Recalcitrant crops budge into new protocol

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Among many other crops, tomato has been known as a highly recalcitrant crop in doubled haploid technology. For a long time, it seemed impossible to obtain doubled haploid plants via gametes. Fytagoras has succeeded in developing a method also for this difficult crop that has already been applied successfully for several customers.

In the breeding strategies and programmes of many crops, genetically pure homozygous lines are highly desired. In pure homozygous individuals, the chromosome pairs (one originating from the father and one originating from the mother) are identical, i.e. the alleles on both chromosomes are identical. An allele is one of two, or more, versions of the same gene at the same place on a chromosome.
These homozygous lines make qualitative and quantitative phenotypic selection more efficient, as no hidden properties are present (all genes are present in just one version, in contrast to heterozygous individuals). In addition, these lines form the basis for F1 hybrid seed production, i.e. a crossing between two homozygous parents resulting in identical offspring. In homozygous parent lines, lethal and weak alleles are eliminated as they cannot survive since no strong counter allele is present.
Finally, pure homozygous lines are desired for establishing chromosome maps, whole genome sequencing, bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations and for mapping of quantitative trait loci (QTLs). There are different methods in plant breeding to obtain (pure) homozygous lines. For different crops, different methods may be applied. In this article, the different methods will be briefly discussed and, subsequently, the focus will be on the production of doubled haploids (DH) via androgenesis.

Methods

Generally, there are three ways to obtain (pure) homozygous plants, more or less applicable to different plant species: back crossing breeding, doubled haploid technology and the haploid inducer system. Back crossing is a relatively simple way to obtain nearly homozygous lines in breeding programmes. These back crossings (inbreeding) result in a more homozygous offspring every cycle, however, complete (100%) homozygosity is never achieved. To obtain an applicable level of homozygosity, up to ten back crossing cycles are required. This is labour intensive, as well as time-consuming (depending on the crop, these back crossings may take up to ten years). Methods like single seed descent (SSD) may be used to speed up the propagation and selection process, but still several years are needed to obtain stable lines.
Hence, the need for methods that deliver 100% homozygous lines in a faster way. The solution to this is the development of doubled haploid (DH) lines via DH technology.

Doubled haploids

DH lines are produced in tissue culture from haploid cells (containing only one copy of the genome) that are forced to divide, duplicate their chromosomes (become doubled haploid = homozygous diploid) and form embryos. The embryos can germinate and form new DH plants. In this respect, DHs are a revolutionary achievement in plant breeding, because completely homozygous plants can be produced within one generation. DH production can be achieved through androgenesis, gynogenesis or parthenogenesis, depending on the species. A chromosome-doubling step is mandatory when spontaneous DHs are not regenerated. This can be achieved by using antimitotic compounds to double the ploidy level of haploid plants.
To obtain DH lines, gametes from meiotic cells are harvested and, from them, plantlets are generated (androgenesis or gynogenesis). Alternatively, haploid embryos can be produced by 'pollination' with pollen (parthenogenesis).
The haploid cell can be either a microspore from an anther or an ovule from an ovary, depending on the species. The regeneration of plants from microspores or ovaries is a one- or two-step process. If the protocol directly induces embryo formation, a plant can be produced in one step. In many cases, first a callus (non-differentiated tissue) is produced and, in a second step, embryos and plants. Gametic cells from meiosis can also be developed into haploid embryos, via parthenogenesis. Thus, the process of DH production always involves a gametic haploid step from which haploid or DH plantlets will be regenerated.
The in vitro culture of gametic cells in androgenesis and gynogenesis techniques requires the original gametophytic pathway of the gamete to be redirected towards a sporophytic pathway, where plantlets can be regenerated. In this technique, androgenesis is the most common method to produce DHs, although, for a number of species, gynogenesis is preferred. In

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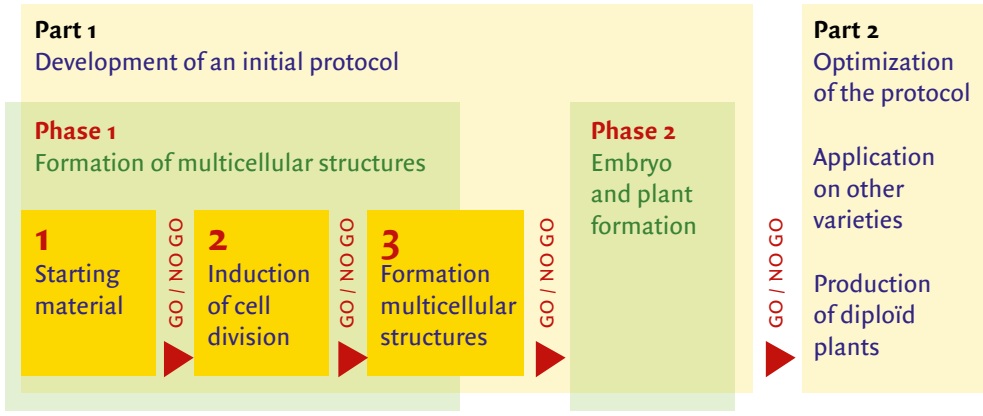

Overview of Fytagoras' approach for doubled haploid plant production

Tomato DH plants in the green house for seed production

androgenesis, isolated microspores or anthers containing microspores are cultured in specific induction media to induce the formation of embryos or callus. The ploidy level of the regenerated plants can differ depending on the cell events related to spontaneous or induced chromosome doubling. Haploids, doubled haploids, mixoploids and tetraploids can be produced during the in vitro DH process.
Potential pitfalls are that some protocols are prone to also produce somatic embryos from anther or ovary tissues. These plantlets are diploid but have an identical genomic background to the mother plants from which DHs are expected to be generated. Also, clones of one individual DH may arise, especially when a callus step is involved in the production of plant material. In parthenogenesis methods, the formation of an embryo from an egg cell without fertilization takes place. Irradiated pollen is used to induce egg cells to form haploid embryos. These embryos only inherit the maternal set of chromosomes. Such embryos germinate in vitro and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants.

Haploid inducer

Finally, the third method to obtain DH plant is the use of haploid inducer (HI) lines. Pollen or ovules from paternal or maternal HI lines allow for fertilization and embryo development but only one set (maternal or paternal) will survive in the process. Hence, haploid embryo's (plantlets) are produced that can be treated with antimitotic chemical to achieve chromosome doubling, resulting in a (pure homozygous) doubled haploid. For example, maize spontaneous developed HI lines are available, while for some other species HI lines are made through genetic modification, e.g. with CRISPR-cas techniques.

Fytagoras approach

Amongst different methods described above,

Tomato DH plants in tissue culture obtained via androgenesis from a single parent line. The high diversity in the phenotype of the different DH plants showing the genetic potential is remarkable

Fytagoras has focused on androgenesis for production of doubled haploids. For this, haploid cells from gametes are collected and provoked for dividing and for doubling of their chromosome store in order to obtain a stable doubled haploid line within only one generation. In contrast, with traditional methods, it may take 6 to 10 generations of self-fertilization to obtain a variety that has fixed major characteristics (which can nonetheless be unstable).

Fytagoras is a research company, which over the last years has become a specialist in doubled haploid technology. As a service to customers, Fytagoras offers DH protocol development as well as production of doubled haploids from existing customer-owned lines on demand. A major milestone has been the realization of protocol for production of tomato doubled haploids in 2017. Tomatoes have been known for many years as a 'very recalcitrant crop' and it seemed impossible to obtain doubled haploid plants through gametes

Obviously, an in vitro cultivation stage is necessary in order to regenerate a complete plant, as the rate of natural mutation from haploid (sterile) to diploid (fertile) is only 1 to 10%, depending on the variety. By careful treatment of isolated reproductive cells and the application of different stress and growth conditions, these cells can double their genetic material and can be stimulated to divide and form multicellular structures, embryos and plants. Typically, for some species, agents are needed to enhance the conversion from haploid to diploid cells and make it useful for practical application. For all species, and often also for every variety, optimal conditions for the generation of doubled haploid must be selected and implemented.

Phasing approach

The strong point from the Fytagoras' approach is the phasing approach (see scheme) combined with practical experience in donor plant selection and treatment selection. Selection and growth of donor plants is the key to success, providing flower material for isolation of viable gametes, which are used for tissue culture activities. Fytagoras, a company active in research projects involving horticulture and seed technology, has implemented a three-step programme. In the first phase, the quality, vitality and responsiveness of the cells are determined. In the following phase, a basic protocol development is carried out, while in the third phase, the protocol is optimized for efficiency.

Crops

Protocols for production of doubled haploids plants have been published for many species already and are commonly used in breeding programmes worldwide. Typically, doubled haploids are produced for open field crops like barley, maize, wheat, rice, tobacco and rape seed, but also for vegetable crops such as cucumber, carrot, pepper, eggplant, tomato and many other crops. Also, with increasing business and specialization over the last years, more breeders of ornamentals make use of doubled haploid technology. However, not all the species respond well enough to doubled haploid technology and even within species, tremendous variation may exist in responsiveness of different varieties for protocols.

Many species are still considered as recalcitrant to these treatments, including many of the most important crops worldwide, such as cotton and coffee. Despite the work of many groups, little is still known about how to overcome recalcitrancy. Initiatives have been launched about identification of genes, and other factors which are related to responsiveness of genotypes for doubling of haploid cells and induction of cell division. Clearly, a systemic approach is needed, strong dedication and an organization which ensures the continuity of the research programme. Commercial agreements with seed companies may be a solid base, ensuring progress over the years and dissemination of the results. At least for tomato, this approach turned out to be successful.