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Modifying Plant Growth the Cisgenic Way

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As genomics has progressed to include a much wider variety of organisms than the few model species that were widely studied in the past, the ability to use native genomic information for transgenic modification has become widely available. In a report in *Plant Biotechnology Journal* on the first use of cisgenes intended to modify the growth of plants, the authors found that the transfer of entire native genes that play roles in biosynthesis or signaling of gibberellic acids (GAs), including their 5' and 3' proximal regulatory regions, impart changes in growth rate and stature in poplars[1]. This essay summarizes their work and evaluates its possible utility for plant breeding.

What are cisgenes and intragenes?

As transgenic technology matures and diversifies, it is useful to have terminology that reflects its growing diversity. Cisgenes are a subset of intragenes. An intragenic plant, produced by insertion of an intragene, is defined as "a transformed plant that only contains genetic elements derived from within the sexual compatibility group"[2], but does not constrain their order, arrangement, or preclude small changes in sequence or expression. Thus, introduced point mutations, promoter/coding region swaps, and the use of RNAi, amiRNA, or antisense suppression, are all legitimate. In contrast, cisgenes are flanked by their native regulatory regions, including their introns, and thus the gene is truly a part of a conventional breeder's gene pool[3]. In intragenics (but not in cisgenics)[3] in which *Agrobacterium* is used, plant-derived T-DNA border sequences (called P-DNA) that closely resemble *Agrobacterium* border sequences are employed so that the claim can be made that all DNAs inserted are of compatible plant origin in sequence. In addition, selectable marker or reporter genes are not included or are removed after transfer by segregation. Recombinases can also be used for marker gene removal, but they do not fully remove all traces of gene presence (the target recombination sequence). However, as they are similar in length to T-DNA borders, it is also likely that P-DNA-like target sequences can be identified if needed. Cisgenes as well as intragenes add to existing genetic diversity due to "position effects" from their insertion; these modify the intensity and pattern of gene expression as a result of their unique chromosomal position and interaction with regulatory elements[1]. Thus, in both cases, genetic diversity in expression is increased compared to that of the progenitor genes.

Why the interest?

Although breeders of many types of annual crops can make dramatic changes in genetic composition in a short time period, many other kinds of plant species are very difficult to breed. Thus, making use of native genetic variation, especially where strong domestication

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phenotypes are sought, can be very slow and difficult. This is obviously mostly true for woody plants, which do not flower for a number of years, are intolerant of inbreeding, and are highly heterozygous (masking the expression of desired recessive alleles). But it is also true for many other types of plant species, especially when they are naturally sterile or are part of a highly desired and commercially widespread clone whose genotype needs to remain intact. Examples include potato, apple, grape, and banana[4]. For example, intragenesis has been applied to the development of non-browning versions of established apple varieties (such as Gala, Fuji, Golden Delicious, and Granny Smith) by the silencing the polyphenol oxidase gene. These apples (named Arctic™ apples because of the color of their skin) have been developed by Okanagan Specialty Fruits and tested in the field since 2004. The company has already petitioned the Animal and Plant Health Inspection Service (APHIS) for deregulation of the product in the USA[5], and plans to do the same in Canada later in the year.

In addition, desired alleles, such as dominant alleles for size reduction, pest tolerance, or specific fruit or nutritional qualities, can be rare or unavailable. Introgression breeding using genetic transformation for disease resistance can benefit from the avoidance of linkage drag[4], and disease resistance alleles can be more rapidly stacked to provide broader or more durable forms of resistance. Because of the lack of linkage drag, cisgenic plants are likely to be as safe as or safer than those produced with the same genes through traditional or mutation breeding[4]. In forest trees such as American Chestnut that have been devastated by exotic diseases, stacking several resistance alleles obtained from interspecies hybrids via conventional and marker-aided breeding, while also restoring the majority of the American Chestnut genome to promote adaptability, would be a very formidable challenge in the absence of transgenic capabilities.

There are also good social reasons to differentiate cis- and intragenes from conventional transgenes. The concept of transgenic organisms and transgenic food is troublesome for many people, which is reflected by their stringent regulation throughout the world. The public is considerably more comfortable with the idea of a cis/intragenic crop when compared with a transgenic crop[6]. For example, a survey in Mississippi showed that 81% would eat a cis/intragenic vegetable, as compared to only 14 – 23% for a transgenic vegetable [containing genes from non-plant sources][7]. Similarly, a nationwide survey in the United States found that 52 – 77% would eat a cis/intragenic vegetable (depending on number of genes inserted and source of the gene); whereas only 17 – 25% would eat the same vegetable if it contained a gene from a microbe (bacterium/virus/fungus) or an animal[8].

Modification of tree growth using cisgenesis

In a proof-of-concept study in which only the growth-modifying "active ingredient" genes, and not the entire T-DNAs, were cisgenic, it was shown that tree growth and architecture could be significantly modified using GA-associated cisgenes[1]. The main goal of the study was to examine the feasibility of using cisgenes to modify gibberellic acid (GA) action and hence growth and architecture in poplar tree. Gibberellic acid is a plant hormone with a wide variety of

functions in controlling plant growth and development. Five different cisgenes (*GA20ox7*, *GA2ox2*, *GAI1*, *RGL 1_1*, and *RGL 1_2*) were studied, along with empty vector controls and non-transgenic controls. GA20ox is an enzyme that catalyzes the penultimate step in the biosynthetic GA pathway, and thus tends to promote cell division and elongation, whereas the other genes tend to repress or attenuate active GA actions (GA degradation by *GA2ox2*; the other genes were DELLA domain proteins that attenuate GA signals).

Several interesting, statistically significant results were obtained in this study. Plants transformed with GA20ox7 cisgene had a higher rate and frequency of regeneration of transgenic shoots during antibiotic selection. This suggests that this gene might be useful as a general transformation enhancer. It also dramatically promoted early height and diameter growth on transformants grown in the greenhouse; after six weeks from the date of transplantation, the average stem volume of the *GA20ox7* transformed plants increased by 40% compared with the transgenic (empty vector) controls (**Fig. 1**). *GA20ox7* gene expression was also statistically associated with the growth enhancement (**Fig. 2**). In as yet unpublished work, the researchers also showed that the levels of active GAs increased in the transgenic lines. The growth improvement due to the *GA20ox7* gene, however, diminished over time. The authors concluded that this might have occurred due to the rapid growth and limited pot size in the greenhouse.

Figure 1. Comparison of *GA20ox7* transformed plants (left group) to the empty vector controls (right group) after six weeks from the date of transplantation to begin the greenhouse trial. The transgenic plants had an average stem volume that was increased by 40% compared with the controls.

However, it might have also resulted from a transitory effect of the cisgene, such as from stimulation of cell division but not cell enlargement. The faster growing trees had similar internode lengths to the control trees.

The GA inhibitory genes had variable effects, but generally retarded plant growth. Plants transformed with *RGL 1_2* gene had a reduced rate of regeneration of transgenic shoots and a reduction in growth rate, but had longer stem fiber lengths. *GA2ox2* and *GAI1* transformed plants also had semi–dwarf phenotypes in the greenhouse, while *RGL 1_1* plants appeared similar to wild type. *RGL1_1* transformed plants, however, had reduced leaf size. In their discussion, the authors emphasized that the results were preliminary and require verification in field environments and with a greater diversity of genotypes, as would occur with normal plant breeding.

Potential uses of cisgenics

Clearly, cis/intragenics can be used to modify plants similarly to conventional breeding, but in many cases appear to be able to do it faster and with more specificity. This efficiency will often enable applications that would otherwise be impractical and unaffordable because of the costs and time frames involved (e.g., when very rare recessive mutants must be sought, many alleles stacked, or difficult hybrids generated and backcrossed). For trees, the ability to speed breeding by transfer of genes among related species for resistance to pests could have very high value, given the proliferation of pest

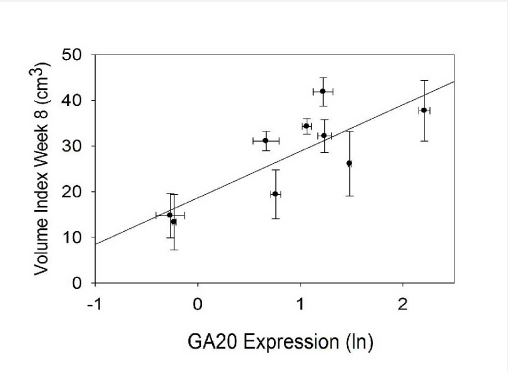

Figure 2. Statistically significant association of growth rate with *GA20-oxidase* gene expression ($p < 0.05$). Bars represent standard errors.

problems in planted and native forests due to climate and other anthropogenic stresses, and the growing proliferation of exotic pest species. Other possible uses include speeding growth rate for bioenergy applications; reducing stature of well-known varieties for ornamental or horticultural applications; improving abiotic stress tolerance by modifying expression of stress tolerance pathway control genes; modification of flowering and induction of sterility; and modification of the quality and nutritional value of ornamental and food products. For example, work is currently underway to produce apples with red flesh[9]. These apples are more pleasing to the eye than the normal apples and contain antioxidants which may provide a direct health benefit to consumers.

Can cis/intragenics avoid the regulatory thicket of transgenics?

Despite many possible uses, the realm of application of cis/intragenics, when compared to transgenics, is highly limited. For example, cisgenics clearly cannot impart new pest tolerance mechanisms, new industrial and pharmaceutical products, or new metabolic pathways to enhance plant nutrition and adaptation. Thus, cis/intragenics should not be viewed as an alternative to transgenics, but as a tool for extension of traditional breeding when dealing with difficult traits and species. It is also a tool with which the public has more comfort and thus might be used with much more freedom and lower cost than transgenics. A strong case has been made for cisgenic plants to come under a new regulatory tier with reduced regulatory oversight or to be exempted from GM regulation[10]. Of the current regulatory systems, to our knowledge, only Australia excludes intragenics from regulation[6]. The Animal and Plant Health Inspection Service (APHIS) in the United States had considered a lower regulatory tier for cisgenic plants in its revised regulations[14], but more recent actions suggest that this proposal is no longer viable. If, instead of improved efficiency, cis/intragenics bring the enormous regulatory, political, and market obstacles of transgenics to what is in essence a modification of conventional breeding, it is unlikely to be pursued for the large majority of potential applications. Unfortunately, some authors have indeed suggested just this[6]. Thus, despite their obvious benefits and high level of familiarity, unless accompanied by regulatory reform, cis/intragenics may be largely avoided rather than embraced.

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