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Review

Biotechnology of temperate fruit trees and grapevines*

Margit Laimer[1]✉, Duarte Mendonça[2], Fatemeh Maghuly[1], Gorji Marzban[1], Stephan Leopold[1], Mahmood Khan[1], Ildiko Balla[3] and Hermann Katinger[1]

[1]Plant Biotechnology Unit, IAM, Deptartment of Biotechnology, BOKU, Vienna, Austria, [2]Centro Biotecnologia dos Açores, Departamento Ciencias Agrarias, Universidade dos Açores, Angra do Heroismo, Azores, [3]Research Institute for Fruit Growing and Ornamentals Budapest, Hungary; ✉e-mail: m.laimer@iam.boku.ac.at

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Challenges concerning fruit trees and grapevines as long lived woody perennial crops require adapted biotechnological approaches, if solutions are to be found within a reasonable time frame. These challenges are represented by the need for correct identification of genetic resources, with the foreseen use either in conservation or in breeding programmes. Molecular markers provide most accurate information and will be the major solution for questions about plant breeders rights. Providing healthy planting material and rapid detection of newly introduced pathogens by reliable methods involving serological and molecular biological tools will be a future challenge of increases importance, given the fact that plant material travels freely in the entire European Union. But also new breeding goals and transgenic solutions are part of the biotechnological benefits, e.g. resistance against biotic and abiotic stress factors, modified growth habits, modified nutritional properties and altered processing and storage qualities. The successful characterization of transgenic grapevines and stone fruit trees carrying genes of viral origin in different vectors constructed under ecological consideration, will be presented. Beyond technical feasibility, efficiency of resistance, environmental safety and Intellectual Property Rights, also public acceptance needs consideration and has been addressed in a specific project. The molecular determination of internal quality parameters of food can also be addressed by the use of biotechnological tools. Patient independent detection tools for apple allergens have been developed and should allow to compare fruits from different production systems, sites, and genotypes for their content of health threatening compounds.

Keywords: plant biotechnology, molecular markers, patogen detection

Plant biotechnology as an interdisciplinary science is able to provide impulses and solutions to agricultural challenges, particularly in the case of woody fruit crops, e.g. by rapid propagation of selected cultivars, conservation of valuable germplasm, phytosanitary and genetic improvement and safeguarding human health, not only through nutritional, but also through ecological aspects.

Since many of these aspects, particularly in the case of perennial crop plants are long term efforts, the application of biotechnological methods can make considerable contributions, including:

- the application of molecular markers for the identification and conservation of valuable genetic resources
- pathogen detection and elimination by *in vitro* methods
- new breeding goals in fruit trees and grapevines, e.g. resistance breeding against pathogens
- molecular determination of internal quality parameters of food, e.g. fruit allergens.

This review summarises the experience of the Plant Biotechnology Unit at the Institute of Applied Microbiology, in the field of fruit tree and grapevine research over the past eighteen years.

MOLECULAR MARKER ANALYSES FOR GRAPEVINE AND FRUIT TREES

One of the major concerns of modern agriculture is the conservation and utilisation of valuable genetic resources of crop plants. The need for correct identification applies to cultivars and acces-

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sions, independently of their mode of conservation, i.e. whether they are maintained in an *in situ* or *ex situ* field gene bank or an *in vitro* gene bank. Tools developed for the characterisation for biodiversity may allow clarifications of synonyms and detection of the origin of species and cultivars.

The European Union requests from fruit tree planting material a certain level of genetic quality. Characterisation and determination of fruit tree and grapevine cultivars are sometimes difficult using conventional methods. Since morphological markers are prone to equivocal interpretations and time consuming, molecular approaches should be implemented in cultivar identification and breeding programmes. Molecular markers help to distinguish labeling mistakes, identification of the genuine owner of the cultivar in question, routine identification of cultivars in nurseries. Further it simplifies work in breeding programmes by accelerating the breeding process by allowing a selection before the first fruit crop, by tracking certain genes or genotypes among offspring of crosses.

Initially isoenzyme markers were developed and applied in *Prunus* crops like peach (Messeguer *et al.,* 1987; Monet & Gribault, 1991), almond (Cerezo *et al.,* 1989; Arús *et al.,* 1994a) cherry (Santi & Lemoine, 1990; Boskovic *et al.,* 1997) and apricot (Pedryc *et al.,* 1996).

DNA markers are currently used for apricot cultivar identification using arbitrary primers (Eldredge *et al.,* 1992; Gogorcena & Parfitt, 1994; Hurtado *et al.,* 1999). RAPD, RFLP and AFLP (Arús *et al.,* 1994a; 1994b; Hurtado *et al.,* 2001) are available, besides isoenzyme markers, to clearly distinguish the different stone fruit cultivars present on the international fruit market. RFLP markers developed for European and North American apricots produce unique profiles for most cultivars. Spanish cultivars cluster together, distinct from the remaining European and North American apricots (Hurtado *et al.,* 2001; Romero *et al.,* 2003).

Recently microsatellite or single sequence repeat (SSR) markers have been developed for peach (Cipriani *et al.,* 1999) and apricot (Lopes *et al.,* 2002; Messina *et al.,* 2004) and applied for characterisation of cultivars, confirmation of geographic origin, pedigree, and identification of synonyms (Maghuly *et al.,* 2004[1]; 2005a).

The cultivar Hungarian Best is cultivated in the Central European Region under different designations, possibly representing synonyms. A precise genetic characterisation and distinction of clones with molecular polymorphisms demonstrated a challenging task. Initially for the analysis and classification of the major apricot cultivars a group of 190 accessions were analysed with 10 newly developed microsatellite loci (Lopes *et al.,* 2002; Messina *et al.,* 2004; Maghuly *et al.,* 2005a). The genetic distance was reflected in the grouping of cultivars in agreement with their geographic origin and pedigree. Eastern European (Hungarian) cultivars belong to four subgroups and share the same parents. Asian (Pakistan, Central Asia) and American (USA, Canada) cultivars were intermediates showing a different genetic basis than the European cultivars. The evaluation of results revealed the existence of three major groups with several synonyms within the Hungarian apricot cultivars (Maghuly *et al.,* 2005b). Further it was clearly possible to separate the cultivars according to their geographic origin (Mehlenbacher *et al.,* 1991).

PATHOGEN DETECTION AND ELIMINATION BY *IN VITRO* METHODS

Viruses and phytoplasmas are widely distributed plant pathogens, and there is no effective cure for already infected plants in the field. They cause considerable economic losses and are therefore a major concern to worldwide phytosanitary agencies. *Rosaceae* in general and *Prunus* species in particular are prone to varying degrees to infections by a range of pathogens (Laimer, 2003a).

The most important viral pathogen is *Plum pox virus* (PPV), however, other viruses, e.g. *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV), and phytoplasmas like *European Stone Fruit Yellows* (ESFY), represent a major threat. Several strains and even recombinant strains of PPV are present in Central Europe (Glasa *et al.,* 2003).

The EU requests from fruit tree planting material a certain level of phytosanitary quality. To achieve this objective it is necessary to have at hand improved strategies for the production of elite plants of pathogen-free stone fruit cultivars. This includes the application of rapid, reliable, user-friendly, sensitive and cost effective methods for the detection and elimination of the major stone fruit tree viruses and phytoplasmas.

An *in vitro* collection of apricots and peaches containing cultivars testing highly positive for the selected target pathogens PPV and ESFY, quarantine organisms in the European Union (OEPP/EPPO 1986, CABI/EPPO, 1991/1992) was established, to serve as models for host-pathogen relationships and pathogen elimination experiments (Balla *et al.,* 2002).

Dealing with pathogens categorised as quarantine organisms, special care was taken. Initially biological test by greenhouse indexing allowed to determine the infection levels of the mother trees used as source for the shoots of the cultivars established

[1]Maghuly F, da Câmara Machado A, Ruthner Sz, Pedryc A, Bisztray G, Katinger H, Laimer M (2004) IV CHB Symposium. Book of Abstracts: 204.

in vitro. Parallely disease-indexing of *in vitro* culture collections was carried out under conditions optimised for micropropagated plants for PPV, PNRSV and PDV using ELISA (using antisera from Löwe, Bioreba, Agritest), Immuno Tissue Printing (ITP) (Knapp *et al.,* 1995) and RT-PCR, and was extended to PCR for the detection of ESFY and phytoplasmas affecting grapevines (Heinrich *et al.,* 2001; Laimer *et al.,* 2001; Laimer, 2003a).

Different *in vitro* techniques, i.e. meristem culture and heat therapy *in vitro* were applied either alone or in combination and results compared. Protocols were validated for high survival rates of plants and for their effectiveness for pathogen elimination.

The Vienna Collection currently contains:

1) an *in vitro* gene bank with 189 accessions of fruit tree and grapevine cultivars (51 apple, 59 plum/cherry, 18 apricot and 61 grapevine)
2) an *in vitro* collection of tissue cultures with well characterised pathogen isolates with 114 accessions (www.boku.ac.at/iam/pbiotech/phytopath)
3) an *in vivo* collection of pathogen-free mother plants with 50 accessions kept under insect-proof screenhouse conditions.

NEW BREEDING GOALS IN FRUIT TREES AND GRAPEVINES

Different traits have been modified in transgenic fruit trees, which comprise a) altered processing and storage qualities, b) modified nutritional properties, i.e. the influence of desirable/undesirable components, c) modified growth habit and vigor, d) resistance to abiotic stresses, e.g. soil factors, drought, low temperature, and e) resistance to biotic stresses (Laimer, 2003b). The breeding and cultivation of virus resistant plants is a major contribution to the control of viral diseases, since there do not exist chemical control strategies.

Work on pathogen-mediated resistance in woody species, focusing on virus resistance breeding in fruit trees and grapevines, started in 1988 at the IAM. No control of these pathogens by chemical means exists (CABI/EPPO 1991/1992), and the chemical control of their vectors, e.g. aphids, nematodes, etc. is ecologically questionable. Following the pathogen-mediated protection approach (Beachy *et al.,* 1990; Lomonossoff, 1995; Baulcombe, 1996, Waterhouse *et al.,* 1998; 2001), we isolated the coat protein gene of the stone fruit pathogen PPV and transformed different explants, i.e. cotyledons, leaf discs and embryogeneic callus cultures, of different *Prunus* species (Laimer da Câmara Machado *et al.,* 1992). Several transgenic lines were regenerated and subjected to genetic characterization and evaluation of conferred protection. Additionally, sequences of the PPV genome involving structural and non structural genes, were introduced in different plasmids in both sense and antisense orientation and used for transformation, showing good levels of resistance in herbaceous model plants (Korte *et al.,* 1995; Mendonça, 2005).

Embryogenic lines of 14 different grapevine cultivars and rootstocks have been established and used for transformation experiments (Gölles *et al.,* 2000; Gribaudo *et al.,* 2003; 2005; Gambino *et al.,* 2005; Maghuly *et al.,* submitted), including the Austrian cultivars Grüner Veltliner and Zweigelt, and the rootstocks Börner and RPG1, a French rootstock with enhanced resistance to nematodes (Bouquet *et al.,* 2000).

Currently at the IAM we have transformed many different apricot, plum, cherry and grapevine lines with different sequences of the PPV genome, the *Prunus necrotic ringspot-virus* genome, the genome of different grape viruses, e.g. GFLV, ArMV, GVA and GVB, and with different marker genes, e.g. GUS, GFP or NPTII (Laimer da Câmara Machado *et al.,* 1992; da Câmara Machado *et al.,* 1995; Korte *et al.,* 1995; Gölles *et al.,* 2000; Gribaudo *et al.,* 2003; Mendonça, 2005; Gambino *et al.,* 2005; Maghuly *et al.,* submitted). These plants represent valuable tools to improve our understanding of host pathogen interactions and may possibly allow the development of alternative defense strategies for crop plants.

However, beyond technical feasibility, efficiency of resistance, environmental safety and Intellectual property Rights (IPRs), also public acceptance needs to be considered (Laimer, 2003c; 2004; 2005). As a matter of fact Graff *et al.,* (2004) have defined the access to Intellectual Property Rights (IPRs) as a major obstacle in the development of transgenic fruit and vegetable crops. Efforts are required to create public understanding and acceptance for these crop plants. To build public confidence the project "Characterisation of transgenic fruit trees and analyses of direct and indirect biological interactions" was initiated to demonstrate the step-by-step principle of working with Genetically Modified Organisms (GMOs) on the case of transgenic fruit trees (http://www.boku.ac.at/sicherheitsforschung/open-e.htm). Transgenic trees of the genus *Prunus* were selected as model organisms to study the performance of woody GMOs over a period of five years.

A "trait/construct dependent focus" for the evaluation in a case by case approach, as suggested by Metz and Nap (1997), seems to be the most appropriate way to evaluate risks and benefits of a genetically modified tree. Much potential exists in the optimisation of constructs, by limiting the expression of transgenes in time and space, e.g. in a certain tissue at a certain developmental stage of a plant, e.g. using inducible promoters (Pühringer *et al.,* 2000; Mendonça, 2005).

MOLECULAR DETERMINATION OF INTERNAL QUALITY PARAMETERS OF FOOD

Apples are the most widely grown and consumed fruit in Europe (a total of 9.1 million metric tons in the year 2000). Regular consumption of fruits and vegetables is generally encouraged in the EU to enhance health and prevent cancer and cardiovascular diseases. However, certain fruits or vegetables may pose a risk to atopic individuals (Steinmetz & Potter, 1996; van´t Veer *et al.*, 2000), since up to 70% of birch pollen allergic persons react with proteins homologous to pollen allergens that are expressed in Rosaceous fruits. On the basis of several epidemiological studies at national levels it can be calculated that at least two millions EU citizens are affected by various fruit allergies (Schäfer *et al.*, 2001).

The molecular mass of the different apple allergens ranges from 9 kDa to > 60 kDa (Hoffmann-Sommergruber, 2002). The 17.5 kDa protein Mal d 1 is the major allergen in pollen-associated fruit allergies, showing a high sequence homology to the major birch pollen allergen Bet v 1 (Vanek-Krebitz *et al.*, 1995). At least two further proteins are involved in the birch-apple syndrome: Mal d 2, a 31 kDa thaumatin-like protein with anti-fungal activity (Hsieh *et al.*, 1995; Krebitz *et al.*, 2003), and Mal d 4, a 14 kDa proline-binding protein, well known as the pan-allergen profilin (Ebner *et al.*, 1995). Mal d 3 — a 9 kDa nsLTP in apple — cross-reacting with homologous proteins in other *Rosaceae* pear, peach and apricots (Schäfer *et al.*, 2001; Fernandez-Rivas & Cuevas, 1999) is encoded by a multigene family (Gao *et al.*, 2005).

The importance of apple allergens, in particular Mal d 1, a Bet v 1 homologue for the pollen-fruit syndrome in Northern European countries and Mal d 3 responsible for true fruit allergies in Southern European countries, has been repeatedly emphasised by researchers during the past two decades. To understand the distribution pattern of major allergens in fruits, we developed tools for investigating the expression of the major allergens in apple pulp and peel, as well as the variations in allergen expression of selected cultivars and their allergenicity for differently sensitised patients across Europe (Marzban *et al.*, 2005). Mal d 1 was measured in 38 apple cultivars by ELISA involving both pAbs and mAbs raised to recombinant and native Mal d 1 respectively, allowing to gather patient independent data. An immuno-tissue-print (ITP) assay was developed, and adopted to localise allergens in apple fruit tissue. The ITP assay allowed a clear mapping of allergens within apple tissues, which confirmed data obtained by real time-PCR and Northern analysis, respectively (Pühringer *et al.*, 2003; Marzban *et al.*, 2005). Mal d 1 and Mal d 2 are distributed throughout the apple pulp and peel, while Mal d 3 is restricted to the peel. The peel-specific expression of Mal d 3 underlines the most likely involvement of nsLTPs in epicuticular wax or cuticle biosynthesis (Kader, 1997).

Different apple cultivars show a markedly different expression of major allergen. This finding may influence the development of diagnostic tools as well as the management of allergic patients. However, all apple cultivars analysed so far by RT-PCR express major apple allerges, Mal d 1 and Mal d 3. Therefore we can assume that there is no naturally occurring apple cultivars without these genes and respective proteins (Marzban *et al.*, 2005).

CONCLUSIONS

Fruit tree planting material in the European Union needs to fulfil requirements for a certain level of genetic and phytosanitary quality. To accomplish this, a well tuned interaction between different players from international specialists developing new technologies, e.g. universities, to national phytosanitary services and accredited laboratories, carrying out the requested tests, to nurserymen and finally to farmers is essential. This will result in improved products for the consumer, that can be traced back on their way of production.

The application of plant biotechnology can make considerable contributions towards the genetic and phytosanitary improvement of temperate fruit trees and grapevines. Entering a phase of market-driven approaches for planting material, where differences about IPRs and protected cultivars will require reliable diagnostic tools to determine cultivar identity are foreseeable. In the field of plant health future challenges we should get prepared for, are certainly new pathogens/vectors requiring the establishment of detection tools. However, every new introduction of a pathogen from abroad, which in times of global mobility and material flow is not avoidable, will require an adaptation to the new situation. Further there exist still diseases of unknown etiology representing future challenges. New breeding goals lie ahead of us. And securing the food quality and contributions to human health will be major issues in the upcoming framework programmes of the EU. The new EU Programme "Plants for the Future" (http://europa.eu.int/comm/research/biosociety/pdf/plant_genomics.pdf) will hopefully render plant biotechnology the appropriate position also in public understanding, it actually would deserve.

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