Review Article
Genetic Transformation in Citrus

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Citrus is one of the world’s important fruit crops. Recently, citrus molecular genetics and biotechnology work have been accelerated in the world. Genetic transformation, a biotechnological tool, allows the release of improved cultivars with desirable characteristics in a shorter period of time and therefore may be useful in citrus breeding programs. Citrus transformation has now been achieved in a number of laboratories by various methods. Agrobacterium tumefaciens is used mainly in citrus transformation studies. Particle bombardment, electroporation, A. rhizogenes, and a new method called RNA interference are used in citrus transformation studies in addition to A. tumefaciens. In this review, we illustrate how different gene transformation methods can be employed in different citrus species.

1. Introduction

Citrus species are the most widely grown fruit crops. Despite substantial genetic diversity and interspecific fertility, the genus Citrus includes some of the most difficult species to breed [1, 2]. This is due to several obstacles for conventional breeding. For example, most species are highly heterozygous and produce progeny that segregate widely for many characters when crosses are made. The juvenile periods are often very long, self- and cross-incompatibility and pollen and/or ovule sterility are relatively common, and the presence of adventitious somatic embryos in the nucellus of developing ovules of the most of Citrus greatly limits hybrid production [2, 3].

The genus Citrus possesses several undesirable characteristics including salt and cold sensitivity [4, 5]; they are also susceptible to diseases caused by fungi, bacteria and viruses, such as Citrus exocortis viroid (CEV), Citrus infectious variegation virus (CIVV), Citrus cachexia viroid (CCaV) and Citrus tristeza closterovirus (CTV) [5, 6]. Classical genetic selection, gene transfer, grafting, and micrografting techniques can contribute to the improvement of Citrus and propagation of selected species. Therefore, in vitro manipulation procedures leading to a rapid, direct bud regeneration for efficient micropropagation as well as genetic transformation are needed as a first step towards Citrus improvement. Practical benefits resulting from in vitro culture methods have already been reported in Citrus [5, 7, 8]. Recent developments in gene transfer techniques via the classical regeneration method have been applied to this genus and have opened the way to induce a specific genetic change within a period of time shorter than using the classical genetic selection method [5, 9, 10].

Conventional breeding methods have demonstrated limitations with respect to citrus improvement due to some of the biological characteristics of woody plants such as nucellar polyembryony, high heterozygosity, long juvenile period, and autoincompatibility [11, 12]. The development of biotechnological tools has made it possible to overcome some of these problems. In the specific case of citrus breeding programs, somatic hybridization [12–14] and genetic transformation [12, 15, 16] have been applied in many countries [10, 12, 17, 18].

In recent years, there has been a major thrust in citrus improvement as competition from international citrus markets, disease, and pest pressure and other abiotic and biotic stress conditions stimulate worldwide interest [19, 20]. Several strategies exist for the genetic improvement of citrus including conventional breeding and genetic transformation [20, 21]. Currently, genetic transformation of citrus as a tool...
for citrus improvement is gaining popularity. This method is especially useful in cases where it is not possible to introduce a particular trait of interest to another elite cultivar using conventional breeding. Citrus cultivars vary in their response to in vitro organogenesis and genetic transformation. This results in the need for cultivar-specific optimization of in vitro protocols [20, 22].

Among the several methods available for the genetic transformation of citrus, the most popular method to transform a wide range of citrus cultivars is Agrobacterium-mediated transformation using epicotyl explants as target cells for incorporation of the T-DNA [20, 23]. However, this method is not suitable for the transformation of any seedless cultivar. Also, special cultivars in the mandarin group remain robust to transform using this method [20, 22, 24].

2. Transformation Studies in Citrus

Genetic transformation and somatic hybridization studies are already integrated in Citrus breeding programs in several countries. Genetic transformation of Citrus is a promising tool that enables the introduction of desirable traits without altering the genetic background [25]. Genetic transformation of citrus has been reported, by using several methods (Table 1).

Agrobacterium has been the most frequently used genetic transformation method in Citrus with explants collected from seedlings germinated in vitro or under greenhouse conditions [68].

Transformation studies have been done for two decades in citrus. In the last few years, different transformation methods such as RNA silencing are used. In order to carry out successful gene transformation studies in citrus, optimized in vitro regeneration protocol is needed. Researchers should optimize efficient regeneration protocol before starting transformation studies. There are also many efficient regeneration protocols published in different citrus species.

Orbovic et al. [36] investigated the effects of seed age on shoot regeneration potential and transformation rate of “Duncan” and “Flame” grapefruit cultivars, along with “Hamlin” sweet orange cultivar. Genetic transformation of citrus explants was carried out as previously described [93] using A. tumefaciens strain EHA105 [94] containing a binary vectors derived from pD35s [22]. In conclusion, the regeneration potential and transformability of citrus juvenile explants are different among cultivars and also change within the fruit harvest season. Because of these findings, especially the latter one, it will be extremely difficult to develop a universal protocol for genetic transformation of citrus. Optimal transformation efficiency will require flexible procedures that account for cultivar variability and timing of seed collection.

In another study, a protocol was developed for regeneration of transgenic plants via A. tumefaciens-mediated transformation of leaf segments from “Valencia” sweet orange (C. sinensis L. Osbeck) using gfp (green fluorescence protein) as a vital marker [27]. The transformation methodology described by Khan et al. [27] was an important finding for generating transgenic plants using leaf segments as explants.

In addition to transformation studies via A. tumefaciens, recently, A. rhizogenes has been used. Many reports suggest the use of A. rhizogenes for expression of the rol genes and also to deliver foreign genes to susceptible plants [95]. The hairy root harbouring the T-DNA segment of Ri-plasmid within its nuclear genomes [96]. A. rhizogenes are also capable of transferring the T-DNA of binary vectors in trans, thereby facilitating the selection of transgenic plants from screened hairy roots [95]. A. rhizogenes-mediated transformation system was found to be very useful in genetic manipulation of plants for the production of phytochemicals [97], large scale secondary metabolite production [98], monoclonal antibody production [99], and phytoremediation [100]. There are many reports that suggest the successful use of A. rhizogenes harbouring binary vectors with desired gene constructs [95] for plant genetic transformation [101]. Due to low transformation efficiency of A. rhizogenes, many researchers have worked to optimize transformation methods.

Chávez-Vela et al. [72] used A. rhizogenes A4 agropine-type strain to develop the transformation system. A4 contains wild-type plasmid pRI A4 which confers hairy-root genotype and binary vector pESC4. In the study seventy-five-day-old sour orange seedlings were used and transgenic sour orange (C. aurantium L.) plants were regenerated from A. rhizogenes transformed roots. 91% of explants produced transformed roots with an average of 3.6 roots per explant.

In another study transgenic Mexican lime (C. aurantifolia (Christm.) Swing) plants were regenerated from tissues transformed by A. rhizogenes strain A4, containing the wild-type plasmid pRI A4 and the binary vector pESC4 with nos-npt II and cab-gus genes. More than 300 Mexican lime transgenic plants were obtained, 60 of which were adapted to growing in soil [2].

In addition to the indirect gene transfer methods, there are studies performed by direct gene transfer methods in citrus. Bespalhok Filho et al. [69] carried out to optimize the conditions for transient gene expression through particle bombardment on Carrizo citrange (C. sinensis × Poncirus trifoliata) thin epicotyl sections. The best conditions for transient GUS expression were M-25 tungsten particles, 1550 psi helium pressure, 9 cm distance between specimen, and DNA/particle holder and culture of explants in a high osmolarity medium (0.2 M mannitol + 0.2 M sorbitol) 4 h prior and 20 h after bombardment. Under these conditions, an average of 102 blue spots per bombardment (20 explants/plate) were achieved. It is stated that protocol is currently being used for transformation of Carrizo citrange and sweet orange (C. sinensis).

Electroporation is an effective direct gene transfer system used for citrus transformation. Hidaka and Omura [90] used electroporation methods for gene transformation in citrus. Protoplasts were prepared from embryogenic callus of “Ohta” ponkan (C. reticulata Blanco) and electroporation with exponential decay pulses was carried out in the solution containing the β-glucuronidase (GUS) chimeric gene coupled to the CaMV 35S promoter (pBI221). At 24 h after incubation, significant GUS activity was detected in the cells by fluorometric assay. Another alternative method for direct gene transformation had been developed in sweet
| Species | Transferred genes | Transformation method | References |
|---------|-------------------|-----------------------|------------|
| C. sinensis L. Osb. | GUS and nptII | A. tumefaciens | [26] |
| C. sinensis L. Osb. | gfp | A. tumefaciens | [27] |
| C. sinensis L. Osb. | GUS | A. tumefaciens | [28] |
| C. sinensis L. Osb. and Carrizo citrange | uidA, nptII | A. tumefaciens | [29] |
| C. paradisi Macf. | RdRp, Gfp, and Gus | A. tumefaciens | [30] |
| C. sinensis L. Osb. | CTV-CP | A. tumefaciens | [31] |
| C. aurantifolia | p25, p20, and p23 | RNA interference | [32] |
| C. aurantifolia Swingle | AtSUC2, RSt1, RTBV, GUS, rolC | A. tumefaciens | [33] |
| C. paradisi | attE | A. tumefaciens | [34] |
| C. unshiu Marc | miraculin | A. tumefaciens | [35] |
| C. sinensis L. Osbeck and C. paradisi Macf. | GFP | A. tumefaciens | [36] |
| C. sinensis L. | CTV-GFP | A. tumefaciens | [37] |
| C. sinensis Osb. | Shiva A and Cecropin B | A. tumefaciens | [38] |
| C. sinensis | CPsV cp (ihpCP), 54K (ihp54K), and 24K (ihp24K) | A. tumefaciens | [39] |
| C. sinensis L. Osb. | GFP and nptII | A. tumefaciens | [40] |
| C. sinensis L. Osb. | pthA-nls | A. tumefaciens | [41] |
| Poncirus trifoliata L. Raf. | AhBADH | A. tumefaciens | [42] |
| C. sinensis L. Osb. | Cy-GFP and Er-GFP | A. tumefaciens | [43] |
| C. aurantifolia Swingle | gus-egfp | A. tumefaciens | [44] |
| Tetraploid citrus rootstock selection | egfp-nptII | A. tumefaciens | [45] |
| “Orange #16” | manA and egfp | A. tumefaciens | [46] |
| Carrizo citrange | nptII, hptII, and GFP | A. tumefaciens | [20] |
| C. sinensis, C. reticulata C. amblycarpa and C. depressa | Gfp | A. tumefaciens | [46] |
| Carrizo citrange and C. sinensis L. Osb. | Gfp | A. tumefaciens | [46] |
| Carrizo citrange, C. paradisi Macf., C. aurantifolia Swingle | EGFP | A. tumefaciens | [23] |
| C. sinensis L. Osb. | GUS and nptII | Sonication-assisted A. tumefaciens (SAAT) | [47] |
| C. sinensis cv. Hamlin | hrpN | A. tumefaciens | [48] |
| Poncirus trifoliata [L.] Raf. | uidA and nptII | A. tumefaciens | [49] |
| Poncirus trifoliata [L.] Raf | GFP and MAC12.2 | A. tumefaciens | [50] |
| Carrizo citrange and C. sinensis L. Osb. | uidA and iaaM/H marker genes | A. tumefaciens | [51] |
| C. sinensis L. Osb. | cp and nos genes | A. tumefaciens | [52] |
| C. sinensis L. Osb. | Nospro-nptII-Noster | A. tumefaciens | [53] |
| Carrizo citrange and C. sinensis L. Osb. | ipt gene | A. tumefaciens | [54] |
| C. paradisi Macf. | CTV-derived candidate resistance | RNA-mediated resistance | [55] |
| Poncirus trifoliata L. Raf. | gfp | A. tumefaciens | [56] |
| Carrizo citrange | nptII | A. tumefaciens | [57] |
| C. aurantium, C. macrophylla, C. limon and Troyer citrange | CTV-p61 and p23U | A. tumefaciens | [58] |
| C. sinensis L. Osb. | attA | A. tumefaciens | [59] |
| C. limonia Osb. | bO | A. tumefaciens | [60] |
| C. paradisi | RdRp | A. tumefaciens | [61] |
Table 1: Continued.

| Species                      | Transferred genes             | Transformation method | References |
|------------------------------|-------------------------------|-----------------------|------------|
| *C. jambhiri* Lush           | GUS and nptII                | *A. tumefaciens*      | [62]       |
| *C. sinensis* L. Osbeck      | gfp and pme                  | PEG                   | [63]       |
| Swingle citrusmelo           | *uidA*, *nptII*, and GUS      | *A. tumefaciens*      | [64]       |
| Carrizo citrange             | GUS and *nptII*              | *A. tumefaciens*      | [65]       |
| Carrizo citrange and *C. aurantifolia* | GUS, GFP, and *nptII* | *A. tumefaciens*      | [66]       |
| Carrizo citrange             | Citrus blight-associated     | *A. tumefaciens*      | [67]       |
| *C. sinensis* and *C. limonia* | GUS                          | *A. tumefaciens*      | [68]       |
| Carrizo citrange             | *uidA* and *nptII*           | Particle bombardment  | [69]       |
| *C. sinensis*                | *pTA29-barnase*              | *A. tumefaciens*      | [70]       |
| *Citrus sinensis*            | PMI                           | *A. tumefaciens*      | [12]       |
| *Citrus sinensis*            | GUS and *nptII*              | *A. tumefaciens*      | [71]       |
| *Citrus aurantium* L.        | GUS and *nptII*              | *A. rhizogenes*       | [72]       |
| *Citrus paradisi* Macf.      | *cp* and GUS                  | *A. tumefaciens*      | [73]       |
| *C. sinensis* L. Osbeck      | GUS                           | Electroporation       | [74]       |
| Carrizo citrange and *C. sinensis* L. Osbeck | GUS | *A. tumefaciens*      | [75]       |
| *Citrus sinensis* L. Osbeck  | *GUS*                         | *A. tumefaciens*      | [18]       |
| *C. reticulata* Blanco       | *pTA29-barnase*              | *A. tumefaciens*      | [76]       |
| *C. paradisi* Macf.          | Carotenoid biosynthetic genes | *A. tumefaciens*      | [16]       |
| Carrizo citrange             | *LFY* and *API*              | *A. tumefaciens*      | [77]       |
| *C. aurantium* L.            | *cp*                          | *A. tumefaciens*      | [78]       |
| *C. paradisi* Macf.          | *CP* and *T36*               | *A. tumefaciens*      | [79]       |
| Troyer citrange              | *Bar* and *uidA*             | *A. tumefaciens*      | [80]       |
| *C. aurantifolia* Swing.     | *cp*                          | *A. tumefaciens*      | [81]       |
| *C. sinensis* (L.) Osbeck    | *Gfp*                         | PEG                   | [82]       |
| *C. aurantifolia* Swing.     | *GUS*                         | *A. tumefaciens*      | [83]       |
| *C. paradisi* Macf.          | *GUS*, *uncp*, *gna*          | *A. tumefaciens*      | [84]       |
| Carrizo citrange             | *uidA* and *nptII*           | *A. tumefaciens*      | [85]       |
| *C. sinensis* L. Osbeck      | *GUS*                         | *A. tumefaciens*      | [86]       |
| *C. aurantifolia* (Christm.) Swing. | *GUS* and *nptII*   | *A. rhizogenes*       | [2]        |
| *C. aurantium* L.            | *cp*                          | *A. tumefaciens*      | [10]       |
| Tangelo                      | *GUS* and *nptII*            | Particle bombardment  | [87]       |
| Carrizo citrange             | *GUS* and *nptII*            | *A. tumefaciens*      | [88]       |
| *C. sinensis* L. Osbeck      | *GUS* and *nptII*            | *A. tumefaciens*      | [89]       |
| *C. reticulata* Blanco       | *GUS*                         | Electroporation       | [90]       |
| *Citrus spp.*                | *GUS* and *nptII*            | *A. tumefaciens*      | [91]       |
| *Citrus spp.*                | cat and *nptII*              | PEG                   | [92]       |

As well as the transformation studies conducted for gene expression, several studies conducted for gene silencing. RNA interference (RNAi) are a posttranscriptional gene-silencing phenomenon induced by double-stranded RNA. It has been widely used as a knockdown technology to analyze gene function in various organisms. Although RNAi was first discovered in worms, related phenomena such as posttranscriptional gene silencing and coat protein-mediated protection from viral infection had been observed in plants prior to this. In plants, RNAi is often achieved through transgenes that produce hairpin RNA. For genetic improvement of crop plants, RNAi has advantages over antisense-mediated
gene silencing and cosuppression, in terms of its efficiency and stability [102]. Soler et al. [32] stated Citrus tristeza virus (CTV), the causal agent of the most devastating viral disease of citrus, has evolved three silencing suppressors proteins acting at intra- (p23 and p20) and/or intercellular level (p20 and p25) to overcome host antiviral defence. Mexican lime was transformed with an intron-hairpin vector carrying full-length, untranslatable versions of the genes p25, p20, and p23 from CTV strain T36 to silence the expression of these critical genes in CTV-infected cells. Three transgenic lines presented complete resistance to viral infection, with all their propagations remaining symptomless and virus-free after graft inoculation with CTV-T36, either in the nontransgenic rootstock or in the transgenic scion. Accumulation of transgene-derived siRNAs was necessary but not sufficient for CTV resistance. Inoculation with a divergent CTV strain led to partially breaking the resistance, thus showing the role of sequence identity in the underlying mechanism. Results are a step forward to developing transgenic resistance to CTV and also show that targeting simultaneously by RNA interference (RNAi) the three viral silencing suppressors appear critical for this purpose, although the involvement of concurrent RNAi mechanisms cannot be excluded.

3. Conclusion

Genetic transformation is an attractive alternative technique for citrus genetic improvement. However, transformation efficiencies are generally low, and protocols are dependent on species, or even cultivar dependent. One of the limitations within this technology is low plant regeneration frequencies especially for many of the economically important citrus species [65]. In addition, difficulty in rooting transgenic shoots for some citrus cultivars has been reported [10, 89, 91]. Development of effective genetic transformants therefore requires specific studies on in vitro regeneration conditions for each genotype.

The development of direct genetic manipulation techniques has provided new opportunities for plant improvement. Plant transformation has made it possible to modify just one or two traits, while retaining the unique characteristics of the original cultivar. The characters that could potentially be manipulated by genetic transformation of Citrus include pest and disease resistance, growth habit, and fruit quality. In order to use this technology, it is essential to develop efficient genetic transformation systems for Citrus.

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