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Abstract
Increasing knowledge of plant genome sequences requires the development of more reliable and efficient genetic approaches for genotype-phenotype validation. Functional identification of plant genes is generally achieved by a combination of creating genetic modifications and observing the according phenotype, which begins with forward-genetic methods represented by random physical and chemical mutagenesis and move towards reverse-genetic tools as targeted genome editing. A major bottleneck is time need to produce modified homozygous genotypes that can actually be used for phenotypic validation. Herein, we comprehensively address and compare available experimental approaches for functional validation of plant genes, and propose haploid strategies to reduce the time needed and cost consumed for establishing gene function.

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Haploid strategies for functional validation of plant genes

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Abstract

Increasing knowledge of plant genome sequences requires the development of more reliable and efficient genetic approaches for genotype-phenotype validation. Functional identification of plant genes is generally achieved by a combination of creating genetic modifications and observing the according phenotype, which begins with forward-genetic methods represented by random physical and chemical mutagenesis and move towards reverse-genetic tools as targeted genome editing. A major bottleneck is time need to produce modified homozygous genotypes that can actually be used for phenotypic validation. Herein, we comprehensively address and compare available experimental approaches for functional validation of plant genes, and propose haploid strategies to reduce the time needed and cost consumed for establishing gene function.

Genetic modification-based functional validation for plant genes

Recent advances in plant genomics and sequencing technology revealed numerous associations between phenotypes and candidate genes. However, definitive functional annotations after *in vivo* validation have been thoroughly established for only few of these genes [1]. Genetic approaches for further validation of gene functions aim to create genetic modifications (See Glossary) that cause phenotypes of interest [1], including physical or chemical mutagenesis, insertional mutagenesis, Targeting Induced Local Lesions in Genomes (TILLING), gene over-expression, gene silencing [2-5]. Most recently, genome editing comprised by Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPR/Cas) enables investigators to manipulate any sequence in plant genomes in situ for validating gene and motif functions [6, 7].

Classic and current approaches to establish plant gene function

The classic approach for plant gene function establishment began with loss-of-function mutagenesis after treatment with mutagens such as radiation with X-rays or neutrons, or chemicals that introduce random small deletions or point mutations in plant genomes [5, 8]. Chemical mutagens such as ethyl methanesulfonate (EMS) were more popular as they are less destructive, easier available, and have a higher efficiency than physical mutagens [9]. Theoretically, we can find a EMS mutation in any given gene by screening no more than 5000 plants from the mutagenized M_1 generation for the model plant *Arabidopsis* [3, 8]. Conventional

mutagenesis has been widely used in forward genetic strategies that start with a phenotype of interest and address identification of genes affecting this phenotype [9-11].

As an alternative forward-genetic tool, insertional mutagenesis, including T-DNA (Transferred DNA) and transposon tagging, facilitate the identification of genes disrupted by these elements [5]. Currently, T-DNA-tagged lines have been generated in large numbers, becoming a popular resource for plant gene function [12, 13]. Superior to T-DNA, mobilizable transponsons can provide a verification about mutational effects of insertions when they are remobilized from the insertion site to recover a potential phenotype [14]. Insertional mutagenesis is less practicable for species, for which a systematic transformation platform has not been established [15].

As the number of characterized plant genes increases, reverse-genetic methodologies play an increasingly important role in gene function validation [16]. Targeting Induced Local Lesions in Genomes (TILLING) is the first reverse-genetic tool, in which chemical or physical mutagenesis is followed by a high-throughput screening for point mutations [3, 4, 16]. TILLING is practicable for plant species with large-sized genomes and without transformation system because it is not different from traditional chemical or physical mutagenesis in creating mutations [12].

Changes of gene expression levels may result in modified phenotypes, which can be another powerful approach for elucidating gene function [5]. Gene silencing or down-regulation induced by RNA interference (RNAi) can be achieved by expressing gene-specific double-stranded RNA (known as siRNA) or single-stranded RNA

(known as microRNA) in plant cell, which in turn generates loss-of-function phenotypes [17, 18]. RNAi is of great value for functional studies in polyploid plants because of its potential of silencing multigene families and homologous genes [19]. However RNAi seldom leads to complete suppression of target gene expression, thus loss-of-function phenotypes cannot be observed by RNAi when the residual expression is still sufficient for gene function [20]. In contrast to RNAi, overexpression or misexpression of an inactive gene or a gene coding a limiting protein will lead to gain-of-function phenotypes in transformants [21]. In this way, even the phenotypes of individual members in a gene family are observable without interference from functionally redundant genes [5].

Targeted mutagenesis can be achieved by ZFNs, TALENs or CRISPR/Cas in which custom DNA binding motifs direct non-specific nucleases to cleave a double strand in the genome at a specific site that further stimulates error-prone nonhomologous end joining or homology-directed repair at specific genomic locations [22-24, 6]. More applicable and easier to manipulate than ZFNs and TALENs, the CRISPR/Cas system only requires a single short RNA to generate target specificity [6], which even allows the genome-wide functional identification [25]. For these reasons CRISPR/Cas is becoming a popular technique for gene targeting [26-29]. Relevant bioinformatic tools for selecting optimal CRISPR/Cas target sites have been developed and are available online [24].

A comparison of different approaches for functional validation of plant genes is displayed in Supplementary Table 1. As recent developed techniques, ZFNs, TALENs,

and CRISPR/Cas can generate custom mutagenesis effectively, resulting in both, targeted gene knockouts and knock-ins [6, 25], which cannot be achieved by any other traditional method. Current research on genome editing addresses increase in precision and efficiency of gene targeting [24, 30]. Targeted mutagenesis still depends on plant transformation, by which T-DNA carrying chimeric enzymes and binding motifs are integrated into the plant genome and expressed for targeted double-strand breaks [27，28, 31]. The methods independent of genetic transformation for genome editing, such as direct delivery of these reagents or transient expression of these enzymes in plant cells, will substantially simplify the process of gene editing, even in species with large genomes [32, 33].

Time and resources required for current approaches

The approaches for validating gene function can be classified into mutagenesis- and transformation-based genetic modification (Figure 1, Figure 2). Independent of the approach, diploid plants are usually heterozygous for the modified region in the first generation (T_0 or M_1) [25, 34]. Altered genes and sequence motifs are often recessive and, therefore, without phenotypic effect in mutagenized T_0 or M_1 plants [34]. One or more additional generations are thus required to obtain homozygous genotypes for altered genes or sequence motifs that can actually be used for validation of their phenotypic effect [34, 35]. Even the most recently developed CRISPR/Cas method requires production of the T_1 generation for functional evaluation of modified plant genes [36, 37]. When multiple genes in a gene family are knocked out to understand gene function redundancies, additional generations and a larger population will be

needed for obtaining homozygous genotypes [34]. The required resources are an important constraint and bottleneck of functional validation in plants.

Strategies: Using haploids for functional validation of plant genes

Plant haploids, sporophytes with gametic chromosome numbers, can be obtained from regeneration of plants from pollen, microspores, eggs or other cells of the gametophyte [38-42]. Some plant species such as maize, *Arabidopsis* and barley can produce haploids by uniparental genome elimination via a male inducer [34, 43, 44]. Efforts to obtain haploid plants have been undertaken in more than 250 plant species of almost all families in the plant kingdom including several crop species [38]. Chromosome doubling of haploids generates doubled haploids and result in genetic homozygosis in a single generation [38]. In recent years, scientists have successfully developed haploid mutagenesis and established haploid transformation for plant species including wheat, triticale, maize, *Brassica napus,* liverwort and tobacco [34, 38, 45-50]. Notably, haploid strategy of *Arabidopsis* mutagenesis has become a successful approach for direct phenotypic characterization of recessive mutations [34]. Diploid *Arabidopsis* was irradiated with γ rays for mutagenesis and further used to pollinate a haploid inducer, generating three phenotypically distinct mutants from 240 haploids in M_1 generation. In addition, exploitation of the haploid *Arabidopsis* toolbox decreased the cost and complexity of screening mutants, accelerating substantially genetic analysis of multiple mutant combinations [34]. A quintuple heterozygous recessive mutant was crossed to a haploid inducer, obtaining 2 individuals homozygous for all 5 loci out of 113 haploids (1/56.5) which was much

higher than 1/1024 (1/32×1/32), the rate expected for the classical approach. [34]. Moreover, transformation for somatic cells of haploid maize embryo has been established for producing homozygous transgenic plants, seeds, and plant cells in a single generation. The average efficiency of transformation for haploid Hi-II (an excellent maize genotype for transformation) embryos reached 35% by *Agrobacterium*-mediated transformation [50], which was even higher than that of diploid embryos of Hi-II [51, 52]. Chromosome doubling frequencies of the transgenic haploid plants exhibited were about 60%. These findings pave the way for unconventional but powerful strategies for accelerated gene function validation in smaller populations.

Herein we propose haploid routes for mutagenesis and transformation-based approaches, respectively. The general processes of mutagenesis-based approaches are outlined in Figure 1 and Figure 2. Figure 1 illustrates the haploid scheme for functional validation of a single mutation which includes two alternatives. It can be used for both forward and reverse genetic screening. In the first alternative (Figure 1B), the pollen collected from diploid plants is directly used for chemical or physical mutagenesis [34, 49], and then hybridized with a paternal haploid inducer as female parent, generating haploid M_1 plants via uniparental genome elimination [34, 49]. Selection of phenotypes of interest in M_1 generation is followed by diploidization and self-pollination [34], producing double haploid M_2 lines. Compared to the conventional scheme (Figure 1A) [53, 54], the time needed is reduced by one generation in approach B. For the other option, an *in vitro* culture-based strategy for

haploid mutagenesis is shown in Figure 1C. Microspores or pollen extracted from diploid plants are treated with a chemical mutagen or radiation for mutagenesis [34, 49], resulting in M_1 haploid lines after embryogenesis [55]. The selected M_1 lines are then chromosome-doubled [56] and available for functional validation. The proposed procedure could be a major breakthrough to reduce cycle time and conducted even in a minimal space in a laboratory setting [57], unless the phenotype of interest has to be identified in the field or greenhouse.

These two alternatives have strengths and weaknesses for phenotypic validation of mutants. *In vitro* culture in Approach C can be carried out with minimal space requirement with 15 plantlets occupying approximately a 250 ml flask [58] and needs only a single M_1 generation for functional validation. However, the required microspore/pollen embryogenesis may be constrained to some particular genotypes [59]. While not requiring cell or tissue culture, pathway B involves generation of haploid cells via uniparental genome elimination from wide hybridization which is currently limited to a few plant species [38].

The scheme in Figure 2 is proposed for functional validation of multiple mutations. Validation of the combined function of multiple mutations at different loci often requires introgression of these selected allels at multiple loci by crossing the single mutants. For the conventional route (Figure 2A), a heterozygous F1 individual is self-pollinated and then F2 generation is screened for the desirable homozygous genotypes. The workload and cost increases exponentially with the increase of number of loci according to the frequency $1/4^n$, where n represents the number of loci

[34]. However, when it comes to the haploid scheme (Figure 2B), a paternal haploid inducer is used as female parent and pollinated with pollen from heterozygous multiple mutant (male parent), and haploid plants are generated by uniparental genome elimination at a probability of $1/2^n$ [34, 53]. And double haploid lines are then generated by diploidization and self-pollination. Compared to the conventional approach (Figure 2A) [53, 54], application of this system does not accelerate the process but significantly reduces the resources needed (BOX1).

Haploid strategies for transformation-based approaches contain two optional experimental routes (Figures 3B, 3C). In route B, microspores or pollen of diploid plants are transformed with the vector containing a foreign DNA fragment and a resistance marker gene which confers a selectable phenotype on plant cells [54, 48], and then be induced into calli. After that, the calli are put on a resistance-screening medium. Only positive transgenic calli will grow, as non-transformed calli will be killed. By embryogenesis, multiple T_0 haploid somatic embryos are generated from each transgenic multicellular callus [55]. Homozygous transgenic diploid lines are further produced from the somatic embryos after experiencing regeneration and diploidization [47]. For the alternative route (Figure 3C), a maternal haploid inducer is used as male parent to pollinate donor plants as female parents. The resulting haploid immature embryos are then transformed [50] and then cultured on a screening-inducing medium for generating transgenic haploid calli from single positive cells. Via clonal propagation followed by embryogenesis and regeneration, every haploid callus generates several plantlets. In the conventional transgenic route

(Figure 3A), the T_1 generation is required to obtain homozygous genotypes at the predicted 1/4 frequency [60]. However only the T_0 generation is required for routes B and C, time and resources are consequently reduced for these haploid transformation-based cycles (BOX 2). Routes B and C mainly differ in the sources of haploid cells, derived from gametophytic cells or haploid embryos, respectively.

Concluding remarks and future challenges

Haploid mutagenesis and transformation have been reported earlier, but have so far hardly been applied in functional validation of plant genes. The objectives of this review paper are to combine the current experimental methods for gene function validation and haploid strategies that could substantially help to accelerate plant gene functions at lower costs. However, the application of haploid strategies needs to overcome obvious limitations (BOX 3).

First, microspore/pollen embryogenesis is limited by various factors and is usually genotype-dependent [61]. Efforts toward improving microspore culture have been made on "stress" treatments such as starvation, heat shock, ethanol stress, water stress, anaerobic conditions, low temperature treatment to induce microspore/pollen embryogenesis by switching the preprogrammed gametophytic to sporophytic development pathway [62-66]. Other methods such as irradiation, treatment with cochicine and auxin can also be applied to induce microspore embryogenesis [38, 61], for example, about 50% of treated microspores were successfully redirected for the predicted pathway by a chemical inducer formulation in wheat [67]. However, genotype dependency strongly limited the wide application of microspore/pollen

embryogenesis [68]. The swollen rate of pepper microspores during embryogenesis from different genotypes varied from 3.11% to 29.56% [69]. In *Brassica rapa*, 22 of 24 genotypes produced somatic embryos ranging from 0.02 to 15.0 per 2×10^5 microspores, and two failed to respond [70]. Beaumont et al. mapped the maize genes controlling embryo induction during anther culture on chromosomes 1, 3, 5, 7 and 8 [71]. Expression of some specific proteins or protein families such as the BnmNAP subfamily and phosphoproteins were found to contribute to microspore/pollen embryogenesis [72, 73]. It would be beneficial to conduct new researches revealing molecular pathways involved in microspore/pollen embryogenesis.

Second, application of haploid inducer technique in generating haploids is still limited to particular species for biological or technical reasons. Up to now, it is only available in a few species such as in maize, barley, wheat, *Arabidopsis*, and potato [38]. Successful rate of haploid seed production relies on different genotypes of haploid inducers as well as different growing conditions [74]. In maize, good haploid inducers can produce 5-16% haploid seeds [75]. QTL mapping studies based on RFLP-markers revealed that *in vivo* haploid induction was controlled by a few recessive genes in maize. Remarkably, two major QTL on Chromosome 1 and 9 might help to increase haploid induction rate [76]. Further efforts on identifying candidate genes and revealing molecular mechanism of haploid induction ability in maize may lead to a promoted application of *in vivo* haploid induction in maize as well as other plant species.

Third, chromosome doubling is a critical step in all the proposed methods. Over the

past decades, different methods have been applied. The most popular method is treatment with colchicine or herbicides such as amiprophos-methyl, trifluralin, pronamide and oryzalin, which inhibits microtubule polymerization [38]. Kato et al. developed treatment with nitrogen oxide for maize chromosome doubling [77], and the mechanism was found in *Lillium* to be depolymerization of microtubules [78]. However, the chemical treatments have the potential to damage plant cells. Spontaneous genome doubling can avoid the chemical damage, which was found during microspore/anther culture in plant species such as maize, barley, wheat and onion with up to 70% doubling efficiency [79-81]. To avoid chimeras individuals, *in vitro* chromosome doubling at early stage of microspore/pollen embryogenesis will be desirable [80, 81].

Fourth, retaining regenerative capacity of microspores/pollen during transformation is essential to obtain regenerated transformed haploids. Previous studies showed that electroporation-based transformation led to the decrease of viability of microspores and pollen. [82-84]. Obert et al. investigated influence of electroporation media on microspore viability and embryogenesis in maize, which showed MES medium is superior to BK and HBS media [85]. However, as the most stable and widely used plant transformation system, *Agrobacterium*-mediated transformation has some obvious problems in microspores/pollen because of co-cultivation of microspores/pollen cells and *Agrobacterium* which results in the decrease of microspore viability and embryoid production [48]. In a recent study, Brew-Appiah et al. suggested that strict control of *Agrobacterium* cell concentration and starting time

for co-cultivation as well as use of timentin for killing remaining *Agrobacterium* after co-cultivation will be advantageous for wheat microspore embryogenesis [48]. More intensive studies should be carried out to determine optimized conditions for *Agrobacterium*-mediated microspores/pollen transformation in other species.

Fifth, another challenge is to develop markers for early identification of haploids. Currently, the available dominant markers of haploid inducers mainly allow haploids to be screened in dry seed, seedlings and mature plants [86]. Furthermore, expression of these genes has a strong maternal component, sometimes impairing the screening of haploids [86]. It will be critically important to be able to effectively screen haploids at an early stage of embryo development and during plant transformation. Consequently, identification of marker genes with good expression in immature embryos for haploid induction would simplify and accelerate the process of haploid transformation. An inducible lethal gene specifically expressed in embryos controlled by an inducible promoter would be desirable as direct marker for haploid selection among immature embryos. By inducing the expression of the lethal gene, the diploid F_1 embryos would be killed and only haploid embryos could survive because they do not contain the marker gene. Without induction of the marker gene, haploid inducer genotypes carrying this marker can be maintained by self-pollination [50].

In spite of these challenges, a major benefit of using haploid strategies for gene validation would be a significant reduction in time and costs needed for the process of functional revealing for plant genes.

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BOX1. Comparison of costs between the conventional and haploid mutagenesis

The cost advantage to adopt haploid strategie in functional validation of multiple alleles can be estimated based on the formula $n_{min}=\ln(1-Q)/\ln(1-P)$ suggested by Lubberstedt et al. (2012) [56]. Here, n_{min} represents the minimal population size need; n equals identical numbers of the unlinked target mutations; P equals to $(1/4)^n$ or $(1/2)^n$ for conventional genetic scheme (Figure 2A) and the haploid genetic scheme (Figure 2B), respectively; Q denotes the probability of finding at least one genotype carrying all the n mutations. For example, the minimal M_2 population size to find a single homozygous plant carrying three independently mutations at a probability of 99% is 293 for conventional method (Figure 2A). However, the minimal M_2 haploid population size decreases to 35 for the haploid approach (Figure 2B), with a 8.5× advantage in costs as compared to the traditional approach.

BOX 2. Comparison of costs between diploid and haploid transformation

To reveal gene function redundancies or the combined function of genes, multiple genes in a gene family may need to be knocked out or edited [34]. For example,

knockout of five unlinked genes results in the expected $1/4^5$ quintuple homozygotes in T_1 generation for traditional diploid transformation (Figure 3A). According to the formula $n_{min}=\ln(1-Q)/\ln(1-P)$ [56], over 4714 individuals in T1 generation are required to find at least one homozygous plant carrying all the five edited genes at a probability of 99%. Comparatively, transgenic plants from each event in the T_0 generation (without the need of producing T1 generation) can be directly used for phenotypic validation after transforming haploids (Figure 3B, 3C).

Glossary

Clonal propagation: an asexual process to reproduce plant cells by tissue culture

Custom mutagenesis: the process to create any site-directed mutation by ZFNs, TALENs, or CRSPR/Cas

Embryogenesis: the process by which the embryo forms and develops in situ.

Forward genetics: an approach of determining the genetic basis responsible for a phenotype which was initially done by generating mutants by physical, chemical, or insertional mutagenesis and subsequently followed by isolation of mutant individuals and identification of functional gene.

Gain-of-function mutation: A mutation that leads to new or enhanced protein function.

Gene knock-in: a genetic engineering method that involves the insertion of a protein coding cDNA sequence at a particular locus in an organism's chromosome.

Gene knockout: a genetic technique where genes in an organism are made inoperative in functions.

Genetic modification: a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation.

Haploid inducer: a specific plant genotype used for the production of haploid plants by cross with a donor plant.

Haploid transformation: a process where haploid cells or tissues are genetically transformed.

Inducting medium: a kind of medium containing hormones which is used for dedifferentiation of plant cell or tissue.

Loss-of-function mutation: A mutation that results in reduced or abolished protein function.

microRNA: a class of single-stranded RNA molecules containing about 22 nt found in plants, animals, and some viruses, which functions in RNA silencing by post-transcriptional regulation of gene expression.

Misexpression: expression of a gene in a cell type or developmental stage or condition where it normally is not expressed.

Overexpression: excessive expression of an endogenous gene in an organism by genetic transformation to enhance its phenotypic effect, resulting in mutant phenotypes.

Regeneration: the process of growing an entire plant from a single cell or cell mass

Reverse genetics: an approach to reveal the gene function by analyzing the phenotypic effects of specific gene sequences. This investigative process proceeds in the opposite direction of forward genetics.

siRNA: a class of double-stranded RNA molecules with 20-25 nt in length, which plays an important role in the RNAi pathway by interfering with the expression of specific genes based on complementary nucleotide sequences.

Transposon tagging: a process where transposable DNA elements are introduced into biological cells for random tagging gene sequences and isolating genes.

Uniparental genome elimination: Chromosome elimination of one parental genome after fertilization of the egg by the sperm of another species (interspecies cross), which results in the formation of haploid embryos.

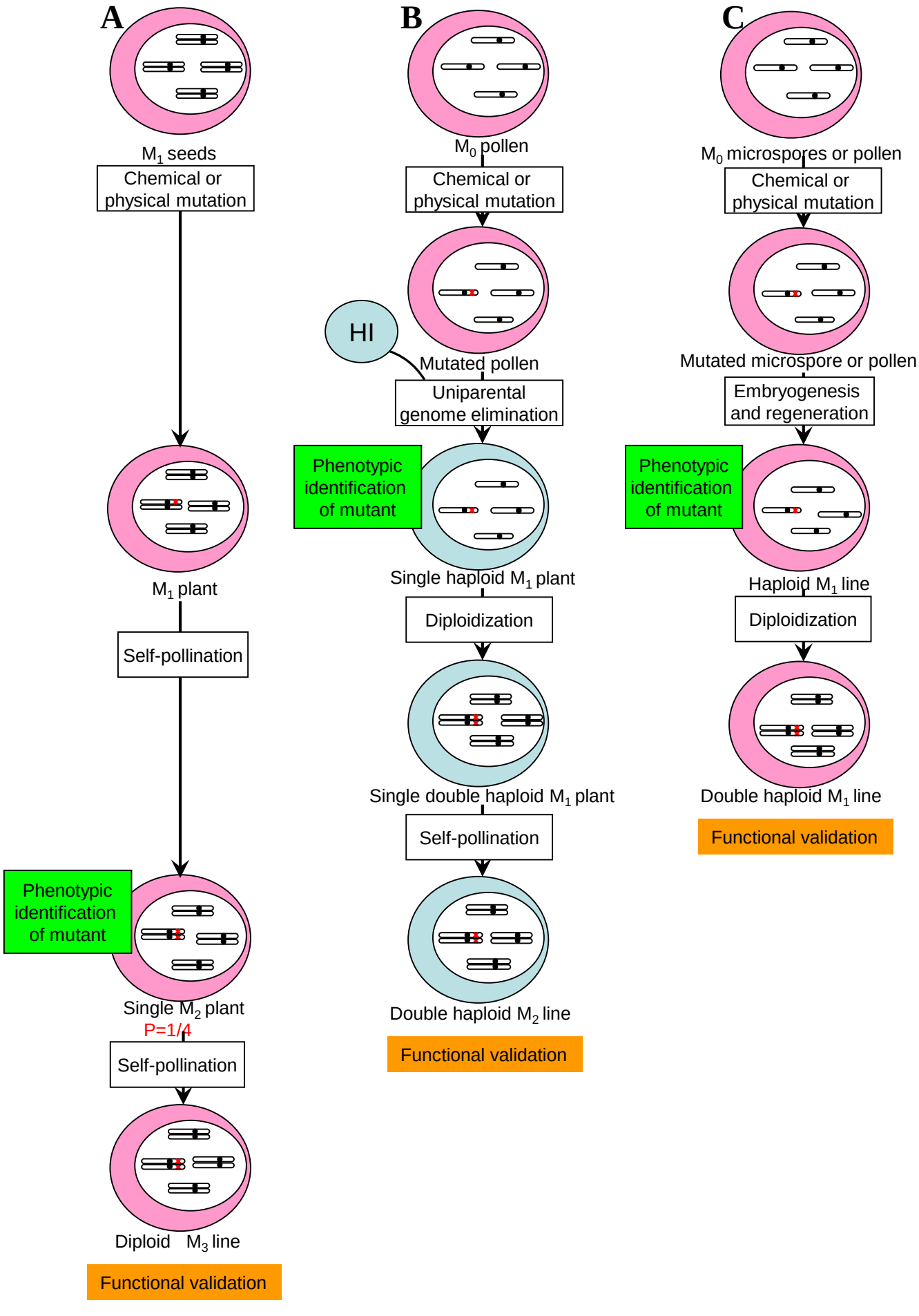
Figure legends

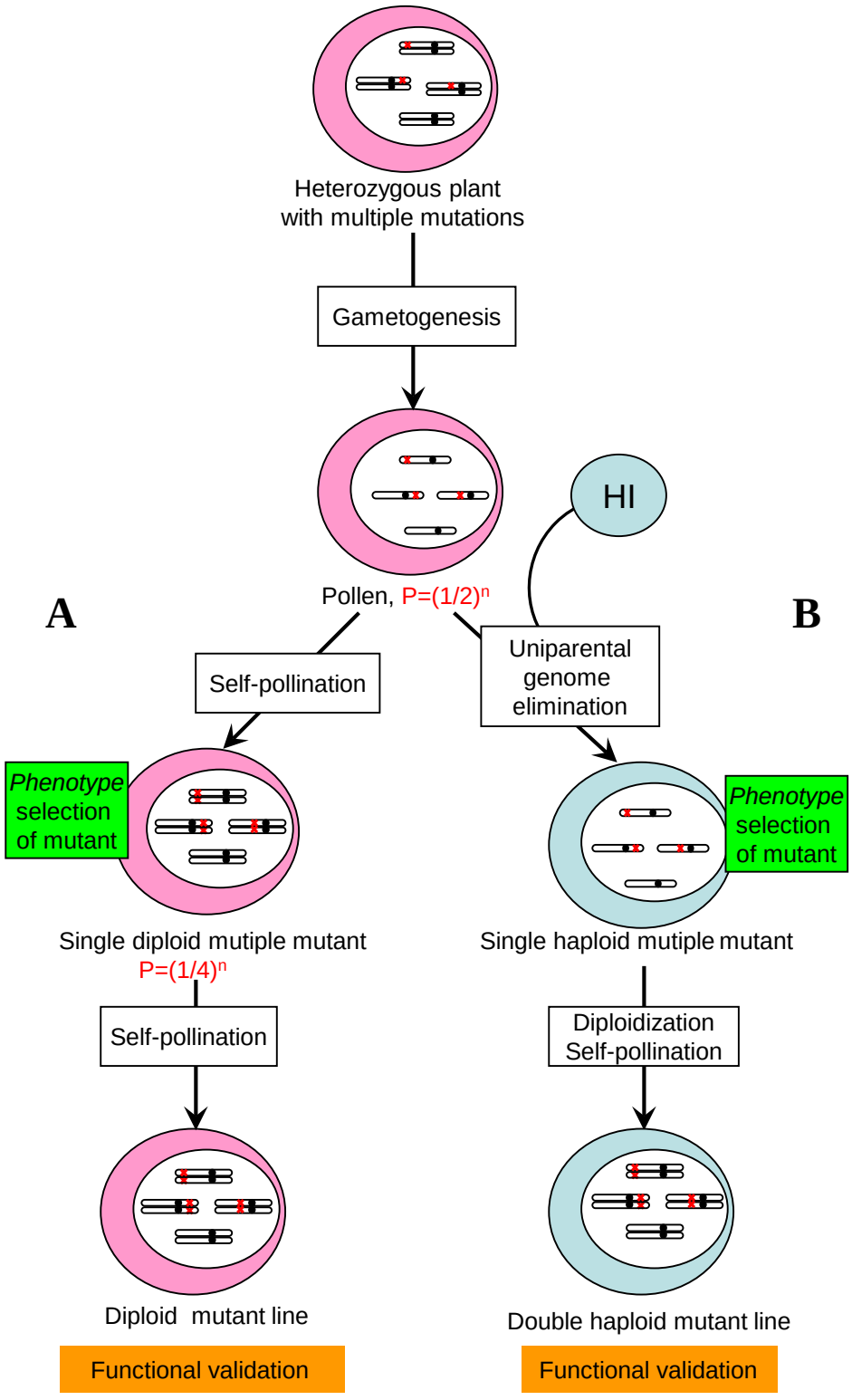
Figure 1. Comparison of diploid and haploid mutagenesis-based procedures for phenotype validation of a single mutant. Generally, a homozygous plant family (with several plants) at the target locus/loci needs to be produced rather than a single plant for final phenotype validation. (A) Conventional diploid mutagenesis followed by self-pollination, where the M_3 generation is required to obtain a desirable homozygous family. (B) Pollen mutagenesis followed by genome elimination, in which the M_2 generation is used for phenotype validation. HI represents a haploid inducer. (C) Microspore or pollen culture-based approach, for which M_1 generation can be used for genotype-phenotype validation.

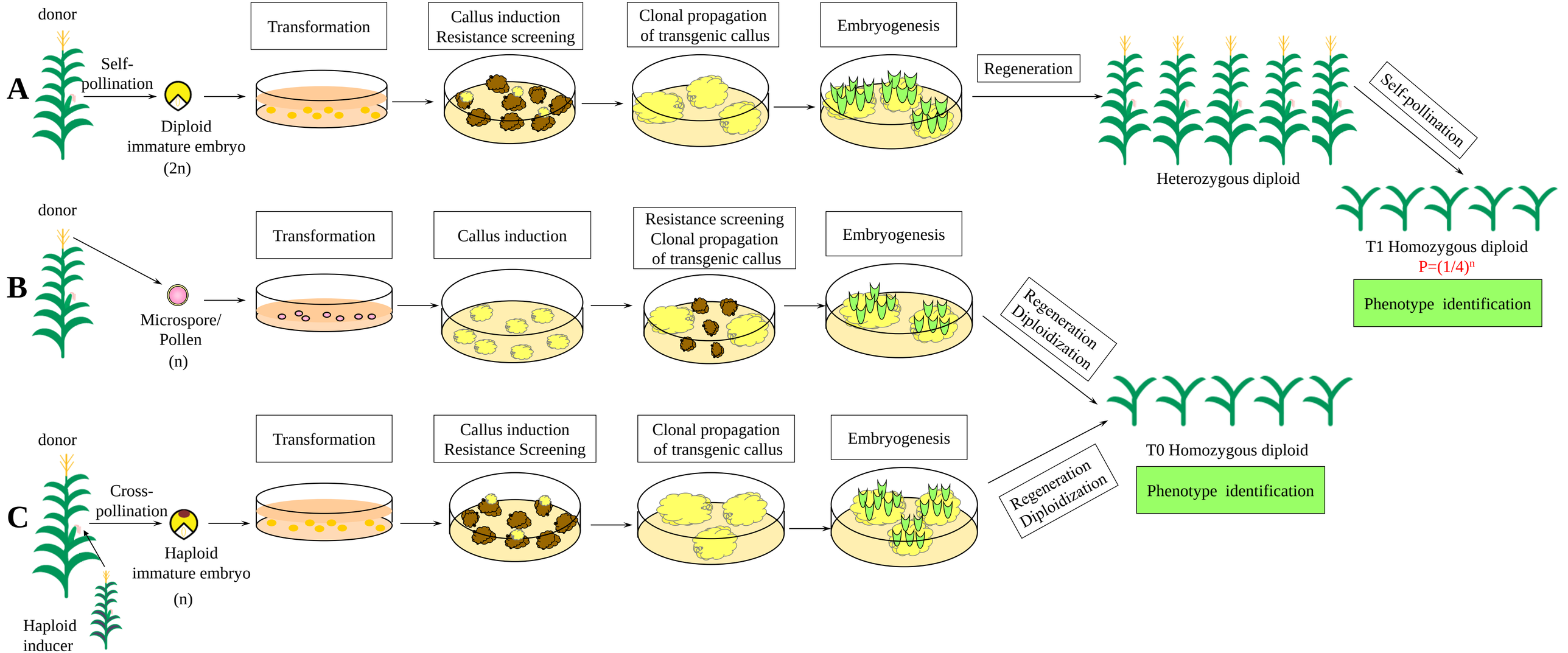
Figure 2. Comparison of diploid and haploid mutagenesis-based procedures for phenotype validation of a triple mutant. (A) Conventional genetic method, where a heterozygous F1 carrying multiple mutations (generated by crossing three single mutants) is followed by self-pollination, and produce the homozygous genotype for

the three alleles at a probability of 1/64 in F2 population. The desirable homozygous genotype is further self-pollinated to generate a homozygous mutant line for functional validation. (B) Haploid scheme, in which a paternal haploid inducer is used as female parent and pollinated with the heterozygous triple mutant, and the desirable haploid genotype is generated by uniparental genome elimination at a probability of 1/8. A double haploid multiple mutant line is then produced by diploidization and self-pollination. HI represents a haploid inducer.

Figure 3. Comparison of diploid and haploid transformation-mediated functional validation of plant genes. Here, transformation includes bombardment, electroporation and *Agrobacterium*-mediated transformation. n denoates the number of target genes transferred or edited. (A) Conventional procedure of immature embryo transformation, where one additional generation following transformation is required to obtain homozygous diploids for the altered gene or motif, which can then be applied for phenotype validation. (B) Microspore or pollen transformation, where a homozygous (for target locus/loci) line can be obtained in the T_0 generation for phenotypic validation. (C) Haploid embryo transformation, positive transgenic single cells originate from callus after induction culture and resistance screening. Homozygosis of target locus/loci is then achieved in the T_0 generation.


A
M1 seeds
Chemical or physical mutation
M1 plant
Self-pollination
Phenotypic identification of mutant
Single M2 plant
P=1/4
Self-pollination
Diploid M3 line
Functional validation
B
M0 pollen
Chemical or physical mutation
HI
Mutated pollen
Uniparental genome elimination
Phenotypic identification of mutant
Single haploid M1 plant
Diploidization
Single double haploid M1 plant
Self-pollination
Double haploid M2 line
Functional validation
C
M0 microspores or pollen
Chemical or physical mutation
Mutated microspore or pollen
Embryogenesis and regeneration
Phenotypic identification of mutant
Haploid M1 line
Diploidization
Double haploid M1 line
Functional validation


Heterozygous plant
with multiple mutations
Gametogenesis
Pollen, P=(1/2)n
HI
A
B
Self-pollination
Uniparental
genome
elimination
Phenotype
selection
of mutant
Phenotype
selection
of mutant
Single diploid mutiple mutant
P=(1/4)n
Single haploid mutiple mutant
Self-pollination
Diploidization
Self-pollination
Diploid mutant line
Double haploid mutant line
Functional validation
Functional validation


A
donor
Self-
pollination
Diploid
immature embryo
(2n)
Transformation
Callus induction
Resistance screening
Clonal propagation
of transgenic callus
Embryogenesis
Regeneration
Heterozygous diploid
Self-pollination
T1 Homozygous diploid
P=(1/4)n
Phenotype identification
B
donor
Microspore/
Pollen
(n)
Transformation
Callus induction
Resistance screening
Clonal propagation
of transgenic callus
Embryogenesis
Regeneration
Diploidization
T0 Homozygous diploid
Phenotype identification
C
donor
Cross-
pollination
Haploid
immature embryo
(n)
Haploid
inducer
Transformation
Callus induction
Resistance Screening
Clonal propagation
of transgenic callus
Embryogenesis
Regeneration
Diploidization