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In Vivo and *in Vitro* Mutation Breeding of Citrus

Rodrigo R. Latado[1*] • Augusto Tulmann Neto[2] • Antonio Figueira[2]

[1] Centro APTA Citros Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil
[2] Laboratório de Melhoramento de Plantas, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, SP, Brazil
Corresponding author: * **rodrigo@centrodecitricultura.br**

ABSTRACT

Spontaneous mutations are important for the development of new *Citrus* varieties, but these mutations appear at a low frequency in nature. In contrast, induced mutagenesis using radiation or chemical mutagens can increase the rate of mutation many fold, which increases the genetic variability that is available for selection. However, few induced mutants have been officially released for cultivation possibly because *Citrus* plants are vegetatively propagated, with a long reproductive cycle and require large areas for cultivation. The most common methods of mutagenesis used were physical mutagens (e.g., gamma-rays, X-rays or thermal neutrons), and buds or seeds as starting materials. Chemical mutagens were used less often, possibly because there are difficulties of penetration in tissues and the target propagules are not seeds. Most projects using induced mutagenesis in *Citrus* aimed to develop new cultivars with the following traits: seedless fruits or fruits with fewer seeds, resistance to diseases, improved fruit color in the peel and pulp, reduced plant height, and trees with alteration in harvest time. In several countries, induced mutants of *Citrus* have been described. The development of new areas of biology (e.g., functional genomics and bioinformatics) and new high-throughput technologies (e.g., high performance sequencing, TILLING (targeting induced local lesions in genomes), microarray, real-time PCR and others) that are associated with induced mutagenesis can help gene discovery programs to elucidate the function and mechanisms of gene action.

Keywords: improvement, mutagenesis, mutant, mutagen, method, vegetative
Abbreviations: CGH, comparative genomic hybridization; **PCR**, polymerase chain reaction; **RT-PCR**, reverse transcription polymerase chain reaction; **TILLING**, targeting induced local lesions in genomes

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INTRODUCTION

Citrus spp. are perennial fruit species of great economic importance in many countries. Within fruit crops, *Citrus* contains the largest cultivated area in the world, which reached 8.89 million ha in 2009 and increased nearly 15.2% in six years (FAO 2012). Sweet oranges are planted most often and occupy approximately 55% of the total cultivated area in the world followed by mandarins with 28.3%, limes and lemons with 13.3% and grapefruit with 3.4% (Neves *et al.* 2010).

Several regions of *Citrus* production, which include the two largest orange producing areas of São Paulo and Florida, are in jeopardy because of the predominance of commercial groves containing a limited number of cultivars, with restricted genetic diversity in continuous areas. For example, approximately 55%, 23% and 22% of the cultivated area of sweet oranges in São Paulo contains the late cultivar 'Valencia', the early cultivar 'Hamlin' and the mid-season cultivar 'Pera', respectively (Neves *et al.* 2010).

Using monoculture within groves and increasing grove size are two strategies implemented to increase the scale of production, which leads to cuts in production costs. However, the combination of these strategies can increase the risk of outbreaks or epidemics that involve novel pests or diseases, which could increase production costs or limit *Citrus* production in some areas. A recent example is the emergence and spread of *Huanglongbing* disease in some areas of São Paulo, Brazil and Florida, USA.

SPONTANEOUS AND INDUCED MUTATIONS IN CITRUS

Various biological barriers, such as seed polyembryony, sexual incompatibility, high heterozygosity and sterility make traditional breeding of *Citrus* using controlled crosses a long and costly process (Cameron and Frost 1968). For these reasons, it is believed that most commercially-adopted sweet orange cultivars originated from spontaneous mutations, which were selected from sports derived from seedling or shoot selections of commercial cultivars (Hodgson 1967). Good examples of cultivars selected from spontaneous mutation include the many grapefruit cultivars selected in Texas (Hensz 1981), sweet oranges cv. 'Washington Navel', 'Baianinha' and the acid-less 'Lima', all selected in Brazil (Domingues *et al.* 1995).

Spontaneous mutations are important for the development of new *Citrus* varieties, but they occur at a low frequency. In contrast, induced mutations using radiation or chemicals compounds can increase the rate of mutation by

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Invited Mini-Review

Table 1 *Citrus* mutant varieties officially released and communicated to FAO/IAEA Mutant Variety Database (MVGS) (adapted from Maluszynski *et al.* 2000)*.

Species	Country of Release	Mutant Variety	Year of release	Mutagen (dose)	Main character induced
Lemon	Argentina	'Eureka 22 INTA'	1987	X-rays (10 Gy)	Fruit set
Grapefruit	USA	'Rio Red'	1984	Thermal Neutrons (NC)	Fruit color
	USA	'Star Ruby'	1970	Thermal Neutrons (NC)	Seedless
Orange	Argentina	'Valência 2 INTA'	1987	X-rays (20 Gy)	Fruit set
Orange/mandarin	China	'Hongju 418'	1983	Gamma-rays (100 Gy)	Seedless
	China	'Hongju 420'	1986	Gamma-rays (100 Gy)	Seed number
	China	'Huegan 9-12-1'	1983	Gamma-rays (100 Gy)	Seedless
	China	'Zhongyu 7'	1985	Gamma-rays (NC)	Seedless
	China	'Zhongyu 8'	1985	Gamma-rays (NC)	Seedless

NC – Not cited.
* No *Citrus* mutant varieties were officially released and communicated to FAO/IAEA database after 1987

many fold, which increases the genetic variability available for selection (Broertjes and Van Harten 1988). Compared with other crops, few *Citrus* mutants have been officially released. According to the last survey conducted by the Food and Agriculture Organization (FAO) / International Atomic Energy Agency (IAEA) Mutant Variety Database (MVGS; http://mvgs.iaea.org/Search.aspx), only nine *Citrus* cultivars that were developed using induced mutagenesis were released (**Table 1**), as cited by Maluszynski *et al.* (2000) and available at the site. However, the available information on mutagenesis is far from complete, as it occurs with cultivars developed by conventional methods.

Although *Citrus* species produce viable seeds, they have a lower rate of success in induced mutation programs because they are perennial plants, vegetatively propagated, with long reproductive cycles and they require large areas to be planted. Long reproductive cycles can hinder breeding by mutagenesis or conventional methods. *Citrus* plant propagation by grafting of adult buds demands approximately two years to initiate production, five years to offer a reasonable preview of yield potential (three consecutive harvests), and a total of eight years to conclude the analysis (six consecutive harvests). Seed propagation of *Citrus* plants requires even longer time periods because the plants have to go through the juvenile stage. In some *Citrus* species, the juvenile stage of seed-derived plants can last from 6 to 20 years (Endo *et al.* 2005).

One of the main obstacles to obtaining *Citrus* mutants is the formation of chimeras. When multicellular shoot apical meristems are used for mutagenesis, a mutational event occurs by chance in each isolated cell. To avoid chimeras, vegetative propagation of mutagenized plants (from M1V1 to M1V4) is performed to amplify and stabilize mutated sectors before mutant selection (Broertjes and Van Harten 1988). In sexually propagated plants, it is easier to circumvent chimeras by selfing because chimeras will disappear in the M2, M3 or M4 segregating population.

The high level of heterozygosity in some *Citrus* species, especially sweet oranges (Hearn 1994) and grapefruits, can facilitate the detection of mutations in the first generation of mutagenized plants (M1 or M1V1) because mutagens induce recessive alleles (from *Aa* to *aa* or *_a*) more frequently, which may enable the identification of mutant phenotypes (Spiegel-Roy 1990).

METHODS OF INDUCED MUTAGENESIS IN CITRUS

Various reviews have described the methods of induced mutagenesis used in plants (Maluszynski *et al.* 1995; Ahloowalia and Maluszynski 2001; Ahloowalia *et al.* 2004), tree fruit species (Donini 1982; Spiegel-Roy 1990; Predieri 2001) and *Citrus* species (Spiegel-Roy and Kochba 1973; Broertjes and Van Harten 1988). Thus, only the main methods of mutation breeding in *Citrus* will be summarized and discussed in this review.

Regarding the type of mutagen used, most studies reported the use of ionizing radiation, such as gamma-rays and X-rays (Spiegel-Roy and Padova 1973; Hearn 1984; Spina *et al.* 1991; Chen *et al.* 1991; Tulmann Neto *et al.* 1996; Gulsen *et al.* 2007), or heavy particles, which were primarily thermal neutrons (Hensz 1960). Chemical mutagens were used less often, possibly because there are difficulties with tissue penetration and the target propagules are not seeds.

The exception is colchicine, which has been extensively used in *Citrus* to produce autotetraploids by *in vivo* or *in vitro* treatments that induce mitotic polyploidy (Barret 1974; Gmitter and Ling 1991; Wu and Mooney 2002; Wakana *et al.* 2005; Latado *et al.* 2007; Zhang *et al.* 2007; Aleza *et al.* 2009; Dutt *et al.* 2010). Colchicine is a powerful anti-mitotic agent that inhibits chromosomal separation during metaphase of mitosis, which results in the doubling of chromosomes in somatic tissues (Dhooghe *et al.* 2011).

To evaluate the physiological effects on the growth rate of propagules, tests for sensitivity to mutagens are conducted at distinct dosages. The results are expressed as plant growth reduction (GR) or lethal dose (LD). Sensitivity to a certain dose of mutagen can vary between species, cultivars, or target tissues (propagules), such as buds, shoot apical meristems, seeds, or *in vitro* explants (Broertjes and Van Harten 1988).

There is a direct correlation between the magnitude of biological effects and increasing dosages; for certain thresholds, increases in dosages also correlate with mutation frequencies. For this reason, a quick pilot experiment is conducted to establish the appropriate dose, which will later be used in the experiment. In *Citrus*, the best option is to use mild doses to treat a large number of propagules; this option will induce mutations without causing serious physiological damages to M1 or M1V1 plants (Tulmann Neto *et al.* 1991) or undesirable occurrences of multiple mutations, especially for vegetatively propagated plants (Broertjes and Van Harten 1988).

The results of experiments evaluating the *in vivo* and *in vitro* sensitivities of *Citrus* propagules to mutagens, mainly gamma-rays, colchicine and chemical mutagens, are presented in **Table 2**.

A large area, currently between 400 to 700 plants ha^{-1} in commercial groves of Brazil (Neves *et al.* 2010), is required for *Citrus* cultivation because these tree species have a medium to large plant size. When grown in greenhouses, *Citrus* plants might require less space, but growth in these spaces is costly. Before mutant selection, which requires a large experimental area, it is recommended to use large populations of 2,000 to 4,000 irradiated plants (M1V1) to obtain M1V2, M1V3 and M1V4 generations because the frequency of mutation is generally low even in programs using induced mutagenesis (Tulmann Neto *et al.* 1996). In this case, the alternatives adopted by mutation breeding programs in Brazil are to keep irradiated plants in greenhouses, to plant *Citrus* at high densities (up to 0.5 m between plants) in the field, or to employ *in vitro* methods (Latado 2011; pers. comm.). After selection of putative mutants, there is a substantial reduction in the number of genotypes to be evaluated, and experiments estimating the mutational stability and yield potential are conducted in the field using conventional plant densities.

Table 2 Sensitivity of the *in vivo* and *in vitro* propagules of *Citrus* to mutagens.

Cultivar/Species	Target tissue	Mutagen	Sensitivity dose (level)	References
'Shamouti' Sweet Orange	Seeds	Gamma-rays	80 – 100 Gy (LD 50)	Spiegel Roy and Kochba 1973
'Pineapple' Sweet orange and Grapefruits	Seeds	Gamma-rays	100 – 150 Gy (LD 50)	Hearn 1984
'Shamouti' Sweet Orange	Decapitated young seedlings	Gamma-rays	20 – 40 Gy (LD 50)	Spiegel Roy and Kochba 1973
'Shamouti' Sweet Orange	Buds	Gamma-rays	50 Gy (LD 50)	Spiegel Roy and Kochba 1973
'Pera' Sweet Orange	Buds	Gamma-rays	40 Gy (LD 30)	Tulmann Neto *et al.* 1996
'Kutdiken' Lemon	Buds	Gamma-rays	50 Gy (LD 50)	Gulsen *et al.* 2007
Mandarins and 'Murcott' Tangor	Buds	Gamma-rays	16.3 – 33.5 Gy (GR 30)	Gonzaga *et al.* 2011
Rough lemon	Seeds (*in vitro* germination)	Gamma-rays	62 Gy	Kaur and Rattanpal 2010
'Shamouti' Sweet Orange	Calli (*in vitro*)	Gamma-rays	240 Gy (LD 50)	Spiegel Roy and Kochba 1973
Rough lemon	Calli (*in vitro*)	Gamma-rays	20 Gy	Kumar *et al.* 2010
'Pera' Sweet Orange	Protoplasts (*in vitro*)	Gamma-rays	41 Gy (LD 50)	Cristofani *et al.* 1993
Lemon	Protoplasts (*in vitro*)	Gamma-rays	200 Gy (LD 50)	Helaly and Hanan El-Hosieny 2011
Mandarins and 'Murcott' Tangor	Epicotyl segments (*in vitro*)	Gamma-rays	16.2 – 20.7 Gy (GR 30)	Gonzaga *et al.* 2011
Clementines, Tangor, Grapefruit	Embryogenic calli (*in vitro*)	Colchicine	0.01 – 0.1% (8 weeks)	Wu and Mooney 2002
'Ponkan' Mandarin	Calli (*in vitro*)	Colchicine	0.1% (4 days)	Dutt *et al.* 2010
Tangor, Mandarin, Sweet orange	Epicotyl segments (*in vitro*)	Colchicine	0.025 – 0.1% (1 – 2 days)	Latado *et al.* 2007
Clementines, hybrids	Micrografted shoot tips (*in vitro*)	Colchicine	0.1% (one application)	Aleza *et al.* 2009
Rough lemon	Seeds (*in vitro* germination)	EMS	0.64%	Kaur and Rattanpal 2010
Rough lemon	Calli (*in vitro*)	EMS and MMS	0.1%	Kumar *et al.* 2010

The vast majority of work reported for *Citrus* mutagenesis has used acute doses of irradiation because it is easier to execute compared with chronic irradiation, which does not demonstrate any significant advantages (Sigurbjörnsson 1977).

Because *Citrus* is a vegetatively propagated plant, the method chosen after mutagenic treatment depends on the expected occurrence of chimeras. In the case of mutagenesis of multicellular propagules (e.g., shoot apices, buds), some vegetative propagation is needed to amplify the mutated sectors before selection because chimeras can occur. When unicellular propagules (or multicellular ones that were further induced from *in vivo* or *in vitro* shoots of unicellular origin) are treated, only solid (i.e., stable) mutants are expected. Therefore, plants can be evaluated directly (Broertjes and Van Harten 1988).

The decision about the best method to use for *Citrus* mutagenesis requires consideration of the timeframe. If buds of adult plants are used as starting material, then time is needed to perform several vegetative propagations before mutant selection. However, in this case, putative mutants are developed from adult tissues; this approach reduces the maturation period required for fruiting. In contrast, if *in vitro* methods are used at the single-cell stage (e.g., the culture of protoplasts or the induction of *in vitro* or *in vivo* adventitious shoots), then chimeras will be avoided. However, the resulting plants will be restored to a juvenile stage, which leads to a longer maturation period.

If spontaneous mutants for early flowering are used, the long period required for *Citrus* mutation breeding can be shortened. For example, mutants, such as sweet orange 'X11' (Latado 2011; pers. comm.) and *P. trifoliata* L. Raf. (Liang *et al.* 1999), exhibit early flowering (i.e., less than 1 to 2 years) and flower several times during the same year. These mutants will facilitate chemical mutagenesis of *Citrus* seeds for breeding purposes and genetic studies because a large population of solid mutants can be easily obtained and reverse genetic methods, such as TILLING (Till *et al.* 2007), can be developed for *Citrus*.

Most projects using induced mutagenesis in *Citrus* have aimed to develop new cultivars with the following traits: seedless fruits or fruits with fewer seeds, resistance to diseases, improved fruit color of the peel and pulp, reduced plant height and trees with alteration in harvest time.

SUCCESSFUL EXAMPLES OF INDUCED MUTAGENESIS IN CITRUS

In the last fifty years, the *Citrus* breeding program with the most impact on commercially-released mutant cultivars and the largest implementation of mutants has been developed in Texas for grapefruits and sweet oranges. After the successful selection and propagation of various spontaneous grapefruit mutants, the Texan program started an induced mutagenesis program in 1960 (Hensz 1960). An induced mutant, the cultivar 'Star Ruby', was released in 1977 because it had a low seed set (between 0 and 3 units per fruit) and a more intense fruit peel and pulp color than the original cultivar 'Hudson' (Hensz 1977). The seeds of 'Hudson' were treated with thermal neutrons, and resulting mutants were evaluated seven years later in the field.

The 'Rio Red' grapefruit cultivar was obtained in a manner similar to 'Star Ruby', but it was derived from the cultivar 'Ruby Red' (Hensz 1985). Budwoods from 'Ruby Red' were treated with thermal neutrons or X-rays, grafted onto sour orange rootstocks and then planted in the field. A mutant was selected because it produced fruits with no seeds and the pulp color was 3 to 5 times more intense than the original cultivar. Currently, these mutant cultivars are the most cultivated among pigmented grapefruits and are commercially important in Texas and other countries.

By irradiating buds with 40 and 60 Gy of gamma-rays and then producing 600 plants by grafting, Spiegel-Roy *et al.* (1985) obtained mutants bearing seedless fruits from the 'Eureka' lemon (*C. limon*). After vegetative pruning, the M1V2 buds obtained from 60 Gy gamma-rays treated plants were evaluated, and one seedless fruit mutant was selected. After a few vegetative propagation cycles, it was demonstrated that the seedless character was stable. Spiegel-Roy *et al.* (1990) reported a similar work using the 'Villafranca' lemon and had the same positive results using 50 Gy gamma-ray treatments. In both cases, there is no information about the commercial release of the mutants.

Chen *et al.* (1991) studied the stability of the seedless character of two sweet orange mutants ('Jin Cheng' lines 7 and 9) that were obtained in China from 100 Gy gamma-rays treatment of seeds. The results indicated that the seedless trait was stable and could be transmitted vegetatively. However, the mutants presented anomalous meiosis that was caused by translocations and inversions, which might have caused the observed sterility. These mutants were commercially released in various producing regions of China.

Seedless mutants of mandarins were also obtained in the USA, South Africa, Italy and other countries (Russo *et al.* 1981; Starrantino *et al.* 1988; Du Plooy *et al.* 1992; Froneman *et al.* 1996). Khalil *et al.* (2011) reported the development of sparse-seeded mutant of Kinnow through budwood irradiation.

Tulmann Neto *et al.* (1996) treated buds of the most im-

portant cultivar of the Brazilian citrus industry, the 'Pera' sweet orange cultivar, with 40 Gy of gamma-rays and obtained around 1,000 M1V1 plants. These mutagenized plants were vegetatively propagated to the M1V4 generation, and approximately 7,600 of these plants were planted in the field. A total of 127 putative mutants were selected for different characteristics, such as dwarf plants, altered canopy architecture, fruits with reduced seed set and fruits with changes in form and maturation period. Working with the same 127 putative mutants, eight mutated clones were characterized as producing seedless fruits with great potential for commercial cultivation (Latado *et al.* 2001); other clones were evaluated because they presented changes in the fruit maturation period or reductions in plant size (Latado *et al.* 2005). For some of the 127 mutants, Latado *et al.* (2004) concluded that the low pollen viability was associated with a reduction or absence of seeds in fruits. Belasque Jr. *et al.* (2009) evaluated six mutant clones and identified three (9-1, 9-2, and 9-3) that showed enhanced resistance to an endemic disease of Brazil, citrus canker.

Gulsen *et al.* (2007) reported the identification of seedless mutant lines of 'Kutdiken' lemon that were tolerant to *mal secco*, a disease caused by *Phoma tracheiphila*, after selection of 478 M1V3 plants derived from buds irradiated using gamma-rays.

The absence of seeds is one of the most important traits in the fresh fruit *Citrus* market. For this reason, most efforts are focused on developing seedless cultivars. Currently, one strategy commonly used by *Citrus* breeding programs is the development of triploid seedless cultivars from crosses between tetraploid and diploid plants (Reforgiato Recupero *et al.* 2005) or occasionally from crosses between two diploid plants, but in this case, at lower frequency (Ollitrault *et al.* 2007). When crosses between diploid parents generate superior hybrid genotypes that produce seeds, mutagenesis is of great interest at later stages of the breeding program, because it allows the elimination of undesirable traits, primarily fruits with seeds, without changing other features.

An interesting strategy that can save time and labor is conducting an early evaluation to determine if the *Citrus* cultivar to be used for mutagenesis is sufficiently parthenocarpic to produce fruits in the absence of seeds. This condition can be verified by pollination experiments using sterile pollen grains, pollen from diploid cultivars treated with mutagens or from triploid cultivars and further evaluation of fruit set (Vardi *et al.* 1988). If a cultivar displays low parthenocarpic ability, it should not be used in mutagenesis for seedless fruits because the chances of obtaining a mutant with high yields are minimal.

Although there is a series of reports using *in vitro* mutagenesis (Spiegel-Roy and Kochba 1973; Broertjes and Van Harten 1988; Predieri 2001), none has resulted in the release of a commercial mutant cultivar. As previously mentioned, the use of induced mutagenesis combined with some *in vitro* culture techniques might avoid the production of chimeras. Although this result is positive, this strategy is recommended only for cultivars with a short juvenile stage because the plants recovered from *in vitro* culture display restored juvenile features. In a practical example, Gonzaga *et al.* (2011) irradiated epicotyl segments of the 'Rangpur' lime cultivar, which is the most important rootstock used in Brazil, and then performed *in vitro* shoot regeneration and *in vitro* micrografting to rapidly develop and harden plantlets. Using this strategy, the first fruiting of irradiated plants was observed two to three years after planting in the field, which indicated that the 'Rangpur' lime has a short juvenile phase. The final objective of this ongoing work is to select mutants that are dwarf and/or resistant to *Citrus* Sudden Death. Starrantino *et al.* (1988) described thorn-less lemon mutants that developed after *in vitro* mutagenesis of nucellar callus.

Tetraploid plants are becoming increasingly interesting to *Citrus* breeders. To improve scion cultivars, a tetraploid plant is crossed with a diploid plant to obtain seedless triploid cultivars (Reforgiato Recupero *et al.* 2005; Ollitrault *et al.* 2007). To improve rootstocks, tetraploid cultivars are used directly because they induce trees of short stature (Lee *et al.* 1990; Grosser and Gmitter 2011). The most common method used to develop *Citrus* autotetraploids is the *in vitro* chromosome-doubling method. In this method, explants are treated *in vitro* using the anti-mitotic agents colchicine or oryzalin for a certain time period, and the shoots are subsequently regenerated (Dhooghe *et al.* 2011). To efficiently generate autotetraploid plants, different explants and methods of applying mutagens have been used in canopy and rootstock cultivars. The explants used most often were embryogenic callus (Gmitter and Ling 1991; Wu and Mooney 2002; Zhang *et al.* 2007), cell suspension cultures (Dutt *et al.* 2010), micrografted shoot-tips (Aleza *et al.* 2009) and epicotyl segments (Latado *et al.* 2007).

FUTURE PROSPECTS

During the last decade, the development of new areas of biology, such as genomics, functional genomics and bioinformatics, and new high-throughput technologies, such as Next Generation Sequencing or TILLING, have increased interest in the development of mutant populations using mutagenesis. This interest is focused on gene discovery programs that are aimed to elucidate the function and mechanisms of gene actions and is not restricted to the development of new cultivars using plant breeding.

Mutant analysis is a widely used approach in biology. Large-scale mutagenesis using chemical or physical agents can generate random mutations in plant populations. Forward genetics involves the identification and characterization of isolated mutants to find the corresponding mutations at the molecular level in DNA or RNA. It is the tool most frequently employed to understand the genetic control of plant development.

However, this approach does not always fill the gaps between the knowledge of genes and their functions. This limitation can be overcome by the use of reverse genetics. Mutated plant populations can be evaluated using high-throughput molecular tools that search for mutations in known genes or complete genomes, or insertional mutagenesis and subsequent phenotypic characterization of mutants can be performed (Wang *et al.* 2009).

The TILLING technique combines chemical mutagenesis with an efficient method of screening for mutations in genomic DNA, which allows the identification of individuals that exhibit allelic variation, such as single base pair changes, in genes of interest. If fast neutrons are used to induce mutations in plants, then deletion mutants can be identified using the De-TILLING technique. When groups of genotypes (e.g., cultivars, ecotypes, land races or accessions) are analyzed to identify natural variants, the technique is called EcoTILLING (Till *et al.* 2009, Wang *et al.* 2009).

Techniques known as microarrays, subtractive libraries or massive sequencing of RNA or DNA can also be used to search for mutations in the genome of a plant of interest. These techniques are becoming more important after the development of new affordable methods of high-throughput sequencing, such as the GS-20 or GS-FLX, Illumina's Solexa and the SOLiD systems.

Citrus has been the target of various genomic studies that usually involve EST sequencing, cDNA or oligonucleotide microarrays, BAC library end-sequencing (BES), and genetic transformation (Flachowsky *et al.* 2009). However, functional genomic studies using reverse genetics and genetic transformations on a large scale are in the beginning stages because of a lack of highly efficient methods to genetically transform *Citrus*. Methods of genetic transformation in *Citrus* would enable insertional mutagenesis or gene knock-outs, RNA interference, activation-tagging using enhancer elements, gene-trap T-DNA insertional mutagenesis, or transposon tagging systems. Thus, the approach with better chance of success would be development of a *Citrus* mutant population, using chemicals or fast neutrons, fol-

lowed by identification of mutated DNA using microarray analysis, TILLING or De-TILLING (Rios *et al.* 2009).

Rios *et al.* (2009) reported the successful identification of deleted gene sequences in the genomes of *C. clementina* mutants that exhibited a delay in the change of fruit peels during ripening. These mutants were obtained after exposure to fast neutrons, and the identification of mutated genes was conducted using Microarray-based CGH (Microarray Comparative Genomic Hybridization) and quantitative RT-PCR.

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