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Melon Biotechnology

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Introduction

The Cucurbitaceae family comprises more than 900 species within approximately 130 genera (Jeffrey, 1980). Melon belongs to this family and has been classified by Jeffrey (1962) as the subfamily Cucurbitoideae, tribe Melothrieae, genus *Cucumis*, species *melo*. Its polymorphism in leaf, flower, fruit shape, and colour (Kirkbride, 1993), allowed the classification of horticulturally important melons into seven groups:

- *C. melo* var. *cantaloupensis* Naud.: medium size fruits, round shape, smooth surface, marked ribs, orange flesh, aromatic flavour and sweet.
- *C. melo* var. *reticulatus* Ser.: medium size fruits, netted surface, few marked ribs, flesh colour from green to red orange.
- *C. melo* var. *saccharinus* Naud.: medium size fruits, round or oblong shape, smooth surface with grey tone sometimes with green spots, very sweet flesh.
- *C. melo* var. *inodorus* Naud.: smooth or netted surface, flesh commonly white or green, lacking the typical musky flavour. These fruits are usually later in maturity and longer keeping than *cantaloupensis*.
- *C. melo* var. *flexuosus* Naud.: long and slender fruit eaten immature as an alternative to cucumber.
- *C. melo* var. *conomon* Mak.: small fruits, smooth surface, white flesh. These melons ripen rapidly, develop high sugar content but little aroma.
- C. *melo* var. *dudaim* Naud.: small fruits, yellow rind with red streak, white to pink flesh.

Conventional breeding methods have led to a considerable varietal improvement, with current work mainly focused on obtaining a uniform melon with high organoleptic

Abbreviations: ABA, abscissic acid; BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 6-(γ,γ-dimethylallylamino)-purine; NAA, α-naphthaleneacetic acid; TDZ, thidiazuron; IAA, Indole-3-acetic-acid; GA_3, gibberellic acid A_3; GUS, β-glucuronidase; KIN, kinetin; NPTII, neomycin phosphotransferase II; PEG, polyethyleneglycol; QTL, quantitative trait loci; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA.

Biotechnology and Genetic Engineering Reviews – Vol. 15, April 1998
0264–8725/98/15/289–312 $20.00 + $0.00

traits, good yield and disease resistance. However, strong sexual incompatibility barriers at the interspecific and intergeneric levels limits the use of the genetic potential through conventional breeding methods. The development of plant biotechnological methods for melons offers the opportunity to develop new varieties, more rapidly, avoiding natural genetic barriers. Biotechnological strategies have been developed to increase the genetic diversity by somatic hybridization or gene transfer and to optimize conventional breeding programmes by the construction of a genetic map. In this review, development of current biotechnological methods for the melon are presented and the first transgenic melons that are virus resistant or with improved shelf-life are described.

Melon regeneration specificities

The development of efficient regeneration processes from *in vitro* cultures is absolutely necessary for the development of biotechnological techniques. Over the last few years, regeneration by organogenesis or somatic embryogenesis has been described for a large number of *C. melo* varieties. Several factors should be taken into account for melon regeneration.

GENETIC CONTROL

Genotype is the most important factor determining regeneration potential. The two subgenera *reticulatus* and *inodorus* behave differently depending on the regeneration protocol used. Oridate *et al.* (1992) and Gray *et al.* (1993) listed *reticulatus* varieties as being more embryogenic than *inodorus* ones. The ability to form somatic embryos is, in most cases, not an intrinsic property of a species, but rather a property under genetic control such that individual genotypes within a species differ in their ability to undergo somatic embryogenesis (Parrot *et al.*, 1991). In melon, Oridate *et al.* (1992) showed that the embryogenic regeneration potential can be transferred from superior responding cultivars to inferior ones by sexual crossing, indicating that ability to regenerate is in this case under genetic control. Regeneration through organogenesis also varies among genotypes (Molina and Nuedz, 1995a), sex form (Jain and More, 1993), explants (Molina and Nuez, 1995b) and even between seed lots of the same cultivar (Orts *et al.*, 1987). Recently, Ficcadenti and Rotino (1995) compared eleven genotypes and showed that *C. melo* variety *inodorus* had a uniformly high regeneration rate whereas *reticulatus* varieties exhibited wide differences in their organogenic response.

NATURAL ENDO-POLYPLOIDIZATION

Tetraploid melons became a source of interest in the 1930s with the discovery that colchicine produced polyploid plants. Later, several studies described methods for the induction of tetraploids obtained by induction (Kubicki, 1962 and Dumas de Vaulx, 1974) and for the screening of spontaneous mutants (Nugent and Ray, 1992). Tetraploid melons were characterized by large flowers, protruding stigmas, thickened leaves, short internodes and round seeds (Ezura *et al.*, 1992a). Fruit quality of

tetraploid plants (Batra, 1952; Dumas de Vaulx, 1974; Nugent, 1994) was found to be superior (sugar level, firmness) to diploid ones. Nevertheless, these plants were less productive, due to low fertility, and the fruits were not marketable due to their smaller, flat shape and their cracking characteristics. With the development of tissue culture, somaclonal variants showing a 4n genotype were characterized (Fassuliatis and Nelson, 1992). In addition, the generation of tetraploids is a widespread phenomenon (Ezura *et al.*, 1992b) in melon plants regenerated from somatic embryogenesis (Ezura *et al.*, 1992a), organogenesis (Bouabdallah and Branchard, 1986), and from protoplast regeneration (Debeaujon and Branchard, 1992). In order to use these culture systems efficiently for further genetic manipulation, it is important to avoid the conditions that produce tetraploid plants.

Ploidy evaluation can be performed by cytological studies using root tips (Ezura *et al.*, 1992b) or tendrils (Yadav and Grumet, 1994) as a tissue source for chromosome visualization. However, these techniques are laborious and time consuming, and are not reliable due to the difficulty of observing the chromosomes. Flow cytometry is now a widely used technique (Galbraith *et al.*, 1983), however, the requirement of specialized equipment limits its use. Alternative techniques have been developed which indirectly determine ploidy levels. Pollen from diploid plants appeared typically triangular and tripolar while tetraploid plants contain many square tetraporous, round-monoporous or oval biporous pollen grains (Adelberg *et al.*, 1994). Chloroplast number counted on the upper epidermal layer of young leaves is another way to discriminate 2n (average of 8.5 chloroplasts per guard cell) from 4n (average of 15.8) melon plants (Fassuliatis and Nelson, 1992).

The ploidy of regenerated plants is dependent upon the ploidy level of the initial explant. For example, in experiments conducted in our laboratory, we found that 2-day-old cotyledon explants containing a majority of multiploid cells generated, through organogenesis, at a high frequency of tetraploid plants (81%). Conversely, young leaf explants containing mainly diploid cells allowed us to generate diploids at a high frequency (85%). The DNA content of cotyledon cells changes from 2°C to 4°C rapidly upon germination of cucumber seeds (Gilissen *et al.*, 1993). It is therefore essential to use quiescent cotyledons if polyploids are to be avoided in cucumber. A similar phenomenum occurs in melon cotyledons. We have found that quiescent seeds exhibit mostly 2n cells, while 2-day-old seedlings contain more than 60% tetraploid cells. Moreover, the potential of cotyledon cells to regenerate is dependent on its ploidy level. According to Ezura and Oosawa (1994a), the ability of diploid cells to form *in vitro* shoots seems greater than that of tetraploid cells. In contrast, the capacity of tetraploid cells to differentiate into somatic embryos is higher than for diploid cells (Ezura and Oosawa, 1994b).

VITRIFICATION

Melon tissues *in vitro*, are very sensitive to vitrification. Explants placed in a liquid medium enlarge up to five-fold and the regeneration rate is much lower than explants cultured on solid medium. However, most regenerated shoots or somatic embryos deriving from liquid culture exhibit a glassy appearance and fail to develop further (Ziv, 1991). A number of causes have been proposed to explain this phenomenon including high relative humidity, low agar, high NH_4 concentrations, ethylene and

Table 1. Regeneration of *Cucumis melo* by direct organogenesis

Reference	Year	Variety	Explant source	Induction medium
Kathal *et al.*,	1988	Pusa Sharbati	Young leaf of 14-day-old seedlings	BAP 0.22 mg/l 2iP 0.2 mg/l
Dirks and Van Buggenum	1989	Accent, Galia Reco, Viva	Cotyledons from quiescent seeds leaf	BAP 1 mg/l
Leshem *et al.*,	1989	no mention	4-day-old cotyledons	BAP 0.2 mg/l
Niedz *et al.*,	1989	Super Star Hearts of Gold Hale's Best Jumbo Goldstar	4-day-old cotyledons	IAA 0.88 mg/l BAP 1.13 mg/l
Tabei *et al.*,	1991	Earl's Favourite Harikei N°3	Cotyledon of quiescent seeds Leaf	2,4-D 0.01 mg/l or IAA 1 mg/l BAP 0.1 mg/l
Roustan *et al.*,	1992	Tezier genotypes	7-day-old cotyledons	NAA 0.1 mg/l BAP 0.5 mg/l
Fassuliatis *et al.*,	1992	Cantaloupe	8-day-old cotyledons	BAP 1.13 mg/l ABA 0.26 mg/l
Aldelberg *et al.*,	1994	Miniloup L14, B line	Cotyledons from immature seeds	BAP 2.25 mg/l
Ficcadenti and Rotino	1995	11 varieties	4-day-old cotyledons	BAP 0.63 mg/l ABA 0.26 mg/l
Yadav *et al.*,	1996	Hale's Best Jumbo Ananas El Dokki	3–4cm expanded leaves	IAA 0.87 mg/l BAP 1.13 mg/l

cytokinin (Ziv, 1991). Leshem *et al.* (1988) characterized vitrification of melon buds as being induced mainly by cytokinin. They stressed that on solid media, it was a cumulative process whereas on liquid media, it is an all-or-nothing process with regards to all factors mentioned above that can induce vitrification. However, Kathal *et al.* (1992), showed that this physiological process could be reversed by frequent subcultures.

Regeneration by organogenesis

De novo shoot formation from various explants has been extensively described (*Table 1*). Several factors can affect the efficiency of this regeneration method, such as explant source, culture conditions, and physical factors.

EXPLANT SOURCE

Direct shoot formation can be induced in both cotyledon (Dirks and van Buggenum, 1989; Niedz *et al.*, 1989; Aldelberg *et al.*, 1994) and leaf explants (Kathal *et al.*, 1988; Yadav *et al.*, 1996). Cotyledons from 4- to 5-day-old seedlings are usually used (Niedz *et al.*, 1989; Leshem, 1989, Ficcandeti and Rotino, 1995). Several authors have shown that shoot formation mainly proceeds from the basal edge of the cotyledons, suggesting a polar phenomenon potentially due to the accumulation of an endogenous growth factor such as auxin at the basal cut (Leshem, 1989). A friable callus can also appear, but regeneration generally occurs directly from cotyledon tissue. Bud initiation can also be achieved using leaf explants (Kathal *et al.*, 1988, Yadav *et al.*, 1996), with the efficiency of regeneration from melon leaves generally decreasing with increasing leaf size. Optimum leaf size appears to be dependent on the plant donor and growth conditions. For plants grown *in vitro*, very young leaves (less than 2 cm length) are preferred while for plants grown in the greenhouse, 3–4 cm leaves are more responsive. Yadav *et al.* (1996) reported a higher frequency of regeneration when leaves were excised from plants grown in pots rather than *in vitro*.

Regeneration (*Table 2*) can also occur indirectly through callus. Regeneration through callus induction can be achieved using cotyledon (Moreno *et al.*, 1985), hypocotyl (Kathal *et al.*, 1986) and root (Kathal *et al.*, 1994) explants. In each case, the age of the donor explant is critical. For example, highest growth callus and nodule formation is obtained with roots excised from 21-day-old seedlings, while no nodule development occurs in 7- or 35-day-old seedling root segments (Kathal *et al.*, 1994).

COMPOSITION OF THE MEDIUM

For direct regeneration, an auxin/cytokinin ratio of less than 1 is used to induce bud formation. For bud development auxins can be used in combination with cytokinins (Tabei *et al.*, 1991; Fassuliatis and Nelson, 1992), but are not essential (Niedz *et al.*, 1989; Moreno *et al.*, 1985; Dong *et al.*, 1991). BAP is the cytokinin most frequently used to induce shoot formation. Buds are obtained after a month of culture and are subcultured for two weeks to elongate and then transferred to a hormone-free rooting medium for another 15 days.

Table 2. Regeneration of *Cucumis melo* by indirect organogenesis

Reference	Year	Variety	Explant source	Callus induction medium	Shoot induction medium
Moreno *et al.*,	1985	Amarillo Oro	cotyledon callus culture 11 to 13-day-old seedlings	IAA 1.5 mg/l KIN 6 mg/l	NAA 0.01 mg/l BAP 0.1 mg/l
Bouabdallah *et al.*,	1986	Doublon Ogon N°9 Piboule Charentais T	cotyledon or hypocotyl callus 7 to 9-day-old seedlings	IAA 2 mg/l KIN 2 mg/l	no hormone
Kathal *et al.*,	1986	Pusa Sharbati	hypocotyl derived callus 7-day-old seedlings	IAA 1 mg/l KIN 0.5 mg/l	BAP 0.5 mg/l 2iP 0.5 mg/l
Kathal *et al.*,	1994	Pusa Sharbati	root derived callus 21-day-old seedlings	BAP 0.68 mg/l 2iP 0.61 mg/l	BAP 0.22 mg/l

A two-step protocol is required for indirect regeneration. Induction of callus was obtained on a medium containing two cytokinins, generally including BAP. The callus exhibiting green nodules was then transferred to another regeneration medium for shoot differentiation. Afterward, shoot elongation can be promoted by reducing cytokinin concentration. Several subcultures are needed to recover a whole plant and three to four months are generally required to recover rooted plantlets (Moreno *et al.*, 1986 ; Kathal *et al.*, 1994). This is approximately twice the time required to obtain plantlets from direct regeneration. Moreover, vitrification is more frequently observed in indirect regeneration protocols (Kathal *et al.*, 1986).

The presence of ethylene in the culture vessel also affects shoot regeneration from melon cotyledons (Roustan *et al.*, 1992). Addition of the silver ion, a potent inhibitor of ethylene action, allows a two-fold increase in shoot regeneration. Moreover, cotyledons from a transgenic antisense ACC oxidase line, where ethylene production is strongly reduced, exhibit a 3.5-fold increase in the regeneration frequency (Ben Amor *et al.*, 1998).

PHYSICAL FACTORS

Environmental conditions can affect the rate of shoot induction. Niedz *et al.* (1989) emphasize the importance of light intensity on bud induction, with highest bud initiation obtained under a light intensity of 5 to 300 µmol m^{-2} s^{-1}. No buds are induced in the dark and bud initiation is reduced under higher intensities. For these authors, increasing the temperature from 22°C to 29°C under an optimum light intensity improves the regeneration frequency from 10 to 50%.

The nature of the media solidifying agent also plays an important role in melon regeneration. Agar (Sigma Chemical Co, St Louis, USA) is superior to 'gelrite' (Serva Feinbiochemica GmbH and Co, KG, Heidelelberg, Germany) for cotyledon organogenesis (Ficcandeti and Rotino, 1995), while Yadav *et al.* (1996) 'phytagel' (Sigma Chemical Co, St Louis, USA) is preferred to agar for leaf organogenesis. However, the use of 'gelrite' or 'phytagel' instead of agar may promote vitrification (Guis *et al.*, unpublished data).

REGENERATION FREQUENCY

Efficiency of direct organogenesis is usually very high (more than 80% of regeneration) using cotyledon or leaf explants (Kathal *et al.*, 1986; Tabei *et al.*, 1991; Ficcadenti and Rotino, 1995; Yadav *et al.*, 1996). It has also been observed (Molina and Nuez, 1995b) that, if the organogenic response can be improved for one explant type, similar improvements can be obtained for other explant types from the same cultivar. These data suggest that the regeneration capacity of all types of explant is under the same genetic control (Molina and Nuez, 1995b).

Regeneration by somatic embryogenesis

The first attempt to regenerate whole melon plants by somatic embryogenesis was reported by Blackmond *et al.* (1981) using callused cantaloupe hypocotyl explants. Subsequently, several authors reported the production of somatic embryos and the

first regenerated melon plants from somatic embryos were obtained by Oridate and Oosawa (1986). In recent years, particular attention has been paid to factors controlling somatic embryogenesis (see review of Debeaujon and Branchard, 1993). The main factors that play a role in the control of *C. melo* somatic embryogenesis are reviewed here (*Table 3*).

EXPLANT SOURCE

The embryogenic response is affected by the nature of the explants. A diversity of starting material has been described with cotyledon explants being the most efficient tissue for the induction of somatic embryogenesis. Cotyledons from quiescent seeds or from 1-day-old seedlings are particularly efficient.

COMPOSITION OF THE MEDIUM

Generally, the embryogenic process requires a two-step protocol: the explants are first cultured on an induction medium in the presence of auxin; then the formation and development of embryos occurs on the expression medium, in the absence of auxin. The Murashige and Skoog (1962) medium, supplemented with 2–3% sucrose is commonly used as the basal medium. Several reports indicate that the nature of the carbohydrate source may play a key role in the induction of somatic embryogenesis. Oridate and Yasawa (1990) have showed that a combination of sucrose, glucose, fructose and galactose leads to the highest rate of embryogenesis. Recently, Guis *et al.* (1997a) showed that the use of glucose enhances the embryogenic rate by two-fold, while the use of maltose completely inhibits embryo formation in the *C. melo* cultivar Védrantais.

A requirement for auxin for induction of somatic embryogenesis has been clearly demonstrated (Tabei *et al.*, 1991). Moreover, the efficiency of embryo formation appears to depend upon the specific auxin used and its concentration. Among the auxins, 2,4-D (4.5 to 22.5 μM) is particularly efficient (Oridate and Oosawa, 1986; Branchard and Chateau, 1988; Tabei *et al.*, 1991). The use of other auxins such as NAA or IAA, at high concentrations, induces abnormal embryos which are unable to develop into normal plantlets (Tabei *et al.*, 1991; Guis *et al.*, unpublished data). Often, 2,4-D is used in combination with a cytokinin, with BAP being the most frequently used. Recently, Gray *et al.* (1993) found that TDZ produced significantly more embryogenic explants and more embryos per explant then other cytokinins. Somatic embryo maturation was commonly achieved on a hormone-free medium although cytokinins (Kageyama *et al.*, 1990) or gibberellins (Tabei *et al.*, 1991) have also been used.

PHYSICAL FACTORS

Melon embryogenesis induction can occur in the light (Branchard and Chateau, 1988; Tabei *et al.*, 1991), however, Gray *et al.* (1993) and Guis *et al.* (1997a) only obtained embryo formation after a dark induction step, generally for a two-week period.

The physical state of the media can also affect embryo quality. Best results are obtained on solid medium (Branchard and Chateau, 1988) and the use of 'phytagel' or

Table 3. Regeneration of *Cucumis melo* by somatic embryogenesis

Reference	Year	Variety	Explant source	Induction medium	Expression medium
Trulson and Shahin	1986	Hale's Best Rocky Ford	Cotyledons germinated 1-day-old	2,4-D 1 mg/l NAA 1 mg/l BAP 0.5 mg/l	NAA 1 mg/l BAP 0.5 mg/l
Branchard and Chateau	1988	Charentais T	Expanded cotyledons	2,4-D 4.52 mg/l BAP 0.44 mg/l	no hormone
Homma *et al.*,	1991	Green Pearl Earl's Favourite	Cotyledons and hypocotyls of quiescent seeds	2,4-D 4 mg/l BAP 0.1 mg/l NAA 2 mg/l	no hormone
Kageyama *et al.*,	1991	Earl's Favourite Haru 1	1-day-old cotyledons	2,4-D 1 mg/l NAA 1 mg/l BAP 0.1 mg/l	no hormone
Tabei *et al.*,	1991	Earl's Favourite Harukei N°3	Cotyledons of quiescent seeds Hypocotyls Petioles and leaves	2,4-D 1 or 2 mg/l	no hormone
Debeaujon and Branchard	1992	Charentais T F1 hybrid cv Preco	Protoplast from cotyledons	2,4-D 1 mg/l BAP 0.1 mg/l	no hormone
Oridate *et al.*,	1992	18 cultivars	Cotyledons and hypocotyls of quiescent seeds	2,4-D 3 mg/l BAP 0.1 mg/l	no hormone
Gray *et al.*,	1993	52 varieties	Cotyledons of quiescent seeds	2,4-D 5 mg/l TDZ 0.075 mg/l	no hormone
Guis *et al.*,	1997	Védrantais	Cotyldedons of quiescent seeds	2,4-D 2.2 mg/l BAP 0.1 mg/l	no hormone

'gelrite' instead of agar can improve the development of embryos. The production of somatic embryos from cell suspension culture has been reported (Moreno *et al.*, 1985; Oridate and Oosawa., 1986; Kageyama *et al.*, 1991; Hosemans *et al.*, 1993). However, most of the somatic embryos exhibited abnormal development (Oridate and Oosawa, 1986; Moreno *et al.*, 1986). Furthermore, there have been frequent reports of vitrification of the regenerated plantlets (Kageyama *et al.*, 1991; Moreno *et al.*, 1985) and the development of polyploidy in long-term callus culture (Hosemans *et al.*, 1993). To reduce this problem, the removal of or a decrease in auxin concentration improves somatic embryo development (Oridate and Oosawa, 1986; Kageyama *et al.*, 1991). Similarly, washing somatic embryos with 0.5% activated charcoal results in an improvement of somatic embryo development (Kageyama *et al.*, 1991).

REGENERATION FREQUENCY AND EMBRYO DEVELOPMENT

Cotyledon explants can be highly embryogenic, up to 100%, with an average of 20 embryos produced per explant (Gray *et al.*, 1993). However, development of somatic embryos into plantlets is a limiting step, and is highly dependent on the genotype. Branchard and Chateau (1988) reported that only 12% of the embryos developed in the plantlets, however, Guis *et al.* (1997a) reported a 50% conversion rate of embryos to plantlets.

Abnormalities during development of somatic embryos and in the morphology of the regenerated plantlets are common in melon. These abnormalities include precocious germination, lack of root or stem meristem, growth arrest, hypertrophy of the cotyledons or first leaf, vitrification and fusion between two embryos. However, these phenotypic variations (leaf mosaic, leaf goffering, stikness) are not transmitted to the progeny (Branchard, 1991).

Production of synthetic seeds in an automated mass production of hybrid plants has been proposed. Cryopreservation of melon somatic embryos in liquid nitrogen after preculture with ABA and controlled dessication has been described by Shimonishi *et al.* (1991). This technique could be applied for safe long-term conservation of germplasm or of synthetic melon seeds. Although this species could benefit from the technique to reduce the cost of hybrid production, adaptation of cultural techniques to incorporate this technology would be difficult (Gray and Purohit, 1991).

Protoplasts

Melon protoplasts can be prepared from a variety of tissues including leaves (Moreno *et al.*, 1980; Moreno *et al.*, 1984), cotyledons (Roig *et al.*, 1986a), and cell suspension cultures (Moreno *et al.*, 1980). Yield varies depending on the genotype and the explant source but generally ranges from 10^6 to 10^7 protoplasts per gram of starting tissue. Protoplasts can then be embedded in agarose gel (Li *et al.*, 1990) or plated on MS with 2,4-D alone or in combination with NAA and BAP associated with a high osmotic pressure (450 to 700 mOsmol. kg) (Moreno *et al.*, 1984; Roig *et al.*, 1986a; Debeaujon and Branchard, 1992). Cotyledon and leaf protoplasts of cantaloupe Charentais all exhibit a similar division rate of 45 and 52%, respectively (Roig *et al.*, 1986a, Moreno *et al.*, 1986). Protocolonies are then transferred to a medium with a reduced osmotic

pressure in order to produce microcallus from the newly dividing cells. These calli are then fragmented and placed on a regeneration medium to induce organogenesis or somatic embryogenesis.

Melon plants were first recovered from protoplasts obtained from leaves (Moreno *et al.*, 1986) and cotyledons (Roig *et al.*, 1986a) via organogenesis. Since then, there have been few reports of successful regeneration of melon plants from protoplasts (Bokelmann *et al.*, 1990; Li *et al.*. 1990; Debeaujon and Branchard, 1992; Tabei *et al.*, 1992; Jarl *et al.*, 1995). Tabei *et al.* (1992) found that several subcultures of the protoplast-derived shoots in the presence of BAP and GA_3 allowed the recovery of normal plants at high frequency. Debeaujon and Branchard (1992) were the first to report the recovery of melon plants from embryogenic callus derived from leaf and cotyledon protoplasts. However, the reported regeneration rates remain very low and abnormal plants are frequently obtained. More efficient procedures with regards to the regeneration rate need to be developed.

Other biotechnological applications

HAPLOID PRODUCTION

The inbred-hybrid concept, on which commercial melon breeding is based, requires the ongoing production of homozygous lines to evaluate potential parents of new productive hybrids. However, the production of such lines requires several generations of inbreeding to achieve the desired level of homozygosity. The main advantage of haploids is that they can be used to rapidly produce true-breeding lines, after chromosome doubling to obtain a stable line.

Dumas de Vaulx (1979) reported the successful recovery of haploid melon plants obtained from an interspecific cross between *C. melo* (2n=2x=24) pollinated with a wild tetraploid species *C. ficifolius* A. Rich (2n=4x=48). However, the yield was very low – three plants recovered for 1000 seeds – and the results were difficult to repeat. To date, androgenesis and gynogenesis have been unsuccessful in the production of haploid melon plants. However, induction of *in situ* haploid parthenogenesis can be achieved by using irradiated pollen. This technique is an approach routinely used in breeding programmes. Gamma irradiated pollen from ^{60}Co at a dose of 300 Grays, selected to preserve pollen germination, is used to pollinate female flowers (Sauton and Dumas de Vaulx, 1987). Three weeks after pollination, seeds are individually opened and embryos are cultured on a specific medium (Sauton and Dumas de Vaulx, 1987). A high frequency of regenerated haploid plants can be obtained with an average of 2.5 haploid embryos per 100 seeds expected for the cantaloupe genotype Védrantais and with a maximum of 70% of these embryos developing into haploid plants (Cuny *et al.*, 1992). These haploid plants show a normal phenotype. Chromosome doubling of haploid plants to obtain homozygous diploid phenotypes is performed by treating the *in vitro* cuttings with 0.5% colchicine for two hours (Sauton, 1990).

TRIPLOID PRODUCTION

Since tetraploid plants grow more vigorously than diploid plants, this characteristic is considered to be useful for melon cultivation. But disadvantageous characteristics of

the fruits, as discussed above, prevent the production of tetraploid varieties. However, the situation may be improved if the ploidy is decreased to a triploid level.

Suzuki (1959, 1960) showed that crossing diploids with spontaneous tetraploid plants leads to the production of triploid melons. However, only 0.22 triploid plants were obtained per fruit. Efficient production of triploids is possible after 2n × 4n crosses and *in vitro* culture of abnormal embryos excised from dried seeds (Ezura *et al.*, 1993) or from ovule culture (Fujishita and Shibata, 1990). Of the embryos obtained from 2n × 4n crosses, 20% are expected to grow into normal plants. As anticipated the plant vigour of the triploid plants is similar to the tetraploid ones and the fruit characteristics of the triploid plants were improved. However, the low fertility of triploid plants means that a growth regulator treatment is required for fruit set before self-pollination. Aneuploid melon plants can be recovered by crossing 3n with 2n plants (Ezura *et al.*, 1994). Aneuploids could be further used for studies of genetic analysis and breeding. Trisomic plants may also be useful for genetic analysis but have not yet been reported.

SOMATIC HYBRID PRODUCTION

Genetic diversity in the species *C. melo* allows the introduction of agronomic or resistance traits of various melon types into cultivated varieties by sexual hybridization. However, strong incompatibility barriers at the intergeneric and interspecific level present in the Cucurbitaceae familly generally prevent sexual hybridization (Niemirowicz-Szczytt and Kubicki, 1979), and few interspecific crosses have been reported. Norton and Granberry (1980) and Granberry and Norton (1980) reported obtaining hybrids from the interspecific cross between *C. melo* and *C. metuliferus*, but this result has not been reproduced. Lebeda *et al.* (1996) described the recovery of viable interspecific hybrid embryos between *C. sativus* and *C. melo*, but whole regenerated plants have not yet been obtained. Somatic hybridization may be an alternative to sexual hybridization through protoplast fusion. Roig *et al.*, (1986b) reported obtaining somatic hybrids between two incompatible species, *C. melo* and *C. myriocarpus* after mesophyll protoplast fusion. Fusion was achieved by conditioning of *C. melo* protoplasts in the presence of PEG and Ca^{2+} at basic pH, and inactivation of *C. myriocarpus* protoplasts with UV irradiation. Among the 53 plants recovered, 6 showed an intermediate phenotype estimated at both the morphogenetic level and at the isozyme level using a peroxidase marker. However, the putative hybrid state has not been confirmed by further analysis. Almost the same conditions have been used for fusion of *C. melo* protoplasts with a *Cucumis* wild species (*C. metuliferus* and *Cucurbita martinezii*; Roig *et al.*, 1986c). Attempts of symmetrical protoplast fusion between *C. melo* and *C. sativus* (Jarl *et al.*, 1995) led to the recovery of a few calli, but no plants. However, molecular analysis by PCR of the calli indicated that some of them incorporated a limited amount of the genetic material from cucumber. Yamaguchi and Shiga (1993) recovered putative somatic hybrids between melon and pumpkin by protoplast fusion. However, although the isozyme pattern, chromosome number and shape indicated that hybridization was successful, traits and taste of the fruits were almost the same as the melon, indicating that hybridity disappeared in the late stage of growth. These data illustrate the difficulties of transferring genes by somatic hybridization in *C. melo*. However,

improvement of this technique may eventually allow the sexual incompatibility barriers to be bypassed enabling disease resistance or horticultural traits from other species to be introduced into the melon.

Genetic Transformation

The successful genetic transformation of melon has been reported as summarized in *Table 4*. The primary focus has been on the introduction of genes for disease resistance. Other attempts to improve resistance to environmental stresses or to enhance fruit quality have also been reported.

TECHNIQUE OF TRANSFORMATION

Genetic transformation of melon is commonly achieved through *Agrobacterium tumefaciens* using cotyledon explants (Dong *et al.*, 1991). Among several strains available, LBA 4404 is the most frequently used. When comparing several strains harbouring the *gus* reporter gene between the T-DNA borders, we have found a two-fold higher GUS expression with the C58 strain rather than for LBA 4404 after one month of culture in infected cotyledon explants. This suggests the presence of a greater number of transfection events with C58. The EHA 101 strain was found to be inefficient (Guis *et al.*, unpublished data), however, transformation frequencies of the three strains must be compared to confirm these results. Particle gun transformation has been described by Gaba *et al.* (1992) and Gonsalves *et al.* (1994) using organogenic explants and by Gray *et al.* (1995) using embryogenic explants. Comparing *Agrobacterium* transformation and particle bombardement, Gonsalves *et al.* (1994) and Gray *et al.* (1995) indicates that a similar percentage of transgenic shoots and embryos is obtained by both methods.

In the tranformation protocols reported to date, *nptII* is the most frequently used selectable marker gene, however, escapes are particularly high in melon (30%, Fang and Grumet, 1990, 75–90%, Dong *et al.*, 1991). Gonsalves *et al.* (1994) used 150 mg/l while Ayub *et al.* (personal communication) used up to 300 mg/l kanamycin on the bud induction media to select for transformants. Kanamycin sensitivity of non-transformed melon tissues may be related to the explant source. For example, 100 mg/l kanamycin is enough to inhibit leaf regeneration of non-transformed tissue, but is unable to completely inhibit cotyledon regeneration (Guis *et al.*, unpublished data). For further bud development, the kanamycin concentration is lowered to 100 mg/l and to 50 mg/l for rooting. The rooting phenotype is different in non-transformed and transformed plants (Dong *et al.*, 1991 and Ayub, personal communication). Non-transformed plants produce roots that are short and have no branches. The typical root morphology for transgenic melon plants is branched, strong and elongated. Dong *et al.* (1991) have found that this is also typical of transgenic cucumber plants and suggests that this morphological criterion could be a useful marker for easy identification of transgenic plants in the Cucurbitaceae family. The use of other antibiotics also detoxified by the *nptII* gene like gentamycin or hygromycin has been found to improve selection efficiency (Guis *et al.*, unpublished data), and may be used as an alternative to kanamycin selection.

Table 4. Gene transfer to *Cucumis melo* by *Agrobacterium tumefaciens* and particule bombardment

Reference	Year	Variety	Explant source	Method of transfer	Gene introduced
Fang and Grumet	1990	Hale's best Jumbo	4–5-day-old cotyledons	*A. tumefaciens* (LBA4404)	*nptII* gene
Dong *et al.*	1991	Orient Sweet	Quiescent cotyledons	*A. tumefaciens* (GV3111SE)	Dihydrofolate reductase
Gaba *et al.*	1992	Galia	Cotyledons	Particle bombardment	*gus* gene
Yoshioka *et al.*	1993	Prince Green Pearl Sunday Ahi		*A. tumefaciens*	CMV coat protein gene
Gonsalves *et al.*	1994	Burpee Hybrid Hale's best Jumbo Harvest Queen Hearts of Gold Topmark	3-day-old cotyledons	*A. tumefaciens* (C58 Z707) Particle bombardment	CMV coat protein *GUS* gene *nptII* gene
Vallés and Lasa	1994	Amarillo Oro	5-day-old cotyledons	*A. tumefaciens* (LBA4404)	*gus* gene
Clough and Hamm	1995	Don Louis Galleon Hiline Mission Parental inbreeds		*A. tumefaciens*	ZYMV, WMV2 and CMV coat protein
Gray *et al.*	1995	Eden Gem	Cotyledons	*A. tumefaciens* and particle gun	*nptII* gene
Ayub *et al.*	1996	Védrantais	5-day-old cotyledons	*A. tumefaciens* (LBA4404)	*ACC oxidase* gene in antisense orientation *nptII* gene
Bordas *et al.*	1997	Pharo Amarillo Canario	7-day-old cotyledons Leaves	*A. tumefaciens* (LBA 4404)	Yeast salt tolerance gene *nptII* gene

The transformation frequency for both *Agrobacterium* and particle gun techniques is generally low and ranges from 1% (Gaba *et al.*, 1992) to 7% (Fang and Grumet, 1990). Moreover, in many cases, most of the transgenic plants recovered are tetraploids or mixiploids (61%, Gonsalves *et al.*, 1994; 75%, Ayub *et al.*, 1996).

IMPROVEMENT OF DISEASE RESISTANCE BY GENETIC TRANSFORMATION

More than 25 viruses are able to induce disease in the melon under natural conditions (Provvidenti *et al.*, 1986). Among them, five have an important economic impact in the world. Cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus-2 (WMV2) are the most widespread. These viruses affect the development of the plant, which subsequently produce abnormal fruits or no fruit at all. Traditional breeding techniques allow the selection of resistant melon plants, but few resistant commercial varieties are currently available. Cultural practices or control of insect vectors using insecticides or mineral oil sprays have a limited effectiveness. Transformed melon plants over-expressing CMV coat protein or zucchini yellow mosaic virus coat protein have been produced by genetic engineering (Gonsalves *et al.*, 1994 ; Clough and Hamm, 1995, Yoshioka *et al.*, 1993). CMV coat protein has been overexpressed in several cultivars of cantaloupe type melons (Yoshioka *et al.*, 1993, Gonsalves *et al.*, 1994). Under greenhouse conditions, transgenic melon plants inoculated with CMV did not develop symptoms during a 46-day observation period whereas non transgenic plants showed visible symptoms three days after inoculation (Yoshioka *et al.*,1993). However, at a high inoculum concentration, the appearance of the symptoms is only delayed in transgenic plants (Yoshioka *et al.*, 1993). Similar observations were made by Gonsalves *et al.* (1994), but they also found among their 45 transgenic plants, five resistant ones that exhibited no symptom, even six months after inoculation. Variation among the expression of resistance observed between the different lines may be due to different sites of insertion of the transgene (Yoshioka *et al.*, 1993). Extensive field trials have been conducted to test the level of resistance of five cantaloupe lines genetically modified with WMV2 and ZYMV-CP under natural infection conditions. At the end of the trial, transformed lines exhibited only a low infection rate whereas more than 60% of the control plants developed symptoms. All the transformed cultivars exhibited significant disease reduction under extreme disease pressure and produced normal fruits. The growth rate, physical appearance and fertility were the same as in the non-transformed type. Recently another strategy has been used to produce genetically modified melons. Transgenic plants including a polyribozyme construction directed toward CMV coat protein, exhibit a good level of resistance (Plages, 1997).

IMPROVEMENT OF POST-HARVEST BEHAVIOUR BY GENETIC TRANSFORMATION

The Charentais type melon is the most important melon produced in France. It is characterized by yellow-orange flesh, strong aromatic flavour and abundant sweetness. However, its storage capabilities are low, due mainly to rapid ripening. Melon fruits with improved shelf-life have been generated by traditional breeding. Genetic manipulation has also allowed the regeneration of transgenic fruits expressing low levels of ethylene (Ayub *et al.*, 1996), the key hormone responsible for fruit ripening.

Ripening is greatly delayed in transgenic fruits expressing an antisense copy of the 1-aminocyclopropane-1-carboxylic-acid oxidase gene. Several parameters of ripening, including colour, firmness and sugar level have been determined in the transgenic fruit. The first difference observed between transgenic and wild-type fruit is the colour of the rind. Wild-type melon develop a yellow colour throughout ripening, whereas transgenic fruit remain green, even at the latest stages of ripening. Only exogenous treatment with ethylene allows the recovery of the yellow colour (Guis *et al.*, 1997b). Improvement in firmness is also observed in transgenic fruit. Flesh of transgenic fruit exhibits an almost complete inhibition of softening, whereas, wild-type fruit soften rapidly. Evolution of sugar content and flesh colour is the same in both transgenic and wild-type melon fruit.

Transgenic melon fruit are able to be stored for at least two weeks at 25°C without over-ripening or fungal attack. Meanwhile, wild-type fruit completely rots and liquefies during this period. The shelf-life of the transgenic melon fruits was greatly extended relative to that of control fruit. In addition, growers will be able to harvest the fruit later, when more sugars have accumulated. Losses of marketable fruit should decrease and the geographical distribution of harvested fruits can be expanded. Exposure to exogenous ethylene allow the recovery of the original quality attributes (i.e. colour of the rind, pattern of aroma volatiles) typical of wild-type fruit harvested at a suitable ripening stage.

IMPROVEMENT OF SALT TOLERANCE

A water soluble protein involved in yeast salt tolerance was transferred to two cultivars of *C. melo* by *Agrobacterium* mediated transformation (Bordas *et al.*, 1997). Sensitivity of shoots to NaCl was evaluated on the resulting plantlets. Under stress conditions (10 g/l NaCl during 16 days), transgenic plants expressing the transgene presented a higher level of tolerance than control plants. Behaviour of these plants under greenhouse and field conditions remains to be determined.

Biochemical and molecular markers

Melon, as most of the *Cucumis* species, is a diploid plant with 12 chromosomes (2n=2x=24) (Jeffrey, 1980). Caryotype is composed of eight median chromosomes, two submedian and two subterminal with two satellite pairs. Their size is around 1.06 and 1.88 μm (Ramachandran and Seshadri, 1986), and due to this small size, they are rather difficult to observe. The size of melon genome is 0.94–1.04 pg/1C (Arumanagathan and Earle, 1991), which is around 3.5 times the *size of the Arabidopsis thaliana* genome.

Conventional breeding programmes have improved agronomic traits by combining characters of different parental lines. The selection of simply inherited characters can be achieved by repeated backcrosses. Combination of complex characters encoded by multiple genes (quantitative trait loci) or recessive genes is more difficult to achieve with classical methods that are mainly based on phenotypical characters and require large population sizes. The use of molecular markers has allowed the connection of phenotypic characters with enzyme activity or genomic loci responsible for them, and are indispensable for plant improvement (Winter and Kahl, 1995).

Genetic variability in melon has been studied using biochemical isozyme markers or molecular markers, such as restriction fragment length poplymorphisms (RFLP) or random amplified polymorphic DNA (RAPD). Several authors (Esquinas, 1981; Dane, 1983; Staub *et al.*, 1987) examined melon accessions for isozyme variability and found few allelic variations. Characterization of six varieties using a 29-enzyme system also revealed a low level of polymorphism (24%) (Perl-Treves *et al.*, 1985). However, the use of molecular markers like RFLP has allowed the detection of sufficient polymorphism (33% polymorphism within 44 *C. melo* accessions) to discriminate varieties belonging to different groups within the germplasm of cultivated melons (Neuhausen, 1992). Unfortunately, discrimination of varieties belonging to the same group was not possible. Neuhausen (1992) found that 40% of RFLP genomic probes contained repetitive sequences and suggested that the use of RAPD markers or microsatellites may be more accurate, as they can also detect polymorphism in repetitive DNA sequences. Garcia-Rodrigez *et al.* (1996) used RADP markers to determine genetic relationships between nine genotypes of *C. melo* belonging to different varietal types. Only 39 amplification products, among the 107 generated using 19 polymorphic primers, revealed polymorphism. Baudracco-Arnas and Pitrat (1996) found that RFLP and RADP markers detected a similar level of polymorphism (around 24%) in the progeny of an F2 cross between a Charentais and a Korean melon type. Microsatellite markers detected a high level of polymorphism (77%) in a sample of 8 varieties, while RAPD primer analysis undertaken on the same melon varieties was only able to detect 38% polymorphism (Danin-Poleg *et al.*, 1996). According to these results, microsatellite markers are a very promising tool for the detection of polymorphism in the Cucurbitaceae family.

To date, more than 90 melon genes have been described, including genes controlling morphological characteristics and those involved in resistance (Pitrat, 1994). Identification of genetic linkages between various genes has allowed the correlation between some disease resistance genes and vegetative or flower biology characteristics. Eight independent linkage groups, containing 23 genes have been described (Pitrat, 1991). These results were the first step towards a genetic map of *C. melo*. The first molecular map of the melon genome was generated from the analysis of an F2 population resulting from an intraspecific cross between two divergent inbred lines, a Charentais type and a Korean type. About 100 markers, mainly RFLP and RAPD markers, were used to define 14 linkage groups (Baudracco-Arnas and Pitrat, 1996). Eleven microsatellite markers were recently added to the map which is now estimated to cover 1522 cM (~60% of the melon genome) (Katzir *et al.*, 1997). Another map based on a new type of marker (Amplified Fragment Length Polymorphism = AFLP) has been built from a back cross progeny between an Indian accession (MR-1) and an Ananas type (Ananas Yokneam). Fourteen major and six minor linkage groups were defined with several markers still unlinked. This map covers 1942 cM (Wang *et al.*, 1997). The four types of markers used seem to be relatively well distributed along the genome. A saturated map will be a useful tool for marker-assisted selection for the improvement of monogenic or quantitative traits.

Conclusions

Biotechnological strategies have been of increasing importance in melon breeding

programmes since the 1980s. Interest has mainly focused on plant transformation and on the development of new breeding tools. Transgenic melon plants have been successfully produced and several laboratories are currently using the method to improve disease resistance or fruit quality. Although transformation efficiency remains low and is still regarded as a difficult problem, there is no doubt that, in the near future genetically engineered melon fruit will be commercialized.

Haploid plants are already used routinely in melon breeding programs in order to fix parental lines of commercial F1 hybrids. A molecular map of melon is being actively developed with several teams now contributing to this work, and finally molecular markers linked to agronomic genes will help to optimize breeding programmes by allowing marker assisted selection strategies.

While efficient systems of somatic hybridization and protoplast regeneration still need to be developed, these new technologies will increase the available genetic variation.

Acknowledgements

The authors are grateful to Brian Jones (ENSA Toulouse, France) and Alan Bennett (UC Davis, USA) for critically reading the manuscript.

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