Development of a Molecular Breeding Strategy for the Integration of Transgenic Traits in Outcrossing Perennial Grasses

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Abstract: Molecular breeding tools, such as genetic modification, provide forage plant breeders with the opportunity to incorporate high value traits into breeding programs which, in some cases, would not be available using any other methodology. Despite the potential impact of these traits, little work has been published that seeks to optimize the strategies for transgenic breeding or incorporate transgenic breeding with other modern genomics-assisted breeding strategies. As the number of new genomics assisted breeding tools become available it is also likely that multiple tools may be used within the one breeding program. In this paper we propose a strategy for breeding genetically-modified forages using perennial ryegrass as an example and demonstrate how this strategy may be linked with other technologies, such as genomic selection. Whilst the model used is perennial ryegrass the principles outlined are valid for those designing breeding strategies for other outcrossing forage species.

Keywords: forage; transgenic; breeding strategy

1. Introduction

"Roundup Ready" alfalfa was the first, and currently only, commercially available transgenic forage in the world [1], with a large range of transgenic traits being developed in a number of different forage species including white clover, subterranean clover, alfalfa, Italian ryegrass, perennial ryegrass, tall fescue, red fescue, creeping bentgrass, bahiagrass, and switchgrass. These traits include forage quality, tolerance to biotic and abiotic stress, and the manipulation of growth and development [1–5]. Genetic transformation of plants is only the beginning of the pathway towards the commercialization of a transgenic product and has not diminished the need for cultivar development through breeding [6].

Notwithstanding the technical and regulatory challenges that are sometimes associated with the development of forage cultivars using new technologies the fact remains that the targeting of high impact traits related to forage quality, productivity and persistence has the potential to greatly increase the profitability of grazing industries [7–9].

Transgenic breeding is an extension of conventional plant breeding technologies and, although it shares the same basic principles and guidelines with conventional plant breeding, it has its own challenges and breeding objectives [10]. These objectives include the selection of transgenic events that exhibit the targeted attribute across generations, whilst retaining all other non-target agronomic

qualities [10,11]. Although the methodologies are in place to generate new transgenic events within forage crops, they have not been assembled or optimised to be integrated into a transgenic breeding program for commercial development.

There is very limited literature related to breeding strategies for the development of transgenic forages from transformation to commercialisation [6,12], with no literature on the breeding strategies for transgenic *Lolium* grasses. Both Kalla et al. [2] and Woodfield and White [6] described the introgression of a transgene within a molecular breeding program in white clover, an outcrossing forage legume, and are focused on the incorporation of a novel trait into a known genetic background using repeated crossing to a transgenic donor plant to derive plants that are homozygous for the transgene. With minor adjustments, both of these breeding strategies could be applied to transgenic *Lolium* grasses. However, since these studies were published, progressive advances in molecular genetic and genomic technologies, and the ongoing expansion of phenomics capabilities, have allowed for designs of genome editing, marker assisted selection [4], genomic selection [13], and hybrid breeding strategies [14] in commercially-relevant breeding programs of temperate outcrossing grasses that could potentially change breeding strategies in forages [15–18]. These new methodologies can potentially increase the rate of genetic gain over generations, reduce time between generations and reduce the cost of phenotyping. The implementation of these technologies has the potential to address a number of short-falls of conventional breeding programs, when it comes to the integration of a transgene into a wider breeding population.

Some of these considerations and technologies will be discussed and an optimum strategy for the transgenic breeding of *Lolium* grasses is proposed. This transgenic breeding strategy will not replace the need for standard aspects of commercial breeding programs, such as the choice of adapted parental germplasm and multi-site screening for genotype $\times$ environment interactions, but rather allow the efficient introgression of transgenic technologies into such a program in a way that is also compatible with other molecular breeding tools.

The breeding strategy described aims to reduce the time-lag between transformation and commercialisation through the creation of market-ready transgenic events in perennial ryegrass. Elements of the strategy were validated during the development of transgenic perennial ryegrass with altered fructan biosynthesis [19]. However, the strategy proposed is not a description of what was done in that experiment, but rather how elements that were used there could be used more broadly in a breeding strategy, such as:

- screening agronomically superior populations for tissue culture responsive (TCR) genotypes to be used in genetic transformation;
- screening and selecting both T_0 events and recipient genotypes concurrently;
- ensuring agronomically fit, genetically-diverse, recipient genotypes were used in crossing,
- introducing endophytes in the T_1 generation via the recipient parent; and
- attempting to complete two cycles of crossing within one year.

This strategy allowed for the development of transgenic perennial ryegrass *Syn*0 germplasm for two transformation within five years of transformation. The strategy included four years of field evaluation on the primary T_0 parents and two years of field evaluation on the transgenic T_1 progeny. During these experiments, a number of areas, as listed below, were highlighted for consideration when attempting to create an optimized transgenic breeding strategy in *Lolium* grasses. These areas need to be considered when planning and developing transgenic *Lolium* for commercial purposes:

- selection methods of tissue culture responsive genotypes;
- development, evaluation, and selection of primary T_0 transgenic events in *Lolium*;
- integration of the gene technologies into the wider breeding population; and
- evaluation of progeny for trait stability and agronomic performance.

2. Selection Methods for Tissue Culture Responsive Genotypes

Callus production and regeneration protocols to produce fertile plants in *Lolium* are pre-requisites to *Lolium* transformation [20]. Thus, the development of an optimized tissue culture protocol is essential for successful genetic transformation [20]. However, not all agronomically elite cultivars/populations are amenable to genetic transformation, due to either poor callus induction or poor callus regeneration. Most genotypes selected for genetic transformation are, thus, selected based on their tissue culture responsiveness rather than agronomic performance. This can have a genetic drawback during initial cultivar development due to the presence of agronomically unfavourable alleles within the selected genotype used for genetic transformation [6,10]. Visarada [11] concluded that it is more realistic to use highly responsive genotypes for genetic transformation, and then use promising transgenic events in a backcrossing strategy with agronomically superior populations/cultivars. It will take at least five generations of backcrossing to regain roughly 97% of the backcrossed parents' genetic background [6]. In outcrossing species, such as *Lolium*, it is essential to backcross with a number of diverse recipient genotypes in each successive generation, to avoid inbreeding depression. This backcrossing strategy will maintain the transgene in a heterozygous state and so it will be present in only 50% of the progeny. Progeny will, thus, have to be screened for the transgene in each generation. Although achievable, this backcrossing strategy will add significant cost and an additional 2–5 years to the breeding process for commercialisation.

Genotypes selected for genetic transformation should, thus, not only be screened for TCR, but for agronomic performance under field conditions prior to transformation, as well. This could easily be implemented in a commercial breeding program, by screening selected superior genotypes from breeding populations for tissue culture responsiveness. This would allow for the identification of TCR genotypes that are agronomically superior well in advance of any transformation work, and would enable continues improvement of TCR genotypes through recurrent selection strategies. Screening for tissue culture responsiveness in a genomic selection reference population would enable the estimation of the marker effects that would allow for the development of genomic selection prediction equations. These prediction equations could then be used to estimate genomic estimated breeding value (GEBVs) for selection candidates for tissue culture responsiveness, as well as for important traits, such as biomass yield, nutritive quality, and persistence in a sward [16,17].

Another consideration in choosing the correct genotype for genetic transformation would be floral induction requirements. Floral induction marks the transition from a vegetative state to a reproductive state and requires a dual induction in most *Lolium* grasses [21–23]. The primary induction is brought on by short days and/or low temperatures (vernalisation), with an obligatory requirement of at least two weeks, followed with the secondary induction in response to a transition to longer days and moderately high temperatures [21,24,25]. The requirements for both primary and secondary induction can, however, vary greatly within perennial ryegrass germplasm [24,26]. The selection of agronomically fit TCR genotypes with short primary induction requirements will assist greatly with the crossing strategies in the initial cultivar development phase. Shorter obligatory PI requirements of 4–6 weeks, will allow for multiple crosses to be done in succession, allowing for a larger number of crosses to be completed within a year. In some of our research, the TCR genotype had a 12 week obligatory PI requirement. This long PI requirement restricted the number of crosses that could be produced within a year, and limited of generations that could be created to three generations every two years. As perennial ryegrass requires both primary and secondary flowering induction to occur, selection on shorter PI requirements alone should not alter heading dates. It would still be important to ensure that heading dates are in line with the breeding objectives. Crossing would have to be done in contained glasshouse facilities, which would most likely have space restrictions depending on the regulatory requirements of the jurisdiction in which the crosses are performed.

All of these considerations and technologies can assist with the selecting of an agronomically fit TCR genotype that would allow for the generation of two generations per year under glasshouse conditions, which could shorten breeding time requirements for commercialisation by 2–3 years.

3. Development, Evaluation and Selection of Primary T_0 Transgenic Events in *Lolium* Grasses

In the past two decades, enabling methodologies for the application of genetic transformation have been developed or improved for many important forage, turf, and bioenergy crops [1,5,27,28]. Genetic transformation technology allows for the introduction of novel genetic variation through the introgression of genes from related or unrelated species through transformation [1] and have been extensively reviewed [29–33]. The uncontrolled nature of both *Agrobacterium*-mediated transformation and biolistic transformation could lead to multiple insertion sites and complex integration patterns that could potentially lead to insertional mutagenesis [34]. This uncontrolled nature of transgene insertion could mean that each transformant has a different insertion site and potentially has a variable number of inserts of the transgene as well [10]. Somaclonal variation during tissue culture and insertional mutagenesis during transformation could also lead to significant phenotypic variation in primary T_0 transgenic events [10,11]. This could become a significant barrier in transgenic breeding but could be overcome by generating large numbers of primary T_0 transgenic events, to screen for T_0 events with low transgene copy number, that exhibit the desired trait without negatively affecting the agronomic performance of the plant [10]. Recent developments in genome editing techniques will also be able to address these concerns through the precise, targeted deletion, substitution, or addition of nucleotides within a specific site in the genome [15,35]. The advantage of these new techniques over *Agrobacterium*-mediated transformation and biolistic transformation is that the integration is site specific, which has the potential to reduce insertional site effects, complex integration patterns, and multiple copy insertions [35] although depending on the technology used there can be a large effort to define and characterize suitable introgression sites. It will also reduce the burden of identifying and characterising insertion sites for the deregulation of a selected transgenic event for commercial use [36]. There is currently no literature available that reports targeted genome editing in any forage species, but the use of genome editing has been reported in other plant species, including maize [37], rice [38], wheat [39], barley [40], and soybean [41].

For a transgenic event to be successful as a cultivar, it is expected that the trait will be stably inherited, consistently expressed, and with no significant negative impact on the agronomic performance [10,11]. In perennial ryegrass, the transgenic breeding strategy starts with the production, evaluation and selection of primary T_0 events for the targeted trait, followed by the introgression of the transgene into the larger population. The cost of running a field trial with transgenic events can be substantial due to regulatory requirements [36,42]. To reduce environmental risks that may arise from the release of transgenic material into the environment, restrictions may be placed on the number of T_0 events that can be evaluated, the length of the trial and the methods used for evaluation (e.g., no animal grazing) by the regulatory governing body. For these reasons, it is necessary to reduce the number of primary T_0 transgenic events to progress to field evaluation, by pre-screening the T_0 events in containment glasshouse facilities. Pre-screening can include gene expression analysis, estimation of transgene copy number, marker gene copy number, and phenotypic analysis of the target trait. For qualitative traits (e.g., disease resistance, herbicide tolerance, etc.), it is possible to pre-screen large numbers under containment conditions in the glasshouse and only take promising T_0 events for evaluation in targeted environments under field conditions. Although it is possible to pre-screen for quantitative traits (e.g., fructan biosynthesis) in the glasshouse as well, it is advisable to screen for the trait in targeted environments under field conditions, as promising events might not express the trait under glasshouse conditions. Due to the cost of deregulation of a transgenic event [43], it is most likely that only one primary T_0 event will be selected for deregulation. However, it is critical that between 4–20 of the most promising T_0 events be advanced in the breeding program, as transgenic events might be deemed unfit due to low T_0 fertility, limited seed production, and irregular trait heritability [10].

4. Introgression of the Transgene into the Wider Breeding Population

Genetic transformation and selection of elite T_0 transgenic events that exhibit the trait of interest is only the start of making a commercial transgenic product and has not diminished the need for cultivar

development through breeding [6]. The introgression of individual genes for cultivar development have been limited in outcrossing species, such as perennial ryegrass, as repeated backcrossing to a single parent is required [44] which, in turn, can lead to inbreeding depression. In this study, the method used to introgress the transgene within a breeding population was to use the clonal ramets of ryegrass to facilitate the simultaneous pair-crossing of a single transgenic event to a number of selected genotypes. Using multiple pair-crosses allowed for the introgression of the transgene into a range of genetically diverse genotypes of single/multiple breeding population(s), in the most cost-effective and controlled manner. To create a transgenic synthetic cultivar, while reducing the risk of inbreeding, around 4–20 recipient genotypes would need to be pair-crossed with each selected primary T_0 transgenic event [45]. This reduces the chance of a founder effect within the transgenic population and will reduce inbreeding depression in the following generations [46]. Kidwell et al. [47] have shown that fewer parents that are genetically diverse are more suited for cultivar development than highly related plants. Plant breeders have, however, until recently, been unable to distinguish genetically-diverse parents within breeding populations and had to rely on larger numbers of parents as a precaution against inbreeding [48,49]. New advances in molecular genetics have enabled breeders to determine the genetic distance between parents and, thus, select smaller subsets of parents, while maintaining genetic diversity and desired characteristics within the population [18]. Recipient genotypes can, thus, be selected based on genetic diversity between genotypes that will be used in crossing activities [47].

In recent years, genomic selection strategies suitable for use in *Lolium* have been proposed [13,16,17]. In a genomic selection breeding program, recipient parents could be selected based on GEBVs, as well as on phenotypic selection [17]. GEBVs could allow for the rapid identification of genetically-diverse parents that contain the desired genotypes for agronomic performance. Although the use of genomic selection is novel in plant species it is likely that for species where appropriate resources are placed into the development of genomic selection algorithms that the technology may be widely used. If genomic selection if combined with a transgenic breeding strategy it is import that both the transgenic genotype and the recipient genotypes should, however, be selected from the breeding population that is linked to the genomic selection training population, to ensure that the genomic predictions of the progenies are accurate [13,17].

The recipient parents could also be a vehicle to introduce endophyte strains into the breeding population. Endophyte/ryegrass symbiota have shown to have a competitive advantage over ryegrass without endophytes [50].

5. Evaluation of Progeny for Transgenic Trait Stability and Agronomic Performance

One of the characteristic features of biolistic transformation is the integration of both full length transgenes and possibly rearranged fragments of the transgenes, with variable copy numbers of both full-length transgenes and transgene fragments [51]. The multiple copies of the full transgenes and possibly rearranged fragments are most frequently inherited as a single locus [51] and is, thus, expected to exhibit a monogenic Mendelian segregation ratio of 1:1. However, studies have shown that between 10%–50% of transgenic events produced by either *Agrobacterium*-mediated transformation or biolistic transformation will show non-Mendelian inheritance of the transgene [10,51,52]. Yin et al. [52] have attributed the non-Mendelian segregation of the transgene to the nature of the recipient genome, the nature of the transgene and the interaction between them. The consequences of selecting a transgenic event that shows non-Mendelian inheritance for the genetic transgene is that an increased number of crosses should be produced after transformation [52]. The selection of a transgenic event that exhibits Mendelian segregation is, thus, preferable.

In this study, the method used to select for monogenic Mendelian inheritance in transgenic events was to pair-cross the primary T_0 event to a recipient genotype. This allowed for the calculation of segregation ratio within the T_1/F_1 progeny. All transgenic events that had a 1:1 segregation ratio in their T_1/F_1 progeny were progressed for further crossing. Using pair-crosses also allowed for cost

savings in molecular screening of progeny as the segregation ratio was likely to be 1:1. This would not have been the case if the transgenic events were placed in poly-crosses with multiple recipient genotypes in the initial integration step. A 1:1 segregation ratio was observed for three primary T_0 events. Using pair-crosses also allowed for full-sib or half-sib progeny testing, and for the selection of genetically-diverse parents to reduce the chances of inbreeding depression. To introgress the transgene into a diverse genetic background, the clonal propagation ability of ryegrass was used to allow for the simultaneous pair-crossing of a single transgenic event to a number of selected genotypes. Using multiple pair-crosses allowed for the introgression of the transgene into a range of genetically diverse genotypes of single/multiple breeding population/s. This crossing step was also used to integrate a novel fungal endophyte in the T_1/F_1 progeny, by only collecting seed from the recipient endophyte-containing maternal parents.

The introduction of a transgene into a recipient genome is complex and can potentially alter the transgene expression levels, depending on a number of factors, including the transgene integration site, transgene duplication, deletion, repeated sequence recombination, rearrangement, and gene interactions [10,52]. It is, thus, critical to screen the T_1 progeny for transgene expression and to evaluate the trait under field conditions. To reduce the number of T_1 progeny that requires screening, GEBVs can be used to select the T_1 progeny with the desired genotype first and then only screen those plants for transgene expression. In this study, transgenic T_1 progenies derived from three primary T_0 events were screened under field conditions, as GEBVs were not available.

A sub-selection of transgenic T_1 plants from each transgenic event can be used in a poly-cross to create T_2/F_2 progeny. The T_2/F_2 progeny should exhibit a 1:2:1 segregation ratio for the transgene as all T_1s used in the poly-cross would have been heterozygous for the transgene. T_2 progeny, homozygous for the transgene, can be used as *Syn*0 parents in a synthetic breeding program. Choices in regards to which event to deregulate can be made at this point. If the trait is deregulated, the progeny of the *Syn*0 parents could be used in a recurrent breeding nursery or genomic selection nursery, where sub-selections can be made for varietal development.

6. An Optimum Transgenic Breeding Strategy in *Lolium* Grasses that Is Compatible with Genomic Selection

An optimised transgenic breeding strategy in *Lolium* grasses will now be outlined. This strategy will address all of the abovementioned requirements, while making use of new technologies that have come to light in the last few years. This transgenic breeding strategy for *Lolium* grasses will allow for an efficient method to deploy transgenes into the wider perennial ryegrass breeding population for commercial cultivar development. This strategy is adapted from a genomic selection strategy described by Hayes et al. [16] (Figure 1) and reviewed by Simeão Resende et al. [17] and assumes that the genomic selection program for non-transgenic breeding material is well established, and that accurate GEBV's can be predicted on selection candidates for important traits, such as biomass yield, nutritive quality, and persistence in a sward [16,17]. With these basic genomic selection steps, a transgenic breeding strategy can be simplified to the following steps:

Year 1:

(i)   **Selection of TCR and agronomically superior genotypes for transformation—Figure 1, number 1.**

   Selection of superior genotypes from the genomic breeding nursery (Figure 1A) for transformation, based on genomic predictions of performance for a range of agronomic traits, as well as the TCR trait that is required both for callus induction and callus regeneration during transformation.

(ii)   **Creation of primary T_0 transgenic events in *Lolium* grasses through targeted genome editing—Figure 1, number 2.**

Generation of primary T_0 events through targeted genome editing technology. Genome editing can accurately delete, substitute, or add nucleotides at a specific site within the genome [35]. This technology has distinct advantages over *Agrobacterium*-mediated transformation and biolistic transformation in that the transgene integration is site specific, which has the potential to reduce insertional mutagenesis, complex integration patterns, and multiple copy insertions [35]. It will also reduce the burden of identifying and characterising insertion sites for deregulation purposes, as the integration site is known and targeted for [35].

(iii) Application to Regulator for the limited release of transgenic *Lolium* grasses for evaluation.

Transgenic technology is governed by the Gene Technology Act 2000 and regulated by the Office of the Gene Technology Regulator in Australia. A licence by the Regulator is required for the intentional release of a transgenic event into the environment.

Year 2–3:

(i) Evaluation and selection of primary transgenic T_0 events in *Lolium* grasses—Figure 1, number 3.

Phase A: Pre-screening of T_0 events for transgene expression and qualitative traits under containment conditions, if possible.
Phase B: Establishment and evaluation of pre-screened T_0 events in a clonally-replicated, space-planted field trial for agronomic performance and performance of the targeted trait.
Phase C: Select 5–10 primary T_0 events for progression, based on "transgene traits" and transgene expression levels under field conditions. Data is based on phenotypic evaluation.

(ii) Selection of recipient genotypes to cross with the primary T_0 transgenic events—Figure 1, number 4.

Selection of genetically-diverse recipient genotypes from the genomic breeding nursery (Figure 1, box A) based on genomic predictions of diversity and performance for a range of traits and endophyte presence.

Year 4:

(i) Pair-crossing of recipient genotypes and primary T_0 transgenic events—Figure 1, number 5.

Four to eight genetically-diverse recipient genotypes (with endophyte) are to be pair-crossed to each of the selected T_0 events (5–10 events). Harvest seed from each recipient parent (endophyte present) as a half-sib family.

(ii) Selection of T_1 progeny for trait stability and agronomic performance.

Phase A: Screen all half-sib families for transgene presence. Transgenic events that exhibit a monogenic Mendelian segregation ratio of 1:1 for the transgene can be progressed. Using targeted genome editing, trait inheritance and trait expression should be more predictable in the progeny compared to alternative transformation methods.
Phase B: T_1 plants from selected events can be screened using genomic predictions for a range of traits/all traits. 4–6 T_1 plants that are genetically-diverse and show high transgene expression, can be selected for synthetic cultivar development through poly-crossing.

(iii) Poly-crossing of selected T_1 events—Figure 1, number 6.

Phase A: Poly-crosses are made for each selected transgenic event (3–5 events). All crosses are 4–6 parent synthetics. Seed should be harvested from each parent as a half-sib family. Molecular analysis for each half-sib family should indicate a segregation ratio of 1:2:1 for the transgene in the T_2/F_2 progeny.

Phase B: T$_2$ progeny homozygous for the gene of interest GOI can be used as Syn0 parents in a synthetic breeding program. The *Syn0* seed from the 3–5 transgenic events can progress to mini-sward evaluations.

Year 5–6:

(i) **Mini-sward field evaluation of *Syn0* progeny for cultivar development—Figure 1, number 7.**

All Phases: Mini swards are sown as *Syn0* half-sib populations. Each of the potential 4–6 parent synthetic populations, (i.e., the 4–6 mini-swards) will need to be observed together for assessment of flowering time uniformity. Phenotypic evaluation of the transgenic trait is measured on a population basis. Choices in regards to which event to deregulate can be made at this point. If the trait is deregulated, the progeny of the *Syn0* parents could be used in a recurrent breeding nursery or genomic selection nursery, where sub-selections can be made for varietal development.

The ability to do whole genome selection has been proven in the cattle industry and although the specific population structures and breeding strategies differ between species the general principles have been used to develop theoretical genomic selection strategies for ryegrass breeding as well [16,53,54]. These technologies have the power to maximise genetic gain whilst reducing the generational cycle time and have become practical within forage breeding [16,17], the result that the potential in commercial breeding programs is being evaluated [13,55]. All of these technologies have enabled a paradigm shift in the way that we evaluate *Lolium* grasses and will ensure a future for both forage development and the grazing industries.
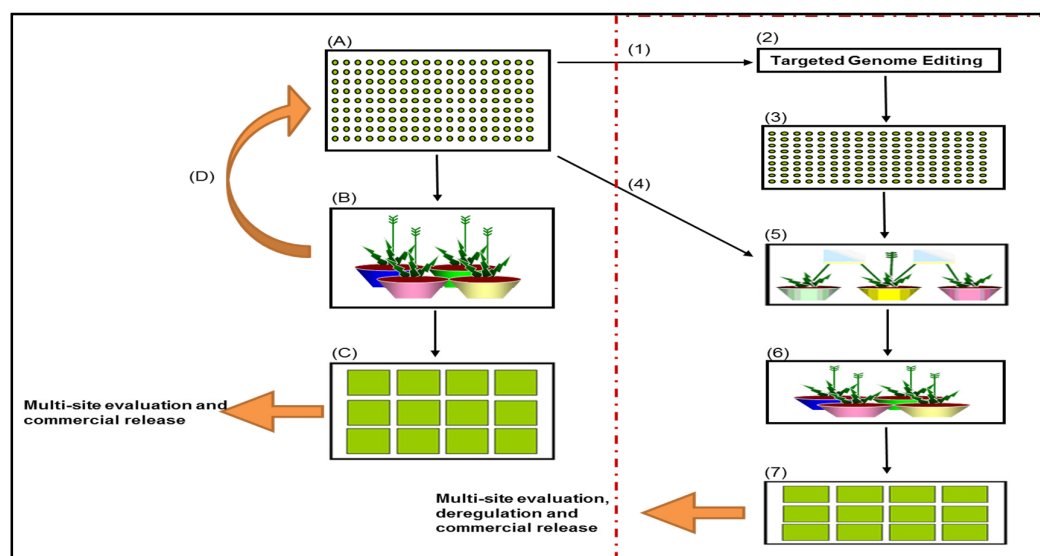

Figure 1. An optimum breeding strategy for transgenic *Lolium* grasses based on a genomic selection approach described by Hayes et al. Sections (**A**–**D**) are the phases of the genomic selection schema as proposed by Hayes et al. Sections 1–7 relate to the transgenic breeding strategy defined in this paper.

7. Conclusions

For novel forage traits to efficiently reach the marketplace, new breeding programs must evolve that optimize the rate of genetic gain, cost of implementation, and are compatible with current commercial breeding practices. Genetic simulation allows the modelling of multiple scenarios before they are implemented, as has recently been demonstrated for genomic selection of perennial ryegrass [13]. Similar challenges exist for transgenic breeding strategies with the added complexity of operating in a regulated environment. However, transgenic technologies offer the

potential to incorporate novel high impact traits into forage breeding programs. To reach this potential value, the technology needs to be incorporated into the wider perennial ryegrass breeding population for cultivar development. The challenges associated with using conventional breeding strategies have been discussed and an optimised transgenic breeding strategy in *Lolium* grasses was formulated, using a genomic selection strategy to deploy transgenes into the wider perennial ryegrass breeding population for commercial cultivar development. The strategy that is proposed is adapted from a genomic selection strategy described by Hayes et al. [16] and is compatible with modern breeding strategies, like F1 hybrid breeding and genomic selection, providing the opportunity for a fully-integrated molecular breeding strategy for perennial forage grasses.

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Abbreviations

The following abbreviations are used in this manuscript:

TCR	Tissue Culture Responsive
T_0	Primary Transformant
T_1	First Generation Transformant
GEBV	Genomic Estimated Breeding Value
GOI	Gene of Interest

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