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The molecular biology of grafting

Recent research may provide new applications for a millennia-old agricultural technology

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Plant grafting is one of the oldest agricultural technologies that has been practiced for at least two and a half Millennia across Europe and Asia in horticulture and fruit orchards (Fig 1). Largely used for trees and shrubs, grafting combines two plant varieties with desirable traits into one plant without the need for tedious crossing and backcrossing. One major use has been to graft fruit trees on dwarfing rootstocks so as to increase yield by accommodating more plants in a given amount of land. Another widespread application of grafting is to optimize pollination. Some fruit trees are not self-pollinating and require a second tree of the opposite sex nearby. It can be more efficient and effective therefore to graft a scion from a male plant onto a female plant. Still, there are many cases where grafting would be highly desirable but has not worked due to incompatibility between the plants. It is here that research to understand the molecular processes underlying grafting could indeed make it possible to extend its scope beyond current applications in viniculture, horticulture and fruit trees.

An ancient technique

The basic principle is simple enough, often involving attachment of the shoot of one plant, known as the scion, onto the stem of another plant—the rootstock—after peeling back the bark of each. Wound healing processes then join the two plants into a hybrid. Alternatively, a single bud rather than a whole shoot of one plant may be grafted on to the rootstock of another, which can be more efficient when the scion is in short supply. Generally, it is the bud or scion that has desirable new traits, such as taste or colour, but requires the rootstock for improved vigour, growth control, protection against pests and disease or some other factor.

The most celebrated example of grafting came in 19th-century Europe when indigenous vines were saved from extinction by grafting with rootstock from a North American variety that had evolved resistance against an aphid commonly known as phylloxera (Fig 2). These microscopic insects had themselves been imported from North America and devastated European vines by feeding on roots, causing deadly secondary bacterial or fungal infections to take hold. American vine species, the rootstocks of which came to the rescue, had evolved several defences against phylloxera: exuding a sticky sap that repels the nymph form of the insects by clogging their mouths, and a protective layer of tissue to protect the wound from secondary infections.

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It has long been known from observation and experience that grafts between different genera of the same family are rarely compatible but are usually possible between species of the same genus. Some combinations require slightly more sophisticated techniques to enable the grafts to take: careful cutting and strong binding such that surfaces contact snugly, with the use of wax and cloth or moist peat moss to protect against drying. Throughout its history though grafting has been developed effectively by trial and error, with no scientific basis either for determining which combinations would work or improving the chances of success. This may be about to change though as research has begun to uncover the molecular mechanisms and evolutionary basis of grafting.

Lateral gene transfer via plasmids

The first major discovery was that whole genomes can move across a graft, which might have evolved as an alternative asexual method of plant adaptation, in addition to sexual reproduction and gene duplication. Apart from the nucleus, plant cells contain genomes in their organelles, the plastids and mitochondria, which could potentially move across a graft to transfer genetic material. One of the first clear demonstrations of organelles being transplanted laterally between organisms came from a 2012 study in which sexually incompatible species had been grafted together (Stegemann *et al*, 2012). It presented phylogenetic evidence from analysis of DNA sequences among related species that the plastid genome in the recipient plant had been replaced by the alien genome.

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Then, a more recent paper identified some of the mechanisms involved (Hertle *et al*,

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Figure 1. Orchard apple tree (*Malus domestica*) near Reilingen, Germany. Wikimedia / AnRo0002.

2021). The authors grafted callous tissue that forms around healing plant wounds rather than full stems, and stained cells with a fluorescent dye to observe cells at the border of the two tissue types for possible gene transfer using live-cell confocal microscopy. This revealed pronounced protrusions between connecting callus cells, with irregular and patchy staining of walls between adhering cells, indicating that substantial rearrangements of the cell wall structure occurred upon formation of the graft union.

Next, the grafted tissue was cryofixed for high-resolution structural analysis by sectioning scanning electron microscopy and tomographic 3D reconstruction. These images revealed cytoplasmic connections—known as plasmodesmata—across the cell walls and plastids and mitochondria in these channels. Furthermore, the organelles in the vicinity of these microscopic channels through the cell walls were smaller than the pores themselves. “Our main conclusions are firstly that genome transfer occurs by cell-to-cell migration of whole organelles, as we have shown for plastids, and secondly that there is a previously unknown intercellular transport pathway that allows exchange of very large cellular structure, including entire organelles, from cell to cell”, explained Ralph Bock from the Max Planck Institute of Plant Physiology in Potsdam, Germany, and lead author of the work. Their paper has been widely acknowledged as having advanced understanding of plant grafting at the molecular level, building on the earlier work showing that genetic material was exchanged. “Yes, I think that this work represents a major contribution to our understanding of intercellular organellar transfer”, said Margaret Frank, a specialist in grafting and plant trait analysis at Cornell College’s School of Integrative Plant Science Plant Biology in the USA.

A role in plant evolution?

There is less agreement though over what this means for plant evolution. Bock proposed that it has revealed an alternative method of adaptation through plant hybridization, even if its full significance has yet to be established. “We believe that horizontal transfer of nuclear genomes represents an asexual pathway of speciation, that is formation of new species that are allopolyploid [with genomes derived from at least two different species]”, he explained. “Horizontal transfer of plastid and mitochondrial genomes is an asexual pathway that produces new combinations of

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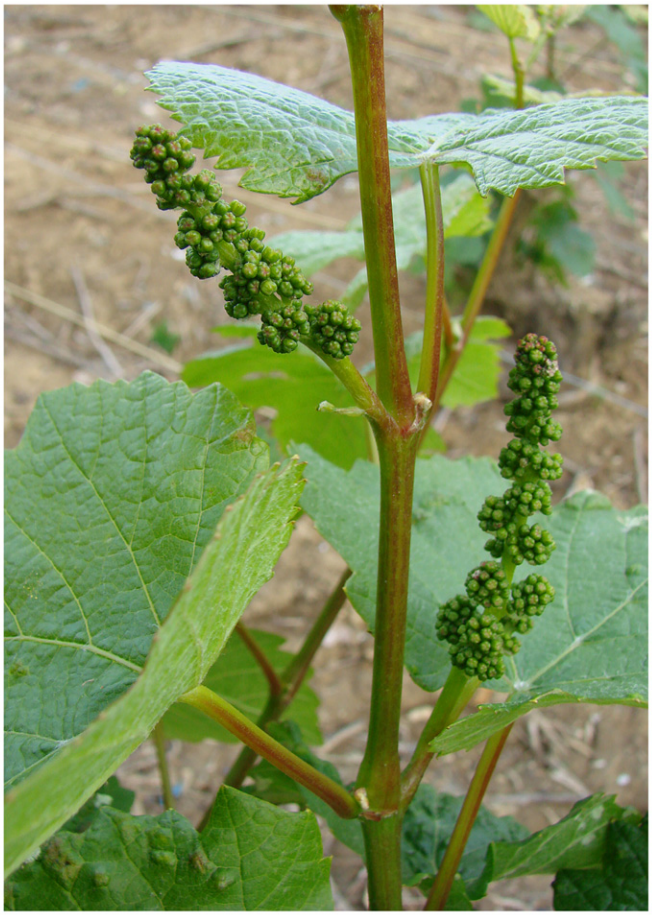

Figure 2. Inflorescence on a grapevine (*Vitis vinifera*). Wikimedia / Vassil.

nuclear and organellar genomes, a phenomenon referred to as organelle capture, or chloroplast capture. [. . .] Again, it is conceivable that, in some cases, organelle capture has occurred sexually, through interspecific hybridization followed by multiple rounds of backcrosses with one of the two parental species, so both mechanisms may have played a role in evolution".

Not everyone is convinced though. "My understanding is that grafts in nature occur somewhat infrequently and do not play a significant role in terms of plant evolutionary adaptations", Frank said. Her view is supported by Pal Maliga, whose lab at Rutgers University in the USA confirmed Bock's discovery that both plastids and mitochondria move through graft junctions. He agreed that grafting does occur in nature, particularly with parasitic plants such as mistletoe and holly, but is careful about ascribing it a wider role. "Hybridization during grafting is likely to play a minor role, because there is no pre-selection for compatible nuclear genomes, which would be the case in sexual hybridization. Gene duplication and sexual selection are major, universal sources of variability", he added.

Whether or not grafting plays a role in plant hybridization or adaptation, it does not appear to have been a primary driver of evolution. "I don't think plants have evolved to graft", said Charles Melnyk at the Swedish University of Agricultural Sciences (SLU) in Uppsala. "Instead, I think grafting is a combination of wound healing and vascular development that people have adapted to successfully graft".

Differences in wound healing

Melnyk's team has been using artificial grafting to study the mechanisms of wound healing, which in turn can help advance the field of grafting. "We have a better understanding of the transcriptional landscape and genes involved with graft formation, for instance what processes are activated and when", Melnyk said (Melnyk *et al*, 2018). He and his team observed the sequential activation of genes important for vascular development, including genes implicated in development and growth, especially of the phloem that transports soluble compounds made by photosynthesis, the xylem that carries water and nutrients in the opposite direction from roots to stems and leaves, and the cambium comprising partially differentiated cells in between these two layers. They observed massive but temporary changes in gene expression, allowing rapid cell differentiation between the rootstock and the scion. Crucially, the communication between tissues occurs independently of the normal functional vascular network and acts as a signal to activate appropriate regeneration of such vascular connections.

There was also a systemic response beyond the graft similar to what happens in wound healing. This involved increased signalling via auxin, a class of plant hormones involved in growth and development, which was perceived by the root within hours of tissue attachment to activate the vascular regeneration process. Yet, a subset of genes was expressed only in grafted tissues, indicating that healing proceeded via different mechanisms depending on the presence or absence of adjoining tissues. The authors speculated that such a recognition process could have broader relevance for tissue regeneration, inter-tissue communication and tissue fusion events, which could be exploited in applied graft research.

Melnyk has a rather different take on natural grafting, which he argues is strictly distinct from the artificial variety, because it did not involve actual damage via cutting into stems. "However, tissue fusions are fairly common in plants, for instance in flower development", he added. "Stem and root fusion also appears, particularly root fusions. It is tough to say if plants evolved this ability, but natural root grafts are quite common and is probably a great way for trees to communicate and support each other through nutrient exchange". Melnyk gave the striking example of the albino redwood of California, which has lost its ability to produce chlorophyll and has white needles. It exists only in forests surrounded by other redwoods and survives by obtaining sugar through root connections with its photosynthesizing neighbours.

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Graft compatibility

There is more to do though. “A challenge will be to confirm that the mechanism of nuclear genome transfer is the same, which is what we suspect”, said Bock. “This will be technically much more difficult”. Although Bock believes that nuclear genomes are also transferred through cell pores, they are much larger than plastids so another mechanism may be involved. Nuclei or nuclear genomes could, for example, be transferred through partial cell fusion, where a cell bud combines with a neighbouring cell in the graft junction.

But, as Bock said, direct observation of cell-to-cell movement by nuclei, or even mitochondria, is much harder technically than in the case of plastids. Plastids do not normally fuse and recombine, which makes it possible to follow the fate of individual organelles. Second, plastids can be genetically transformed, facilitating expression of fluorescent marker proteins within the organelle. These proteins usually stay within the organelle, whereas they tend to leak out of the nucleus into the cytosol, where they could create a source of observational error if they were taken up by another nucleus.

There are also largely unanswered questions around graft compatibility, of which species can hybridize this way and which cannot. “The question relates to the evolutionary distance that allows for firstly horizontal transfer of nuclear genomes, and secondly horizontal transfer of plastid genomes”, Bock explained. “So far, we’ve shown that transfer is possible between closely related species. It is conceivable that, with increasing phylogenetic distance between the two species involved, we will see cases of genome incompatibility that result in infertility or other mutant phenotypes”.

Applications

Nonetheless, the better understanding of molecular mechanisms leads on to practical applications, either to establish grafts between hitherto incompatible plants, or making grafting as it has been practiced for centuries more efficient, with improvements in yields and reduction in costs. “The knowledge can be shifted to conventional intrafamily grafting”, commented Michitaka Notaguchi at Nagoya University in Japan. “Even among closed plant species, we frequently meet graft incompatibility. For commercial purposes, graft efficiency or success rate and yield need to be maintained at a high level. Even a small graft incompatibility cannot be accepted. Therefore, even though new cultivars harbouring better traits have been developed, because of graft incompatibility they sometimes cannot be utilized”.

He pointed to recent work, including his own lab’s, on grafting inducers to create new combinations of potential agronomic value. His team looked in particular at β-1,4-glucanases, one of the glycosyl hydrolases that help break down cellulose, the primary component of plant cell walls. In this way, these enzymes encourage plant cells to contact with each other in grafts, with β-1,4-glucanase playing a crucial role for cell–cell adhesion in the *Nicotiana* family.

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Notaguchi’s group used transcriptomic approaches to identify a specific clade of β-1,4-glucanases that is upregulated in successful grafting but not in incompatible grafts. This was found to precede graft adhesion and was facilitated by an overexpressor of the β-1,4-glucanase. Notaguchi’s team demonstrated this by successfully grafting tomato scions onto rootstocks from other plant families to potentially increase yield, quality or abiotic tolerance. In general, it shows that improving direct cell–cell adhesion could enhance plant grafting to develop new combinations (Notaguchi *et al*, 2020).

In the same paper, Notaguchi and his co-authors also discuss so-called supergrafters, plants that are capable of hybridizing with a wide range of other species. One example is the tobacco relative *Nicotiana benthamiana*, which is capable of acting as an intermediary between pairs of plant species that are otherwise incompatible for grafting. In this case, the expression of β-1,4-glucanases secreted into the extracellular region turned out to facilitate cell wall reconstruction. These insights at the molecular level hold much potential for using grafting as a short cut in conventional plant breeding to establish novel traits on robust stocks more quickly. “Repeated back-crossing can be avoided by one-step transfer of chloroplasts of economic value by graft junction”, Maliga commented. As a result of this economic potential, brought about by those recent advances, the field of grafting research, which has been quite close knit for some years, is expanding.

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