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Genetic Transformation of Carrot (*Daucus carota*) and Other *Apiaceae* Species

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ABSTRACT

Carrot (*Daucus carota* L.) is well known as a model species for plant tissue culture systems. It is also amenable to genetic modifications using both vector and non-vector methods. Several works have been commenced to study factors affecting transgenesis in this species. As a result, optimized transformation protocols have been established. Genetically modified *Ammi*, *Anethum*, *Apium*, *Bupleurum*, *Carum*, *Centella*, *Coriandrum*, *Foeniculum*, *Levisticum*, *Petroselinum*, *Peucedanum* and *Pimpinella* belonging to the *Apiaceae* family have also been obtained. However, unlike carrot, most of them were mainly used for hairy root development after *Agrobacterium rhizogenes*-mediated transformation. Extensive research in carrot has focused on studying gene function and regulation, and plant metabolism. Applied research includes the development of plants resistant to both abiotic and biotic stresses, and modifications of biosynthetic pathways. As the *Apiaceae* family is an important reservoir of condiments and medicinal plants, some species were studied for the ability of their hairy roots to produce pharmaceutically principal secondary metabolites as well as for phytoremediation. This review provides an overview on the essential genetic transformation studies conducted in the *Apiaceae* family with insight into the application of these genetically modified species for basic and applied research.

Keywords: *Agrobacterium*, hairy root, non-vector transformation, *Umbelliferae*
Abbreviations: CaMV, cauliflower mosaic virus, **EP**, electroporation; **GM**, genetically modified; **GUS**, β-glucuronidase; **NPTII**, neomycin phosphotransferase II; **PB**, projectile bombardment; **PEG**, polyethylene glycol; **PGR**, plant growth regulator; **SE**, somatic embryogenesis

CONTENTS

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Invited Review

INTRODUCTION - CARROT, A MODEL SPECIES FROM *APIACEAE* FAMILY

Apiaceae (*Umbelliferae*) is a cosmopolitan family comprising 455 genera and over 3500 species, which makes this family one of the largest taxon among higher plants (Pimenov and Leonov 1993). They can be distinguished by characteristic morphology of the inflorescence, a compound umbel, with flowers occurring in unbellets arranged radially. The most commonly cultivated members of the family are carrot (*Daucus carota*), celery (*Apium graveolens*), parsley (*Petroselinum crispum*) and parsnip (*Pastinaca sativa*) used as vegetables or feed crops grown on more than 1.2 million ha worldwide with annual production estimated at over 25 million tonnes. The importance of root vegetable carrot, which is the primary source of pro-vitamin A in the human diet, is denoted by the fact that its production has increased over 40% during the last decade (FAOSTAT 2006, http://faostat.fao.org).

The *Apiaceae* taxa are also distinguishable by the occurrence of umbelliferose, petroselinic acid and polyacetylenes, which are characteristic compounds in this family. They also contain several specific phenols, phenylpropanoids, terpenes, saponins and coumarins in fruits, leaves or roots (Hegnauer 1990). These bioactive compounds make *Apiaceae* species well recognised in traditional medicine. Also several *Apiaceaeous* condiments are desired for culinary purposes. Despite the large diversity of this family, the use of biotechnological methods for basic research is restricted to a small number of species only. Achievements in applied research, which would lead to the development of new technologies substantial for industry, are still in the shadow of top economically important crops. Nevertheless, substantial progress can be noticed as the number of studies employing modern biotechnology in this family is increasing. Certainly carrot is the most notable *Apiaceae* species, to which biotechnology is widely utilized. Biotechnology of parsley and celery is far less advanced, while that of other species is considered as occasional.

Genetic manipulations of higher plants (except *Arabidopsis*) rely nowadays almost exclusively on the use of plant tissue culture and the establishment of protocols for stable, efficient and genotype-independent *in vitro* culture systems has become essential. Thus it is not surprising that carrot has been used extensively in biotechnology research for decades. In fact, it was one of the pioneer species used to develop tissue culture systems. The first two independent reports came in 1939 from French scientists R. J. Gautheret and P. Nobècourt who produced stable callus tissue from root discs. The second milestone, reported by Steward 20 years later, revolutionized the future of biotechnology. His group developed a system for freely suspended cells (Steward *et al.* 1958) and proved the theory of totipotency showing the ability of a single somatic cell for development into the whole plant (Steward 1958). Those findings opened the door for the development of tissue culture techniques for other plant species, too. Nowadays, we experience that virtually any part of a carrot plant can be a source for successful tissue culture, independent of its stage of development. Relatively simple callus initiation and maintenance as well as high potential for both organogenesis and somatic embryogenesis made carrot a model species suitable for studying various processes, like morphogenesis, somatic embryogenesis, somaclonal variation or protoplast recovery. The successful development of carrot tissue culture techniques and establishment of effective protocols were also essential prerequisites for genetic modifications, the next step in carrot biotechnology. Since the 1980s, gene transfer has become commonly used for modification of the carrot genome to study gene function and regulation, plant metabolism as well as for the improvement of agronomic and quality traits, in more recent years.

This review describes methods of genetic transformation conducted in *Apiaceae* species with stress placed on carrot. The second part is devoted to paths of genetic manipulation that show the applications of genetically modified (GM) carrot and other *Apiaceae* species in both basic and applied research. Selected aspects of carrot transformation, particularly dealing with the use of *A. tumefaciens* and the production of herbicide as well as disease-resistant plants, have been also reviewed in a book chapter by Punja *et al.* (2007).

TRANSFORMATION METHODS – CARROT

Most of the available transformation techniques have been exploited in carrot research. The most common system utilizes *Agrobacterium* as a vector for gene transfer including both *A. tumefaciens* and *A. rhizogenes* species. In contrast, non-vector methods of direct DNA transfer like, microprojectile bombardment or DNA uptake into protoplasts mediated by polyethylene glycol or electroporation are only occasionally used.

Agrobacterium tumefaciens-mediated transformation

A. tumefaciens is definitely the most commonly used vector for transformation of higher plants, which is also reflected in the number of studies conducted on carrot. The use of *A. tumefaciens* for carrot transformation was a natural consequence of successes demonstrated in other species like tobacco. The first report on *A. tumefaciens*-mediated carrot transformation was published in 1987 by Scott and Draper who used highly embryogenic suspension culture of cultivar 'Early Nantes'. The choice of a genotype capable of developing many plants via somatic embryogenesis (SE) allowed an elevated production frequency of transgenic cell colonies estimated on 60% when compared to the non-transformed control and assessed as the number of colonies surviving on 100 mg/l kanamycin-enriched selection medium. The transgenic cells underwent embryogenesis and phenotypically normal plants were subsequently recovered. Both callus and plants that were formed could synthesize

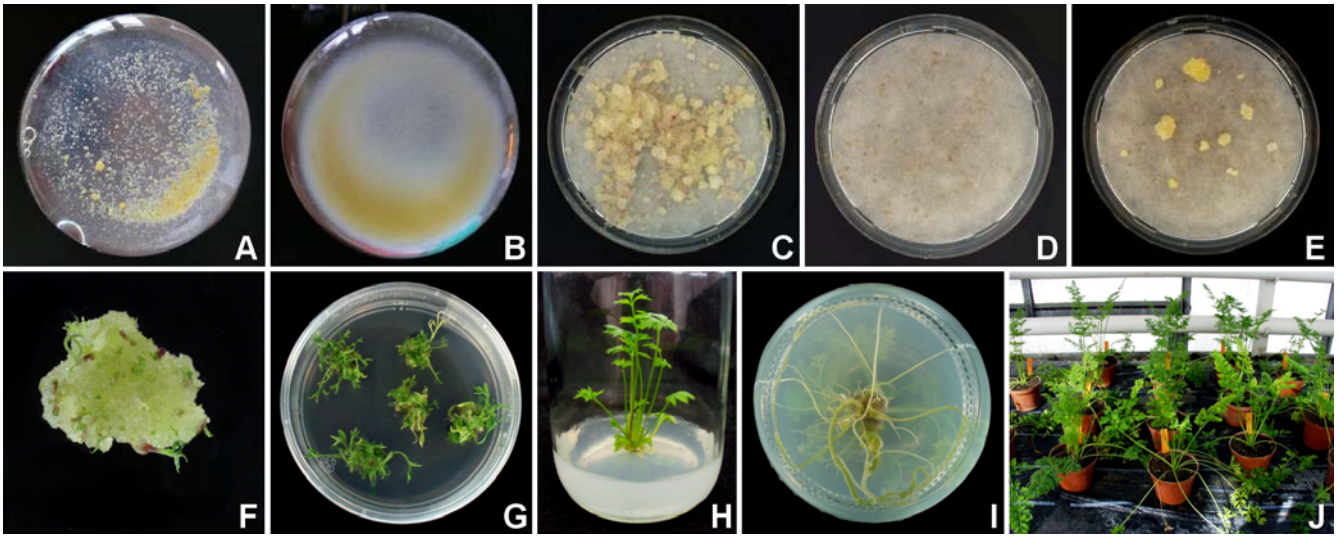

Fig. 1 *A. tumefaciens*-mediated transformation of carrot cell suspension and the development of transgenic carrot plants: one-week-old initial cell suspension derived from callus (**A**), established 12 week-old cell suspension (**B**), growth of the non-transformed callus on a solid medium (**C**), died non-transformed cells on the kanamycin selection medium (**D**), selection of the transgenic calli on the kanamycin enriched medium (**E**), shoot regeneration from the kanamycin resistant callus (**F**), micropropagation (**G**), shoot development (**H**), rooting (**I**) and transgenic plants in the glasshouse (**J**).

nopaline and the transgene integration into carrot genome was confirmed by 1 to 8 hybridization signals of the *npt*II (neomycine phosphotransferase II) probe after Southern blot analysis.

As almost all carrot explants can be used for tissue culture, an obvious variety of *in vitro* culture techniques were subsequently utilized in carrot genetic transformation (**Fig. 1**). Several studies on the development of various methods enabled the study of factors influencing the transformation process and, in consequence, more efficient protocols were established (**Table 1**). However, they cannot be considered as versatile, which was emphasized by Hardegger and Sturm (1998) who compared published methods available at that time and concluded that they did not support the development of a large number of independent transgenic cell lines. To overcome this problem factors affecting transgenesis were evaluated and the most important ones were indicated. Certainly two of the most crucial factors affecting carrot transformation are plant genotype and bacterial strain.

Plant material

Depending on carrot genotype, the transformation rate varied considerably from 1–6% (Thomas *et al.* 1989), 0–47% (Pawlicki *et al.* 1992), 2–12% (Gilbert *et al.* 1996) and 1–12% (Takaichi and Oeda 2000). However, direct comparison of these values is ambiguous as they were obtained from different tissue culture systems and were calculated in various manners. Pre-cultured hypocotyls of cv. 'Danvers 126' responded to a 2–3 day co-cultivation with *A. tumefaciens* LBA4404 and produced 5.8% kanamycin-resistant calli, while in the same experiments, 'Imperator 58', 'Chantenay Red Cored' and 'Nantes Scarlet' gave a 3.8%, 2.1% and 0.9% transformation rate, respectively. Hypocotyls excised from seedlings and used directly for co-cultivation did not respond at all (Thomas *et al.* 1989). In another study (Takaichi and Oeda 2000), hypocotyls incubated with *A. tumefaciens* C58C1 and LBA4404, both possessing pNGL2 binary plasmid, also reacted in a different way depending on their genotype. The efficiency of transgenic plant production was estimated at 1.2–6.7% for 'Kurodagosun' and 6.1–11.8% for 'Nantes Scarlet'. Petiole segments were co-cultivated with *A. tumefaciens* C58C1 possessing pGV2260 helper vir plasmid and a binary plasmid pGSTRN943 carrying the *npt*II gene or pGSGluc1 carrying *npt*II and *gus* (β-glucuronidase) genes. Petioles of cv. 'Nanco' were the most susceptible to *Agrobacterium* (46.7–35.8% depending on the binary plasmid used), cv. 'Gold Pack' did not react to bacteria at all and the genotypes 'De Chantenay', 2027H and 840217 showed an intermediate response (Pawlicki *et al.* 1992). Comparison of hypocotyl and petiole explants of cv. 'Nantes Coreless' co-cultivated with *A. tumefaciens* LBA4404 possessing a modified pCambia binary plasmid revealed that the latter could give a higher percentage of transgenic callus, 1.4% and 3.3%, respectively (Chen and Punja 2002). The data gathered on petioles' response only indicated that cvs. 'Danvers Half Long' and 'Nantes Coreless' behaved similarly (3.4% and 3.3%, respectively) while cv. 'Nantes' was four times less efficient (0.8%). Epicotyl explants of the same three cultivars also showed various reactions to EHA105 strain with cv. 'Nanco' being the most responsive (12.1%) followed by 'Golden State' (6.1%) and 'Danvers Half Long' (1.8%) (Gilbert *et al.* 1996).

The type and origin of plant explant and its age can also influence transformation efficiency. Cell suspension originated from hypocotyls and cotyledons were expedient for transformation unlike the suspension established from callus or root discs (Hardegger and Sturm 1998). On the other hand, Pawlicki *et al.* (1992) found petioles to be much more favourable explants for inoculation than cotyledons, hypocotyls or roots. They also observed that the use of hypocotyls from three-week-old aseptic seedlings almost doubled the transformation rate in comparison with two-week-old seedlings, but the use of seedlings older than 3 weeks reduced the efficiency. Comparison of epicotyl and petiole explants from three cultivars indicated a genotype × explant interaction; efficiency of transgenic callus production was the highest for 'Nanco' and the lowest for 'Danvers Half Long' epicotyls; an opposite trend was onserved in petioles (Gilbert *et al.* 1996; Chen and Punja 2002).

Bacterial strain

Efficiency is also highly dependent on the bacterial strain used, which is related to the type of vir plasmid, binary plasmid or bacterial chromosomal background. Comparison of *A. tumefaciens* LBA4404 strain containing the pAL4404 helper and p35SGUSint binary plasmids and GV3101 strain containing the pPM6000 helper and pLRG binary plasmids indicated that the transfer of T-DNA from GV3101 was much more efficient in transformation of seedling root segments (5% and 95%, respectively), hypocotyls (112% and 128%, values over 100% resulted from more than one GUS positive focus per explant identified) and vacuum-infiltrated tap root slices (2% and 41%) (Hardegger and Sturm 1998).

In callus transformation with bacterial strains differing in their helper plasmids but all having the same C58 chromosomal background, an at least two-fold increase in the

Table 1 References concerning optimisation of carrot (*D. carota*) transformation protocols.

Method	Bacterial strain	Plasmid	Transgene[a]	Explant source	Other evaluated factors	Reference
At	A281	pGA471	*npt*II	hypocotyls	co-cultivation period, medium	Balestrazzi *et al.* 1991
	EHA105 MOG101	pGA492-CHN pMOG196 pMOG198	*chit*	epicotyls	genotype, explant age, co-cultivation period	Gilbert *et al.* 1996
	GV3101 LBA4404	p35SGUSINT pLRG	*uid*A	callus cell suspension hypocotyls roots	pre-treatment, medium	Hardegger and Sturm 1998
	GV2260	pGSGluc1 pGSTRN943	*npt*II *uid*A	cotyledons hypocotyls petioles roots	genotype, inducers, explant type and age, pre-culture and co-cultivation period	Pawlicki *et al.* 1992
	GV3850	pGV1103	*npt*II	cell suspension	co-cultivation period	Scott and Draper 1987
	LBA4404	35SCAT NosCAT pBI101.1 pBI121.1 pRGUSII	*cat* *npt*II *uid*A	hypocotyls	genotype	Thomas *et al.* 1989
	EHA101	pIG121Hm	*hph* *npt*II *uid*A	hypocotyls	co-cultivation period	Tokuji and Fukuda 1999
	A281 A348 EHA101 GV3850	pC19+ pGA472	*Bt* *npt*II	callus	injury, phenolic inducers	Wurtele and Bulka 1989
Ar	A4 A4T LBA1334 LBA9402	pBIN-*mgfp5*-ER	*gfp* *npt*II	root discs	genotype, acetosyringone, pre-culture	Baranski *et al.* 2006
	1855 2659 8196	wild type	*aux*+ *aux*-	root discs	disc side, auxins	Cardarelli *et al.* 1987b
	n.a.	p35SGUS-INT	*uid*A	root discs	disc side, auxins, acetosyringone, pre-treatment	Guivarc'h *et al.* 1993
EP	-	pTiC58	*nos*	protoplasts	DNA amount, voltage	Langridge *et al.* 1985
	-	pCATTi	*cat*	protoplasts	DNA amount, voltage, PEG supplement	Boston *et al.* 1987
MB	-	pRT99-GUS	*npt*II *uid*A	callus	microcarrier, pressure, DNA amount, pre-culture	Deroles *et al.* 2002
PEG	-	pNUN7	*npt*II	protoplasts	genotype, hormones, protoplast density	Dirks *et al.* 1996
	-	pBI221.1	*uid*A	protoplasts	genotype, PEG conc. component order	Rasmussen and Rasmussen 1993

Abbreviations of transformation methods: At – *A. tumefaciens*, Ar – *A. rhizogenes*, EP – electroporation, MB – microprojectile bombardment, PEG – polyethylene glycol.
[a] Transgenes: *aux* – auxin, *Bt* –*Bacillus thuringensis* toxin, *cat* – chloramphenicol transacetylase, *chit* – chitinase from petunia, tobacco and bean, *gfp* – green fluorescent protein, *hph* - hygromycin B phosphotransferase (hygromycin resistance), *npt*II – neomycin phosphotransferase (kanamycin resistance), *nos* – nopaline synthase, *uid*A –β-glucuronidase (GUS).

number of transgenic callus clumps was observed for plasmid pGV3850 than for pTiA6. Also the presence of the binary plasmid pGA472 was about 10-fold more advantageous than pC19+, a derivative of pGA472 with an extra 4.1 kbp insert in T-DNA carrying 35S::Bt fusion (Wurtele and Bulka 1989). Pawlicki *et al.* (1992) reported various efficiencies of petiole transformation depending on the binary plasmid carried by *A. tumefaciens* C58C1 possessing the same pGV2260 helper plasmid. Transfer of T-DNA from pGSTRN943 plasmid was more efficient than from pGSGluc1 (with an additional fragment of the β-glucuronidase gene in T-DNA) for all four responsive cultivars and the difference could reach as much as 50%. This relationship was observed only when petioles were collected form young (two-week-old) seedlings. Older seedlings (three- or four-week-old) showed similar susceptibility to both plasmids; also no difference could be observed for other explants, such as cotyledons or hypocotyls. Also no significant differences between three binary plasmids were reported by Gilbert *et al*. (1996). The process of T-DNA transfer can be enhanced by multiple copies of *virG*. Insertion of octopine-type *virG* into nopaline *A. tumefaciens* strains gave about a three-fold increase of GUS transient expression in carrot calli. However, this effect was neither observed when octopine *virG* was introduced into agropine Ti plasmid nor when agropine *virG* was inserted into nopaline Ti plasmid, which was in contrast to observations made on celery (Liu *et al.* 1992).

More complex data were provided by Takaichi and Oeda (2000) who showed that there was a strong interaction between plant genotype and bacterial strain. The use of LBA4404 strain gave two-fold more transgenic plants of cv. 'Nantes Scarlet' than C58C1 (11.8% and 6.8%, respectively) while C58C1 strain gave an almost six-fold increase over LBA4404 (6.7% and 1.2%, respectively) when applied to cv. 'Kurodagosun'. A similar observation was reported earlier, i.e. that MOG101 strain was more virulent to cv. 'Danvers Half Long' (5.1%) than to 'Nanco' (1.8) while EHA105 gave opposite results (0.5% and 12.1%, respectively) (Gilbert *et al.* 1996).

Inoculum and media composition

Other group of factors are related to the medium composi-

tion and conditions of tissue culture. In particular, the time of pre-culture as well as the duration of co-culture with *Agrobacterium* are essential. These factors have meaning for overall efficiency estimated not only by the number of transformation events but also by the ability of transformants to develop into embryos or plants. In general, pre-culture of the explants before co-cultivation enhances transformation efficiency. Using scanning electron microscope, Tokuji and Fukuda (1999) showed that the transformation strictly relied on the ability of *Agrobacterium* to pass through the cuticle. As the cuticle of fresh hypocotyls is a difficult barrier for bacterial cells, prolonged incubation of explants prior to co-cultivation results in breaks of this natural layer and easier bacteria attachment to the host tissue. Pre-cultivation also enables the accumulation of phenolics required for the induction of *vir* genes (Balestrazzi *et al.* 1991). The use of chemical stimulants like acetosyringone (AS), *p*-hydroxybenzoic acid or mechanical wounding is often crucial for successful plant transformation. However, it seems that carrot is not very sensitive to such inducers, probably due to the fact that carrot tissues are rich in endogenous phenols. Thus, cell chopping before inoculation or addition of *p*-hydroxybenzoic acid to the medium used during co-cultivation had a very small or no effect (Wurtele and Bulka 1989). Similarly, the application of 100 µM AS to bacterial suspension, inoculum or co-cultivation medium did not increased transformation frequency of petioles (Pawlicki *et al.* 1992) and cell suspension (Hardegger and Sturm 1998).

Composition of culture media is essential not only during early steps of transformation i.e., co-cultivation with bacteria, but also during later steps of cell recovery, embryogenesis and conversion into plants. Such conclusions came from experiments where GUS-positive transgenic events were identified, but only a small fraction was able to grow and develop into callus. The change of media composition from MS (Murashige and Skoog 1962) to B5 (Gamborg *et al.* 1968) resulted in higher frequency of callus formation on hypocotyl and root explants by two and three-fold, respectively (Hardegger and Sturm 1998).

Cell suspension can be conveniently used for *Agrobacterium*-mediated transformation. It can be a constant source of rapidly dividing single cells or small cell aggregates, easily exposed to bacteria during co-cultivation. Moreover, the establishment of embryogenic suspension allows the fast production of somatic embryos capable of conversion into plants. It was found that low initial cell density can be a limiting factor of cell recovery and embryogenesis (Higashi *et al*. 1998). Usually only a small fraction of cells is transformed by *Agrobacterium* thus selection and subsequent development of transgenic cells takes place at suboptimal conditions. Stimulation of cell proliferation can be enhanced by the use of a nurse or feeder layer. Although laborious, such an approach was utilized by Scott and Draper (1987) who embedded carrot cells in agar and covered such feeder medium with two filter papers a 'guard disc' and 'transfer disc', and incubated the plates for three days. After such pre-treatment cell suspension was spread over the 'transfer disc' and co-cultivated with *Agrobacterium*. Another interesting solution is synchronization of the cell cycle. Incubation of cells for 24 h in the presence of fluorodesoxyuridine arrests the cells in the G1 phase. Transition to the S phase is then initiated on fresh medium supplemented with thymidine. Co-cultivation of the treated cells with *Agrobacterium*, which was introduced simultaneously with thymidine, resulted in a two to four-fold increase in transgene expression in comparison with unsynchronised culture (Imani *et al.* 2002).

An effect of nursing components was also evaluated in the transformation of hypocotyls. Phytosulfokine, a peptide plant hormone, was identified as an active factor secreted by cells *in vitro* that binds a membrane receptor kinase and induces proliferation (Matsubayashi *et al.* 2002). Addition of phytosulfokine directly into growing medium at a low concentration (10^{-6} M) increased the frequency of transgenic callus formed on carrot hypocotyls by over five-fold (Matsubayashi *et al*. 2004).

A final essential point to consider is the selection of transformants. It has been well documented that the concentration of the antibiotic used as a selection agent considerably affects the transformation efficiency. On one hand, high antibiotic concentration ensures reliable selection while on the other hand, it decreases potential for embryogenesis and plant development. In the majority of reports, the addition of kanamycin to medium was used as a selection agent, usually at 100 mg/l. However, it was shown that the ability of the cultured tissue for embryogenesis decreased at the concentrations above 50 mg/l (Hardegger and Sturm 1998). Only Wurtele and Bulka (1989) used high level of kanamycin (300 mg/l), but they did not perform comparative studies at lower concentrations. Gilbert *et al.* (1996) applied 25 mg/l kanamycin to the selection media to ensure that the antibiotic would not decrease embryogenesis, then the developed embryos were transferred to fresh media with 100 mg/l kanamycin for final selection of the transgenics.

Agrobacterium rhizogenes-mediated transformation

Since the discovery that the Ri T-DNA is transferred to plant cell nucleus (Ackermann 1977; Chilton *et al.* 1982; Willmitzer *et al.* 1982), *A. rhizogenes* has been considered as an alternative transformation vector to *A. tumefaciens*. This species causes hairy root disease, which is manifested by the development of adventitious roots at the infection site (**Fig. 2**). In carrot transformation, the term 'adventitious roots' is often used for the description of newly formed roots instead of commonly accepted term 'hairy roots'. This is due to the fact that developing roots resulting from expression of Ri genes do not exhibit typical hairy phenotype observed in other species. In the case of neoplastic roots developed after infection with wild type *A. rhizogenes* the term 'transformed roots' are often used, particularly when the roots are later transferred to liquid culture for biomass production. Nevertheless, the term 'hairy root' is commonly applied in *in vitro* culture systems. Thus for clarity, in this review, 'hairy root' is used thoroughout.

Although the mechanism of neoplasy caused by *A. rhizogenes* is analogous to crown gall tumour in which the hairy roots posses bacterial T-DNA, *A. rhizogenes*-mediated carrot transformation was mainly used for studying the mechanism of induction and growth of hairy roots. Only a few studies concerned factors that might be essential for the establishment of effective carrot transformation method and then plant production (**Table 1**).

Protocol

A standard procedure has been developed and commonly applied with minor modiffications by various research groups. In the majority of reports, tap roots are a source tissue. They are surface sterilized by disinfectants, washed out, peeled and cut transversally into 0.5–0.7 mm discs and placed in containers or Petri dishes for incubation. The discs can be set on filter paper moistened with sterile water (Epstein *et al.* 1991), agar medium (1–10% agar in water, Cardarelli *et al.* 1985; Fründt *et al.* 1998) or culture medium, usually MS with reduced amount of macro- and microelements (Cardarelli *et al.* 1987b) without plant growth regulators (PGRs). Commonly, discs are orientated apical side (towards root tip) up, but basal side is also capable of hairy root development when the right bacterial strain is used in the presence of exogenous auxins. The inoculation is performed by spreading bacteria suspension on the disc cut surface, either over the whole area (Cardarelli *et al.* 1987b) or along the secondary cambium only (Boulanger *et al.* 1986), which is composed of meristematic cells. The evidence indicating the competence of this tissue for DNA uptake was provided by Guivarc'h *et al.* (1993)

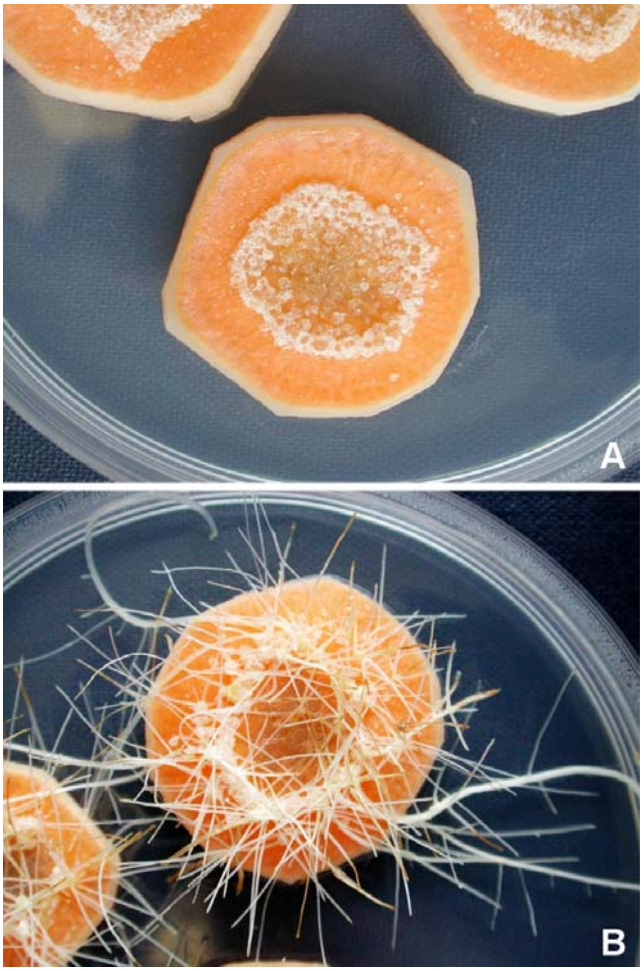

Fig. 2 Aseptic carrot root discs four weeks after the inoculation with *A. rhizogenes*: the non-inoculated control (**A**) and the inoculated discs developing hairy roots from meristematic cells of the secondary cambium (**B**).

who identified blue GUS positive cells in the cambial ring and actively dividing phloem. Their experiments supported previous macroscopic observations on the localization of hairy root origin (Bercetche *et al.* 1987). Application of the inoculum along cambial ring prevents unintended suspension leakage onto the medium and the subsequent bacteria growth outside the target tissue. After inoculation, the discs are incubated in the dark, rarely in light (Bercetche *et al.* 1987), at 22–26°C. Disease symptoms appear usually two weeks after inoculation and the scoring of hairy roots is done after the next two to four weeks. The excised hairy roots or only root meristems are transferred to a fresh solid or liquid growing media free of PGRs and incubated in the dark where they grow vigorously. On solid media, they show plagiotropic growth and highly branching lateral roots that cover the Petri dish surface. In liquid media, even a small tissue inoculum, usually a root tip, can be used for the establishment of the culture, which can be maintained for years with the biomass increase rate much exceeding that of other tissues (Araújo *et al.* 2006). However, after a long period of culture, instability of the transgene and altered morphology can be observed as well (Guivarc'h *et al*. 1999).

The development of hairy roots can be enhanced by choosing an appropriate virulent bacterial strain capable of infection a susceptible carrot genotype or by modification of inoculum chemical composition.

Bacterial strain

Bacterial strains differ in their Ri plasmid architecture. Mannopine and cucumopine strains have a single T-DNA region while agropine strain has two regions, T_L and T_R. The T_L region containes a series of *rol* genes responsible for hairy root phenotype while T_R region contains genes for agropine and auxin synthesis. In general, two types of strains exist: the first is able to induce hairy roots on both apical and basal surfaces of the tap root disc and the second type is able to induce hairy roots on the apical surface only. The latter type is denoted as basal attenuated Bas^{att} (e.g., TR7, 2559, 8196) in contrast to the basal positive (Bas^{+}, e.g., A4, 1855, 15834, TR105) one. This phenomenon is now explained by the activity of the *aux* genes. The polarity in root disc response is related to unidirectional transport of endogenous auxins from leaves to the root tip. Thus, when performing disc inoculation, it is essential to place the apical surface facing up. The application of 10^{-6}–10^{-5} M naphthaleneacetic acid (NAA) to the inoculum can partially compensate auxin deficiency at the basal surface and the development of hairy roots can be initiated (Ryder *et al.* 1985; Bercetche *et al.* 1987).

Virulence of *A. rhizogenes* strains depends not only on the type of Ri plasmid, but also on the chromosomal background. Strains possessing the same plasmid, but differing in bacterial chromosome induced various numbers of hairy roots. Transformation with strain *A. rhizogenes* LBA9402 containing pRi1855 was less effective than using strain LBA1334, a derivative of *A. tumefaciens* C58 with pRi1855 plasmid. The same relationship in favour of C58 background was observed for A4T strain with pRiA4 plasmid in comparison to *A. rhizogenes* A4 strain (Baranski *et al.* 2006).

Carrot genotype

A. rhizogenes-mediated transformation of carrot was extensively done by several groups, but usually each of them used one variety only. However, the observed differences in explant response suggested that genotype effect was significant. A broad comparison of carrot cultivars done recently confirmed that all 12 genotypes used in that study responded to *A. rhizogenes* infection. The most susceptible genotypes showed hairy roots on 93% of the inoculated discs after inoculation with strain LBA1334 while the most resistant ones on 25%, only. Interestingly, within a given cultivar, individual tap roots differed considerably in their reaction, but the susceptibility of the discs originating from the same tap root was independent of the bacterial strain used, which emphasizes the importance of genotype factor (Baranski *et al.* 2006).

Media composition

Growth regulators and AS can be considered as chemical stimulants used for hairy root induction. It was shown that the presence of auxins is necessary for successful transformation. However, the level of endogenous auxins is usually sufficient to induce hairy roots. Pre-incubation of discs in the presence of 0.5 µM NAA increased the frequency of transgenic events six-fold on the apical side, but not on the basal side and was effective on weakly susceptible discs while not on very susceptible ones. Moreover, 0.5 µM and 1.0 µM NAA decreased efficiency when applied to apical side of very susceptible discs (Guivarc'h *et al.* 1993).

A stimulating effect (45-fold) of 0.25 µM AS pretreatment was observed for weakly susceptible discs on their apical side; other concentrations were much less effective. However, pretreatment with 0.25 or 0.5 µM AS in combination with NAA (0.5 or 1.0 µM) increased transformation efficiency significantly on both disc sides. Very susceptible discs did not respond to AS treatment (Guivarc'h *et al.* 1993). Some improvement was observed when AS was added directly to the inoculum. Discs inoculated in the presence of 50 µM AS produced 45% more hairy roots independently on the bacterial strain used (Baranski *et al.* 2006).

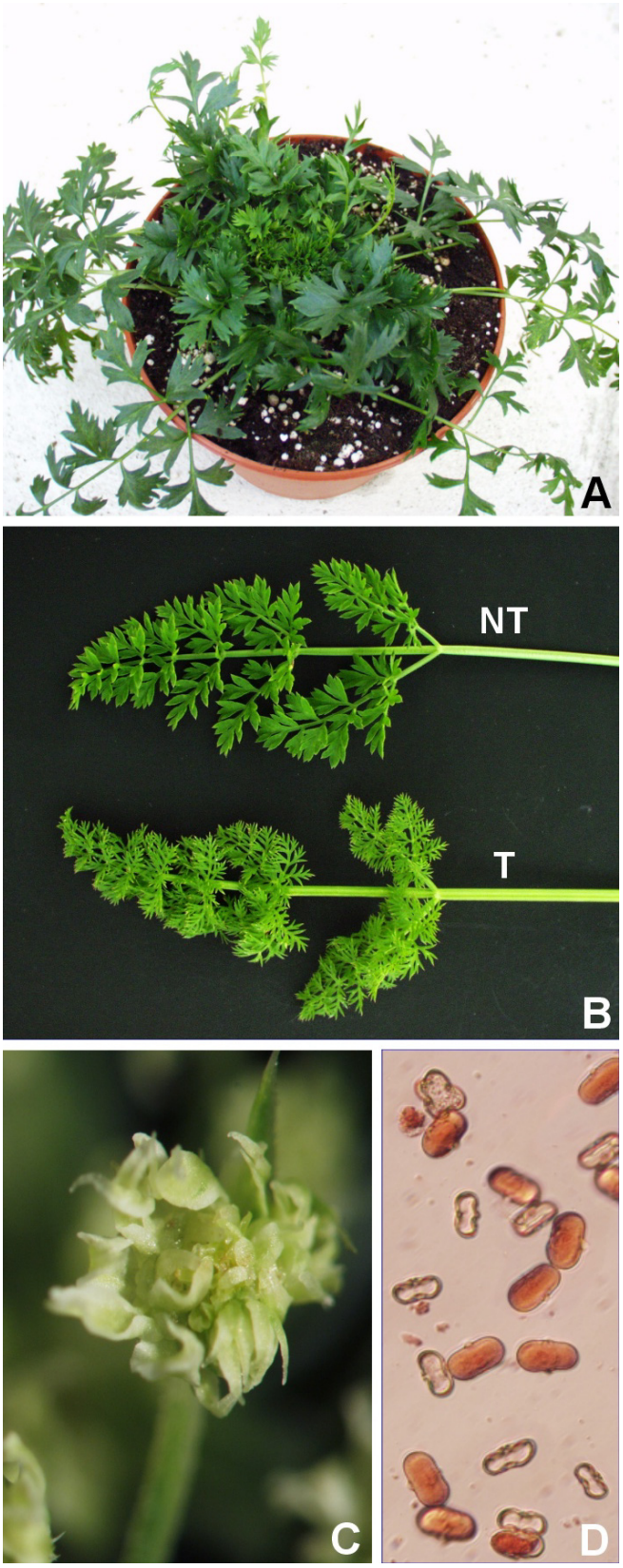

Fig. 3 Side effects of *A. rhizogenes*-mediated transformation on plant phenotype: plant dwarfism (**A**), changed leaf morphology (**B**), distortions of flower structure (**C**) and reduced pollen viability (pollen grains stained with acetocarmine) (**D**). NT: non-transformed control; T: transgenic plant.

Regenerated plants

Hairy root tissue can be also used as starting material for shoot formation and then transgenic plant development. Indeed, several authors describe a high potential of hairy roots for regeneration, which makes *A. rhizogenes*-mediated transformation an interesting alternative for the production of transgenic plants (for review see Christey 2001). However, only a few works report the production of mature plants from hairy roots in carrot as the majority of the studies concentrated on the induction of hairy root tissue. Tepfer (1984) obtained carrot plants from axenic hairy root culture growing well via the establishment of a cell suspension and then somatic embryogenesis. Regeneration of shoots via callus was reported by Baranski *et al.* (2006). Acclimatised plants were grown in a glasshouse for phenotypical observations. Various side effects of *A. rhizogenes* on plant phenotype could be noticed (**Fig. 3**). In vegetative plant parts the most distinct aberration was seen on leaves, which had wrinkled lamina or curved petioles. Additionally, some plants produced two to three meristems or showed dwarfism (Tepfer 1984; Limami *et al.* 1998; Baranski *et al.* 2006). Other biometric distortions, often observed in several species, were not reported in carrot probably due to the fact that carrot leaves are arranged in a rosette, they are also alternate and compound with pinnate blades and the leaflets are divided into small, highly lobed segments thus difficult for assessment. Also changed morphology of storage roots can not be fully justified in T_0 plants as tissue culture condition directly modifies root phenotype. The abnormalities of vegetative organs are not deleterious, but those affecting generative organs can be crucial for plant maintenance and reproduction. Ri plasmid genes can alter flowering period; in carrot, which is a biennial crop, transgenic plants flowered also at the end of vegetative growth after 8 or 12 months without vernalization (Limami *et al.* 1998). A similar switch from biennial to annual habit was reported by Tepfer (1984), but was not observed by Baranski *et al.* (2006). However, flower distortions as well as reduced pollen viability and seed production were also noticed (the author's unpublished data, Tepfer 1984).

Non-vector systems

DNA uptake by protoplasts

Naked protoplasts are useful targets for direct transfer of foreign DNA particles. Carrot protoplasts culture methods have been developed since Kameya and Uchimiya (1972) obtained embryoids from isolated protoplasts. Later, Matthews *et al.* (1979) showed that they are capable of liposome-mediated uptake of the *E. coli* pBR322 plasmid. Carrot protoplasts were transformed using either an electroporation or a treatment with polyethylene glycol (PEG) (**Table 1**). The first stable transformation using electroporation was described by Langridge *et al.* (1985). The authors obtained protoplasts from a suspension culture and transferred pTiC58 plasmid DNA isolated from *A. tumefaciens* LBA4301 to them by applying electrical pulses. The transformed protoplasts were capable of cell wall recovery and became hormone-independent unlike the untreated control. The cells cultured on hormone-free medium developed into embryos producing opines and then regenerated into plants possessing integrated plasmid DNA. Definitely parameters of the electric shock influence membrane permeability and successful DNA delivery, but also the viability of the protoplasts. Voltage, capacitance and pulses must be determined empirically as they depend on an electroporator construction and cuvette type. It was demonstrated that using various conditions for electroporation of carrot WOO1C protoplasts in the presence of a plasmid containing the chloramphenicol acetyl transferase (CAT) gene, the higher the voltage used the lower the protoplast viability and the higher the CAT activity (Boston *et al.* 1987). Thus the optimum balance between the number of viable protoplasts and expressing ones must be established. Using an ISCO power supply and cuvettes with aluminium electrodes at a distance of 1 cm about 40% of protoplasts remained viable after a shock of 2 kV/cm and in these conditions the expression of CAT was highest. In contrast to the cited work, Langridge *et al.* (1985) showed the positive effect of

carrier thymus DNA on transformation efficiency. Both linear and supercolid DNA were electroporated into protoplasts with a similar frequency and the expression of the delivered gene was not affected by the DNA conformation. However, the experiments provided evidence that linear DNA ligates and recircularizes after the uptake into protoplasts (Bates *et al.* 1990). They also showed that PEG present in the medium during electroporation favoured DNA uptake into protoplasts, but the process was hardly dependent on PEG concentration and the recorded efficiencies for 2.5% and 13% PEG were comparable to each other.

Uptake of foreign DNA by protoplast can also occur in the presence of PEG, which disturbs membrane integrity. In contrast to electroporation, this method does not require any sophisticated apparatus thus seems more attractive. After the first experiments conducted by Ballas *et al.* (1987), Dröge *et al.* (1992) obtained transgenic plants. Protoplasts were released from cell suspension of cv. 'Rote Risen' by cell incubation in enzyme solution (cellulase, macerozyme, driselase in 0.4 M mannitol), filtered and washed in $CaCl_2$ solution. 10^6 protoplasts were mixed with 20–40 μg pWD26.41 plasmid DNA carrying the *pat41* (phosphinothricin-*N*-acetyl-transferase) gene in the presence of PEG and then cultured on MS medium with 0.4 M mannitol and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. The transgenic callus was selected on 20 mg/l phosphinothricin and after eight weeks the resistant calli were directed to somatic embryogenesis for plant production. Further experiments provided data on several crucial factors influencing successful DNA uptake by carrot protoplasts (reviewed by Gallie 2001). The efficiency of DNA delivery measured by transient expression of the transgene depends considerably on PEG concentration. In general, the higher the concentration the higher is the activity of transformed cells observed up to 25% PEG (Rasmussen and Rassmusen 1993). However, using GFP (green fluorescence protein) fluorescence, it was recently shown that at 24% PEG more transformed cells die within the first days after treatment than at 20% and the latter is more suitable for stable transformation (Baranski *et al.* 2007b). Another mandatory factor is the presence of Ca^{2+} cations. The lack of these ions drastically reduced transformation efficiency and could not be recompensated by Mg^{2+} ions; the highest efficiency was obtained in the presence of both ions. Although this relationship was demonstrated for mRNA delivery it can be anticipated for DNA uptake, too (Gallie 1993). It was also shown that DNA must be added to protoplast solution and incubated shortly prior to PEG treatment. The reverse order of the component application almost completely inhibited transformation (Rasmussen and Rasmussen 1993). The delivery of target DNA or mRNA to protoplasts can be enhanced by the addition of carrier DNA from herring or salmon sperm. Comparison of luciferase activity in PEG treated protoplasts incubated with luciferase mRNA showed the use of 50 mg and 100 mg salmon sperm increased the transgene activity by 3.6 and 5.2-fold, respectively (Gallie 1993). Studies of GUS activity revealed that initial protoplast density is crutial for sucesfull transformation. The use of 10^6 protoplast of cv. 'Risø' per 1 ml of transfection solution resulted in the highest efficiency while the use of 10^5 protoplasts was an about 10-fold less effective. Also increasing protoplast density above 10^6/ml reduced the efficiency (Rasmussen and Rasmussen 1993). Analogous conclusions were reported by Gallie (1993) who observed proportional increase of luciferase activity with cell density in the range of 0.25-1.0 x 10^6 per 1 ml. Protoplasts sensitivity to culture conditions is highly dependent on carrot genotype (Dirks *et al*. 1996). This factor affects also transformation efficiency of PEG-treated protoplasts. It was found that after the delivery of GFP to different carrot genotypes, breeding stock AS884 had an about three-fold higher proportion of fluorescing cells than cv. 'Dolanka' (Baranski *et al*. 2007b). However, all used genotypes showed similar trend of declining transient GFP expression during the next week after PEG treatment, which was in agreement with previous observations by Rasmussen and Rasmussen (1993) on transient GUS activity. An improved PEG protocol was proposed by Dirks *et al*. (1996) who embedded PEG-treated protoplasts in 1.4% sodium alginate. Immobilization triggered cell divisions and a great number of embryos were produced, which were released by washing in sodium citrate solution. Also new CPP and CPPD media based on Kao and Michayluk basal salts and sugar composition were used instead of MS medium.

Microprojectile bombardment

Microprojectile bombardment is an advanced method of DNA delivery to the target cells successfully used in many species, particularly those resistant to *Agrobacterium*. As vector systems are commonly used for carrot transformation, biolistic methods received much less interest. Nevertheless, this method allowed transformation of carrot callus cells and gave insight into basic factors affecting biolistic transformation (Deroles *et al.* 2002). The effect of the type and size of the microcarrier, helium pressure, shoot distance and the amount of DNA used per shot were evaluated. Carrot cells were bombarded with DNA containing the GUS gene and the efficiency of delivery was determined based on GUS expression. The use of 1.0 μm gold particles resulted in more blue foci than 1.6 μm gold or tungsten particles. Helium pressure of 600 kPa and the distance of 12 cm were optimal for cell line 7, while another genotype, cell line 8, almost did not respond to the bombardment even at much lower pressure and distance. The amount of DNA used for preparation had no or little effect while the amount of DNA delivered per shot was significant and a higher amount increased the efficiency for recalcitrant line 8. Transient GUS expression directly after treatment was low, but increased in time over the next 2–3 days and remained at a constant level for the next three days. After a week, stable expression was observed in cell clusters indicating active divisions of transformed cells; however the number of blue foci was drastically reduced by about 5-fold within the next two weeks. Microprojectile bombardment was also used to deliver DNA to root chromoplasts using root slices as target tissue (Hibberd *et al.* 1998) and to callus pieces (Kumar *et al.* 2004). The latter authors compared various bombardment conditions using a helium shot gun and chose 7,500 kPa and 12 cm distance as the most suitable for successful delivery of DNA coated onto 0.6 μm gold particles. Using these parameters they obtained one event per 7.5 plates (13.3% efficiency) expressing spectinomycin resistance.

TRANSFORMATION METHODS – OTHER *APIACEAE* SPECIES

Apiaceae species other than carrot were transformed primarily using *Agrobacterium* (**Table 2**). However, in contrast to carrot, *A. tumefaciens* was used less frequently than *A. rhizogenes*. Only celery, caraway, coriander and anise were co-cultivated with *A. tumefaciens*.

A. tumefaciens–mediated transformation

Apium graveolens L. (celery)

The first report on transgenic celery came from Catlin *et al.* (1988) who inoculated petiole explants with nopaline strain carrying pMON200 plasmid and co-cultivated them for 2 days on a tobacco or celery feeder layer or on B5 medium. Neither of the feeder layers was advantageous over basic medium for callus formation or transformation efficiency. The highest frequency of callus resistant to kanamycin (43.6%) was achieved using a depleted antibiotic level (50 mg/l) in comparison to 100 mg/l (17.2%). As a consequence, 20 plants were produced via somatic embryogenesis. A very efficient protocol has recently been reported by Song *et al*. (2007). Cotyledon and hypocotyl explants of two cultivars XP166 and XP85 were exposed to three bacterial strains.

Table 2 Genetically transformed species belonging to the *Apiaceae* family.

Species / common name	Method / *Agrobacterium* strain	Source explant	Transgenic tissue	References
Ammi majus large bullwort	Ar / A4, LBA9402	stem, leaves	hairy roots	Królicka *et al*. 2001; Sidwa-Gorycka *et al*. 2003
Ammi visnaga toothpick weed	Ar / A4, 15834, R1601	plant	hairy roots	Kursinszki *et al*. 1998, 2000
Anethum graveolens dill	Ar / LBA9402	seedlings	hairy roots	Santos *et al*. 2002
Apium graveolens celery	At / n.a. / EHA105, GV3101, LBA4404	cotyledons, hypocotyls, leaves, petioles	T_1 plants	Catlin *et al*. 1988; Song *et al*. 2007
Bupleurum falcatum thorow-wax	Ar / A4	seedlings	hairy roots	Ahn *et al*. 2006; Kim *et al*. 2006
Carum carvi caraway	At / AGL0	cotyledonary nodes, hypocotyls, leaves, petioles	T_0 plants	Krens *et al*. 1997
Centella asiatica Asiatic pennywort	Ar / R1000	leaves, petioles, stem	hairy roots	Aziz *et al*. 2007
	/ R1601	leaves, petioles		Kim *et al*. 2007
Coriandrum sativum coriander	Ar / A4	stem, hypocotyls	hairy roots	Mugnier 1988
	At / GV3850		T_0 plants	Wang and Kumar 2004
Daucus carota carrot	see Table 1	see Table 1	see Table 1	see Table 1
Foeniculum vulgare fennel	Ar / A4	stem	hairy roots	Mugnier 1988
Levisticum officinale lovage	Ar / A4	seedlings	hairy roots	Santos *et al*. 2005
Petroselinum crispum parsley	EP	protoplasts	callus	Loyall *et al.* 2000
	MB	cell suspension	callus	Frohnmeyer *et al*. 1999
	PEG	protoplasts	protoplasts	Torres *et al.* 1993
Peucedanum terebinthaceum	MB	callus	shoots	Wang *et al*. 1999
Pimpinella anisum anise	Ar / n.a.	seedlings	hairy roots	Andarwulan and Shetty 1999
	/ A4	stem		Mugnier 1988
	/ A4	seedlings		Santos *et al*. 1998, 1999
	At / T37	stem	shoots	Salem and Charlwood 1995

Abbreviations of transformation methods: At – *A. tumefaciens*, Ar – *A. rhizogenes*, EP – electroporation, MB – microprojectile bombardment, PEG – polyethylene glycol.

Independently on the genotype and explants type, a similar susceptibility to either EHA105 or LBA4404 strains was observed with 16–18% efficiency calculated as the percentage of explants with at least one transgenic event. Nopaline strain GV3101 was less effective and the transformation frequency for leaf sections was much lower (5%). Each individual explant produced tens to hundreds of transgenics growing on B5 medium supplemented with 100 mg/l serine and 0.5 mg/l 2,4-D and 0.6 mg/l kinetin. Stimulation of somatic embryogenesis resulted in the production of plants, of which 60% were phenotypically normal. The remaining plants exhibited reduced apical dominance and altered leaf morphology. Similar abnormalities, but also including chlorophyll deficiency and lower fertility were observed by Catlin *et al*. (1988). They found that transgenic plants had changed ploidy, chromosome additions or translocations. Both research groups were able to produce offspring, which segregated for kanamycin resistance in a Mendelian fashion indicating stable integration of a single transgene copy, which was in agreement with molecular data obtained from the analysis of T_0 plants.

Carum carvi L. (caraway)

Only one report is available to date on caraway transformation published in 1997 by Krens *et al*. After screening the regeneration capacity of various explant types, the authors chose hypocotyls and cotyledonary nodes, which were co-cultivated for two or four days with AGL0 strain containing pMOG410 vector with a *gus*-intron gene. The cotyledonary node explants gave a much higher response to agroinfection than hypocotyls (usually about five-fold) observed either as transient or stable *gus* gene integration. The time of co-cultivation had no effect on transient expression, 7.6% and 6.3% for 2 and 4 days co-cultivation, respectively. In contrast, the frequency of stable integration estimated by histochemical staining of plant tissue was four-fold higher after only two days co-cultivation (13.0%) in comparison to four days (3.0%).

Coriandrum sativum L. (coriander)

A protocol for coriander transformation consisting of three steps was proposed by Wang and Kumar (2004): two days co-cultivation of hypocotyls with *A. tumefaciens* strain GV3850, then callus stimulation on MS medium in the presence of 2,4-D and antibiotic selection, and final shoot regeneration and rooting on PGR-free medium. Using this procedure 23% of hypocotyls developed resistant callus and finally 20 plants were produced, which gave 6.6% transformation efficiency. Coriander explants were more sensitive to kanamycin than other species and selection was performed at 50 mg/l.

Pimpinella anisum L. (anise)

Anise transformation using *A. tumefaciens* was entirely restricted to the production of shoot culture *in vitro* only. Such transgenic shoots were obtained after inoculation of anise stem explants with nopaline strain T37 (Salem and Charlwood 1995). Transformation of anise was mainly performed using *A. rhizogenes* (see below).

A. rhizogenes-mediated transformation

A. rhizogenes readily promotes hairy root development in carrot and this fact definitely inspired many authors to utilize the pathogen in several *Apiaceae* species as well. Two approaches can be distinguished in *A. rhizogenes*-mediated transformation of these species. The first one relies on the use of wild-type bacteria, which is exploited for hairy root induction and the establishment of long-term axenic culture. In the second approach, engineered strains, which carry a recombinant plasmid with a reporter gene are used. Thus,

Table 3 Growth rate of hairy root tissue in liquid medium after 30 days culture in the dark.

Species	Medium	Inoculum of hairy root tissue (g/ml medium)	Dissimilation value [a] (mg)	Maximum growth rate	Reference
Ammi majus	MS	0.08 / 75	n.a.	200	Królicka *et al.* 2001
Anethum graveolens	½ MS	1.0 / 50	600	13	Santos *et al.* 2002
Bupleurum falcatum	MS	0.5 / 30	n.a.	2 after 45 days	Kim *et al.* 2006
Centella asiatica	MS	0.05 / 50	n.a.	~30 after 36 days	Aziz *et al.* 2007
Daucus carota	½ B5	0.05 / 100	n.a.	77	Araújo *et al.* 2002
Levisticum officinale	SH	0.5 / 50	400	35	Santos *et al.* 2005
	½ MS	0.5 / 50	180	10	
Pimpinella anisum	SH	0.5 / 50	600	n.a.	Santos *et al.* 1998
	½ MS	0.5 / 50	200	n.a.	

Media: B5 – Gamborg *et al.* (1968), MS – Murashige and Skoog (1962), SH – Schenk and Hildebrandt (1972)
[a] Dissimilation value determined according to Schripsema *et al.* 1990

the transgenic tissue can be additionally identified despite its characteristic phenotype. The first approach was applied to *Ammi*, *Anethum*, *Bupleurum* and *Foeniculum* while the second one to *Centella*, *Levisticum* and *Pimpinella*. A comparison of the protocols indicates various sensitivities of *Apiaceae* species to *A. rhizogenes* and that several factors affect hairy root induction. Moreover, the obtained roots have different growth rates even when cultured under similar conditions (**Table 3**). It is therefore crucial to consider each species, case by case, to determine their potential for efficient hairy root development and culture. However, *A. rhizogenes* was often used for hairy roots induction only and to enable biomass production for studying their secondary metabolites, so no details on *A. rhizogenes*-mediated transformation is provided.

Ammi majus L. (large bullwort)

The starting materials were two- to three-week-old plantlets of *A. majus* grown *in vitro* on MS medium with phytohormones. Two types of explants, stem segments and leaves, were inoculated with bacterial suspension incubated in the presence of 200 μM AS. Agropine strains A4 and LBA9402 were capable of hairy root induction on stems at the first node only. Hairy root induction with mannopine strains ICPB TR7, NCPPB 8196, nopaline ATCC 11325, cucumopine ICPB TR107 and agropine ATCC 15834 was unsuccessful. The obtained roots were subcultured several times on fresh MS media with 500 mg/l claforan and 500 mg/l carbenicillin, and free of PGRs, which took five passages to eliminate bacteria and the next few to establish a uniform hairy root culture of a typical plagiotropic and branching phenotype. The growth rate of the culture was about 30 times higher than that of callus growing on MS medium supplemented with 5 mg/l indole-3-acetic acid (IAA) and 1 mg/l 6-benzylaminopurine (BAP), and the fresh weight increased 200-fold during 30 days (**Table 3**). The transgenic character of tissue was confirmed by PCR with specific primers for *rol*B and *rol*C genes (Królicka *et al*. 2001). *A. majus* hairy roots were also able to grow in a dual system together with cell suspension of *Ruta graveolens* (*Rutaceae*) for furanocoumarin production. A high proportion of *R. graveolens* cells in the culture inhibited *A. majus* hairy root growth but by choosing the optimal proportion of starting materials (*A. majus*:*R. graveolens*, 6:1), culture fresh weight increased 40 times after 25 days incubation. An inhibitory effect on hairy roots was also observed when *R. graveolens* shoots were used instead of cells, but the growth rate of the shoots increased in comparison to the single culture of *R. graveolens* (Sidwa-Gorycka *et al.* 2003).

Ammi visnaga Lam. (toothpick weed)

Development of hairy roots was initiated by inoculation of two-months-old aseptic plants with three strains, A4, 15834 and R1601. The roots were further grown on solid B5 or MS medium with 800 mg/l carbenicillin or 250 mg/l cefotaxime with 1000 mg/l ampicillin mixture for bacteria elimination at 24°C in the dark. Bacteria-free roots were cultured in liquid B5 or MS media. The authors concluded that *A. rhizogenes* strain influenced hairy root development, but no detailed data was made available. Root growth was faster on B5 than on MS medium and in liquid rather than solid medium. The addition of 500 mg/l $MgSO_4$ increased biomass production almost three-fold, but in contrast, it reduced a valuable secondary metabolite visnagin by 50% (Kursinszki *et al*. 1998, 2000).

Anethum graveolens L. (dill)

The method relied on the inoculation of three- to four-week-old dill seedlings by wounding the tissue along the hypocotyl and internodal regions using a needle, which was immersed in LBA9402 bacteria suspension. After co-cultivation for two days, the seedlings were transferred to fresh half strength (½) MS medium with antibiotics cefotaxime and carbenicillin 250 ml/l each and cultured in the dark. Developed roots were excised and used for new culture in liquid ½ MS medium free of antibiotics and PGRs. The progress of hairy root growth was the highest during the first two weeks of culture. After that, fresh weight reached a maximum while dry weight continued to increase over the next two weeks (Santos *et al.* 2002).

Bupleurum falcatum L. (thorow-wax)

Hairy roots of thorow-wax were obtained using *A. rhizogenes* A4 strain. The roots were maintained in liquid MS medium by agitation at 25°C in the dark. The replacement of MS by 3×RCM (White and Nester 1980 medium with triple the amount of macronutrients) did not affect the root fresh weight but doubled the dry weight (Kim *et al.* 2006). The dry weight, but not the fresh weight, also increased with higher sucrose concentrations with an optimum set at 8% (Ahn *et al.* 2006). The optimized conditions were further used for the production of triterpene saikosaponin in hairy root culture.

Centella asiatica L. (Asiatic pennywort)

Various explants were used for hairy root initiation: stems and leaves (Aziz *et al.* 2007) or petioles and distal or proximal parts of the leaf blade (Kim *et al.* 2007). Stem and leaves were injured with a needle and inoculated with strain R1601 carrying pRiA4b plasmid with the *npt*II gene and an additional pTVK291 plasmid determining supervirulence. After 3 days co-cultivation in a 16 h photoperiod and subsequent incubation on MS medium at 25°C, 75–84% of stems obtained from different genotypes produced hairy roots. Transformation efficiency of leaves was five-fold lower. The hairy roots developed usually 10 days after inoculation and were resistant to kanamycin at concentrations up to 200 mg/l when cultured in liquid MS medium. However, at 75–200 mg/l callus development was observed and 50 mg/l was sufficient to select transgenic roots (non-transgenic roots did not survive at 25 mg/l kanamycin). Liquid culture was

maintained in MS medium under a 16 h photoperiod. The maximum growth of hairy roots was in medium with 6% sucrose (Aziz *et al.* 2007). Another method was used by Kim *et al.* (2007) who submerged petioles or leaf parts in a bacterial suspension of R1000 strain carrying pCambia1302 plasmid with *hpt* (hygromycin resistance) and *mgfp5* (green fluorescence protein) genes for 40 min and then placed them on half-strength MS medium with 50 µM AS and co-cultivated at 19°C in darkness. The developed hairy roots were selected on 20 mg/l hygromycin-enriched medium. Liquid culture was established in 3% sucrose MS medium from root tips at 25°C in the dark. Hairy roots developed most frequently at the site of the junction between the petiole and the leaf blade (14.1% calculated as the ratio of hygromycin-resistant roots), and 4.4- and 10.1-fold more frequently than from the distal leaf part and petiole, respectively. Prolonged co-cultivation from 3 to 7 days increased transformation efficiency 7.5-fold (36.1%), but further co-cultivation for 14, 21 and 28 days decreased the efficiency. The roots developed only after co-cultivation on medium with cefotaxime but free of hygromycin, which in other cases caused explant necrosis. The transgenic character of the roots was monitored by GFP expression in root tips starting from the fifth day after induction of hairy roots. PCR analyses using specific *hpt* and *rol*B gene primers and Southern blotting was used for confirmation of transgenic nature of the hairy roots (Kim *et al.* 2007).

Foeniculum vulgare Mill. (fennel)

After carrot, fennel was the second species where hairy roots were obtained after *A. rhizogenes* inoculation. Mugnier (1988) inoculated stem sections of 1–2-months-old aseptic plants with A4 strain. Emerging hairy roots were excised after 3–4 weeks and grown further on 2% MS medium without PGRs and with 500 mg/l carbenicillin or 250 mg/l cefotaxime to kill bacteria. Transformation was confirmed by an opine assay.

Levisticum officinale W. D. J. Koch (lovage)

Aseptic three-week-old lovage seedlings were wounded with a scalpel and dipped in a culture of A4 strain carrying pRiA4::70GUS plasmid. After two days co-culture in the dark, the seedlings were incubated on a half-strength MS medium with 0.5 mg/l NAA, 0.1 mg/l benzyladenine and 300 mg/l cefotaxime. The roots developed from epicotyls and first leaves and were then transferred to antibiotic- and PGR-free liquid SH (Schenk and Hildebrandt 1972), B5, ½B5 or ½MS media in the dark. SH and B5 media appeared to be the best for fast hairy root growth (Santos *et al.* 2005).

Pimpinella anisum L. (anise)

Despite anise having being transformed with *A. tumefaciens* (see above), several reports concerned *A. rhizogenes*-mediated hairy root development. The very first report came from Mugnier (1988) who obtained hairy roots in the same way as in fennel (see *Foeniculum vulgare*). The next reports were published 10 years later. Santos *et al*. (1998) applied A4 strain carrying pRiA4::70GUS plasmid by immersion of scalpel-wounded, four-week-old aseptic seedlings into bacterial suspension and then placed the seedlings on B5 medium containing half-strength MS macro- and micronutrients. After four days co-cultivation the seedlings were transferred to a fresh medium where hairy roots developed. Excised roots were subcultured and checked for transgene expression by histochemical GUS assay. The growth rate of hairy roots was assessed in four liquid media. B5 and MS media stimulated callus growth; the highest biomass production was obtained in SH medium (Santos *et al.* 1998). After two years, morphological instability of the culture was observed. B5, ½B5 and ½MS media promoted de-differentiation, callus production and also greening when cultured in 16 h photoperiod. Additionally, spontaneous organogenesis appeared in ½MS medium in light. Only SH medium ensured stable hairy root phenotype and a fast growth rate (Santos *et al.* 1999). SH medium was also used by Andarwulan and Shetty (1999) who transferred fast growing hairy roots on MS medium for embryogenesis and shoot induction.

Non-vector methods

Except for carrot, only two other *Apiaceae* species were transformed using non-vector methods to date. DNA was delivered to *Peucedanum* protoplasts by PEG treatment while to *Petroselinum* using PEG, electroporation and microprojectile bombardment.

Petroselinum crispum (Mill.) Nyman. ex A.W. Hill (parsley)

Microprojectile bombardment was used to deliver a pSP*CHS/LUC* plasmid into 3-days-old parsley cells obtained from the established suspension in B5 medium. Two µg DNA were bound to 0.5 mg gold particles of 1.5–3 µm in the presence of spermidine and shot with 900 kPa acceleration at –80 kPa chamber pressure. The transgenic cells were selected on 30 ppm hygromycin medium and grown for callus development for 4–6 weeks (Frohnmeyer *et al.* 1999). The same method was used by Blume *et al*. (2000) who delivered the apoaequorin gene into parsley cells. Twenty-seven of 37 hygromycin-resistant calli showed expression of apoaequorin by bioluminescence in the presence of Ca^{2+}. Stable transformation was observed for over a year and was confirmed by detection of one to six copies of the transgene using Southern blotting. Parsley protoplasts isolated from cell suspension culture were also transformed in the presence of PEG (Torres *et al.* 1993) or by electroporation (Loyall *et al.* 2000) using 10–20 µg DNA per 10^6 protoplasts. The PEG-treated protoplasts were assayed for transient GUS expression and not cultured further; the electroporated protoplasts derived stably transformed callus.

Peucedanum terebinthaceum Fisch. ex Turcz.

Segments of young petioles were used for callus development and subsequent establishment of cell suspension. In the optimized protocol, the cells were enzyme digested to release protoplasts that were incubated with 50 µg DNA in the presence of 10% PEG and incubated at 26°C in the dark. Dividing cells formed developing aggregates, which were cultured for plantlet formation via somatic embryogenesis (Wang *et al.* 1999).

APPLICATION OF GM *APIACEAE* FOR BASIC RESEARCH

Promoter function

Expression of heterologous genes requires the delivery of a construct containing a gene of interest fused to a regulatory sequence. Regulatory elements ensure stable gene expression or switch on and off the expression depending on the environmental conditions, tissue type or developmental stage. Thus the effect of transgenesis is highly dependent on the role of the promoter used to drive the transgene. In most works on carrot transformation, commonly used promoters also in other species are utilized such as CaMV 35S or nos (nopaline synthase). In one of the very first works devoted to carrot the usefulness of two promoters were evaluated for protoplast transformation. Boston *et al.* (1987) investigated the mannopine synthase promoter from Ti plasmid, which is constitutively expressed in crown gall tumour and the promoter of a seed storage 19 kD zein gene from maize, which is active in the endosperm of developing kernels only. Both promoters were fused to the sequence coding for chloramphenicol acetyltransferase (CAT) and the vectors were

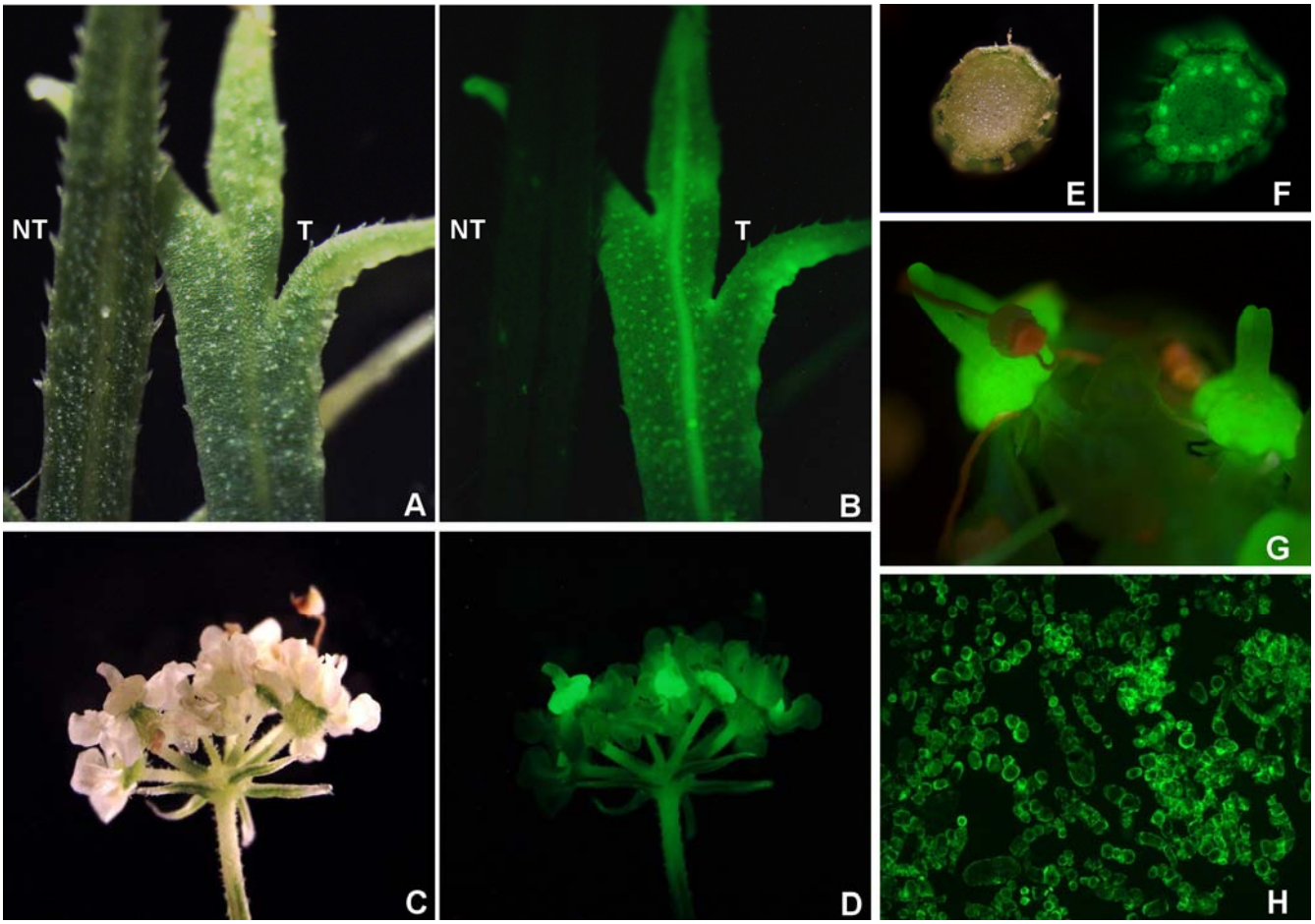

Fig. 4 Fluorescence of carrot expressing green fluorescent protein (GFP) under the control of CaMV 35S promoter. Leaf (**A**, **B**), umbel (**C**, **D**) and transversely cut stem (**E**, **F**) observed in white light (**A**, **C**, **E**) and showing green fluorescence when exposed to blue light (**B**, **D**, **F**); strong fluorescence of styles contrasting to non-fluorescing petals and stamens in blue light (**G**); fluorescence of callus cells (**H**). NT: non-transformed control; T: transgenic plant.

delivered to protoplasts by electroporation. The experiments with control vectors containing the *cat* sequence and lacking promoters clearly indicated that the presence of the promoter region and its correct orientation are essential for CAT expression. Both *mas* and *zein* promoters activated *cat* expression in carrot protoplasts (Boston *et al.* 1987). A comparison of two commonly used promoters fused to the β-glucuronidase *uid*A gene and delivered to carrot protoplasts showed that the *nos* promoter drove a four-fold higher level of expression than CaMV 35S. The expression was doubled when the octopine synthase enhancer (OCS) was fused upstream of the 35S promoter in either orientation (Rathus *et al.* 1993). Further enhancement of the GUS reporter gene occurred by insertion of a G-box tetramer upstream of the 35S promoter. The G-box tetramer sequence with GC-rich flanking sequences increased 35S activity about 13-fold in transgenic carrot cotyledons in comparison to the 35S promoter lacking a G-box insertion (Ishige *et al.* 1999).

A function of the promoter sequence of *D. carota* thaumatin-like protein (DcTLP) fused to β-glucuronidase reporter gene was investigated in callus obtained after *A. tumefaciens*-mediated transformation. Carrot TLP is a pathogenesis-related protein with a high homology to osmotin from tobacco, which shows a protective role against water deficiency. The observations of GUS expression in carrot callus revealed that the dcTlp promoter was induced by drought stress and that the level of expression gradually increased with the duration of the drought treatment. The experiments also showed that the promoter function was not induced by abscisic, salicylic or jasmonic acid. The gathered data indicates that the dcTlp promoter is drought specific and its activity is independent of the organ and developmental stage (Jung *et al.* 2005).

Various carrot organs can be the targets of genetic modifications. For example, storage roots can be modified for carotenoid biosynthesis, leaves for the expression of pathogenesis-related proteins while whole plant expression can be important for herbicide tolerance. Therefore, the knowledge of how different regulatory elements act in the carrot plant is essential for future crop improvement. Recent work is dedicated to compare the usefulness of various promoters for high transgene expression in carrot plants or its particular organs. A preliminary study showed that in leaves the strongest was *Arabidopsis* ubiquitin (ubi-q) promoter followed by maize ubiquitin (ubi-1) and CaMV 35S. Root-specific *A. rhizogenes rol*D promoter had low activity and *A. rhizogenes* agropine synthase (ags) promoter was inactive in leaf tissue. In roots, the same constitutive promoters were more active than the root-specific ones although the order of activity was different (ubi-q, 35S, ubi-1, rolD, ags). In both plant parts, a double 35S promoter was more active than ags but less than *rol*D promoters (Punja *et al.* 2007). Visual reporters e.g., green fluorescent protein (GFP), are convenient tools for evaluation of tissue-specific expression. GFP under the constitutive 35S promoter controls fluorescence with various intensities depending on the carrot tissue and organ assayed (**Fig. 4**). The highest expression was observed in vascular bundles of petioles and stem, and young leaves. Particularly bright green fluorescence was found in flowers in the style, which can be utilized for monitoring transgenic plants *in planta* directly before pollination using a hand-held UV lamp (Baranski *et al*. 2007b).

A novel technology using a quorum-sensing component for inducible gene regulation has been recently proposed. Quorum-sensing component is a system of gene switching

found in *A. tumefaciens*, which utilizes TraI/TraR inducer/ receptor proteins. Carrot protoplasts were treated with PEG in the presence of the vector-containing *traR* sequence and co-transformed with a vector possessing *traI* and *gus* genes. Significant GUS expression was achieved in the protoplasts after treatment with the inducing molecule, 3-oxooctanyl-L-homoserine lactone. Although no carrot plants were produced for further analyses, analogous experiments on *Arabidopsis* plants revealed that the expression of the reporter gene could be induced by the application of this lactone compound on leaves (You *et al.* 2006).

Gene function and metabolism

Gene function was extensively investigated using carrot as a model species. Much attention was devoted the role of *Agrobacterium* genes, particularly those involved in hairy root development and auxin synthesis. Carrot root discs inoculated with *A. rhizogenes* were particularly suitable for studying the mechanism of neoplastic tissue development, gene expression and further metabolic changes after T-DNA transfer. Much attention was placed on understanding the function of the series of *rol* genes responsible for hairy root phenotype and identification of the most important *rol*B gene, which is capable of inducing hairy roots independently on the other *rol* genes (Boulanger *et al*. 1986; Cardarelli *et al*. 1987a; Capone *et al.* 1989, 1994; Serino *et al*. 1994). A comprehensive description of *rol* gene function and regulation was review by Altamura (2004). The incidence of hairy root development is highly auxin-dependent as they activate the *rol*B promoter. Thus root induction can occur only in the presence of an elevated amount of auxins, which is therefore often supplemented to the inoculum if the endogenous auxin level is insufficient (Bercetche *et al.* 1987; Cardarelli *et al* 1987b). This is particularly important in the case of root disc transformation as neoplastic tissue development can be restricted on the basal disc side. This phenomenon is strictly dependent on the unidirectional auxin transport towards the apical side, but bacterial strains capable of non-polar virulence on both root disc sides were also identified (Cardarelli *et al.* 1985; Ryder *et al.* 1985). Moreover, a carrot root disc system was utilized for studying *Agrobacterium* opine genes and identification of border sequences flanking T-DNA (Jen and Chilton 1986; Hansen *et al*. 1991).

The next step forward in carrot transgenesis was a shift to research fields related to plant metabolism. Transgenic carrot cells, tissues or whole plants were utilized for the investigation of plant gene function and understanding the processes they are involved in. Metabolism of sucrose was studied in carrot plants expressing antisense sucrose synthase gene fragment driven by the CaMV 35S promoter. The transgenic plants had inhibited sucrose synthase in storage roots, but its activity in leaves was not altered. The plants were smaller, with both reduced root and leaf mass indicating that the enzyme is not only involved in the conversion of sugars, but is also one of the important factors affecting plant growth (Tang and Sturm 1999). Similar studies were performed with carrots expressing antisense invertase sequences, vacuolar invertase and cell wall invertase. The restricted enzymes' activity influenced carbohydrate composition and their amounts in plant organs. Moreover, plant development was highly affected; inactive cell wall invertase stimulated leaf growth, sucrose and starch accumulation in leaves and a slower root growth with reduced sucrose content. In contrast, suppressed activity of vacuolar invertase did not affect root growth, but stimulated leaf development and promoted carbohydrate accumulation in leaves rather than in roots. Also distinctive morphological distortions were evident at early developmental stages (Tang *et al.* 1999).

Ethylene regulates many plant processes, including senescence. One of the site-mutated ethylene receptors (ERS1) suppresses activity of this phytohormone in *Arabidopsis*. Transgenic coriander plants expressing mutated *ers1* gene exhibited delayed senescence. Six-week-old leaves contained 40% less chlorophyll than fresh leaves while the non-transformed control plants lost over 80% chlorophyll. Also flower life time of the transgenic plants was two-fold longer than that of the control (Wang and Kumar 2004). The effect of nitrogen supply on carrot hairy root senescence indicated that under stress conditions or excess ammonium the glutamine synthetase activity decreased in favour of glutamate dehydrogenase suggesting a switch to an alternative metabolic pathway (Souza *et al*. 2007). Programmed cell death was also investigated in carrot cell suspension in which cells were transformed with an antisense *tip1b* sequence of topoisomerase I under the control of the 35S promoter. The cells expressing the antisense transgene had lower topoisomerase I activity and induced apoptosis resulting in a slow growth of the cell suspension and precocious cell death. The reduced activity of ascorbate peroxidase and ascorbate content also suggested that an apoptotic pathway can function independently of the mechanism involving reactive oxygen species (Locato *et al*. 2006). Antisense vacuolar ATPase was used to study transport mechanisms across cell organelle membranes. Its expression in carrot cells blocked tonoplast-specific vacuolar ATPase A subunit, which disturbed proton pumping and, as a consequence, osmotic uptake of water into the vacuole. The plants exhibited strong distortions of leaf morphology (Gogarten *et al.* 1992). Ion transport and accumulation in the cells is important for both plant behaviour and utilization of plant tissue as the source of macro- and microelements in human or animal diets. The *Arabidopsis* cation exchanger 1 (CAX1) transporter is involved in vacuolar Ca^{2+} accumulation. Carrot plants with an introduced CAX1 gene accumulated up to 50% more Ca^{2+}, but not other minerals (Cu^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}) in storage roots than the wild type. This ability was transmitted also to T_1 and T_2 progeny, which paved the way for further research towards the development of quality improved crops (Park *et al*. 2004).

Parsley cell suspension was used as a model for studying the regulation of light-induced expression of chalcone synthase (CHS), the key enzyme in biosynthesis of stress protective flavonoids. Parsley cells possessing a CHS promoter and luciferase (LUC) gene fusion, and exposed to millisecond flashes of UV-B light had seven-fold enhanced luciferase expression. That response was correlated with an increase of free Ca^{2+} released to the cytosol (Frohnmeyer *et al.* 1999). UV light stress also induced the activity of glutathione *S*-transferase (PcGST1), which together with glutathione synthase (GSH) took part in a signal cascade required for subsequent CHS expression (Loyall *et al*. 2000). Activation of cytosolic-free Ca^{2+} was also observed after the response of parsley cells to treatment with a phytopathogen-derived oligopeptide elicitor Pep-13 (Blume *et al.* 2000).

Somatic embryogenesis (SE) is the process of plant development from somatic cell analogous to embryogenesis of a zygote. Carrot cells easily undergo SE, which is regulated by changing the level of PGRs. Efficient SE can be stimulated by the treatment of cell suspension with 2,4-D followed by culture in medium devoid of PGRs. The role of several genes involved in the process of SE was investigated using transgenic carrot cells. Carrot early somatic embryogenesis 1 (*C-ESE1*) gene was found to be specifically active in embryo primordial cells at early developmental stages. The expressed glycoprotein was an essential cell wall component and its suppression by transformation with the *C-ESE1* gene in the sense orientation caused strong modification of cell attachment and delayed embryo development (Takahata *et al.* 2004). At a late developmental stage, a transcription factor *C-ABI3* was found to be involved in regulation of LEA (late embriogenesis abundant proteins) genes essential for tolerance to desiccation (Shiota and Kamada 2000). SE system was also utilized to study the effect of *Agrobacterium rol* genes. Expression of a GUS reporter fused to the *rol*B promoter was observed in the central part of the globular SEs independent of the auxin

level indicating that *rol*B promoter activity was developmentally regulated (di Cola *et al*. 1997). Protein extract analyses revealed also that nuclear proteins interact with *rol*C activating its expression during SE (Fujii 1997).

Fast growing hairy root culture is a convenient system for elucidating accumulation mechanisms of root secondary metabolites. It was used for characterization of triterpene saikosaponin biosynthesis in *Bupleurum falcatum*. Five genes of the isoprenoid pathway (*hmgr*, *ipi*, *fps*, *ss* and *osc*) were isolated from hairy roots based on the homology to sequences available in other species and their expression level was determined. HPLC analysis of saikosaponin showed that its increasing content in roots correlated to the activity of those five genes during culture and that the role of individual enzymes in biosynthesis regulation was proposed (Kim *et al*. 2006).

Genomics

Several reports are available in the field of gene cloning and sequencing, mapping and genome characterization of carrot and also related species, but the results presented were seldom obtained by the utilization of transgenesis. Transposon tagging can be a valuable tool for screening the carrot genome and for cloning genes. The maize transposable elements (TEs), *Activator* (*Ac*) and *Dissociation* (*Ds*) were incorporated into the carrot genome using *Agrobacterium*-mediated transformation. *Agrobacterium* C58C1 strains containing the Ti plasmid with either the *Ac* or a defective *Ds* element, and the pRi plasmid were used for root disc transformation. Co-transformation of both plasmid types allowed the selection of hairy roots, which were then checked for the presence of the TEs. The presence of the *Ac* and *Ds* TEs was confirmed in all selected root lines by Southern analysis. Additionally, an empty donor fragment was identified indicating on the *Ac* and not *Ds* excision, which was transposition-defective. The excision of *Ac* element was found in 28% of carrot root lines and occurred early after transformation (van Sluys *et al*. 1987). After the demonstration of *Ac* transposase activity the *Ac/Ds* system was delivered to carrot callus by *A. tumefaciens*-mediated co-transformation where the plasmid T-DNA contained either 35S::*Ac* transferase construct or the *Ds* element separating the 35S promoter from the acetolactate synthase (ALS) gene conferring chlorsulfuron resistance. Transformation of callus with both plasmids resulted in identification of chlorsulfuron-resistant lines, which provided evidence of *Ds* excision. Moreover, the reinsertion of this TE was observed in new genome sites. Analyses of the flanking sequences revealed that 38% of *Ds* insertions happened in gene regions of the carrot genome (Ipek *et al.* 2006b). In further research F_1 carrot plants were obtained from crosses between the parents each bearing transcriptase or the *Ds* element. The F_1 plants showed no *Ds* excision while *Ds* was transposed in callus derived from those plants. Sequence analyses of DNA from F_1 plants revealed that one of the transcriptase introns was incorrectly spliced preventing transposition. The correct splicing in callus indicated tissue-specific processing of transcriptase, which is an important aspect for the future application of the *Ac/Ds* system for gene tagging and genome analysis (Ipek *et al*. 2006a).

So far, several heterologous genes were targeted to carrot plastid for their product sequestration like carotenoids or osmoprotectants (Hauptman *et al.* 1997; Kumar *et al*. 2004). Recently, the complete carrot plastid genome was sequenced opening new possibilities for transformation research (Ruhlman *et al.* 2006). There are several advantages of targeting transgenes to plastids including high expression and maternal inheritance. Knowledge of the complete plastid sequence will stimuli precise transgene detection and site insertion as well as challenge the control of the plastid genes.

UTILIZATION OF GM *APIACEAE* FOR APPLIED RESEARCH

Herbicide resistance

Phosphinothricin (PPT, glufosinate ammonium) is an active ingredient of total herbicides BASTA® and Liberty® that reduce the glutamine level and as a consequence the ammonia content increases leading to cell membrane disruption, termination of photosynthesis and final plant death. *Streptomyces viridochromogenes* and *S. hygroscopicus* genes code for enzyme phosphinothricin N-acetyltransferase (PAT) inactivating PPT by acetylation. Several economically important crops like, maize, rapeseed, rice, soybean and sugar beet were successfully engineered to express the PAT enzyme. Carrot resistance to PPT was demonstrated by Dröge *et al*. (1992) who introduced a modified *S. viridochromogenes pat*41 gene under CaMV 35S promoter control into PEG-treated protoplasts for studying PPT metabolism in plants. Resistant callus was selected on a medium supplemented with 20 mg/l PPT. Susceptibilty of carrot tissue to PPT was further utilized in several research programs for the selection of transgenic material. Herbicide resistant plants were obtained after expressing the *bar* gene driven by a CaMV 35S promoter (Chen and Punja 2002). The transgenic callus survived on medium containing 10 mg/l PPT, but the development of callus on explants was highly restricted. Lower concentrations were chosen as optimal for the initial culture. The regenerated plants survived 0.2% Liberty® spray and were also resistant to a higher dose of 0.4% herbicide. This selection procedure involving an increase of PPT in the medium from 1 to 10 mg/l and then brushing the leaves of obtained plants with 0.2% Liberty® using cotton swabs was used in further studies (Jayaraj and Punja 2007).

Herbicide resistance was also used to control broomrape (*Orobranche aegyptiaca*), a carrot root parasite. Mutated acetolactate synthase (ALS) gene from *A. thaliana* conferring resistance to imidazolinone herbicides was introduced into PEG-treated protoplasts of cvs. 'Tiptop' and 'Nandrin'. Transgenic cells were selected on a medium with 0.1 μM Imazapyr and the plants were regenerated in the presence of 0.15 μM Imazapyr. The potted plants tolerated the herbicide in a wide range of concentrations up to 500 μM Imazapyr. Most importantly, broomrape did not appear in pots treated with only 100 μM Imazapyr. The plants remained free of the parasite, and whose seed did not germinate in the presence of the herbicide. This approach may result in an alternative to expensive and highly toxic methyl bromide control of broomrape (Aviv *et al.* 2002). The same *als* gene was also used for selection of transgenic *Peucedanum terebinthaceum* cells after DNA uptake of PEG-treated protoplasts. The cells were selected on 10 mg/l chlorsulfuron while the regenerated plants could grow in the presence of 60 mg/l herbicide (Wang *et al.* 1999).

Tolerance to salt stress

Glycine (Gly) betaine is an osmoprotectant found in many organisms exposed to high salinity. Betaine aldehyde dehydrogenase (BADH) is the main enzyme involved in Gly betaine biosynthesis, which is active in chloroplasts. The BADH gene was delivered to carrot protoplasts via microprojectile bombardment of a vector containing sequences homologous to chloroplast DNA and the *badh* sequence regulated by a ribosome-binding site region of the bacteriophage T7 gene 10 leader, which allowed its expression in green and non-green plastids in the dark. The obtained transgenics had high BADH activity measured in cell suspension as well as in roots and leaves of the regenerated plants. About eight times higher BADH activity in the transgenic plants than in the non-transgenic control resulted in an over 50-fold increase of betaine accumulation. The plants exhibited high tolerance to salt stress and could grow in soil containing 400 mM NaCl while the control plants

Table 4 Transgenic carrot expressing pathogenesis-related genes.

Protein	Gene	Source	Pathogen assayed	Resistance assay [a]	Resistance level [b] (%)	References
BAX inhibitor-1	*HvBI-1*	barley	*Botrytis cinerea*	leaves	30	Imani *et al*. 2006
			Chalara elegans	root	60	
chitinase (acidic)	*Pach1*	petunia	*Alternaria radicina*	petioles	none	Punja and Raharjo 1996
			B. cinerea		none	
			Rhizoctonia solani		none	
			Sclerotium rolfsii		none	
			Thielaviopsis basicola (*Ch. elegans*)		none	
chitinase	*TbCh1*	tobacco	*A. radicini*	petioles	none	
			B. cinerea		50	
			R. solani		60	
			S. rolfsii		70	
			T. basicola		none	
chitinase	*chit36*	*Trichoderma harzianum*	*A. dauci*	leaflets	40	Baranski *et al*. 2008
			A. radicina	petioles	50	
			B. cinerea	leaflets	50	
chitinase	*Chi-2*	barley	*A. radicicola*	T_0 plants	40	Jayaraj and Punja 2007
			B. cinerea	leaves	25	
lipid-transfer protein	*ltp*	wheat	*A. radicicola*	T_0 plants	50	
			B. cinerea	leaves	40	
	Chi-2 + ltp		*A. radicicola*	T_0 plants	20	
			B. cinerea	leaves	10	
chitinase and β-1,3-glucanase	*TbCh1 + Glu-1*	tobacco	*A. dauci*	plants	n.a.	Melchers and Stuiver 2000
			A. radicina			
			Cercospora carotae			
			Erysiphe heraclei			
lysozyme	*hly*	human	*A. dauci*	petioles	30	Takaichi and Oeda 2000
			E. heraclei	T_0, T_1 plants	40	
microbial factor 3	*mf3*	*Pseudomonas fluorescence*	*A. dauci*	leaflets	70	Baranski *et al*. 2007a
			A. radicina	petioles	70	
			B. cinerea	leaflets	70	
thaumatin-like protein	*tlp*	rice	*A. carotiincultae*	leaves	none	Chen and Punja 2002; Punja 2005
			A. dauci		50	
			A. petroselini		50	
			A. radicini		40	
			B. cinerea		60	
			R. solani		70	
			Sclerotinia sclerotiorum		90	
			S. rolfsii		40	
snakin-1	*StSn1*	potato	research in progress			Baranski, unpublished

[a] an inoculated detached organ or the whole plant
[b] the mean disease score of the transgenic plants expressed as the percentage of the control (estimated by the author from the published data); none - 100% or more

exhibited strong growth retardation at 200 mM NaCl (Kumar *et al*. 2004).

Enhanced resistance to phytopathogens

A vast number of studies published in recent years indicate that crop tolerance to phytopathogens can be improved by introduction of antimicrobial genes into plants. Potentially useful are genes of the pathogenesis-related (PR) proteins that are known to be non-specific i.e., involved in plant resistance response to several pathogens. Both plant and microbial PR genes proved to be effective in the development of transgenic carrot plants resistant to fungal pathogens (Punja *et al*. 2007).

In most studies, plant chitinases were evaluated for their enhancing effect on carrot resistance to phytopathogens. The results of laboratory assays with detached petioles indicated that carrot tolerance to fungi can be increased by expressing tobacco or barley genes (**Table 4**). Plants with an introduced single tobacco *TbChi1* chitinase gene were more tolerant to three pathogens, *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotium rolfsii*, although some differences in the acquired resistance level were observed between two carrot cvs. 'Nanco' and 'Golden State'. This chitinase offered no protection against two other pathogens, *Alternaria radicina* and *Thielaviopsis basicola* (Punja and Raharjo 1996). The authors also showed that chitinase from petunia (PaCH1) failed to increase tolerance of carrot challenged by the fungi. This difference resulted from the fact that petunia chitinase belongs to acidic class II functioning in the extracellular space and has lower antifungal activity than class I chitinase of tobacco, which is a basic type accumulated intracellularly.

A two-gene approach was also tried in carrot by combining in a host organism the *TbChi1* chitinase and β-1,3-glucanase genes from tobacco (Melchers and Stuiver 2000) or by introduction of barley *Chi-2* and wheat lipid-transfer protein (*ltp*) genes (Jayaraj and Punja 2007). In both cases, the results indicated that combination of two different genes ensures more effective protection against fungal pathogens in comparison to transformants expressing a single gene alone. Chitinase and glucanase are both enzymes degrading the fungal cell wall thus their combined action strengthens the destruction of phytopathogen cells. Barley CHI-2 and wheat LTP seem to act via complementary mechanisms as LTP is involved in secretion and deposition of extracellular lipids and transport of cutin monomers to the leaf surface. It may also have a role in signal transduction in response to pathogen attack thus affecting systemic acquired resistance. Transgenic carrots expressing either CHI-2 or LTP singly showed enhanced resistance to *A. radicicola* and *B. cinerea* with disease indexes (DI) of 1.3–3.0 and 1.9–4.2, respectively that were significantly lower than the control (DI = 5.5). Plants expressing both

genes had a DI of about two-fold lower than plants expressing a single gene, so for the most resistant plants, the disease severity was reduced by about 90% and 95% for both pathogens, respectively.

Microbial chitinase has been recently used for the improvement of carrot resistance, too (Baranski *et al.* 2008). One *Trichoderma harzianum* chitinase, CHIT36, gene was introduced into cv. 'Koral' protoplasts treated with PEG. The transgenic plants we obtained had enhanced resistance to *A. radicina* and *B. cinerea* and, additionally, a tendency for tolerance to *A. dauci* was found. The transgenic clones had on average reduced symptoms of about 50% and the resistance level was highly correlated with chitinolytic activity determined in leaf extracts.

Three other genes were used for enhancing systemic carrot resistance. Plants expressing a rice thaumatin-like protein (TLP) gene driven by the maize ubiquitin promoter increased tolerance of some carrot plants to *B. cinerea* and *Sclerotinia sclerotiorum*, but the other plants were more tolerant to only one of these pathogens (Chen and Punja 2002). Further experiments allowed identification of the transgenic clone being more tolerant than the control to three *Alternaria* species, *B. cinerea*, *R. solani* and *S. sclerotiorum* (Punja 2005). TLP-expressing cv. 'Nanco' plants were less diseased than those expressing tobacco chitinase. Takaichi and Oeda (2000) evaluated the effect of human lysozyme (HLY), which has antibacterial and antifungal activity, the latter due to its ability for degrading chitin. Transgenic plants expressing HLY were more tolerant to foliar diseases caused by *Erysiphae heraclei* and *A. dauci*. HLY was also active in T_1 progeny enhancing carrot resistance to these two pathogens. Severity of foliar diseases was also reduced by expressing the *Pseudomonas fluorescence* Microbial Factor 3 (MF3) gene, which is homologous to FK506-binding protein and probably takes part in induced systemic resistance (Baranski *et al*. 2007a). Similar to previous reports, the transgenic plants had slower disease progress and the symptoms caused by *A. dauci*, *A. radicina* and *B. cinerea* were reduced by 20–40%. The effect of snakin-1 from potato, a cysteine-rich peptide of antimicrobial activity *in vitro*, is currently studied in transgenic carrot expressing *StSn1* gene (Baranski, ongoing research).

Hypersensitive reaction is another mechanism of plant defense to pathogen attack. The protein, BAX Inhibitor-1, involved in induced cell death in barley was expressed in carrot plants under the mannopine synthase promoter. Excised leaves challenged to *B. cinerea* showed various progress of disease symptoms depending on the clone; however, in some clones the development of the fungus was almost completely inhibited. The effect of the same protein on the development of black root rot symptoms in carrot roots was also evaluated. In fact, it was the first case of resistance screening of root tissue in transgenic carrot. Two out of three transgenic lines were more tolerant to *Chalara elegans* than the control. The most resistant line had three-fold less clamydospores per centimeter root length than the control (Imani *et al.* 2006).

Carrot breeding for resistance to virus diseases is still challenging. A group from the University of Melbourne have commenced research on expressing the nuclear inclusion protein a (NIa) region of *Carrot virus Y* in carrot and celery. So far, optimization of the transformation protocol and preparation of a construct suitable for RNAi technology was described, but no further report on the research progress is currently available (McCormick *et al.* 2004).

The majority of the resistance assays were performed on detached foliar organs i.e., petioles, leaflets or whole leaves, that were inoculated by either spore suspension or agar plug overgrown with the mycelium. These approaches are chosen because of the high reproducibility of the assay conditions like temperature and humidity. Also little space is required and several repetitions can be done on material collected from one plant. Laboratory assays also allow several pathogens to be evaluated simultaneously. However, the results obtained using such methods are not always congruent with the resistance level observed in field conditions as different defense mechanisms may function in the detached organs than in the whole plant. The confirmation of enhanced resistance under natural conditions is therefore essential for objective description of PR gene effects. Takaichi and Oeda (2000) evaluated whole transgenic carrot plants challenged by *E. heraclei*. The HLY-expressing plants were placed among pots with non-transgenic powdery mildew diseased plants to allow for natural infestation in a glasshouse. The authors found that the *hly* gene conferred a high level of resistance in both T_0 and T_1 plants. Jayaraj and Punja (2007) also used a whole plant assay for evaluation of resistance to *A. radicicola*. T_0 plants were inoculated with conidial suspension and incubated in a humid chamber followed by growth in a glasshouse. An interesting assay was described by Melchers and Stuiver (2000) who evaluated carrot in an open field where they observed a broad spectrum resistance, however, no detailed data from these experiments were published.

Modification of carotenoid biosynthesis

Carrot is grown and consumed worldwide due to a high level of β-carotene accumulating in storage roots. This provitamin A carotenoid is essential for the health of human beings who are not capable of its biosynthesis. Several breeding programs were dedicated to increase β-carotene content in carrot roots. Carotenoid biosynthetic pathway functions in higher plants as well as in microbial organisms. Thus the improvement of carotenoid synthesis in carrot can be achieved by expressing bacterial gene(s). One of the limiting steps in β-carotene synthesis is phytoene synthase (PSY) which converts geranylgeranyl diphosphate to phytoene, the first carotenoid compound in this pathway. Analogous to PSY enzyme, phytoene synthase *crt*B, from bacteria *Erwinia herbicola* was introduced into carrot via *A. tumefaciens*-mediated transformation. The construct contained a fused sequence of the *crt*B gene and N-terminal plastid transit peptide sequence driven by mannopine synthase activator and promoter, which allowed root plastid expression of the chimeric polypeptide. Transgenic plants exhibited a higher phytoene synthase activity leading to an increased level of phytoene in roots. As a consequence, the total amount of carotenoids increased 2.6-fold, in particular, β-carotene content doubled. Additionally, transversely cut roots had deepper and uniformly distributed colour across the cut surface (Hauptmann *et al.* 1997).

Punja's group at Simon Fraser University, Canada, has recently commenced research towards modification of the carotenoid biosynthetic pathway in carrot. They introduced an algal *Haemotococcus pluvialis* β-carotene ketolase and *A. thaliana* β-carotene hydroxylase genes. These enzymes catalyse the conversion of β-carotene into zeaxanthin and ketokarotenoids, canthaxanthin and astaxanthin, which are strong antioxidants and are also used as feed supplements enhancing pink colour of cultured salmon and trout. The algal β-carotene ketolase gene was fused to the ribulose bisphosphate carboxylase-oxygenase (RUBISCO) signal peptide targeting root chromoplasts and leaf chloroplasts. Transgenic carrot with ketolase has been already obtained and the results of the carotenoid profile are expected next (Punja *et al.* 2007).

Production of secondary metabolites in hairy roots

Several *Apiaceae* species have been studied for biosynthesis of secondary metabolites in *in vitro* culture systems. Cell, tissue and organ cultures were found to be interesting sources of important compounds used for pharmacological purposes (for review see Ekiert 2000). Hairy root cultures have also received much attention in recent years for the production of such valuable phytochemicals. Fast biomass growth and higher metabolite stability of hairy roots make them advantageous over other tissue or organ cultures, des-

Table 5 Secondary metabolites studied in hairy root culture of *Apiaceae* species.

Species	Secondary metabolites	Main compounds	Main properties	Maximum amount	References
Ammi majus	furanocoumarins	umbelliferone	absorbs UV	0.019 mg/g dry wt.	Królicka *et al*. 2001
		xanthotoxin; bergapten	photosensibilitic	1.0 mg/g dry wt. 0.7 mg/g dry wt.	Sidwa-Gorycka *et al*. 2003
Ammi visnaga	furanochromone	visnagin	antispasmodic	0.14 mg/g dry wt.	Kursinszki *et al*. 1998, 2000
Anethum graveolens	essential oils	apiole; falcarinol; dill apiole miristicin	anti-inflammatory	0.2 mg/g f.w.	Santos *et al*. 2002
Bupleurum falcatum	saponin	saikosaponin	analgesic anti-inflammatory	40 mg/g dry wt.	Ahn *et al*. 2006; Kim *et al*. 2006
Centella asiatica	saponin	asiaticoside	anti-inflammatory	7.1 mg/g dry wt.	Ahn *et al*. 2007
Levisticum officinale	essential oils	falcarinol; ligustilide	antitumour antispasmodic	0.2 mg/g f.w.	Santos *et al*. 2005
Pimpinella anisum	essential oils	EPB[a]; geijerene; pregeijerne; zingiberene; β-bisabolene	anti-inflammatory	1.0 mg/g f.w.	Santos *et al*. 1998
	phenols	total phenols	antioxidant	2.2 mg/g f.w.	Andarwulan and Shetty 1999
	phenylpropanoid	EPB	anethole precursor	1.0 mg/g f.w.	Duval and Shetty 2001

[a] EPB – epoxypseudoisoeugenol-2-methylbutyrate

pite some morphological instability also being reported (Guivarc'h *et al.* 1999). They also inherit biosynthetic capabilities of their origin plant, although often with modified yield and profile (reviewed by Gómez-Galeria *et al.* 2007). Hairy roots of *Apiaceae* species were also studied for their potential as a source of pharmaceuticals (**Table 5**).

Essential oils are important compounds of fruits, leaves and roots of many *Apiaceae* species. Their composition and yield was studied in hairy roots of anise, dill and lovage. The results clearly indicate that the oil composition as well as total yield depend considerably on the culture conditions. The amount of individual compounds can be either lower, at the same level or even higher than in other plant organs of parent plants, including roots. Yield of these metabolites is certainly much lower than in fruits (20–100 fold) and roots (2–6 fold) calculated per both fresh and dry weight; mass production of hairy roots in bioreactors can however, easily compensate that difference. Interestingly, the composition of essential oil main components in hairy roots is different than in roots of parent plants. This has two consequences, first, the optimization of culture conditions is required for the efficient production of the desired compound and, secondly, new main phytochemicals can be identified and produced in hairy roots (Santos *et al.* 1998, 2002, 2005; for review see Figueiredo *et al*. 2006). Stability of a metabolic process is critical for large-scale production. A three-year study showed that anise hairy roots cultured in optimized conditions ensure not only morphological stability but also unaffected essential oil production (Santos *et al.* 1999).

Anise is also a valuable condiment due to a high level of antimicrobial and antioxidant compounds. Anise hairy roots efficiently synthesized phenolic compounds at a higher level than untransformed roots and whose production was stable even in prolonged culture from 30 to 60 days. One of the main compounds identified was epoxypseudoisoeugenol-2-methylbutyrate (EPB), an anethol precursor. EPB is known to be involved in seed germination thus a large scale production of hairy roots can be utilized for efficient extraction of EPB and its subsequent use for improving germination of food crops. Hairy roots with a stable level of EPB could be much more suitable for this compound production than seeds where a high variability in EPB concentration was found (Andarwulan and Shetty 1999). Anise hairy root extract was used to imbibe pea seeds for 24 h. Shoot weight of the developed seedlings increased about 50% in comparison to shoots from non-treated seeds. The EPB-treated seedlings showed higher phenolic content and enhanced antioxidant protection (Duval and Shetty 2001).

A precious triterpene saponin, saikosaponin, with analgesic, anti-inflammatory, antipyretic and antitussive properties is receiving much attention in medicine. It is obtained from young roots of *B. falcatum*, which differ considerably in saponin content depending on plant age, genetic variation as well as on climate and cultivation techniques, so the extraction of saikosaponin is expensive. Hairy root culture can be an alternative source of saikosaponin. The recent optimization experiments of *B. falcatum* showed that its hairy roots can contain up to 40 mg saikosaponin per g dry weight, which supersedes the level detected in field-grown plants (6–25 mg/g dry wt.; Ahn *et al.* 2006).

An anti-inflammatory triterpenoid saponin, asiaticoside, is another example of the potential use of hairy root culture. Asiaticoside is synthesized in *C. asiatica* plants and its accumulation is highly tissue-specific. *In vitro*-cultured plantlets showed higher asiaticoside content in *in vitro*-grown plantlets than in glasshouse ones. The content depended on the *C. asiatica* genotype but was always highest in leaves. In contrast, hairy roots developed from an inoculated stem were free of this saponin (Aziz *et al.* 2007). However, independent experiments by Kim *et al.* (2007) indicate that *C. asiatica* hairy roots have the potential for asiaticoside biosynthesis. Although the authors also did not detect this compound in hairy roots, they stimulated its biosynthesis by applying an elicitor, 0.1 mM methyl jasmonate. β-amyrin synthase gene expression was observed 12 h after the treatment and, as a consequence, after the next three weeks asiaticoside was produced at a high level (7 mg/g dry wt.).

A. majus is a well known species of pharmacological importance. Its fruits are rich in coumarins like umbelliferone, psoralen, xanthotoxine and bergapten, which are useful in the therapy of dermatological disorders. As *A. majus* plant growth is strictly dependent on climate seed production is limited. Thus hairy roots are an interesting alternative for production of these compounds. An established hairy root culture of *A. majus* contained 19 μg/g dry wt. umbelliferone, which was similar to the amount determined in seeds (17 μg/g dry wt.). Other important furanocoumarins were not detected in hairy roots (Królicka *et al.* 2001). However, psoralens are derivatives of umbelliferone and are synthesized in the same biosynthetic pathway, which can be stimulated by biotic stress. The same research group co-cultivated *A. majus* hairy roots with *Ruta graveolens* (*Rutaceae*) shoots. A stimulating effect of *R. graveolens* shoot metabolites on hairy roots was observed and, as the result, the latter started to synthesize xanthotoxin and bergapten in significant amounts (1.0 and 0.7 mg/g dry wt., respectively) (Sidwa-Gorycka *et al.* 2003). An enhanced level of umbelliferone and bergapten was also found in callus derived from hairy roots, which could be further stimulated for furanocoumarin production by exposure of the media to a magnetic field generated by a ADR-4® magnetizer (Królicka *et al*. 2006).

Another *Ammi* species, *A. visnaga*, was evaluated for the use of its hairy roots for visnagin production. Visnagin is a furochromone of high antispasmodic activity and *A.*

visnaga is a valuable medicinal herb. *A. visnaga* hairy roots are capable of visnagin synthesis although its content depends on the root clone. Comparison of several cultures showed that visnagin level varied from 24–136 µg/g dry wt. (Kursinszki *et al.* 1998, 2000).

Edible vaccines

Carrot is a vegetable, which can be consumed raw and is available all year round as the roots can be kept in soil after vegetation or stored in a cool house after harvest for several months. Thus it may be a valuable crop for production of edible vaccines. So far, several antigens have been expressed in carrot plants or tissue culture *in vitro*. Porceddu *et al*. (1999) produced carrot plants expressing human glutamic acid decarboxylase (GAD65), which is a major autoantigen in insulin dependent diabetes mellitus. The GAD65 gene under the CaMV 35S promoter was introduced into the carrot genome where it was correctly transcribed and translated into the protein. Plant-derived antigen retained enzymatic activity and correct immunoreactivity although the expression level was low (0.012% of total soluble plant proteins). Utilization of these carrot roots for oral immunization would require further complex studies on administration dose. Also carrot explants inoculated with *A. tumefaciens* carrying 35S::*mpt64* construct developed into plants expressing MPT64 protein. MPT64 is a native protein of *Mycobacterium tuberculosis*, the causal agent of tuberculosis (Wang *et al*. 2001). Cell suspension and then carrot plants expressing a small hepatitis B virus surface protein (SHBs), a major component of hepatitis B virus antigen, were successfully produced, too. The level of protein in roots depended on auxin treatment as the coding gene was regulated by the mannopine synthase (MAS) promoter (Imani *et al*. 2002). Unfortunately, no further reports are available on the usefulness of the described transgenic carrots for oral vaccination.

More promising were works on vaccine against measles. Two neutralizing immunogenicity factors were expressed in carrot i.e., polyepitope antigen and hemagglutinin glycoprotein. The plant-expressed hemagglutinin had lower molecular weight than the viral protein, but the transgenic protein folded properly. Immunization of mice with leaf and root extracts induced production of antibodies that cross-reacted with and neutralized the measles virus *in vitro* (Marquet-Blouin *et al*. 2003). Also a chimeric polyepitope antigen combining tandem repeats of four copies of hemagglutinin noose epitope and four copies of promiscuous T cell epitope of tetanus toxoid ($[L_4T_4]_2$) was produced in carrot plants. $[L_4T_4]_2$ protein induced high levels of measles antibodies in mice neutralizing several virus isolates originating from Asia and Africa (Bouche *et al.* 2003). The broad activity of anti-$[L_4T_4]_2$ antibodies was also shown in relation to eight wild-type mutated viruses (Bouche *et al.* 2005).

Diarrhea is a common fatal disease in developing countries caused by enterotoxigenic *E. coli*. A pathogenic factor, the subunit B of heat-labile toxin (LTB), is used as an immunogen for vaccine production. For the development of edible vaccine, the LTB gene with an optimized codon for efficient expression in plants was engineered and introduced into carrot hypocotyl explants by *A. tumefaciens*-mediated transformation. The root tissue of the obtained plants expressed TLB in high amounts reaching 0.3% TLB of the total soluble proteins (Rosales-Mendoza *et al.* 2007). Pure TLB or a hydrated freeze dried root powder containing 23 µg carrot-derived TLB were oral administered to two mice groups in three doses. In both groups IgG and IgA specific antibody level increased although in case of carrot-derived TLB the level was lower. The experiments demonstrated that carrot-derived TLB induced antitoxin systemic and intestinal immunity suggesting potential application of transgenic carrot for the production of edible vaccines against diarrhea and cholera (Rosales-Mendoza *et al.* 2008).

Utilization of carrot-derived pharmaceuticals may be soon realized at an industrial scale. Carrot cell suspension is used for efficient production of recombinant human glucocerebrosidase (GCD) using ProCellEx™, a bioreactor-based production system. Recombinant plant-derived GCD (prGCD) may be an interesting alternative option to Cerezyme®, the unique and expensive enzyme used in therapy of Gaucher's disease today. prGCD exhibits similar activity to Cerezyme®, does not require posttranslational modifications and has no adverse effect on prGCD-treated mice (Shaaltiel *et al.* 2007). In 2007, the U.S. Food and Drug Administration approved a phase III clinical trial of prGCD performed on a randomized, large group of patients.

Phytoremediation

Extensive root proliferation resulting from *A. rhizogenes*-mediated transformation is a desirable character, which can be utilized for toxin phytoremediation. Phenol and its chlorinated derivatives are dangerous compounds occurring in industrial waste or as a component of chemicals used in plant protection. The established carrot hairy root culture retained its fast growth even at a high level of phenol (1000 µmol/l) independently of the selected root clone. Tolerance to chlorophenols was much lower, but the roots could still grow at 50µmol/l (Araújo *et al.* 2002). Persistence of the roots in the medium containing these toxins was due to a high peroxidase activity, which is even higher than that of field-grown horseradish roots (6.0–12.4 and 5.8 U/g f.w., respectively) used as the standard in experiments on peroxidases (Araújo *et al.* 2004). It was shown that peroxidases extracted from carrot hairy roots were able to oxidize phenol and some derivatives, 2-chlorophenol, catechol and guaiacol, with similar efficiency. That indicates the potential use of carrot hairy roots for phytoremediation of phenolic compounds. The amount of exogenous phenol, 2,6-dichlorophenol and 2,4,6-trichlorophenol added to the medium of actively growing culture was fast diminished. It was demonstrated that roots were able to uptake these compounds and, within the first five days, over 90% of the toxins were metabolized. The kinetics of the removal depended on the compound, which was due to various chemical structures and therefore their toxicity. It should be also noted that other plant enzymes may be involved in transformation of phenolic compounds as well (Araújo *et al.* 2002, 2006).

FINAL NOTE

In the late 1980s and 1990s, work on carrot transformation focused on the methods suitable for heterologous gene delivery. As the result, several efficient protocols were established with *Agrobacterium*-mediated ones dominating worldwide. However, the efficacy of transgenesis is highly dependent on the carrot genotype, thus having a particular genotype in mind one must be aware of poor success unless the transformation method has already been optimized. In the past decade, *in planta* transformation methods were developed with a floral dip method for *Arabidopsis* being the most common (Clough and Bent 1998; Kojima *et al*. 2006). Theoretically, *Apiaceae* species are potential candidates benefiting from this technique as their umbel inflorescences host a large number of exposed flowers and produce many seeds. Unfortunately there is no successful report on floral dip or vacuum infiltration in *Apiaceae*, so far. However, a simple *ex vitro* transformation technique using Fibrgro® cubes soaked with *A. rhizogenes* suspension may be an interesting approach for heterologous DNA and RNAi delivery and for studying root functional genomics (Collier *et al.* 2005). This approach could simplify the transformation procedure by omitting *in vitro* tissue culture.

After two decades of research devoted to understanding the role of *Agrobacterium* genes in the transformation process, nowadays more attention is paid to revealing the function and regulation of plant genes. Simultaneously, carrot genotypes with altered or improved characters have been developed, but have not yet been commercialized. Con-

siderable public awareness of GM crops in many regions of the world, including Europe, restrains large-scale research on the usefulness of those genotypes at an industrial scale. Nevertheless, still new reports emerge in the field of basic and applied research concerning not only carrot, but also other *Apiaceae* species. Particular interest is directed to the utilization of hairy root cultures as plant bioreactors for the production of pharmaceutically important secondary metabolites. Additionally, achievements in genomics like the recent sequencing of the complete carrot plastid genome (Ruhlman *et al*. 2006) pave the way towards crop genetic improvement in *Apiaceae* species.

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