

The Application of Combinatorial Biotechnology in Improving Potato Resistance to Biotic and Abiotic Stress

Elena Rakosy – Tican¹✉, Ramona Thieme², Adriana Aurori¹,
Imola Erdely-Molnár¹, Enikő Besenyey¹, Raluca Alina Mustata¹, Antonia-
Maria Mărgineanu¹ and Daniel Cruceriu¹

SUMMARY. The concept of combinatorial biotechnology was introduced in previous presentations to international conferences in order to emphasize the importance to combine many tools of biotechnology, including phenomics, genomics and metabolomics, for the final goal to improve plant resistance to biotic and abiotic stress. This concept is exemplified here by a few examples in improving potato crop, one of the most important security crops worldwide and the third important crop as productivity at global scale. This crop is amenable for such improvement for some reasons: it responds well to tissue culture, somatic hybridization and transformation *in vitro*, its genome was sequenced and has got a rich resource of wild resistant relatives in the center of origin of potato crop. Moreover, potato is one of the crops facing great losses because of many diseases and pests, some of them causing total loss of production. The case studies presented in our work involve the use of sexually incompatible *Solanum bulbocastanum* and sexually compatible *S. chacoense* species as resources of multiple resistance genes, such as resistance to late blight caused by blight potato famine agent *Phytophthora infestans*, Colorado potato beetle and the abiotic stress caused by drought. Another example is genetic transformation with a marker free hair pin construct for PVY resistance combine with stress selection *in vitro* for tolerance to draught.

Keywords: Colorado potato beetle, DNA mismatch repair deficiency, draught tolerance, *Potato virus Y*, resistance to late blight, *Solanum bulbocastanum*, *S. chacoense*, Somatic hybridization

¹ "Babeș-Bolyai" University, Plant Genetic Engineering Group, Cluj-Napoca, Romania.

² Julius Kühn-Institut, Institute for Breeding Research on Agricultural Crops, Groß Lüsewitz, Germany.

✉ **Corresponding author: Elena Rakosy-Tican**, "Babeș-Bolyai" University, Plant Genetic Engineering Group, Cluj-Napoca Romania,
E-mail: arina5744@yahoo.com

Introduction

Potato ranks third in global crop productivity, and is recognized as an important security crop. Climate change and exponential growth of world population are posing new challenges to plant breeding (Vreugdenhil, 2007).

In order to obtain an increased crop production it is mandatory to use the most valuable cultivars, but such crops as potato with tetrasomic inheritance and vegetative propagation are lacking genetic diversity and might be difficult to improve further by classical breeding. Genetic resources which are useful to improve the quality of commercial species were exploited to a limited degree mainly because sexual incompatibility with many of the related wild species. Potato, in particular, has got a great diversity of tuber bearing wild *Solanum* species, distributed in South and North America, which represent a rich reservoir of resistance genes for potato improvement (Hawkes, 1990). The new technologies available today, mostly biotechnological tools and new data brought about by genomics, phenomics and metabolomics, allow us to find new possibilities to combine different methods toward the improvement of resistance traits in potato crop (Bradshaw et al. 2006). This is the reason why we introduced the concept of combinatorial biotechnology for crop improvement, exemplifying it in potato (Rakosy-Tican *et al.*, 2012).

In this short review the goal is to present schematically some examples how one can combine somatic hybridization through protoplast fusion or genetic transformation, with molecular markers of resistance genes, molecular characterization of resistant wild species, the use of other tools of *in vitro* culture (embryo rescue, medium term conservation, stress selection), and different tools for somatic hybrids or transgenic lines characterization from molecular analysis, cytogenetic studies (flow cytometry, chromosomes counts, genomic *in situ* hybridization.GISH), resistance analysis, trichome analysis, up to phenomics studies. The final goal is to transfer and combine many resistance genes and traits in pre-breeding clones available for further plant breeding.

The use of *Solanum bulbocastanum* as a source of resistance to *Phytophthora infestans*, Colorado potato beetle and drought stress

The first example is the use of a diploid wild species which is sexually incompatible with potato, *Solanum bulbocastanum* (Rakosy-Tican *et al.*, 2015). In this combinatorial biotechnology scheme for the transfer of: more resistance genes to *Phytophthora infestans* (*Pi*), the oomycete causing agent of late blight disease, also known as Irish potato famine disease; resistance to Colorado potato beetle (CPB) and

the tolerance to drought stress, many biotechnological tools were combined as illustrated in Fig. 1. In the first step, the accession of the wild species was selected by analyzing the presence of *Pi* resistance genes. From four resistance genes characterized to date, the genes *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* were searched for by using gene specific markers (data not published). One accession coded *GLKS* 31741 (*blb* 41, Gross Lüsewitz Potato Collection of the IPK Gene Bank, Leibniz Institute of Plant Genetics and Crop Plant Research, Germany) was identified, which carries two resistance genes *Rpi-blb1* and *Rpi-blb3* (data under publication).

Potato cultivars (Agave, Baltica, Delikat, Quarta and Rasant) and *blb41* were multiplied *in vitro* and used for protoplast isolation and electrofusion (Rakosy-Tican *et al.*, 2015). Both symmetric and asymmetric somatic hybrids were regenerated as it was demonstrated by flow cytometry, molecular markers (SSR, AFLP) and cytogenetic studies (Rakosy-Tican *et al.*, 2015) (Fig. 1). Moreover, only two combinations of potato cultivars with *blb41* i.e. Delikat and Rasant were fertile and by embryo rescue back-cross progenies were successfully produced (Fig. 1). Fertile somatic hybrids were further studied by using gene specific molecular markers and two resistance tests, leaf detached assay (DLA) and field resistance (area under disease progress curve – AUDPC) to identify resistant somatic hybrid clones. Finally, seven somatic hybrids and BC₁ clones were characterized as carrying both *Rpi-blb* genes transferred from the wild *blb41* accession and expressing phenotypically the resistance trait in greenhouse and field (data under publication). Some of the somatic hybrids were also evaluated by a laboratory bioassay for CPB resistance. It was demonstrated that many of the somatic hybrids and their progenies are resistant to CPB (Thieme *et al.* 2014). Further on *in vitro* selection on PEG containing medium and on a phenotyping semi-automated platform (SSDS-HAS Szeged Hungary - http://www.plant-phenotyping-network.eu/eppn/has_hungary) for tolerance to mild drought stress, demonstrated drought tolerance of the wild species accession and some of derived somatic hybrids and BCs progenies (Dénes, 2015; Dénes *et al.*, 2016 – abstract in this volume). These examples demonstrate the successful combination of somatic hybridization based on mesophyll protoplast electrofusion, with marker assisted selection (MAS), cytogenetics (classical and molecular techniques), *in vitro* micropropagation and cloning of resistant wild species accession, embryo rescue for back- crossing and further introgression of resistance genes, *in vitro* stress selection for drought tolerance and semi-automated phenotyping and finally but most important the analysis of resistance in the laboratory, greenhouse and field. All these tools allowed the selection of pre-breeding material combining multiple genes (two major *Rpi-blb* genes) for late blight resistance, with unknown factors inducing CPB resistance and biochemical strategies (data to be published) for drought tolerance.

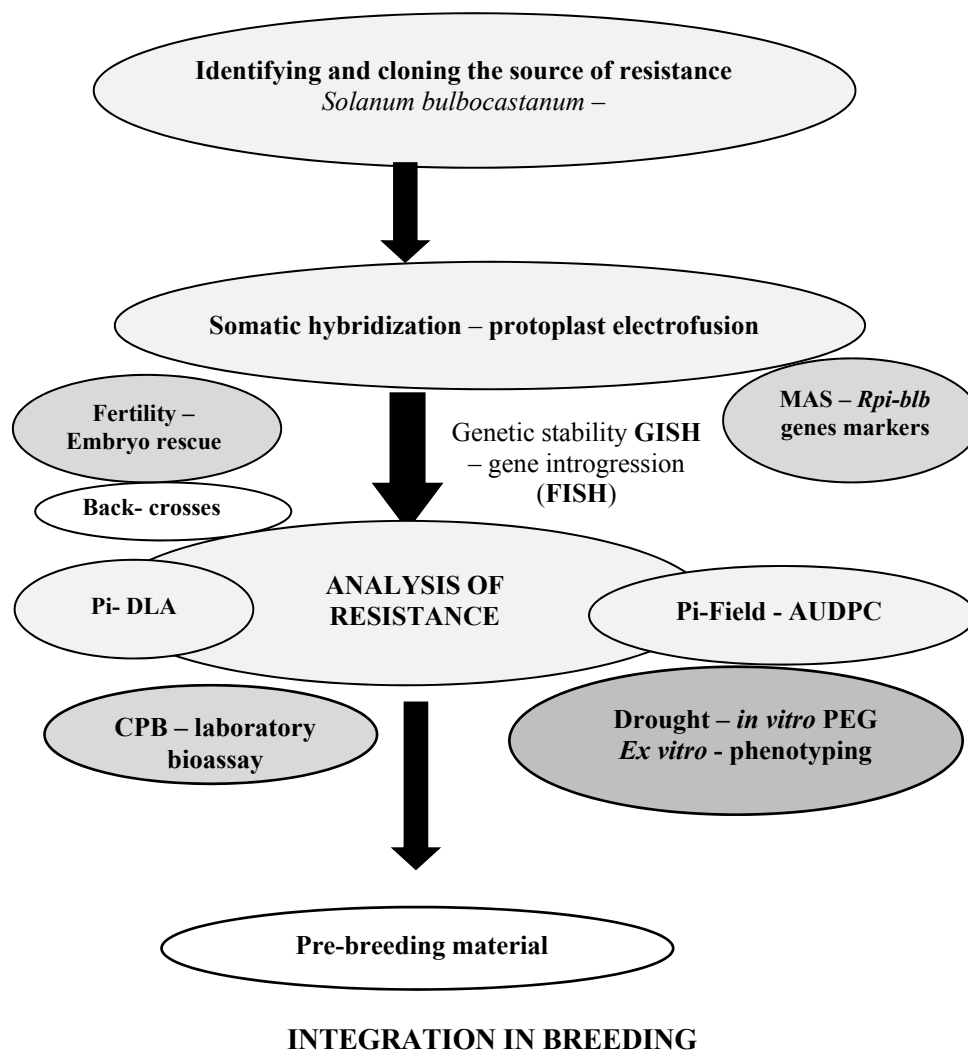
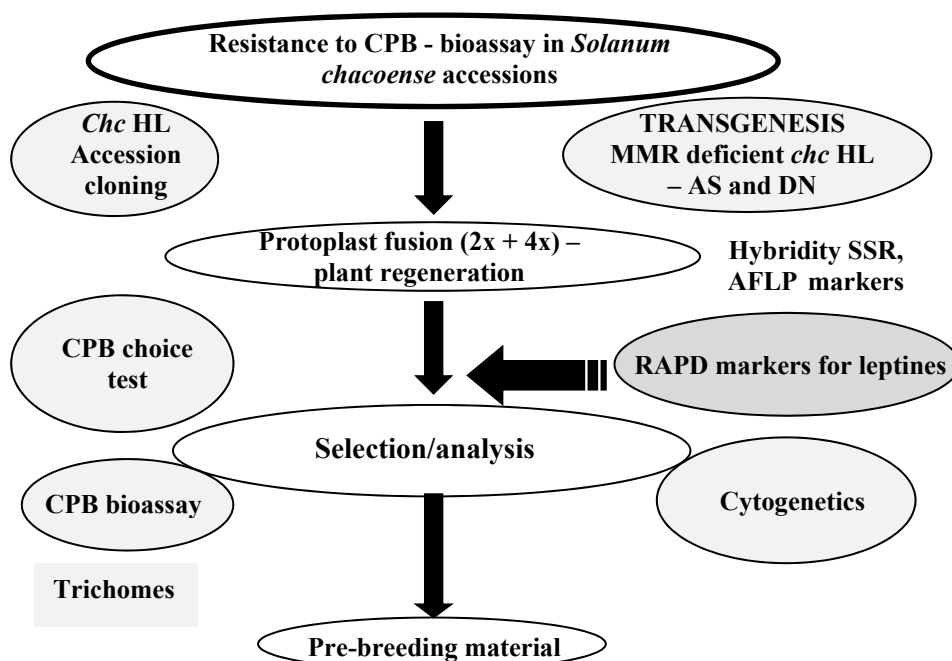


Figure 1. Schematic representation of combinatorial biotechnology applied to improve potato resistance to late blight, Colorado potato beetle and tolerance to drought stress, by using the wild incongruent species *Solanum bulbocastanum* (accession GLKS *blb41*)

The wild diploid species *Solanum chacoense*, sexually compatible with potato, used as a source of resistance to Colorado potato beetle – combining MMR deficiency with somatic hybridization

The second case study presented here involves another diploid wild species, *Solanum chacoense* (*chc* HL), which is a 2 EBN (endosperm balance number – Johnston *et al.*, 1980), and hence sexually compatible with potato tetraploid cultivars, but the classical scheme takes a very long time and needs many steps to transfer only partially resistance from this species into potato gene pool.

One example of potato cultivar incorporating after many crosses genetic material from *chc* is cv. Pannonia (Cernák *et al.*, 2008). This is why we chose somatic hybridization by mesophyll protoplast electrofusion. In the first step the accession of the wild species was chosen i.e. the highest leptine producer (HL) accession PI 458310 (NPGS Sturgeon Bay, USA), and cloned *in vitro*. *S. chacoense* was also transformed, by *Agrobacterium*-mediated gene transfer, with the gene *Atmsh2* – the key gene involved in mismatch repair of DNA (MMR) isolated from *Arabidopsis thaliana* and used as antisense (AS) or dominant negative mutant (DN) form (Rakosy-Tican *et al.*, 2004). Both transgenic, MMR deficient, or wild type *chc* were used in the somatic hybridization experiments (Fig. 2; Rakosy-Tican *et al.* under publication). Regenerated plants were multiplied *in vitro*, analyzed for 'mutator' phenotype and microsatellite instability (MSI) (data under publication). The hybrid status of all somatic hybrids was analyzed by using flow cytometry, cytogenetic indirect (number of chloroplasts in guard cell) or direct methods (chromosome counts), molecular cytogenetics (GISH) and RAPD markers for leptine biosynthesis. Leptines I and II are very specific glycoalkaloids synthesized only in the green tissues of *S. chacoense*, from common glycoalkaloids as solanine and chaconine. These glycoalkaloids are well known as repellent and toxic for CPB (Flanders *et al.*, 1992). Three RAPD markers were described by other authors (Bouarte-Medina *et al.*, 2002), but only one marker was giving positive results in our studies, OPT-20 (Molnar *et al.*, – data under publication). Moreover, to date there were two mechanisms known to interact with the voracious pest, CPB, leptines and glandular trichomes (Pelletier *et al.*, 2011; Mărgineanu *et al.*, 2014; 2015). Besides leptines, we also analyzed the presence and density of the glandular trichomes considering them as the first level of interaction between the insect pest, in our case CPB, and the plant leaf (Fig. 3). Since, not only glandular trichomes but also non-glandular ones can interfere with the insect, at least as mechanical barrier, we also studied this type of trichomes (Fig. 3, Mărgineanu *et al.*, 2014; 2015). In order to reveal first the resistance of somatic hybrid plants with wild type *S. chacoense* to CPB, two assays were applied: the choice test and a laboratory bioassay.



INTEGRATION IN BREEDING

Figure 2. Schematic representation of the combinatorial biotechnology applied in improving resistance to Colorado potato beetle by using transgenesis for MMR deficiency, somatic hybridization and specific molecular markers for leptine biosynthesis along with different techniques for somatic hybrid plant characterization.

The choice test, also used by Cheng *et al.* (1995), allows the adult beetle to choose between one of the parent's leaf or one somatic hybrid leaf. After two hours the preference of the beetle can be quantified by measuring the quantity of leaf which was eaten by the beetle. The test shows the repellence of the wild species and its derived somatic hybrids. Moreover, the laboratory bioassay is done on 25 larvae, grown and fed with leaves of the parents and derived somatic hybrid plants. In this test, the viability, growth curve of larvae, the pupae formation and development of adults as well as adult fertility are assayed. By using those two tests, it was possible to identify somatic hybrids with both antibiosis and antixenosis effects on CPB (Molnar *et al.*, under publication) and it was shown that the proportion of resistant somatic hybrids involving *S. chacoense* with MMR deficiency is higher than when wild type *chc* was used. Those data sustain the hypothesis that MMR deficiency increases homeologous recombination between the two related species.

Moreover, the somatic hybrids with MMR deficiency were also phenotyped after a preselection *in vitro* on polyethylene glycol (PEG) 6000 containing media. It was surprising that although the parental species were not tolerant to drought, some of the somatic hybrids with MMR deficiency were tolerant and accumulated the same biomass as the control plants under normal watering. These results open new possibilities for the exploitation and integration in breeding of these somatic hybrid clones.



Figure 3. The interaction between Colorado potato beetle larvae and one somatic hybrid leaf shows the first level of defense based on glandular (black arrow) and non-glandular (open arrow) trichomes, as well as accumulation of anthocyanins on abaxial leaf surface (purple color).

Combining transgenesis with *in vitro* stress selection for the transfer of PVY resistance and tolerance to drought

Another example of combining genetic transformation with *in vitro* stress selection allowed us to select three clones integrating the marker free hairpin construct of coat protein gene from potato virus Y (PVY) with tolerance to drought,

after two steps of selection *in vitro* on media supplemented with PEG 6000, first at callus and then at plant level on increased concentrations of PEG (5, 10 and 15%) (Rakosy-Tican *et al.*, 2010; Mustață *et al.*, 2014). In this strategy, first the transformation procedure based on *Agrobacterium*-mediated protocols and using *gfp* reporter gene and *nptII* selectable marker gene were used to improve gene transfer and select the potato cultivars with the best regeneration abilities (Fig. 4, Rakosy-Tican *et al.*, 2007).

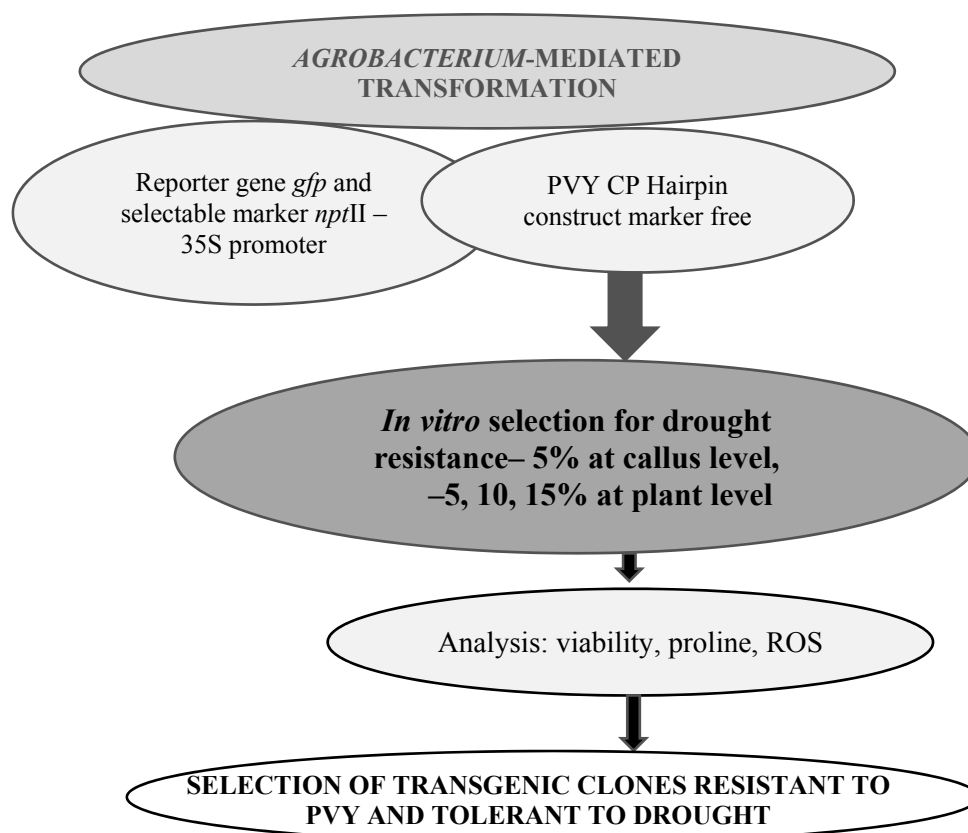


Figure 4. Combining transgenesis with a marker-free hairpin construct for PVY resistance and *in vitro* stress selection for drought tolerance in potato.

Then potato cvs. Baltica and Désirée were transformed by using a marker-free construct with hairpin structure containing PVY coat protein genes separated by an intron (Rakosy-Tican *et al.*, 2010). The transgenic clones were analyzed by PCR to confirm they integrated the hairpin marker-free construct and then were multiplied and stored *in vitro* as minitubers. The transgenic clones were then selected *in vitro* by

transferring internodes on media containing 5% PEG 6000 (Mustață *et al.*, 2014). The regenerated plants on PEG containing media, as well as controls were further selected on increased concentrations of PEG: 5, 10 and 15% PEG 6000. Plant regeneration and viability, proline biosynthesis and H₂O₂ were determined and three clones integrating PVY construct and showing tolerance to drought were selected (Mustață *et al.*, 2014).

Conclusions

The case studies presented in this mini-review on the application of combinatorial biotechnology for potato improvement shows how different biotechnological tools like somatic hybridization or transgenesis can be combined with *in vitro* techniques, molecular analysis including marker assisted selection, cytogenetics and phenotyping (for resistance traits) for the transfer and integration of multiple genes and traits into potato crop. This new concept opens new ways for the integration of all modern tools of genomics, phenomics and metabolomics with *in vitro* technologies for better improvement of crops.

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A. –M. MĂRGINEANU, D. CRUCERIU

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