Agrobacterium-Mediated Transformation of Tree Fruit Crops: Methods, Progress, and Challenges

Guo-qing Song1*, Humberto Prieto2 and Vladimir Orbovic3*

1 Department of Horticulture, Plant Biotechnology Resource and Outreach Center, Michigan State University, East Lansing, MI, United States, 2 Biotechnology Laboratory, La Platina Station, Instituto de Investigaciones Agropecuarias, Santiago de Chile, Chile, 3 Citrus Research and Education Center, Institute of Food and Agricultural Sciences (IFAS), University of Florida, Lake Alfred, FL, United States

Genetic engineering based on Agrobacterium-mediated transformation has been a desirable tool to manipulate single or multiple genes of existing genotypes of woody fruit crops, for which conventional breeding is a difficult and lengthy process due to heterozygosity, sexual incompatibility, juvenility, or a lack of natural sources. To date, successful transformation has been reported for many fruit crops. We review the major progress in genetic transformation of these fruit crops made in the past 5 years, emphasizing reproducible transformation protocols as well as the strategies that have been tested in fruit crops. While direct transformation of scion cultivars was mostly used for fruit quality improvement, biotic and abiotic tolerance, and functional gene analysis, transgrafting on genetically modified (GM) rootstocks showed a potential to produce non-GM fruit products. More recently, genome editing technology has demonstrated a potential for gene(s) manipulation of several fruit crops. However, substantial efforts are still needed to produce plants from gene-edited cells, for which tremendous challenge remains in the context of either cell’s recalcitrance to regeneration or inefficient gene-editing due to their ploidy. We propose that effective transient transformation and efficient regeneration are the key for future utilization of genome editing technologies for improvement of fruit crops.

Keywords: Agrobacterium, genome editing, genetic engineering, genetic transformation, woody fruit and nut crops

INTRODUCTION

Fruits and nuts (F&N) provide essential nutrients for human growth and health. There are 494 culinary fruits (excluding fruit vegetables such as watermelons and tomatoes) and 14 nuts listed as of submission in Wikipedia. The increasing consumer awareness regarding health benefits and growing population worldwide are boosting a market for more and higher-quality fruits and nuts. For example, the total global production of fresh fruit increased from 13.6 million metric tons in 1996 to 33.3 million metric tons in 2016 (FAO). On the other hand, many adverse impacts, including emerging diseases (e.g., citrus greening and papaya ringspot virus), abiotic stress (e.g., salt, drought, and extreme temperatures) due to global warming, and natural resource depletion (e.g., land and water) due to the growing population, have threatened fruit yield and quality of F&N crops (Gottwald, 2010; Castillo et al., 2011; Fiore et al., 2018). Thus, the major task for F&N breeders is to develop new cultivars with improved resistance to diseases and abiotic stress,
and higher productivity. Conventional breeding for many F&N crops often takes a few to over 10 years due to their long juvenile periods and asexual propagation nature (Janick, 2005). Utilization of biotechnology in breeding is an efficient alternative allowing for the manipulation of gene(s) of interest (GOI) through genetic engineering in shorter period of time, relative to conventional breeding. The efficiency of biotechnology application in breeding has become evident with the successes achieved via Agrobacterium-mediated transformation (Figure 1 and Table 1), and carries a great potential with increased availability of sequenced genomes of F&N crops that can be used by technical advances such as gene editing for trait improvement (Table 2).

New biotechnological tools revolutionized plant breeding and offered new and effective ways for plant breeders to manipulate traits at the levels of individual gene(s) or gene blocks (Gelvin, 2012; Hiei et al., 2014; Nester, 2014). Except for the widely commercialized virus-resistant papaya produced in 1992 through biolistic-mediated transformation (Fitch et al., 1992), virus-resistant plum (Ravelonandro et al., 1997; Scorza et al., 2001, 2007) and non-browning apples (Waltz, 2015) have been both produced by Agrobacterium-mediated transformation. The transgenic plum is on the horizon to be commercially released and the non-browning apples are in the stores. Agrobacterium-mediated transformation protocols for stable and transient expression remain a major platform for gene editing technologies in F&N species (Table 1). In this review, we summarize the availability of reliable transformation protocols and discuss recent progress, current constraints, and future perspectives of application of Agrobacterium-mediated transformation for the improvement of woody F&N crops.

HISTORY OF GENETIC TRANSFORMATION OF F&N CROPS (FIGURE 1)

Phase I (1985–2000)
Development of transformation protocols. After success in Agrobacterium tumefaciens-mediated stable transformation of tobacco plants (Horsch et al., 1985), substantial effort was made to develop A. tumefaciens-mediated transformation protocols for F&N crops (Kole and Hall, 2008), such as apple (Yao et al., 1995), pear (Mourgues et al., 1996), plum (Mante et al., 1991), cherry rootstock (Gutierrez-Pesce et al., 1998), grapes (Mullins et al., 1990), walnuts (Migranahan et al., 1988), kiwifruits (Uematsu et al., 1991), citrus (Cervera et al., 1998), and European chestnuts (Seabra and Pais, 1998).

Phase II (2001–2015)
RNA interference (RNAi) technologies. The efforts to develop/improve transformation protocols for more F&N crops or cultivars continued and as a result blueberry (Song and Sink, 2004) and sour cherry (Song and Sink, 2006) were transformed. RNAi was used to suppress either virus RNAs or plant endogenous RNAs in plum (Scorza et al., 2004, 2013), and apple (Saurabh et al., 2014; Waltz, 2015). On the other hand, driven by advances in sequencing, cloning, and RNAi technologies, functional gene analysis became the major focus for the F&N crops, and workable transformation protocols had been developed, including transient expression systems (Scorza et al., 2013). The recombination/excision systems [e.g., Cre/LoxP and Flp-FRT (flippase recognition target)] have been demonstrated to be effective in producing selectable marker gene (SMG)-free apple (Kost et al., 2015; Krens et al., 2015), apricot (Petri et al., 2012), and citrus (Zou et al., 2013). The most significant progress at this stage include: (1) Deregulation of transgenic plum with plum pox virus (PPV) resistance (Scorza et al., 2007, 2013); and (2) Commercialization of non-browning apples (Waltz, 2015).

Phase III (2015–Present)
Precision breeding. Gene editing technologies have become powerful tools to precisely manipulate nucleic acids in a plant cell. The very first attempts of these technologies in apple (Nishitani et al., 2016), grape (Ren et al., 2016; Nakajima et al., 2017; Wang X.H. et al., 2018), sweet orange and grapefruit (Jia and Wang, 2014; Zhang F. et al., 2017), and kiwifruit (Wang Z. et al., 2018) have relied on the use of Agrobacterium to produce stable transgenic plants expressing either editing reagents or small RNAs inducers. Ideally, transient expression of editing reagents leading to stable editing of a GOI or a regulatory DNA sequence, similar to those demonstrated in annual crops (Svitashev et al., 2016; Liang et al., 2018), will be the next step for F&N plants.

TRANSFORMATION PROTOCOLS FOR WOODY FRUIT AND NUT CROPS

The current transformation protocols rely on procedures mainly developed between 1990 and 2000. Within the group of F&N species, the majority (over 95%) are still recalcitrant for transformation, and most of the transgenic F&N crops were produced using A. tumefaciens-mediated transformation (Singh and Sansavini, 1998; Kole and Hall, 2008). The scarce availability of regenerable explant sources and the time required to produce transgenic individuals are the major constraints (Figure 2). For example, only 16 papers on developing or optimizing Agrobacterium-mediated transformation of F&N crops have been published since 2014, but regardless of these excellent efforts, little break-through in terms of transformed species and transformation frequency has been made (Table 1).

KEY LIMITATIONS FOR TRANSFORMATION OF F&N CROPS

Efficient protocols for transformation rely on effective gene delivery, easy but accurate selection, and prolific regeneration from treated explants (Figure 2). Agrobacterium-mediated gene transfer is a powerful tool for delivery of transgenes although optimal conditions for efficient gene delivery vary for different species, genotypes and strains of...
Agrobacterium (Wang, 2015). Transfer DNA (T-DNA) has been shown to be a consistent carrier for a considerable variety of cargoes ranging from conventional expression cassettes used for GOIs, to the current RNA hairpin inducers (Song et al., 2013) or shuttle vectors for secondary DNA-replicons used in gene editing (Baltes et al., 2014). A. tumefaciens is preferable to biolistic guns for stable transformation of F&N crops due mainly to its low cost in operation and the high potential in producing transformations with a low-copy number of the inserted sequence (such as GOI) (Gelvin, 2012). A. tumefaciens with ACC deaminase activity has been developed to improve transformation frequency of annual plants through reducing ethylene levels in plants (Nonaka and Ezura, 2014), although it has not been tested in F&N crops. Up to now, gene delivery is not a key limitation for transformation of F&N crops, A. tumefaciens-mediated transformation remains the major approach (Tables 1–3).

Within the protocols for transformation of F&N crops, the effective selection is achieved mainly by using a SMG

### TABLE 1 | Recent successes (2014-present) in Agrobacterium tumefaciens-mediated transformation of F&N crops for transformation technology development.

| Species              | Agrobacterium strain | Source explants                                               | Transformation frequency | Reference                        |
|----------------------|----------------------|---------------------------------------------------------------|--------------------------|----------------------------------|
| Citrus maximma       | EHA105               | In planta transformation of 3–4-week old seedlings            | 3.2–20.4%                | Zhang Y.Y. et al., 2017          |
| Citrus spp.          | EHA105               | Microshoots cultured in temporary immersion bioreactor (TIB)  | 0.25%                    | Zhang Y.Y. et al., 2017          |
|                      | EHA105               | Internodal stem segments from 30-day old in vitro seedlings   | 3–15-fold increase       | Hu et al., 2016                  |
|                      | EHA101 or EHA105     | Mature explants (stems) from bud-grafted mother plant         |                          | Orbovic et al., 2015            |
| Diospyros kaki       | GV3101               | Leaves (3–7-week old)                                        | NA                      | Orbovic et al., 2015            |
| Malus × domestica    | EHA105               | Cotyledons                                                    | 11.7%                    | Mo et al., 2015                  |
| Malus spp.           | GV3101               | Young leaves from plantlets                                   | 5.7–32.4%, results       | Dai et al., 2014                 |
| Persia americana     | AGL1                 | Somatic embryos (globular stage)                              | 1.2%                     | Zhang et al., 2016               |
| Prunus avium         | EHA105               | Leaves from in vitro plants                                   | 1.2%                     | Palomo-Rios et al., 2017         |
|                      | EHA105               | Young leaves                                                  | 21.7%                    | Sgambarra et al., 2015           |
|                      | EHA105               | Leaf explants                                                 | 21.7%                    | Wang and Pijut, 2014             |
|                      | CBE21                | Leaf explants                                                 | 1.4%                     | Sidorova et al., 2017            |
| Pyrus ussuriensis    | EHA105               | Buds-leaves from one-year old branches                         | 11.7%                    | Yang et al., 2017                |
| Rubus fruticosus     | LBA4404              | Cut ends of petioles                                           | 5.7–32.4%, results       | Sidorova et al., 2017            |
| Rubus fruticosus     | GV3101               | Somatic embryos from whole flower cultures                     | NA                      | Dai et al., 2015                 |
TABLE 2 | Application of CRISPR-Cas9 gene editing technologies for F&N crops.

| Traits | Crop | Gene description | Principle results | Reference |
|--------|------|------------------|-------------------|-----------|
| Stable gene transformation-mediated genome editing | Apple | Analysis of four separated gRNAs targeting apple phytoene desaturase gene (PDS) | 31.8% of regenerated transgenic plants for one gRNA induced targeted mutation by Cas9 | Nishitani et al., 2016 |
| | Grape | Targeting grape L-idonate dehydrogenase gene (IidDH) | Three of six transgenic plants regenerates from 21 stable transgenic cell lines showed targeted mutation | Ren et al., 2016 |
| | Grape | Targeting grape PDS | Stable transgenic (T₀) plants showed targeted mutation in the VvPDS gene although chimeric phenotype was observed | Nakajima et al., 2017 |
| | Grape | Targeting grape transcription factor WRKY52 | 22 targeted mutants were obtained from 72 stable transgenic plants | Wang X.H. et al., 2018 |
| | Kiwifruit | Targeting kiwifruit PDS gene (AcPDS) | A demonstration of Cas9-mediated genome editing using paired gRNAs approach for gene fragment deletion in the AcPDS gene | Wang Z. et al., 2018 |
| | Citrus | Targeting the promoter region of the susceptibility gene CsLOB1 and the gene itself for citrus canker resistance | 11.5–64.7% mutation rates for five Cas9-constructs (orange), 23.8–89-4% mutation rate for a single Cas-9 construct (grapefruit) | Jia et al., 2017b; Peng et al., 2017 |
| Non-transgene -involved genome editing | Apple and grape | Targeting MLO-7, a susceptible gene (S-gene) in order to increase resistance to powdery mildew (PM) in grape cultivar and DIPM-1, DIPM-2 and DIPM-4 in the apple to increase resistance to fire blight disease | PEG-mediated delivery of preassembled Cas9-gRNA reagents resulted targeted mutagenesis in protoplast cells, but no plants with targeted gene editing was obtained | Mainoy et al., 2016 |

conferring resistance to the plant against either an antibiotic or an herbicide (Miki and McHugh, 2004). Three major SMG for plant transformation include the neomycin phosphotransferase II (nptII) gene that confers kanamycin resistance, the hygromycin-B-phosphotransferase gene (hph) conferring hygromycin B resistance, and the bar gene conferring resistance to phosphinotricin, a glutamine synthetase inhibitor produced by plants from the herbicide bialophos (Miki and McHugh, 2004). The nptII is, to date, the most widely used SMG for the transformation of F&N crops (Kole and Hall, 2008). However, effective selection of transformed cells without assistance of SMGs remains a key limitation for plant transformation.

In general, plants with high regeneration capability from non-meristem containing explants are amenable for genetic transformation. Unlike annual crops (e.g., wheat, maize, rice, and soybean), the cultivated F&N crops, for example, blueberries (Song and Hancock, 2012) and cherries (Song, 2014; Zong et al., 2018), are often clonally propagated; to maintain genetic stability, regeneration from explants of clonally propagated tissues is preferable. Lack of available explants sources (e.g., seedlings) makes plant regeneration studies for F&N crops a lengthy process (Figure 3), because in many instances an efficient micropropagation system has to be established for obtaining regenerable explants (e.g., leaves and petioles) prior to regeneration studies (Kole and Hall, 2008). Optimal conditions for both micropropagation and regeneration are determined by many factors (e.g., blueberries) (Liu et al., 2016; Figure 3). In addition, high selection pressure on plant regeneration. Finally, for some extremely recalcitrant species in the group (e.g., Prunus persica) species, regeneration can be achieved mostly on the basis of the use of seed explants (Song, 2014; Petri et al., 2018; Zong et al., 2018). In terms of using transgenes to improve regeneration efficiency, one report demonstrated that constitutive expression of the class I KNOX gene of maize increased production of adventitious shoots from leaf explants of plum (Srinivasanan et al., 2011). More recently, overexpression of morphogenic regulators, i.e., maize Baby boom and maize Wuschel2, has been demonstrated to be effective in improving monocot transformation (Artlip et al., 2016); and the potential for use of these genes for F&N crops has been recently demonstrated in citrus (Miki and McHugh, 2004).

RECENT DEVELOPMENTS IN GENETIC ENGINEERING OF F&N CROPS

Genetic transformation of F&N crops might be necessary when a breeding goal is not easily achievable through traditional breeding approaches (Singh and Sansavini, 1998).
Of many approaches developed, gene addition and gene subtraction are two basic strategies for GM trait development (Pena and Seguin, 2001).

Transgrafting

Grafting by artificially conjoining different vascular systems (i.e., rootstock and scion) is a widely used agricultural practice with over 3000-year history for horticultural crops, especially for F&N crops (Jensen et al., 2012; Nawaz et al., 2016; Chitarra et al., 2017). Traditionally, grafting is used to produce plants for asexual propagation, altered plant vigor and architecture, increased tolerance to biotic/abiotic stresses, precocity, and higher yield. The term transgrafting was introduced when GE rootstocks were used in grafting in 1990 (Haroldsen et al., 2012; Figure 4). To date, long-distance transportation of transgene-derived small interfering RNAs (siRNA) from rootstock to non-transgenic sweet cherry scions have been verified by small RNA sequencing (Zhao and Song, 2014). For large mRNA molecules, qRT-PCR analysis detected short-distance transported mRNAs of the (Zhao and Song, 2014). For large mRNA molecules, qRT-PCR analysis detected short-distance transported mRNAs of the (Zhao and Song, 2014). For large mRNA molecules, qRT-PCR analysis detected short-distance transported mRNAs of the (Zhao and Song, 2014).

The interaction of transgenic rootstocks and non-transgenic scions in transgrafted plants through either the mobile transgenic products or immobile transgenic products has been demonstrated to be effective in facilitating changes in non-transgenic scions directly or indirectly (Smolka et al., 2010; Song et al., 2015; Artlip et al., 2016); this is also well-supported by recent studies of grafting in non-transgenic rootstocks and scions (Jensen et al., 2012; Chitarra et al., 2017). Using transgenic apple rootstock expressing the root-inducing rolB gene of Agrobacterium rhizogenes T-DNA reduced the vegetative growth of nontransgenic scions (Smolka et al., 2010). Thus, transgenic rootstocks have the potential to expand the use of transgenesis for production of non-transgenic F&N crops (Haroldsen et al., 2012).

**FastTrack Breeding**

The long juvenile phase exhibited by several F&N crops can severely limit the traditional breeding efforts which are dependent on the ability to make genetic crosses (Janick, 2005). “FastTrack breeding” as demonstrated in many recent publications is done through the manipulation of flowering pathway genes to hasten flowering (Figure 5 and Table 3; Petri et al., 2018). Stable transformation of elite cultivars by either overexpression of flower promoting genes (e.g., FLOWERING LOCUS T, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1, LEAFY, and APETALA1) or repression of flower repressing genes (e.g., TERMINAL FLOWER 1) is an effective approach to enable a fast introduction of genes of interest from wild germplasm through the early flowering seedlings from both crosses and backcrosses (Figure 5A). This approach relies on efficient transformation systems for elite cultivars and low cross-incompatibility between the transformed elite cultivars and donor plants. Transgrafting on transgenic rootstocks can affect flowering in non-transgenic scions (Artlip et al., 2016), suggesting that there is a potential to use a rootstock overexpressing FT to promote early flowering of the scions from juvenile seedlings, although this potential has not yet successful been demonstrated for trees (Zhang et al., 2010; Srinivasan et al., 2012; Figure 5B). Alternatively, transient transformation using virus vectors either to enhance expression of flower promoting genes expression or to repress flower repressing genes has shown a potential in promoting flowering of juvenile plants (Yamagishi et al., 2014; Velazquez et al., 2016).

**Selectable Marker Gene-Free Plants and Intragenesis**

The negative perception of transgenic plants by consumers spurred research to develop either plants modified with plant derived sequences (intragenesis) or transgenic plants from which the SMG was removed (Figure 6). While these strategies have the potential to allay consumer fears, both approaches are currently regulated as transgenic plants worldwide (Holme et al., 2013). Transgenesis without a SMG or with a plant-derived SMG is preferable to SMG-containing transgenic products by the public (Miki and McHugh, 2004). To date, plant-derived reporters, for example, the genes regulating anthocyanin biosynthesis have been successfully used as alternative to

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### TABLE 3: Recent successes (2014-present) in A. tumefaciens-mediated transformation of F&N crops for agronomic trait improvement.

| Traits                  | Crop                    | Gene description                                                                 | Principle results*                                                                 | Reference                  |
|-------------------------|-------------------------|----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|----------------------------|
| Tree architecture       | Apple                   | Apple double-strand RNA-binding protein (MdDRB1) functions in mRNA processing and maturation | Overexpression of MdDRB1 promotes adventitious root production and results in columnar-like tree architecture | You et al., 2014          |
| Seed abortion           | Grape                   | The MADS-box gene VvAGL11 functions in female gametophyte development and fertilization, and seed formation | The mutation of the VvAGL11 leads to seed abortion                                 | Royo et al., 2018         |
| Non-browning fruit      | Apple                   | Apple polyphenol oxidase (PPO) catalyzes enzymatic browning                       | Silencing/knock-down the expression of PPO leads to non-browning apple            | Waltz, 2015                |
| Yield                   | Blueberry               | VcSOC1-K: Keratin-like domain of the MADS-box SUPPRESSOR of OVEREXPRESSION OF CONSTANS 1 (SOC1) gene of blueberry | Overexpression of the MADS-box gene K-domain increases the yield potential of blueberry | Song and Chen, 2018       |
| Flowering time          | Kiwifruit               | Kiwifruit SHORT VEGETATIVE PHASE3 (SVR3) functions in chilling-mediated flowering | Overexpression of the kiwifruit SVR3 gene affects reproductive development         | Wu et al., 2014           |
| Abiotic                 | Apple                   | Apple transcription factor bHLH104 (MdbHLH104) regulates transcription            | Overexpression of MdbHLH104 increase apples tolerance to iron deficiency           | Zhao et al., 2016         |
|                        | Apple                   | Peach C-repeat/DRE binding factor 1 (PpCBF1)                                     | Overexpression of PpCBF1 increases freezing tolerance in transgenic plants         | Antlip et al., 2016; Wisniewski et al., 2015 |
|                        | Apple                   | Apple dehydration-responsive element binding factor 6.2 (MsDREB6.2) in stress signaling pathway | Overexpression of MsDREB6.2 enhances drought tolerance in transgenic apple plants | Liao et al., 2017         |
|                        | Blueberry               | Blueberry DWARF AND DELAYED FLOWERING 1 (VcDDF1)                                | Overexpression of VcDDF1 increases freezing tolerance in transgenic blueberry plants | Song and Gao, 2017; Walworth and Song, 2018 |
|                        | Plum                    | Plum stress-associated protein (PpSAP1)                                         | Overexpression of PpSAP1 increases water retention under drought stress in transgenic plum plants | Lloret et al., 2017       |
|                        | Citrus                  | P35 anti-apoptotic protein                                                       | Overexpression of P35 improved cold tolerance                                      | Orboviˇc et al., 2017     |
|                        | American chestnut       | Wheat oxalate oxidase (oxo)                                                      | Expression of the OxO enhances blight resistance                                   | Newhouse et al., 2014; Newhouse et al., 2018 |
|                        | Apple                   | Apple Mildew Locus O19 (MoMLO19)                                                | The knock-down of the expression of MoMLO19 increases apple’s resistance to powdery mildew | Pessina et al., 2016a      |
|                        | Apple                   | Fire blight resistance gene of Malus × robusta S (FB_MR5)                       | Expression of the FB_MR5 increases fire blight disease resistance                  | Kost et al., 2015         |

(Continued)
TABLE 3 | Continued

| Traits     | Crop     | Gene description                                                                 | Principle results*                                                                 | Reference                                      |
|------------|----------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|------------------------------------------------|
| Apple      | Apple    | Chalcone 3-hydroxylase (CH3H) from Cosmos sulphureus                             | Overexpression of the CH3H reduces susceptibility to fire blight and scab in transgenic apples | Hutabarat et al., 2016                         |
| Apple      | Apple    | Apple cytosolic malate dehydrogenase gene (MdcyMDH)                              | Overexpression of the MdcyMDH enhances tolerance to salt and cold stresses          | Wang Q.J. et al., 2016                         |
| Banana     | Banana   | Disease resistance protein (RGA2)                                                | Expression of the RGA2 increases resistance to fungus TR4                            | Dale et al., 2017                              |
| Cherry     | Cherry   | PNRSV-CP: a partial coat protein gene of Prunus necrotic spot virus               | Rootstock-to-scion transfer of transgene-derived small interfering RNAs enables virus resistance in non-transgenic sweet cherry scions | Zhao and Song, 2014                            |
| Citrus     | Citrus   | Mthionine: a synthetic gene encoding the modified thionin                         | Overexpression of the Mthionine enhances disease resistance to citrus canker and Huanglongbing | Hao et al., 2016                               |
| Citrus     | Citrus   | Citrus sinensis lateral organ boundary 1 (CsLOB1)                                 | The knock-out of the CsLOB1 enhances disease resistance to citrus canker             | Jia et al., 2017b                              |
| Citrus     | Citrus   | AtNPR1 regulator of systemic acquired resistance                                  | Tolerance to HLB disease                                                            | Robertson et al., 2018                         |
| Grape      | Grape    | Grapevine E-(β)-caryophyllene synthase (VvGwECar2)                               | VvGwECar2 overexpression results in higher E-(β)-caryophyllene emissions             | Salvagnin et al., 2016; Salvagnin et al., 2018 |
| Grape      | Grape    | Grape Mildew Locus O (WvMLOs)                                                    | The knock-down using RNAi of VvMLO7 in combination with WvML06 and WvML011 enhances grape’s resistance to powdery mildew | Pessina et al., 2016b                          |
| Grape      | Grape    | A GFLV (grapevine fanleaf virus)-specific nanobody gene Nb23                      | Overexpression of the Nb23 increases virus resistance to GFLV in grapevine           | Hemmer et al., 2018                           |
| Kiwifruit  | Kiwifruit| Synthetic chimeric gene (SbtCry1Ac) encodes the insecticidal protein bCrylAc.     | Expression of the SbtCry1Ac results in insect-resistant plants                      | Zhang et al., 2015                            |

*Except non-browning apple that has been commercialized, all the others are proof-of-concept studies.
non-plant-derived reporters [i.e., green fluorescent protein (GFP), β-glucuronidase (GUS)] for transformation (Krens et al., 2015; Kandel et al., 2016; Dutt et al., 2018). Co-transformation enables production of SMG-free GM crops through the crosses for the segregation of SMG-removal transformants in next generation, but this is not desirable for asexually propagated F&N crops. The recombination/excision systems [e.g., Cre/LoxP and Flp-FRT (flippase recognition target)] are effective in generating SMG-free apple (Kost et al., 2015; Krens et al., 2015), apricot (Petri et al., 2012), grape (Dalla Costa et al., 2016), and citrus (Zou et al., 2013).

Intragenesis is desirable for food crops because it relies on the gene pool for conventional breeding. Technically, intragenesis is more challenging than SMG-assisted transgenesis (Figure 6). Previous studies have demonstrated the potential of production of intragenic apples and citrus (An et al., 2013; Kost et al., 2015; Krens et al., 2015).

**Genome Editing**

Genome editing technologies provide powerful tools for precise manipulation of targeted genome sequence(s) for crop improvement (Arora and Narula, 2017; Limera et al., 2017). Remarkably, these technologies make it possible to edit or excise a specific gene in a genome without introduction of any extra DNA.

Programmable DNA binding proteins such zinc finger (ZF) and transcription activator-like effector (TALE) emerged as the first generation engineered nucleases to create targeted mutagenesis, which is an alternative to classical protocols for random mutagenesis. These tools have a recognition capability of specific target DNA sequences based on customized arrangements of one (TALE) or three (ZF) nucleotides, and in such way bringing to these places a nuclease (for instance C-terminal domain of FokI) which disrupts DNA adjacent to the recognition zones. Both ZF- and TALE-nucleases, require two effectors (left and right) in order to define the nuclease cutting site (Gaj et al., 2013). Prior to the wide application of these technologies for F&N crops, a more powerful gene editing tool -The clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 mediated gene editing technology was developed (Jinek et al., 2012). The CRISPR/Cas9 has been a revolutionary molecular tool since its discovery as an adaptive line of defense against viral infection in Archaea (Mojica et al., 2000). This system operates through guide RNAs (gRNAs) that contain specific sequences designed according to their targets in the genome. The Cas nuclease (commonly Cas9), when directed by the gRNA generates a double strand break adjacent to the gRNA’s annealing location allowing for a target-specific mutagenesis. More recently, CRISPR/Cpf1, another CRISPR/Cas system that overcomes some of the CRISPR/Cas9 system limitations, has been found more efficient at DNA editing (Ledford, 2015; Zetsche et al., 2015; Fonfara et al., 2016; Jia et al., 2017a).

Delivery of CRISPR/Cas9 components into the plant cell has been achieved by either transgenic or non-transgenic approaches (Table 2). To date, as proof-of-concept, CRISPR/Cas9 guided DNA editing through stable transformation has been demonstrated in four major fruit crops [i.e., apple...
FIGURE 5 | Different approaches for FastTrack breeding – an example for blueberries. (A) Stable gene transformation into elite cultivars for overexpression of flower-promoting genes (e.g., *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, *LEAFY*, and *APETALA1*) or repression of flower-repressing genes (e.g., *TERMINAL FLOWER 1*). The early flowering seedlings enable blueberry to skip 2–3 years of juvenile period for each cross. (B) Transgrafting on *FT*-overexpressing rootstock promotes early flowering of the scion.

FIGURE 6 | Transgenesis without a SMG and intragenesis. A strategy to obtain SMG free and backbone free transgenic and intragenic F&N crops. The T-DNA region is drawn according to the construct used for intragenic apple (De Paeppe et al., 2009; Kost et al., 2015; Collier et al., 2018). This construct allows production of SMG-free T<sub>0</sub> transformants. RB: right T-DNA border, LB: left T-DNA border, P: promoter, T: terminator, CDS: coding sequence, GOI: gene of interest. Green boxes show plant-derived intragenic/cisgenic components. *Cisgenesis has not been demonstrated in *A. tumefaciens*-mediated transformation of plants because RB and LB are difficult to replace or remove.

(Nishitani et al., 2016), grape (Ren et al., 2016; Nakajima et al., 2017; Wang X.H. et al., 2018), sweet orange and grapefruit (Jia and Wang, 2014; Zhang F. et al., 2017), and kiwifruit (Wang Z. et al., 2018), suggesting that the CRISPR/Cas9 is suitable for precise gene knockout. The main constraint using stable transformation is that unlike annual crops, from which gene editing associated transgenes can be effectively segregated post editing through traditional crosses, breeding out the transgenes through crosses for F&N crops is undesirable and will eliminate the identity of the clonally propagated variety. Transient transformation without stable integration of the CRISPR/Cas9 components is possible and desirable for
gene editing (Chen et al., 2018); whereas technically it is very challenging to identify the targeted mutant cells caused by the CRISPR/Cas9 due to the lack of SMG for transformed plant cells (Table 2). Non-transgene-involved gene editing by using guide gRNA-Cas9/Cpf1 ribonucleoprotein (RNP) complex is ideal for protoplasts of F&N crops and has been demonstrated in apple and grape cells (Malnoy et al., 2016). An alternative approach has been the use of DNA-replicon strategy (Baltes et al., 2014), based on the Bean yellow dwarf virus (BeYDV) genome structure in the absence of proteins required for its infection and mobility (i.e., disarmed virus). This allowed a high copy number in the cell without the insertion of the replicon into the plant genome (Gil-Humanes et al., 2017). Despite these improvements in the genetic engineering area, it is extremely challenging to induce plant regeneration from protoplast cells in F&N species (Table 2). Again, the main challenge for using gene editing technologies to improve the F&N crops remains the lack of efficient regeneration systems.

**Genes and Traits**

Regardless of the approach, genetic engineering of the F&N crops aims to modify selected traits through manipulation of targeted gene(s) or gene regulatory sequence(s). The progress made in genetic engineering for fruit crops prior to 2013 has been well-documented (Rai and Shekhawat, 2014).

More recently (2014-present), progress has been made for *A. tumefaciens*-mediated transformation of nine F&N crops using 24 genes for improving seven traits (Table 2). This progress has demonstrated that *A. tumefaciens*-mediated transformation is a powerful tool and remains a major approach for improvement of F&N crops. In this regard, the availability of genome drafts in several F&N species allow for the advance toward improved and safer application of techniques such as RNAi and CRISPR/Cas9 editing. Interference of target mRNAs using artificial microRNAs, or editing of on-target and effect over off-target loci can be assessed by *ex-ante* analyses using dedicated designers which predict these activities in an efficient rate (Doench et al., 2014; Castro et al., 2016). For instance, computing of protospacer adjacent motifs (PAMs) for Cas9 over a target genome allows for a first-dimension analysis of the putative cut sites for the nuclease upon gRNA leadership. In that way, Wang et al. (Peng et al., 2017) described the occurrence of more than 35 million PAMs in the grapevine genome and similar developments for other species are becoming available tools (Wang Y. et al., 2016; Pulido-Quetglas et al., 2017).

**CONCLUDING REMARKS**

Low success rate and recalcitrance of some species to transformation remain as major challenges for adoption of the new breeding techniques to fruit crops. As those challenges are overcome, several new technologies based on *Agrobacterium*-mediated transformation, such as transgrafting, fast-track breeding, intragenesis, and genome editing will be employed more frequently for solving problems facing tree fruit industry worldwide.

**DATA AVAILABILITY**

All datasets generated for this study are included in the manuscript and/or the supplementary files.

**AUTHOR CONTRIBUTIONS**

HP wrote the gene editing part. G-qS and VO drafted the rest of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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