Transformation of Recalcitrant Crops: Progress and Remaining Challenges

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Technologies in transformation and tissue culture, when coupled with classical techniques, could play an increasingly important role in the solutions to challenges in agriculture and the environment. These might include agronomic traits, such as those for pest and stress (cold and drought) resistance, herbicide resistance for stand management, and for value-added traits, such as the introduction or modification of enzymes involved in sugar biosynthesis or the possibility of making other value-added products in sugarbeet. However, before such technologies can be used effectively for agronomic improvement significant challenges exist in genetic engineering of crop species. New insights into these problems can be gained by studying those encountered with other recalcitrant species. Many of the same issues and areas of research for cereals are the same as for sugarbeet biotechnology. These include (but are not limited to): Lack of reproducible and efficient transformation systems, which include:

1. Identification of reliable selection schemes
2. Development of compatible gene introduction methods
3. Genotype limitations
4. Lack of regenerability of transformed tissue
5. Transgene instability
6. Inactivation of transgene expression
7. Somaclonal variation

Cereals are an important food crop; however, they were not the first crop to be transformed. Dicotyledonous plants were first and utilized *Agrobacterium*-based transformation methods that focused on using totipotent leaf tissue. But the application of these methods to cereals was ineffectual. First, because leaf tissue in cereals is not totipotent and will not give rise to division-competent embryogenic cells. This problem was solved by utilizing other explants for transformation, such as embryos or anthers. Second, the utilization of *Agrobacterium* was ineffectual. This was solved by going to more direct methods of DNA introduction, such as electroporation and bombardment. Thirdly, selection methods used on dicots, namely the use of antibiotic resistance genes, led to problems with cereals either because their use did not result in the survival of transformed tissue or because it adversely affected regenerability of the transformed tissue. The use of the herbicide resistance gene, *bar*, proved very valuable in early transformation efforts with cereals. Although each of these problems was an obstacle in the initial stages of transformation efforts each has been overcome and in fact, once reproducible transformation efforts were perfected, the latter two problems have been revisited and found to be non-issues. That is, *Agrobacterium* has been found to be useful in cereals and antibiotic resistance genes can be used effectively. The issue of explant and totipotency at least for cereals still remains. Genotype limitations still remain, although in barley we have made much progress in this arena. The progress relates to strides made in identifying and being able to manipulate tissues from

recalcitrant genotypes. For example, for some barley genotypes, embryos plated to generate callus tissue for bombardment or selection do not respond very well. Few embryos form callus and the majority of callus is not embryogenic (incapable of leading to regeneration of plants). However, it is usually possible by manipulating culture conditions (hormones, embryo size, other media components, temperature, light) to increase the quantity and quality of the tissue. In thinking about transformation success it is important to remember that it is really a numbers game. Only a small number of cells will receive exogenous DNA (either by bombardment or *Agrobacterium* infection) and only a certain percentage of the cells are capable of sustained division and embryogenesis. Therefore, whatever you can do to improve either will improve your chances of success. In barley, once we succeeded with a model cultivar, we focused on recalcitrant, commercially important genotypes attempting to optimize their culturability and regenerability. Coupling this advance with changes in gene delivery and selection, we were able to succeed in transformation with these recalcitrant varieties. I believe that this will also prove to be true with other recalcitrant crop species. The next issue we were forced to address in barley was somaclonal variation, which is the propensity of plants regenerated from in vitro growth to accumulate heritable mutations. These mutations might be highly visible, chlorophyll-less sectors, shortened plant height, or more subtle effects like lowered plant yield. This phenomenon occurs in all systems to differing extents. What did this look like in barley? When transgenic plants, second and fourth generation, were taken to the field and compared to their wild-type siblings for agronomic performance, 100-seed weight, yield and plant height, the transgenic plants were severely affected. This can be solved by outcrossing the transgenic plants with wild-type germplasm for most species, but this can be tedious and time-consuming. We are approaching this problem through the utilization of a different culturing system which we suspect might minimize the mutation frequency.

One of the additional issues that compounds the problem of inefficiency of transformation and somaclonal variation is transgene instability. What does this mean? Instability can arise in two forms: the gene itself can be lost in future generations or the ability to express the gene can be lost. Either can be disastrous for the commercial potential of transgenic crops. Physical introduction of exogenous DNA by microparticle bombardment usually results in multiple copies of the introduced DNA being inserted, frequently leading to gene inactivation and instability. But this also occurs in Agrobacterium mediated transformation and likely results from the fact that genomic DNA in plant species is heavily methylated. Obviously genes are expressed in plants and they are stable but it means that we must understand the basic biochemistry and biology of the system before we can exploit it fully. Because of the presence of heavily methylated areas of DNA, in some species, e.g. 80% in wheat and barley, it is necessary to find methods to deal with this. My laboratory is approaching this by using certain mobile genetic elements, transposons, from maize which are known in that species to have a preference for transcriptionally active DNA. We are attempting to use these elements to deliver our favorite gene to a transcriptionally active area. Others are working on utilizing homologous recombination systems from other organisms, such as the cre/lox system from bacteria. In this system, a bacterial recombinase, *cre*, is being used to target genes to precise locations in the plant genome demarcated by the lox sites. This will permit researchers to identify empirically regions of the genome that support stable integration and expression of the transgene and then to use that site, marked by lox sites, to serve as the location for integration of other transgenes through homologous recombination.

In summary, genetic engineering of other recalcitrant crops has made significant progress in the past few years in solving many of the early problems involved in the process. However, significant problems remain in order to be able to utilize the technologies most efficiently for commercialization. Sugarbeet research does not have to solve all of these problems since the problems and their solutions are in general very similar. What we learn in cereals will help solve problems in sugarbeet. What is needed is a dedication to performing the basic biological, biochemical and genetic work in sugarbeet that will form the basis for the genetic manipulations of the future. It is imperative that we understand specific processes in detail in particular crops in order to be able to manipulate them effectively. This is the real challenge facing us in manipulating crop species through genetic engineering in the future.