Genetic Engineering Strategies for Seedlessness Breeding

Subjects: Horticulture

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Seedless fruit occurs naturally and can be produced using hormone application, crossbreeding, or ploidy breeding. However, the two types of breeding are time-consuming and sometimes ineffective due to interspecies hybridization barriers or the absence of appropriate parental genotypes to use in the breeding process. The genetic engineering approach provides a better prospect, which can be explored based on an understanding of the genetic causes underlying the seedlessness trait.

genome editing molecular breeding ovule abortion parthenocarpy seedlessness stenospermocarpy

1. Introduction

Seedlessness is one of the most valuable agricultural traits in fruit crops that consumers appreciate for fresh consumption and value-added processed products [1][2]. It enriches the eating quality of the fruits due to their expanded edible pulp and the absence of hard seeds with an awful taste. Further, seedlessness could prevent browning and bitterness caused by seeds [3]. Moreover, it improves many other fruit biometric characteristics regarding acid/sugar levels, dry matter, firmness, and overall shelf-life qualities of climacteric fruit due to reduced ethylene generated by seeds [4]. Seedlessness can also mitigate fruit yield losses caused by environmental stresses affecting pollination and fertilization [5]. Finally, it occurred independently of pollination and fertilization, which increases fruit production, particularly in dioecious species, due to the uselessness of the pollen source staminate trees. Studies on fruit seedlessness suggest that the trait is coordinated by intricate systems involving hormonal, genetic, and environmental factors [6][7]. Therefore, there are many causes underlying the seedless fruit set program [8]. The most classical reasons include male sterility, degradation of mother pollen cells, embryonic abortion, and chromosomal irregularities during meiosis leading to triploidy.

In typical seeded fruit, the ovary proliferates after fertilization through a coordinated program of molecular, biochemical, and structural changes that stimulate fruit size enlargement due to the interplay of cell division, differentiation, and expansion of sporophytic and gametophytic tissues [9]. Research on mechanisms underlying fruit seedlessness has highlighted the potential involvement of two distinct strategies, parthenocarpy and stenospermocarpy [10]. In parthenocarpy, true seedlessness occurs, and the ovary develops into fruit independent of pollination and fertilization [11]. However, two different procedures were identified for the parthenocarpic fruit set program. The obligatory-parthenocarpy, where a plant always produces seedless fruits (i.e., pineapple), and the facultative-parthenocarpy, where seedless fruits only develop if pollination is prevented (i.e., watermelon) [12]. Parthenocarpic fruit development is triggered by the deregulation of the hormone balance in ovary tissues, mainly auxin, gibberellins (GAs), and/or cytokinins (CKs). Applying these hormones to unpollinated ovaries at anthesis can stimulate pollination-independent ovary growth and produce parthenocarpic seedless fruit, strongly supporting their individual and overlapped roles during early fruit development [13]. An earlier study reported that the growth of tomato fruit is coordinated by a delicate balance between auxin and GA, whereby auxin is needed to mediate cell division and GA is required to organize cell expansion [7]. The parthenocarpy trait stability in fruit crops primarily occurs through elective pressure for seedlessness during domestication and breeding [8]. However, parthenocarpic genotypes were also identified in wild species and non-fruit crops [14].

In stenospermocarpy, pollination and fertilization typically occur; however, the seed growth is prematurely aborted due to the cessation of seed coat and endosperm development, resulting in expanded fruit size with seminal rudiments or seed traces [15]. The fact that different degrees of seedlessness were observed in progeny grapevines resulting from crossing seeded and stenospermocarpy seedless parents adds more complexity to the integrative regulatory network and signaling pathways underlying the stenospermocarpy seedless fruit set machinery [16]. Despite recent advances in grape biology, the molecular basis that triggers stenospermocarpy fruit development is largely unknown [10]. The efforts to unravel the molecular basis for stenospermocarpy in grapes were able to identify and functionally characterize several genes that can be potentially involved in the procedure [17][18][19]. Although the results did not show an ultimate gene network, they at least shed light on potential molecular mechanisms that synchronize stenospermocarpy machinery.

Fruit size and weight are positive commercial attributes, through which the number of developed seeds per fruit is positively correlated with the two characters [20]. Parthenocarpy fruit set results in considerably smaller fruit size than seeded fruit due to the absence of seed initiation, leading to reduced hormone levels necessary to sustain fruit growth [7]. However, stenospermocarpy does not compromise or, in the worst-case scenario, slightly reduce the fruit size because the ovary-growth event occurs after pollination and fertilization, making stenospermocarpy seedlessness a more attractive trait for breeding (**Figure 1**). CKs are essential to determining ovary size before fertilization. However, the slightly compromised size of the stenospermocarpy fruit is due to the availability of CKs post-fertilization, which negatively regulates cell expansion during fruit development [21].

Figure 1. Close-up cluster views of Thompson seeded mutant (DVIT 1334), Thompson seedless, and Centennial seedless grape genotypes exhibiting seeded, stenospermocarpy, and parthenocarpy fruit set programs, respectively.

Parthenocarpy seedlessness can be induced by applying hormones to unpollinated inflorescences at anthesis, via fostering self-incompatibility, or through generating triploid plants using conventional breeding practices [4][22]. Nevertheless, all the strategies are laborious, time-consuming, and sometimes impossible to use due to the absence of proper parental genetic resources. In the meantime, no treatment or application that can induce stenospermocarpy seedlessness has been identified yet. Accordingly, both seedlessness mechanisms are important, depending on growth conditions and commercial value. This has opened up the opportunity for genetic engineering approaches that have given encouraging results, both in the quality and quantity of seedless fruit production.

2. Genetic Engineering Strategies for Seedlessness Breeding

Parthenocarpy seedless fruits can be accomplished either by exogenous application of plant growth regulators, conventional breeding, interspecies hybridization, or polyploidy breeding. However, none of these strategies is feasible to induce stenospermocarpy seedless. For instance, muscadine seedless grape breeding is not viable due to the absence of the trait within the species. The only available seedless muscadine genotype, “Fry Seedless”, is parthenocarpic with limited commercial value and cannot be used as a crossing parent in the breeding program due to male sterility (**Figure 2**) [23]. Muscadine and bunch grape are classified under the *Euvitis* genera; however, the pronounced differences in their phenomic, metabolomic, and genomic characteristics represented by the dissimilarities in stress responses, horticultural and reproductive growth characteristics, and genome structure enabled us to classify them into two different genera, *Muscadinia* and *Vitis* [24]. Accordingly, introducing the stenospermocarpy seedless trait to muscadine grapes via generating *Vitis* x *Muscadinia* interspecific hybrids is challenging due to the differences in chromosome number and genetic incompatibility [25]. Alternatively, developing triploid seedless muscadine grapes might be an option that avoids the genetic barrier between species. However, the attempt to establish a triploid seedless muscadine grape did not produce satisfactory genotypes that can be promoted into new cultivars due to limited reproductive growth qualities [26]. Hence, genetic engineering could be a promising strategy for introducing a seedless trait.

Figure 2. Close-up cluster view of muscadine cultivars Majesty (female flower, seeded), Floriana (perfect flower, seeded), and Fry seedless (perfect flower, parthenocarpy seedless).

Advanced genome-editing tools, such as CRISPR-TSKO, precise base editing, or prime editing approaches, were efficiently applied in different crops for precise genome editing that prevented or minimized the pleiotropic effects [27][28]. However, this approach is considered GMO and may affect consumer acceptance. Interestingly, the Cas9-free lines can be selected by crossing out the transgenes from the segregating populations or through RNP-mediated protoplast transformation and regeneration [29][30]. Moreover, the CRISPR reagent (RNP) can be delivered to the germline cells using viral vectors like the Tobacco rattle virus (TRV). Thus, an inherited mutation could be achieved using the seeds from the germline-edited plant [31].

The model presented in **Figure 3** illustrates the suggested strategy for introducing a seedless trait using genome editing technology.

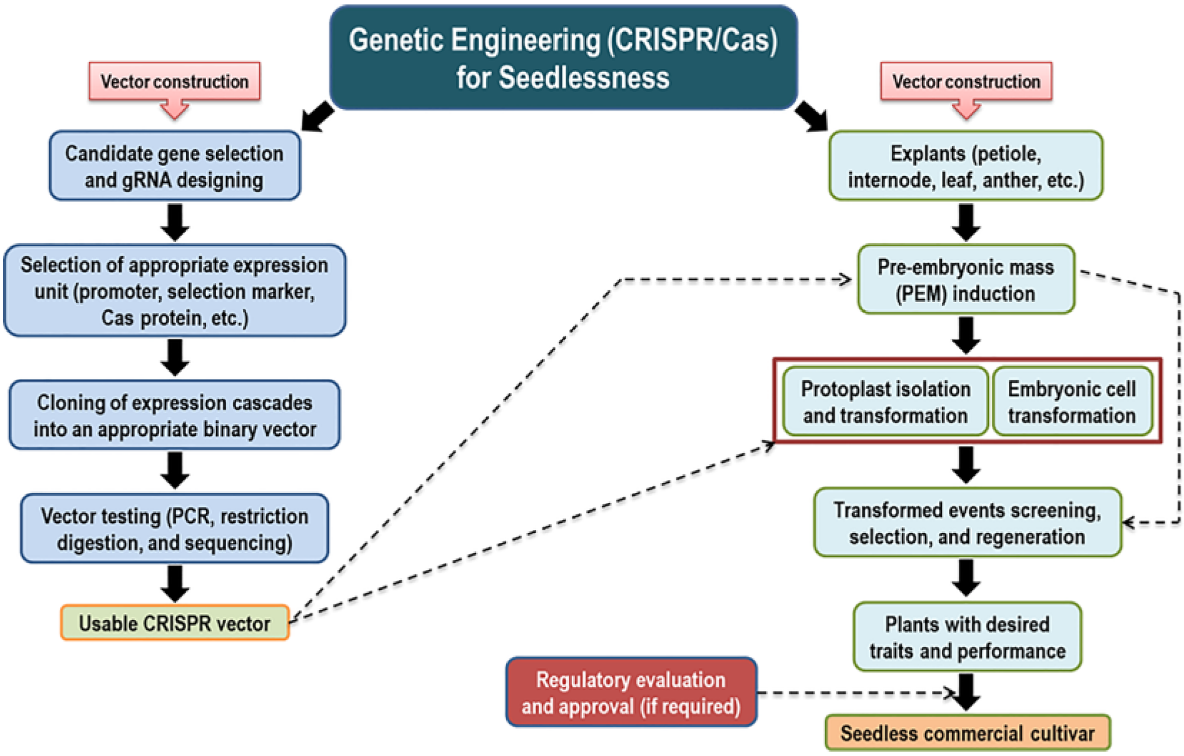

Figure 3. Visual scheme of gaining the seedlessness trait using genetic engineering (CRISPR/Cas) strategy.

(I) Establishing a plant regeneration system based on embryonic suspension cell culture. Genetic transformation using embryogenic cell suspension cultures is a good opportunity because of their higher organogenetic potential [32][33][34][35]. Regeneration of putatively transformed cells and subsequent grafting of transgenic micro-shoots on rootstocks may shorten the juvenile period for flowering and fruiting [36].

(II) Selecting appropriate candidate gene(s) and regulatory elements for targeted genome editing. The *AGL11* is considered the only identified upstream regulatory gene that controls ovule/seed development, and its *Arabidopsis* mutant phenotype (*stk*) displayed compromised seed characteristics. [37]. In the case of *V. vinifera*, stenospermocarpy seedlessness is associated with an SNP mutation in *VviAGL11* (R197L) [16]. The availability of whole genome sequence (i.e., muscadine whole genome sequence) [38] facilitates target gene selection. Researchers highlighted many other genes associated with seed development/abortion. These genes could be target candidates for genome editing. Organ-specific promoter-driven Cas protein expression has been reported on the CRISPR platform [39][40][41][42]. Using a seed-specific promoter could achieve the goal more effectively and efficiently because the desired expression will occur only in seeds. Seed development-specific promoters have been characterized using various genes and different plant species [43][44][45][46].

(III) Cloning and assembly of a binary vector. Guide RNA (gRNA) design for the particular gene and cloning the gRNAs into appropriate vector backbones are the primary tasks for genome editing vector construction. Different online tools for gRNA design (i.e., CRISPOR [47], CRISPR-P [48], CCTop [49], CHOPCHOP [50], and GuideMaker [51]) with customized features are openly accessible. High-efficiency cloning technology (MoClo) [52] and readymade cloning materials of different expression modules are readily available from addgene (https://www.addgene.org/)

and other sources. Adopting the MoClo cloning technology would accelerate vector construction efficiency. Recently, it has been shown that gRNA possessing the same restriction site as the type II restriction enzyme used for the GG reaction does not affect MoClo cloning and subsequent genome editing efficiency, which expanded the gRNA selection options [53].

(IV) Transformation of embryogenic cells or protoplasts and regeneration of putatively transformed cells into a complete plant. Using the appropriate transformation system increases the chances of getting transformed events. Among the different types of transformation processes, *Agrobacterium*-mediated or direct protoplast transformation could be adopted. Interestingly, RNP (ribonucleoprotein) mediated genome editing of protoplasts could avoid current GMO regulations, as the USDA does not consider the plant a GMO if the engineering involves a plant self-repair mechanism.

(V) Screening for potential transgenic events and securing approval from regulatory agencies. The procedure is associated with both molecular and phenotypic evaluations. Molecular screening means genomic and proteomic studies of the desired genome engineering plant(s) to confirm that desired change(s) in the genome, and phenotypic screening means the study of visual changes (either positive or negative) in the plants. If satisfactory performance is achieved, the new plant genotype needs approval from regulatory authorities before releasing for commercialization.

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