Review Article

Generating transgenic plants by minimal addition of exogenous DNA – a novel selection marker based on plant tubulins

Peter Nick[*1]**, Paul Christou**[2] **and Diego Breviario**[3]
[1]Institut für Biologie II, Schänzlestr. 1, D-79104 Freiburg, Germany
[2]Institut für Molekularbiologie und angewandte Ökologie, FhG/IME,
Grafschaft, Auf dem Aberg 1, D-57392 Schmallenberg, Germany
[3]Istituto Biosintesi Vegetali, CNR, Via Bassini 15, I-20133 Milano, Italy
Tel: +49 761 203 2646/2669
Fax +49 761 203 2612
Email peter.nick@biologie.uni-freiburg.de

Abstract

Most transgenic crops available on the market contain antibiotic resistance genes as selection markers. Although scientifically not justified, this raises public concern about a potential spread of antibiotic resistance genes in soil and intestinal bacteria, possibly accentuated by the agricultural use of transgenic crops. These markers are necessary to generate the transgenic crop, but they are dispensable for the agricultural use of these plants. We propose a selection system for plant transformation that is exclusively based on genetic information already present in the host plant and that does not require antibiotic resistance genes. The approach exploits the natural variability of plant tubulins as essential components of cell growth, cell division and stress tolerance in plants. There exist a panel of tubulin promoters that can drive expression either constitutively or, alternatively, in different tissues or in response to different signals. They can be fused with a panel of different tubulin isotypes with distinct physiological features to produce a versatile kit of combinations that will confer distinct properties such as stress or herbicide tolerance to specified target tissues. This is used to construct a selection marker that is based upon truncated tubulins lacking the binding site for aryl-carbamate herbicides. The expression of this truncated tubulin is driven by the homologous tubulin promotor within a DNA fragment free of exogenous sequences (minimal expression unit) using *clean DNA* technology. The suitability of this selection system is demonstrated for rice as a model system for Graminaceous crops.

Selection markers and the public perception of GM plants in Europe

The production of new transgenic crops is currently limited by low transformation efficiencies. Moreover, it is difficult to regenerate transformed plants from dedifferentiated cells. Therefore, efficient selection is vital. Routinely, genes that confer resistance to antibiotics such as kanamycin, hygromycin or neomycin are utilized for this purpose and most of the transgenic crops that have been commercialised to date invariably contain antibiotic (or herbicide) resistance marker genes.

These markers raise public concern about potential risks of horizontal gene transfer to soil or intestinal bacteria. Although the actual risk that antibiotic resistance might spread in bacteria due to the agricultural use of transgenic plants is generally estimated to be fairly low, if existing at all, it is difficult to actually quantify this risk. Therefore, this issue has become a central element in the public debate concerning "green biotechnology". Public sensitivity is especially high when antibiotics such as kanamycin or amikacin are involved, which are of medical importance in the treatment of otherwise resistant pathogens.

Irrespective of the actual risk posed by transgenic crops, the development of new selection systems that work independently of

---

[*]Author for correspondence.

antibiotic resistance genes is a key element for the public acceptance of green biotechnology in general.

What would be the ideal selection marker for genetic transformation?

Selection markers can be designed either for positive or negative selection. For positive selection, the marker confers a new trait that allows the transformed cell to survive on a minimal medium that otherwise would not support survival. For instance, a gene for bacterial xylose isomerase allows a transformed plant cell to grow on agar where sucrose has been replaced by xylose (Haldrup *et al.,* 1998). In the commonly used negative selection, the transformed cell acquires resistance to an otherwise toxic substance that will kill all untransformed sister cells. For instance, the *PAT* gene from *Streptomyces viridochromogenes* allows the transformed cell to metabolise phosphinothricin, a product of the herbicide glufosinate (Dröge *et al.,* 1992)

Recently, it has become possible to remove marker genes once they have become dispensable by using inducible promoters that lead to the excision of target sequences (Zuo *et al.,* 2001). However, the induction is difficult to handle, leading to chimaeras of marker-free and marker-containing cells that in addition would contain genetic material (estrogen receptor, recombinase), whose effects and/or risks are difficult to assess.

An ideal selection system should therefore fulfil the following requirements:

Minimal risk by minimal manipulation: undesired side-effects for the environment or consumers are avoided by eliminating everything which is superfluous in terms of function.

Optimal use of natural resources: only DNA already present in the crop plant is used and its potential exploited simply by recombining genes and promoters in a novel manner.

The versatile tubulin molecular kit – exploiting the natural potential of tubulins

Plant tubulins exist in numerous isotypes that convey different but essential functions during plant life such as signal-triggered morphogenesis, axial cell division, resistance to abiotic stresses and non-host pathogen resistance. They are driven by a relatively large number of different promoters with different activity patterns in relation to development, tissue and signal specificity and they are targets for antimitotic herbicides, which can be used for selection. The versatility of plant tubulins combined with their strong impact on many aspects of plant life are ideal preconditions for biotechnological manipulations that are both subtle and powerful (for review, see Breviario and Nick, 2000).

Point mutations in individual tubulin genes can lead to a resistance against antimitotic herbicides. For instance, a tubulin mutant from the goosegrass *Eleusine indica* has been shown to confer tolerance to dinitroaniline herbicides (Anthony *et al.,* 1998). However, the respective mutation is situated relatively far from the C-terminus, in a region of essential tubulin functions.

In contrast, resistance based on mutations in the C-terminus of tubulins, where a high natural variability is observed, would open a large field for engineering new selection markers. The aryl carbamate herbicides such as phenyl urethane, propham or chlorprophame that have been used extensively as sprouting inhibitors and pre-emergence herbicides (Bartels and Hilton, 1973) bind to the C-terminus of α-tubulins. They can be coupled to sepharose and used to purify plant tubulins. Moreover, it is possible to discriminate between subpopulations of α-tubulin with different affinity for these herbicides. The presence of a carboxyterminal tyrosine that is present in all α-tubulins is crucial for the affinity to these herbicides (Wiesler *et al*., 2002), and the carboxyterminal 13 amino acids can confer the affinity to an ovalbumin carrier. These findings imply that resistance to aryl carbamates can be generated without interfering with the essential functions of tubulin that are located further upstream of the molecule. In fact, the rice mutant *ER31*, that is resistant to aryl carbamates (Nick *et al*., 1994) has been found to harbour a tubulin isotype with reduced affinity for these herbicides due to a truncation of the carboxyterminus in consequence of a precocious stop codon. When the mutated tubulin, lacking the binding site for aryl carbamates, is transferred into calli, these calli will acquire tolerance to otherwise inhibitory doses of the aryl carbamate phenyl urethane (Figure 1).
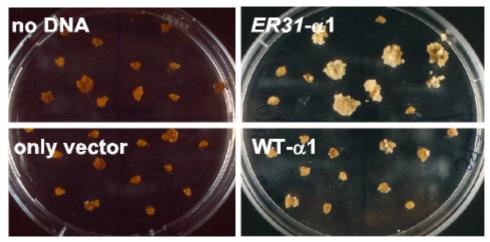

Figure 1. Performance of rice calli transformed with rice α1-tubulin in the wild-type version (WT-α1) or in a C-terminally truncated version (*ER31*-α1) under selective pressure with 0.75-1 mM of ethyl-N-phenylcarbamate (EPC) in comparison to calli that were bombarded with uncoated gold particles (No DNA) or with the empty vector p4517 (only vector). Plates are shown 2 months after bombardment.

Based on the mode of action of all microtubule blockers known so far (Mizuno and Suzaki, 1991), it is not necessary to express such new selectable markers at high abundance. The aryl carbamates and other microtubule-eliminating drugs bind the αβ-heterodimers and prevent their incorporation into the microtubule. In a situation where wild-type tubulin (which binds the drug with high affinity) and mutated tubulin (which does not bind the drug) coexist, the assembled microtubules would preferentially consist of the mutated tubulin. In fact, the resistance to aryl carbamates has been found to be a dominant-negative trait (Nick *et al.,* 1994), indicating that the modified tubulin is progressively trapped in the microtubules. It is therefore possible to use the marker at moderate expression levels provided by the native tubulin promoters.

Tubulin promoters can provide a great variety of expression profiles that can be modulated in amplitude and tissue specificity or in response to different stimuli. For both the α- and the β-tubulins, promoters can be found that drive expression ranging from semi-constitutive to patterns that are confined to specific cells at specific developmental stages (for review see Breviario and Nick, 2000). A further level of specificity is added by intron sequences that mediate a high level expression along with tissue- and organ-specificity. This has been shown for the rice isotypes α1 (Jeon *et al.,* 2000) and β16 (Morello *et al.,* 2002) but appears to be a general feature of all rice α-tubulin isotypes (Breviario, unpublished results). Such tubulin introns that modulate amplitude and localisation of tubulin transcription can be located within the coding sequence as well as within the untranslated sequence (UTR) 5' upstream of the first translational codon (ATG).

This modulatory function of introns allows for further flexibility in the biotechnological use of tubulin promoters: For any given tubulin isotype, pattern and amplitude of expression could be adjusted by the simple subtraction (or addition) of its own or cognate intron sequences. This would allow one to tune expression over a broad range from very weak to high, depending on the requirements of the actual application. It should be noted that this adjustment could be achieved without any DNA sequence that is not already present in the plant of interest.

A further regulatory feature of plant tubulins is a relatively strict homeostasis of tubulin mRNA and protein abundance. When exogenous tubulin genes are introduced into a given plant cell, this will suppress the expression of endogenous tubulin (Anthony and Hussey, 1998). This means that even a mild over-expression of transferred tubulin would result in a *de facto* replacement of endogenous tubulin. In other words: the natural homeostasis of the tubulin system can be utilized to obtain a more or less complete replacement of the endogenous gene product by its designed counterpart, an effect that for other genes would be achieved only through homologous recombination.

This short survey demonstrates that promoters, introns and UTR-sequences of plant tubulins present in any crop plant of interest are versatile enough to generate almost any desired pattern of expression. In combination with designed coding sequences for plant tubulin that confer tolerance to selective agents targeted to microtubules, it should be possible to obtain a selection marker that exclusively consists of DNA that is already present in the plant. This culminated in the EcoTub approach (standing for Ecologically safe selection markers based on plant Tubulins).

Towards the minimum – *clean DNA* technology and the EcoTub philosophy

To fulfil the two EcoTub principles, a gene system is required that carries essential functions (to allow selection after transformation) and a high degree of natural variability (to allow developmental homeostasis). As shown above, both preconditions are met by the plant tubulin system. Coding sequences of plant tubulins are modified to generate resistance against tubulin-eliminating drugs. They are fused to appropriate tubulin promoters from the host plant itself. The transformed host plant will thus acquire resistance against these drugs without the introduction of alien (cross-species) genetic information. This resistance is then used for selection after transformation.

To pinpoint the first principle, any additional extraneous DNA vector backbone sequences, (that include antibiotic resistance markers used for plasmid selection in bacteria and bacterial origins of replication) are avoided. This is guaranteed by using a novel *clean DNA* technology (Fu *et al.,* 2000) involving transformation with minimal expression units (promoter, open reading frame and terminator, no antibiotic-resistance genes, no bacterial origins of replication). This technology has the additional advantage of generating transgenic plants with low transgene copy numbers, virtually no transgene silencing and a very high multiple transgene coexpression frequency, even when the different transgenes are introduced separately.

Prospects – cold tolerance

In temperate climates, crop yield is limited by low temperatures. This is caused by a high sensitivity of growth to low temperatures that is more pronounced than that of photosynthesis. As a consequence, it is the retarded unfolding of leaves that confines productivity during the spring season (Watson, 1952). However, chilling sensitivity is an important issue outside the temperate regions as well. Most crop species of tropical or subtropical origin such as cucumbers, melons, cotton, rice, tobacco and many tropical fruits suffer severely, when they are exposed to cool temperatures that are still far above the freezing point. This so-called chilling damage has to be distinguished from the generally known freezing damage. Microtubules disassemble in response to low temperature and have therefore been considered for a long time as primary targets for chilling stress (for review see Nick, 2000). In fact, in various crops, the cold stability of microtubules has been found to correlate with chilling sensitivity, and disassembly of microtubules by antimicrotubular compounds was observed to increase chilling sensitivity (Rikin *et al.,* 1980). Cold hardening or treatment with abscisic acid improve the performance under low temperature and are accompanied by an acquired cold tolerance of microtubules (Wang and Nick, 2001).

It appears that the cold-induced disassembly of microtubules depends on the carboxyterminal region: cold-induced tubulin isotypes of tubulin possess a truncated C-terminus (Chu *et al.,* 1993), and cold-sensitive maize tubulin could be rendered cold-stable by proteolytic cleavage of the C-terminus (Bokros *et al.,* 1996). Interestingly, the microtubules of the *ER31* mutant of rice (Nick *et al.,* 1994), where the C-terminus of TUBA1 is truncated, are resistant at temperatures, where the microtubules of the wild type are rapidly eliminated (Figure 2).

In addition to aryl-carbamate resistance (Wiesler *et al.*, *Plant Journal in press*), mutations that truncate the C-terminus of tubulins are therefore expected to produce cold tolerance of microtubules. In
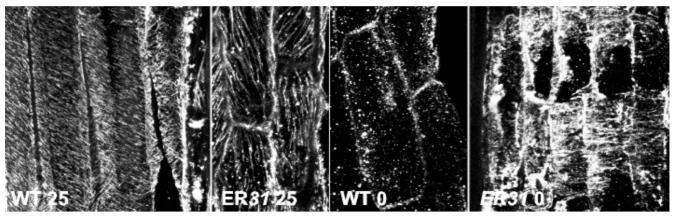
Figure 2. Response of cortical microtubules in rice cells challenged by chilling (0) as compared to controls kept at 25°C (25) for microtubules consisting of the WT-version of the α1-tubulin (WT) as compared to microtubules containing the mutated version of α1-tubulin, where the C-terminus is truncated at amino acid 414 (*ER31*).

chilling-sensitive species, such as rice, tobacco or cotton, the cold tolerance of growth limits the performance of the crop at low temperature. In other words: the cold sensitivity of microtubules might be the bottleneck for the cold tolerance of the whole plant. In the future we therefore plan to generate plants with improved chilling tolerance by engineering the carboxy terminus of α-tubulins.

Acknowledgements

References

Anthony, R.G.; Hussey, P.J. (1998) Suppression of endogenous α- and β-tubulin synthesis in transgenic maize calli overexpressing α- and β-tubulins. *Plant Journal* **16**, 297-304.

Anthony, R.G.; Waldin, T.R.; Ray, J.A.; Bright, S.W.J.; Hussey, P.J. (1998) Herbicide resistance caused by spontaneous mutation of the cytoskeletal protein tubulin. *Nature* **393**, 260-263.

Bartels, P.G.; Hilton, J.L (1973) Comparison of trifluralin, oryzalin, pronamide, propham, and colchicines treatments on microtubules. *Pesticide Biochemistry and Physiology* **3**, 462-472.

Breviario, D.; Nick, P. (2000) Plant tubulins: a melting pot for basic questions and promising applications. *Transgenic Research* **9**, 383-393.

Bokros, C.L.; Hugdahl, J.D.; Blumenthal, S.S.D.; Morejohn, L.C. (1996) Proteolytic analysis of polymerized maize tubulin: regulation of microtubule stability to low temperature and Ca2+ by the carboxyl terminus of β-tubulin. *Plant, Cell & Environment* **19**, 539-548.

Chu, B.; Snustad, D.P.; Carter, J.V. (1993) Alteration of β-tubulin gene expression during low-temperature exposure in leaves of *Arabidopsis thaliana. Plant Physiology* **103**, 371-377.

Dröge, W.; Brore, I.; Pühler, A. (1992) Transgenic plants containing the phosphinothricin-N-acetyltransferase gene metabolize the herbicide L-phosphinothricin (glufosinate) differently from untransformed plants. *Planta* **187**, 142-151.

Fu, X.; Duc, L.T.; Fontan, S.; Bong, B.B.; Tinjuangjun, P.; Sudkhakar, D.; Twyman, R.M.; Christou, P.; Kohli, A. (2000) Linear transgene constructs lacking vector backbone sequences generate low-copy number transgenic plants with simple integration patterns. *Transgenic Research* **9**, 11-19.

Haldrup, A.; Petersen, S.G.; Okkels, F.T. (1998) The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. *Plant Molecular Biology* **37**, 287-296.

Jeon, J.-S.; Lee, S.; Jung, K.-H.; Jun, S.-H.; Kim, C.; An, G. (2000) Tissue-preferential expression of a rice α-tubulin gene, OsTubA1, mediated by the first intron. *Plant Physiology* **123**, 1005-1014.

Mizuno, K.; Suzaki, T. (1991) Effects of anti-microtubule drugs on *in vitro* polymerisation of tubulin from mung bean. *Botanical Magazine (Tokyo)* **103**, 435-448.

Morello, L.; Bardini, M.; Sala, F.; Breviario, D. (2002) A long leader intron of the *OsTub16* rice β-tubulin gene is required for high level of gene expression and can autonomously promote transcription both *in vivo* and *in vitro. Plant Journal* **29**, 33-44.

Nick, P.; Yatou, O.; Furuya, M.; Lambert, A.-M. (1994) Auxin-dependent microtubule responses and seedling development are affected in a rice mutant resistant to EPC. *Plant Journal* **6**, 651-663.

Nick, P. (2000) Control of the response to low temperatures. In *Plant Microtubules*. [*edited by* P. Nick] Heidelberg, Germany: Springer Verlag, pp. 121-135.

Rikin, A., Atsmon, D.; Gitler, C. (1980) Chilling injury in cotton (*Gossypium hirsutum* L.): Effect of antimicrotubular drugs. *Plant and Cell Physiology* **21**, 829-837.

Wang, Q.Y. and Nick, P. (2001) Cold acclimation can induce microtubular cold stability in a manner distinct from abscisic acid. *Plant and Cell Physiology* **42**, 999-1005

Watson, D.J. (1952) the physiological basis of variation yield. *Advances in Agronomy* **4**, 101-145.

Wiesler, B.; Wang, Q.Y.; Nick, P. (2002) The stability of cortical microtubules depends on their orientation. *Plant Journal* **32**, in press.

Zuo, J.; Niu, Q.W.; Møller, S.G.; Chua, N.H. (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. *Nature Biotechnology* **19**, 157-161.