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A CRITICAL STUDY ON GENETIC TRANSFER TECHNOLOGY FOR FUNCTIONAL STUDIES IN GRAPEVINE

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Abstract
The know-how of the genetic determinism of plant phenotypes calls for the practical annotation of genes governing particular trends consisting of the characterization in their regulatory networks. A placing function of the grapevine genome and proteome lies within the life of massive families related to wine attributes that have a better gene copy range than in different sequenced plant life. All through speciation, the arrival of new adaptive features is regularly primarily based at the evolution of orthologous genes in the end related to duplication leading to new proteins and expression profiles. The presence of original capabilities in grapevine, together with perennial status, vegetative structure, inflorescence/tendril, flower enterprise, and fleshy fruit of giant acidity with various flavonoid compounds, makes functional genomics a critical approach to hyperlink a gene to a trait. For grapevine, the modern-day loss of high throughput genetic techniques and the difficulties associated with genetic mapping highlights the important function of transgenic generation for characterizing gene feature. Exclusive techniques are to be had to reap data approximately gene functioning, but the preference of a selected approach depends at the technique investigated and the experimental motive.

Keywords: Plant phenotypes, grapevine, compounds

Introduction

Transient expression assays provide a rapid and convenient tool for basic research in plant biology. They have been developed for gene function studies (Hellens et al., 2005; Lee and Yang, 2006) and have also proved helpful for assessing the activity of gene constructs before undertaking stable transformation (Sparkes et al., 2006). Recently, many sequencing data sets have been released within the grapevine community, prompting research in the development of efficient transient expression systems in this species. Grapevine (Vitis vinifera L.) is one of the most economically important fruit crops of the world, and it is widely cultivated for fruits, juice and especially for wine. Its genetic improvement relies on conventional breeding and genetic engineering, depending on the availability of germplasm resources and the identification of agronomically important genes (Burger et al., 2009; Reisch et al., 2012). The completion of the grapevine genome sequence project 7 years ago has opened the door to in-depth genetic studies (Jaillon et al., 2007; Velasco et al., 2007). Very recently, Di Genova et al. (2014) sequenced a table grape cultivar and compared it to the reference genome of the genotype PN40024 (Jaillon et al., 2007), leading to the identification of 240 novel genes, as well as numerous structural variants and SNPs. In addition, transcriptome analyses were performed by RNA-seq (Venturini et al., 2013; Zenoni et al., 2010) and small RNAs libraries were obtained (Carra et al., 2009; Han et al., 2014; Mica et al., 2010; Pantaleo et al., 2010; Wang et al., 2011). This genetic information could be exploited to identify genes or elucidate pathways involved in traits of agronomic importance (Di Gaspero and Cattonaro, 2010).

Functional genome analysis is an experimental approach of assigning a function(s) to genes and determining how genes interact. Functional genomics focuses on the dynamic aspects of a biological system such as gene transcription, translation, and protein-protein interactions, as opposed to the static aspects of the genomic

information such as DNA sequence. There are different methods for studying plant gene function (Grotewold 2003). Forward genetics, based on the analysis of segregating populations to map loci linked to specific traits is difficult for grapevine due to the size of the plant, the delay in flowering, and heterozygosity. The analysis of somatic variants is another way to develop forward genetics but no artificially induced mutant collections are available so far in grapevine. The use of natural mutants has, in some cases, proven useful (Rathjen and Robinson 1992, Boss and Thomas 2002, Chatelet et al. 2007, Fernandez et al. 2007) but this resource is limited and the chimeric status of somatic variants hampers the analysis of phenotypes and genotypes (Franks et al. 1998).

Growth of grapevine biotechnology

After the first report of successful *Vitis* tissue culture by Morel (1944), many efforts were made to develop biotechnologies for propagation and regeneration. Somatic true-regeneration was developed through both embryogenesis and adventitious organogenesis for a large number of *Vitis* genotypes (Torregrosa 1995, Bouquet and Torregrosa 2003, Martinelli and Gribaudo 2009). The first report of gene transfer into grapevine dealt with the induction of galls and roots with wild-type *Agrobacterium tumefaciens* (Hemstad and Reisch 1985). Although Baribault et al. (1989) obtained vegetative organs of Cabernet Sauvignon expressing transgenes, the first transgenic grapevine plants were obtained by Mullins et al. (1990).

However, despite many improvements, the recovery of transgenic grapevine plants remains a complex and long process (Figure 1) because it is based on the selection of regenerative structures that need several months to form whole plants. Nevertheless, various other alternative techniques have been developed to introduce transgenes into single cells, cell suspensions, organised tissues or organs.

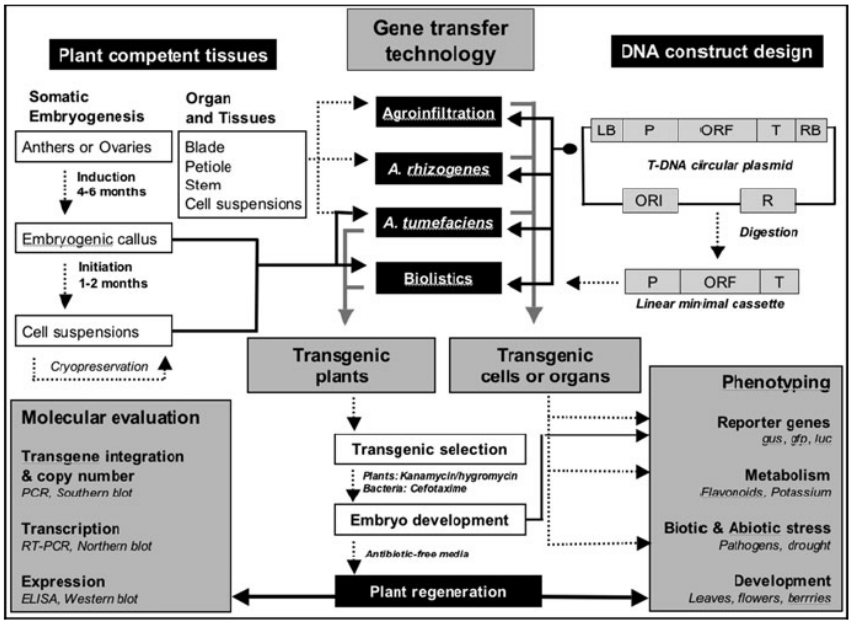

Figure 1. Schematic representation of multi-disciplinary and inter-disciplinary techniques for approaching the study of plant gene function that includes preparation of competent plant tissue, design of DNA constructs, genetic transformation methods, and tissue culture techniques for molecular and phenotypic evaluation of the target transgene in cells, tissues or regenerated plants. LB: left border; RB: right border; P: promoter; T: terminator, ORF: open reading frame; ORI: origin of replication; R: bacteria resistance gene.

Non-regenerative transgenic technologies

There are a large number of methods that allow transient gene expression in grapevine single cells or tissues, or the stable expression of a transgene in organs without the regeneration of whole transgenic plants. *Gene transfer into single cells or cell suspensions* Biolistics or micro-particle bombardment is a physical method commonly used for gene transfer into plants cells that employs DNA constructs coated onto the surface of gold particles that are propelled to high-velocity at target cells or tissues using a biolistic device (Taylor and Fauquet 2002). Once

inside the cells, the DNA elutes off the particles. If the foreign DNA reaches the nucleus, then transient expression will likely result and the transgene may be stably incorporated into host chromosomes. In grapevine, several tissues have been found suitable for biolistic-mediated gene transfer experiments: e.g. undifferentiated callus (Torregrosa et al. 2002b) or embryogenic cell suspensions (Hébert et al. 1993, Kikkert et al. 2004). This technology was initially developed by Bruce Reisch's group at Cornell University (NY-USA) as an alternative to *Agrobacterium* to regenerate transgenic plants from embryogenic tissues of *V. vinifera* and *Vitis* hybrids. However, embryogenic tissues or somatic embryos can also be used for transient assays (Kobayashi et al. 2002). Biolistic-mediated gene transfer has also been applied to undifferentiated cell suspensions to induce transient gene expression. For instance, this strategy was used to analyse the function of the *VvAdh* promoter (Torregrosa et al. 2002b) and the transcription factors regulating the flavonoid pathway (Bogs et al. 2007, Deluc et al. 2008). Another approach to introduce genes into grapevine single cells or cell suspensions is the use of *Agrobacterium*. For instance, this technology was used to obtain apoaequorin-expressing cell suspensions of *V. vinifera* to study elicitor mechanisms and resistance-inducing activities of cellodextrins (Aziz et al. 2017) and to investigate the signalling network involving calcium, nitric oxide, and active oxygen species in BcPG1-elicited cells (Vandelle et al. 2006).

Agro-infiltration or agro-injection

Direct *Agrobacterium*-mediated transformation of an organized organ is a simple technology that has proven very useful for functional studies when the recovery of transgenic plants were not required (Orzaez et al. 2006). This technology that allows investigations at the plant organ level appears particularly interesting for species like grapevine where the regeneration of transgenic plants is difficult. First report of gene transfer assays into grapevine organs via *Agrobacterium* was by Kobayashi et al. (2005) studying transcription factors involved in anthocyanin accumulation in detached berries. For vegetative organs, there have been recent reports showing that grapevine leaves are suitable for Agroinfiltration-mediated transient expression (Santos-Rosa et al. 2008, Zottini et al. 2008).

Hairy roots

Guellec et al. (1990) were the first to obtain hairy roots encoding engineered transgenes (*nptII*). To avoid the tedious construction of co-integrated vectors, Torregrosa and Bouquet (1997) developed a system based on co-inoculation with *A. rhizogenes and A. tumefaciens*. Recombinant binary plasmids could also be introduced into wild-type *A. rhizogenes* strains where pRi virulence genes act in *trans* with T-DNA binary plasmid. A large number of genotypes were found suitable for hairy root induction, including *Vitis* rootstocks, *V. vinifera* and *V. vinifera* ¥ *Muscadinia rotundifolia*. The A4 strain proved to be a very efficient vector to recover a large number of hairy roots being co-transformed with both pRi and binary T-DNAs without antibiotic-based selection (Ageorges et al. 2008).

However, despite many efforts to induce bud organogenesis or embryogenesis from hairy root tissues (Torregrosa 1994), the regeneration of plants was never achieved. Therefore, the use of *A. rhizogenes* remains a convenient system to generate a large amount of transformed tissues in a fully organised root context. The possibility to graft hairy roots with other stem explants provided the opportunity to associate a specific transgenic roots line with any other type of material (Torregrosa and Bouquet 1997). Hairy root technology has already been used in several functional studies dealing with grapevine development and pathogen interactions (Lupo et al. 1994, Franks et al. 2006, Cutanda-Perez et al. 2009, Gomez et al. 2009, Terrier et al. 2009).

Transgenic plants

Despite some interesting advances in direct gene transfer into grapevine meristems (Dutt et al. 2007), the most common method for obtaining a whole plant is based on regeneration. The regeneration of transgenic grapevine plants involves four main steps: (i) the initiation of regenerative cultures, i.e. embryogenic tissues; (ii) the introduction of transgenes into cells or small embryogenic cell clusters; (iii) the selective induction of embryo-like structures; and (iv) the germination of the structures into non-chimeric plantlets (Figure 1). There are several

comprehensive reviews dealing with the regeneration of transgenic grapevine plants (Martinelli and Mandolino 2001, Kikkert et al. 2004, Perl et al. 2004, Bouquet et al. 2006, 2008). The following sections will only provide an overview of the main technological features.

Selection procedures

Generally, for the selection of transgenic tissues, two types of markers are commonly introduced in addition to the sequence of interest: a selectable marker that allows the selection of cells containing transgenes and a visual or reporter marker that enables the observation of transgenic events. The more common selectable markers are: neomycin phosphotransferase gene (*npt*II) that confers resistance to kanamycin; hygromycin phosphotransferase gene (*hpt*) that confers resistance to hygromycin; and the phosphinothricin amino-transferase gene (*pat*) that confers resistance to the Basta® herbicide. Attempts to use the phosphomannose-isomerase gene (*pmi*) as an alternative selectable marker have been disappointing (Kieffer et al. 2004, Vaccari and Martinelli 2009).

However, whatever the antibiotic used as selective agent, a balance between effective selection of transformed cells and the inhibitory effects on cell growth need to be precisely defined (Torregrosa et al. 2000a,b). Since the first gene transfer experiments, bglucuronidase (GUS) enzyme encoded by *uidA* gene has been extensively used as a reporter marker (Mullins et al. 1990), but its major drawback is that assays are destructive. For this reason, the green fluorescent protein (GFP) from jellyfish (*Aequora victoria*) has become the favoured visual marker (Torregrosa et al. 2002a, Li et al. 2006). The luciferase enzyme, which is encoded by the firefly *Photinus pyralis luc* gene and uses luciferine as substrate, offers the capability of monitoring and quantifying reporter expression patterns non-destructively in real time (Verriès et al. 2004, Bogs et al. 2007).

Several strategies were recently proposed to remove reporter and selection genes during transgenic plants regeneration process. Dutt et al. (2008) reported a very efficient and simple system based on the co-transformation with a mixture of two *Agrobacterium* strains, one containing a standard binary plasmid with a gene of interest and a second strain harbouring the *nptII* gene for positive selection and the cytosine deaminase (*codA*) gene for negative selection, linked together by a bi-directional, dual promoter. Another strategy based on the use of vectors carrying *cre/loxP* sites and *cre* recombinase regulated by 17-beta-estradiol was also recently proposed by Dalla Costa et al. (2009).

Biolistic-mediated gene transfer

In plant research, the initial application of the biolistic method was for transient gene expression studies (Vain et al. 1996). In addition to biolistic parameters, three factors (embryogenic cultures, DNA constructs, and selection systems) are required to be optimised for the process to have maximum effect. In grapevine, embryogenic cell suspensions growing at exponential phase were found to be the most convenient tissue for biolistic experiments when the final goal is the regeneration of transgenic plants. With optimal parameters, the process can be easy and highly effective. Vidal et al. (2003) reported in detail the evolution over time from transient to stable transgenic plants from grapevine embryogenic cell suspensions.

During the selection process, the number of transgenic structures decreased from 7883_1928 transient expression spots per 55 mm plate (2 day after bombardment) to 46_32 stable transgenic structures per plate (95 days after bombardment) to result in five transgenic embryos per plate an average (Figure 2) with more than half that able to be regenerated.

With this technology, the first regeneration of a transgenic grapevine plant was reported by Kikkert et al. (1996) using Chancellor (*Vitis* hybrid). But the method has also been successfully and routinely used for *V. vinifera* cultivars such as Chardonnay and Merlot (Reisch et al. 2003, Vidal et al. 2006a,b) and an optimised procedure was recently reported by Vidal et al. (2009).

Agrobacterium-*mediated gene transfer*

Initial experiments to target gene transfer into adventitious buds were inconsistent (Colby et al. 1991, Torregrosa 1994). A successful protocol was recently reported (Mezzetti et al. 2002), but the potential of this system as an alternative to somatic embryogenesis remains to be confirmed. So far, the most widely used system consists of

the co-culture of disarmed *A. tumefaciens* vectors with somatic embryogenesis callus or embryogenic cell suspensions.

It has been a successful method for many grapevine genotypes, but efficiency and reliability vary considerably from one genotype to another. Good results have been obtained with *Vitis* species, such as *V. rupestris* and *V. riparia* or *V. rotundifolia* and interspecific hybrids (Bouquet et al. 2008, Dhekney et al. 2008).

Results have been more variable with *V. vinifera* (Iocco et al. 2001). The choice of *Agrobacterium* strain is critical. LBA4404 that was the first successfully used strain (Mullins et al. 1990) and continues to be extensively used (Bornhoff et al. 2005). Strain GV3101 was also successfully used in past experiments and recently improved by the addition of the gene T-6b (Fan et al. 2008).

However, the most efficient strain for *Agrobacterium* gene transfer into grapevine is probably EHA105 and derivatives that have been widely used during the last decade (Franks et al. 1998, Torregrosa et al. 2002a, Li et al. 2006). EHA105 strain is a C58C1 chromosomal background with an oncogene-deleted pTiBo542 supervirulent plasmid from A281. During or immediately after co-cultivation with *Agrobacterium*, grapevine tissues become partially brown, with some cultivars being more prone to this phenomena than others. Techniques to reduce browning include pre-culture on activated-charcoal enriched medium, addition of antioxidants like PVP or DTT (Perl et al. 1996, Li et al. 2006, Bouquet et al. 2008). In order to reduce excessive bacterial growth after co-culture, and avoid irreversible necrosis, co-cultivated tissues require careful washing and subsequent culture in the presence of high levels of antibiotics (Bouquet et al. 2006).

Agrobacterium *versus biolistic*

Grapevine has often shown a hypersensitive response to *Agrobacterium* that causes cell browning and death (Bouquet et al. 2008). The physical nature of the biolistic method avoids the necessity to eliminate *Agrobacterium* after co-culture and the potential occurrence of false positives arising from growth of *Agrobacterium* in host tissues

(Kikkert et al. 2004). Operation of the biolistic device is easy and there are only a few instrument parameters to adjust. Furthermore, plasmid construction is often simplified and co-transformation with multiple transgenes is easy.

The possibility to use minimal linear expression cassettes eliminates the chance of plasmid backbone DNA insertion (Vidal et al. 2006b, Figure 2). On the other hand, some disadvantages of the biolistic method include the lower gene transfer efficiency compared with *Agrobacterium*-mediated transformation, the high cost of the device and consumables, and the tendency of complex integration patterns and multiple copy insertions that may cause unexpected gene silencing.

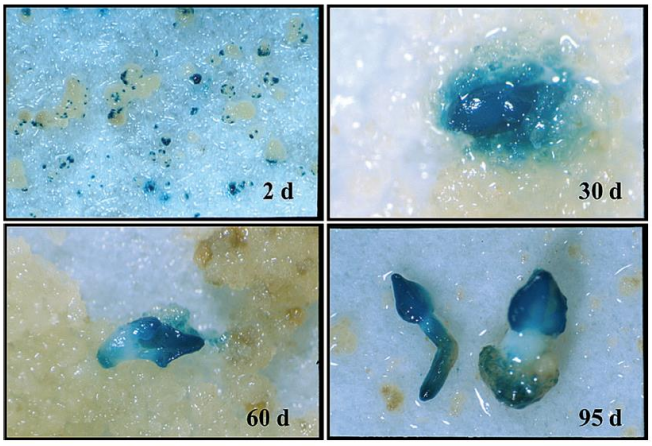

Figure 2. Visual time course of a biolistic experiment with the reporter gene *gus* (®-glucuronidase). Many cells transiently expressing GUS (blue spots) within an embryogenic cell suspension of Chardonnay, 2 days after biolistic, to only a few stable transformed developed embryos expressing GUS, 95 days after bombardment.

Strategies for expression cassette engineering

To obtain scientific evidence about the role of a specific sequence, the transgenic approach aims to analyse phenotypic changes resulting from gene expression changes. Gain-of-function can be achieved by increasing the level of transcription of the target gene and loss-of-function can be obtained by either co-suppression or expression of anti-sense or RNA hairpin sequences (Ferreira 2000, Grotewold 2003). Another approach to reveal the mechanism of gene expression regulation is the use of promoter-reporter constructs. Information about the subcellular location of the predicted protein often involved translational fusion containing reporter genes (Figure 3).

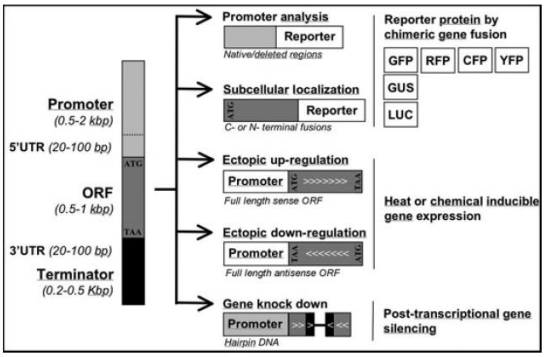

Figure 3. Schematic representation of a multiple strategy to construct expression cassettes for gene transfer. Kbp: kilo base pair, bp: base pair, ORF: open reading frame, UTR: untranslated sequence, (G-Y-C-R) FP:(green-yellow-cyan-red) fluorescent protein, GUS: ®-glucuronidase, LUC: luciferase, ATG: start codon, TAA: stop codon. Number in bracket gives the usual size of grapevine gene sequences.

GATEWAY: a new and versatile cloning system

Recent progress in transgenic research relied to a large extent on the evolution of DNA cloning technologies to facilitate the construction of gene expression cassettes and the simple-to-use *Agrobacterium* binary vectors (Karimi et al. 2005, 2007, Komari et al. 2006). Classically, the insertion of interesting genes into the T-DNA region of a binary vector has been carried out by standard subcloning techniques, using DNA restriction and ligase enzymes. Recombination cloning methods have recently provided a significant improvement (Marsischky and LaBaer 2004). In particular, the Gateway system that has been designed to join fragments in a predefined orientation and reading frame in only two quick and simple steps (Karimi et al. 2007). A large number of plasmids flanked by two attL sites are now available to build entry clones able to transfer cloned sequences into any destination vector (binary) carrying attR sites. Several entry and destination plasmids (Karimi et al. 2002, Curtis and Grossniklaus 2003, Helliwell and Waterhouse 2003, Nakagawa et al. 2007) have been successfully used in grapevine for either gene over-expression or silencing, promoter studies and protein sub-cellular cell localization (see section 5).

Promoter analysis and protein subcellular localization

The transcriptional activity of a promoter can be characterized by the temporal and spatial expression variations of a reporter gene. In such studies, the promoter sequence must be cloned upstream of the corresponding reporter gene. In a translational fusion, when the added domain codes for a fluorescent protein or an epitope tag, the protein of interest can be localised within the cell by microscopic analysis of living or fixed cells. Several binary vectors have been designed with a recombinant site upstream or downstream of ORFs coding for LUC or GUS, a fluorescent protein, a purification tag (polyhistidine [6xHis]), an epitope tag (haemagglutinin [HA], FLAG, c-Myc, or AcV5) or a combination of these (Karimi et al. 2007).

Gene over-expression

For gene overexpression, transcript abundance is increased by cloning the whole ORF after a constitutive promoter such as from CaMV 35S, Cassava vein mosaic virus, nopaline synthase, maize ubiquitin or *A. rhizogenes* rolD. To control ectopic expression level, several inducible transcription activation sequences

activated by either heat treatment or a chemical agent were also developed (Curtis and Grossniklaus 2003). This collection of promoters can be complemented with several terminator sequences such as CaMV 35S, nos or ocs.

Gene down-regulation

Detailed descriptions about the molecular mechanisms implicated in RNA silencing can be found in Eamens et al. (2008). One of the most effective methods to silence an endogenous gene is to encode a hairpin RNA (hpRNA). Gateway LR recombination has considerably facilitated the design of such constructions, which can be performed in a single LR reaction using a cloning cassette with two opposite recombinant sites (attR1-ccdB-attR2-*intron*attR2-ccdB-attR1). Several destination vectors differing in backbone, intron spacer, and promoter are available (Karimi et al. 2007). In grapevine, this technology has been recently applied for functional analysis of *VlMybA1* (Torregrosa et al. 2008b), *VvANR* and *VvLAR* (Harris et al. 2008), and *VvCCD1* (Lashbrooke et al. 2008).

Another method for gene silencing is the virusinduced gene silencing (VIGS). VIGS use viral vectors that generate double-stranded RNA to induce the posttranscriptional gene silencing (PTGS) of a target gene (Burch-Smith et al. 2004). This approach is potentially very interesting as the phenotype can be obtained at the plant level after only a few days if the virus can spread to the targeted organ. In grapevine, a significant advance has been reported by Muruganantham et al. (2008) who engineered a VIGS vector based on the Grapevine virus A (GVA). An *Agrobacterium*-mediated method for inoculating *V. vinifera* plantlets via their roots proved efficient in silencing endogenous Phytoene desaturase in leaves, demonstrating this system may constitute an important future tool.

Using transgenic approaches for functional studies

Each transgenic approach has its advantages and weaknesses. Transient expression is an attractive method because of its simplicity, but this system may not be appropriate when specific co-factors are required or if the physiological status of the background is not compatible with the investigated function. For instance, in cell suspension, bHLH co-transformation was found to be required to study some MYB genes of the flavonoid pathway (Bogs et al. 2007). Furthermore, the mechanical or biological procedures to introduce transgenes can induce a tissue response impairing the analysis of the phenotype. For instance, Agro-infiltration of grapevine leaves induced a strong defence reaction potentially interfering with the analysis of fungus resistance genes (Santos-Rosa et al. 2008). Moreover, transient expression studies are not suitable for large-scale gene expression analysis as the few transgenic events are diluted within many wild-type cells. The hairy root system appears to be very interesting for obtaining stabilised transgenic organs but its use is restricted to functions operating in roots.

Finally, stable expression in plants allows the manipulation of genes at the whole plant level. However, this process is lengthy, with 16–24 months required to establish a vine in a greenhouse and 12–18 additional months to complete reproductive development. In the following sections, several studies will be detailed to illustrate how transgenic grapevines have been used to obtain information on gene function.

Inflorescence and flower development

Flowering and flower development are complex biological process regulated by a number of genetic factors. In grapevine, a number of genes putatively involved in the regulation of the reproductive organ have been identified and isolated (Carmona et al. 2008) and some of them have already been studied through grapevine genetic transformation. For instance, the expression pattern of the MADS-box gene *VvMADS1* suggested a dual role in both flower and berry development (Boss et al. 2001).

Subsequent over-expression of *VvMADS1* in transgenic grapevine altered the development of flower organs confirming the ‘C’ function of this candidate gene (Boss et al. 2003). Another example is *DefH9-iaaM*, a gene involved in auxin synthesis. The ectopic upregulation of *DefH9- iaaM* in transgenic Thompson Seedless and Silcora plants increased the number of inflorescences per cane and the number of berries per bunch (Costantini et al. 2007), suggesting a role for auxin in reproductive development of grapevine.

Fruit development

Micro-array analysis of the berry transcriptome has revealed many genes whose expression specifically varies with the stage of fruit development (Pilati et al. 2007). However, these data are not enough to assign function to a specific isogene. For instance, in grapevine, 3 isogenes of alcohol dehydrogenase (*Adh*) have been described (Tesniere and Verriès 2000), with *VvAdh2* showing a huge increase of transcription at the onset of ripening (Tesniere et al. 2006a). To study the function and the specific role of *VvAdh2* in grapevine, several transgenic approaches were investigated. The functioning of the *VvAdh* 5′ and 3′ UTR sequences in relation to anaerobic conditions and ethylene signalling was studied via transient expression in cell suspensions with both *luc* and *gus* reporter genes (Tesniere et al. 2004, Verriès et al. 2004, Tesniere et al. 2005). The role of VvADH2 in grapevine development was investigated with stable transgenic plants ectopically up- or down-regulating ADH activity. The phenotyping of transgenic leaves revealed some major changes of sugar content, proanthocyanidin polymerisation degree, and the level of specific glycosidic volatile compounds such as monoterpenes, C13-norisoprenoids and shikimates (Tesniere et al. 2006b). However, transgenic berries with modified ADH levels showed no major morphological changes with acidity and sugar uploading being similar to the control. Conversely, a significant reduction in benzyl alcohol and 2-phenylethanol content was found in berries over-expressing *VvAdh2* (Torregrosa et al. 2008a). These experiments suggested that ADH is not only involved in berry primary metabolism but may have some impact on secondary metabolite pathways.

Berry acidity and pH

Potassium is involved in many important functions in grapevine. For instance, the regulation of potassium flux in stomata is associated with water transpiration and respiration control. In grapes, the accumulation of potassium can depreciate the quality of the wine due to its negative impact on pH and on tartaric acid stability. VvSIRK, the first K channel identified in grapevine (Pratelli et al. 2002) was found to be a member of the Shaker channel family. This protein was electrophysiology characterized as an inward rectifying channel. The activity of the SIRK promoter region fused to the *gus* gene was analysed in both grapevine and *Arabidopsis*. Like other KAT-like channels, SIRK was found expressed in stomata guard cells in both grapevine and *Arabidopsis*. In berries, *VvSirk* expression was found to decrease during ripening in association with the evolution of stomata into lenticels and changes in vascular tissue properties, suggesting a role in the regulation of transpiration and water fluxes. VvSOR is another K channel identified in grapevine berry as an outward rectifying channel (Pratelli 2000). The functional analysis of *VvSor* was done using grapevine transgenic plants encoding dominant negative mutated proteins (i.e. overexpression of VvSOR mutant proteins that results in the production of non-functional chimeric channels) compared with over-expressing lines (i.e. increasing the number of functional channels). Analysis of the transgenic berries suggested an involvement of *VvSOR* in berry acidity balance in relation with fruit growth (WO/2005/078101 patent).

Flavonoid pathway

In grapevine fruit, flavonoids are mainly represented by anthocyanins, pro-anthocyanidins (PAs, condensed tannins) and flavonols. Among plant species, there is some conservation of the structural schema of this pathway, but some grapevine genes appear to be unique (Bogs et al. 2005, 2006). Critical steps determining metabolite forms such as methylation, acylation or polymerization are not yet elucidated. Mechanisms for the transport of intermediate and final metabolites and the storage of these compounds into the vacuole remain unknown. At the regulatory level, many transcription factors (TFs), potentially involved in the control of the flavonoid pathway, have been putatively identified (Matus et al. 2008). However, only a few of them have been characterised so far (Kobayashi et al. 2002, Deluc et al. 2006, 2008, Bogs et al. 2007, Walker et al. 2008, Terrier et al. 2009). The introduction of these sequences (promoter, partial or full length cDNA) through various gene transfer techniques has proven to be a valuable approach for functional annotation. For instance, Bogs et al. (2007) used transient expression in cell suspensions to screen for genes activated by *VvMYBPA1*. This study showed that VvMYBPA1 controls *LAR* and *ANR* promoters, as well as the promoters of other gene of the general flavonoid pathway but not the *VvUFGT* promoter, suggesting *VvMYBPA1* is specific to the regulation of PA biosynthesis.

Terrier et al. (2009) ectopically up-regulated VvMYBPA1 and VvMYBPA2, a new putative TF of the PA pathway, to observe important qualitative and quantitative changes in the proanthocyanidin profile. High throughput transcriptomic analyses of transgenic organs allowed the identification of a large set of putative targets of VvMYBPA1 and VvMYBPA2. Both genes significantly activated enzymes of the flavonoid pathway, including ANR and LAR1, the terminal steps in the biosynthesis of respectively epicatechin and catechin, but not LAR2. In addition, this study revealed putative new players in the PA pathway, such as specific glucosyltransferases and transporters.

Berry pigmentation is another example were transgenic grapevines were essential for assigning gene function. Several transient expression technologies (biolistic and *Agrobacterium*-mediated) were performed to characterize the ability of *VvmybA1*, *VvmybA2*, and *VvmybA3* to drive anthocyanin pigmentation (Kobayashi et al. 2002). The up-regulation of *VlmybA1-2* in hairy roots allowed the identification of new components of the anthocyanin pathway (Cutanda-Perez et al. 2009) such as MATE-type proteins acting as vacuolar H+-dependent acylated anthocyanin transporters (Gomez et al. 2009). Tonoplast localization of some of these proteins, namely anthoMATE1 and 3, was also performed in transgenic grapevine organs.

Deluc et al. (2006, 2008), who identified VvMYB5a and VvMYB5b in grapevine fruit, used transient expression assays to screen for genes potentially controlled by these TF. Additional experiments with transgenic tobacco plants up-regulating VvMYB5b revealed a dual function of this TF in both the anthocyanin and proanthocyanidin pathways.

Future challenges for grapevine transgenic technology

The transfer of genes into grapevine either for transient or stable expression is a powerful technology for gaining information on gene function. Until mutant grapevine plant collections become available for both forward and reverse genetic studies, gene transfer technologies will remain the preferred and often the only means of studying and confirming gene function at a spatial, temporal, cellular, and whole plant level. However, after 20 years of technological developments, there still are some challenges that need to be addressed to gain a wider use outside of the relatively few laboratories skilled in the various techniques of gene transfer into grapevine.

Genotypes highly competent for genetic transformation

The selection of grapevine genotypes that can be easily manipulated for gene transfer experiments is a critical step. Several studies showed varied response of the grapevine genotypes and cultivars to genetic transformation (i.e. browning, cell or tissue ability for gene integration, etc.) through biolistic or *Agrobacterium*-mediated transformation and regeneration (production of regenerative structures, stability of embryogenic tissues and germination capacity of embryos after the selection process). A large number of grapevine genotypes suitable for gene transfer have been identified: e.g. *V. vinifera* cv. Portan (Torregrosa et al. 2002a), cv. Sultanine and Chardonnay (Franks et al. 1998, Iocco et al. 2001, Dutt et al. 2008), *Vitis* roostock cv. 110 R (Le Gall et al. 1994) or 41B (Coutos-Thévenot et al. 2001) or non-*Vitis* species such as *Muscadinia rotundifolia* (Dhekney et al. 2008). These cultivars are genetically diverse and differ in many features including plant stature and anatomy, types of reproductive organs (female, male or hermaphroditic flowers), size, colour and types of fruit, response to abiotic stress (temperature, water deficit) and tolerance to pathogens. However, the genetic determinism of the traits determining competency for genetic transformation and regeneration is still unknown and the selection of improved highly competent genotypes will require empiric and laborious multifactor experiments.

Expression cassettes

The development of new cloning technology based on recombination is highly beneficial for engineering expression vectors. Currently, the main limitation is certainly the availability of a suitable library of promoters targeting gene expression to specific cells and organs in grapevine. Enhanced expansion of this library would include inducible sequences combined with tissue-specific promoters allowing the researcher to control both temporal and spatial transgene expression. This aspect is particularly critical if ectopic transgene overexpression

interfere with related (iso)genes or when the manipulation of gene expression needs to be organ-, tissues-, or stagedependant.

The recent development of high throughput expression screening technologies makes the identification of specific gene expression profiles easier (Pilati et al. 2007, Tesniere et al. 2006a) but detailed studies of expression patterning require transgenic plants or *in situ* analysis of transcript abundance, both presently being very laborious in grapevine. As a result, these types of studies are very rare (Coutos-Thévenot et al. 2001, Gollop et al. 2002, Pratelli et al. 2002, Torregrosa et al. 2002a, Verriès et al. 2004, Chervin et al. 2009).

Another challenge that needs to be addressed in the near future is the requirement in some cases to introduce multiple transgenes into a single plant. Multiple transgenes can be introduced within a single transfer experiment by co-transformation (Dhekney et al. 2008).

However, the preferred approach would be to successively introduce transgenes linked to the same selectable marker (if the technology of marker removal is operative; Dutt et al. 2008, Dalla Costa et al. 2009) or linked to different selectable markers. The last approach would not only require the development of a vector family that contains different selectable markers but also grapevine genotypes that are easy to select with different agents such as kanamycin, hygromycin, and phosphinothricin. Another alternative would be to sexually combine transgenes introduced through separate gene transfer experiments, but an essential requirement would be rapid flowering of transgenic lines.

Conclusion

In grapevine, the selection and establishment of nonjuvenile stabilised transgenic plants requires 2–3 years. From this point, 2 or 3 additional growth cycles are required to obtain phenotypically consistent data as grapevine only produces one set of inflorescences per vegetative cycle. This represents a major bottleneck for functional studies when reproductive organs (e.g. genes involved in berry development) or further generations (e.g. to sexually combine transgenes or establish homozygous transgenic loci) are required. To boost the flowering of grapevine plants, a classical way is to optimise plant growth after acclimatisation. For instance, Huglin and Julliard (1964) proposed a culture system encouraging main shoot growth to get highly fertile latent buds from seedlings which could be adapted for transgenic vitroplants.

Another possibility could be to engineer specific improved grapevine genotypes. In several plants, the over-expression of some flowering genes, such as LFY, AP1, or FT, was found to decrease the delay in flowering. For instance, in trifoliate orange, the up-regulation of an FT homolog from *Citrus* conferred an early flowering phenotype on transgenic T1 lines and F1 progenies (Endo et al. 2005). In grapevine, both *VvFT* and VvMADS8 introduced into *Arabidopsis* also lead to rapid flowering (Sreekantan and Thomas 2006). Thus, one strategy could consist of engineering specific lines over-expressing FT grapevine homologs to be used in gene transfer experiments or to use *VvFT* for co-transformation studies. Another possibility, as FT signal can translocate within organs (Corbesier et al. 2007), would be to introduce *VvFT* into grapevine lines to be used as rootstocks for transgenic lines harbouring genes of interest.

Finally, another option could be the development of lines containing the *Vvgai1* mutation (Boss and Thomas 2002, Franks et al. 2002). Phenotypic effects associated with the *Vvgai1* mutation, i.e. dwarfism and continuous production of inflorescences in place of tendrils, were found genetically heritable. Thus, *Vvgai1* could be introduced either through gene transfer or in sexual progeny to generate lines associating high competency for genetic transformation and regeneration and rapid flowering.

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