Progress toward the production of transgenic grapevines by *Agrobacterium*-mediated transformation

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Summary: Grape possesses the basic prerequisites for *Agrobacterium*-mediated transformation – it is a host for *Agrobacterium* and plant regeneration can be induced from cultured grape explants. Leaf explants were cocultivated with disarmed *Agrobacterium* vectors carrying kanamycin resistance and GUS genes and cultured on shoot-inducing medium containing kanamycin. After 21 d, intense and sharply-defined blue regions were observed, including some blue organized meristematic structures, consistent with plant-driven GUS gene expression. No GUS activity was detected in control explants. Among single leaf tips excised from over 200 regenerated shoots, one was GUS positive. The recovery of transgenic shoots might be improved by increasing the frequency or modifying the site of transformation and/or regeneration.

Key words: transgenic grapevine, Agrobacterium, crown gall, tissue culture, kanamycin resistance, β-glucuronidase, gene expression, genetic engineering, genetics.

Introduction

Of the several different transformation strategies that have now been successfully employed to produce transgenic plants, the most common is *Agrobacterium*-mediated transformation (KLEE et al. 1987). This approach exploits the natural ability of this bacterium to introduce genes into plant cells. *Agrobacterium* strains from which the disease-causing properties are removed ('disarmed') can be endowed with new genes of academic or agricultural interest from virtually any other organism. When such a strain is allowed to infect a plant cell, the new gene is introduced and integrated into the plant's own genetic material and becomes a permanent part of the genotype of the recipient plant cell. Because the descendents of such a transformed cell inherit the new gene, a plant regenerated from transformed cells will be transgenic: it will carry the new gene in every cell.

The basic requirement for successful *Agrobacterium*-mediated transformation is an explant that is both transformable and regenerable. The plant species must be a host for *Agrobacterium* and the particular explant tissue used must be susceptible to infection. The recovery of a transgenic plant is dependent on a means to regenerate an entire plant from the explant. These requirements have been fulfilled in systems as diverse as tomato leaf disks from which adventitious shoots develop (HORSCH *et al.* 1985) and walnut somatic embryos that produce secondary embryos on their surface (MCGRANAHAN *et al.* 1988). Grape (*Vitis vinifera* L.) is a well-known host for *Agrobacterium* and, in fact, crown gall disease is a serious viticultural problem (PEARSON and GOHEEN 1988). Entire plants can also be regenerated from a variety of grape explant types via either adventitious shoots or somatic embryos (MONETTE 1988). Because the basic requirement is met, it is reasonable to expect that *Agrobacterium*-mediated transformation can be successfully adapted to grape.

The AGROBACTERIUM strains associated with grapevines in nature are predominantly of the biovar 3 type (PEARSON and GOHEEN 1988). However, the strains generally used as plant gene vectors are biovar 1, a distinctly different group with different properties (KNAUF *et al.* 1982). To establish whether the biovar 1 strains in general use might be employed as gene vectors for grape, we investigated the ability of several biovar 1 strains to incite galls on *in vitro*-grown plants of

Results of a typical cocultivation experiment with 'Thompson Seedless' leaf explants. Data was collected after 15 d of incubation, at which time all tissue was sacrificed for analysis and none was allowed to develop further

	Number of explants	Percentage
Total	193	
Producing adventitious buds and shoots	120	62
Containing GUS-positive regions	183	95
GUS expression in meristematic structures	14	7
Structures partially GUS-positive	7	4
Structures entirely GUS-positive	7	4

several *vinifera* cultivars. Although differences were observed among strains, galls were produced on all cultivars and the presence and expression of introduced genes could be detected in gall tissue, thus demonstrating that biovar 1 *Agrobacterium* strains could infect and transform grape cells (MARTIN 1987).

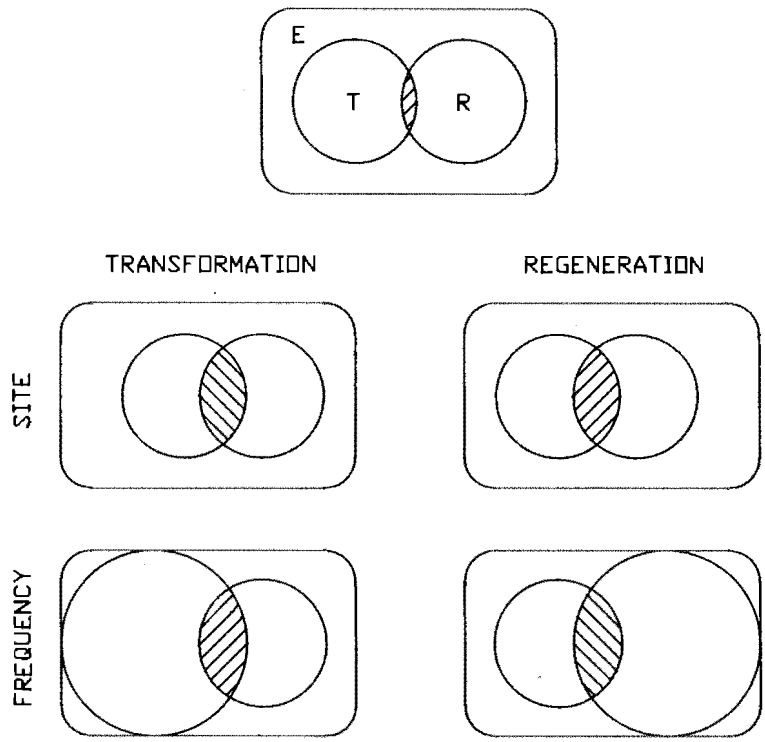
Materials and methods

Employing disarmed *Agrobacterium* vectors carrying chimeric genes encoding aminoglycoside phosphotransferase (kanamycin resistance) and β-glucuronidase (GUS), an enzyme whose activity can be detected as a blue color in a histochemical assay (JEFFERSON *et al.* 1987), we conducted a series of studies with leaf explants from which the development of adventitious shoots can be induced. Leaves of *V. vinifera* cvs Thompson Seedless (syn. Sultanina) and French Colombard were obtained from *in vitro* nodal cultures and either cultured intact or bisected perpendicular to the midrib. Under the appropriate conditions, this explant type will produce adventitious shoots on the petiolar stub and at the basal end of severed veins (STAMP *et al.* 1989). Leaf explants were cocultivated with *Agrobacterium* and then incubated on shoot-inducing medium containing carbenicillin and kanamycin. At periodic intervals, explants were sacrificed and sectioned for histochemical analysis of GUS activity. Other explants were incubated until adventitious shoots developed. A single leaf-tip was removed from each adventitious shoot for GUS assay and the shoots were also excised and tested for their ability to root in kanamycin-containing medium.

Results and discussion

Analysis of GUS expression by both stereo- and compound microscopy at 4 d post cocultivation revealed diffuse pale blue patches on the lamina and petiole regions and within the leaf veins, suggestive of *Agrobacterium*-driven GUS gene expression. After 11 d, however, intense and sharply defined blue-staining regions could be seen with the aid of a stereomicroscope. Closer examination of these regions with a compound microscope revealed both individual cells and groups of cells containing dark blue cytoplasm, consistent with plant-driven GUS expression in clones of transformed plant cells. Many of these regions were internal but some occurred on the explant surface. Infrequently, organized meristematic structures associated with the early stages of shoot formation were observed that were either partially or entirely intensely blue-staining, suggesting that, in these explants, cells that can be transformed (or their descendants) can also participate in regeneration (Table). No GUS activity was detected in control explants that had not been cocultivated with *Agrobacterium*.

Explants that were allowed to continue until adventitious shoots formed (28-56 d) exhibited bleaching typical of kanamycin toxicity. Islands of green tissue and, less commonly, green shoots were observed on some cocultivated explants. Histochemical analysis of leaf tips from over 200 of these green shoots revealed one GUS-positive leaf tip. The putatively transgenic shoot from which this leaf tip was obtained was destroyed in a laboratory accident before a Southern analysis could be performed.



Improving the recovery of transgenic plants. The relative number of cells in the explant (E) that participate in both transformation (T) and regeneration (R) is indicated by the shaded area. Increasing the frequency or changing the site of either transformation or regeneration will increase the size of the shaded area.

Although the approach employed here possessed the two requisite components – an explant that is both transformable and regenerable – it has not yet resulted in the production of transgenic plants at an acceptable frequency. There are a number of avenues by which the results might be improved.

A transgenic plant is the result of the coincidence of transformation and regeneration in the same cells (Fig.). The larger the number of transformed cells that also participate in regeneration, the higher the probability of recovering a transgenic plant. Both transformation and regeneration are phenomena that are amenable to manipulation. Critical parameters of both processes are frequency and site. An increase in frequency or a change in the site of either transformation or regeneration may increase the size of the coincident cell population (Fig.). The site and/or frequency of transformation might be improved by choice of *Agrobacterium* strain, cocultivation conditions, or explant type. The site and/or frequency of regeneration might be modified by choice of explant or culture conditions. A histological study of adventitious shoot development from leaf explants has revealed that the coincidence of transformation and regneration is likely very small under our conditions (COLBY, S. M.; JUNCOSA, A.; STAMP, J. A.; MEREDITH, C. P.; in preparation) and we are currently investigating several approaches by which we hope to improve our success.

Literature cited

HORSCH, R. B.; FRY, J. E.; HOFFMANN, N.; EICHHOLTZ, D.; ROGERS, S. G.; FRALEY, R. T.; 1985: A simple and general method for transferring genes into plants. Science **227**, 1229-1231.

JEFFERSON, R. A.; KAVANAGH, T. A.; BEVAN, M. W.; 1987: GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901-3907.

KLEE, H.; HORSCH, R.; ROGERS, S.; 1987: *Agrobacterium*-mediated plant transformation and its further applications to plant biology. Ann. Rev. Plant Physiol. **38**, 467-486.

KNAUF, V. C.; PANAGOPOULOS, C. G.; NESTER, E. W.; 1982: Genetic factors controlling the host range of *Agrobacterium tumefaciens*. Phytopathology **72**, 1545-1549.

MARTIN, L. A.; 1987: Genetic Transformation and Foreign Gene Expression in Tissue of Different Woody Species. Master of Science Thesis, Univ. of California, Davis, USA.

MCGRANAHAN, G. H.; LESLIE, C. A.; URATSU, S. L.; MARTIN, L. A.; DANDEKAR, A. M.; 1988: *Agrobacterium*-mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Bio-Technology **6**, 800-804.

MONETTE, P. L.; 1988: Grapevine. In BAJAI, Y. P. S. (Ed.): Biotechnology in Agriculture and Forestry, Vol. **6**, Crops II, 3-37. Springer-Verlag, Berlin.

PEARSON, R. C.; GOHEEN, A. C. (Eds.); 1988: Grape Disease Compendium. American Phytopathological Society, St. Paul, Minnesota, USA.

STAMP, J. A.; COLBY, S. M.; MEREDITH, C. P.; 1989: Direct shoot organogenesis and plant regeneration from leaves of grape (*Vitis vinifera* L.). Plant Cell Tissue Organ Culture (submitted).