A preliminary study into the genetic transformation of carrot (daucus carota) using agrobacterium derived vectors

Sigurdardottir, Dagmar Regina

A PRELIMINARY STUDY INTO THE GENETIC TRANSFORMATION OF CARROT (DAUCUS CAROTA) USING AGROBACTERIUM DERIVED VECTORS

DAGMAR REGINA SIGURDARDOTTIR

DISSERTATION
SUBMITTED IN PARTIAL FULFILMENT OF REQUIREMENTS
FOR DEGREE OF MASTERS OF SCIENCE
UNIVERSITY OF DURHAM

1989?

ABSTRACT

Agrobacterium tumefaciens infection has been used to transform different species of dicotyledonous plants.

In this study experiments were carried out leading to a transformation protocol for the carrot (daucus carota). Resistance to the antibiotic kanamycin was transferred into suspension culture cells and root discs by cocultivation with *Agrobacterium* harbouring a binary vector system.

CONTENTS:

4. Discussion

4.1. Discussion of the Methods

4.2. Discussion of the Results

1. INTRODUCTION

Cloning of DNA into prokaryotes and yeast cells has been successful and has illuminated many new possibilities in manipulating biological organisms. These opportunities were research carried out on prokaryotic organisms for a long time due to the lack of suitable vectors for introducing foreign DNA into higher eukaryotes.

The discovery of the identity of the tumour-inducing principle in *Agrobacterium tumefaciens* lead to preliminary experiments in the genetic transformation of plants (Schell and Van Montagu, 1977; Wullems et al., 1981). When the tumour-inducing (Ti) plasmid was investigated in more detail (Thomashow et al., 1980; Zambryski et al., 1980), it was discovered that only a limited number of genes of the large plasmid were necessary to control the incorporation of bacterial DNA into the plant cell. New vectors were designed which were capable of introducing reporter genes into dicotyledonous plants or cultures of these plants (Herrera-Estrella et al., 1983; Bevan et al., 1983; Murai et al., 1983). In some cases, transformed plants were regenerated. (Leemans, 1982; Zhi, 1987).

This development opened up new opportunities in improving the characteristics of crop plants. The possibilities of manipulating crop plants which are exploited in traditional plant breeding programs are restricted by genetic incompatibility barriers. For carrots, which have been selectively bred for more than 200 years, only a narrow range of varieties exists from which genes can be transferred by traditional methods.

Genes for resistances to the major pests, such as Soft-Rot-causing bacteria or nematodes, are not available within this gene pool. By using *Agrobacterium* derived vectors it may be possible to transfer such resistance genes from other crop plants, such as potatoes or cabbage to carrots.

Before this can be done, a transformation system will have to be set up which must produce a high number of viable cells from which whole transformed plants can be grown. The gene must be present in the plant in a stable form and it must be driven by a suitable promoter to ensure expression at the right time in the correct tissue.

When establishing such a system, the biology of the carrot and its treatment as a crop must be considered. Many of the opportunities of plant tissue culture and genetic manipulation procedures can be exploited. Thus it is necessary to carry out preliminary experiments in combining these methods to establish a simple and effective transformation system.

1.1 THE CARROT AS A CROP

The carrot is very common vegetable. The total world production in 1980 was over 10 million metric tonnes. The carrot is grown all over the world, even in the tropics, eventhough it originated from areas with mild to cold, dry climate. It has proven to be a highly adaptable plant and an ideal crop.

The carrot has been known to man for a long time (Simmonds, 1976). In earliest times it was used for medical purposes. The first use of the carrot for human consumption is dated back to 300 BC and there are signs that both the Greeks and the Romans ate the vegetable.

The domestic carrot was first grown in the Middle East in the 10th century. In old literature, two types of carrots are mentioned, a red kind and a kind of yellow to green colour. From the Middle East, the carrot spread both towards east and west; the first signs of cultivation in Europe date back to the 13th century in Italy, the same time it became known in China.

In the 16th century, the red or violet carrot was an important part of all European diets (Banga, 1962). The disadvantage of these dark coloured vegetables was that they coloured all soups and dishes violet. This was one of the reasons for both French and Dutch horticulturists to breed varieties of lighter shades. The French had little success with their white carrot, the Dutch yellow and orange subspecies became favourites. All of the carrot varieties grown today originate from four crosses carried out in Holland in the 17th century.

Carrots have lost some of their importance since the 19th century. They are now grown only for human consumption or as additional feed for horses and cows. The nutritional value of the crop is not very high, but as it is easily digestible and an excellent source of vitamins, it is a good supplement to every diet.

FIGURE 1. Nutritional value of the domesticated carrot per 100g edible product (Taylor, 1978; Tindall, 1983)

Water	88 ml	Carotene equivalents	2 - 7 mg
Energy	23 - 40 kcal	Thiamin	0.04 - 0.06 mg
	98 - 170 kJ	Riboflavin	0.04 - 0.05 mg
Protein	0.7 - 1.1 g	Niacin	0.6 - 0.7 mg
Fat	0 - 0.3 g	Nicotinic Acid	
Carbohydrate	5.4 - 9 g	Derivatives	0.7 mg
Fibre	1.4 g	Ascorbic Acid	6 - 8 mg
Ca	35 mg		
P	38 mg		
Fe	0.6 - 1.2 mg		

As carrots are grown in most countries of the world, many countries, e.g. England are self-sufficient (Eddowes, 1976). In the EEC, it is only the Netherlands which export carrots, as they have specialized in the production of very early and small varieties.

In most other First World countries, carrots are cultivated with a high level of mechanisation. In the early spring the fields are prepared by ploughing and treatment with herbicides. Between March and June, seeds pretreated with insecticides are sown by special drilling machines.

In the next 6-12 weeks the fields are continually monitored and, if necessary, sprayed with a wide selection of herbicides and insecticides. In the late summer, or early spring of the following year the crop is harvested by special machines which cut the tops off, pull the root from the ground and frequently also wash them automatically. Thus with selected varieties, optimum spacing between plants and good weather conditions, yields up to 50 t/10 m^2 can be achieved (Pririe, 1975).

1.2. DISEASES AND PESTS OF THE CARROT

Many of their physiological characteristics make carrots especially vulnerable to diseases and pests. A disease is defined as a disturbance to the normal life processes of a plant, affecting either a particular organ or the entire plant (Brooks, 1953). At specific stages of their development carrot plants are likely to be attacked by a whole range of organisms.

As the seeds may take up to 4 weeks to germinate, bacteria and fungi have a long time to develop in the soil and on the seed coat. Dipping the seed in mercuric chloride or other sterilizing solutions or coating the seed with fungicidal powder prevents disease attack before germination (Eddowes, 1976).

Young seedlings often show the symptom of 'damping-off' when they are infected by fungi (Butler, 1969). In conditions of high humidity, the seedlings are attacked at or a little below soil level. Their stems become brown and constricted and cause the young plant to fall over and die. Thus whole fields can be wiped out 6 weeks after the crop was sown.

After the long growing period of up to 4 months, the carrot roots contain high amounts of carbohydrate and nitrogenous material, which make them ideal growth media for bacteria and fungi as well as nematodes (Walker, 1976). In the soil, the carrots are protected by their tough skin but when they are harvested, the roots are frequently slightly bruised or cut. Through these wounds the parasites invade the tissue. If the crop is not quickly used or stored in cold, dry conditions, attacks of soft or semi-soft rot on carrots are common, often spoiling the whole harvest.

In FIGURE 2. common diseases and pests of carrots are listed, along with the symptoms shown by the plant and the possible mechanisms of disease control. Most diseases of carrots are caused by bacteria or fungi. More rarely viruses attack carrots, but their effects are usually not as devastating to the crop.

Nematodes are also considered to be diseases of the carrot (Tindall, 1983). The female nematode lays its eggs into a young root. The young worms hatch, causing the root skin to explode, thus liberating the next generation. As up to 12 generations of nematodes can develop in one carrot growing season, whole fields can easily be destroyed by a nematode epidemic.

FIGURE 2. Diseases and pests of the domesticated carrot (Brooks, 1953; Eddowes, 1976; Tindall, 1983; Walker, 1976)

CAUSE OF DISEASE	SYMPTOMES	CONTROL
VIRUSES		
Cucumber Mosaic Virus	leaves yellow and distorted plant stunted	destroy host (aphid)
Aster Yellow Virus	chlorosis, sterility plant stunted	destroy host (leaf hopper)
BACTERIA		
Bacterium carotovorum (=Erwinia carotororum)	'Soft Rot' through pectic enzymes	crop rotation
FUNGI		
Albugo candida	'White Rust'	crop rotation fungicides
Alternaria dauci	'Leaf Blight', yellow leaves damping-off	fungicides
Cercospora carotae	'Leaf Spot'	fungicides
Chalaropsis thielavioides		
Helicobasidium purpureum	violett mycelium on leaves	
Phytophthera megasperma	rotting of root	not necessary
Plasmopara nivea	yellow-brown leaves	fungicides
Sclerotina ssp.	semi-soft rot	
Stemphylium radicium	'Black Rot' grey-purple spots on leaves	fungicides

NEMATODES		
Meloidogyne (Root-knot nematodes)	root galls, chlorosis stunted growth	resistance through hypersensitivity, soil sterilants
PESTS		
Psila rosae (carrot fly)	larvae destroy roots	pesticides

1.3. CONTROL OF DISEASES AND PESTS OF THE CARROT

The control methods listed in FIGURE 2 represent only the most commonly used approaches to eliminating specific organisms. These methods all fall into one of the six categories of disease and pest control listed by G. W. Ware in 1983:

1.3.1 Chemical control

Control of pests by chemical substances has already been known to the Greeks. After the First, and even more so after the Second World War, many new pesticides were developed from compounds produced for military purposes. These first generation pesticides, ranging from mercuric and arsenic compounds to chlorinated hydrocarbons were widely used in agriculture for treatment and prophyllaxis. The publication of the book 'Silent Spring' by R. Carson in 1962 aroused the public awareness to the widespread effects these chemicals have on the environment and man.

Since then the control of the application of pesticides has become much stricter. New pesticides with lower levels of persistance have been developed, e.g. organophosphates and carbamates. Third generation pesticides based on biological substances such as plant defence chemicals and insect hormones are now being investigated and it is hoped that they will have fewer non-target effects than the traditional pesticides.

Unfortunately, these modern low-persistance substances are expensive, so in many Third World countries traditional pesticides like DDT are still in use (Hassal, 1969).

1.3.2. Biological control

Studies into the ecology of agricultural land have lead to the development of so-called biological pesticides. These can be either predators on the pest or organisms causing diseases of the pest. Bacillus thuringiensis and the polyhedrosis virus have been commercially available for 15 years and have been proven to be effective against many insect pests (Huffaker, 1971). A potential problem is the possibility that the biological pesticides mutate and attack new hosts. Attempts are being made to genetically engineer these organisms to limit their host range and life time.

1.3.3. Cultural control

Traditionally the only way to prevent diseases was the correct management of the field. When growing carrots, organic material was ploughed into the field the year before the carrots were sown and left to decay during the winter to destroy harmful organisms. The soil was prepared with a large amount of sand for good drainage. Carrots were grown in strict rotation with a maximum of one carrot crop in five years (Tindall, 1983). Even now, crop rotation is the only practical way of combatting Soft-Rot-causing bacteria and nematodes.

More generally, all cultural and farming practises associated with crop production, that make the environment less favourable for the survival, growth and reproduction of harmful organisms can be summed up under the category of cultural control.

1.3.4. Host plant resistance

On approximately 75% of the total crop acreage of the U.S. disease resistant varieties of crop plants are used (Hassal, 1968). Most of these exploit resistances which are present in the wild variety and have been especially introduced into the high yielding agricultural variety. Since the domestic carrot varieties all originate from the small gene pool of four crosses and they can be easily cross-bread with the wild varieties, traditional methods of plant breeding have shown some success. Unfortunately even the wild varieties are not resistant to all of the most common diseases, so the genes for reducing the infestation or damage caused by those pathogens must be taken from a different gene pool.

It is not possible to cross the incompatibility barriers between species with the methods of traditional breeding. The chances of generating resistance genes by mutation selection are also faint. The development of genetic engineering in plants has opened up a unique opportunity of introducing only specific, selected genes into the host plant. As it has been shown with tobacco and brassica, it is now possible to screen plant material for resistance to diseases and pests, isolate the responsible gene and transfer it into a crop plant. The resulting transformed plants have the same high yield and quality characteristics as the untransformed ones with the added advantage of resistance.

This method is now being investigated for many crop plants and it is hoped that in the next decade, transformed pest-resistant plants will become available to the farmer.

1.3.5. Physical and mechanical control

To keep insects away from fields, simple nets have proven to be sufficient. Nematodes can be killed by flooding fields with water at 60° C (Tindall, 1983). These are two examples of simple physical methods which have proven useful and cheap. The application of direct or indirect measures that kill the pest, disrupt its physiology other than by chemical means, exclude it from an area, or adversely alter the pest's environment should be exploited further to limit the need for chemical pest control.

1.3.6. Regulatory control

As it has been discovered that many of the major diseases and pests on crop plants have been imported from other areas, strict control on all organisms entering a country or continent can help minimize the danger of epidemics. If necessary, quarantines or regulated wide-spread control will have to be carried out under official supervision to limit the distribution of the pest or disease.

Probably the most efficient way of controlling disease and pest on crops is summarized in the term "Integrated Pest Management" (Sheets, 1979). Instead of pretreating his land with pesticides, the farmer hires a consultant who regularly checks the fields for the quantity of harmful organisms and, if it becomes necessary, prescribes a specific treatment suitable to the conditions of the field, the environment and the extent of infestation, if it becomes necessary. This approach would save the farmer money and limit the harm done to the environment by pest control measures.

1.4. THE BIOLOGY OF THE CARROT

One of the most important criteria when selecting a pesticide treatment for a crop is the elimination of harm done to the plant by the treatment. Thus the biology of the carrot must be considered in detail and the correct time and type of pesticide application evaluated.

Carrot seeds are about 3 mm in length and 1 mm in width. They are halves of a divided fruit, each containing a set of cotyledons. The tiny embryo is connected directly to the large endosperm without a suspendor. The endosperm which provides nutrition for the embryo, contains high amounts of oil and no starch. To make the sowing procedure easier, the hard spikes of the seeds are removed before packaging.

In the field, the seeds germinate after 10 - 20 days. They are stimulated by moisture and alternating temperatures above 4°C. At first, the radicle breaks through the seed coat and within a few days the hypocotyl emerges from the soil. Then the first phase of vegetative growth begins. The plant above ground develops rapidly while the thin taproot extends into the ground.

Depending on genetic and environmental factors, the plant switches to the secondary vegetative growth during which the root width expands. The secondary phloem and xylem swell, starting from a single vascular cambium. The edible part of the vegetable consists mainly of the swollen parts of the taproot and a small portion of the hypocotyl. The average size of the carrot root is 15 cm length and 1 - 2 cm width.

When the carrots are left in the field they will produce flowers. The flat-topped inflorescence is called an "umbel". Individual flower stalks arise from the same point on the rays and are of different lengths, so as to raise all the flowers to the same height. In the centre of the umbel, a few flowers are often darker than their surrounding white or yellowish neighbours, to attract pollinating insects

Each flower is either both male and female or only male. Sterile flowers are rare. To minimize self-pollination, the pollen of each plant matures before the stigma. No other self-incompatibility reactions exist to prevent self-fertilization, which simplifies breeding strategies since inbred lines are easily produced.

1.5. THE HISTORY OF PLANT TISSUE CULTURE

Many of the facts about the biology and more specifically about the physiology and cellular metabolism of the carrot were revealed by studies on cultures of carrot tissues. Ever since Schleiden and Schwann (1838/1839) defined the cell as the single unit of life, scientists have attempted to isolate individual organs or cells of organisms and to proliferate them artificially. First results were achieved in 1922 when Kotte succeeded in cultivating roots for a short time. This was the basis of organ culture. Street (1977) defined organ culture as the culture of isolated organs of plants, including those derived from root or shoot tips or leaf primordia.

The first carrot cultures were calli established independently by two French scientists in 1937. Cultures of undifferentiated growth on solid media are called calli.

In the following years, efforts were concentrated on the production of better growth media, on which higher rates of cell division could be achieved. The medium of Murashige and Skoog (1962) has now been established as the most generally used basic plant tissue culture medium.

During the same period, the technique of suspension culture was discovered. Street's definition of a suspension or cell culture is the proliferation of cells and cell aggregates in stirred liquid medium. In suspension culture, cells have a higher rate of division and are more easily manipulated. Halperin observed in 1966 that regeneration of carrot plants was possible starting from suspension cultures. Under specific conditions, the first step in regeneration was the formation of a structure which was very similar to that of a normal embryo within a seed. These structures are called embryoids. In 1970 it was shown by Backs-Huesemann and Reinert that these embryoids actually develop from one single cell, a result which proved the theory that cells are totipotent.

A further development in plant tissue culture was the production of protoplasts, cells without cell walls. First experiments were carried out in 1892, when Klercker attempted to destroy cell walls mechanically, but it was only in 1960, that an adequate method of protoplast isolation was discovered by Cocking. He made use of crude extracts containing cellulases, pectinases and other cell wall degrading enzymes, which had proven useful in removing fungal cell walls. Protoplast isolation is now a standard procedure for many plants providing easily manipulatable single cells.

1.6 TISSUE CULTURE OF THE CARROT

The fundamental principle of working with cells or tissues from artificial cultures is that the culture provides a stable, constantly regeneratable source of material for experiments. Thus the first step in working with plant tissue is the production of a callus or suspension culture.

Calli can be produced starting with all tissues of the carrot. Usually young seedlings are used, since their division rate is very high and the callus is established very quickly, but cultures derived from fully differentiated leaf or root tissue are also common. Seedlings have the added advantage, that they can be grown sterile, whereas samples from mature plants grown in the field or greenhouse must be thoroughly sterilized.

Once the plant material is sterile, callus production is quite simple. Sections of the tissue are cut and placed on a solid medium in a flask or petri dish. The cultures are kept sterile at approximately 26° C. After 2 - 3 weeks, undifferentiated growth can be observed. A stable callus culture will have to be subcultured every 2 - 4 weeks by transferring small sections from the callus to a new dish.

Suspension cultures are easily established from a callus culture. A sample of callus is passed through a sieve to separate the cells. The cells are cultured at high cell density in a liquid medium of the same composition as the medium for the callus but without the solidifying agent. The flasks are placed on a shaker at low speed. Usually the cells adapt quickly to the new environment and the culture will be stable within a few weeks. As the cells are completely surrounded by nutrient solution, they grow more quickly and will need to be diluted every week.

From these two sources, tissue samples can be removed regularly and used for further experiments. For the production of protoplasts, cells from a suspension culture are transferred to a solution containing cell wall degrading enzymes and incubated for some time.

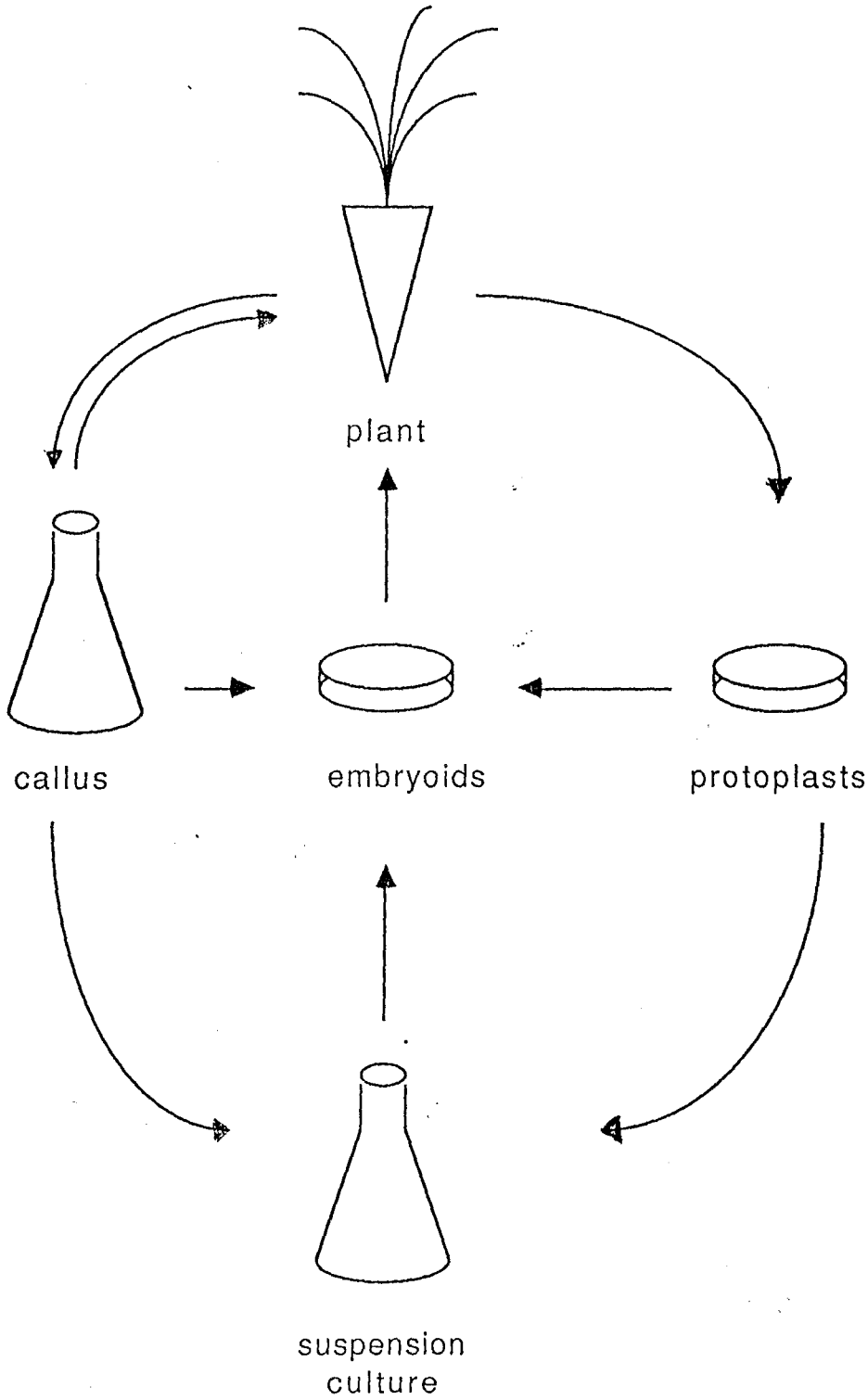


FIGURE 3. Relationships between different types of carrot tissue culture

The protoplasts are washed and cultured in normal medium containing high amounts of mannitol or sorbitol as osmotica. The cells will regenerate their cell walls within a few days and start cell division (Seitz, 1985). If the protoplasts are to be cultured without cell walls, cellulase must be added to the medium.

It has been shown by Kameya (1971) that protoplasts can be isolated directly from carrot roots by using higher concentrations of enzymes. The protoplasts were grown in suspension culture for two weeks and then plated out on solid media. Within three months, whole carrot plantlets were regenerated.

Carrot plants can also be regenerated from callus and suspension cultures. Cells from a suspension culture are transferred to a medium with different plant growth substance composition. In the case of the carrot, auxins are completely removed. In this solution, the cells will start to generate embryoids and after two weeks, these embryoids can be plated out. Callus samples can be directly transferred to a solid medium without auxins on which embryoids will form. Again within 2 - 3 months, whole plants can be observed (Kartha, 1982).

Unfortunately, most cultures which have been cultivated for more than six months loose their embryogenic potential. Experiments have been carried out to try to recover the ability to form embryos, but success has been limited to a few species (Smith and Street, 1974).

1.7. POSSIBLE APPROACHES TO CARROT TRANSFORMATION

As it has been shown in the previous chapter, a wide selection of methods exists for the cultivation of carrot tissue. Carrot cultures are kept in many different laboratories and it is not surprising that when methods for introducing foreign DNA into eukaryotes were developed, many experiments were carried out on carrot tissue. Even though other plants, for example tobacco and Petunia, have now become the standard experimental objects for genetic manipulation of plants, a number of publications report different approaches to transforming carrots. These protocols can be divided into two basic techniques: direct transformation systems and systems using *Agrobacterium tumefaciens* technology.

Before organisms, especially complex ones such as plants are transformed with the actual "useful" genes, preliminary experiments are carried out with socalled reporter genes (Lichtenstein and Draper, 1986). These genes code for proteins which are easily detected, either because they can be recognized by antibodies or because they carry a specific activity which is quickly examined. For plants, the octopine and nopaline synthase genes, a bacterial luciferase gene, or a bacterial glucuronidase gene are generally used (Otten and Schilperoort, 1987; Koncz et al., 1987; Jefferson et al., 1987). In addition, antibiotic resistances are often used to select for transformed cells. The plasmids or DNA fragments which transform the plant tissue can code for resistances against many different antibiotics, including kanamycin and rifampicin. These genes are usually active both in the vector and in the plant, so in both cases the same antibiotic can select for hosts carrying the gene.

Once an adequate method for transforming the plant has been found with the help of the reporter gene, experiments using the "useful" gene will have to be carried out. Unfortunately it cannot be assumed that if the transformation and possibly the regeneration of the plant was successful in the preliminary trials, it will definitely work with different genetic material. Often the actual character of the DNA influences the result of genetic manipulation (Old and Primrose, 1985).

1.7.1. Direct transformation systems

A simple procedure for the transformation of prokaryotes is directly mixing the organisms with a solution containing DNA in circular or supercoiled form in the presence of salts and PEG (polyethyleneglycol). The DNA will "diffuse" into the cells and usually stays in the cytoplasm as a plasmid. Expression of the genes on this plasmid has been reported (Old and Primrose, 1985).

Unfortunately, the DNA can not pass through plant cell walls and direct transformation of cultured plant cells is not possible. On the other hand, protoplasts without cell walls have been shown to take up DNA when stimulated by PEG and Ca^{2+} (Krens, 1982; Paszkowski, 1984). This method has only been only reported for tobacco protoplasts.

One of the most common methods for transforming protoplasts is electroporation (Boston, 1987; Fromm, 1987; Koncz, 1987). Protoplasts are isolated from fast growing cells in a quick procedure taking only 2 - 3 hours.

Aliquots of the washed and concentrated protoplasts are placed in an electroporation cuvette and incubated with isolated supercoiled DNA and PEG. Electroporation is carried out under current limitation at room temperature for 10 minutes. The cells are recovered by centrifugation, washed and cultured under standard conditions. Expression of introduced genes can already be detected after 3 hours. Boston (1987) and Fromm (1987) do not state how long they were able to cultivate their transformed protoplasts but Koncz (1987) reports that stably transformed calli were produced. None of the publications mention regeneration of whole plants.

Electroporation of carrot protoplasts has not been reported for generating transformed carrot plants but it is now a standard procedure for checking for expression of genes introduced into in plant tissue. The isolation of protoplasts can be done quickly and high viabilities are achieved as optimal conditions for the electroporation have been worked out. After the transformation 60% of the cells are still viable and expression of the newly introduced gene can be analyzed the next day (Boston, 1987). Thus this system offers a high number of transformants in comparatively little time.

Another possible approach to the transformation of carrots is microinjection. Single cells are directly injected with DNA through a micromanipulator. This procedure is difficult to carry out and only very few transformations can be achieved in a given period of time. Since it is to be expected that not all of the injected cells will survive the operation, and not all of the surviving cells will correctly express the gene, microinjection does not seem to be a very promising technique for producing transformed plants.

1.7.2. Systems using *Agrobacterium tumefaciens* technology

The most well developed system for the introduction of genes into dicotyledonous plants is the use of vectors derived from *Agrobacterium tumefaciens*. This soil bacterium usually attacks wounded plants and injects a piece of DNA from the Ti-plasmid into the plant cell, where it is integrated into the chromosomal DNA and expressed, thus inducing crown gall tumor. By genetic manipulation, the oncogenic region has been removed from the Ti-plasmid rendering a disarmed bacterium, which can attack cells and introduce the T-region of the Ti-plasmid but cannot cause a tumor (Barton et al., 1983).

Since the Ti-plasmid is too large to manipulate genetically, two derivatives of the Ti-plasmid have been produced (Matzke and Chilton, 1981). In the first system, sequences from the plasmid pBR 322 have been cloned into the T-DNA region. The gene which is to be introduced into the plant is cloned into a normal pBR 322 plasmid and the resulting plasmid is then introduced into a host carrying the Ti-plasmid. The pBR 322 regions will cross over between the two plasmids and the cloned gene is transferred to the Ti-plasmid. The complete Ti-plasmid is then translocated into the *Agrobacterium* by conjugation.

The second system, the type that is used in the experiments reported here, is a binary system. Two plasmids are present in the *Agrobacterium*, one carrying only the T-DNA with the foreign gene and another carrying the other regions of the Ti-plasmid which are important for the infection of the plant cell and the introduction of the T-DNA into the plant genome. As these genes function in trans, they will also transfer a T-DNA region on a completely separate plasmid.

The genetic manipulation steps for establishing either of these two systems are rather complex and time consuming, but when the construct is complete and present in a suitable *Agrobacterium* transformation of dicotyledonous plants is simple.

The most common protocol for transforming plant cells with *Agrobacterium* again uses protoplasts (It has been shown that in nature *Agrobacterium* only attacks wounded plants. The regenerating cell wall of a protoplast seems to copy the "wounded" status of the plant cell and *Agrobacteria* attach to these cells. (Sen, 1986). Sen concludes from experiments of using different lengths of time of enzyme digestion, that the cell wall and not the cell membrane is essential for bacterial attachment.

The general transformation protocol states that protoplasts are isolated from fast growing suspension cultured cells in a quick procedure. They are then washed and plated out at a density of 10^5cells/ml, usually in petri dishes.

After 20-30% of the cells have divided, or when regeneration of the cell wall can be seen, *Agrobacteria* are added at 100 bacteria per plant cell. This mixture is cultured for two days. The cells are washed and plated in a selection medium including an antibiotic to kill any *Agrobacteria* that are left. Sen reports transformation frequencies of 1.5 - 2% for their experiments on carrot cells.

Transformation frequencies of 7×10^{-2} (Sen, 1986) or 3×10^{-4} (Zhi, 1987) with virulent *Agrobacterium*) have been achieved in experiments in which suspension cultured cells were directly transformed with *Agrobacteria*. The experiments are carried out similar to the protoplast transformation. Zhi mentions that from their transformed cells, whole plantlets were regenerated. These plantlets produced opines, whereas untransformed cells only developed into calli which do not produce opines. This is the only report which states that whole carrot plants can be regenerated from transformed tissue. It is important to note that the transformation was carried out with oncogenic bacteria and the resulting plants were derivatives of tumorous tissue.

An approach to transformation which is generally used for tobacco plants but has not been reported for carrot is the use of tissue explants. Horsch introduced the method in 1985 by showing that tobacco, petunia and tomato leaf explants which were sterilized, submerged in a solution containing *Agrobacteria*, cocultivated and placed on a selection medium could redifferentiate into shoots after two weeks. When placed on rooting medium, whole plants resulted which when analyzed proved to carry the introduced gene. This method seems a plausible procedure for obtaining transformed carrot plants.

A rather different method for transforming whole plants was published by Feldmann and Marks in 1987. Instead of transforming cultured tissue they added *Agrobacteria* to germinating seeds of Arabidopsis. In the first generation only partially transformed plants developed but their seeds were collected which germinated to produce completely transformed plants. These plants were selected by antibiotic resistance as well as an enzyme activity. The transformation rates are rather low (0.32%), but since the procedure can easily be carried out with large numbers of seeds, adequate numbers of transformed plants can be produced.

1.8 AIMS OF THIS PROJECT

In this project, preliminary experiments were to be carried out which might reveal a simple and effective method for producing whole transformed carrot plants. Since the direct transformation systems demand a high standard of technology, result in low transformation frequencies and have not been shown to be effective for regeneration experiments, the *Agrobacterium* system was used for all experiments. Simple tests on suspension cultured cells, protoplasts, root discs and seeds were carried out as they are described here.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals

Acridine Orange

Amino acids

Antibiotic A3 medium (Difco)

Augmentum

Auramin

Bacto-tryptone

Bacto-peptone

Bovine serum albumin (BSA)

Bromo-chloro-indolyl β-D glucuronic acid (x-glu)

B-5 medium

Calcofluor white (American Cyanamid Co)

Cellulase (Onozuka)

2,4-Dichlorophenoxyacetic acid (2,4-D)

Fluorescein diacetate

Indole acidic acid (IAA)

Inorganic salts

Kanamycin

Kinetin

KOH

Mannitol

Mercaptoethanol

Murashige and Skoog medium (MS medium)

Myo-inositol

Na_2EDTA

NaOH

Nicotinic acid

Pectinase

Phenosafranin

Pyridoxine · HCl (Vitamine B1)

Rhozyme

Rifampicin

Sodiumdodecylsulphate (SDS)

Sorbitol

Sucrose

Thiamine · HCl (Vitamine B6)

Triton X-100 (detergent)

Yeast extract

2.1.2. Biological material

Carrot cell culture

The carrot cell culture which was used in these experiments is a subculture of the B1 cell line established in Tubingen, W-Germany in the 1950s. It is a derivative of root callus selected for anthocyanine deficiency.

Redifferentiation experiments were carried out with these cells some years ago without success. Cells from this line grown in suspension culture have been used for protoplast regeneration studies and calli have been obtained from protoplasts by transferring them step by step into a medium without osmotica and then plating out the cell clusters onto agar (Seitz, personal communication). These cells were provided in flasks of suspension culture and flasks of callus. The cells were cultured as described in chapter 2.3.1.

Carrot seeds

The first experiment with carrot seeds was carried out with seeds of the variety Nantes Early Spring supplied by Suttons Seeds Ltd. For the second experiment F1-hybrid seeds of the variety Nandor from the same company were used.

Agrobacteria

Two strains of *Agrobacteria,* C58 and LBA 4404 were used in the experiments described here.The strain LBA has proven effective for transforming tobacco tissue (Jefferson, 1987), C58 has been shown to transform suspension cultured carrot cells (Zhi, 1985). Both strains were supplied as colonies growing on agar plates. The methods used for storing and multiplying the bacteria were listed in chapter 2.3.2. .

Both strains of *Agrobacterium tumefaciens* harbour the same T-DNA plasmid, pSIT73, supplied by Dr. P. Mullineaux, John Innes Institute. In FIGURE 5., a map opf this plasmid is shown. Between the two Cauliflower Mosaic Virus 35 s subunit promoters and terminating sequences, the GUS gene, coding for the enzyme b-D-glucuronidase and the aph-4, a herbicide selector, have been cloned. Both of these regions as well as the NPT II (bacterial neomycin phosphotransferase) gene, driven by the nos (nopaline synthase) promoter are present within the boundaries of the T-DNA.

In the strain C 58, the T-DNA plasmid is supported by the plasmid PGV 3850, which carries the genes necessary for the infection of the plant cell and the T-DNA transfer. Both plasmids were brought into the *Agrobacterium* by triparental mating between *E.coli* and *Agrobacterium.*

In the strain LBA 4404, the T-DNA plasmid is supported by the plasmid PGV 4404.

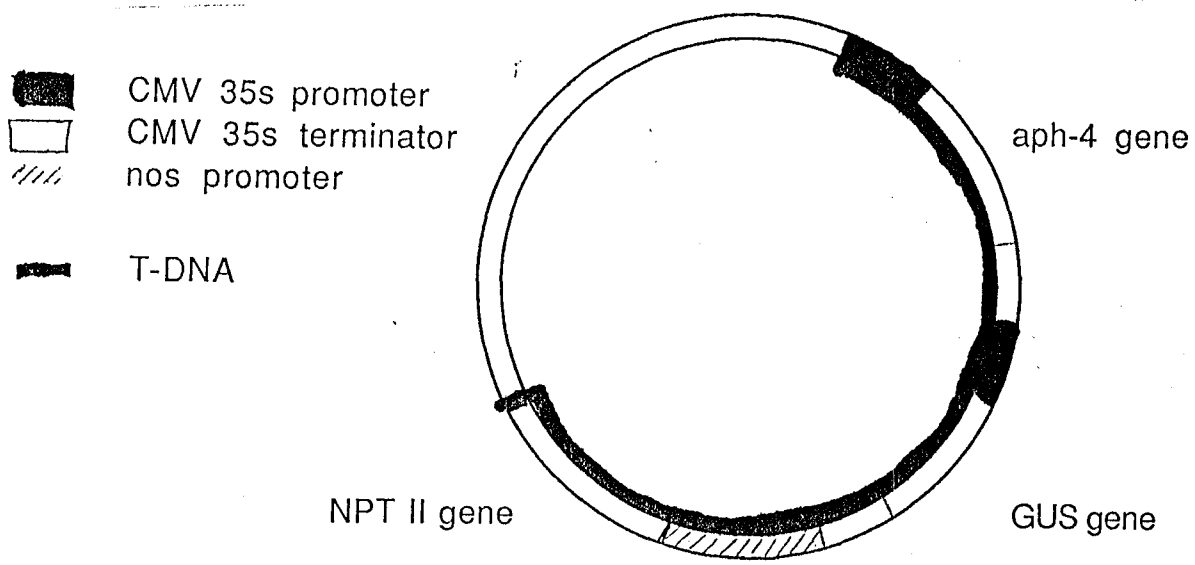


FIGURE 4. Map of plasmid pSIT73

2.1.3. Special equipment

Mira cloth

Petri dishes (small = 6 cm diameter, medium = 9 cm diameter, large = 14 cm diameter)

Sterile cabinet

Sterile Filter (pore size 0.2 µm)

2.2. MEDIA AND SOLUTIONS

2.2.1. Tissue Culture

Medium for the maintenance of callus and suspension cultures

I,2a Medium (Seitz, 1985)

Sucrose	30 g	
Myo-inositol	100 mg	
Amino acid stock solution	10 ml	
Vitamin stock solution	1 ml	
Micronutrient stock solution	10 ml	
Macronutrient stock solutions	10 ml	each
2,4-D stock solution	1 ml	
IAA stock solution	10 ml	
Kinetin stock solution		10 ml

adjust pH to 5.6 with KOH

fill to 1 l with distilled water

autoclave

add sterilly

Fe-EDTA stock solution 5 ml

Amino acid stock solution

Alanine	297 mg	Leucine	50 mg
4-Amino butyric acid	260 mg	Lysine	20 mg
Arginine	31 mg	Methionine	0.5 mg
Asparagine	38 mg	Phenylalanine	0.5 mg
Aspartic acid	17mg	Proline	19 mg
Cysteine	30 mg	Serine	128 mg
Glutamine	3 mg	Threonine	41 mg
Glutamic acid	157 mg	Tyrosine	0.5 mg
Glycine	27 mg	Valine	23 mg
Histidine	0.5 mg		

add distilled water to 200 ml

store in small portions at -20° C

Vitamine stock solution

Nicotinic acid	50 mg
Pyridoxine · HCl	10 mg
Thiamine · HCl	10 mg

add distilled water to 100 ml

store in small portions at -20° C

Micronutrient stock solution

H_3BO_3	62 mg
$MnSO_4 \cdot 4\ H_2O$	223 mg
$ZnSO_4 \cdot 7\ H_2O$	86 mg
KI	0.3 mg
$Na_2MoO_4 \cdot 5\ H_2O$	2.5 mg
$CuSO_4 \cdot 5\ H_2O$	0.25mg
$CoCl_2 \cdot 6\ H_2O$	0.25mg

add distilled water to 100 ml

Macronutrient stock solution

add distilled water to 100 ml to each of the following salts

KNO_3	19 g
NH_4NO_3	16.5 g
$CaCl_2 \cdot 2\,H_2O$	4.4 g
$MgSO_4\ 7\,H_2O$	3.7 g
KH_2PO_4	1.7 g

Fe-EDTA stock solution

$FeSO_4 \cdot 7\,H_2O$	2.78 g
$Na_2 \cdot EDTA$	3.72g

add double distilled water to 100 ml

bring to boil

autoclave

add to complete, sterilized medium

Phytohormone stock solutions

IAA 20 mg

dissolve in small amount of ethanol
add distilled water to 100 ml
store in small portions at -20° C

Kinetin 2 mg

dissolve in 1 ml 0.5 M HCl at 40° C
add distilled water to 100 ml
store in small portions at -20° C

2,4-D 10 mg

dissolve in small amount of ethanol
add distilled water to 100 ml
store in small portions at -20° C

To make a solid medium for callus culture
add 1% agar before autoclaving
pour 10-20 ml of the autoclaved solution into medium size petri dishes

Solution for the isolation of protoplasts from suspension culture

Iso-medium (Seitz, 1985)

Sucrose	15 g	
Myo-inositol	0.1 g	
Mannitol	45.5 g	
Sorbitol	45.5 g	
Amino acid stock solution	10 m	
Vitamine stock solution	1 ml	
Micronutrient stock solution	10 ml	
Macronutrient stock solution	10 ml	each except $CaCl_2$
$CaCl_2$ stock solution	14 ml	

adjust pH to 5.6 with KOH

fill to 1 l with distilled water

autoclave

Enzyme solution for the isolation of protoplasts from suspension culture

BSA	0.1 g
Cellulase	1.5 g
Rhozyme	0.2 g

adjust pH to 5.6 with KOH

fill to 100 ml with Iso-medium

filter over night at 4° C

sterilize by filtration through sterile filter

Solution for the propagation of protoplasts from suspension culture

PC 6 (Seitz, 1985)

2,4-D stock solution	1 ml

fill to 1 l with Iso-medium
adjust pH to 6.1 with KOH
autoclave
add sterilly

Fe-EDTA stock solution	1 ml

Enzyme solution for the isolation of protoplasts from carrot root (Kameya, 1972)

Pectinase	0.1 g
Cellulase	5 g
KCl	3.5 g
$CaCl_2$	0.5 g

add distilled water to 100 ml
filter through paper filter
sterilize by filtration through sterile filter

Washing solution for protoplasts isolated from carrot root (Kameya, 1972)

Mannitol	14.58 g
KCl	0.25 g
$CaCl_2$	0.25 g

add distilled water to 100 ml

autoclave

Culture medium for protoplasts isolated from carrot root (Kameya, 1972)

$Ca(NO_3)_2 \cdot 4\,H_2O$	280 mg	$MnCl_2 \cdot 4\,H_2O$	6 mg
KNO_3	80 mg	Ferric citrate	10 mg
KCl	65 mg	Na_2MoO_4	0.002 mg
$MgSO_4 \cdot 7\,H_2O$	740 mg	Thiamin	0.35 mg
NaH_2PO_4	20 mg	Pyridoxin	0.15 mg
Na_2SO_4	455 mg	Nicotinic Acid	0.15 mg
$CuSO_4$	0.02 mg	2,4-D	0.1 mg
H_3BO_3	1.5 mg	Kinetin	0.2 mg
$ZnSO_4 \cdot 7\,H_2O$	2.7 mg	Mannitol	127.5 g
KI	0.8 mg		

adjust pH to 5.8 with KOH

add distilled water to 1 l

autoclave

Alternative medium for the propagation of protoplasts isolated from carrot root

IAA stock solution	10 ml
Kinetin stock solution	10 ml
Fe - EDTA	1 ml

filtersterilize

add sterilly to 1 l Iso-medium

2.2.2. Microbiology

Medium for the propagation of *Agrobacterium*

YEB-broth

Bacto-tryptone	5 g
Yeast extract	1 g
Beef extract	5 g
$MgSO_4$	0.5 g

adjust pH to 7.2 with NaOH

add water to 1 l

autoclave

add sterilly

Sucrose stock solution	10 ml
Kanamycin stock solution	5 ml
Rifampicin stock solution	5 ml

Sucrose stock solution

autoclave 50 g sucrose

dissolve in 100 ml sterile distilled water

Antibiotics stock solutions

Kanamycin	100 mg

add distilled water to 10 ml

filtersterilize

store at 4° C

Rifampicin	200 mg

add methanol to 10 ml

shake

store at -20° C in the dark

Augmentum	100 mg

add distilled water to 10 ml

filtersterilize

use immediately

Alternative medium for the propagation of *Agrobacterium*

Antibiotics medium 3	17.5 g
Sucrose stock solution	5 g

adjust pH to 7.2 with NaOH
add water to 1 l
autoclave
add sterilly

Kanamycin stock solution	5 ml
Rifampicin stock solution	5 ml

2.2.3. Microscopy

Auramine O

Stock solution	1 mg/10 ml	in distilled water
Final concentration	0.1 ml/10 ml	in distilled water or Iso-medium

Calcofluor White

Final concentration	1 mg/10 ml	in distilled water or Iso-medium

Fluorescein diacetate

Stock solution	5 mg/ml	in acetone
Final concentration	0.2 ml/10 ml	in 0.05 M phosphate-buffer pH 5.8

Phenosafranine

Final concentration	10 mg/10 ml	in Iso-medium

store at -20 C

2.2.4. Analysis of transformed cells

Selection medium for the analysis of transformed cells

Rifampicin stock solution	10 ml
Kanamycin stock solution	10 ml
Augmentum stock solution	20 ml

add sterilly to 1 l PC6 medium

Solutions for the fluorescence GUS assay

MUG minus buffer

NaH_2PO_4	0.78 g
Na_2 - EDTA	0.336 g
Triton X-100	10 µl
Mercaptoethanol	69 µl

adjust pH to 7.0

add sterile distilled water to 100 ml

MUG substrate

MUG	10 mg

dissolve in 10 ml MUG minus buffer

Stop solution

Na_2CO_3	2.12 g

dissolve in 100 ml distilled water

Solutions for the colourimetric GUS assay

GUS substrate

X-glu 2 mM

dissolve in 0.1 M phosphate buffer pH 7.0

2.3. METHODS

2.3.1. Tissue culture

As sterility is one of the most important aspects of tissue culture, special care was taken to ensure that all flasks, tools, media and solutions were sterilized. If possible, all solutions and equipment were autoclaved for 20 minutes at 121° C and 1 bar. Unstable solutions, e.g. enzyme solutions were sterilized by filtration through a sterile filter. If reusable filters were used, they were autoclaved after every application. Tools such as scissors, tweezers and spatulas were either autoclaved or they were sterilized by dipping them into 99% ethanol and burning off the alcohol.

Once the plant tissue was sterilized, all manipulations were carried out in a sterile cabinet. Before every use, the cabinet was allowed to run for at least 15 minutes to sterilize the air. It was then cleaned out with alcohol and all the equipment was placed inside the cabinet. After 5 more minutes the air in the cabinet was expected to be sterile.

The usual methods of sterile technique were used even when working in a clean bench. These measures included sterilizing hands before working, flaming the tops of all bottles, covering containers when they were not used, removing equipment which was not needed any more, pipetting with a sterile pipettor, and so on.

When new cultures were to be established, there was always a problem that some fungus spores survived even a very thorough sterilization procedure. Thus all new cultures were checked at least daily and contaminated tissue was removed.

Sterility of the cultures was checked by plating out 0.1 ml samples of liquid medium onto petri dishes with YEB-broth agar and incubating them over night at 28° C. If any colonies became visible, the culture was considered contaminated and was discarded.

Other influences on the tissue culture were the culture conditions. Eventhough plant tissue cultures were much less demanding than animal cell cultures, only a specific set of conditions will lead to optimal growth. In the case of the cultures used here these conditions were 26° C and dark unless otherwise stated. To avoid contamination, only sterile material was kept in the culture room.

Maintenance of callus and suspension cultures

The carrot suspension cultures were grown in 250 ml conical flasks mounted on a shaker at 120 rpm. Every 7 days, 8 ml of culture were transferred to a new flask containing 60 ml I,2a medium.

Callus tissue was cultured in 250 ml conical flasks which contained approximately 50 ml I,2a agar. These cultures were subcultured every 3 weeks for optimum growth. For long term storage, they could be left for up to six weeks without harm.

Isolation of protoplasts from suspension culture

Cells from a five day old suspension culture were collected on a mira cloth. They were placed in a sterile 250 ml flask and weighed. To each gram wet weight of cells, 10 ml of enzyme solution were added. The flask was sealed and placed on a shaker at 28° Cfor 4.5 - 5 h. The conditions were adequate to digest over 90% of the cell walls.

The solution was then filtered through 140 µm and 60 µm pore size nylon nets to hold back any undigested material. To sediment the cells, the solution was centrifuged at 100 g for 5 min. The supernatant was removed and replaced with Iso-medium. The solution was centrifuged at the same setting and the cells were washed one more time. After removing the washing solution, the cells were taken up in a small amount, e.g. 20 ml of PC 6 and counted. They were diluted to $1x10^5$ cells/ml and 10 ml portions were pipetted into medium size plastic petri dishes. The dishes were sealed with laboratory cling film and placed in a 22° C culture room in the dark.

Isolation of protoplasts from carrot root

For the isolation of protoplasts from carrot roots a method based on the procedure reported by Kameya and Uchimiya in 1972 was used. The following changes were made in the protocol to cope with missing facilities, safety standards and problems which became apparent during the experiments:

- The carrots were bought in a supermarket. The variety was unknown and the age was guessed by their size and appearance.

- Instead of a 0.2% solution of mercuric chloride, a 10% solution of commercial bleach was generally used.

- As a hand microtome was not available, the slices were cut as thin as possible using a new scalpel blade.

- The sterile filter was usually clogged up by the enzyme solution. Thus the solution was always prefiltered through normal filter paper in a funnel.

- The incubation temperature was 26° C.

- As a stainless steel mesh was not available, in the first experiments a fine nylon mesh of undefined pore size was used. Later, a nylon mesh with a pore size of 0.1 mm became available.

- The coconut milk listed as an ingredient for the growth medium was replaced by kinetin as mentioned in the article.

- 1 g of carrot was about equal to 2 carrot slices of approximately 0.5 cm height and 1 cm^2 surface area. Two slices were used in all experiments.

- As the cells did not sediment well, they were centrifuged in small laboratory tubes at 100 g for 1 min.

The adapted procedure is described here:

From mature carrot roots, slices of 0.5 - 1 cm thickness were cut. They were sterilized by shaking them first in 10% commercial bleach solution for 5 min and second in 70% ethanol for 1 min. They were rinsed 5 times with sterile water. The skin was removed with a scalpel and slices of about 0.1 mm were cut. These slices were placed into a flask containing the enzyme solution at a concentration of 1 g carrot tissue/10 ml enzyme solution. The digestion takes place over night (15 h) on a shaker.

Large debris was removed from the solution by filtering it through a nylon mesh with 100 µm pores. The cells were centrifuged at 100 g for 5 min and washed twice with washing solution. They were then taken up in culture medium, counted and either plated on small petri dishes or poured into 50 ml flasks which were sealed with cling film. The cells were cultured at 26° C in the presence of light.

Preparation of carrot discs for cocultivation experiments

Mature carrot roots were washed thoroughly and divided into 0.5 cm slices. They were dipped into 96% ethanol for 15 s, washed with sterile water and placed in a flask with a 10% solution of commercial bleach. The slices were sterilized by the bleach for 30 min on a shaker. Then they were rinsed 3 times with sterile water before the skin was removed and the slices were divided into 4 segments. 4 - 8 segments were placed on agar in a 9 cm petri dish. The dishes were sealed and kept at 26° C in the dark.

Sterile seed germination

Commercial carrot seeds were sterilized by shaking in 96% ethanol for 7 min, sterile water for 8 min and a 10% solution of sterile bleach for 30 min. They were collected on a filter and washed thoroughly with sterile distilled water. The seeds were germinated either on sterile moist filter paper in petri dishes or on medium size petri dishes with approximately 10 ml I,2a agar. About 20 seeds were spread on one dish. The containers were sealed with laboratory cling film.

2.3.2. Microbiological techniques

Propagation of *Agrobacterium*

Agrobacterium can be grown and stored on petri dishes with YEB or A3 agar. A platinum loop was either dipped into a solution of *Agrobacterium* scratched on the surface of a frozen glycerol sample of bacteria or touched to a colony growing on another dish. It was passed over the top of the new agar. The petri dish was sealed and kept at 37° C for propagation over night and then at 4° C for storage.

To multiply the bacteria, a 10 ml universal bottle or a 100 ml conical flask with liquid medium was inoculated with *Agrobacterium* . Within 2 - 3 days the cells had multiplied to maximum density.

Long term storage of *Agrobacterium*

One colony from a petri dish was transferred to 5 ml liquid medium and grown over night. Into a sterile vial with 0.15 ml of glycerol 0.85 ml of the culture were pipetted. The vial was closed, mixed on a vortex and transferred immediately to a -70° C freezer. To recover the bacteria, the surface of the frozen mixture was scratched with a platinum needle or loop.

2.3.3. Microscopical techniques

The cells were examined under special conditions to avoid crushing the fragile protoplasts. Either a sample of the culture was placed on a haemocytometer and viewed under a normal microscope or a drop of culture was spread on a normal glass slide and was examined using an inversion microscope. No differences between the pictures taken by either method could be detected.

For staining the cells, samples of 0.1-0.2 ml of culture were mixed with equal amounts of staining solution, incubated for 30 - 60 s and placed on a haemocytometer or a slide. The following stains were used:

For establishing the viability of the cells Auramine O, Fluorescein diacetate and Phenosafranin were used. Auramine O is a basic dye which was first used for plant microscopy by Heslop-Harrison in 1977. It binds to lipids, especially unsaturated acidic waxes and cutin precursors (Considine and Knox, 1979). Viable cells fluoresce in a bright greenish yellow colour, while dead cells remain unstained. Fluorescein diacetate is a stain that diffuses into the cells. Living cells split the diacetate from the fluorescein, liberating the stain which fluoresces light green. Phenosafranin has also been introduced as a fluorochrome for viability studies on plant cells and it works on the dye exclusion principle, staining only dead or dying cells which become visible in normal light as red spots.

The presence of cell walls was detected by staining with the fluorescent brightener Calcofluor White (Huges and McCully, 1975). This fluorochrome selectively stains cellulose, white fluoresces blueish white.

A quick simple test for cell walls was adding a drop of a 5% SDS solution to cells stained for viability. SDS will destroy the cell membrane. If the cytoplasm stays within the cell shape, a cell wall was present, otherwise the cytoplasmic contents will flow out of the cell (Emmerling, personal communication).

Cell densities were counted in a haemocytometer of 0.1 or 0.2 mm height. A minimum area of eight large squares was counted to reduce statistical errors.

2.3.4. Transformation

All experiments described in this thesis use the method of cocultivation for transforming the plant cells. For this procedure only the plant cultures or explants and a solution of *Agrobacterium* at high density was needed. The bacteria were added directly to the plant cells by pipetting a small amount of the growth medium in which the bacteria were present in large amounts to the plant culture medium. The cells and bacteria were cocultivated for 2 days, during which the bacteria attached to the cells and transformed them. The *Agrobacteria* were then removed by washing the cells. The cells were transferred to a selection medium which selects for transformants through antibiotic resistance and also includes an antibiotic to kill any bacteria left on the cells.

Protoplasts were transformed by adding 200 µl of bacteria to a medium size petri dish with cells at a density of $1x10^6$ cells/ ml. The dishes were kept in the dark at 26° C without agitation. After two days of cocultivation, the cells were centrifuged at 100 g for 5 min and the supernatant containing most of the bacteria was removed. The cells were washed once with Iso-medium. Then 10 ml of the selection medium were added to each batch and the cells were poured into medium size petri dishes. The cells were analyzed for transformation at different stages of growth.

Suspension cultured cells were cocultivated with the *Agrobacteria* similar to the protoplasts. Instead of washing off the bacteria, antibiotics in the following concentrations were directly added to the cocultivation mixture.

Augmentum	200 µg/ml
Rifampicin	100 µg/ml
Kanamycin	100 µg/ml

Tissue explants and seeds were transformed by placing 1 - 2 drops from a 200 µl pipette (= 20 - 40 µl) directly on top of the sample. They were cultivated for two days. The bacteria were removed by washing every single explant or seed in 2 baths of sterile water. Explants were placed on selection agar containing all antibiotics, whereas the seeds were transferred to I,2a agar containing only augmentum which destroys the *Agrobacterium* but does not harm untransformed plant cells.

2.3.5. Analysis of transformed tissue

Different methods were used to check the cells for successful transformation. The first selection step was culturing the cells in a selection medium containing antibiotics which are fatal to normal carrot cells. Into the T-DNA of the Ti-plasmid in the *Agrobacterium* sequences for proteins were cloned which confer resistance to rifampicin and kanamycin. By incorporating these genes the plant cells become resistant to the antibiotics and will survive in the selection medium.

The other methods of checking for transformation use the other gene product of the T-DNA. Bacterial glucuronidase was transported to the plant genome by the transformation. The presence of this enzyme can be proven either by exploiting its activity or through specific antibodies.

Fluorescence GUS assay

The plant tissue or cells were ground with sand in 0.1 ml MUG minus buffer in a reaction tube which was kept on ice to minimize proteolysis. After centrifugation at 10 000 g the supernatant was pipetted into another reaction tube to which 0.21 ml MUG minus buffer were added. 35 µl of MUG substrate solution were pipetted into the mixture which is then incubated at 37° C for 1 h.

The reaction was stopped by transferring 0.1 ml of the reaction mixture to a tube with 0.9 ml stop-solution. For qualitative analysis, the solution was viewed on a transilluminator. For the analysis of suspension cultured cells or protoplasts, the cells were concentrated by centrifugation, washed with medium and pelleted at 500 g for 5 min before the supernatant was removed. 0.1 ml MUG minus buffer were then added and the mixture was left on ice for a few minutes. The other steps were carried out as described above.

Colourimetric GUS assay

To 10 - 50 µl samples of concentrated cells or protoplasts or small fragments of callus tissue 10 µl 2 mM GUS substrate and 10 µl of the phosphate buffer were added. After thoroughly mixing the cells, the mixture was incubated at 37° C over night.

3. RESULTS

3.1. TRANSFORMATION OF PROTOPLASTS FROM CARROT ROOTS

3.1.1. Isolation of Protoplasts

Many different attempts were made to isolate protoplasts from carrot roots using the method described in chapter 2.3.1. The results of these experiments are listed in FIGURE 5.

FIGURE 5 Isolation of protoplasts from root

Experiment No.	Special conditions	Result
1	none	total lysis after 5 d
2	none	bacterial contamination
3	none	total lysis after 6 d
4	addition of 0.1 M sucrose to medium	total lysis after 5 d
5	MS medium	total lysis after 5 d
6	B5 medium	total lysis after 5 d
7	smaller, younger carrots	total lysis after 6 d
8	cultivation in small tubes	no regeneration of cell wall after 10 d, bacterial contamination
9	cultivation in petri dishes	bacterial contamination

10	cultivation in universal bottle	bacterial contamination
11	new sterilized solutions	bacterial contamination
12	20% bleach	lysis after incubation
13	20% bleach, no ethanol	lysis after incubation
14	none	lysis after incubation
15	younger, smaller carrots	lysis after incubation
16	purification in sucrose gradient	lysis after gradient
17	sterile seedling roots cultivation on petri dishes	used for transformation
18	new solutions, cultivation on petri dishes	used for transformation

The first three experiments were carried out exactly as described in the methods chapter. PICTURES 1 - 4 show newly isolated protoplasts after they have been filtered and washed.

PICTURE 1 shows unstained cells. It is interesting to note that the cells have different sizes, ranging from approximately 10 - 30 µm diameter. The cells have large vacuoles and the cytoplasm containing the carotene pigments is concentrated at one side. Large bits of debris can be seen which are probably undigested cell walls.

PICTURE 2 shows the same cells stained with FDA and illuminated by UV light. One can see that a high percentage of cells are viable. Also it can be recognized that a few cells do not stain up. These are dead cells which can usually be identified in PICTURE 1 because of their irregular shapes and rough cell surfaces.

In PICTURE 3 protoplasts of the same batch are shown, stained with calcofluor white and illuminated by normal light.

For PICTURE 4 UV light is used on the same PICTURE. When comparing the two PICTURES it can be seen that only very little cellulose is left in the cells. Most of the stained cellulose seems to be floating around as debris, whereas most of the round, healthy looking protoplasts have no cell wall around them.

As the protoplasts from experiments 1 and 3 lysed after 5 - 6 days without any visible contamination, it was suggested that the composition of the medium might not be adequate for the regeneration of the protoplasts. The first change was the addition of sucrose to the medium, supposing that the cells had used up their store of carbohydrates and were not able to digest the mannitol present in the medium. The second attempt was to use well established culture media, MS and B5, supplemented by 0.7 M mannitol. All of these approaches were without success (experiments 4 - 6).

In experiment 7, so-called new young carrots were used. It was hoped that younger cells would have better chances of survival. This was shown not to be the case.

The next approach was that the growth conditions were not adequate for the regeneration. In experiments 8 - 10, different containers were used for cultivating the protoplasts. The best result was achieved with 1.5 ml plastic laboratory test tubes, but even there the cells died after 10 days.

Samples from the cultivation in plastic tubes taken on day 5 are shown in PICTURES 5 and 6. In PICTURE 5 the contaminating bacteria are visible as small spots in the medium. On the surface of the cells small circles can be seen, which are a typical sign of contaminated cells. The staining for viability with FDA, seen in PICTURE 6, shows that most of the cells are still alive.

On day 8, the cells were examined again. On PICTURE 7 it can be seen that some of the cells have lysed. Most of the protoplasts show the typical spots on the surface, enhanced by the phase contrast optics. PICTURE 8 illustrates that the cells show less fluorescence when stained with FDA on day 5, eventhough the staining condition and incubation period was the same. This fact suggests that the cells are not healthy. Two days later, all the protoplasts had lysed, probably due to bacterial infection.

From these experiments it was concluded that the lack of sterility is the major problem of the isolation. For the next attempt (experiment 9), all solutions were newly prepared and sterilized. To test for sterility, samples from all solutions were taken and plated out on YEB medium. All of the incubation steps were checked for sterility the same way, also samples from the protoplasts were taken on a daily basis. All of the solutions proved all sterile, but already the sample taken from the overnight incubation showed colonies on the YEB agar plate.

The following experiments, 12 and 13, were designed to evaluate whether the contamination coming from the carrot roots could be controlled by more rigorous sterilization.

Unfortunately, these experiments were negatively influenced by the use of cellulase from a different company. As experiments 13 and 14, which were carried out following the original procedure but using the new cellulase show, isolations carried out with this cellulase resulted in the destruction of the protoplasts during the isolation process.

After these experiments, the emphasis of the research was shifted from the isolation of regenerating protoplasts to the production of concentrated solutions of protoplasts for transient transformation assays. An attempt was made to collect the protoplasts in a sucrose gradient after they were washed, but all the cells lysed in the sucrose solution. It was not investigated further, whether this effect was due to the high osmolarity of the solution or the acceleration-force of 500 g.

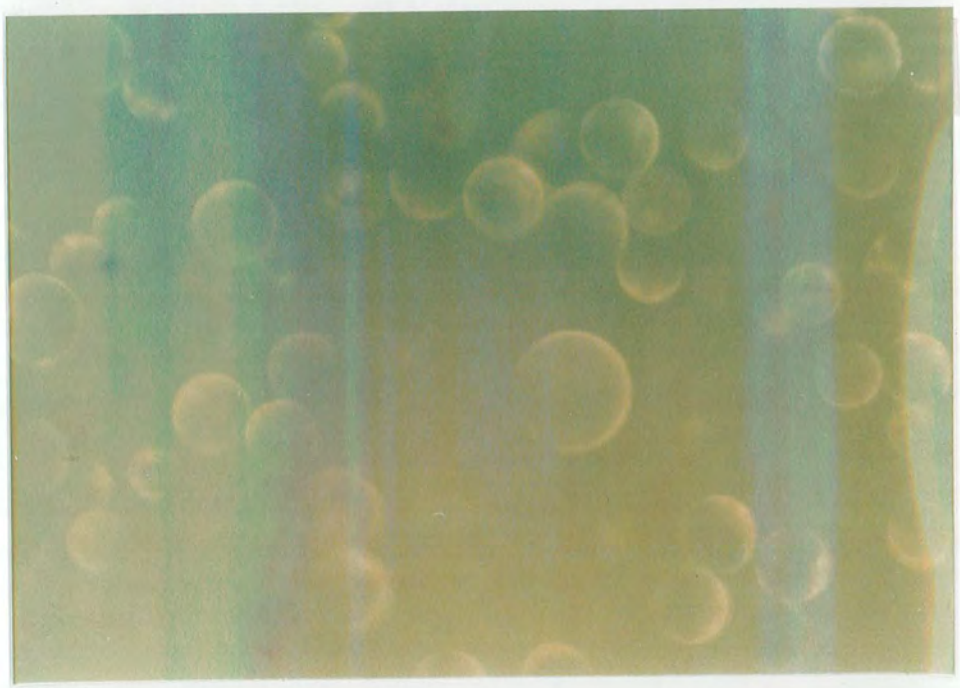
Another attempt to produce protoplasts was made when sterile seedlings became available. The cells isolated in experiment 17 were used directly for the transformation.

When the isolation of protoplasts from suspension cultured cells proved successful, one last experiment with carrot roots was carried out. The protoplasts were cultivated in the alternative medium. In PICTURE 9 the cells are shown after one day of regeneration, when about 50% of the cells were still alive. The presence of the cell wall was examined by adding SDS solution to the cells. It can be seen in PICTURE 10 that the protoplasts immediately lysed, indicating that no cell wall regeneration had started.

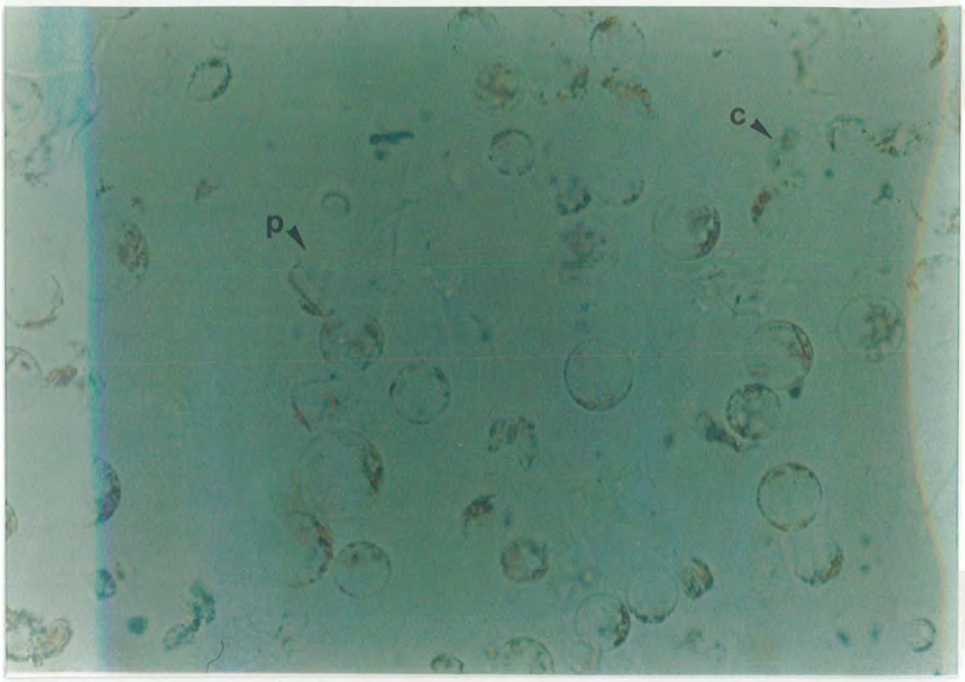
PICTURE 1 Newly isolated protoplasts from carrot root

s = small cell d = debris

l = large cell i = cell with irregular shape

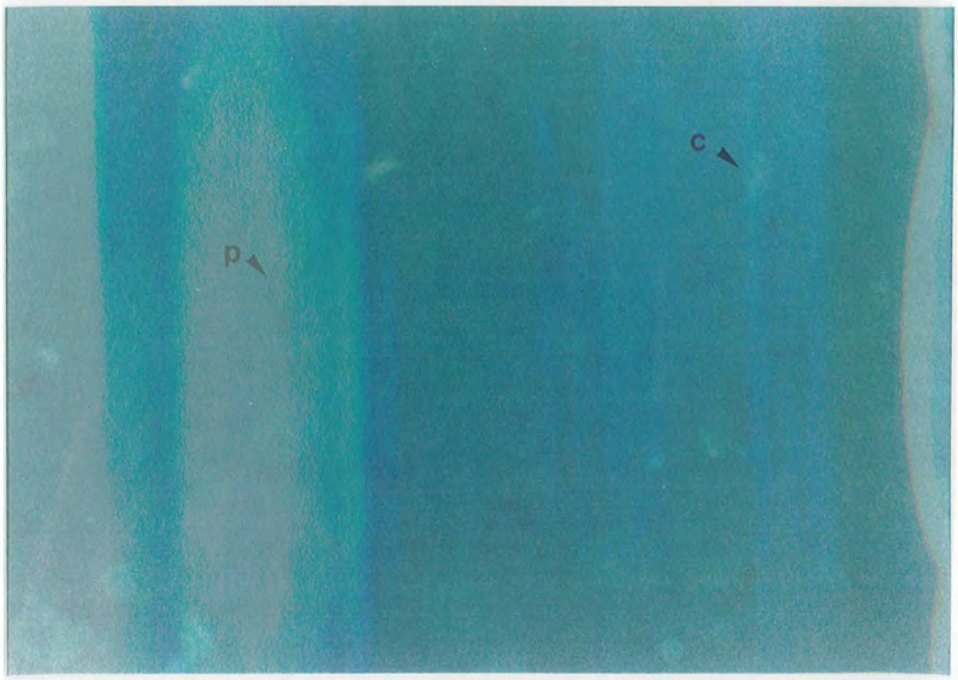


PICTURE 2 Newly isolated protoplasts, stained with FDA illuminated by UV light



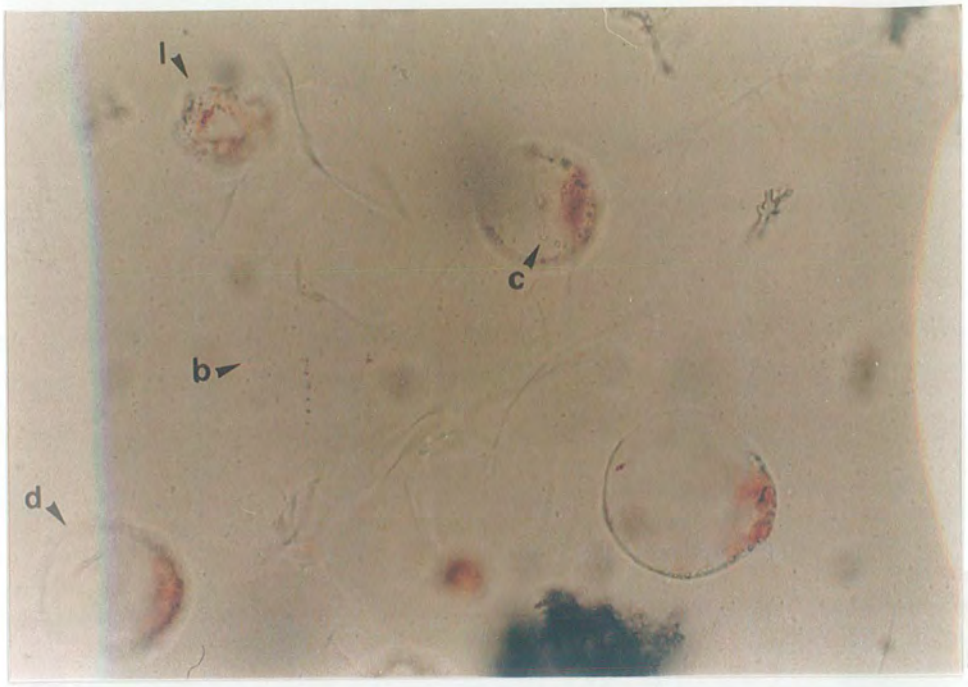
PICTURE 3. Newly isolated protoplasts, stained with calcofluor

p = protoplast c = cellulose



PICTURE 4. Newly isolated protoplasts, stained with calcofluor illuminated by UV light

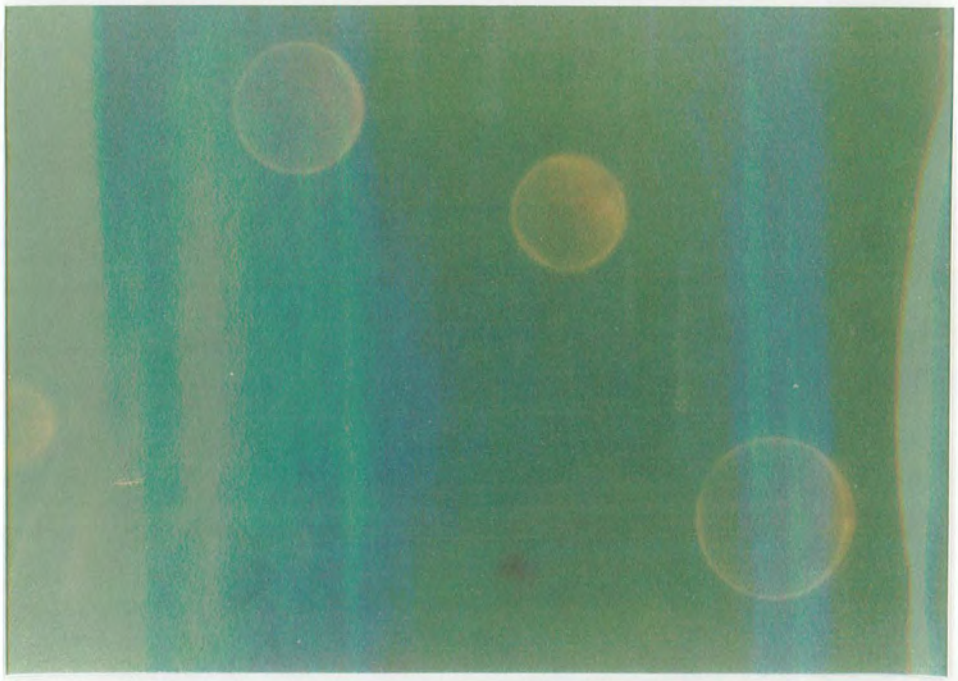
p = protoplast c = cellulose



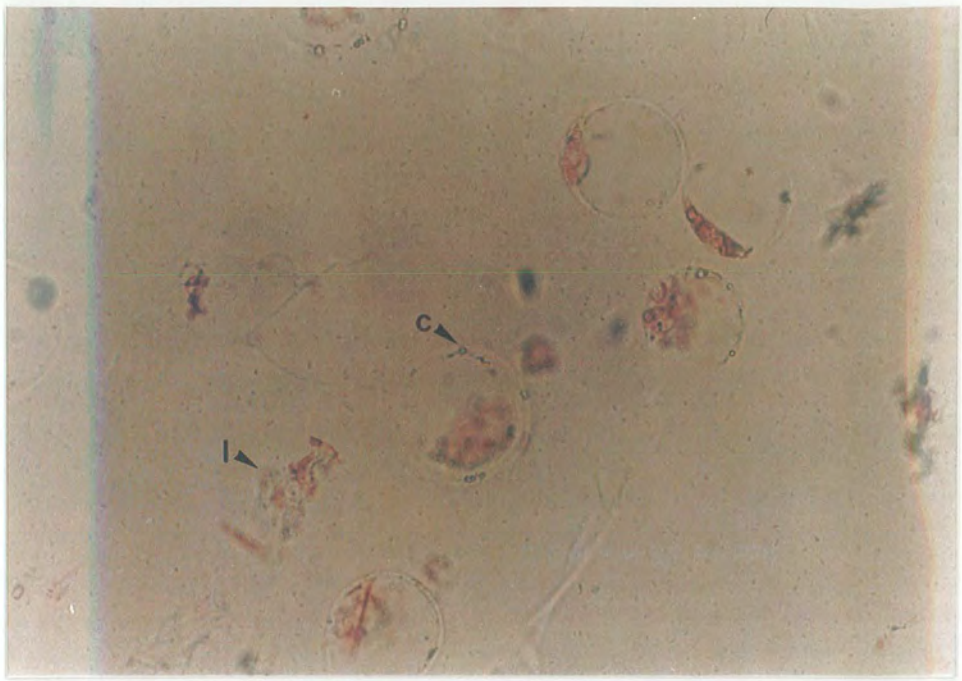
PICTURE 5 Protoplasts from carrot root on day 5

b = bacteria d = dieing cell

c = circle on cell surface l = lysed cell



PICTURE 6 Protoplasts from carrot root on day 5, stained with FDA illuminated by UV light

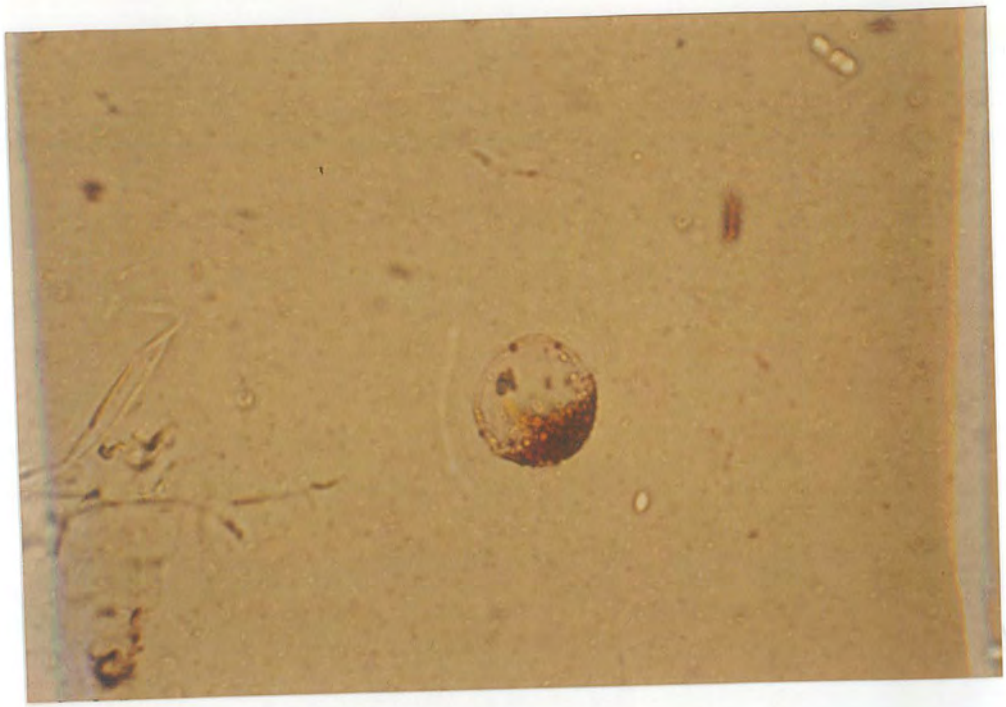


PICTURE 7 Protoplasts from carrot roots on day 8

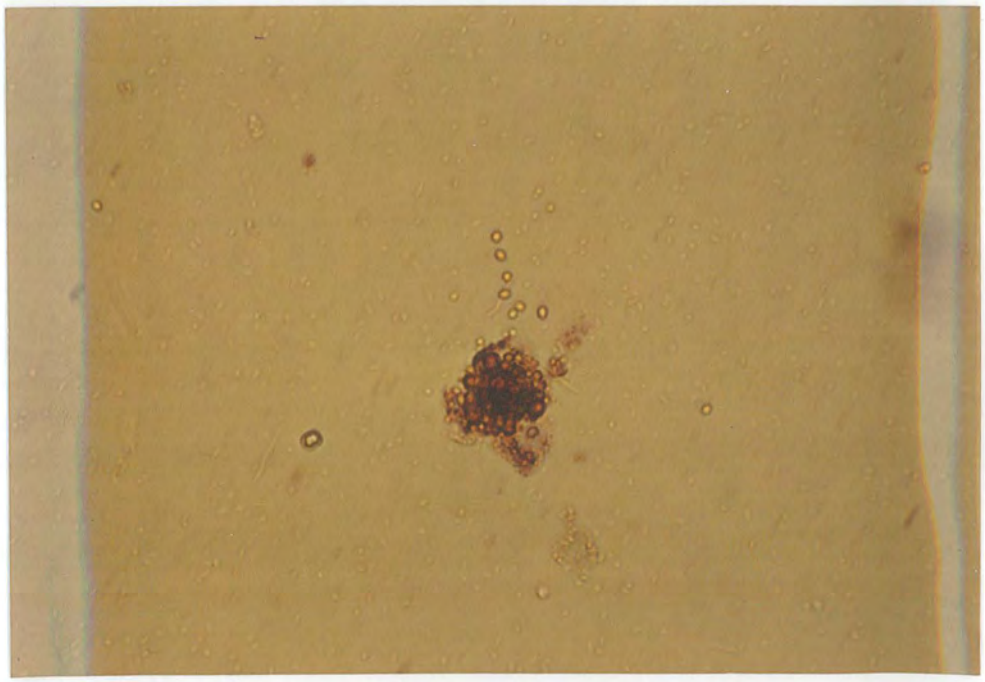
c = circle on cell surface l = lysed cell

PICTURE 8 Protoplasrs from carrot root on day 8, stained with FDA illuminated by UV light

w = weak fluorescence



PICTURE 9 Protoplasts from carrot roots



PICTURE 10 Protoplasts from carrot roots, SDS added

Lysis, indicating that cell wall regeneration had not started

Eventhough the cells were contaminated by bacteria, the protoplasts were used for transformation experiments, since it was hoped that the infecting bacteria would be destroyed either through competition with the *Agrobacteria* or by the antibiotics in the selection medium.

3.1.2. Transformation of protoplasts

The protoplasts from the isolation experiments 17 were cultivated in petri dishes for one day after the isolation. Then 20 µl of bacterial solution were added. After 2 days of cocultivation, all of the plant cells had lysed. It was not investigated, whether it was due to the presence of the *Agrobacteria* or the antibiotics in the bacterial solution that the protoplasts had died.

The cells from experiment 18 were also inoculated with the bacteria after 1 day of regeneration. After 2 days of cocultivation, about 20% of the protoplasts were still alive. They were directly used for the GUS fluorescence assay .

3.2. TRANSFORMATION OF PROTOPLASTS FROM SUSPENSION CULTURED CELLS

3.2.1. Isolation of protoplasts

Two sets of experiments were carried out to isolate protoplasts from suspension cultured cells. In the first set, changes to the original procedure had to be made.

The cells were incubated in universal bottles in batches of 2-4 g of cells (wet weight) to 10 ml of enzyme solution. The protoplasts were not filtered through the nylon meshes. Minor changes in the composition of the medium were made because not all of the ingredients were available.

Four batches of protoplasts were started simultaneously. The newly isolated protoplasts looked healthy when stained with FDA and observed through the microscope. After one day of cultivation, about 50% of the cells were viable, of which approximately 20% had already started with the regeneration of the cell wall. This could be seen by staining with calcofluor. These cells were used for the following experiments:

FIGURE 6 Isolation of protoplasts from suspension culture

Experiment No.	*Agrobacterium* strain
1	+ 20 µl C 58
2	+ 20 µl LBA 4404
3	+ bacterial medium
4	Control

The cells were checked after two days of incubation. At this point all the cells had lysed. Under the microscope, contamination was visible in experiments 3 and 4.

Two more isolations were carried out, both of which showed bacterial infections after 3 - 5 days. When the sterility of the sterile cabinet was examined YEB agar plate open in the bench over night, colonies were found on the plate, indicating that the air in the clean bench was unsterile.

The second set of protoplast isolation experiments was carried out under sterile conditions under supervision of Professor Seitz. The procedure described in the methods chapter was followed.

In the first experiment in this series, 2 g of newly harvested cells were incubated with 18 ml enzyme solution. 2 · 10 protoplasts were recovered after the purification procedure and divided into 18 petri dishes. Already after 2 hours the typical cell clusters could be observed. After the first day 84% of the protoplasts were still alive and all of these had cell walls. On the second day of incubation, antibiotics were added to the cells to examine which amounts were necessary to kill the cells.

FIGURE 7 Antibiotic resistance of protoplasts

Experiment No	Amount of Antibiotic	Type	Viability	Cultivation Period
1	100 µg/ml	rifampicin	49%	3 d
2	10 µg/ml	rifampicin	not checked	
3	1 µg/ml	rifampicin	52%	5 d
4	50 µg/ml	kanamycin	55%	3 d
5	10 µg/ml	kanamycin	not checked	
6	1 µg/ml	kanamycin	not checked	
7	200µg/ml	augmentum	45%	3 d
8	20 µg/ml	augmentum	not checked	
9		control	50%	3 d

When the cells were examined for viability three days later, no difference could be detected between the counts of the samples containing antibiotics and the control samples. Two explanations for this effect are possible. First, both the kanamycin and the rifampicin solutions were stored at 4° C for longer periods of time. This comparatively high temperature might have reduced their activities. The solution containing augmentum was new, which suggests that augmentum does not harm the plants at concentrations of up to 200 µg/ml. The second explanation is that the concentration of all the antibiotics are too low to affect the cells.

The control cells were kept in the same medium for 2 weeks. By then the viability had decreased to about 20% and the cells had formed large clusters.

In the last experiment, 7 . 10 protoplasts were isolated from 10 g of suspension culture. 84% of the cells were alive when stained with phenosafranin. The cells were cultivated in petri dishes and used for transformation experiments.

3.2.2. Transformation of protoplasts

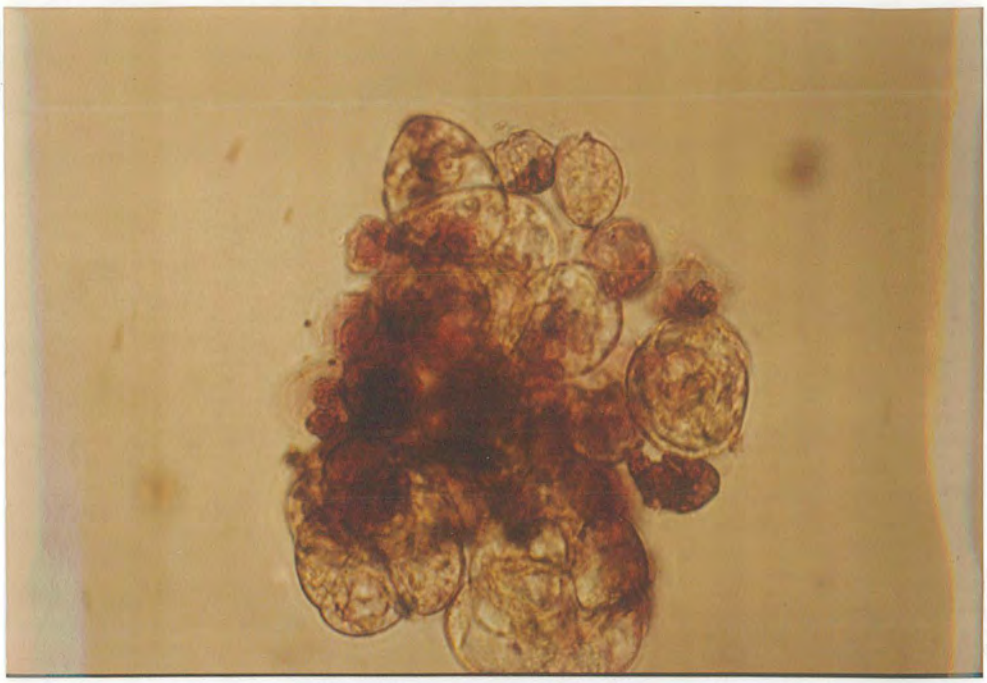
The protoplasts produced in the last isolation procedure weretransformation was carried out in duplicate, using the method described in chapter 2.3.4.

FIGURE 8 Transformation of protoplasts

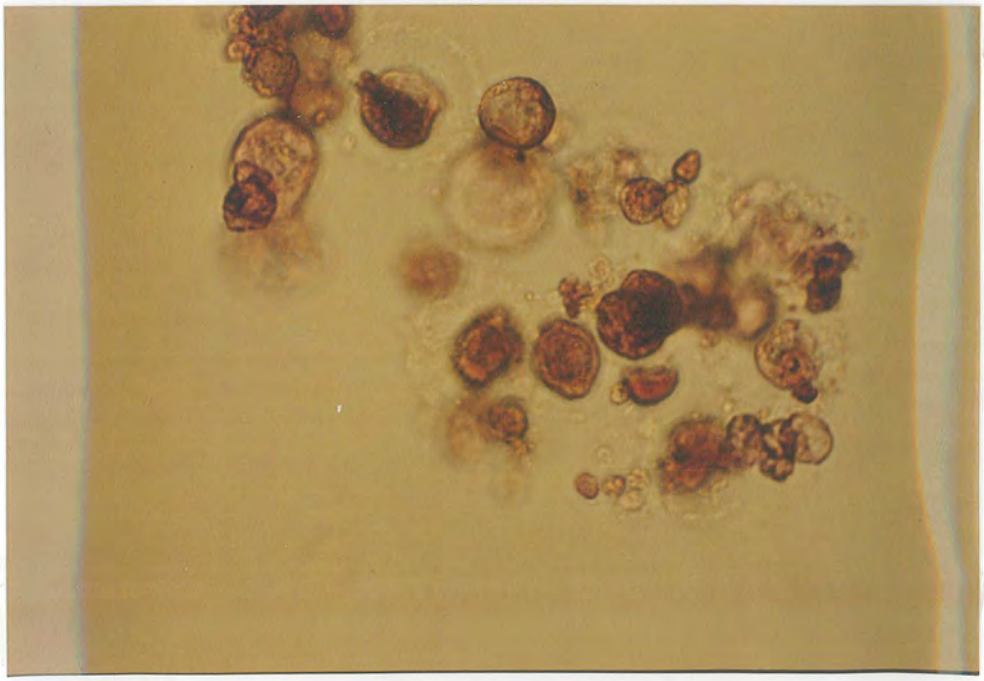
Experiment No	Time of cultivation before transformation	*Agrobacterium* strain
1	0 d	C 58
2	0 d	LBA 4404
3	1 d	C 58
4	1 d	LBA 4404
5	2 d	C 58
6	2 d	LBA 4404
7	2 d	bacterial medium
8	3 d	C 58
9	3 d	LBA 4404
10	3 d	bacterial medium
11	4 d	C 58
12	4 d	LBA 4404
13	4 d	bacterial medium

All of the transformations were tested in the GUS fluorescence assay. It is possible that the reason why none of the samples had any GUS activity was that too few cells had survived the selection process.

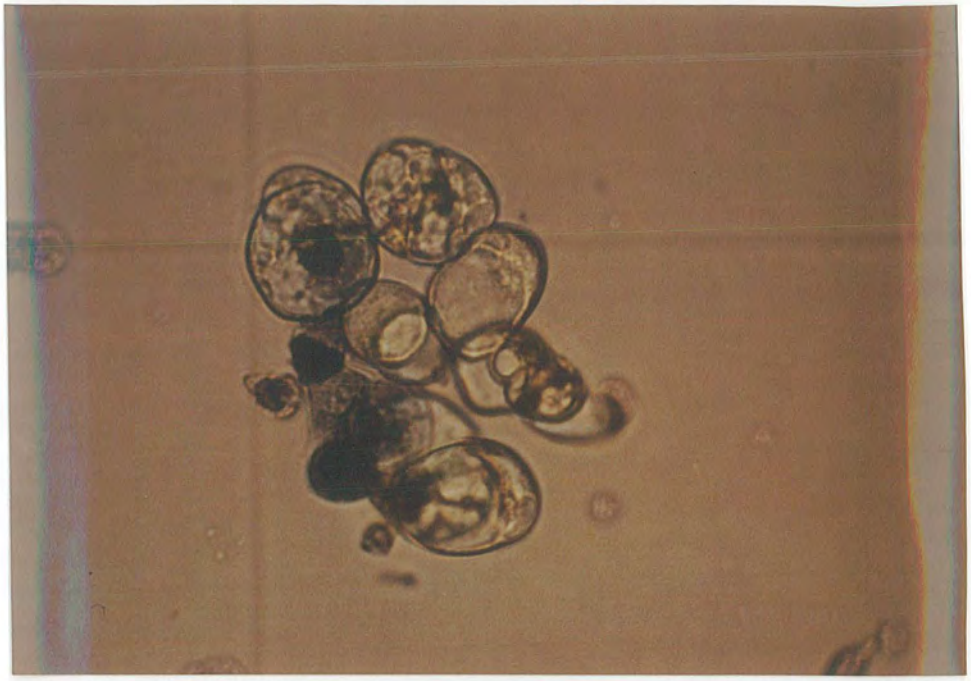
The cocultivation was monitored with the microscope. PICTURE 11 shows control protoplasts after 7 days of cultivation. By then the typical cell clusters have formed and through the phenosafranin stain one can see that about 40% of the cells are still alive.



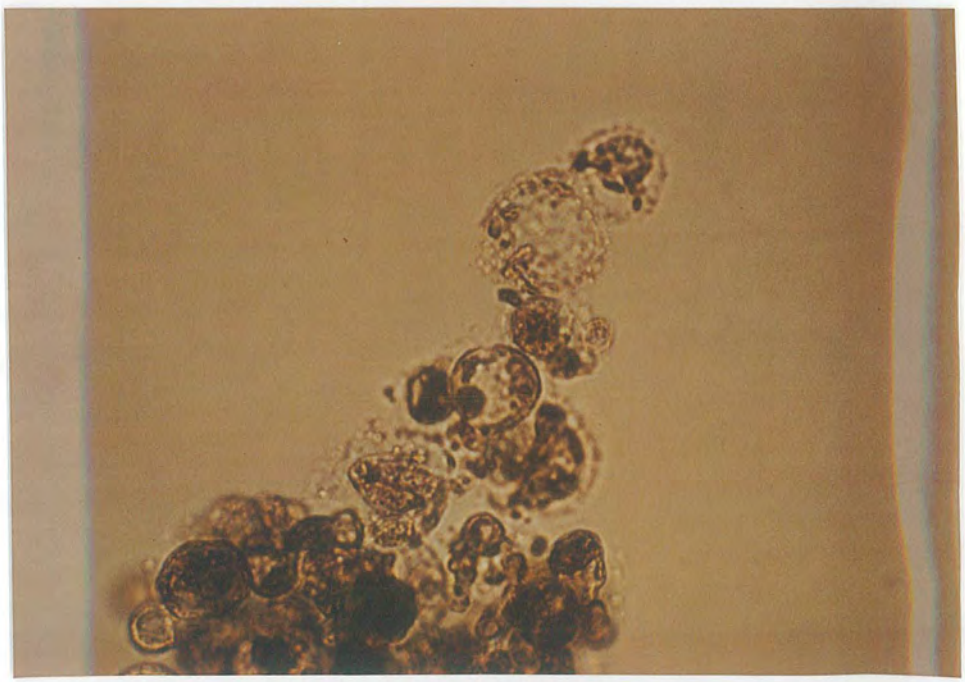
PICTURE 11 Protoplasts from suspension culture after 7 days stained with phenosafranin



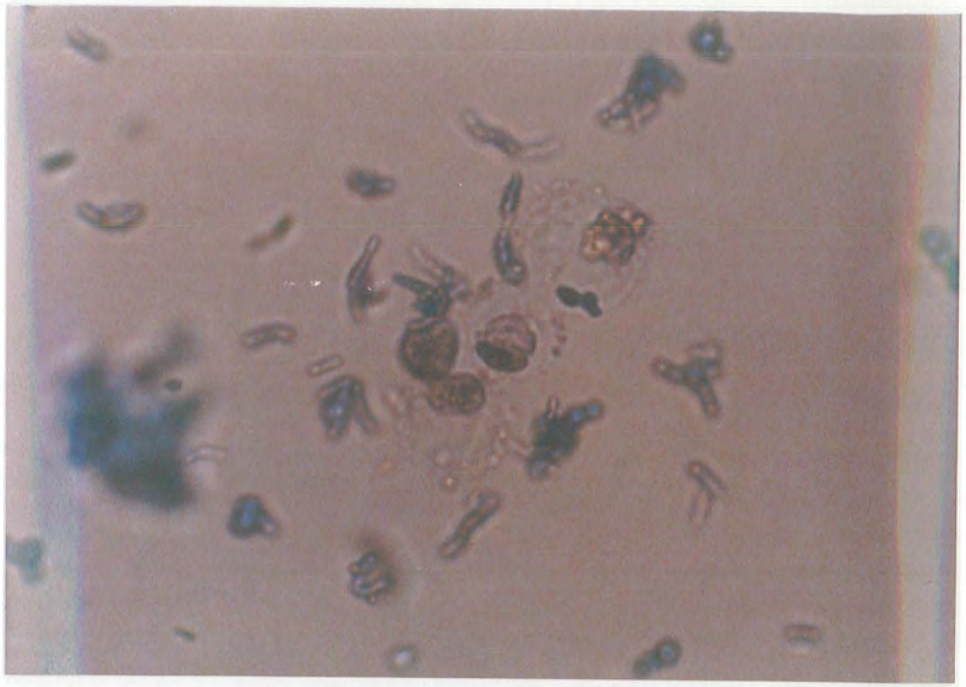
PICTURE 12 Protoplasts from suspension culture after 7 days cultured in selection medium



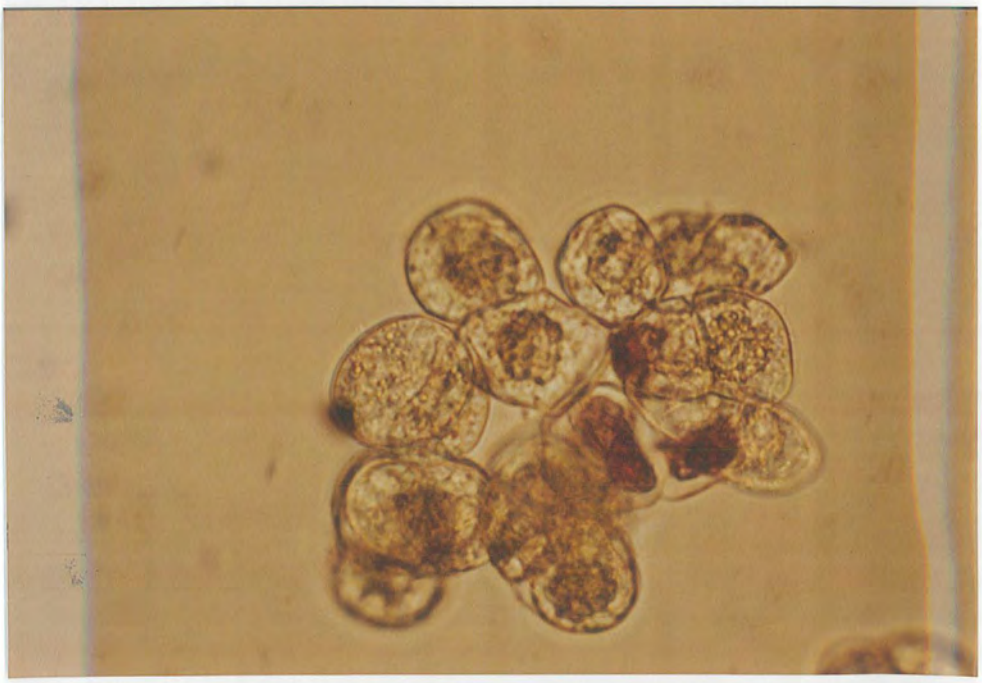
PICTURE 13 Protoplasts from suspension culture after 3 weeks stained with Thymolblue



PICTURE 14 Transformed protoplasts after 3 weeks in selection medium



PICTURE 15. Transformed protoplasts after 3 weeks contaminated



PICTURE 16 Suspension culture cells stained with phenosafranin

In PICTURE 12, cells from experiment 7 are seen after they have been washed and cultivated in the selection medium for 4 days. The untransformed cells have died during the selection.

PICTURES 13 - 15 were taken after 3 weeks of cultivation. The control cells, shown in PICTURE 13, have differentiated and resemble the original suspension culture cells. PICTURE 14 shows typical transformed cells. Most of the cells have died, leaving a small shrivelled cytoplasm surrounded by a cell wall which can be seen because of the attached bacteria. The cells in PICTURE 15 are contaminated by fungi.

In experiments 5 - 7 the cells from one of the petri dishes were plated out on a petri dish with selection medium agar. This was done to examine whether the cells would grow directly into calli. All of these cells died, in part because they were infected by a mold, but even in the uninfected areas, the cells did not recover.

3.3. TRANSFORMATION OF SUSPENSION CULTURE CELLS

On sterile petri dishes 10 ml of a solution of cells of the concentration $1 \cdot 10^5$ cells/ml were plated out. They were suspension cultured cells taken three and five days after subcultivation.Representative cells are shown in PICTURES 16 and 17. The cells show a high degree of differentiation, which increases with the age of the culture.

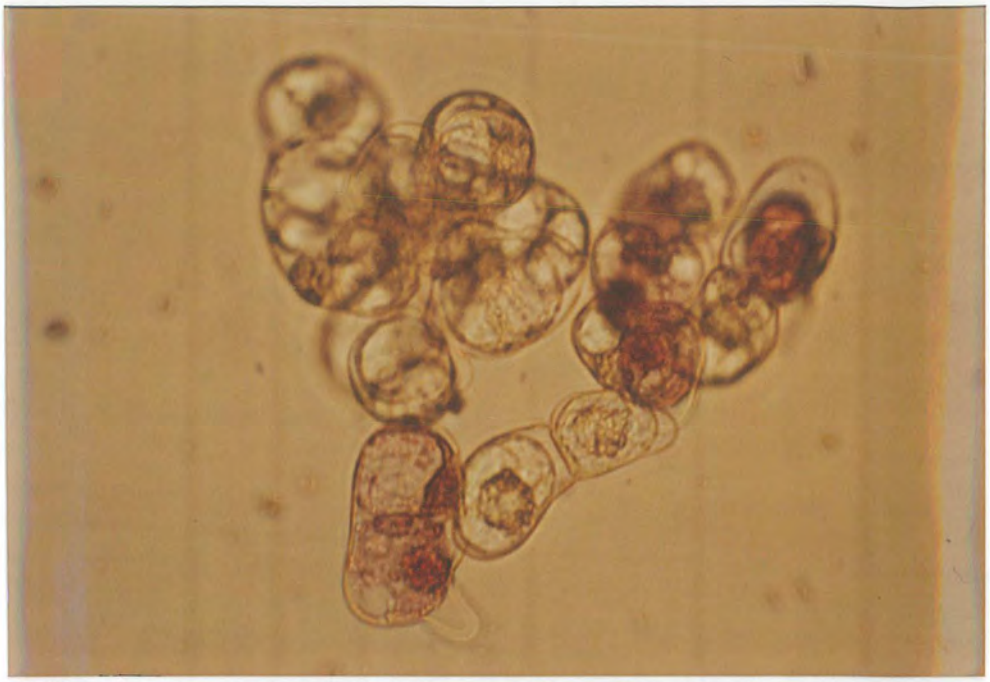
The transformation experiments which were carried out with these cells are listed in FIGURE 9

FIGURE 9 Transformation of suspension culture cells

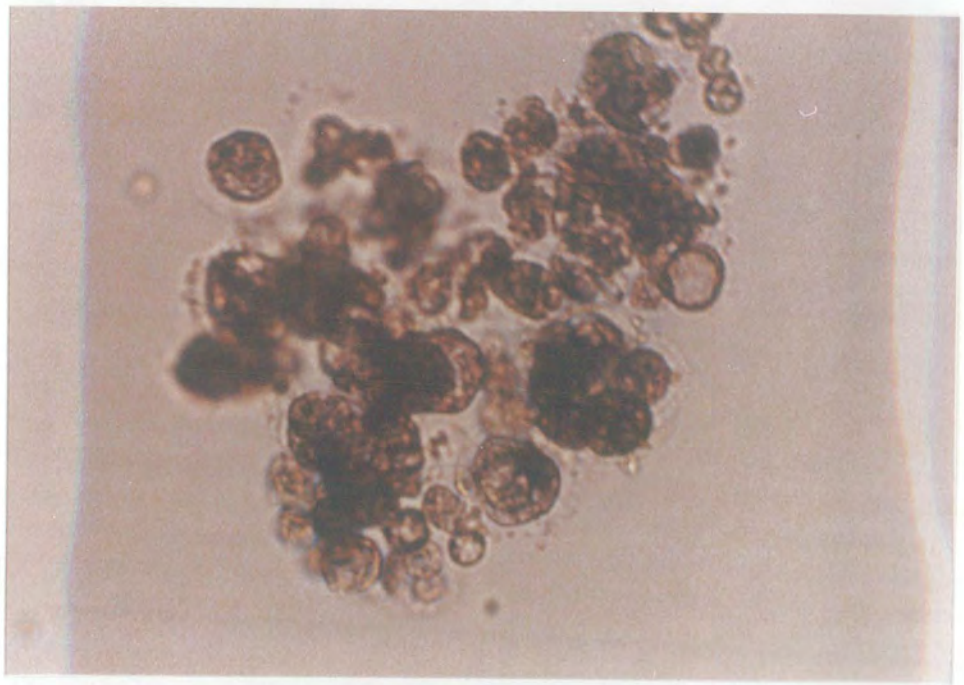
Experiment No.	"Age" of cells	*Agrobacterium* strain
1	3 d	C 58
2	3 d	LBA 4404
3	3 d	bacterial medium
4	4 d	C 58
5	4 d	LBA 4404
6	5 d	C 58
7	5 d	LBA 4404
8	5 d	bacterial medium

The cocultivation was examined both by microscopy and glucuronidase activity tests. PICTURE 17 shows untreated control cells after 3 weeks of cultivation. On PICTURE 18 untransformed cells from experiment 3 are shown. One can see that all of these cells have died in the selection medium. Transformed cells from experiment 2 growing in selection medium have been photographed for PICTURE 19. Since the cells have survived, it could be expected that they have acquired resistance against the antibiotics. This was checked by the GUS assay.

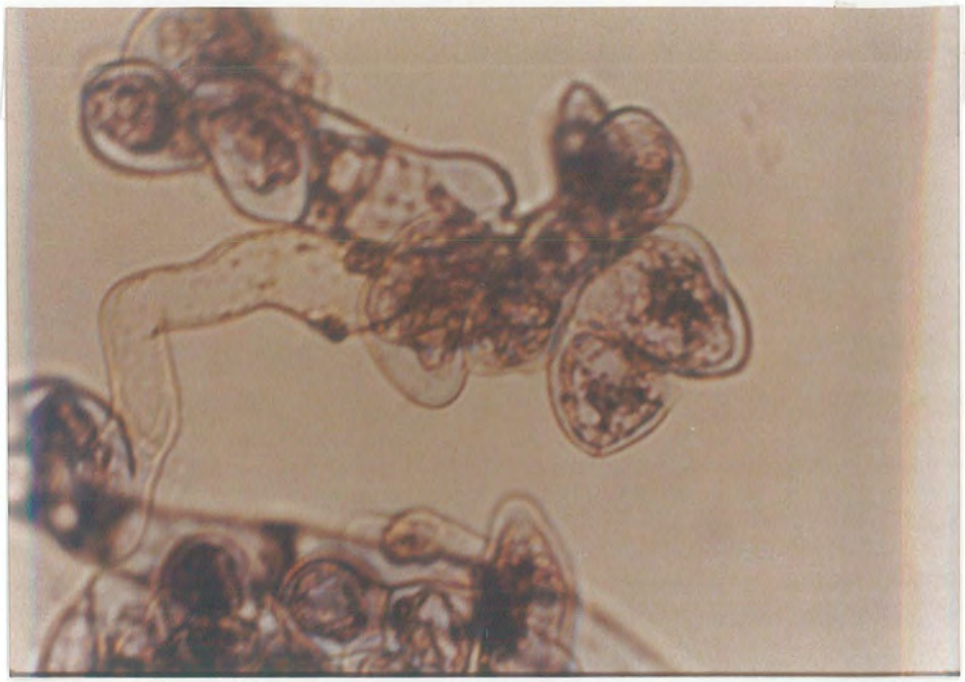
When samples from experiments 2 and 3 were tested in the fluorescence assay 2 the samples to which the antibiotics had not been added gave weak positive signals, while those in the selection medium gave negative results.



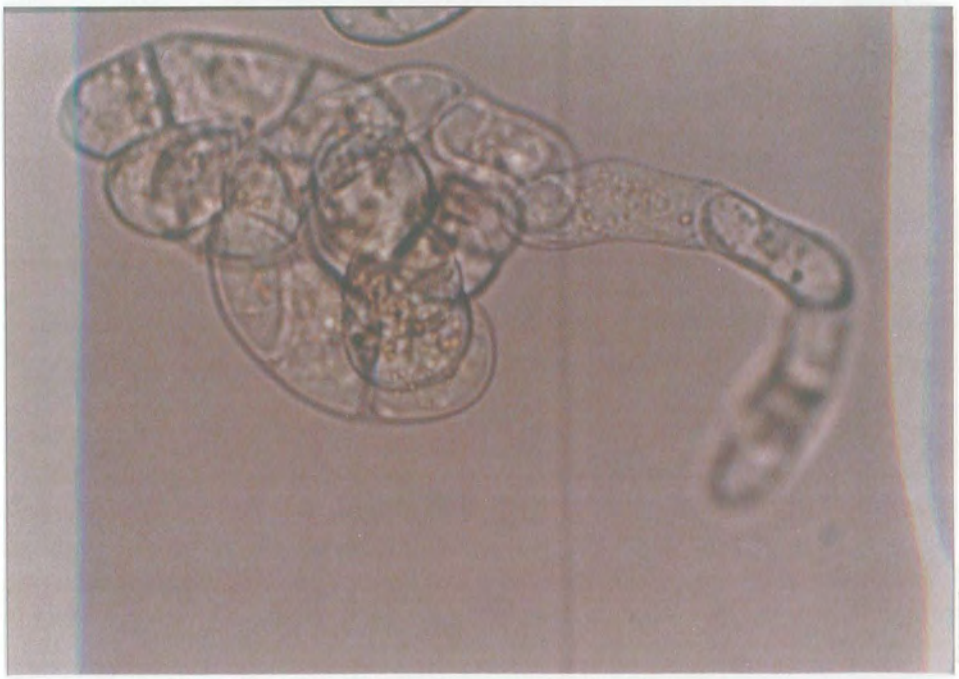
PICTURE 17 Suspension culture cells



PICTURE 18 Suspension culture cells after 3 weeks in selection medium



PICTURE 19 Transformed suspension culture cells after 3 weeks in selection medium



PICTURE 20 Suspension culture cells after 3 weeks

3.4 TRANSFORMATION OF CARROT DISCS

Carrot discs, which had been prepared as described, were transformed by *Agrobacterium* by placing 2 drops of bacterial solution on top of the explants. The following experiments were set up, each with 2 petri dishes containing 8 root disc fragments:

FIGURE 10 Transformation of carrot discs

Experiment No	Time of Cultivation before Transformation	*Agrobacterium* strain
1	0 d	C 58
2	0 d	LBA 4404
3	0 d	bacterial medium
4	1 d	C 58
5	1 d	LBA 4404

During the first week, the only effects that could be observed were that the control discs, which had been cultivated without treatment on the I,2a medium had turned brown and bacterial colonies had developed on their surfaces. All of the samples, which had been transferred to the selection medium remained sterile.

After ten days, callus development became visible on some discs of experiments 1 and 2. Callus development did not take place only in specific areas of the tissue explants, but at various different points. Many of the other explants became brown and seemed to die. The dishes were photographed 3 weeks after the transformation.

Samples of these calli were examined in GUS assays.

3.5. TRANSFORMATION OF GERMINATING CARROT SEEDS

Twenty petri dishes with 20 seeds each were prepared for the transformation experiments. For each of the following experiments, 2 dishes were used.

FIGURE 11 Transformation of germinating seedlings

Experiment No.	Time of Germination before Transformation	*Agrobacterium* strain
1	1 d	C 58
2	1 d	LBA 4404
3	1 d	bacterial medium
4	2 d	C 58
5	2 d	LBA 4404
6	3 d	C 58
7	3 d	LBA 4404
8	4 d	C 58
9	4 d	LBA 4404

As described in the methods chapter, the seeds were placed on an agar containing I,2a and augmentum. Thus, germination itself does not select for transformation. The first steps of germination became visible after 4 days in the control dish.

It was interesting to observe that the germination process was interrupted by the presence of the bacteria, but after the seedlings were washed, germination continued.

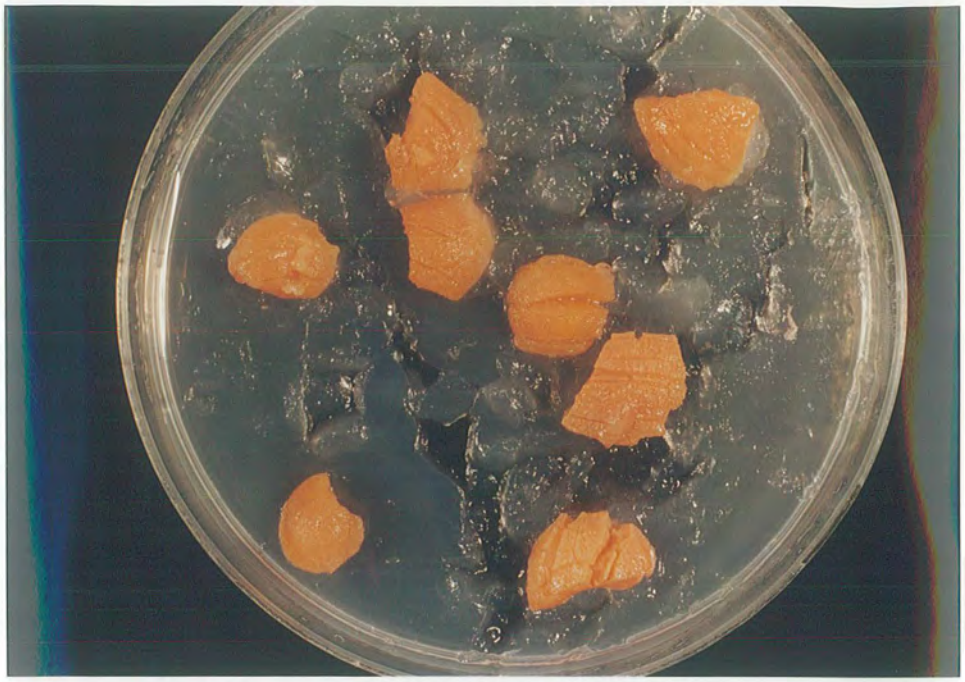
Many of the seeds and seedlings showed signs of contamination with a mould, so more than half of the plantlets had to be removed. Those that remained developed quickly.

When the radicle touched the medium, calli immediately developed. (PICTURE 22). After the first small callus had formed, the radicle turned its growth direction and extended in to the air. (Not seen, as the seedlings were shaken, before the picture was taken.) The developing leaves did not show any callus development. This can be seen on PICTURE 23.

After 10 days of germination, 5 seedling from each petri dish were transferred to 50 ml clear sterile plastic tubes containing 5 ml of Iso-medium. This step was done to stop the callus development and to induce faster growth of the seedlings. In a preliminary experiment it had been shown that seedling can grow quickly, even if they are submerged in liquid medium.

When the tubes were examined after three weeks, no further growth could be detected. Two of the flasks were contaminated by molds. It was not investigated, why the seedlings in the other tubes had neither developed any further nor had they deteriorated.

Samples from the surviving seedlings on the petri dishes were used for GUS assays.



PICTURE 20 Carrot disks on selection medium, some calli visible

PICTURE 22 Carrot seedlings forming calli on roots

PICTURE 23 Carrot seedlings

3.6. ANALYSIS OF TRANSFORMED TISSUE

3.6.1. Fluorescence GUS assay

To analyze the cells which had survived in the selection medium, fluorescence a GUS assay was carried out following the procedure described in section 2.3.5. The results are listed in FIGURE 12

FIGURE 12 Fluorescence GUS assay

Experiment No.	Sample	Result
1	positive control	++
2	C58 in YEB medium	-
3	LBA in YEB medium	-
4	Suspension culture cells, control	+
5	Suspension culture cells with LBA	+
6	Suspension culture cells, experiment no	-
7	Suspension culture cells with C58	-
8	Suspension culture cells, experiment no.	-
9	Protoplasts, control	+
10	Protoplasts, in selection medium	-
11	Protoplasts, experiment no. 2	-
12	Protoplasts, experiment no. 1	-
13	Protoplasts, experiment no. 4	-
14	Protoplasts, experiment no. 3	-
15	Protoplasts, experiment no. 9	-
16	Protoplasts, experiment no. 8	-

17	Protoplasts, experiment no 12	-
18	Protoplasts, experiment no. 11	-
19	Protoplasts from root, experiment no. 18	
	+ LBA	++
20	Protoplasts from root, experiment no. 18	
	+ C58	++

It was noticed that the bacteria showed a weak white fluorescence, while the positive control gave off a blueish light.

As most of the cultures showed negative results, a negative control was carried out to establish whether something was erasing a possible positive signal. When sterile selection medium was added to the positive control, the fluorescence was immediately erased. It was shown that the rifampicin was disturbing the fluorescence. This result is consistent with the fact that none of the samples containing rifampicin gave positive signals.

When it was examined why the the control cells and control protoplasts (experiments 4 and 9) gave positive results, it was noticed that the substrate itself had a weak fluorescence activity, possibly due to contamination with enzyme from the positive control.

No other fluorescence tests could be carried out to establish which of the cultures expressed the GUS gene as neither the enzyme nor the substrate was available.

3.6.2. Colourimetric GUS assay

Samples from the calli growing on the carrot discs and from the transformed suspension cultures were treated as described in the methods chapter. Eventhough the experiments were done twice, all samples gave negative results. It was not examined whether this was due to the experimental set up or the samples themselves.

4. DISCUSSION

In this thesis, preliminary studies into the genetic transformation of carrot using *Agrobacterium* derived vectors were carried out. Two transformation systems, the cocultivation of suspension cultured cells and the inoculation of carrot root discs with *Agrobacterium* gave the best results. Regeneration of whole plants has been reported from both suspension cultures and root discs (Seitz, 1985). Thus either of these systems may lead to the production of whole transformed carrot plants.

4.1. DISCUSSION OF METHODS

For the development of a carrot transformation protocol different tissue culture methods were tested. The direct isolation of protoplasts from mature carrot roots and the regeneration of plantlets as described by Kameya and Uchimiya in 1972 could not be repeated. Eventhough different parameters, such as medium composition, tissue source, cultivation conditions and sterilization procedure were varied, the cells only survived for a maximum of 10 days. Regeneration of the cell wall and cell division could never be shown with certainty.

It is possible that Kameya and Uchimiya used a special carrot variety which grows vigorously, but this chance is not very likely. The other possibility, that the addition of coconut milk had a great advantage over kinetin does not sound promising, since none of the other protoplast regenerating media contain coconut milk (Seitz, 1985, Wetter and Constabel, 1982). This method, which could have been a very quick way of regenerating transformed carrot plants (3 months), was abandoned.

Protoplasts from suspension cultures were isolated and proved to be viable over more than 4 weeks. Seed germination experiments proved to be difficult since a procedure for successful sterilization of all carrot seeds could not be found. Both suspension cultures and root discs proved to be easily cultured.

For the transformation of solid plant tissues, the inoculation procedure described by Horsch (1974) was used. The drawback in this procedure is that only a few cells on the surface of the explant which have previously been injured by cutting are susceptible to attack by the *Agrobacterium*. The regeneration of untransformed tissue cannot be eliminated, even if the explants are cultivated on a selection medium (Hinchee et al., 1985). This effect was not significant in the carrot root discs transformation experiments described here, since no development of calli took place on untransformed discs placed on selection medium agar. Possibly some untransformed calli might have developed adjacent to transformed calli. This chance can be eliminated by subculturing the calli frequently on selection agar, or by transferring the callus culture to a suspension culture in which every cell would be separate from the others.

A very significant effect was noticed when the time of inoculation was varied. Only those discs which were inoculated one day after they had been prepared and cut gave rise to transformed calli. Later infection did not cause any callus development. A possible explanation for this phenomenon is that the cells had started to recover after the first day and thus they were still accessible to the *Agrobacterium* on day one, while later the cells had either recovered completely or they had died, and the bacteria were not able to infect the tissue.

This theory fits with the report published by Sen et al. in 1986 in which they postulate that partial cell walls are necessary substrates for *Agrobacterium* attachment.

A similar time dependence is reported by Feldmann and Marks (1987) for the inoculation of germinating seed. For *Arabidopsis* a specific time span exists during which the seedlings are maximally susceptible to *Agrobacterium*-mediated transformation. An experiment similar to the one reported was carried out with carrot seeds (data not shown) but it could not be analyzed since the seeds were contaminated by a fungus which quickly infected all seeds in the liquid medium. The procedure was changed to germinating and inoculating the seeds on solid agar plates on which infected seeds could be quickly identified and removed. The results from this experiment will only become available when the plantlets have reached adequate size so that GUS assays can be carried out on leaf tissue without disturbing the plant's growth.

The method of cocultivating suspension cultures seemed more promising since the *Agrobacterium* would have direct access to all cells and transformed cells can easily be identified by selection with kanamycin and through GUS assays. The transformation procedure has been described in detail by Zhi et al.(1987). They report transformation frequencies of 10-4 but no development of untransformed calli. For the experiments described in this thesis, no transformation frequencies can be defined, since the effect of the kanamycin selection was not strong enough to destroy untransformed cells before the transformed cells had divided.

When the time of cocultivation was varied, it was shown that the age of the culture was not important for the transformation.

This result is consistent with Zhi's report (1987). The second factor which was varied during the experiments was the *Agrobacterium* strain. The results with the strain C58, which Zhi had also used, were not different from those with the strain LBA 4404.

The last transformation system which was examined was the cocultivation of *Agrobacterium* with carrot protoplasts. This method seemed very promising, since protoplast transformation has been reported for many plants, such as tobacco, petunia, soy bean and regeneration of carrot plants from protoplasts, reported by Grambow in 1972, has now developed to a standard method (Seitz, 1985).

In the experiments carried out here, none of the transformed cells survived longer for than ten days, while control cells survived for longer than 4 weeks. The infected cells seemed to be filled with bacteria, which were seen as moving particles within the shape of the cell. Possibly the infection with *Agrobacterium* disrupted the cell wall regeneration process. Complete regeneration of the cell wall is necessary before the cells can divide.

It is not very likely that the *Agrobacterium* infection affects any other part of the cells metabolism since it has been shown that suspension cultured cells infected under the same conditions as the protoplasts can survive. It might be necessary to use a less virulent strain of *Agrobacterium*, such as GV 3103 described by Koncz et al.(1987).

They report that transgenic carrot calli were obtained from protoplasts but it is not clear whether they used the cocultivation or the electroporation method. So far no report has been published which describes a successful regeneration of carrot protoplasts transformed by cocultivation with *Agrobacterium*. This fact combined with the results from this project leads to the conclusion that cocultivation of *Agrobacterium* with protoplasts does not produce viable transformed carrot tissue.

4.2. DISCUSSION OF THE RESULTS

From the carrot root disc transformation experiment and the suspension culture cocultivation experiment cells were obtained which could survive in the selection medium containing 100 µg/ml kanamycin. When samples of the calli and the suspension cultures were analyzed for expression of the inserted GUS-gene, both the fluorimetric and the colourimetric assay gave negative results. These data seem inconsistent because only transformed cells should survive in the selection medium and those cells should also have acquired GUS activity through the transformation. The discrepancy can be explained in many different ways:

Kanamycin resistance, which has been introduced as a selectable marker for plant cell transformation analysis by Herrera-Estrella et al. in 1983, is dependent upon the promoter. Herrera-Estrella and Lichtenstein and Draper (1986) report that the NPT II gene driven by the nos promoter can convey resistance to up to 500 µg/ml kanamycin. But they also state , as does Hinchee et al.(1988), that plant cells differ in their reaction to kanamycin.

Hinchee mentions that for soy-bean selection by 100 µg/ml kanamycin only served as an aid to finding transformed plants. Only 6% of their resistant plants proved to be transgenic and gave positive results in the colourimetric GUS assay.

From the sensitivity experiments mentioned in this thesis it may be deduced that carrot cells are naturally resistant to high amounts of kanamycin, which would lead to the conclusion that the cells growing the selection medium must not necessarily be transformed cells. However, control cells of the suspension culture transferred to selection medium died within 3 weeks. Also, no callus formation could be detected on carrot discs grown on selection medium without prior inoculation with *Agrobacterium*. These facts suggest that the cells growing in the selection medium must actually have acquired resistance to kanamycin, in this case through the transformation.

Another explanation as to why the cells show such divided characteristics could be due to lack of control of the genetics of the plasmid and the transformed cells. It is possible that under tissue culture conditions only the nos promoter is activated, leading to the expression of the NPT II gene, while the CaMV 35s promoter is quiescent. Also, rearrangements or duplications of the genes might have occurred during the insertion of the T-DNA into the plant chromosome. Both Feldmann and Marks and Hinchee et al. analyzed progeny of their transformed plants by Southern blot for the presence of multiple copies of T-DNA inserts but they came to different conclusions.

Hinchee et al. describe that all of their plants showed the same pattern of putative T-DNA junction fragments at a level consistent with one or a few copies of T-DNA. From these data in combination with the fact that the progeny of the transgenic plants co-segregated in a 3:1 ratio when selfed, they concluded that the same transformation event had taken place in all of their cells. Feldmann and Marks however report that they found multiple copies of NPT II genes in some of their plants and by combining Southern blot analysis with genetic crossing experiments they concluded that many of these copies are silent.

To examine which of the two cases is present in the carrot transformation experiments, genetic analysis would have to be carried out. The first step would be to isolate DNA from the putative transformed cells, digest it with a restriction enzyme, separate the fragments on a gel and to hybridize labelled probes of the T-DNA of the plasmid to them. This Southern blot will prove whether the T-DNA has actually been inserted into the plant cell chromosome and how many copies are present. By using different probes, one specific for the NPT II gene and one specific for the GUS gene it might also be possible to investigate whether the two genes on the T-DNA were split during the insertion.

If only the NPT II gene was found it would explain why the cells did not show GUS activity. In this case it would need to be investigated whether the GUS gene was never inserted or whether it was deleted. By growing transformed plants and checking the characteristics of their progeny more information about the genetic composition of the plants can be collected.

Both of these experiments would supply data on the efficiency and the course of the transformation independent of whether the genes are expressed or not. If the GUS gene can be found in the plant chromosome but no GUS activity can be shown it would have to be examined whether any transcription or translation of the gene was taking place. This could be done by Northern blots and by using polyclonal antibodies against the GUS enzyme. Before a resistance gene can be successfully engineered into the carrot plants, expression of the resistance gene must be ensured.

Another possibility why the samples of the transformed plants gave negative results could be that the GUS assays were not sensitive enough, The fluorescence assay, which is expected to be the more sensitive test could not be repeated. Even if it could have been done, all the cells would have to be thoroughly washed to remove the rifampicin which was shown to erase the fluorescence. The colourimetric assay which is now frequently used (Jefferson, 1988; Hinchee et al., 1988) is usually carried out on callus tissue. The amount of medium present in the cell pellet after the centrifugation might have diluted the substrates to a measure at which it is no longer detectable. Positive results might be achieved if the cells were freeze-dried before they were stained.

Another approach is to follow the original procedure described by Jefferson and to fix the cells before the GUS assay is carried out. Both of these methods should not influence the result achieved by analyzing the callus growing on the transformed carrot discs. However it might be possible that the substrate was not able to penetrate far enough into the callus tissue to crate a visible amount of staining.

When the tests mentioned here have been carried out it should be possible to determine, why the cells which were cocultured with the *Agrobacterium* proved to be resistant to kanamycin but did not show GUS activity. If it can be shown that a transformation had taken place, investigations in two directions should be carried out. First it would have to be examined whether whole plants can be regenerated from the transformed calli or suspension cultures. Second, attempts should be made to improve the transformation efficiency. With the results from these experiments it will be possible to set up a system for producing transformed plants.

REFERENCES

Backs-Husemann, D., Reinert, J., (1970) Protoplasma 70, 49-60

Banga, O. (1962) in Roemer, T., Rudorf, W., Handbuch der Pflanzenzuchtung, 2. ed.

Bevan, M.W., Flavell, R.B., Chilton, M.-D. (1983) Nature 304, 184-187

Boston, R.S., Becwar, M.R., Ryan, R.D., Goldsborough, P.B., Larkins, B.A., Hodges, T.K. (1987) Plant Physiology 83,4, 742-756

Carson, R. (1962) Silent Spring

Cocking, E.C. (1981) Philosophical Transactions of the Royal Society London, Series B, 292, 557-568

Fraley R.T., Rogers, S.G., Horsch, R.B., Eichholtz, D.A., Flick, J.S., Fink, C.L., Hoffmann, N.L., Sanders, P.R. (1985) Biotechnology 3, 629-635

Fromm, M., Taylor, L.P., Walbot, V. (1985) Proceedings of the National Academy of Sciences USA 82, 17, 5824-5828

Feldmann, K.A., Marks, D.M., (1987) Molecular and General Genetics 208, 1-9

Grambow, H.J., Kao, K.N., Miller, R.A., Gamborg, O.L. (1972) Planta, 103,.348-355

Halperin, W. (1966) American Journal of Botany 53,5, 443-453

Herrera-Estrella, L., Depicker, A., VanMontagu, M., Schell, J. (1983) Nature 303, 209-213

Herrera-Estrella,.L., DeBlock, M., Mesens, E., Hernalsteens,J-P., VanMontagu,M., Shell, J. (1983) EMBO Journal 2, 6, 987-995

Hinchee, M.A.W., Connor-Ward, D.V., Newell, C.A., McDonnell, R.E., Sato, S.J., Gasser, C.S., Fischhoff, D.A., Re, D.B., Fraley, R.T., Horsch, R.B. (1988) Biotechnology 6, 915-920

Horsch, R.B., Fraley, R.T., Rogers, S.M., Sanders, P.R.,Lloyd, A., Hoffmann, N.(1984) Science 223, 496-498

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholz, D., Rogers, S.G., Fraley, R.T (1985) Science 227, 1229-1231

Jefferson,R.A., Kavanagh, T.A., Bevan, M.W. (1987) EMBO Journal 3, 13, 3901-3907

Kameya, T., Uchimiya,H. (1972) Planta 103, 356-360

Kartha,K.K. (1982) in Wetter, L.R., Constabel, F. Plant Tissue Culture Methods

Klercker, J. (1892) Ofvers. K. Vetensk. Akad. Forh. Stockholm, 49, 463-475

Koncz, C., Olsson, O., Langridge, W.,Schell, J., Szalay, A.A. (1987) Proceedings of the National Academy of Sciences 84, 131-135

Kotte, W. (1922) Berichte der Deutschen Botanischen Gesellschaft 40, 269-272

Krens, F.A., Molendijk, K.L., Wullems, G.J., Schilperoort, R.A.(1982) Nature 296, 72-74

Lichtenstein, C., Draper, J (1986) in Glover, D.M., DNA cloning Volume II

Marton, J., Wullems, G.J., Molendijk, K.L., Scilperoort, R.A. (1979) Nature 277, 129-131

Murashige, T., Skoog, F., (1962) Physiology of the Plant 15, 473

Otten, L.A.B.M., Schilperoort, R.A. (1987) Biochimica Biophysica Acta 527, 497

Paszkowski,J., Shillito, R.D., Saul, M., Maucak, V., Hohn, T., Hohn, B., Potrykus, I. (1984) EMBO Journal 3 , 2717-2722

Seitz, H.U., Seitz, U., Alfermann, W. (1985) Pflanzliche Gewebekultur

Sen,P., Chatterjee,G., Kumar, D.M., Sen, S.K. (1986) Indian Journal of Experimental Biology 24, 3, 153-155

Smith, S., Street, H. (1974) Annales of Biology 38, 223-241

Solederer, H. (1962) Systematic Anatomy of Dicots

Street, H.E. (1977) Plant Tissue and Cell Culture, volume 2

Thomashow, M.F., Nutter, R., Postle, K., Chilton, M.-D., Blattner, F.R. (1980) Proceedings of the National Academy of Sciences USA 77, 6448-6452

Wetter,L.R., Constabel, F. (1982) Plant Tissue Culture Methods, 2. ed.

Wullems, G.J. Molendijk, l., Ooms, G., Schilperoort, R.A. (1981) Proceedings of the National Academy of Sciences USA, 78,4344

Zambryski, P., Joos, H., Genetello, C., Leemans,J. Van Montagu, M., Schell, J. (1983) EMBO Journal, 2, 2143

Zhi, L., MengKang,F., Cheng, C. (1987) Scientia Sinica Series B 30, 11