Applied Microbiology and Biotechnology (2020) 104:2435–2451
https://doi.org/10.1007/s00253-020-10403-7

MINI-REVIEW

Rhizogenic agrobacteria as an innovative tool for plant breeding: current achievements and limitations

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Received: 27 November 2019 / Revised: 16 January 2020 / Accepted: 20 January 2020 / Published online: 30 January 2020

Abstract

Compact plant growth is an economically important trait for many crops. In practice, compactness is frequently obtained by applying chemical plant growth regulators. In view of sustainable and environmental-friendly plant production, the search for viable alternatives is a priority for breeders. Co-cultivation and natural transformation using rhizogenic agrobacteria result in morphological alterations which together compose the Ri phenotype. This phenotype is known to exhibit a more compact plant habit, besides other features. In this review, we highlight the use of rhizogenic agrobacteria and the Ri phenotype with regard to sustainable plant production and plant breeding. An overview of described Ri lines and current breeding applications is presented. The potential of Ri lines as pre-breeding material is discussed from both a practical and legal point of view.

Keywords *Agrobacterium rhizogenes* · Compact growth · Hairy root phenotype · Plant breeding · Rhizogenic agrobacteria · Ri phenotype · Ri plasmid

Introduction

Plant breeding is a continuous process in which the creation of novelty and new traits are primary goals (Louwaars et al. 2012; Morgan and Funnell 2018). Classical breeding by intra- and interspecific crosses has been used to obtain new traits and develop new varieties (Kuligowska et al. 2016). This strategy meets several restrictions related to time investment, available diversity in the gene pool, and fertility barriers (Horn 2002; Long et al. 2018). *Agrobacterium*-mediated transformation can be used to obtain new traits in crops that are not present in the existing gene pool (Casanova et al. 2005). However, this technique is often associated with the use of genetic vectors that typically results in a genetically modified organism (GMO) and is therefore limited in terms of its applicability for practical plant breeding in Europe (Gelvin 2003). Another strategy to increase variability in plants is *Agrobacterium*-mediated transformation by the use of wild type rhizogenic agrobacteria (Otten 2018a).

Rhizogenic agrobacteria are a group of bacteria with a pathogenic lifestyle and root-inducing ability, and consists of strains belonging to the *Rhizobium radiobacter* and *Rhizobium rhizogenes* species both carrying the root-inducing (Ri) plasmid (pRi) (Bosmans et al. 2017; White and Nester 1980b). The Ri plasmid confers pathogenicity to the bacteria and contains the mechanism to initiate a horizontal gene transfer event by which a part of the Ri plasmid, namely the transfer DNA (T-DNA), is transferred to the host plant (Chilton et al. 1982; Lacroix and Citovsky 2016). After successful transfer and integration of the T-DNA genes in the nuclear DNA, the host plant will undergo changes that are instigated by the foreign DNA with the primary symptom being the proliferation of roots from the infection site. This phenomenon was first described by Riker et al. (1930) as infectious or hairy roots. It was later understood that the hairy roots are synthesizing and excreting specific compounds, termed opines, that serve to create an ecological niche in which nutrients are provided for the agrobacteria (Moore

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et al. 1997; Petit et al. 1983). The fact that opine production is plasmid encoded suggested that also the rhizogenic response of infected tissues could be evoked by pRi T-DNA-specific genes (White et al. 1982). Multiple genes involved in hairy root formation were later identified and documented to have strong morphogenic effects (Huffman et al. 1984; Spena et al. 1987). These genes were consequently defined as "plast" genes by Levesque et al. (1988). The hairy roots, because of their genetic stability and vigorous growth in hormone-free media, became a popular tool for plant metabolic engineering and studying root biology in the form of hairy root cultures (Hamill and Lidgett 1997; Shanks and Morgan 1999). Tepfer (1984) found that, following pRi T-DNA integration, plant regeneration was possible, resulting in a modified phenotype of the regenerated plant termed the hairy root or Ri phenotype.

This knowledge was applied to obtain phenotypically distinct plants with useful agronomical traits. Initially, this was used to create composite plants consisting of a transformed root system and wild type aerial parts (Lambert and Tepfer 1992). Regeneration from transformed tissues resulted in the development of complete natural transgenic plants, which also allows sexual transmission of the phenotype (Costantino et al. 1984; Ooms et al. 1985; Tepfer 1984). In some plant species, such as *Nicotiana glauca*, *Nicotiana tomentosiformis*, and *Ipomoea batatas*, spontaneous regeneration from hairy roots under natural circumstances occurred, and the resulting genotypes were characterized (Chen et al. 2014; Kyndt et al. 2015; White et al. 1983). The natural occurrence of gene transfer to plants combined with the plethora of useful traits makes the technique of using wild type rhizogenic agrobacteria very promising in terms of practical plant breeding applications (Casanova et al. 2005; Otten 2018b; Tempé and Schell 1977). Moreover, Ri lines of *Kalanchoe blossfeldiana* have already been successfully applied in commercial plant breeding (Christensen et al. 2014; Lütken et al. 2012b).

The global trend towards a more sustainable plant production comes with many challenges. Plant breeders today are faced with restrictions on the use of plant growth regulators, crop protection substances, and GMO techniques. The search for viable alternatives is a priority. In this work, we highlight the potential of wild type rhizogenic agrobacteria in the light of sustainable plant production, how this technique can lead to the creation of novel phenotypes in a non-GMO setting, and its application in practical plant breeding.

Introduction of pRi oncogenes in crops

Transformation of plants using rhizogenic strains carrying a wild type Ri plasmid often relies on an in vitro co-cultivation protocol (Christensen and Müller 2009a; Karami 2008). The process is started by a selection of a wild type genotype that will be transformed (Fig. 1a). Ideally, an axenic culture of the plant is established, allowing fast propagation and year-round availability of plant material for protocol development. Specific parts, called explants, are harvested from the plantlets and used for further inoculation. Different types of explants vary in their susceptibility to bacterial infection, thus, multiple explant types are commonly tested (Cao et al. 2009; Ooi et al. 2013; Weber and Bodanese-Zanettini 2011). In some crops, the plant tissue culture can be quite challenging. For this reason, the tissue culture step is sometimes omitted by direct inoculation of surface-sterilized plant material or by the use of in vitro-germinated seedlings (Hegelund et al. 2017; Mugnier 1988). The second major component of the transformation protocol is the selection of a virulent rhizogenic strain and the preparation of an inoculum in a standardized way. Host range studies of *R. rhizogenes* have exhibited significant differences in plant susceptibility and revealed a specific strain relationship in terms of virulence with the plant host (De Cleene and De Ley 1981; Porter and Flores 1991). Therefore, multiple strains are often compared in terms of their virulence and transformation potential. In addition to the type of strain, also the preparation and density of the bacterial inoculum are important parameters influencing the success rate of co-cultivation and transformation (Desmet et al. 2019). Next, explants are submersed in a bacterial suspension for a fixed amount of time before being transferred to a co-cultivation medium. During the co-cultivation, the bacteria will infect the explants and transfer of pRi T-DNA occurs. Afterwards, the explants are submitted to an antibiotic treatment, which serves to eliminate residual *R. rhizogenes* from the explants. Successfully transformed explants will produce hairy roots from the sites of infection that serve as a source material for shoot regeneration (Tepfer 1984). Since no selectable markers are used, regenerated shoots are individually tested to confirm the transgenic nature. Transgenic-regenerated shoots are clonally propagated as unique Ri lines and are, after acclimatization to greenhouse conditions, further grown to facilitate phenotypic evaluation. Ri lines with interesting morphological features can then be further used in plant breeding (Fig. 1b).

Ri plasmid

Rhizogenic agrobacteria carry an extrachromosomal circular DNA structure referred to as the Ri plasmid. This virulence plasmid is essential for the pathogenicity of the bacterium and contains several regions with high homology to the tumor inducing (Ti) plasmid (pTi) of tumorigenic agrobacteria (Huffman et al. 1984; White and Nester 1980b). Ri plasmids are classified as mega-

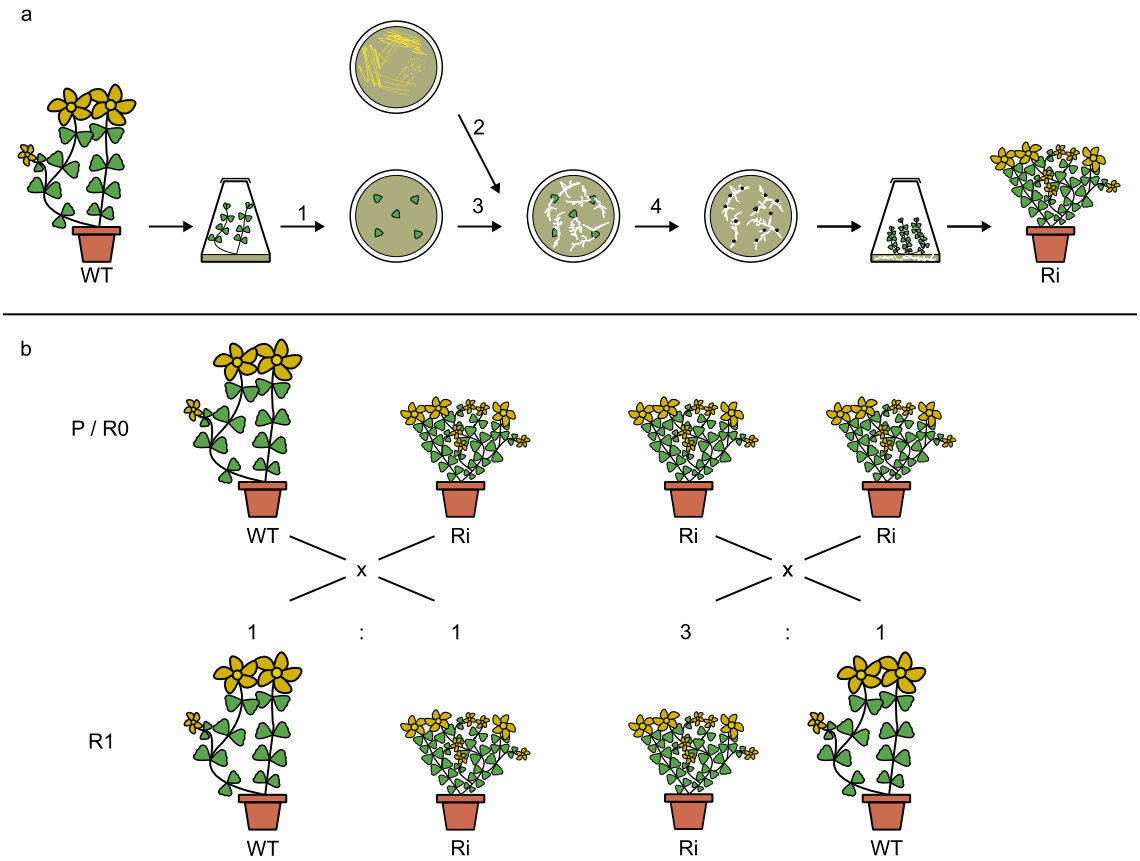

Fig. 1 Schematic overview of (**a**) natural pRi transformation. Numbers 1–4 depict steps in which optimization is needed: (1) selection of explant type and pre-culture; (2) selection of the bacterial strain and inoculum preparation; (3) method of inoculation and co-cultivation; (4) hairy root culture and regeneration. **b** application in plant breeding (WT, wild type; Ri, Ri line, plant derived from hairy root tissue; P, parental lines; R0, plants regenerated from hairy root tissue carrying pRi T-DNA genes; R1, first generation progeny of an Ri plant, ratios given apply to offspring of a single locus T-DNA insertion Ri line)

plasmids because of their relative large size, ranging from 180 to 250 kbp (Petersen et al. 1989; Suzuki et al. 2009). Different types of plasmids are classified based on the type of opine produced by transformed tissues (Moore et al. 1997). To date, four different opine and Ri plasmid (pRi) types have been described: agropine (pRiA4 of type strain A4), cucumopine (pRi2659 of type strain NCPPB2659), mannopine (pRi8196 of type strain NCIB8196), and mikimopine (pRi1724 of type strain MAFF301724) (Fig. 2) (Vladimirov et al. 2015). Parts of the plasmids with high similarity that are not transferred to the host plant include the virulence region (Hooykaas et al. 1984; White and Nester 1980a), the origin of replication (Jouanin et al. 1985), and the opine catabolism region (Moore et al. 1997). The transferred part of the Ri plasmid, the T-DNA, carries genes for opine biosynthesis, genes necessary for the initiation of hairy roots, among which the *root oncogenic loci* (*rol* genes) and other genes of unidentified function, i.e., open reading frames (ORFs) (Sinkar et al. 1987; Slightom et al. 1986). The T-DNA of agropine-type strains is physically split into the two parts: T_L-DNA and T_R-DNA. The T_L-DNA of agropine strains shares homology with the T-DNA of cucumopine, mannopine, and mikimopine strains, which consists of a single T-DNA (Jouanin 1984). In comparison, the T_R-DNA shares homology with pTi T-DNA in loci responsible for agropine synthesis and the auxin biosynthesis genes (*aux1* and *aux2* of pRi being homologous to *tms1* and *tms2* of pTi) (Camilleri and Jouanin 1991).

The phenotypic effects observed in Ri lines are the direct consequence of transfer, integration, and expression processes of pRi T-DNA to the host plant cell (Costantino et al. 1994). Upon successful completion of these steps, many of the T-DNA genes and ORFs will cause strong morphogenic effects that originate from underlying shifts in plant hormone metabolism. Through the last almost 40 years, numerous studies have addressed the phenotypes of the *rol* genes and ORFs in relation to the morphological changes observed in transformed plants. In comparison, the knowledge of the

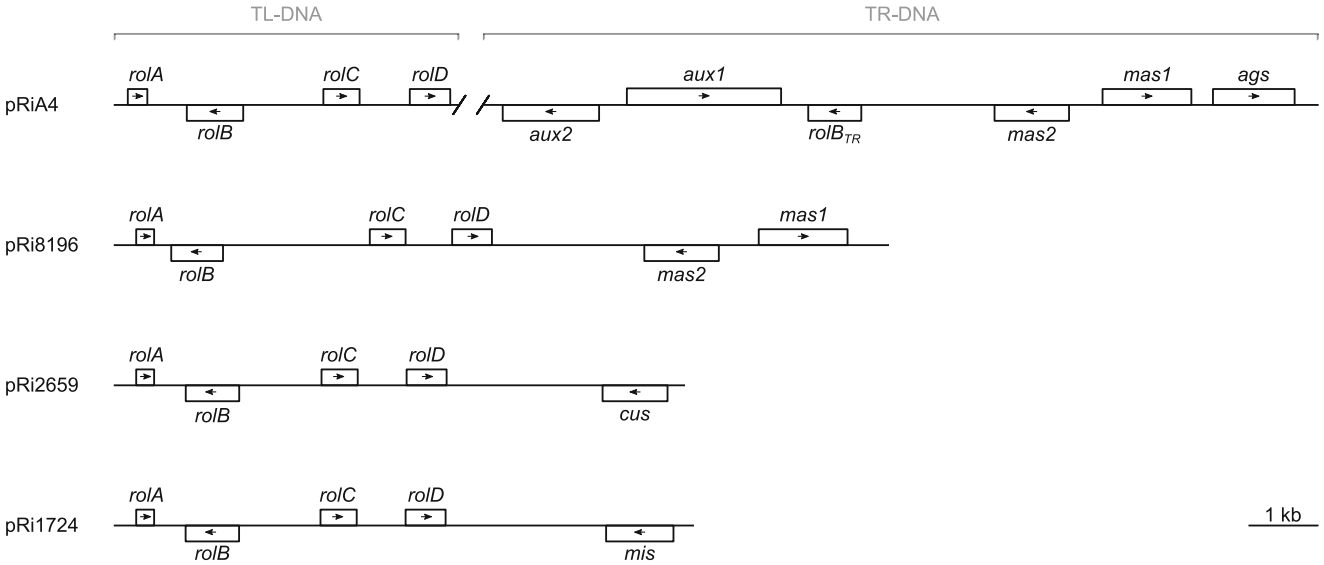

Fig. 2 Gene position schematic representation of the four types of root-inducing (Ri) plasmids (*ags agropine synthase*, *cus cucumopine synthase*, *mas mannopine synthase*, *mis mikimopine synthase*, scale bar 1 kb)

mechanisms per se and the protein functions mostly remain elusive. Many of the *rol* genes and ORFs lead to hormonal changes and altered cell division in plants. Some of the overall mechanisms are briefly described in the following. The *rolB* gene is the most well-characterized gene and known as a strong denominator of root induction as well as promoting floral meristems. Several modes of action have been proposed for the gene: tyrosine phosphatase (Filippini et al. 1996), indoxyl-beta-glucosidase stimulation of auxin binding to membranes (Estruch et al. 1991) as well as interaction with a 14-3-3 protein and subsequent localization in the nucleus (Moriuchi et al. 2004). *rolC* has been shown to have cytokinine beta-glucosidase activity. In respect to plant growth, ORF13 has been demonstrated to induce callus on carrot roots (Fründt et al. 1998), and when overexpressed, it led to extreme dwarfing as observed in *Arabidopsis* (Kodahl et al. 2016). Furthermore, *rolB*, *rolC*, and ORF13 resulted in dark green leaves. Moreover, leaf wrinkling was described for *rolA*, *rolB*, *rolB*$_{TR}$, and ORF13. In this respect, it can be speculated that there is a connection with cell division. In contrast, the function of *rolD* has been elucidated; it encodes a functional cyclodeaminase converting ornithine to proline, and it has been described as a stress-related osmoprotectant (Trovato et al. 2001). It seems that the *rol* genes and ORFs act synergistically in altering the transformed plants phenotypically (Spena et al. 1987; White et al. 1985). Further studies are needed to unravel the exact mechanisms of the *rol* genes and ORFs. One interesting avenue to elaborate on the functions of *rol* genes and ORFs will be targeting their corresponding gene products and studying protein interactions. For more thorough information of the mechanisms, we refer to Britton et al. (2008); Bulgakov (2008); Costantino et al. (1994); Mauro et al. (2017); Nilsson and Olsson (1997); and Otten (2018a).

Rhizogenic agrobacteria and plant breeding

Ri phenotype

Plants regenerated from hairy roots have an altered phenotype, which is commonly referred to as the "Ri phenotype" or "hairy root (HR) phenotype" (Christey 2001). Features of the Ri phenotype originally listed by Tepfer (1984) include wrinkled leaves, increased rooting ability, reduced apical dominance, and changes in flower morphology. Numerous studies have since been performed to obtain naturally transformed plants for which similar traits have been described. In the current study, an extensive literature review was conducted to compile a list of described Ri phenotypes. However, only studies which met the following criteria were selected: (1) a rhizogenic strain with a wild type Ri plasmid was used for transformation, (2) full plant regeneration from hairy root tissue was described, and (3) a clear description of the obtained Ri phenotype was given. A total of 91 studies provided a clear description of the Ri phenotype of 80 distinct plant species. From the 95 descriptions, several commonly recurring alterations of plant morphology can be derived. Based on the original description of Tepfer (1984), four definitive Ri traits were defined that influence different parts of the plant: (1) compact growth, (2) altered leaf morphology, (3) altered root morphology, and (4) altered flowering and fertility. Each definitive trait is broadly defined, such that a gradient of variation is encompassed (Table 1).

Table 1 Definitive traits of the Ri phenotype of different plant species transformed using a rhizogenic strain carrying a wild type Ri plasmid. Studies listed in this table all use rhizogenic strains with a wild type Ri plasmid for transformation, describe full plant regeneration from hairy root tissue, and give a clear description of the Ri phenotype

Plant species	Bacterial strain	Definitive trait				Reference
		Compact growth	Leaf morphology	Root morphology	Flower morphology and fertility	
Actinidia deliciosa	ArM123, IFO14555	x	x	x		Yamakawa and Chen 1996
A. deliciosa	MAFF301724	x	x	x		Yazawa et al. 1995
Ajuga reptans	MAFF301724	x	x		x	Tanaka and Matsumoto 1993
Alhagi pseudoalhagi	A4			x		Wang et al. 2001
Allocasuarina verticillata	A4, NCPPB2659	x	x	x		Phelep et al. 1991
Amaranthus spinosus	LBA9402	x	x	x		Pal et al. 2013
Angelonia salicariifolia	A13, D6	x	x			Koike et al. 2003
Antirrhinum majus	A4	x			x	Handa 1992a
A. majus	A13	x	x		x	Hoshino and Mii 1998
Apocynum venetum	LBA9402	x		x		Jia et al. 2008
Aralia elata	ATCC15834	x	x	x		Kang et al. 2006
Armoracia lapathifolia	ATCC15834	x	x	x		Saitou et al. 1991
A. lapathifolia	A4		x	x		Noda et al. 1987
Artemisia annua	LBA9402	x	x	x		Banerjee et al. 1997
Astralagus melilotoides	A4	x	x	x		Zhang et al. 2008
Atropa belladonna	ATCC15834, MAFF301724	x	x			Jaziri et al. 1994
Bacopa monnieri	A4, LBA9402	x	x	x		Majumdar et al. 2011
Brassica napus	A4	x	x		x	Hegelund et al. 2018
B. napus	A4	x	x	x	x	Guerche et al. 1987
Brassica oleracea	A4T	x	x		x	Christey and Sinclair 1993
B. oleracea var. *acephala*	ArM123	x		x		Hosoki et al. 1989
B. oleracea var. *botrytis*	ATCC15834, A4	x	x	x	x	David and Tempé 1988
Calibrachoa excellens	ATCC15834	x	x		x	Gennarelli et al. 2009
Catharanthus roseus	R1000	x	x	x		Choi et al. 2004
C. roseus	ATCC15834	x	x	x		Brillanceau et al. 1989
Centaurium erythraea	LBA9402	x	x	x	x	Piatczak et al. 2006
Cephaelis ipecacuanha	ATCC15834	x	x	x		Yoshimatsu et al. 2003
Cichorium intybus	A4, NCIB8196		x		x	Sun et al. 1991
Convolvulus arvensis	A4	x	x	x	x	Tepfer 1984
Datura arborea	NCPPB1855	x	x	x		Giovannini et al. 1997
Datura sanguinea	NCPPB1855	x	x	x	x	Giovannini et al. 1997
Daucus carota	A4	x	x	x	x	Tepfer 1984
D. carota	NCIB8196			x		David et al. 1984
Digitalis purpurea	A13	x	x		x	Koga et al. 2000a
Diospyros kaki	A4	x		x		Tao et al. 1994
Duboisia myoporoides x *Duboisia leichhardtii*	A4	x	x			Celma et al. 2001
Eustoma grandiflorum	A13, MAFF301724	x	x		x	Handa 1992b Handa et al. 1995
E. grandiflorum	NCPPB1855	x		x	x	Giovannini et al. 1996
Gentiana purpurea	ATCC15834	x	x			Momčilović et al. 1997

Table 1 (continued)

Plant species	Bacterial strain	Definitive trait				Reference
		Compact growth	Leaf morphology	Root morphology	Flower morphology and fertility	
Gentiana scabra	MAFF301724	x	x	x	x	Suginuma and Akihama 1995
G. scabra	A4	x	x		x	Mishiba et al. 2006
Gentiana triflora x *G. scabra*	A4	x	x		x	Hosokawa et al. 1997
G. triflora x *G. scabra*	A4	x	x		x	Mishiba et al. 2006
Helichrysum stoechas	ATCC15834	x			x	Giovannini 2006 Giovannini et al. 2008
Hybanthus enneaspermus	A4, NCIB8196	x	x	x		Behera et al. 2016
Hyoscyamus muticus	LBA9402	x	x		x	Sevón et al. 1997
H. muticus	A4	x	x	x	x	Oksman-Caldentey et al. 1991
Hypericum perforatum	ATCC15834	x				Bertoli et al. 2008
H. perforatum	ATCC11325, ATCC15834	x	x			di Guardo et al. 2003
H. perforatum	ATCC15834	x	x		x	Koperdáková et al. 2009
H. tomentosum	ATCC15834, A4	x	x	x		Henzelyová and Čellárová 2018
Ipomoea batatas	MAFF210266	x				Otani et al. 1993
Ipomoea trichocarpa	A13, NIAES1724	x	x	x	x	Otani et al. 1996
Kalanchoe blossfeldiana	ATCC15834	x	x		x	Christensen et al. 2008 Christensen and Müller 2009b
Larix decidua	ATCC11325					Huang et al. 1991
Lavandula x *intermedia*	A5, A13	x		x	x	Tsuro and Ikedo 2011
Linum usitatissimum	NCPPB1855	x	x	x		Zhan et al. 1988
Lotus corniculatus	C58C1	x	x		x	Webb et al. 1990
Lycopersicon peruvianum	NCIB8196	x	x	x		Peres et al. 2001
Macuna pruriens	MTCC2364, MTCC532					Vishwakarma et al. 2017
Malus prunifolia	MAFF210265	x	x	x		Yamashita et al. 2004
Malus x *domestica*	A4	x		x		Lambert and Tepfer 1992
Mecardonia sp.	ATCC15834	x	x	x		Pérez de la Torre et al. 2018
Medicago sativa	A4T	x	x		x	Golds et al. 1991
M. sativa	NCPPB1855	x	x	x		Spanò et al. 1987
Nicotiana glauca	A4	x	x	x		Taylor et al. 1985
Nicotiana hesperis	LBA9402	x	x		x	Hamill and Rhodes 1988
Nicotiana tabacum	A4	x	x	x		Taylor et al. 1985
N. tabacum	A4	x	x	x	x	Tepfer 1984
Nierembergia scoparia	A13	x	x	x		Godo et al. 1997
Ophiorrhiza pumila	ATCC15834	x	x			Watase et al. 2004
Orobrychis viciifolia	A4T	x	x	x		Golds et al. 1991
Panax ginseng	ATCC15834			x		Yang and Choi 2000
Papaver somniferum	MAFF301724	x	x			Yoshimatsu and Shimomura 1992
Pelargonium fragrans	HRi	x	x	x		Pellegrineschi and Davolio-Mariani 1996
Pelargonium graveolens	A4, LBA9402	x	x	x	x	Saxena et al. 2007

Table 1 (continued)

Plant species	Bacterial strain	Definitive trait				Reference
		Compact growth	Leaf morphology	Root morphology	Flower morphology and fertility	
Pelargonium odoratissimus	HRi	x	x	x		Pellegrineschi and Davolio-Mariani 1996
Pelargonium quercifolia	HRi	x	x	x		Pellegrineschi and Davolio-Mariani 1996
Picrorhiza kurrooa	A4	x	x	x		Rawat et al. 2013
Plumbago indica	ATCC15834	x		x		Gangopadhyay et al. 2010
Plumbago rosea	A4	x	x			Satheeshkumar et al. 2009
Pogostemon cablin	ATCC15834	x	x	x		He-Ping et al. 2011
Prunus avium x *Prunus pseudocerasus*	NCPPB1855	x	x	x	x	Gutièrrez-Pesce et al. 1998 Rugini et al. 2015
Rauvolfia serpentina	A4	x	x	x	x	Mehrotra et al. 2013
Rehmannia elata	R1000	x	x		x	Kim et al. 2012
Rehmannia glutinosa	ATCC15834	x		x		Zhou et al. 2009
Rhaponticum carthamoides	A4	x	x	x		Skała et al. 2019
Rudbeckia hirta	A5		x	x	x	Daimon and Mii 1995
Solanum dulcamara	A4T	x	x	x		McInnes et al. 1991
Solanum tuberosum	LBA9402		x	x		Ooms et al. 1985
S. tuberosum	ATCC15834, LBA9402	x		x		Hänisch ten Cate et al. 1988
Taraxacum platycarpum	ATCC15834			x		Lee et al. 2004
Tylophora indica	A4, LBA9402	x	x	x		Chaudhuri et al. 2006
Vaccaria pyramidata	A13	x	x		x	Koga et al. 2000b
Vinca minor	A4	x	x	x		Verma et al. 2017

The most prominent Ri trait is a degree of compact growth resulting from multiple morphological changes. Plants with shorter internodes display a reduced total plant height. Also, increased branching and enhanced axillary bud growth, alongside with reduced apical dominance, further contribute to the compact growth habit of the plant. The change in compactness is one-directional, i.e., all described Ri plants have a similar or more compact growth habit compared with their wild type control. Shorter internodes of Ri lines have been reported in a wide range of plant species such as *Catharanthus roseus*, *Prunus avium* x *Prunus pseudocerasus*, *Allocasuarina verticillata*, and *Lavandula* x *intermedia* (Choi et al. 2004; Gutièrrez-Pesce et al. 1998; Phelep et al. 1991; Tsuro and Ikedo 2011). The reduction in apical dominance is observed through increased branching in Ri lines of *Centaurium erythraea* and *Hyoscyamus muticus* (Piatczak et al. 2006; Sevón et al. 1997). However, in some cases, the changes in terms of plant architecture have a compensating effect on each other. For example, a *Plumbago indica* Ri line exhibited longer shoots; however, the increase in individual shoot length was compensated for by a decrease in internodal length, hence maintaining a similar degree of compactness (Gangopadhyay et al. 2010). A comparable compensatory mechanism for the number of internodes and their individual length has been observed for the Ri phenotype of *Datura arborea* (Giovannini et al. 1997). However, for *K. blossfeldiana*, *Mecardonia* sp. and *Medicago sativa*, this compensation does not occur, and a more compact plant phenotype was obtained (Christensen et al. 2008; Pérez de la Torre et al. 2018; Spanò et al. 1987).

Altered leaf morphology is the second most frequent observed change in Ri phenotypes. In most cases, this consists of strong wrinkling of the leaves, coincided by changes in leaf size. In addition to this, differences in leaf pigmentation are common. Several reports correlate the coloration of darker green leaves with higher chlorophyll content (Jaziri et al. 1994; Koike et al. 2003); however, also more pale and yellowish leaves have been observed (Mehrotra et al. 2013). Discoloration and strong wrinkling effects could potentially have an effect on the growth, photosynthetic capacity, and yield of Ri plants, especially when this phenotype progresses to be more extreme as the plant ages (Giovannini et al. 1997; Webb et al. 1990). More recently, Rugini et al. (2015) investigated chlorophyll a and b content in leaves of *P. avium* x

P. pseudocerasus Ri lines and found no significant difference compared with wild type plants. In some plant species, a change in the number of leaves per Ri plant has been noted: an increased number of leaves was observed in the species *Pelargonium fragrans*, *Pelargonium odoratissimus*, *Pelargonium quercifolia*, *Pelargonium graveolens*, and *Ajuga reptans* (Pellegrineschi and Davolio-Mariani 1996; Saxena et al. 2007; Tanaka and Matsumoto 1993), whereas a decreased number of leaves were found for *Digitalis purpurea* and *Rhaponticum carthamoides* (Koga et al. 2000a; Skała et al. 2019). The leaf number increase is developmentally correlated with the number of nodes and branching pattern. However, for *D. purpurea*, also increased branching was observed (Koga et al. 2000a). An important difference to note here is that both incidences of reported decrease in the number of leaves per plant relate to rosette forming species.

The third foremost trait groups changes to the root morphology of Ri plants. In two-thirds of the Ri phenotypes, a clear alteration in root morphology was reported. This trait relates back to the biological essence of the hairy root syndrome (Moore et al. 1979; Nester 2015). Infected hosts are, by natural means of T-DNA insertion, altered to produce differentiated, neoplastic roots (Costantino et al. 1994). The implication is that hairy roots carry a genetically underlying basis that enhances adventitious root formation. Hairy roots are characterized by fast growth, a high degree of lateral branching, and plagiotropic development (Christey 2001; Tepfer 2016). Likewise, in Ri plants, this genetic basis leads to enhanced rooting ability as observed in many Ri phenotypes, both in vitro as in greenhouse or field conditions. This more extensive root system has pronounced implications for the applicability of Ri plants. Firstly, the increase in rooting ability can lead to efficient vegetative propagation and better adaptation to ex vitro conditions (Casanova et al. 2005). Moreover, Ri plants with better developed root systems are promising in terms of sustainable plant production/agriculture, because of improved water and nutrient management, with for example better drought tolerance (Tepfer 2016). Secondly, increases in root biomass are commonly reported, a trait especially beneficial for root crops or when roots are used for extracting specific metabolites (Chaudhuri et al. 2006; Gangopadhyay et al. 2010; Majumdar et al. 2011; Pérez de la Torre et al. 2018; Sevón et al. 1997).

A fourth major change that can be expected encompasses changes in flower morphology, flowering onset and fertility of the plant. While decreases in flower size have been reported in Ri lines of *Rudbeckia hirta*, *Brassica napus, Rehmannia elata*, *Ipomoea trichocarpa*, and *P. graveolens* (Daimon and Mii 1995; Hegelund et al. 2018; Kim et al. 2012; Otani et al. 1996; Saxena et al. 2007), an increase in flower size has not been reported. Another compensatory mechanism seems to be present in terms of flowering, with the size and number of flowers per plant being inversely correlated, i.e., reduced flower size generally coincides with an increase in the number of flowers. However, Hosokawa et al. (1997) reported Ri lines of *Gentiana triflora* x *Gentiana scabra* to have an increased number of flowers without apparent changes to the size of individual flowers. In this case, the increase in flower number is consequential of increased branching and a reduction in apical dominance. The onset of flowering is also influenced by the presence of pRi T-DNA, with delayed flowering being the most prominent change. Earlier flowering has only been observed for *Helichrysum stoechas* and *G. scabra* Ri lines (Giovannini 2006; Suginuma and Akihama 1995).

An important point to address is phenotypic variability of Ri lines within the same plant species. Pronounced morphological differences in Ri lines of *G. triflora* x *G. scabra* (Hosokawa et al. 1997), *P. avium* x *P. pseudocerasus* (Gutièrrez-Pesce et al. 1998; Rugini et al. 2015), and *B. napus* (Guerche et al. 1987; Hegelund et al. 2018) have been reported. Similarly, the fertility of *Antirrhinum majus* Ri lines varied (Handa 1992a; Hoshino and Mii 1998). The range of phenotypic variation is useful in a plant breeding program; different Ri lines can be used to introduce specific traits in crops. Since it is known that hairy roots are clonal of origin (Chilton et al. 1982; Van Sluys and Tempé 1989), differences in Ri phenotype severity have been ascribed to several influencing variables, such as (1) the plant species and genotype, (2) the bacterial strain used for transformation, and (3) the genetic details of the transformation event itself (Christensen and Müller 2009a; Gelvin 2017; Koncz and Schell 1992). Genetic details which render Ri lines a unique character include the following: the inserted copy number of pRi oncogenes, complete or truncated transfer of the T-DNA, expression levels of the transferred genes, and positional integration effects (Golds et al. 1991; Hänisch ten Cate et al. 1990; Lütken et al. 2012a; Sun et al. 1991).

The importance of the rhizogenic phenotype in breeding applications

Natural pRi T-DNA transformation, through underlying genetic changes, results in plants that display the rhizogenic phenotype. Since wild type strains are used in this technique, the processing, transfer, and integration of the T-DNA are governed by natural means. This implies that the transformation event is pleiotropic in its effects on the host plant. Multiple phenotypic traits are affected by one transformation event, leading to changes which, depending on the point of view of the breeder and the specific use of the plant species, can be interpreted as wanted or unwanted changes (Christey 2001).

The importance of plant architecture and its modification through plant breeding has long been recognized, with compact plant architecture being a desirable trait in agriculture and horticulture (Coyne 1980; Hammerschlag and Smigocki

1998; Hauptli et al. 1990; Lütken et al. 2012a). In many crops, such as legume and cereal grains and sugarcane, compactness and branching pattern are important traits in relationship to lodging and productivity (Berry et al. 2004; Heath et al. 1994; Pribil et al. 2007). In ornamentals, compact growth is inherently linked to plant quality and economic viability of the plant product (Bergstrand 2017; Rademacher 2015). Ri lines of *K. blossfeldiana* with increased branching, reduced apical dominance, and shorter internodes have already been successfully applied in relation to commercial plant breeding to enhance compact growth (Christensen and Müller 2009a; Lütken et al. 2012b). Likewise, Koga et al. (2000a) created *D. purpurea* Ri lines to obtain dwarf cultivars. Alterations of leaf color, flower morphology, or the number of flowers can improve the ornamental value of plants (Casanova et al. 2005; Kim et al. 2012). The increased rooting ability of Ri lines has already been used to facilitate rooting of *Malus* x *domestica* cuttings (Lambert and Tepfer 1992). On the other hand, changes such as severe leaf wrinkling could negatively impact the photosynthetic capacity of crop canopy or reduce the visual appeal of ornamentals. For generatively propagated crops, reduced fertility is unwanted. In addition, a delay in flowering time could negatively affect production time for many ornamentals (Lütken et al. 2012b).

Due to the fact that in Ri lines, both desired as undesired traits can occur; a plant breeding approach based on introgression of specific Ri traits should be used. The implementation of Ri lines in existing breeding programs can offer an elegant way to obtain a phenotype with specific Ri traits, while maintaining the initial commercial/breeding value of the line after subsequent back-crossing (Christensen et al. 2008; Otten 2018b). Moreover, it has been well established that the inserted T-DNA is transmissible through meiosis and that the Ri phenotype is inheritable, mainly in a dominant Mendelian fashion (David et al. 1984; Durand-Tardif et al. 1985). Therefore, the segregation ratio of pRi T-DNA inserts is sufficiently high to allow further breeding.

To date, a limited number of studies have investigated pRi T-DNA inheritance in detail. Out of the aforementioned references with described Ri phenotypes, those with (1) a clear description of a breeding step with an Ri line as one of the parents and (2) described inheritance of the Ri phenotype were selected. In addition to this, the breeding with Ri lines described by Durand-Tardif et al. (1985), Limami et al. (1998), and Lütken et al. (2012b) was included (Table 2). Similarly to the generation nomenclature of plants derived from genetic modification, the generations are designated as R0, R1, and R2 (Yin et al. 2004). From the described R1 and R2 generations, it is clear that inheritance of pRi T-DNA takes place, regardless of whether the fact that progeny was obtained by either selfing or crossing. It is generally accepted that T-DNA insertion leads to plants that are hemizygous for the locus of insertion, both for events in which single or multiple copies (as direct or inverted repeats) are inserted at the same locus (Gelvin 2017; Sridevi et al. 2006). Thus, an R0 plant with a single locus T-DNA insert will transmit this in a dominant Mendelian way with a phenotype/genotype ratio of 3:1/1:2:1 in the case of selfing and 1:1/1:1 for a backcross with a wild type parent (Fig. 1b). Segregation can be confirmed by either phenotypic or molecular methods. Since opines are known to diffuse through plant tissue and variability of the Ri phenotype is common (Hegelund et al. 2017; Zhan et al. 1988), molecular techniques such as polymerase-chain reaction (PCR) and Southern hybridization are preferred to confirm segregation ratios.

Expected Mendelian segregation for single and double locus insert Ri lines has been observed for *Nierembergia scoparia* and *B. napus*, respectively (Godo et al. 1997; Guerche et al. 1987; Jouanin et al. 1987). However, segregation distortion was observed in R1 generations of *Daucus carota*, *Calibrachoa excellens*, and *A. majus* (David et al. 1984; Gennarelli et al. 2009; Handa 1992a) (Table 2).

Plant genotype, Ri phenotype, and segregation are linked and mediated through T-DNA structure and expression. The Ri phenotype in itself, as a direct consequence of the transformation event and therefore the R0 genotype, is not always sufficient to fully explain segregation and heredity. Copy number and the number of insertion sites both influence segregation; as the number of insertion sites increases, so does the complexity of segregation patterns (Jouanin et al. 1987). Additionally, homozygosity for the Ri locus is thought not to lead to direct changes in Ri phenotype (Durand-Tardif et al. 1985; Tepfer 1984), but it is still desired from a breeding point of view because of its influence on segregation ratios (Martin-Tanguy et al. 1990; Passricha et al. 2016). In summary, differences in segregation ratios and subsequent variability of the Ri phenotype can be explained by the nature of the recombination and the genetic makeup of the R0 line used to create the progenies. Detailed molecular characterization of R0 lines thus provides highly relevant information for the breeding process.

Legislation and legal aspects

In respect to legislation, the first and most important issue to address is whether modified or unmodified bacterial strains of *R. rhizogenes* are used. Application of modified strains, i.e., strains where recombination of nucleic acids has been made, results in GMOs according to legislation in the European Union (EU) (European Union 2001, Directive 2001/18/EC, Annex I A, part 1§1). Hence, gene insertions, deletions, and genome editing such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) combined with a CRISPR-associated protein 9 (Cas9), CRISPR/Cas9, all fall under the GMO definition. Consequently, using transformation with

Table 2 Overview of breeding with Ri lines in different plant species

Plant species	Generation[a]	Segregation[b]		Molecular screening	R0 copy number	Reference
		Phenotype	Genotype			
Antirrhinum majus	R1 (S)	36:4	36:4	pRiA4 T_L-DNA	2 copies	Handa 1992a
Brassica napus	R1 (S)	68:27	Ql	pRiA4 T_L-DNA + T_R-DNA	3 + 1 or more copies	Guerche et al. 1987
		87:31			3 + 1 or more copies	
		112:7			4 + 4 copies	
		86:22			3 + 2 copies	
		94:25			3 + 2 copies	
	R1 (BC: P x R0)	42:45	Ql	pRiA4 T_L-DNA + T_R-DNA	3 + 1 or more copies	
		45:47			3 + 1 or more copies	
		63:22			4 + 4 copies	
		77:16			4 + 4 copies	
		31:26			3 + 2 copies	
Brassica napus	R1 (S)	80:42:0:18[c]	80:42:0:18[c]	pRiA4 *rolB* + *aux1*	1 + 1 copy	Hegelund et al. 2018
Calibrachoa excellens	R1 (BC: R0 x P)	4:21	4:21	pRiA4 *rolC*	1 copy	Gennarelli et al. 2009
Cichorium intybus	R1 (S)	Ql	Ql	pRiA4 T_L-DNA + T_R-DNA	2 copies	Sun et al. 1991
	R1 (BC: R0 x P)	Ql	Ql	pRiA4 T_L-DNA + T_R-DNA		
	R1 (BC: P x R0)	Ql	Ql	pRiA4 T_L-DNA + T_R-DNA		
Cichorium intybus	R1 (BC: R0 x P)	Ql	Ql	pRiA4 T_L-DNA	1 complete + 1 truncated copy	Limami et al. 1998
	R2 (R1 x R1)	Ql	Ql			
Daucus carota	R1 (S)	9:8	NT	pRi8196 T-DNA	2 copies	David et al. 1984
Eustoma grandiflorum	R1 (S)	Ql	Ql	pRi1724 T-DNA	1 copy	Handa et al. 1995
Helichrysum stoechas	R1 (S)	4:4	4:4	pRiA4 *rolC*	NT	Giovannini et al. 2008
Ipomoea trichocarpa	R1 (S)	3:1	NT	NT	NT	Otani et al. 1996
Kalanchoe blossfeldiana	R1 (BC: P x R0)	9:10	9:10	pRiA4 T_L-DNA	2 copies	Lütken et al. 2012b
	R2 (R1 x R1)	29:18	29:18			
Lycopersicon peruvianum	R1 (BC: R0 x P)	Ql	NT	ORF13	NT	Peres et al. 2001
Nicotiana glauca	R1 (S)	4:4	4:4	pRiA4 T_L-DNA + T_R-DNA	1 copy	Taylor et al. 1985
Nicotiana tabacum	R2 (R1 x R1)	54:25:42[d]	Ql	pRiA4 T_L-DNA	Ql	Durand-Tardif et al. 1985
	R2 (R1 x P)	64:9:75[d]	Ql	pRiA4 T_L-DNA	Ql	
Nicotiana tabacum	R1 (S)	29:26:14[d]	Ql	pRiA4 T_L-DNA	Ql	Tepfer 1984
		22:36				
		37:33:6[d]				
		19:27:14[d]				
	R1 (BC: R0 x P)	37:32	Ql	pRiA4 T_L-DNA	Ql	
		35:33				

Table 2 (continued)

Plant species	Generation[a]	Segregation[b]		Molecular screening	R0 copy number	Reference
		Phenotype	Genotype			
		38:56				
		34:30				
	R1 (BC: P x R0)	35:33	Ql	pRiA4 T_L-DNA	Ql	
		38:34				
		37:21				
		50:35				
Nierembergia scoparia	R1 (BC: R0 x P)	22:24	Ql	pRi1724 T-DNA	1 copy	Godo et al. 1997

[a] *R0*, plants regenerated from hairy root tissue carrying pRi T-DNA genes; *R1*, *R2*, progenies obtained by using R0 plants as parent(s); *P*, parent plant of the same species; *S*, self-fertilization; *BC*, backcross ♀ × ♂

[b] Ratio, present:absent

[c] Ratio = *rol+/ aux+*: *rol+ / aux-*: *rol−/aux+*: *rol−/ aux−*

[d] Ratio = exaggerated Ri phenotype:Ri phenotype:WT phenotype; *NT*, not tested; *Ql*, qualitative data was collected

modified strains is not applicable as a commercial breeding method in the EU.

Interestingly, the same directive also describes exemptions from the rule defining which techniques do not result in a GMO in the EU; natural processes such as conjugation, transduction, and transformation belong to this category (European Union 2001, Directive 2001/18/EC, Annex I A, part 2§2). Based on the definition of the latter, usage and application of unmodified bacterial strains have also been termed natural transformation (Christensen and Müller 2009b; Christensen et al. 2008; Lütken et al. 2012a). This, therefore, represents an interesting avenue for potential commercial application based on natural transformation. The natural transformation concept is supported by several studies showing that a number of wild plants actually contain remnants of *R. rhizogenes* T-DNAs, i.e., they have been transformed naturally during plant speciation. To this group belongs, e.g., tobacco, sweet potato and several species of *Linaria* (Kovacova et al. 2014; Kyndt et al. 2015; Matveeva et al. 2012; Suzuki et al. 2002). A recent study even indicates that an additionally 23 species contain bacterial T-DNA (Matveeva and Otten 2019).

In Denmark, authorities have confirmed that plants obtained through the biotechnological method of using unmodified strains of *R. rhizogenes* are non-GMOs (Lütken et al. 2012a). Furthermore, in Japan, unmodified strains of *R. rhizogenes* similarly do not fall under the GMO definition (Mishiba et al. 2006). In the USA, plants transformed with potential plant pathogens have to be evaluated by the Animal and Plant Health Inspection Service (APHIS) under the United States Department of Agriculture (USDA). The criteria are described under APHIS's Plant Protection Act 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason To Believe Are Plant Pests." Based on this, APHIS stated that *Kalanchoe* obtained following natural transformation does not fall under the term "altered or produced through genetic engineering," and hence, the plants do not require specific regulations as they do not fall under the Plant Protection Act 7 CFR part 340.

In respect to breeders' rights of plants derived following natural transformation with *R. rhizogenes*, one strategy is to protect naturally transformed plant as new cultivars. This can apply if the plant clearly exhibits new and distinctive traits, which has to be described and registered officially. Moreover, it must be possible to propagate the plant consistently. However, one might be aware that the introduction of *rol* genes in a protected plant is actually leading to the creation of an essentially derived variety (EDV) which cannot be protected itself (Krieger et al. 2019).

Another strategy has also been pursued to protect interspecific hybrids of *Kalanchoe* transformed with unmodified *R. rhizogenes* strains with the aim of obtaining the desired intermediate plant height. The plants produced with this

method are covered by a patent (EP2698432A1, Christensen et al. 2014). The disclosure of the patent specifically includes *Kalanchoe* interspecific hybrid plants, and considers *rol*-transformation in *Kalanchoe* species and hybrids. The methodology discloses production of *rol*-transformed *Kalanchoe* interspecific hybrid plants, as well as resultant *rol*-transformed *Kalanchoe* interspecific hybrid plants with novel phenotypes.

Conclusion

In terms of practical breeding applications, co-cultivation with rhizogenic agrobacteria has potential to deliver pre-breeding material. Having efficient tissue culture protocols for transformation and regeneration ensures the creation of many Ri lines. Thorough genetic identification of lines from unique transformation events will facilitate the implementation in conventional and molecular plant breeding. The exact segregation ratio of the pRi T-DNA and its accompanying phenotype, based on molecular characterization, is essential for accurate application in breeding programs and allows breeders to make informed decisions. To date, many of the studies involving the transformation of plants using wild type rhizogenic agrobacteria and the Ri phenotype end without the continuation to the plant breeding process. Furthermore, in those cases for which Ri breeding is studied, the majority of the generated knowledge is mostly based on qualitative observations that, while valuable, are at best indicative of the usefulness of Ri lines in plant breeding. There is a need for studies with well-defined Ri lines that integrate both phenotypic and molecular based approaches in a range of crops. Such knowledge and detailed examples will help the implementation of Ri breeding on a larger scale.

Funding information This study was funded by VLAIO (LA grant number 150889).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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