover, phylogenetic and evolutionary studies evidenced no homology with any plant sequences but, otherwise, it belongs to the *Plast* family, a group of *rolB*-homologous bacterial genes.

Finally, the finding of sequences similar to *rolB* in plants not infected by *A. rhizogenes* suggests a hypothetical plant origin for this gene, implying different possibilities about its evolution.

1. *Agrobacterium rhizogenes* and *rol* genes

Agrobacterium rhizogenes is a Gram-negative microorganism living in the soil near to plant roots. First identified as *Rhizobium rhizogenes* in the 1930s (Riker et al., 1930) and given the actual name in 1942 (Conn, 1942), this bacterium induces the *hairy root* syndrome, characterized by root overgrowth on plant stem at the infection site. This disease is caused by the integration into the plant genome of a bacterial DNA fragment from the so-called root-inducing (Ri) plasmid (Chilton et al., 1982). The mechanism of this horizontal and inter-kingdom gene transfer mimics a natural genetic engineering process by which the pathogen mainly preserves its own survival.

A complex molecular pathway involving genes present in the bacterial chromosome, in the plasmid and in the plant genome participates in the T-DNA transfer process (Zupan and Zambryski, 1995; Mauro et al., 2017).

The transferred DNA (T-DNA) fragment contains a set of genes that interferes in plant development, physiology and metabolism, also providing bacterium food. This last effect, requiring genes lying in both the T-DNA and the bacterial chromosome, consists in the synthesis of opines, amino acid derivatives secreted by plant cells and utilized by the bacterium as nutriment (Dessaux et al., 1992).

In the 1980s scientists established the T-DNA genes affecting the induction of *hairy root* disease and described their effect and function. Four of these, named *rol* (for *root locus*) *A*, *B*, *C* and *D*, have been object of intense study being the main responsible for the syndrome. Analyses have been largely performed in dicotyledonous plants as tobacco, carrot, tomato and *Arabidopsis thaliana*, transformed with single genes or in combination (Casanova et al., 2005).

Even if their roles are pleiotropic, some characteristic aspects relative to each of these genes have been progressively established (for comprehensive reviews see Casanova et al., 2005; Mauro et al., 2017; De

Abbreviations: ORF, open reading frame; IAA, indole-3-acetic acid; CDPK, calcium-dependent protein kinase; ROS, reactive oxygen species; miRNAs, micro RNAs.

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<https://doi.org/10.1016/j.plaphy.2021.04.037>

Received 18 February 2021; Accepted 28 April 2021

Available online 14 May 2021

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Paolis et al., 2019).

1.1. The *rolB* oncogene

The *A. rhizogenes rolB* gene, identified in 1985 (White et al., 1985), has been thoroughly investigated because of its specific ability to promote, alone, root formation (Fig. 1) (Delbarre et al., 1994), showing fast growth, high branching and ageotropic behavior (Altamura, 2004).

As for the other *rol* genes, the term oncogene refers to its capacity to change the plant genetic programme by rapid neoplastic cell division.

For both temporal and research purposes, *rolB* studies could be ideally divided in two main phases, sometimes overlapping: in the first, the interest has been primarily focused on its role in *hairy root* pathogenesis and on the characterization of transgenic plants (Schmülling et al., 1988; Lemcke and Schmülling, 1998a); in the second phase on its competence to modulate pathways in order to counteract environmental changes improving plant stress tolerance (Bulgakov et al., 2012, 2018).

Besides, these discoveries have allowed the development of useful methodologies to better investigate in both basal research and biotechnology application.

2. First phase

2.1. *rolB*-plant interaction: plant phenotype and hormone content

Insertion of the *rolB* gene in tobacco, one of the first plants utilized for its study, determines a typical organization with abundant adventitious roots, stunted growth, increased size of stigma and flowers, heterostyly, altered leaf morphology, reduced fertility and smaller fruits (Schmülling et al., 1988; Altamura, 2004). Moreover, in flowers the reproductive process is affected (Cecchetti et al., 2004).

These characteristics can, however, slightly vary depending on the species analysed. In the model plant *A. thaliana*, *rolB*-transgenics further show increased number of inflorescences per rosette area with respect to the wild type, and premature senescence/early necrosis of vegetative organs (Kodahl et al., 2016). In tomato, a decrease in apical dominance and a reduction of internode length have been observed (Fig. 2) (Arshad et al., 2014). In the same plant, tissue-specific expression of *rolB* in the ovary induces parthenocarpy, i. e. the development of fruits without fertilization (Carmi et al., 2003).

rolB function was expanded from root-inducing to meristem-inducing following the discovery of its morphogenetic effect in thin cell layer (TCL) systems (Altamura, 2004), where the formation of adventitious roots and flower buds is, respectively, stimulated and enhanced at the stage of meristemoid formation. Thin cell layer experiments with varying auxin and/or cytokinin concentrations have shown even more that the meristem-inducing activity is inherent to the *rolB*



Fig. 1. Leaf disc from *Arabidopsis thaliana rolB*-transformed plant on hormone-free medium.

protein, while phytohormones are involved in determining the type of organ that will be formed. The same is true also in *planta*, where *rolB* induces the formation of ectopic meristems that could give rise to different plant organs (Altamura, 2004).

The modifications induced by *rolB*, in particular in root-inducing activity, led to the suggestion that its action could involve variations in auxin content or sensing. *rolB* root cultures *in vitro* are able to grow independently of this hormone, while wild type ones are not (Bellincampi et al., 1996). This capacity could be mainly ascribed to an enhanced sensitivity to auxin. In fact, in protoplasts from *rolB* transgenic tobacco plants, auxin perception was increased 3,000 to 10,000-fold, significantly more than in protoplasts from plants untransformed or transformed with *rolA* or *rolC* genes (Maurel et al., 1991). However, such altered response did not depend on variations of the intracellular concentration of the hormone (Delbarre et al., 1994). Support to the hypothesis of *rolB* participation in auxin signal transduction came from a proteomic approach that demonstrated *rolB*-induced upregulation of the VHI-interacting kinase VIK, involved in auxin-activated cascade (Bulgakov et al., 2018).

The auxin role in *rolB* action is further sustained by the inhibitory effect on root promotion by oligogalacturonides, cell wall-derived signal molecules acting as auxin-antagonists in defense and developmental processes, defining as well *rolB* as late responsive gene (Mauro et al., 2002).

The tight auxin connection with different aspects of *rolB* function and their specific mode of action is far from being explained. Differently from animals, hormones in plants have less specificity with a widespread influence. Auxin showed, and could still evidence, new aspects of its activities by *rolB*-mediated interaction.

Phytohormone levels were determined in transgenic tobacco, showing minor variations in indole-3-acetic acid (IAA), abscisic acid (ABA) and gibberellic acid 1 (GA1) and a decrease in cytokinin content, which led to a higher auxin/cytokinin ratio with respect to untransformed plants (Schmülling et al., 1993), typical of an *in vitro* condition for root-induction.

Finally, the integration of the *rolB* gene in grape increased chromosome variability (Kiselev et al., 2013). Such influence in genome alteration may suggest a role in decreased control of DNA homeostasis or higher genetic flexibility.

2.2. *rolB* family: DNA structure and function

rolB is present in the T-DNAs of different *A. rhizogenes* Ri plasmids, classified based on the type of opine they produce such as agropine, cucumopine, mannopine and mikimopine (Bevan and Chilton, 1982). In the cucumopine and mannopine strains the T-DNA is transferred as a single fragment, while in the agropine strain 1855, where major analysis has been performed, it consists of two fragments, TL- and TR-DNA, having a size, respectively, of 15–20 kb and 8–20 kb (Slightom et al., 1986).

The 1855 TL-DNA contains 18 open reading frames (ORFs), 11 of which display eukaryotic features for functioning into the plant (Slightom et al., 1986). In particular, ORFs 10, 11, 12 and 15 correspond, respectively, to *rolA*, *B*, *C* and *D* genes (White et al., 1985). *rolB* and ORF13a are in opposite orientation with respect to ORF8, *rolA*, *rolC*, ORF13 and ORF14 (Fig. 3).

The size of the *rolB* ORFs in different *A. rhizogenes* strains ranges between 762 and 837 bp (Table 1). Minor differences have been found at the protein level, with the exception of RolB from pRi1724 having 17 additional amino acid residues at the N-terminus with respect to pRiA4 or pRi8196 (Tanaka et al., 1994).

A number of sequences with homology to *rolB* have been identified and grouped in a highly divergent RolB or *Plast* gene family (Otten, 2018; De Paolis et al., 2019).

In the TR-DNA it has been detected an ORF (*rolB_{TR}*) with significant similarity to the *rolB* gene, both at the nucleotide (52.6%) and at the



Fig. 2. Phenotype of tomato plants transformed with the *rolB* gene (left) and of the corresponding *in vitro* regenerated controls (right). Flowers are covered with paper bags for self-fertilization.

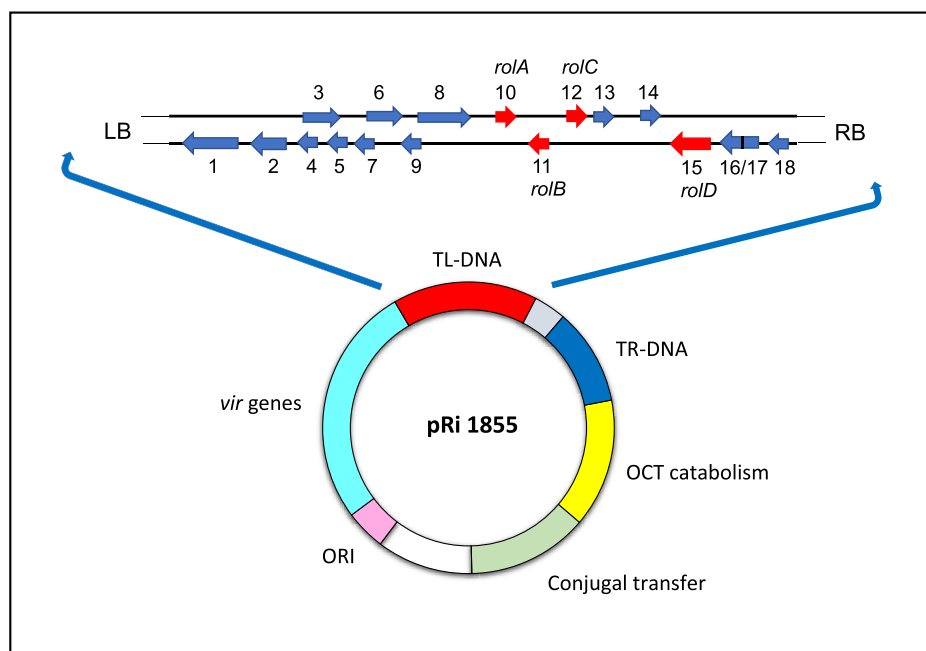


Fig. 3. Schematic representation of agropine-type Ri plasmid of *A. rhizogenes* strain 1855 and map of gene locations and organization on TL-DNA. OCT, octopine; ORI, origin of replication; LB, left border; RB, right border.

amino acid level (40.6%) (Bouchez and Camilleri, 1990). This ORF is 822 bp long and encodes a predicted polypeptide of 274 amino acids. The *rolB_{TR}* gene does not show functional homology with the *rolB* gene,

as it does not support root formation in infected tobacco leaf discs. Transgenic *rolB_{TR}* plants were shorter with respect to wild type, with shoot outgrowth at the base of the stem and smaller, wrinkled leaves

Table 1

rolB genes from different pRi plasmids characterized by specific opine production. ORF, open reading frame.

Ri plasmid, opine type	<i>rolB</i> ORF (bp)	Predicted protein (amino acids)	References
pRi8196, mannopine	762	254	Hansen et al. (1991)
pRiA4, agropine	774	258	Slightom et al. (1986)
pRi1724, mikimopine	837	279	Tanaka et al. (1994)
pRi2659, cucumopine	774	258	Serino et al. (1994)

(Lemcke and Schmölling, 1998b).

Partial similarity (27.9%) has also been found between the RolB protein and the N-terminal region of the ORF 8 polypeptide (Levesque et al., 1988), whose C-terminal fragment shows significant homology to the tryptophan monooxygenase (IaaM) enzyme of *Pseudomonas* and *Erwinia* spp. that catalyzes the synthesis of auxin precursor indole-3-acetamide from tryptophan (Otten and Helfer, 2001). Tobacco plants overexpressing this *rolB*-homologous region show stunted growth, intercostal chlorosis and necrosis (Umber et al., 2002).

Other members of the RolB family include the already mentioned *rolC*, *orf13* and *orf14* from *A. rhizogenes*, the 5, 6 and 6b genes from *A. tumefaciens*, while the b, c', d, e, 3', 4', 7 and *lso* genes are from other *Agrobacterium* spp. (Otten, 2018).

2.3. Regulatory regions and DNA-binding factors

Referring to the 1855-derived *A. rhizogenes* plasmid, *rolB* is expressed from a bidirectional promoter located in the intergenic region between *rolB* and *rolC* and regulating both genes (Schmölling et al., 1989). A 1100 bp sequence upstream to the *rolB* coding sequence has been dissected to determine the relevant regions for specific expression of the gene in cells, tissues and organs (Capone et al., 1991, 1994). Mainly, *rolB* is expressed in the plant vascular system and in most root tissues. Five regulatory domains, named A, B, C, D and E, were identified and their specific competences evaluated in the root. In particular, domain A (located at position –623 to –471) controls the level of transcription and directs the expression to the root cap and to the protoderm; domain B (–341 to –306) is the most crucial for the expression in the root meristem and for conferring auxin responsiveness to the gene; domain C (–216 to –158) is specifically active in the protoderm, while domain D (70 bp surrounding the CAAT box) is needed for expression in the dermatogen and, as domain B, in meristematic cells of the cortex; finally, domain E (80 bp around the TATA box), as domain A, drives expression to the cells of the root apex. The emerging data show the capacity of promoter domains to discriminate between meristematic and non-meristematic tissue for *rolB* activation. Moreover, gene expression in meristematic cells is inducible by auxin, while in the non-meristematic cells does not seem to depend upon the hormone (Capone et al., 1994).

Factors binding to these promoter domains have been described: in domain B an ACTTTA site for an endogenous one zinc-finger transcription factor of the Dof family, NtBBF1 (*Nicotiana tabacum rolB* domain B Factor 1), has been detected, conferring tissue-specific expression in apical meristems and vascular system as well as auxin inducibility (Baumann et al., 1999). Otherwise, in the domain A the factor RBF1 (Rol Binding Factor 1) is involved in controlling the level and/or tissue specific expression of non-meristematic cells in the root apex, in particular protoderm and root cap. A sequence of the RBF1 DNA binding region, TACGTTTA, is complementary to the ATGCAAAT octamer first found as a conserved motif in transcriptional control elements of the immunoglobulin light chain gene, and successively in other animal genes (Filetici et al., 1997).

The evidence that a multipartite promoter of a bacterial gene is regulated by endogenous plant factors needs further work, in order to identify other elements involved in controlling the expression of *rolB*.

The *rolB* regulatory region has been studied also in other *A. rhizogenes* plasmids. In the cucumopine-type pRi2659 (Serino et al., 1994) the A domain is missing, coherently with the lack of expression of the *rolB* homologue *rolβ* in the root cap and the protoderm. In pRi1724 (Handayani et al., 2005) the activity of the promoter is stimulated by exogenous auxin and two auxin-inducible domains have been detected, a NtBBF1 binding site and an auxin-responsive module found in the soybean *SAUR 15A* promoter. The same region conferring auxin inducibility is also responsible for tissue-specific expression in the root apex meristematic cells of the vascular cylinder.

2.4. RolB protein

Until now no data have clearly defined a function and/or mechanism for RolB protein. Moreover, in spite of the presence of eukaryotic regulatory motifs, no sequence homology with plant genes has been found.

However, even if not definitive nor successively supported or reproduced, two different enzymatic activities have been attributed to this gene, i. e. β-glucosidase (Estruch et al., 1991a) and tyrosine phosphatase (Filippini et al., 1996). Both enzymes have in common hydrolysis as main reaction, but a different cellular localization.

The first, identified in tobacco plants, consists in a β-glucosidase able to hydrolyse indoxyl-β-glucoside, similar to the IAA inactive conjugate IAA-β-glucoside, releasing indoles. This suggested that the RolB protein could release active auxins from their inactive conjugates, leading to increased hormone activity and altering physiology and development of transgenic plants.

Beta-glucosidases are widespread all over the living world, ranging from fungi and plants to animals, but all the functions in which they play have not been unravelled yet. Recent progress describes a high substrate specificity for these enzymes. In plants they have an important role in metabolic processes where *rolB* is also involved, from development and hormone-mediated growth regulation, to defense and microbial communication (Morant et al., 2008).

Next, RolB product has been described as a tyrosine phosphatase, a plasma membrane-located protein that showed characteristic sequence domain with catalytic and signalling target sites and a helix structure containing a tyr phosphatase superfamily motif CX₅R. As phosphatases are crucial enzymes in signal transduction cascades, RolB protein has been suggested to affect auxin perception.

Other key aspects of tyrosine phosphatase function are related to the regulation of stem cells and differentiation (Hale et al., 2017). In plants, tyr phosphatases are engaged in post-translational modification, physiology and development, and in abiotic and biotic stress response (Shankar et al., 2015).

Phosphatases are some of the most efficient enzymes, that can catalyze the hydrolysis of different, but related, substrates. Such “catalytic promiscuity” could have paved the way for the evolution of new enzymatic activities (Hengge, 2013), exploited by RolB to extend the range of its abilities.

A clue on the possible function of *rolB* in the production of adventitious roots came from the work by Moriuchi et al. (2004) in tobacco, where direct binding of the RolB protein from the *A. rhizogenes* mikimopine strain 1724 to a protein of the 14-3-3 family, Nt14-3-3, was observed. This association was involved in RolB nuclear localization and rooting activity. As shown for viral oncoproteins in animal cells, the interaction could influence plant development by regulating cell growth (Aoki et al., 2000).

3. Second phase

At the beginning of the XXI century the progress in plant culture methodologies (Ochoa-Villarreal et al., 2016) pointed out to the

cultivation of specific organs or undifferentiated cells, since they significantly promoted metabolic and physiological plant pathways investigation with respect to *in planta* systems, mainly because “controlled” cell growth simplified the analysis.

In this frame, the culture of *hairy roots* and of *rolB* transgenic cells demonstrated more stability and an indefinite growth capacity, eliminating many technical difficulties (Bulgakov et al., 2018).

rolB does not stimulate and even inhibits growth in transgenic cell cultures, high gene expression promoting programmed cell death and necrosis (Kiselev et al., 2007; Shkryl et al., 2008). In *A. thaliana* calli with moderate *rolB* expression level, biomass accumulation did not differ significantly from that of controls, even if growth was reduced (Bulgakov et al., 2018). In this regard, transcription level is an important determinant of the many plant aptitudes attributed to the action of the gene. As intermediate levels of expression supported growth, preventing the negative effect of the oncogene, this condition has been often chosen to investigate the interference of the gene in metabolic pathways (Bulgakov et al., 2018). The new knowledge shifted the interest from *rolB* role in plant pathogenesis to other emerging activities as increase in secondary metabolites production (Bulgakov, 2008), antioxidant capacity (Bulgakov et al., 2012) and stress response modulation (Bulgakov et al., 2013).

From then on, the analyses have been performed in a huge variety of plants species, including those having agronomic, pharmacological or industrial importance, as *Nicotiana*, carrot, tomato, rice, maize, *Vitis amurensis*, *Linaria*, gentian, *Artemisia* spp., *Catharanthus roseus*, *Rodhiola rosea*, *Rubia cordifolia* and *Panax ginseng*.

3.1. *rolB* and secondary metabolites

Secondary metabolites, even if not essential for plant survival, are important for communication and, in particular, are primary elements for the protection against pathogens and abiotic injuries.

Transgenic plants derived from transformation with *rol* genes can yield molecules not found or present in a lower concentration in the wild type plants (Bulgakov, 2008). Although other *rol* genes can produce a greater amount of secondary metabolites, *rolB* is the most active.

As mentioned above, also in this case the level of *rolB* expression is decisive (Grishchenko et al., 2016). In *rolB*-*R. cordifolia*, the increment of the phytoalexin anthraquinone (AQ) accumulation is correlated to *rolB* expression level and, interestingly, to callus color, that can vary from yellow in low *rolB* expressing lines, to orange in moderately expressing and orange-red in highly expressing. This effect is probably due to the increased transcription of the isochlorismate synthase gene, a key enzyme of AQ biosynthesis (Shkryl et al., 2008).

Also, in *Vitis amurensis* cell culture *rolB* induces an increase of up to 100 times of resveratrol, whose biosynthesis is again tightly correlated with the abundance of *rolB* mRNA transcripts (Kiselev et al., 2007). Resveratrol increment is linked with changes in calcium-dependent protein kinase (CDPK) gene expression, suggesting that its synthesis is Ca²⁺ dependent and implicating *rolB* in calcium-dependent pathways (Dubrovina et al., 2009). This molecule, one of the most efficient phytoalexins, is also involved in human protection since, besides its strong antioxidant power, is an important modulator of metabolism (Gambini et al., 2015).

Transgenic tomatoes exhibited a 62% and 58% increase, respectively, in the fruit content of lycopene and phenolic compounds, compared to control, enhancing nutritional quality (Arshad et al., 2014).

In *Artemisia* spp. the amount of artemisinin, one of the most effective treatments for malaria, increased up to ninefold upon *rolB* transformation, in accordance with the increased level of genes of its biosynthetic pathway (Dilshad et al., 2015). Consistent increases were also observed for other phytochemicals, such as rutin, caffeic acid, isoquercetin and quercetin. Last, in *Arabidopsis* calli, a threefold higher level of indolic glucosinolates has been induced by *rolB* through the specific activation of genes encoding MYB and bHLH transcription

factors, demonstrating, for the first time, a specific relationship between *rolB*-induced secondary metabolite production and transcription factors (Bulgakov et al., 2016).

3.2. *rolB* and ROS metabolism

The qualitative and quantitative effects exerted by *rolB* on secondary metabolites production suggested its involvement in antioxidant capacity, because of the well-known role of these compounds in scavenging reactive oxygen species (ROS). For example, in *Artemisia annua* expression of flavonoids biosynthetic pathway genes phenylalanine ammonia-lyase and chalcone synthase was found to be significantly boosted following the insertion of *rolB* (Dilshad et al., 2016). The amount of polyphenols was thus enhanced, playing an important role both in quenching free radicals and in degrading peroxides due to their redox capacity.

rolB reduces free radicals also by acting on their metabolism (Bulgakov et al., 2012, 2013; Shkryl et al., 2013). In transgenic *R. cordifolia*, *P. ginseng*, and *Arabidopsis* cell cultures the level of ROS is controlled through the enhanced activity of genes encoding scavenging enzymes i. e. cytosolic ascorbate peroxidase, catalase, and superoxide dismutase (Bulgakov et al., 2012). In *R. cordifolia* class III peroxidases, belonging to PR-9 family involved in regulating H₂O₂ levels and phytoalexin biosynthesis, were overproduced (Shkryl et al., 2013). In the same plant *rolB* activates also a NADPH oxidase gene apt to maintain redox homeostasis (Bulgakov et al., 2013).

3.3. *rolB* in pathogen defense and stress response

In tomato, *rolB* enhances protection from fungal species *Fusarium oxysporum* and *Alternaria solani* by increasing lycopene, phenolic compounds and ascorbic acid content (Arshad et al., 2014). Moreover, in plants infected with *F. oxysporum* electrolyte leakage, a peculiar indicator of the hypersensitive response induced against pathogen assault, showed a higher tolerance to the fusaric acid toxin when compared to wild type.

rolB cells and plants are more resistant to abiotic stress, as salt and temperature. *Rubia cordifolia* transgenic cell cultures were more tolerant to NaCl than untransformed cells, with IC₅₀ values of 25 mM and 16 mM, respectively (Bulgakov et al., 2012, 2013). Moreover, in transgenic *P. ginseng* cells increased salt resistance was related to CDPK expression (Kiselev et al., 2010). In tomato, *rolB* expression during early fruit development had a protective effect against both high and low temperatures, with plants showing higher yield and number of fruits, increased fruit weight, improved jelly fill and lack of malformations with respect to controls (Shabtai et al., 2007).

Finally, *rolB* could be able to differently modulate the expression of relevant plant genes in both abiotic and pathogen defense suggesting a role in the respective pathways. These genes may be, in part, those previously described to participate in reduction of ROS, induction of scavenging enzymes and modulation of secondary metabolites.

As biotic and abiotic stress response mechanisms are mostly shared in plants, *rolB* transgenics will contribute to identify relative pathways.

3.4. *rolB* and photosynthesis

Light-dependent processes play a fundamental role in plant adaptation, and increasing evidences show their interaction with stress-related pathways (Pandey et al., 2015).

In this regard, for the first time a modulation of photosynthesis, the fundamental and primary plant metabolic pathway, by *rolB* has been highlighted (Bettini et al., 2016, 2020).

Tomato plants expressing the oncogene promote a safer and more efficient photosynthetic process with respect to wild type plants, an effect even more striking when the analyses were performed under far-red-enriched light. Performance of transgenic plants showed increased

non-photochemical quenching, preserving the photosynthetic apparatus from oxidative damage by dissipating excess light energy as heat. The lower chlorophyll *a/b* ratio, typical of sciaphilous (shade-requiring) plants, suggested that these plants could easily acclimate to low light regimen. Coherently with this behaviour, in *rolB* plants exposed to far-red-enriched light a higher efficiency of photoprotection and an improvement in photochemistry were observed, especially at low light intensities. In such condition electron transport between Photosystems I and II is more active in transgenic plants, resulting in higher capacity for ATP and NADPH production.

Such increased tolerance could be related, at least in part, to the activation of genes involved in the photosynthetic apparatus regulation, as small heat shock protein, chlorophyll *a/b* binding protein and carbonic anhydrase.

3.5. *rolB* and epigenetic regulation

The first example of a T-DNA oncogene involvement in epigenetic modulation comes from the 6b oncoprotein of the T-DNA region of *A. tumefaciens* (Ishibashi et al., 2014). This gene, belonging to the *RoLB* family, is able to bind histone H3 in tobacco influencing the host chromatin status and modulating plant gene expression. The 6b protein also participates in ADP ribosylation by targeting microRNAs (miRNAs) processing machinery. In particular, it interacts with the Argonaute 1 (AGO1) and SERRATE (SE) proteins involved in the biogenesis of miRNAs (Wang et al., 2011). The reduced miRNAs molecules in plants overexpressing the 6b protein suggested that it could act as a suppressor of RNA silencing and may participate in the inhibition state observed in *A. tumefaciens* crown gall syndrome.

Moreover, in a non-tumorous *N. glauca* × *N. langsdorffii* hybrid, containing inactive *rolB*, the demethylating agent 5-azacytidine reactivated endogenous *rolB* gene expression and induced hormone autotrophy, again suggesting a control mediated by epigenetic mechanisms (Bogani et al., 2012).

It has been recently discovered that overexpression of *rolB* in *A. thaliana* cells affects miRNA biogenesis and stability, potentially influencing post-transcriptional gene silencing (Bulgakov et al., 2015, 2018). Specifically, core (*DCL1-SE-HYL1*, *AGO1*) and helper (*TGH* and *DDL*) genes of the miRNA processing machinery are upregulated, indicating that the complete module for the transcription and maturation of primary transcripts is enhanced in transformed cells. HEN1, an RNA methyltransferase involved in mature miRNAs protection, was also highly expressed suggesting a role of *rolB* in their stabilization.

Moreover, increased expression of the *AGO4* and *RDR2* genes, the two major effectors in transcriptional gene silencing, has been observed, proposing *rolB* as a player in both processes.

These results suggest that the complex effects of *rolB* on transformed plant cells may be under epigenetic control. For example, the recent demonstration that miRNAs are active in secondary metabolism regulation (Bulgakov and Avramenko, 2015), could indicate a role for miRNA in *rolB*-mediated induction. The same, specific miRNAs identified in plants are also promoted in developmental processes and under biotic and abiotic stress, enhancing tolerance (Bulgakov et al., 2015).

3.6. *rolB* and the other *rol* genes

The *rolB* coexpression with the complex of T-DNA genes, and in particular with other *rol* genes, shows a cooperative or antagonistic activity affecting tumour formation and transgenic plants and cells.

Even if there are no detailed studies, in plants transformed with single *rol* genes or in combination the sum of single effects produces a characteristic physiological and/or developmental frame that can buffer or enhance the traits ascribed to a single gene (Pavlova et al., 2014). For instance, in the *hairy root* syndrome induced by a T-DNA *rolD* mutant, root elongation was prevented (White et al., 1985) suggesting the *rolD* involvement in spite of its role in flowering transition and meristem

induction (Falasca et al., 2010).

Furthermore, the relationship with *rolC* is extremely interesting also in the light of the newly identified activities performed by *rol* oncogenes. Sharing with *rolB* the promoter domain, *rolC* acts, as until now described, as a glucosidase releasing active cytokinins from their glucoside conjugates (Estruch et al., 1991b). The combined action of these two genes could modulate the intracellular concentration of auxin and cytokinin, the main growth factors able to perturb plant growth in both the *hairy root* onset and the transgenic plants. In fact, the phenotypic effects of *rolA* and *rolB* genes, both showing an increased sensitivity to auxin, were not observed in *RoLABC* plants indicating an antagonistic effect of the latter gene (Pavlova et al., 2014). Also in tissue culture *rolC*, promoting vigorous cell growth, balances the inhibition exerted by *rolB* (Schmülling et al., 1988; Shrkyl et al., 2008).

rolB and *rolC* are both able to stimulate secondary metabolism, but the outcome is different. Metabolites produced in *rolC*-transformed tissue, such as tropane alkaloids, pyridine alkaloids, indole alkaloids and ginsenosides (Bulgakov, 2008), are not detected in *rolB*-transgenics.

Finally, an unexpected synergy has been found in the antioxidant capacity, since *rolC* inhibits ROS production while *rolB* activates ROS degradation (Bulgakov et al., 2012, 2013). *rolB* and *rolC* genes mediate, independently, significant changes in the expression profile of *R. cordifolia* CDPK genes. Possible effects on ROS metabolism may be attributed to the altered transcription of particular CDPK isoforms (Shrkyl et al., 2020).

4. Conclusions

Once established its primary function in *hairy root* disease, the *rolB* gene has subsequently been shown to be able to induce many related activities. The control of plant morphology, the hormone modulation and, meaningfully, the induction of meristem formation from any kind of plant cell, demonstrated the transgenic plant ability to follow a determined ex novo genetic programme.

However, *rolB* action influences other aspects amenable to enhancing plant robustness, as the capacity to regulate the production of specific secondary metabolites, to reduce ROS level, to increase tolerance to pathogen and abiotic stress, all playing a central role on plant survival. Moreover, the positive performance of transgenic plants on photosynthetic process in different light conditions, expands its influence to primary metabolism (Fig. 4).

The involvement of this gene in the photosynthetic process gives plants more opportunities to withstand climate changes and injury. In fact, both increased expression of photosynthetic genes and light conditions are known to interfere with pathogen tolerance (Lopez-Kleine et al., 2012) and secondary metabolites modulation (Kuo et al., 2015). Therefore, a *rolB* contribution on these processes could also be mediated by its role in plant photosynthesis.

How can this gene interfere with so many pathways? Suggestions and implications

Why a single gene could be able to promote different metabolic pathways and effects?

What links root promotion, meristem induction, secondary metabolites production, photosynthesis protection and efficiency, epigenetic regulation and stress response?

The significance of promoter domains

A suggestion could derive from the upstream regulatory domains, that could be one of the keys to understand the ways in which the gene works producing different responses, each concerning independent aspects of plant network.

Specific factors, activated only in a given developmental or environmental condition, could bind *rolB* promoter domains to indicate where and when the gene product must exert its function.

In accord with data already obtained, recent proteomic analyses underline the potential *rolB* ability to regulate mainly signaling pathways related to immunity control, miRNA processing, hormone

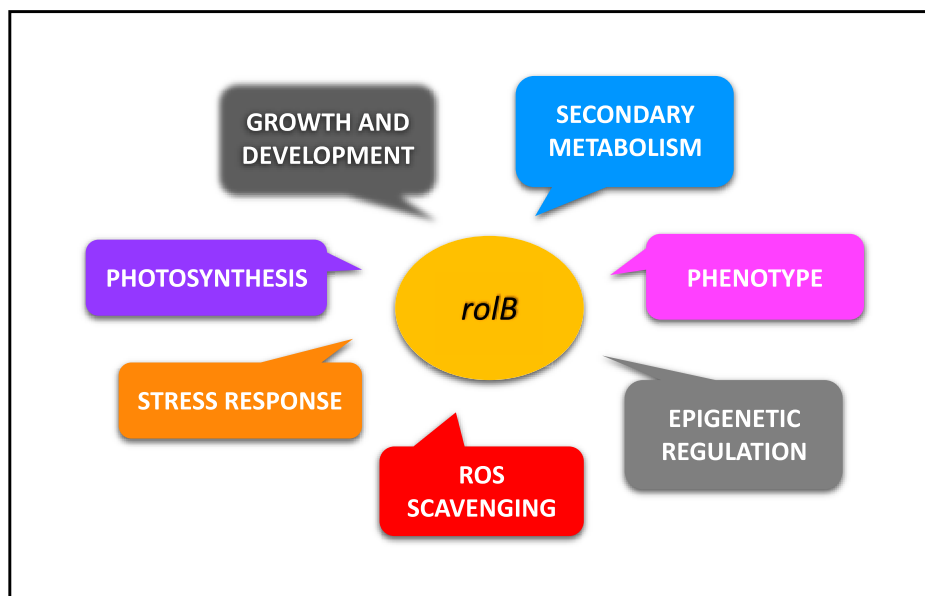


Fig. 4. Multiple roles of *rolB* in plants. ROS, reactive oxygen species.

modulation, calcium sensing, ROS buffering and secondary metabolites production (Bulgakov et al., 2018). In this frame, *rolB* could act also as a signaling factor that can trigger a cascade of events leading to alternative responses.

Therefore, a role could be proposed for *rolB* in creating a “bridge” between plants and the outside world, favouring life in unstable and changeable environment and conveying plant energies to implement a defense approach rather than basal metabolism (Bulgakov et al., 2018).

Can the effect of this gene tell us more on its evolution?

The *rolB* interference on plant miRNA pathway, an evolutionarily selected machinery, together with eukaryotic-type promoter features, further highlights the close relationship between this bacterium-derived oncogene and the plant genome, suggesting its putative plant origin in an ancient era (Mauro et al., 2017).

In the beginning, *rolB* could have conferred a selective advantage to plants because of the wide array of activities that it is able to promote. The actual loss of function of this gene in plants could suggest that, successively, evolution replaced it with new developmental and/or safeguard systems. Acquired by *Agrobacterium*, *rolB* is now utilized to its own needs by producing, in accordance with the other T-DNA genes functions, the characteristic syndrome in order to improve bacterium survival. In particular, improving adventitious root formation increases the mass of tumour tissue and consequently the production of opines that feed the bacterium population, creating an ecological niche not affected by other competitors.

As for other T-DNA genes, in the genome of few not infected dicotyledonous species as *Nicotiana*, potato and *Linaria* (Mauro et al., 2017), sequences homologous to *rolB* have been identified and named cellular T-DNA (cT-DNA). These sequences have been considered the “memory” of a past insertion event, also because they contain at the border site a region important for their integration into plant genome. The meaning of their maintenance is not clear: it could represent a form of “vestigial” genes that, even if rarely transcribed and in general at very low level, may be activated in special conditions (Matveeva et al., 2012).

To date, knowledge on *rolB* does not provide evidence for a consolidated biochemical and functional role, nor for detailed molecular pathway(s) or mechanisms engaged in the many effects promoted in plants. The future challenge to unveil how this gene interferes with plant genome programme will have to take into account its tight interaction with variable plant condition and with phytohormones, in particular with the master plant hormone auxin, for deciphering the elusive

function of this plant regulator still relevant in the technological era.

In this regard, the establishment of *rolB* *in vitro* root culture systems is currently used as a powerful tool to facilitate the production of root mass in recalcitrant plants. The increased biomass is also utilized to obtain large amounts of root-derived compounds such as secondary metabolites and bioactive compounds, both for investigating the molecular process of their biosynthesis and/or improving industrial, pharmaceutical or agronomical products.

The same, metabolites of *rolB* cells or callus cultures differ compared to *in vivo* plants, and can be controlled either through promoter expression level, biosynthetic pathways and environmental context (Bulgakov, 2008), and by selecting appropriate cell lines, elicitors, media components and hormone concentrations (Raj and Saudagar, 2019). The findings suggest that it will be possible to produce plants with a tailored chemical profile, opening a new road for *rolB* application. Large-scale production could be further supported through bioreactor system technology, bringing reduced cost, increased yield and profit (Pavlov and Bley, 2018).

We apologize with all the Authors whose work we could not cite due to the limited reference number to be inserted.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions statement

Maria Luisa Mauro: Conceptualization, Writing - Original Draft, Writing - Review & Editing. **Priscilla P. Bettini:** Writing - Original Draft, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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