

The present invention describes a process for the genetic transformation of plants belonging to the genus *Cucumis*, in particular of melons (*Cucumis melo*). This process involves the transformation of explants with
5 *Agrobacterium tumefaciens* and the in vitro regeneration of the transformed plants.

This transformation and regeneration technique can be used for introducing, for example, a gene for resistance to cucumber mosaic virus into plants belonging
10 to the species *Cucumis melo*.

In many cases, the transfer of genetic characters of a plant species to a different plant species is limited by incompatibility barriers.

These problems are found in particular in the
15 family of the Cucurbitaceae, in the genre *Cucumis* (melon and cucumber) and *Cucurbita* (pumpkin). Thus, interesting agronomical characters, such as resistance to viral diseases or to insects, which are present in wild species, cannot be transferred to cultivated species.

20 Amongst the cultivated species of the genus *Cucumis*, the only sexual crosses which are possible are between *C. sativus* (cucumber) and the related species *C. hardwickii* and *C. sikkimensis* (Deakin et al., 1971; Van Raamsdonk, 1989). Crosses between *C. sativus* and other
25 wild species will in most cases only give sterile fruits; however, melon (*Cucumis melo*) presents itself as a recalcitrant species which cannot be crossed with any other species (Kho et al., 1980; Van Raamsdonk, 1989).

The applications of novel genetic engineering
30 techniques present a promising alternative for introducing novel characters with a view to improving plant species. These techniques include the genetic transformation by introducing a foreign gene, or foreign genes; somatic hybridization by protoplast fusion and the
35 induction of somaclonal variations or mutations for inducing genetic modifications.

The transfer of foreign genes into plant species is carried out fairly routinely using
Agrobacterium tumefaciens strains which contain a

disarmed Ti plasmid (Fraley et al., 1986) (Klee et al., 1987) (Horsch et al., 1985). At present, a large number of transgenic plants has been obtained using *Agrobacterium tumefaciens*. This bacterium allows a foreign gene to be transferred into plant cells which will be able to regenerate transformed plants. Genes which encode interesting agronomical characters have been introduced into plant species. Thus, it has been possible to obtain plants which are resistant to herbicides (Degreef et al., 1989) and to insects (Hilder et al., 1987; Vaeck et al., 1987).

The transfer of genes can be carried out either using a disarmed Ti plasmid after homologous recombination, using intermediate vectors (Fraley et al., 1985) or using a binary vector with the aid of a disarmed Ti plasmid (Bevan, 1984; Fraley et al., 1986).

This transfer of genes can also be effected by using *Agrobacterium rhizogenes* which will induce roots on the transformed tissue instead of transgenic plants. Plants having an abnormal phenotype can be regenerated from these transformed roots (Chilton et al., 1982, David et al., 1984).

A character which is particularly interesting is resistance to viral diseases. Genetically transformed plants have been obtained which are resistant to various viruses (Powell Abel et al. 1986) (Cuozzo et al. 1988) (Tumer et al. 1987) (Hoekema et al. 1989) (Van Dun and Bol 1988). These plants (tobacco, potato and tomato) express a gene which encodes the coat protein of the virus to which they are resistant. The mechanism of protection has not been elucidated as yet.

The traditional process of protecting plants against viral diseases consists in inoculating the plants with an attenuated strain of the virus to prevent infection by more virulent strains. This procedure, which is termed cross-protection, has made it possible to reduce yield losses caused by viral infections (Broadbent 1976) (Fernow 1967) (Costa and Miller 1980).

A system of regenerating plants from cultured

individual cells or explants will be indispensable for the application of the genetic engineering techniques.

Such processes have been described recently for the regeneration of untransformed Cucurbitaceae:

5 The regeneration of cucumber (*Cucumis sativus*)
has been described after the induction of adventive shoot
buds on calli obtained from cotyledons (Msikita et al.,
1988; Kim et al., 1988); Wehner and Locy (1981) already
having described the induction of shoot buds on
10 cotyledons. It was possible to regenerate cucumber plants
by means of somatic embryogenesis. These somatic embryos
have developed either in cellular suspensions from calli
developed from leaf explants (Chee and Tricoli, 1988) or
from hypocotyl explants (Rajasekaran et al., 1983), or
15 directly on cotyledon calli (Cada et al., 1988) or leaf
calli (Malepszy and Nadolska-Orczyk, 1983). In all the
above-described cases, it is required for the material to
undergo a phase of callogenesis and cell dedifferentia-
tion. A prolonged callogenesis passage can induce
20 undesired somaclonal variations. In certain cases, these
variations can cause sterility in the regenerated plants.

 In the case of melons (*C. melo*), regeneration by
organogenesis has already been described either directly
on cultured cotyledons (Smith et al., 1988; Niedz et al.,
25 in press; Dirks and Van Buggenum, 1989), or after passage
through calli from cotyledons (Mackay et al., 1988;
Moreno et al., 1985; Orts et al., 1987; Bouabdallah and
Branchard, 1986), from hypocotyls (Abak and Dumas de
Vaulx, 1980; Kathal et al., 1986) or from leaves (Kathal
30 et al., 1988).

 It has already been reported that melon plants
have been obtained from somatic embryos (Oridate and
Oosawa, 1986; Branchard and Chateau, 1988).

 All these techniques must undergo a more or less
35 long dedifferentiation and callogenesis stage which will
precede the differentiation of shoot buds or embryos.
These shoot buds can develop to give either plants with
an abnormal phenotype or sterile plants (Bouabdallah and
Branchard, 1986). Moreover, the induction of embryos or

shoot buds from calli is weaker compared with the direct induction of shoot buds on cotyledons.

5 A regeneration technique has also been described in another Cucurbitaceae species: pumpkin (*Cucurbita pepo*) (Jelaska, 1972, 1974). This author has obtained plants using somatic embryos from calli which have been cultured for several months.

10 As regards the regeneration of genetically transformed plants, EP-A-0,262,972 describes the transformation of *Cucumis sativus* (cucumber) with *Agrobacterium rhizogenes* followed by regeneration, and EP-A-0,265,100 describes the transformation of *Cucumis sativus* by means of protoplast fusion, followed by regeneration.

15 De Both and Ben Tahar (1989) have reported that calli of transformed melon have been obtained. These calli, which develop on kanamycin and express the β -glucuronidase gene, were incapable of developing transgenic plants despite the use of an experimental protocol which has already been used successfully in the regeneration of untransformed melon.

20 EP-A-0,257,993 describes the production of transformed shoot buds and calli of *Cucumis melo*. Specifically, Example IX describes the transformation of *Cucumis melo* explants with *Agrobacterium tumefaciens* containing the SURB-Hra gene, which encodes a protein "AL", which is resistant to certain herbicides. The explants are 7- to 14-day-old cotyledons. After the transformation stage, the explants are cultured on a medium containing the macroelements of Murashige and Skoog to which 6 mg l⁻¹ of kinetin and 1.5 mg l⁻¹ of indole-3-acetic acid have been added to give morphogenic calli or, possibly, shoot buds. The calli are then transferred to a medium termed "regeneration medium" which contains 0.1 mg l⁻¹ of BAP. The shoot buds obtained are then transferred to a rooting medium. The production of transgenic plants and plantlets has not been described.

35 The fact that in the species *Cucumis melo* there is, as yet, no method of regeneration from transformed

tissues prevents transgenic melon plants which express interesting agronomical characters, such as resistance to viral diseases, from being obtained.

5 One of the objects of the invention is to obtain regeneration of transgenic plants which belong to the species *Cucumis melo*.

10 A further object of the invention is to define the culture media which are required during each stage of the regeneration of transgenic plants belonging to the genus *Cucumis*.

15 More particularly, the present invention relates to the genetic transformation of cotyledons, hypocotyls, and leaves of the species *Cucumis melo* by using *Agrobacterium tumefaciens*, followed by the induction of shoot buds and the production of genetically transformed plants. The genetic transformation of various tissues which are described hereinabove, followed by organogenesis, allows the improvement of cultivated species and, more particularly, of the species *Cucumis*
20 *melo*. The plants regenerated under the conditions described in the present invention have a normal phenotype and are fertile.

A further object of the present invention is the genetic transformation of melons to introduce a gene for
25 resistance to cucumber mosaic virus (CMV).

These objects are achieved by the process according to the invention.

The invention relates to a process for the production of transgenic, phenotypically normal plantlets
30 starting from genetically transformed explants, said plantlets belonging to the species *Cucumis melo* and containing at least one gene which has been introduced through *Agrobacterium tumefaciens*, characterized by induction of genetically transformed shoot buds starting
35 from cotyledons of *Cucumis melo* which have germinated for 0 to 4 days and, after this period, have been brought into contact with *Agrobacterium tumefaciens*, followed by culturing genetically transformed shoot buds in two successive stages, the first of these culture stages

taking place on a plant cell culture medium containing a cytokinin and, more particularly, 6-benzylaminopurine (BAP), and the second stage, which is carried out when the shoot buds have reached a length of at least approximately 3 mm, taking place on a plant cell culture medium containing, as macroelements,

	KH ₂ PO ₄	approximately 50 to	approximately 100 mg l ⁻¹
	MgSO ₄	" 75 to	" 300 "
	CaCl ₂ ·2H ₂ O	" 500 to	" 2500 "
10	KNO ₃	" 750 to	" 1200 "
	NH ₄ NO ₃	" 150 to	" 200 "

The invention also relates to a process for inducing genetically transformed shoot buds starting from genetically transformed explants, which contain at least one gene introduced by means of *Agrobacterium tumefaciens*, said explants originating from a plant of the species *Cucumis melo*, where the induction is carried out on an induction medium for genetically transformed shoot buds which comprises all of the mineral salts and vitamins normally required for the induction of shoot buds starting from genetically non-transformed explants and containing, amongst the mineral salts, calcium chloride and bacto-agar or agar-agar, characterized in that the CaCl₂ content of this medium is from approximately 440 to approximately 2,200 mg l⁻¹ calculated as CaCl₂·2H₂O and the bacto-agar or agar-agar content is from approximately 0.8 to approximately 1.2 %, said induction medium being supplemented with approximately 0.3 to approximately 1.13 mg l⁻¹ of 6-benzylaminopurine (BAP) and 0 to approximately 1.3 mg l⁻¹ of indole-3-acetic acid (IAA).

According to a preferred embodiment of the invention, this process for the induction of transformed shoot buds is carried out on an induction medium whose calcium content is from approximately 1,000 to approximately 2,200 mg l⁻¹, more particularly from approximately 1,750 to approximately 2,200 mg l⁻¹.

By carrying out the processes of the invention, an explant of a plant belonging to the species *Cucumis*

melo can be transformed and regenerated to give transgenic plants. The invention also relates to the transgenic plants, the transgenic plantlets, the transformed shoot buds and the transformed explants which are capable of being produced by the processes of the invention, and also to the seeds.

The regeneration of transformed or non-transformed plants starting from explants involves several cell culture steps, where each step requires a culture medium, additives, for example cytokinins, and very precise culture conditions which frequently vary according to the species to be regenerated and according to the "route" of regeneration (that is to say, via organogenesis or via somatic embryogenesis, for example).

Even if a combination of media and conditions is known for the regeneration of a non-transformed plant, it is impossible to predict if these same media and conditions can be applied successfully for the regeneration of the plant when the latter is in the transformed state. The additional transformation stage requires the use of other media, such as transformation and coculture media, and other additives, for example cefotaxime, which can have an effect on the behaviour of the cells in the media and under the conditions which apply to the later stages. The susceptibility of the transformed plants to vitrification is also a factor which sometimes requires the development of specific conditions.

Vitrified plants are characterized by a series of morphological and physiological anomalies which result from the in-vitro culture conditions. Frequently cited reasons for explaining vitrification are:

- an unduly high concentration of NH_4^+ ions or cytokinins
- an unduly low concentration of agar (or other gelling agent) in the medium
- a sensitivity to the ethylene produced by the plant which can be found in the volume of the culture medium (Kevers et al., 1984).

Vitrification of in-vitro cultures of melon

plantlets due to the presence of cytokinins in the medium have been reported by Leshem et al. (1984).

Thus, all these factors make perfecting a combination of media and conditions which leads to the
5 regeneration of a transgenic plant highly complex.

The inventors have succeeded in formulating a shoot bud induction medium which allows the induction of genetically transformed shoot buds starting from transformed explants. This medium is a shoot bud induction medium which contains all of the mineral salts and
10 vitamins normally required for induction of shoot buds starting from genetically non-transformed explants and containing, amongst the mineral salts, calcium chloride and bacto-agar or agar-agar, the CaCl_2 content of this
15 medium being from approximately 440 to approximately 2,200 mg l^{-1} calculated as $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and the bacto-agar or agar-agar content being from approximately 0.8 to approximately 1.2 %. This induction medium is supplemented with approximately 0.3 to approximately 1.13 mg l^{-1}
20 of 6-benzylaminopurine (BAP) and approximately 0 to approximately 1.3 mg l^{-1} of indole-3-acetic acid (IAA).

A particularly preferred induction medium is an induction medium whose calcium content is from approximately 1,000 to approximately 2,200 mg l^{-1} , more particularly from approximately 1,750 to approximately
25 2,200 mg l^{-1} . These concentrations of calcium represent an increase of approximately four to five times the concentrations conventionally used for induction media. Generally, the inventors have found that elevated calcium
30 concentrations have a highly beneficial effect on the regeneration steps of transformed melon. Figure 4 shows the effect of various calcium concentrations on the induction of shoot buds in different genotypes of melon.

According to an embodiment of the invention, the
35 shoot bud induction medium can be the medium of Murashige and Skoog (1962), known under the term MS-medium, whose CaCl_2 and bacto-agar, or agar-agar, content is modified, if appropriate, within the limits defined hereinabove. Without modification, the MS-medium is composed as

follows:

<u>MS-medium</u>		(mg l ⁻¹)
	(NH ₄)NO ₃	1,650
	KNO ₃	1,900
5	CaCl ₂ .2H ₂ O	440
	MgSO ₄ .7H ₂ O	370
	KH ₂ PO ₄	170
	FeSO ₄ .7H ₂ O	27.8
	Na ₂ EDTA	33.6
10	MnSO ₄ .4H ₂ O	22.3
	ZnSO ₄ .7H ₂ O	8.6
	H ₃ BO ₃	6.2
	KI	0.83
	Na ₂ MoO ₄ .2H ₂ O	0.25
15	CuSO ₄ .5H ₂ O	0.025
	CoCl ₂ .6H ₂ O	0.025
	myo-inositol	100
	nicotinic acid	0.5
	pyridoxine.HCl	0.5
20	thiamine.HCl	0.1
	glycine	2.0
	sucrose	0,000
	pH	5.7

The solidified MS-medium additionally contains
 25 0.8 % of agar-agar or bacto-agar.

A preferred induction medium is the MS-medium which has been supplemented with 0.3 to 1.13 mg l⁻¹ of BAP, more particularly 0.85 mg/L of BAP, in the absence of any other cytokinin.

30 The vitamins which are present in the induction medium can be those which are normally present in the MS-medium. According to another embodiment of the invention, these vitamins can be the "Staba" vitamins (Staba, 1969)

Vitamins of "Staba"

35	nicotinamide	2	mg l ⁻¹
	pyridoxine-HCl	2	mg l ⁻¹
	d-biotin	1	mg l ⁻¹
	Ca panthothenate	1	mg l ⁻¹
	thiamine-HCl	1	mg l ⁻¹

	choline chloride	1	mg l ⁻¹
	p-aminobenzoic acid	0.5	mg l ⁻¹
	folic acid	0.5	mg l ⁻¹
	riboflavin	0.5	mg l ⁻¹
5	cyanocobalamin	1.5	µg l ⁻¹

A particularly preferred shoot bud induction medium is the medium termed "MI-medium", which has been perfected by the inventors. The MI-medium is composed as follows:

10 MI-medium

	Macroelements:	KNO ₃	1900	mg l ⁻¹
		NH ₄ NO ₃	1650	
		CaCl ₂ ·2H ₂ O	2200	
		MgSO ₄ ·7H ₂ O	370	
15		KH ₂ PO ₄	170	
	Na ₂ EDTA	as in MS		
	Microelements	as in MS		
	Vitamins	Staba		
	Myo-inositol	100	mg l ⁻¹	
20	Sucrose	30	g l ⁻¹	
	Agar-agar	0.8	%	
	BAP	3.75	µM	
	ABA	1	µM	

25 The agar content of the solidified MI-medium can be modified from 0.8 % to 1 % (w/v). 0.8 % is preferred. Approximately 1 µM of abscisic acid may be added to the induction media according to the invention.

Another medium perfected by the inventors is the medium which allows transgenic plantlets to be obtained starting from transformed shoot buds.

30 This medium is a plant cell culture medium containing, as macroelements,

	KH ₂ PO ₄	approximately	50 to	approximately	100	mg l ⁻¹
	MgSO ₄ ·7H ₂ O	"	75 to	"	300	"
35	CaCl ₂ ·2H ₂ O	"	500 to	"	2500	"
	KNO ₃	"	750 to	"	1200	"
	NH ₄ NO ₃	"	150 to	"	200	"

The other components of the medium, for example microelements, vitamins and the like... are those

normally used in cell culture media, for example MS-medium, at conventional concentrations. The sucrose content can be reduced to approximately 10 g l^{-1} .

5 In a preferred embodiment of the invention, the medium for the development of transgenic plantlets contains, as macroelements:

	KH_2PO_4	85 mg l^{-1}
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	185 mg l^{-1}
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	$1,720 \text{ mg l}^{-1}$
10	KNO_3	950 mg l^{-1}
	NH_4NO_3	165 mg l^{-1}

It must be noted that the $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ content according to this aspect of the invention is approximately five times higher than in the traditional media.
15 In contrast, the concentration of KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KNO_3 is approximately half of what can be found in other media of this type, and the concentration of NH_4NO_3 is approximately one tenth.

A particularly preferred medium according to the
20 invention for the development of transgenic plantlets is the medium termed MB6, whose composition is as shown in Table 1.

This medium, which has been perfected by the inventors, is essential for obtaining transgenic plantlets starting from shoot buds. However, it is important
25 that the shoot buds are cultured in two stages, the first of these stages taking place on a first plant cell culture medium containing a cytokinin. The cytokinin can be kinetin or BAP. BAP is particularly preferred. A first
30 suitable cell culture medium is, for example, the MS-medium which has been supplemented with $0.1 - 1.2 \text{ mg l}^{-1}$ of BAP, in particular approximately 0.2 mg l^{-1} . This culture medium is normally solidified by the addition of approximately 0.8 % of agar-agar or bacto-agar.

35 When the shoot buds have reached a length of at least approximately 3 mm on this first medium, they are transferred to a second medium, which is the medium which allows transgenic plantlets to be obtained and which is defined in detail hereinabove, for example the medium

MB6. In the following, this medium will be termed "MB6-type medium".

On this MB6-type medium, the shoot buds, which have a length of at least 3 mm, grow in length and, after approximately 4 weeks, the plantlets root. Once the secondary roots have developed on this medium, the plants can be transferred into pots containing a mixture of compost and coarse sand and can be grown in the greenhouse. The transgenic plants thus obtained are phenotypically normal and fertile.

Of course, the media according to the invention are supplemented, if necessary, with substances which allow transformants to be selected, for example antibiotics, and substances which inhibit the growth of *A. tumefaciens*, for example cefotaxime. The concentrations of cefotaxime which may be used are in the order of approximately 200 mg l⁻¹ to approximately 400 mg l⁻¹. Carrying out simultaneously selection with kanamycin allows the use of approximately 200 mg l⁻¹ of cefotaxime for inhibition. It is particularly preferred to use 200 mg l⁻¹ of cefotaxime since the development of the plantlets is not affected by this rather low concentration. The concentrations of kanamycin used can vary between approximately 50 to 400 mg l⁻¹. If the MI medium is used as selection medium, the kanamycin concentration must be from 150 mg l⁻¹ to 400 mg l⁻¹.

According to an embodiment of the invention, the shoot bud induction medium and the MB6-type medium can be used successively for the regeneration of transgenic melon plants starting from transformed explants. The shoot buds which are induced by culture on the induction medium are excised from the explant and transferred to the first plantlet development medium containing cytokinin, for example BAP, and then to MB6-type medium.

According to another embodiment of the invention, explants of plants belonging to the species *Cucumis melo* are transformed with *Agrobacterium tumefaciens*, and the transformed explants thus obtained are then cultured on the shoot bud induction medium. The shoot buds are then

subjected to the stage of transgenic plantlet development which, as has already been explained, takes place in two stages, the latter being performed on the MB6-type medium. Finally, the transgenic plantlets thus obtained
5 are cultured by methods which are already known for the development of non-transformed plantlets, in order to obtain transgenic plants.

The explant used as starting material for the transformation and regeneration of transgenic melons can
10 be any explant which can be transformed with *Agrobacterium tumefaciens*, for example: cotyledons, hypocotyls or leaves. The use of cotyledons as explant is particularly advantageous. The inventors have found that the potential for the regeneration of shoot buds on
15 cotyledons taken from embryos having up to four days of culture, more particularly two days of culture, is superior to the potential of younger or older cotyledons (see Fig. 1). This phenomenon has been observed in a large number of different varieties. It is important to
20 define that the cotyledons are derived from embryos which have germinated during 0 to 4 days, the embryos having been isolated from mature melon seeds. Germination starting from seeds does not lead to the same advantageous results. The germination medium is, preferably,
25 0.8 % (weight/volume) of agar-agar in sterile water, which can be supplemented with 50 μM of CoCl_2 or NiCl_2 , which seems to improve the induction of shoot buds in the following stage. As indicated further above, the germination period is from 0 to 4 days. A period of less than
30 three days and, especially, two days, is particularly preferred. Germination takes place in the normal manner under a light intensity of 60 to 80 $\mu\text{Em}^{-2}\text{s}^{-1}$, and a photoperiod of 16/24 hours at approximately 26°C during the day and 24°C during the night.

35 The genetic transformation of the melon explant is carried out by bringing into contact a strain of *A. tumefaciens*, for example in the form of a bacterial suspension, and the explant which has been wounded, for example by cutting. In the case of cotyledons, they are brought into

contact with the bacteria when the cotyledons are 0 to 4 days old, more particularly 2 days old.

5 The duration of this contact is not critical; for example, it can take from approximately 30 minutes to one hour, and is carried out, for example, in a suspension in an MS culture medium.

10 The explants are then dried on a sterile paper filter and transferred to a coculture medium, which may be MS-medium solidified with agar-agar. The conditions of light intensity and photoperiod which are applied at the time of coculture are, in general, the same as those used at the time of the germination stage. This coculture stage normally takes approximately 48 hours.

15 A large number of nopaline and octopine strains of *A. tumefaciens* are known and can be used for the genetic transformation of the species *Cucumis melo*.

20 The inventors have used the octopine strain LBA4404 which contains the Ti plasmid pAL4404 and the binary vector pGA472 supplied by G. Ann, Washington University (Ann et al., 1985), or the nopaline strain C58'3 (Ben Tahar and De Both, 1988). These strains were cured of their vector, and the binary vector pBI121 (supplied by R. Jefferson, Plant Breeding Institute) (Jefferson et al., 1987) was introduced into strain 25 LBA4404, which does not contain vector pGA472. This binary vector has a kanamycin resistance marker gene and a reporter gene, the β -glucuronidase gene, which encodes an enzymatic activity. This kanamycin resistance gene contains the encoding region of the neomycin phospho- 30 transferase gene, which has been isolated from transposon Tn5 and which is flanked at the 5' and 3' end by the promoter and terminator of the nopaline synthase gene. The region which encodes the β -glucuronidase gene is flanked by the RNA 35S promoter of the cauliflower mosaic 35 virus and by the terminator of the nopaline synthase gene.

Strain LBA4404, which contains vector pBI121, is preserved at -20°C in 15 % glycerol. This strain grows on LB-medium which contains 50 mg/l of kanamycin.

After the coculture stage, the transformed explants are transferred to the shoot bud induction medium which has been described in the preceding paragraphs. This medium is usually supplemented with agents which allow transformants to be selected, for example kanamycin, and agents which inhibit the growth of *A.tumefaciens*, for example cefotaxime.

The inventors have found that the use of "Falcon"™ Petri dishes 9 cm in diameter gives particularly advantageous results at the time of the shoot bud induction stage. The conditions of light intensity and photoperiod which are applied during this stage are the same as those applied at the time of the germination stage, a light intensity of $80 \mu E.M^{-2}s^{-1}$ being particularly preferred.

Shoot buds develop directly on the explant and, when they have reached a size which allows them to be manipulated physically (approximately 0.2 - 1.5 mm, more particularly 0.5 mm), they are excised from the explant and transferred to fresh medium, which is usually a medium containing BAP or another cytokinin and which constitutes the "first" culture medium for the development of plantlets.

In a variant of the process according to the invention, the duration of the induction stage, which is normally approximately 21 days, can be reduced to 14 days. In this case, the shoot buds can be transferred to an MS-medium without hormones for one week before being subjected to the plantlet development stage instead of being transferred directly to the first plantlet development medium.

The plantlet development stage takes place in two parts, as has been explained further above. The shoot buds undergo longitudinal growth on this medium and, when they have reached a length of approximately 3 mm to 1.0 cm, they are transferred to MB6-medium which has been supplemented with selective agents in order to select the transformants, for example antibiotics and agents which inhibit the growth of *A.tumefaciens*. However, these

agents are not essential for the regeneration.

After the transgenic plantlets have remained on this medium for a few weeks, they root. The plants can be transferred into pots which contain compost and coarse sand (2:1 v/v) as soon as the secondary roots have developed.

According to a preferred embodiment of the invention, a gene which encodes the coat protein of cucumber mosaic virus is introduced into the melon explant through *A.tumefaciens*. (See Figures 2 and 3)

This virus is composed of 4 RNAs of different sizes; the CMV coat protein gene is at the 3' end on RNA 3 and on RNA 4. The translation into coat protein is only effected from RNA 4, the 3' end of RNA 3 has strictly the same nucleotide sequence. The coat protein gene of two virulent strains which originate from different geographical regions has been isolated and sequenced by the inventors.

In this aspect of the invention, a French virulent CMV strain termed I17F, and a plasmid (pUC18) containing the DNA complementary to the RNA 3 of a virulent strain, FNY, which has been isolated in New York from infected melons, were used. It goes without saying that it would be possible thus to introduce any other gene into melon explants, for example genes which encode the coat protein of other CMV strains and of other viruses, in which case the process according to the invention allows the regeneration of the transgenic plant containing this gene.

Examples

A) Process for the production of transgenic melons which express the kanamycin resistance gene and the β -glucuronidase gene

1. GERMINATION OF CUCUMIS MELO EMBRYOS

Mature melon seeds (*Cucumis melo* L) are used to obtain mature embryos. The integuments of the seed are opened by means of a scalpel while avoiding to wound the embryos. The embryos are sterilized by immersing them for 5 seconds into pure ethanol and then for 20 minutes with

constant agitation into 200 ml of a saturated calcium hypochlorite solution and 0.05 % of Tween 80. The embryos are then washed for 20 minutes using 200 ml of sterile water. The embryos are then dried on a paper filter and placed into sterile Petri dishes containing 0.8 % weight/volume of agar-agar sterile water. The embryos are germinated for 2 days in growth cabinets under a light intensity of 60 to 80 $\mu\text{Em}^{-2} \text{ s}^{-1}$ and a photoperiod of 16/24 hours at 26°C during the day and 24°C during the night.

2. AGROBACTERIUM TUMEFACIENS STRAINS AND TRANSFORMATION VECTORS

For the transformation experiments, strain LBA4404 containing the vector pBI121 (described on the preceding pages) is cultured in 10 ml of antibiotic-free Luria-Bertani medium for 12 hours at 28°C, with constant agitation (200 rpm). This bacterial solution will act as an inoculum for a larger volume of LB (Luria-Bertani medium) (50 ml) which has been supplemented with 100 mg l^{-1} of rifampicin and 50 mg l^{-1} of kanamycin. The LB medium contains 10 g of bactotryptone, 5 g of yeast extracts and 10 g of NaCl per litre, pH 7.5. This bacterial suspension is kept up for 4 to 5 hours at 28°C, with agitation, until the optical density is 1 at a wavelength of 660 nanometres. Then, the bacterial suspension is centrifuged for 5 minutes at 4,000 rpm, and the pellet is taken up in the same initial volume in MS-medium. This solution is ready for being brought into contact with the plant tissue.

3. TRANSFORMATION PROCEDURE

The cotyledons from the embryos which have germinated for 2 days on the medium described in paragraph 1 are removed carefully so as to avoid the apical meristem tissues. Younger or older cotyledons have a reduced shoot bud regeneration potential compared with cotyledons which have been removed from embryos which have been cultured for 2 days. The result is shown in Figure (1).

These two-day cotyledons are cut into 4 pieces in the bacterial suspension which has been taken up in

MS-medium to guarantee contact with strain LBA4404 containing the binary vector pBI121 after they have been wounded. The cotyledon pieces are left for 30 minutes to one hour in the bacterial suspension. Each fragment is
5 dried on a sterile paper filter and transferred to Petri dishes containing MS medium which has been solidified using 0.8 % of agar-agar.

The explants are cultured for 48 hours in an in-vitro growth cabinet under the conditions of light
10 intensity and photoperiod which have been described in paragraph 1.

4. SHOOT BUD INDUCTION

After this coculture for two days which has been described above, the cotyledon pieces are transferred to
15 fresh medium containing MS which has been supplemented with 400 mg l⁻¹ of cefotaxime (Roussel-Uclaf), 50 mg l⁻¹ of kanamycin sulphate, 1.13 mg l⁻¹ of BAP and 0.88 mg l⁻¹ of IAA. The presence of cefotaxime is necessary to inhibit the growth of the bacterium. Kanamycin sulphate is used
20 as a selecting agent so as to select the transformed cells. Transformed cells will be those which will have the kanamycin resistance gene and the B glucuronidase gene described in paragraph 2 in their genome. The plant hormone BAP which has been added to the culture medium is
25 necessary to induce shoot buds at the concentrations described hereinabove. In contrast, the presence of IAA is optional.

The cells which have been transformed in this manner will induce shoot buds which will develop on
30 50 mg/l of kanamycin.

The cotyledon pieces are incubated in in-vitro growth cabinets under the conditions of light intensity and photoperiod which have been described in paragraph 1.

In the presence of 50 mg l⁻¹ of kanamycin, shoot
35 buds develop directly after 3 to 5 weeks, starting from the cotyledon pieces. With this induction medium, no shoot bud can develop on 50 mg l⁻¹ of kanamycin starting from non-transformed cotyledon pieces. This effect is shown in Figure 2.

5. DEVELOPMENT OF TRANSGENIC PLANTLETS

When the shoot buds which have been induced directly on the cotyledon pieces cultured on the medium described in paragraph 3 in the presence of 50 mg l⁻¹ of kanamycin, have reached a length of 0.5 mm, they are excised from the cotyledon pieces. These shoot buds are transferred singly to a fresh culture medium containing MS which has been supplemented with 0.68 mg l⁻¹ of BAP, 400 mg l⁻¹ of cefotaxime and 50 mg l⁻¹ of kanamycin sulphate. On this medium, the shoot buds undergo longitudinal growth, and, after 2 to 4 weeks, when they have reached a length of 3 mm, they are again transferred singly, this time to 100 ml of MB6-medium (see Table 1) containing 50 mg l⁻¹ of kanamycin sulphate and 400 mg l⁻¹ of cefotaxime, in culture vessels which have a volume of 500 ml.

These shoot buds, which have a length of 3 mm, undergo longitudinal growth on this above-described medium and, after 4 weeks, the transgenic plantlets root. Once the secondary roots have developed on the above-described medium, the plants are transferred to pots containing a mixture of compost and coarse sand (2:1) and are grown in the greenhouse. During the first seven days, the plants are protected by a plastic lid so as to preserve a humid atmosphere. The plants are watered daily. Photo 1 shows a transgenic plant (B) and a non-transgenic plant (A).

One of the plants which have been regenerated in this manner (termed 884-5) was allowed to flower and selfed. The seeds were collected. Four R1 plants germinated, and their transgenic character was confirmed by carrying out the tests described hereinbelow.

The first test, which detects the activity of the enzyme B-glucuronidase (GUS) is performed by the method of Jefferson et al. (1987). Another test involves the induction of calli on a leaf or on a leaf stalk on a kanamycin-containing medium. The formation of a callus indicates that the plant is transgenic. The kanamycin concentration required in a BAP- and NAA-containing

MS-medium varies depending on the hormone concentration and on the tissue. In an MS-medium which has been supplemented with 1 mg l⁻¹ of BAP and NAA, for example, 100 mg l⁻¹ of kanamycin are sufficient to inhibit the formation of calli on leaves and leaf stalks.

The presence of foreign genes in the regenerated plants was also tested using the polymerase chain reaction (PCR) (see Lassner et al. 1989; De Both 1990) using 2.5 units of Taq polymerase. The primers used allowed simultaneous amplification of the GUS gene and of the NPT gene. PCR has only been carried out using the R1 generation so as to avoid false positives due to the presence of Agrobacteria in the regenerated tissue.

B) Production of transgenic melons which express a gene for resistance to the herbicide phosphinotricin

The binary vector pIB16.1 (Hoechst, GmbH, Frankfurt, FRG) contains the neo gene and the pat gene (phosphinothricin acetyl transferase), which imparts resistance to phosphinotricin.

The transformation and regeneration process described in Example A was used for the production of plants of the genotype Vedrantaïs which have been transformed with the vector. The transgenic character of the plants obtained in this manner was confirmed by the callus formation test, and the hybridization of neo probes by the method of Southern.

These plants were selfed, and the seeds obtained in this manner were germinated in the greenhouse. The plants obtained in this manner underwent treatment with the herbicide, and some showed themselves to be resistant.

C) Production of transgenic melons which express the coat protein of cucumber mosaic virus (CMV)

In the examples which follow, a virulent French strain of CMV, termed I17F, and a plasmid (pUC18) containing the DNA which is complementary to the RNA3 of a virulent strain, termed FNY, which has been isolated in New York on infected melons, were used as a source for CMV coat protein genes.

1. CLONING THE PROTEIN COAT OF STRAIN FNY

Starting from the restriction map of the plasmid pUC18 which contains the cDNA of the RNA 3, which was determined by P. Palukaĩtis, a fragment of a size of 1.6Kb which covers at the 3' end the 1Kb sequence of the coat protein was subcloned in an expression vector "Blue scribe". This cDNA was subcloned in the vector Blue scribe (BS+) (Stratagène catalogue), and each subcloned fragment was sequenced following the protocol "séquenase R" (version 2 of USB: United State Biochemical). The sequenced fragments were analysed on a denaturing 6 % acrylamide gel as described in Maniatis et al. 1982. The gel was then fixed for 10 minutes in 10 % acetic acid, dried in vacuo for 30 minutes at 80°C and then autoradiographed (Maniatis et al. 1982). The beginning of the sequence leader of the coat protein of strain FNY have the same length as that of the Japanese strain Y (P. Palukaĩtis personal communication). The partial sequence of RNA 4 of strain Y has been published (Hidaka et al. 1985). We were able to position the beginning of the leader sequence of strain FNY by means of comparison with this sequence.

The sequence of the coat protein which covers the leader sequence, the encoding region and the non-encoding region 3' is shown in Fig. 2. Starting from this sequence, two oligonucleotides of 30 bases which are complementary in 3' and 5' were synthesized, and the sequence of the coat protein was amplified using the PCR technique (Polymerase Chain Reaction) described by Scharf et al. (1986). The amplification product was analysed on 1 % agarose gel to confirm the size of this fragment of 1034bp. The oligonucleotides in 5' and 3' have BamH1 restriction sites at their ends. Thus, it was possible to clone this amplification product in the single BamH1 site of vector pBIOS3. This vector pBIOS3, which has been constructed in the laboratory of the inventors, has a plant promoter and terminator. The 35S promoter originates from the 35S RNA of cauliflower mosaic virus, and the NOS terminator originates from the non-encoding 3'

region of the nopaline synthase gene.

2. CLONING THE COAT PROTEIN OF STRAIN I17F

Tomato plantlets in the 2-leaf stage were inoculated with CMV strain I17F. 15 Days after infection, the virus was purified from infected leaves according to the technique described in the thesis of Dr. G. Marchoux (1975). The quality of the viral RNA (RNA 1, 2, 3 and 4) is checked on 1 % agarose gel. Once checked, a DNA which is complementary to the 4 RNAs is synthesized using the Amersham kit "cDNA Synthesis System plus". The radioactive DNA, which is complementary to the 4 RNAs, is placed on 1 % agarose gel. After migration of these DNAs, the gel is dried in vacuo and then autoradiographed (Maniatis et al. 1982). These complementary DNAs, which have blunt ends, are then cloned in the SmaI site of the dephosphorylated plasmid pUC 18 (Maniatis et al. 1982). This plasmid carries ampicillin resistance. E. Coli JM101 bacteria, which have been made competent by means of the calcium chloride method (Maniatis et al. 1982), are transformed with the pUC18 plasmids which contain the DNAs complementary to the viral RNAs. As the plasmid pUC 18 carries ampicillin resistance, the transformed bacterial colonies are selected, on the one hand, on Luria Broth medium (Maniatis et al. 1982) which has been supplemented with 100 mg/l of ampicillin and, on the other hand, by the blue colour of the colonies (Maniatis et al. 1982). The recombinant plasmid is isolated from each colony and purified by the method of Birnboim and Doly (Maniatis et al. 1982). The recombinant plasmids are digested with the restriction enzymes EcoRI and BamHI; these enzymes, which cut around the SmaI site, allow, on the one hand, plasmid pUC 18 and, on the other hand, the complementary DNA to be separated by means of electrophoresis. The digestion products are placed on 1 % agarose gel, and, after migration, the plasmids containing a complementary DNA of a size of 1kb (size of the complementary DNA) are selected. It was possible to identify the recombinant plasmids which possess the DNA which is complementary to RNA 4 by a restriction map

using the enzymes HindIII, SalI and HindII by means of comparison with the restriction map of strain D which has been published by Cuozzo et al. (1988). This clone which possesses the gene which encodes the coat protein was recloned between the EcoRI and BamHI restriction sites of plasmid Bluescribe (BS+ and BS-) (Stratagène catalogue). Fragments of a smaller size were recloned in vector Bluescribe, and single-strand DNA was prepared from each of these recombinant plasmids by the method described by Stragène. The sequence of these different fragments which have been cloned was determined by the method described in the protocol "séquenase R", version 2, of USB United States Biochemicals. The sequenced fragments were analysed on denaturing 6 % acrylamide gel (Maniatis et al. 1982), the gel is subsequently fixed for 10 minutes in 10 % acetic acid, then dried in vacuo at 80°C for 30 minutes and then autoradiographed (Maniatis et al. 1982). The sequence of the coat protein of strain I17F is shown in Fig. 3.

The sequence of the coat protein is subsequently extracted from the recombinant plasmid by means of digestion with the restriction enzymes EcoRI and BamHI. BamHI linkers are attached to the ends of the sequence (Maniatis et al. 1982) which is subsequently cloned in the BamHI sites of the dephosphorylated plasmid pBIOS3 (Maniatis et al. 1982). This sequence, which encodes the coat protein, is thus positioned between the 35S promoter and the NOS terminator.

3. INTRODUCTION OF GENES WHICH ENCODE CMV COAT PROTEINS (STRAINS I17F AND FNY, RESPECTIVELY) INTO MELON COTYLEDONS, AND REGENERATION OF TRANSGENIC PLANTS

The 2 genes which encode the coat proteins (I17F and FNY) which have been positioned between the control sequences are subsequently cloned separately in the binary vector pBI121 (Jefferson et al. 1987) which has been modified by suppressing the 3' EcoRI site of the GUS gene and by the creation of an EcoRI site at the HindIII site. These modifications allow the cassette promoter - structural gene - terminator from pBIOS3 to be

inserted.

This last vector is finally introduced into strain LBA 4404. Agrobacterium strains containing the binary vector which possesses, on the one hand, the coat protein sequence from strain FNY (pBIOS132) and on the other hand from strain I17F (pBIOS135) are used for infecting melon cotyledons at the 2-day stage as described in Example A. The process for the regeneration of the transformed cotyledons is the same as described in Example A. The melon plants which have been regenerated on selective medium containing 100 mg/l of kanamycin are analysed. The transgenic character of the regenerated plants was confirmed by the detection of GUS activity and by the callus formation technique as described in Example A.

The expression of the coat protein of strain I17F was detected in a plant during a Western blot analysis with antibodies against the intact strain I17F. The coat protein expressed amounted to approximately 0.01 % of the total soluble protein in the leaves.

The transformed plants are subsequently allowed to go to seed. It was demonstrated that these transformed plants are resistant to cucumber mosaic virus.

Figure 5 is a Western blot which demonstrates the immune reaction bands which have been detected with antibodies against CMV strain I17F.

Fifteen R1 plants were tested for resistance by mechanical inoculation with the virus. The inoculation is effected on the leaf of a plantlet in the one-leaf stage. The symptoms which appear on the subsequently formed leaves allow the systemic infection of the virus to be studied. From amongst these fifteen plants, ten were shown to be transgenic by the expression of GUS.

Nineteen days after inoculation, the fourth leaf of at least one of these transgenic R1 plants showed a great reduction of symptoms and an absence of mosaic in comparison with the non-transformed Vedrantaïs controls.

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PATENT CLAIMS

1. Process for the production of transgenic, phenotypically normal plantlets starting from genetically transformed explants, said plantlets belonging to the species Cucumis melo and containing at least one gene which has been introduced through Agrobacterium tumefaciens, characterized by the following steps:
- 5 i) induction of genetically transformed shoot buds starting from cotyledons of Cucumis melo which have germinated for 0 to 4 days and, after this period, have been brought into contact with A. tumefaciens, the induction being carried out on an induction medium for genetically transformed shoot buds which comprises all of the mineral salts and vitamins normally required for the
- 10 induction of shoot buds starting from genetically non-transformed explants and containing, amongst the mineral salts, approximately 440 to approximately 2200 mg l⁻¹ of calcium chloride calculated as CaCl₂.2H₂O and approximately 0.8 to approximately 1.2% of bacto-agar or agar-agar, said induction medium being supplemented with
- 15 approximately 0.3 to approximately 1.13 mg l⁻¹ of 6-benzylaminopurine (BAP) and approximately 0 to approximately 1.3 mg l⁻¹ of 3-indoleacetic acid (IAA);
- 20 ii) culturing the resulting genetically transformed shoot buds in two successive stages, the first of these culture stages taking place on a plant cell culture medium containing a cytokinin and, more particularly, 6-benzylaminopurine (BAP), and the second stage, which is carried out when the shoot buds have reached a length of at least
- 25 approximately 3 mm, taking place on a plant cell culture medium containing, as macroelements,
- 30
- | | | | | |
|----|--------------------------------------|---------------|---------------------|------------------------|
| | KH ₂ PO ₄ | approximately | 50 to approximately | 100 mg l ⁻¹ |
| | MgSO ₄ | " | 75 to | " 300 " |
| | CaCl ₂ .2H ₂ O | " | 500 to | " 2500 " |
| 35 | KNO ₃ | " | 750 to | " 1200 " |
| | NH ₄ NO ₃ | " | 150 to | " 200 " |
2. Process according to Claim 1, characterized in that the medium for inducing the genetically transformed shoot buds is the medium of Murashige and Skoog (1962),



which is known under the term MS-medium, supplemented with 0.3 to 1.13 mg l⁻¹ of BAP and, more particularly, 0.85 mg l⁻¹, said BAP being the only plant hormone present.

5 3. Process according to Claim 1, characterized in that the vitamins present in the shoot bud induction medium are the vitamins of 'Staba' (1969).

4. Process according to Claim 1, characterized in that the calcium content is from approximately 1000 to
10 approximately 2200 mg l⁻¹, more particularly from approximately 1750 to approximately 2200 mg l⁻¹.

5. Process according to Claim 3, characterized in that the induction medium is the medium termed 'MI-medium', which is composed as follows:

15 MI-medium

Macroelements: KNO₃ 1900 mg l⁻¹

NH₄NO₃ 1650

CaCl₂.2H₂O 2200

MgSO₄.7H₂O 370

20 KH₂PO₄ 170

Na₂EDTA as in MS

Microelements as in MS

Vitamins Staba

Myo-inositol 100 mg l⁻¹

25 Sucrose 30 g l⁻¹

Agar-agar 0.8%

BAP 3.75 µM

ABA 1 µM

6. Process according to Claim 1, characterized in
30 that the culture medium used in the second stage of culturing the transformed shoot buds contains, as macroelements:

KH₂PO₄ 85 mg l⁻¹

MgSO₄ 185 "

35 CaCl₂.2H₂O 1720 "

KNO₃ 950 "

NH₄NO₃ 165 "

7. Process according to Claim 2, characterized in that the medium is the MB6-medium, whose composition is



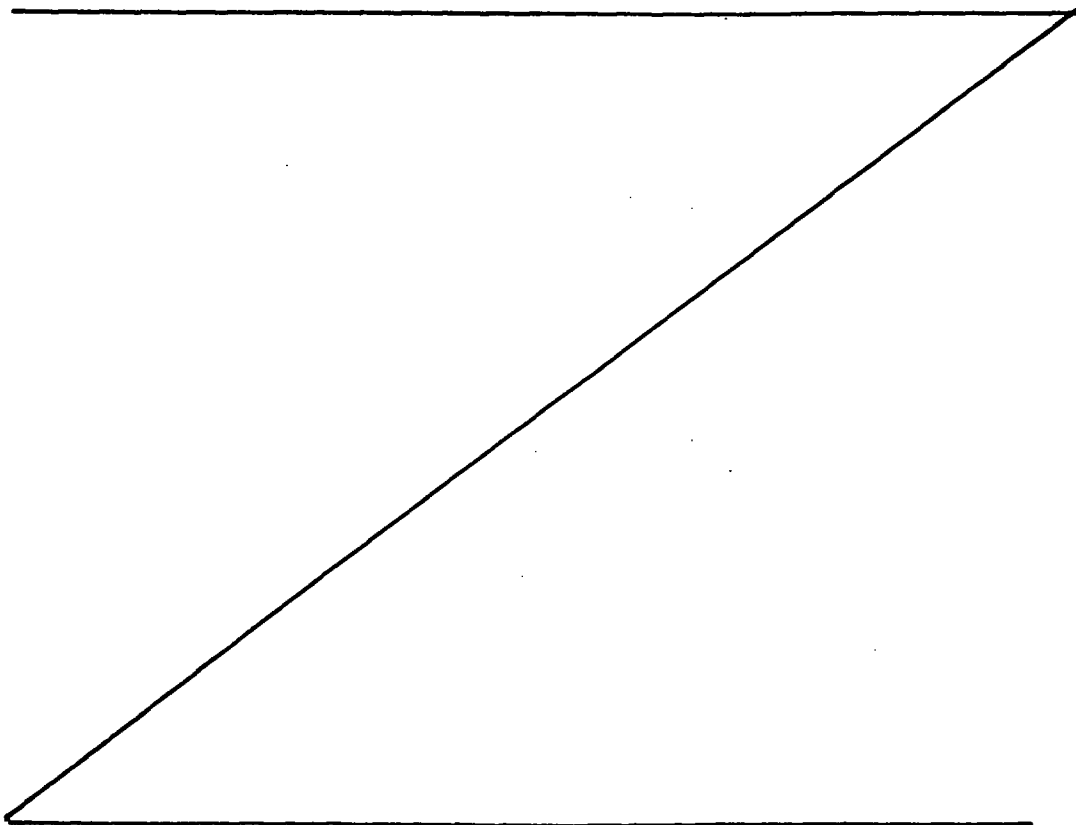
as shown below:

		<u>mg/l</u>
	KH ₂ PO ₄	85
	MgSO ₄ .7H ₂ O	185
5	CaCl ₂ .2H ₂ O	1720
	KNO ₃	950
	NH ₄ NO ₃	165
	MnSO ₄ .H ₂ O	16.9
	KI	0.83
10	H ₃ BO ₃	6.2
	ZnSO ₄ .7H ₂ O	8.6
	CuSO ₄ .5H ₂ O	0.025
	Na ₂ MoO ₄ .2H ₂ O	0.25
	CoCl ₂ .6H ₂ O	0.025
15	FeSO ₄ .7H ₂ O	27.85
	Na ₂ EDTA	37.25
	Nicotinic acid	0.50
	Thiamine-HCl	0.10
	Pyridoxine-HCl	0.50
20	Glycine	2.0
	Myo-inositol	100
	Sucrose	10,000
	Gelrite	4,000
	pH 5.6	
25	8. Process according to any one of Claims 1 to 7, characterized in that said cotyledons are obtained by transforming cotyledons from embryos isolated from seeds with <u>Agrobacterium tumefaciens</u> , said cotyledons having germinated for 0 to 3 days, more particularly for 2 days.	
30	9. Process according to Claim 8, characterized in that the transformation of the cotyledons is carried out by bringing cotyledon fragments into contact with a bacterial suspension of <u>A. tumefaciens</u> , where <u>A. tumefaciens</u> contains a functional gene intended to be	
35	introduced into the plant, followed by co-culture of the cotyledon fragments and the bacteria over approximately 48 hours.	
	10. Process according to Claim 9, characterized in that the bacterial suspension is a suspension of bacteria	



in MS-medium, and the co-culture medium is MS-medium which has been solidified with agar-agar.

11. Method of producing transgenic, phenotypically normal plants which belong to the species Cucumis melo, characterized in that the transgenic plantlets which can be produced according to any one of Claims 1 to 10 and which have secondary roots are transferred to a culture medium which is normally required for the development of non-genetically transformed plants from plantlets.
12. Process according to any one of the preceding claims, characterized in that the gene intended to be introduced into the plant through Agrobacterium tumefaciens is the gene which encodes the coat protein of cucumber mosaic virus (CMV).
13. Process according to Claim 12, characterized in that the gene comprises the following sequence or a fragment of this sequence which is capable of imparting resistance to cucumber mosaic virus:



GTTATTGTCTACTGACTATATAGAGAGTGTTTGTGCTGTGTTTTCTCTTTT	51
GTGTCGTAGAATTGAGTCGAGTC ATG GAC AAA TCT GAA TCA ACC	95
Met Asp Lys Ser Glu Ser Thr	7
AGT GCT GGT CGT AAC CGT CGA CGT CGT CCG CGT CGT GGT	134
Ser Ala Gly Arg Asn Arg Arg Arg Arg Pro Arg Arg Gly	20
TCC CGC TCC GCC CCC TCC TCC GCG GAT GCT AAC TTT AGA	173
Ser Arg Ser Ala Pro Ser Ser Ala Asp Ala Asn Phe Arg	33
GTC TTG TCG CAG CAG CTT TCG CGA CTT AAT AAG ACG TTA	212
Val Leu Ser Gln Gln Leu Ser Arg Leu Asn Lys Thr Leu	46
GCA GCT GGT CGT CCA ACT ATT AAC CAC CCA ACC TTT GTA	251
Ala Ala Gly Arg Pro Thr Ile Asn His Pro Thr Phe Val	59
GGG AGT GAA CGC TGT AGA CCT GGG TAC ACG TTC ACA TCT	290
Gly Ser Glu Arg Cys Arg Pro Gly Tyr Thr Phe Thr Ser	72
ATT ACC CTA AAG CCA CCA AAA ATA GAC CGT GGG TCT TAT	329
Ile Thr Leu Lys Pro Pro Lys Ile Asp Arg Gly Ser Tyr	85
TAC GGT AAA AGG TTG TTA CTA CCT GAT TCA GTC ACG GAA	368
Tyr Gly Lys Arg Leu Leu Leu Pro Asp Ser Val Thr Glu	98
TAT GAT AAG AAG CTT GTT TCG CGC ATT CAA ATT CGA GTT	407
Tyr Asp Lys Lys Leu Val Ser Arg Ile Gln Ile Arg Val	111
AAT CCT TTG CCG AAA TTT GAT TCT ACC GTG TGG GTG ACA	446
Asn Pro Leu Pro Lys Phe Asp Ser Thr Val Trp Val Thr	124
GTC CGT AAA GTT CCT GCC TCC TCG GAC TTA TCC GTT GCC	485
Val Arg Lys Val Pro Ala Ser Ser Asp Leu Ser Val Ala	137
GCC ATC TCT GCT ATG TTC GCG GAC GGA GCC TCA CCG GTA	524
Ala Ile Ser Ala Met Phe Ala Asp Gly Ala Ser Pro Val	150

CTG GTT TAT CAG TAT GCC GCA TCT GGA GTC CAA GCC AAC 563
Leu Val Tyr Gln Tyr Ala Ala Ser Gly Val Gln Ala Asn 163

AAC AAA CTG TTG TAT GAT CTT TCG GCG ATG CGC GCT GAT 602
Asn Lys Leu Leu Tyr Asp Leu Ser Ala Met Arg Ala Asp 176

ATA GGT GAC ATG AGA AAG TAC GCC GTC CTC GTG TAT TCA 641
Ile Gly Asp Met Arg Lys Tyr Ala Val Leu Val Tyr Ser 189

AAA GAC GAT GCG CTC GAG ACG GAC GAG CTA GTA CTT CAT 680
Lys Asp Asp Ala Leu Glu Thr Asp Glu Leu Val leu His 202

GTT GAC ATC GAG CAC CAA CGC ATT CCC ACA TCT GGA GTG 719
Val Asp Ile Glu His Gln Arg Ile Pro Thr Ser Gly Val 215

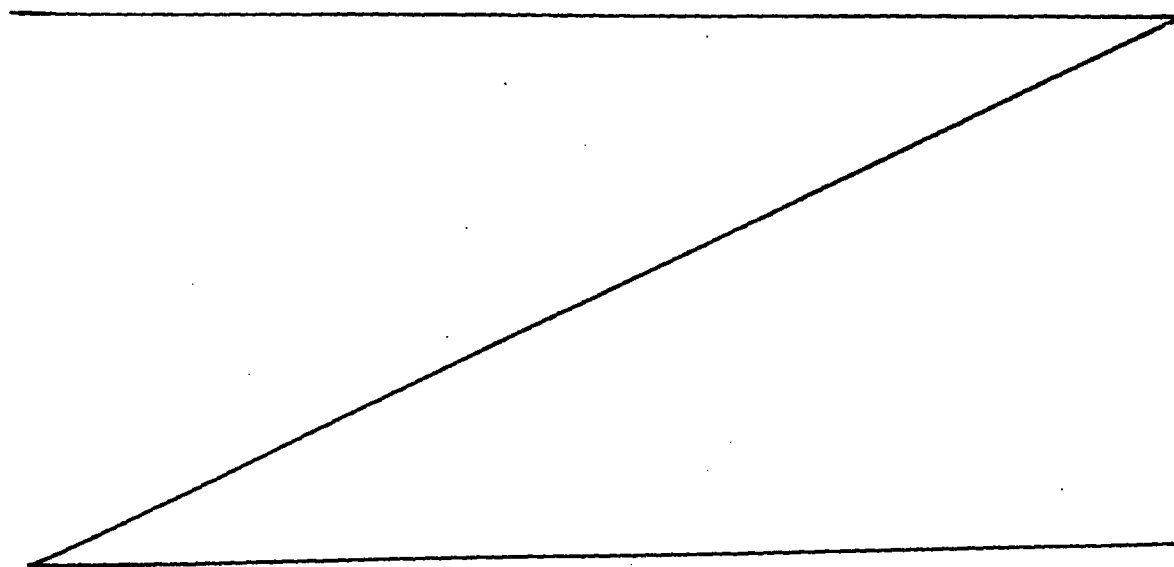
CTC CCA GTC TGATTCCGTGTTCCCAGAATCCTCCCTCCGATCTCTGTGG 768
Leu Pro Val 218

CGGGAGCTGAGTTGGCAGTTCTGCTATAAACTGTCTGAAGTCACTAAACGTT 820

TTTTACGGTGAACGGGTTGTCCATCCAGCTTACGGCTAAAATGGTCAGTCGT 872

GGAGAAATCCACGCCAGCAGATTTACAAATCTCTGAGGCGCCTTTGAAACCA 924

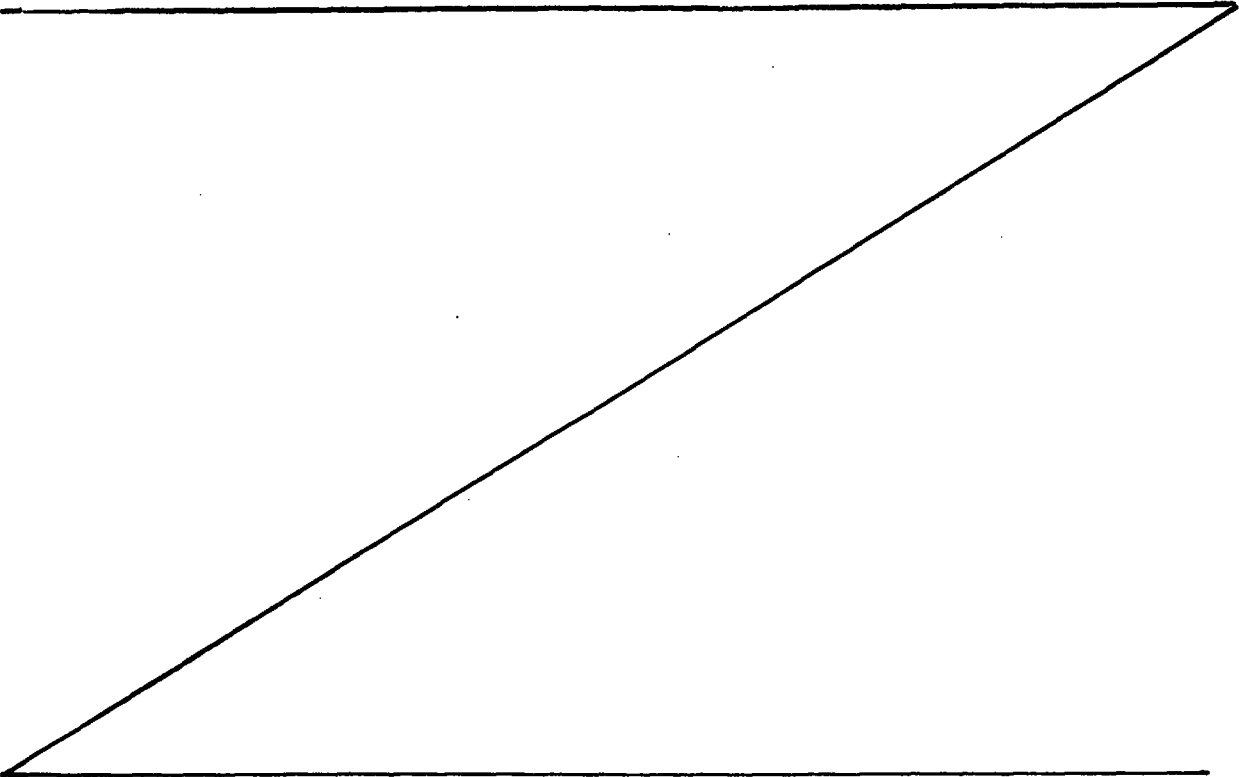
TCTCCTAGGTTTCTTCGGAAGGACTTCGGTCCGTGTACCTCTAGCACAAACGT 976



14. Process according to Claim 12, characterized in that the gene comprises the following sequence or a fragment of this sequence which is capable of imparting resistance to cucumber mosaic virus:

AGAGAGTGTGTGTGCTGTGTTTCTCTTTTGTGTGCTAGAAATTGAGTCGAG	51
TC ATG GAC AAA TCT GAA TCA ACC AGT GCT GGT CGT AAC	89
Met Asp Lys Ser Glu Ser Thr Ser Ala Gly Arg Asn	12
CGT CGA CGT CGT CCG CGT CGT GGT TCC CGC TCC GCC CCC	128
Arg Arg Arg Arg Pro Arg Arg Gly Ser Arg Ser Ala Pro	25
TCC TCC GCG GAT GCT AAC TTT AGA GTC TTG TCG CAG CAG	167
Ser Ser Ala Asp Ala Asn Phe Arg Val Leu Ser Gln Gln	38
CTT TCG CGA CTT AAT AAG ACG TTA GCA GCT GGT CGT CCA	206
Leu Ser Arg Leu Asn Lys Thr Leu Ala Ala Gly Arg Pro	51
ACT ATT AAC CAC CCA ACC TTT GTA GGG AGT GAA CGC TGT	245
Thr Ile Asn His Pro Thr Phe Val Gly Ser Glu Arg Cys	64
AGA CCT GGG TAC ACG TTC ACA TCT ATT ACC CTA AAG CCA	284
Arg Pro Gly Tyr Thr Phe Thr Ser Ile Thr Leu Lys Pro	77
CCA AAA ATA GAC CGT GGG TCT TAT TAC GGT AAA AGG TTG	323
Pro Lys Ile Asn Arg Gly Ser Tyr Tyr Gly Lys Arg Leu	90
TTA CTA CCT GAT TCA GTC ACG GAA TAT GAT AAG AAG CTT	362
Leu Leu Pro Asp Ser Val Thr Glu Tyr Asp Lys Lys Leu	103
GTT TCG CGC ATT CAA ATT CGA GTT AAT CCT TTG CCG AAA	401
Val Ser Arg Ile Gln Ile Arg Val Asn Pro Leu Pro Lys	116
TTT GAT TCT ACC GTG TGG GTG ACA GTC CGT AAA GTT CCT	440
Phe Asp Ser Thr Val Trp Val Thr Val Arg Lys Val Pro	129
GCC TCC TCG GAC TTA TCC GTT GCC GCC ATC TCT GCT ATG	479
Ala Ser Ser Asp Leu Ser Val Ala Ala Ile Ser Ala Met	142
TTC GCG GAC GGA GCC TCA CCG GTA CTG GTT TAT CAG TAT	518
Phe Ala Asp Gly Ala Ser Pro Val Leu Val Tyr Gln Tyr	155
GCC GCA TCT GGA GTC CAA GCC AAC AAC AAA CTG TTG TAT	557
Ala Ala Ser Gly Val Gln Ala Asn Asn Lys Leu Leu Tyr	168
GAT CTT TCG GCG ATG CGC GCT GAT ATA GGT GAC ATG AGA	596
Asp Leu Ser Ala Met Arg Ala Asp Ile Gly Asp Met Arg	181

AAG TAC GCC GTC CTC GTG TAT TCA AAA GAC GAT GCG CTA	635
Lys Tyr Ala Val Leu Val Tyr Ser Lys Asp Asp Ala Leu	194
GAG ACG GAC GAG CTA GTA CTT CAT GTT GAC ATC GAG CAC	674
Glu Thr Asp Glu Leu Val Leu His Val Asp Ile Glu His	207
CAA CGC ATT CCC ACG TCT GGA GTG CTC CCA GTC TGATTCTG	715
Gln Arg Ile Pro Thr Ser Gly Val Leu Pro Val	218
GTTCCCAGAATCCTCCCTCCGATCTCTGTGGCGGAGCTGAGTTGGCAGTTC	767
TGCTATAAACTGTCTGAAGTCACTAAACGTTTTTACGGTGAACGGGTTGTCC	819
ATCCAGCTTACGGCTAAAATGGTCAGTCGTGGAGAAATCCACGCCAGTAGAT	871
TTACAAATCTCTGAGGCGCCTTTGAAACCATCTCCTAGGTTTCCTCGGAAGG	923
ACTTCGGTCCGTGTACCTCTAGCACCAACGTGCTAGTTTCAGGGTACGGGTGC	975
CCCCCCTTTTCGTGGGGGCCCTCCAAAAGGAG	1007



15. Cell culture medium capable of developing shoot buds into plantlets in the course of the regeneration of a plant, characterized in that it contains, as macroelements:

5	KH_2PO_4	approximately 50 to approximately 100	mg l^{-1}
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	" 75 to "	300 "
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	" 1000 to "	2500 "
	KNO_3	" 750 to "	1200 "
	NH_4NO_3	" 150 to "	200 "
10	16.	Cell culture medium according to Claim 15, characterized in that it contains, as macroelements,	
	KH_2PO_4	85	mg l^{-1}
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	185	"
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1720	"
15	KNO_3	950	"
	NH_4NO_3	165	"

17. Cell culture medium according to Claim 15, characterized in that it has the following composition:

		<u>mg/l</u>
20	KH_2PO_4	85
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	185
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1720
	KNO_3	950
	NH_4NO_3	165
25	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.9
	KI	0.83
	H_3BO_3	6.2
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
30	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.85
	Na_2EDTA	37.25
	Nicotinic acid	0.50
35	Thiamine-HCl	0.10
	Pyridoxine-HCl	0.50
	Glycine	2.0
	Myo-inositol	100
	Sucrose	10,000

Gelrite

4,000

pH 5.6

18. Shoot bud induction medium composed of a plant cell culture medium which comprises all the mineral salts and the vitamins normally required for inducing shoot buds starting from non-genetically transformed explants and containing, amongst its mineral salts, calcium chloride, and bacto-agar or agar-agar, characterized in that the CaCl_2 content of this medium is 1000 to 2200 mg l^{-1} calculated as $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, and the bacto-agar or agar-agar content is 0.8 to 1.2%, said medium being supplemented with 0.3 to 2.0 mg l^{-1} of BAP and 0 to 1.3 mg l^{-1} of IAA.

19. Shoot bud induction medium according to Claim 18, supplemented with 0.3 to 1.13 mg l^{-1} of BAP, more particularly 0.85 mg l^{-1} , said BAP being the only plant hormone present.

20. Culture medium according to Claim 18, characterized in that the vitamins present in the induction medium are the vitamins of 'Staba' (1969).

21. Shoot bud induction medium according to Claim 18, characterized in that the calcium content is from approximately 1750 to approximately 2200 mg l^{-1} .

22. Shoot bud induction medium according to Claim 20, characterized in that it is composed is as follows:

MI-medium

30	Macroelements:	KNO_3	1900 mg l^{-1}
		NH_4NO_3	1650
		$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2200
		$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
		KH_2PO_4	170
	Na_2EDTA	as in MS	
	Microelements	as in MS	
	Vitamins	Staba	
35	Myo-inositol	100 mg l^{-1}	
	Sucrose	30 g l^{-1}	
	Agar-agar	0.8%	
	BAP	3.75 μM	
	ABA	1 μM	

23. Genetically transformed shoot buds which belong to the species Cucumis melo and which can be obtained in the course of the process according to any one of Claims 8 to 14.
- 5 24. Genetically transformed cotyledons which belong to the species Cucumis melo and which can be obtained in the course of the process according to any one of Claims 8 to 14.
- 10 25. Transgenic, phenotypically normal plantlets which belong to the species Cucumis melo and which can be obtained according to any one of Claims 1 to 10.
26. Transgenic, phenotypically normal plants which belong to the species Cucumis melo and which can be obtained according to Claim 11.
- 15 27. Transgenic, phenotypically normal melons which can be produced according to Claim 11, characterized in that they express the coat protein of cucumber mosaic virus.
28. Seed of transgenic plants according to Claim 26.