

Either/or selection markers for plant transformation

Ortrun Mittelsten Scheid

The use of D-amino acid oxidase as a marker gene allows positive or negative selection of plants that have incorporated transgenic DNA.

Identifying plants that have successfully incorporated transgenic DNA is somewhat akin to finding a transgenic needle in a non-transgenic haystack. Until now, plant biologists have commonly relied on positive selection of a marker gene—usually cotransferred with a transgene of interest—that provides cells integrating the DNA with a growth advantage over nontransformed cells under selective conditions. In this issue, Erikson *et al.*¹ present a novel marker system that is based on the metabolism of D-amino acids (mirror images of L-amino acids). Their system allows both positive and negative selection, expanding the choice of markers available for creating transgenic plants.

In contrast to the increase in number of transformable species, the choice of widely applicable marker genes has not expanded significantly. Traditionally, selection has been carried out with a handful of antibiotic- or herbicide-resistance genes², most of which, whether justified or not, have become infamous for their potential danger to health or the environment. In addition, repetitive use of these same markers hampers the combination of transgenes and sequential transformation and also increases the risk of unwanted mutual gene silencing.

As an alternative approach, Erikson *et al.* have generated transgenic *Arabidopsis thaliana* plants expressing D-amino acid oxidase (DAAO) from the *DAO1* gene of the yeast *Rhodotorula gracilis*. This enzyme catalyzes deamination of several amino acids with stereospecificity for their D enantiomer (the mirror image, at the asymmetric carbon atom, of the L configuration). Erikson *et al.* have demonstrated that development of non-transgenic *A. thaliana* seedlings is arrested soon after germination in the presence of D-serine or D-alanine, whereas plants expressing *DAO1* detoxify these amino acids and grow without developmental abnormalities. In contrast, D-valine or D-isoleucine are non-

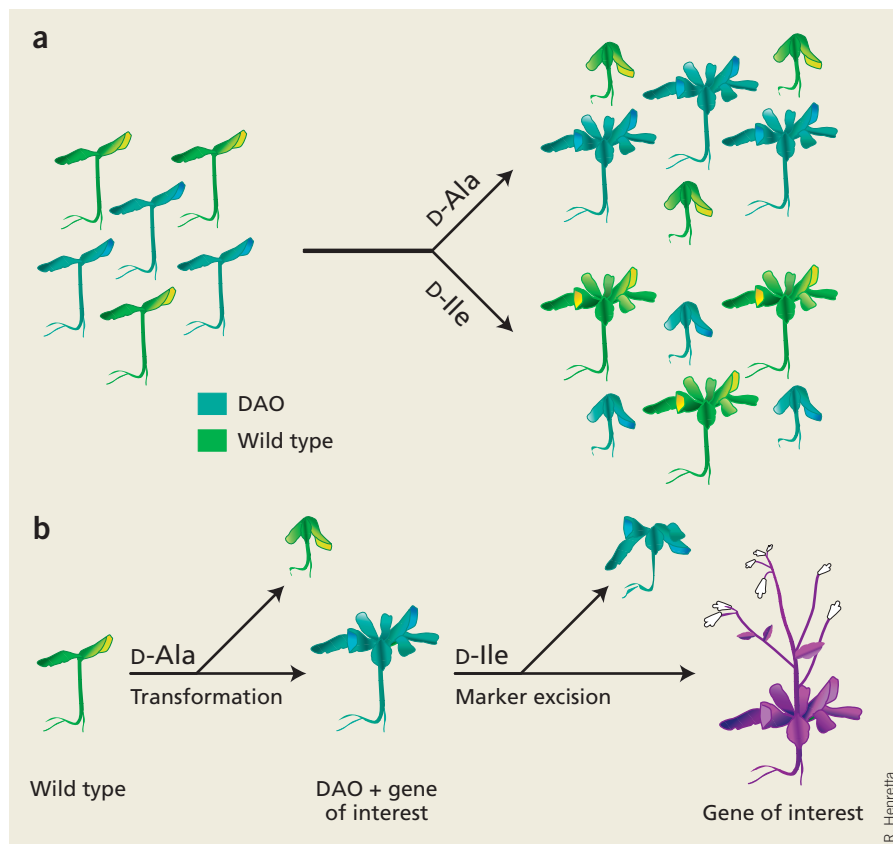


Figure 1 The potential of the D-amino acid oxidase (DAAO)-based selection system. (a) Growth of nontransgenic wild-type plants lacking DAAO activity is inhibited by D-amino acids such as D-alanine (D-Ala) but is not affected by others such as D-isoleucine (D-Ile). In contrast, plants expressing the transgenic *DAO1* gene detoxify D-Ala and survive (positive selection), whereas they metabolize D-Ile to toxic compounds that kill the plants (negative selection). (b) Hypothetical application: plants that have integrated a gene of interest together with the *DAO1* marker are first detected by positive selection. Subsequent negative selection identifies plants from which the no-longer-desirable selection marker has been removed (e.g., by genetic segregation or site-specific recombination systems), leaving the gene of interest as the only transgenic sequence in place.

toxic to wild-type plants but kill plantlets transgenic for *DAO1*. Thus, the same transgene product allows both positive and negative selection, depending on the selective agent applied (Fig. 1a).

This system has obvious advantages. Namely, selective agents can be applied by spraying on to soil-grown seedlings—a procedure that once made the bialaphos resistance genes (*PAT*, encoding phosphinothricin-N-acetyl transferase, or *BAR*, encoding bialaphos resistance) and their selective agent

phosphinothricin superior, for many purposes, to the antibiotic-resistance markers encoding neomycin phosphotransferase (*NPTII*) and hygromycin phosphotransferase (*HPT*)¹. Additionally, the natural levels of D-amino acids and other DAO substrates in plants do not appear to be a problem, because even plants with high DAAO expression are indistinguishable from transgene-free plants under nonselective conditions. Selection rates, and thus transformation, using the *DAO1* gene and D-Ala and D-Ser selection are

Ortrun Mittelsten Scheid is at the Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Dr. Ignaz Seipel-Platz 2, A-1010 Vienna, Austria. e-mail: ortrun.mittelsten_scheid@gmi.oeaw.ac.at

in the same range as those obtained with the well-established *NPTII* gene and kanamycin selection.

But the trump card of DAAO could prove to be its combination with site-specific excision systems: after positive selection of transgenics followed by initiation of recombination, a switch to negative selection could verify the removal of the now redundant marker (Fig. 1b). This scheme has yet to be demonstrated, however, and claims of panaceas should be postponed until the functionality of the selection principle in other plant species has been proven.

Although *A. thaliana* appears to lack DAAO, we know little about endogenous DAAO enzyme activity in other plants, or about the distribution of its substrates among plants (and plant eaters). The enzyme shows a high specificity for the D enantiomer, but its substrate specificity is less strict and side effects of overexpression will have to be carefully excluded from recipient plants.

Nevertheless, the marker gene is of eukaryotic origin and encodes one of the best-known enzymes. The activity of DAAO was discovered more than 60 years ago, and it is a model flavoprotein for which the catalytic

mechanisms and a three-dimensional structure have been described in detail^{3,4}. D-amino acids, although not incorporated into proteins and peptides of eukaryotes, are present in natural and processed food⁵ and are probably easy to handle safely under laboratory conditions.

The potential of the *DAOI* marker will trigger rapid, broad trials in various transformation systems in combination with different regulatory elements, provided that the use of *DAOI* is not inhibited by lengthy regulatory negotiations. The results of such experiments will also allow thorough examination of biosafety aspects. If the selection principle can be transferred to crop plants and subsequent integration and excision prove feasible, we will have gained an elegant and important new tool for the plant biotechnology workshop.

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On the road to therapeutic cloning

Teruhiko Wakayama

Human embryonic stem (ES) cells have been derived from cloned embryos, an important step in the development of therapeutic cloning.

The political tussles surrounding human ES cells have overshadowed discussion of the scientific challenges that lie ahead if the promise of ES cells for regenerative medicine is to be realized. A recent report by Hwang *et al.*¹ in *Science* of ES-cell derivation from a cloned human embryo invites reflection on the obstacles that remain in the development of this technology.

Human ES cells are envisioned as an unlimited source of cells for cell replacement therapies. However, as with any allogeneic material, ES cells derived from fertilized blastocysts, and the progeny of such cells, risk immune rejection after transplantation. It has

been proposed that ES cells derived from embryos cloned from a patient's own cells represent a solution to the problem of rejection, as any replacement cells would be genetically identical to the patient. The work of Hwang *et al.* provides an important proof of principle of the feasibility of creating human ES cells using a patient's own cells as a source. But many challenges remain, including reprogramming without induction of abnormalities, induction and targeting of differentiation, the control of stem cell proliferation and the dilemma of persistent re-rejection in cases of autoimmune disease.

The success of Hwang *et al.* did not rest on a major technical breakthrough; rather, it resulted from a persistent and painstaking optimization of the many seemingly minor, but actually critically important, experimental factors involved in mammalian cloning. A similar experiment was attempted in the United States in 2002, but in that study the

cloned embryos showed abnormalities and none was viable past a very early developmental stage². At the same time, it should be recognized that another key to the achievement of Hwang *et al.* may lie in the enthusiasm with which research on regenerative medicine is greeted in South Korea, which gave the researchers access to the 242 human oocytes used in the study and allowed them to conduct their work in a relatively permissive regulatory environment—a much more favorable climate than in many countries in Europe and North America.

As has been the case with some previous reports of experimental cloning, commentators may challenge Hwang *et al.*'s ES cells as the products of experimental error, suggesting that they may be parthenogenetic in origin rather than derived from a *bona fide* cloned embryo. Those with experience in cloning know, however, that the chance of such an error occurring is vanishingly small. Nuclei are always positioned under the polar body, and it is no more likely for a scientist to misidentify a failed enucleation than for a professional chef to miss the fact that a yolk and egg white have failed to separate. Indeed, Hwang *et al.* confirmed enucleation by Hoechst staining and UV irradiation, which in the end may have actually damaged the cloned embryos and worked to lower their success rate. They report that only one line of ES cells was established from 30 original blastocysts, giving a success rate of 3.3%. Moreover, the donor somatic cell and oocyte were taken from the same individual, a requirement that, if not overcome through technical advances, would mean that only patients possessing a supply of healthy oocytes could benefit from therapeutic cloning.

Although the technique of generating somatic cell-derived ES cell lines by nuclear transfer (which we refer to as ntES cells) is still relatively new, studies in mice have shown that ntES cells can be established using nuclei from fibroblasts—a plentiful and nearly ubiquitous source—and, furthermore, that such ntES cells can be derived at efficiencies of 10–20% using cells from donors of either gender and from lines that have never been successfully used to produce a live-born cloned individual³ (Fig. 1). If the same proves true for humans, improvements in therapeutic cloning techniques may enable any patient to act as his or her own ntES cell donor. In mice, success rates for deriving ntES cell lines can be as much as an order of magnitude higher than those for reproductive cloning, suggesting that the scientific barriers to therapeutic cloning may be lower than the barriers to creating a new individual by cloning.

*Teruhiko Wakayama is at the Center for Developmental Biology, RIKEN, 2-2-3 Minatogima Minamimachi Chuo ku, Kobe, Hyogo 650 0047, Japan.
e-mail: teru@cdb.riken.jp*