



# International Journal of Research in Agronomy

E-ISSN: 2618-0618

P-ISSN: 2618-060X

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2025; 8(2): 316-323

Received: 21-11-2024

Accepted: 27-12-2024

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## Evolutionary insights and cytogenetic solutions for apple orchard sustainability

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DOI: <https://www.doi.org/10.33545/2618060X.2025.v8.i2e.2583>

### Abstract

Apple (*Malus domestica*) is a widely cultivated fruit crop of significant commercial importance. Its domestication has primarily involved hybridization and clonal propagation, enabling the selection of desirable traits. However, the genetic diversity of domesticated apple varieties remains limited, presenting challenges for breeding efforts. Wild relatives of apples serve as a valuable genetic resource for enhancing traits such as stress tolerance, disease resistance, and fruit quality. Advances in cytogenetic techniques, including fluorescence in situ hybridization (FISH), genomic in situ hybridization (GISH), and flow cytometry, have contributed to a deeper understanding of genome structure and chromosomal modifications in apples. Additionally, polyploidization plays a crucial role in stress adaptation, with triploid and tetraploid cultivars showing enhanced resistance to both abiotic and biotic stress factors. This review explores the genetic and cytogenetic mechanisms that influence stress responses in apples while highlighting the role of genomic tools and breeding strategies in developing climate-resilient cultivars. Furthermore, the application of genome-editing technologies such as CRISPR-Cas9 in apple improvement is discussed.

**Keywords:** Chromosome, cytogenetic techniques, hybridization and polyploidization

### Introduction

*Malus* spp., members of the Rosaceae family, there are roughly 61 apples wild species and hybrids, which are often classified based on fundamental physical characteristics, along with one domesticated species, *Malus domestica* Borkh. According to Bramel and Volk, apples were preeminent worldwide commercial fruit crops.

*M. domestica* (Apple) fruit is harvested for various uses as, included being eaten Fresh, Used for cider and processed into other products like applesauce, dried fruit, while the other *Malus* species serve as rootstocks as well as decorative plants. Wild apple trees grow naturally in temperate regions across the northern hemisphere, with China being their main place of origin. (Velasco *et al.*, 2010)<sup>[42]</sup>. *Malus domestica* is thought to have developed as *M. sieversii*, species native in Central Asia and Western China.

Apple trees with desirable characteristics have been identified in their natural habitat. The primary motivators for apple domestication probably involve fruit size & organoleptic properties. The domesticated apple, *Malus domestica*, have been originated from *Malus sieversii*, a wild apple species native to western China, Kazakhstan, and parts of Central Asia. As these apples traveled along the ancient Silk Road, they crossed through regions like the Caucasus, where *Malus orientalis* grows, and into Europe, home to *Malus sylvestris*. Along the way, they mixed with other wild apple species, resulting in the apples we know today. Additionally, a process called whole-genome duplication (WGD), where the plant's genetic material doubles, played a key role in their evolution and adaptation over time. This process could last roughly 200 million years. Apple genome evolution started with extensive genome duplications between fifty and seventy million years earlier (Proost *et al.*, 2011)<sup>[30]</sup>. The *Malus* genus has developed through processes like hybridization, diploidization and polyploidization understanding its genetic makeup, especially genome size as well as ploidy level, is critical in the development of new apple varieties having desirable qualities like as stress resistance,

increased fruit quality, and useful characteristics. This information enables breeders to pick and control genes to get the desired results. Studying both karyotype and chromosome features, both physically and molecularly, is critical effective apple breeding, especially given the problems faced by climate change & population expansion. This article investigates the use of several technologies, including chromosome counting, flow cytometry, through extensive genome wide genotyping, and to identify polyploids and aneuploids, thereby aiding the production of new cultivars. The cytogenetics of the *Malus* genus has an extensive relationship with the domesticated apple (*M. domestica* Borkh.)

Abiotic stress like drought stress, can majorly impair crop growth and development, Leading to decreased yield and quality. Plants cause these stresses mainly at molecular level, affecting every stage of their growth (khan *et al.*, 2019) [17]. Drought stress particularly is a severe form of abiotic stress that mostly restricts the global crop production and is expected to become more prevalent in future (Sun *et al.*, 2020) [41]. Therefore, enhancing crops with genetic traits that improve their tolerance to drought while attaining high yields is a critical aim in crop Management.

In prevailing Maloideae subfamily, (Esumi *et al.*, 2005) [6] used molecular phylogenetic analysis to propose an autopolyploid genesis arising from the progenitor, *Gillenia*, a genus containing 9 base chromosomes, refuting the extensive hybridization theory. (Considine *et al.*, 2012) [4] suggested a three-step idea for creating the *Malus* genome, which includes auto-aneuploidization, tetraploid formation, & diploidization, all of which contribute to the current diploid state (Cornille *et al.*, 2019) [5] reinforced this by proposing that *Malus* evolved by autopolyploidization, diploidization, and aneuploidization.

These studies emphasise the intricate evolutionary history for apples particularly the Maloideae subfamily, shining light over the genetic processes associated in their genesis.

The study looks into the way nuclear DNA content varies among diploid, triploid, as well as tetraploid organisms. It looks on the functions of mitotic & meiotic polyploidization in the evolution of apple varieties. Furthermore, it investigates how genome size & ploidy level influence certain phenotypic features. This study gives an understanding of the mechanisms causing genetic variation including its consequences for the traits of apple cultivars.

### Ploidy levels among *Malus* species

*Malus* species, which include apples, usually have a pair of chromosomes, making them diploid. Other species, however, are triploid, and tetraploid, with different chromosomal numbers. For example, *M. toringoides* & *M. hupehensis* are solely triploids, but *M. angustifolia*, *M. glaucescens*, *M. lancifolia*, and *M. platycarpa* are all tetraploid Species. Scientists discovered that polyploidy, essentially possessing many sets of chromosomes, might help these creatures adapt to their circumstances. It is assumed that newly generated polyploid species occur often through processes that include genomic duplication and diploidization, both of which result in increasing chromosome counts. (Schuster *et al.*, 1995) [35] propose that changes in ploidy levels throughout a species may indicate hybridization.

Triploid individuals, for example, could be the outcome of numerous crossings including diploids and tetraploids, as well as apomixis. Triploid organisms are typically unable to self-pollinate, however in some situations they may create viable reduced and unreduced gametes. Recent study shows that

whenever triploid apple cultivars such as 'Jonagold' are permitted to cross-pollinate spontaneously, the progeny can have a wide range of chromosomal numbers & ploidy levels. This shows that spontaneous cross-breeding among triploid apples produces significant genetic heterogeneity in their offspring.

### Stress Management

Plants are faced with a variety of biotic and abiotic challenges in their natural environment, and to cope with these stresses, numerous signaling pathways are activated.

#### Heat stress

Stress caused by heat is a key abiotic factor that reduces productivity and quality, which leads to severe economic losses. Previous research has shown that high temperatures can impact plant morphology, anatomy, physiology, and biochemistry. Efforts to combat heat stress in crops are hindered by a lack of understanding of its impact on fruit growth. Improving heat stress tolerance via breeding & biotechnology is critical (Foster *et al.*, 2018) [7]. Heat stress influences a variety of biological processes in plants. High temperatures can harm photosynthetic equipment, resulting in slower rates of photosynthesis (Wang *et al.*, 2018) [45].

Autophagy is an ancient and evolutionarily conserved process that is responsible for clearing excess cytoplasmic content while facilitating recycling of proteins. In plants, nutrient cycling has been shown to be important for their resistance against abiotic as well as biotic stressors. Heat stress research has reported proline-rich proteins (PRPs) to be important cell wall proteins responsible for responding to stress. A study of nine PRP genes revealed that \*MdPRP6\* was responsible for enhanced heat stress tolerance in genetically engineered apple varieties. Increased levels of \*MdPRP6\* minimized cellular damage due to stress and increased photosynthesis, emphasizing the significant contribution of PRP proteins towards enabling apple plants to withstand harsh environmental conditions.

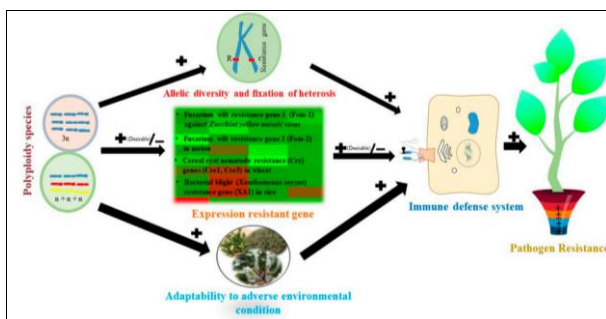
#### Drought Stress

Drought is another key constraint on crop output. Drought is becoming a global worry, compromising future agricultural output as temperatures rise and water resources grow scarce. Plants have evolved a variety of chemical and physiological stress response mechanisms. Autophagy is thought to help people cope with stress. enhanced levels of MdATG18a from apple plants improve drought tolerance, probably due to enhanced autophagosome production. These processes have been proven to assist dissolve protein aggregates and protect against oxidative damage (Zhang *et al.* 2015) [49]. Nitrogen-activated protein kinase genes became more active during drought, indicating that they help strengthen the relationship between AMF (arbuscular mycorrhizal fungi) and apple trees, making the trees more drought-resistant. Additionally, plant transcription factors (TFs) play a key role in controlling how plants respond to stress (Wang *et al.* 2016) [44].

#### Polyploidization

Polyploidy can affect plant species' allelic diversity, expression of genes (overexpression), and physiological status. Polyploids have more alleles at a particular locus, which increases allelic diversity and resistance due to the increased likelihood of heterozygosity. Multiple polyploid chromosomal sets contribute to gene expression. In contrast, it had been hypothesized that increased ploidy level reduces gene expression (at specific loci or throughout the genome, and in rare cases, silences genes).

Polyploid plants gain stress tolerance, which allows them to adapt to a broad spectrum of environmental situations. Invasive plant species tend to be more disease-resistant than humans exposed to environmental stressors. For developing new cultivars, it is important to select polyploid plants that exhibit greater genetic diversity in resistance genes and stronger immune responses. These traits can provide enhanced resilience to diseases. In addition, it is crucial to evaluate how these plants' resistance mechanisms interact with environmental stresses, ensuring that cultivars can thrive in variable conditions. This integrated approach improves the likelihood of producing robust, stress-tolerant crops that meet both agricultural and environmental challenges. Polyploidy can significantly influence a plant's resistance to pathogens. In plants with higher ploidy levels, there is an increased likelihood of improved pathogen resistance. This is primarily due to factors such as greater genetic diversity, dominance of beneficial alleles, and enhanced expression of resistance genes. These elements collectively lead to better adaptability against both biotic (pathogen-related) and abiotic (environmental) stresses. Polyploidy can significantly influence a plant's resistance to pathogens. In plants with higher ploidy levels, there is an increased likelihood of improved pathogen resistance. This is primarily due to factors such as greater genetic diversity, dominance of beneficial alleles, and enhanced expression of resistance genes. These elements collectively lead to better adaptability against both biotic (pathogen-related) and abiotic (environmental) stresses



**Fig 1:** Polyploidy, or having extra sets of chromosomes, can affect a plant's resistance to diseases. A "+" sign means higher ploidy improves resistance, while a "-" sign means it reduces resistance. Overall, polyploidy can influence how well a plant fights off pathogens. In polyploid plants, having more gene variations, stronger gene activity, and a mix of beneficial traits can help defend against diseases. Some disease-resistant genes and the pathogens they target have been identified (Zhang *et al.*, 2019)<sup>[49]</sup>. Additionally, changes in chromosome number can also help plants adapt to different environmental stresses, which may indirectly improve their ability to resist infections.

### Biotic stress

Apple scab (*Venturia inaequalis*) & powdery mildew (*Podosphaera leucotricha*) are two major fungal diseases affecting economically significant crops. (Bolger *et al.*, 2014)<sup>[3]</sup> found that these pests can significantly diminish fruit yield and quality, weaken trees, and reduce their resilience to frost. Fungicides can be used to prevent or treat these diseases based on weather circumstances. To combat these diseases, develop scab-resistant cultivars with the Vf gene. Several countries have registered apple cultivars that are resistant to scab since the 1970s. However, many do not meet growers' and consumers' expectations due to low yields and poor fruit quality, particularly taste. Cultivating cultivars with low scab susceptibility requires minimal plant protection, and in some cases, zero chemical

protection at all, which is their principal advantage. The impact of biotic variables on apple productivity highlights the necessity of creating stress-resistant cultivars. Furthermore, a study on apple cultivars' responses to biotic and abiotic stress conditions helps to understand their resilience.

### Triploids for biotic stress

*Venturia inaequalis*, an ascomycete fungus, causes apple scab, a frequent disease of roses (Rosaceae). Whereas this disease affects various plant species, such as Sorbus, Cotoneaster, and the Pyrus, it is most usually associated with Malus tree infection, which includes flowering crabapple and cultivated apple. Infection often causes fruit distortion and premature leaf and fruit drop, enhancing the host plant's sensitivity to environmental stress and secondary infections. Reduced fruit quality and productivity could end up in production losses of as much as 70 percent, putting apple growers' profitability at risk.

Triploid apple varieties are generally more resistant to diseases and pests due to their genetic composition, which stems from the fusion of unreduced 2n gametes. This unique genetic structure enhances their adaptability to different environmental challenges, including pathogen invasions and pest attacks. Their higher genetic diversity offers a stronger defense mechanism, allowing them to better survive under biotic stress. Additionally, triploids tend to be more vigorous and productive, contributing to their natural ability to withstand stress factors more effectively. triploid apples make an important asset in breeding programs aimed at creating more disease-resistant varieties, which can reduce the reliance on chemical treatments and promote more sustainable farming practices. For instance, a 2020 study discussed how triploid cultivars arise from natural polyploidization through unreduced 2n gametes, contributing to enhanced growth and productivity. The study also highlighted their role in improving genetic diversity within commercial apple cultivars. Another recent investigation explored the contribution of triploids to modern breeding, especially in Poland, where they represent around 10% of cultivars. (Sedov *et al.*, 2014)<sup>[36]</sup> developed several tri-ploid apple cultivars, primarily through interploidy crosses (2x-4x) and some through meiotic polyploidization. Triploid varieties are known for their resistance to apple scab disease, consistent fruiting, great fruit quality, and increased autogamy.

### Tetraploids

Apple tetraploids have higher productivity, quality of fruit, and resistance to both abiotic and biotic stresses. According to Gorbacheva *et al.* Some apple varieties with four sets of chromosomes (tetraploids) have been found to contain higher levels of sugars and vitamin C compared to those with two sets (diploids). Through crossbreeding tetraploids with diploids, certain traits, like increased vitamin C, can be passed down to their offspring with three sets of chromosomes (triploids). Studies show that tetraploid crops are generally better at resisting diseases than diploid crops. This is because their extra sets of genes, resulting from genome duplication, enhance their disease-fighting capabilities. For example, research by (Chen *et al.*) revealed that tetraploid apples, such as 'Hanfu' and 'Gala,' are much more resistant to diseases caused by fungi like *Alternaria alternata* and *Colletotrichum gloeosporioides*. The genetic foundation of enhanced resistance to these two illnesses' invasion was evaluated using an instantaneous quantitative PCR analysis. *Erwinia amylovora*, a bacteria that causes fire blight, is a serious bacterial pest for apple trees. Apple scab is a disease, produced by a fungus called *Venturia inaequalis* (Winterfeld *et*

*al.*) is the most destructive fungal disease, causing losses in yield of up to a few dozen percent. Polyploidization is a significant source of variation in plant breeding. Allopolyploidy & autopolyploidy have a significant impact on plant phenotype and genotype. Genome duplication modifies DNA structure, resulting in chromosomal alterations such as deletions, translocations, inversions, as well as point mutations, in addition to changes in DNA methylation patterns. As a consequence, several autotetraploid clones representing six apple kinds were created.

The study sought to investigate whether tetraploid 'Redchief' apple clone react when exposed to disease, specifically towards *E. amylovora*, leading to fire blight. It investigated several characteristics associated with these tetraploid clones, particularly their development patterns whether grown on its own roots or grafts onto M9 rootstock within orchards. One clone, 4x-25, possessed extreme resistance to fire blight, therefore the researchers investigated its capacity to endure

drought at both the physiological & genetic levels. Furthermore, they examined the pollen from those tetraploid clones to make sure that they may effectively crossbreed with conventional diploid apple trees in future breeding attempts. Overall, the study intended to shed light on how polyploidization influences apple properties and their potential to improve disease resistance including drought tolerance. Tetraploid apple varieties tend to have stronger disease resistance due to their ability to express resistance genes more efficiently, which makes them better at coping with infections and other biotic stresses. The added chromosome sets in tetraploids lead to greater genetic diversity, which improves their overall resilience. These qualities are particularly beneficial for breeding programs focused on creating apple cultivars with enhanced durability, minimizing the need for chemical treatments, and promoting more sustainable agricultural practices.

**Table 1:** Effect of polyploidy in abiotic stress management

Stress	Inducing method	Crop	Methods
Drought	Laboratory conditions induced by polyethylene glycol.	Apple	1. Tetraploid cultivars had a greater relative level of water compared to diploid cultivars (81.76% and 63.84%, respectively, after 3 and 6 hours of treatment, and 69.89% and 48.16%, respectively). 2. Tetraploid cells with lower MDA levels showed improved membrane integrity during drought stress. 3. Tetraploid plants showed decreased levels of aquaporin genes during drought stress (Abdolinejad & Shekafandeh 2022) <sup>[1]</sup>
Salinity	Nacl induced salinity	Orange	1. Tetraploids had increased NPK and proline levels compared to diploids. 2. Tetraploids had reduced MDA and H <sub>2</sub> O <sub>2</sub> levels compared to diploids (Lloyd & Bomblies 2016) <sup>[21]</sup> .
Drought	Nacl induced salinity	Citrus	Antioxidative enzymes were higher in tetraploids (Lloyd & Bomblies 2016) <sup>[21]</sup> .

### Meiotic Analysis of Chromosome Pairing

Diploid apple varieties frequently demonstrate normal chromosomal behaviour, with 17 In diploid apple varieties, chromosome pairing at meiotic metaphase I generally shows 17 bivalents, indicating normal chromosomal behavior. However, research by (Dar *et al.*, 2015) identified meiotic anomalies in several commercial apple cultivars ( $2n = 34$ ). In the study, certain irregularities were observed during cell division, such as unpaired chromosomes (univalents), errors in chromosome separation (non-disjunction), and abnormalities in the later stages of division (anaphase and telophase). These problems were at levels of 5.06% in the 'Raj Ambri' variety to 7.07% in 'Golden Delicious'. The 'Golden Delicious' apple genome was sequenced by an advanced sequencing technique known as whole-genome shotgun sequencing. This process combined Sanger sequencing with paired-end reads, covering 26% of the genome with an average depth of 16.9 times. In addition, 454 sequencing (using both paired and unpaired reads) contributed another 74% coverage of the genome.

### In Situ Hybridization

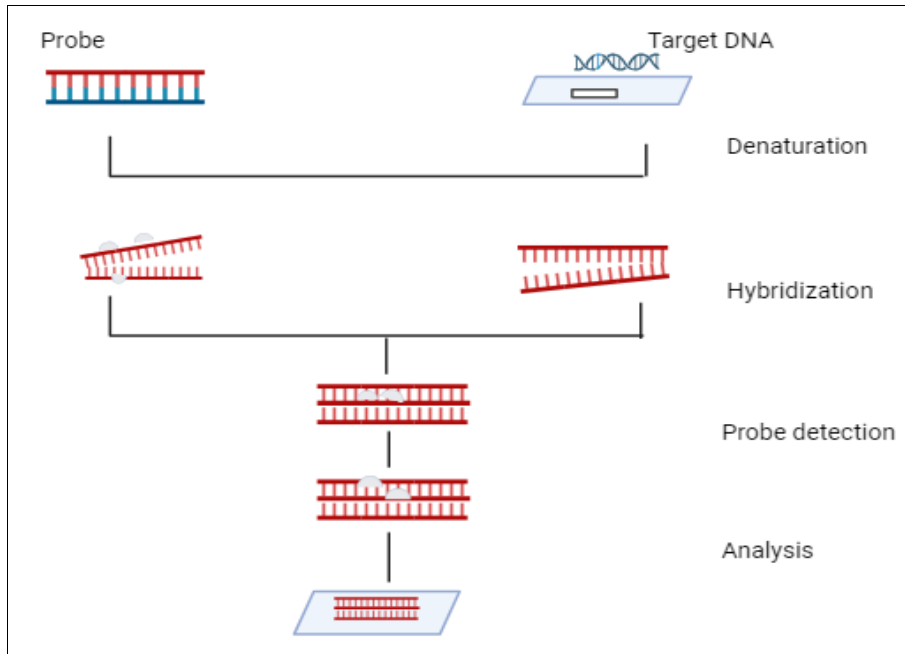
In situ, hybridization is used to localize and detect particular sequences of DNA in cells, as well as identify gene positions. There are several kinds of ISH laboratory methods where single-stranded DNA probes are complementary and hybridized with the desired gene.

There are two types of approaches for detecting fluorescent in situ hybridization: direct and indirect. In the direct technique, the

detecting molecule (reporter) is directly coupled with the nucleic acid probe, allowing the hybridization sites to be visualized microscopically immediately. The indirect approach involves labeling the probe with a reporter molecule, which is only observable after immune cytochemical localization.

### Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization, also known as FISH, is a technique for identifying chromosomal markers and studying evolutionary relationships across organisms (Sharma *et al.*, 1994) <sup>[38]</sup>. This technique locates the actual position of DNA sequences on chromosomes, whether they are unique or repeating. Few studies have used FISH to study *Malus* species' tiny chromosomes. In situ hybridization with the diploid apple root-stock *M. sieboldii* A106 was performed to determine the physical locations of three fruit ripening genes, endopolygalacturonase and ACC synthase. FISH analysis showed that genes related to The EPG, and ACC synthase were spread over Ten pairs of chromosomes. The *Malus* genomes have been advanced by the use of bacterial artificial chromosomes (BACs) and FISH (BACFISH) to identify gene loci. (Minamikawa *et al.*, 2010) <sup>[23]</sup> employed BAC-FISH analysis to determine the relationship between MdFBX11, a monomorphic gene, and the S locus area of apples. MdFBX11 was found to be at a specific locus on the sub-telomeric site of a chromosome using FISH. Additionally, a BAC clone with repeated sequences was discovered at the centromeric region on every of the 17 chromosomes.



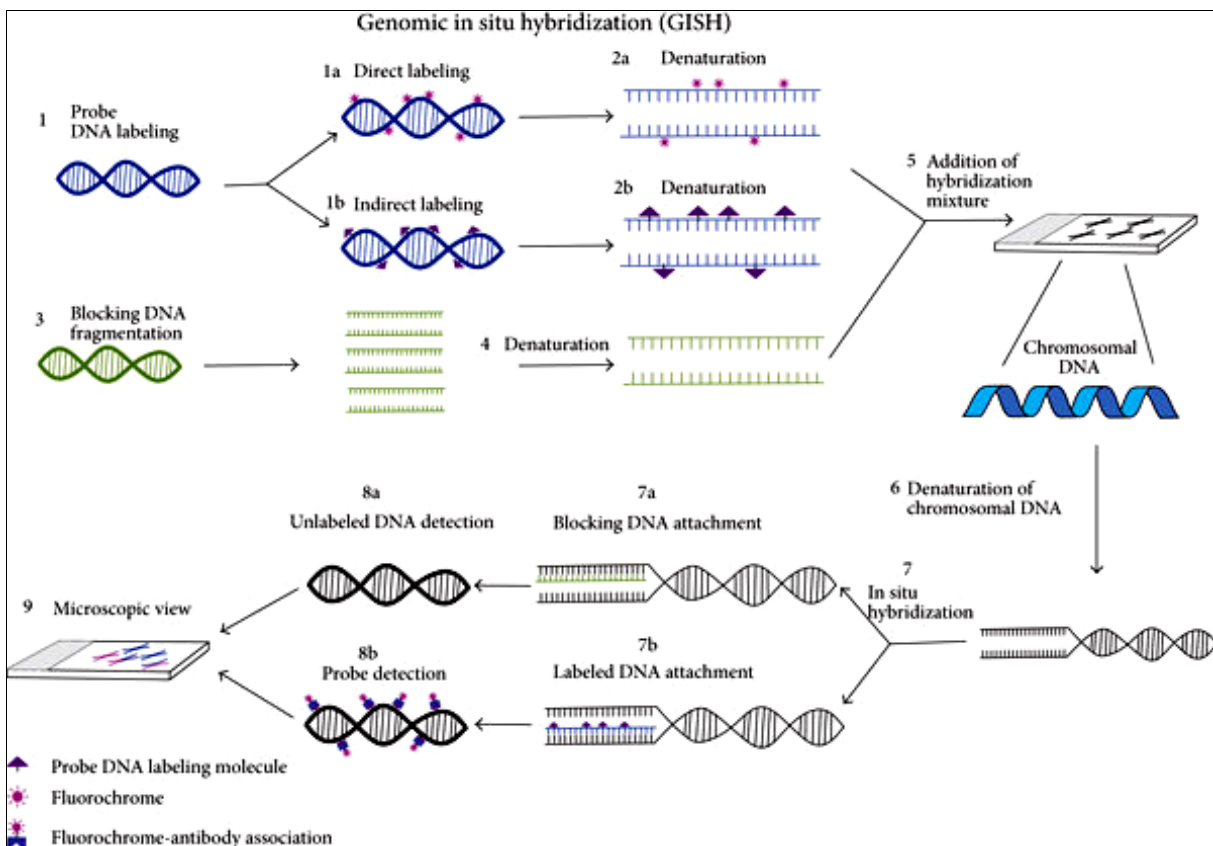
**Fig 2:** Fluorescence in situ hybridization (FISH) is a cytogenetic technique used to identify and locate specific DNA sequences within cells

**Genomic in situ hybridization (GISH)**

Genomic in situ hybridization (GISH) is a genetic detective technology that scientists use to solve plant genetic puzzles. It is used to determine which sections of a plant's DNA originate from which parent. GISH, which uses specific markers on chromosomes, may expose the links between various crops as well as identify genes from different species that have been introduced. Consider it a high-tech paternity test of plants. GISH enables scientists to investigate how plants evolve, comprehend the makeup of their genes, and they can even observe how

chromosomes act when different species marry. This technology has expanded the possibilities for understanding plant genetics and developing superior crops.

GISH (In Situ Hybridization with Genomic DNA) is a technique used to detect foreign chromosomes, chromosome parts, and structural changes in chromosomes. It helps scientists see the genetic composition of a plant and distinguish between chromosomes from different parent species, especially in hybrid plants with multiple sets of chromosomes.



**Fig 3:** In situ hybridisation using genomic DNA (GISH) is a way to identify foreign chromosomes and also chromatin and chromosomal rearrangements, which are created owing to mosaic chromosomes. (Ramzan *et al.*, 2017) <sup>[32]</sup>

### Whole-genome sequencing and Transcriptomics

Whole-genome sequencing (WGS) has greatly revolutionized apple breeding by determining disease resistance (R-genes), drought tolerance (MdPIP1;3), and fruit development (Velasco *et al.*, 2010) [42] genes. Apple genome sequencing has made it possible for scientists to determine genetic variations that improve stress adaptation and fruit quality. Through the determination of genes associated with pathogen resistance, abiotic stress response, and fruit development, WGS gives a critical platform for contemporary apple breeding programs.

The genome of the apple is intricate because of multiple duplication events and polyploidization, and it is difficult to identify major functional genes. Nevertheless, WGS has made it possible to find quantitative trait loci (QTLs) that are responsible for stress tolerance, enabling breeders to choose varieties with enhanced resistance to drought, salinity, and high temperatures. Furthermore, knowledge obtained from WGS is useful in generating molecular markers for marker-assisted selection (MAS), which accelerates the breeding process.

Transcriptomics serves as a supplement to WGS through the evaluation of differentially expressed genes (DEGs) in diverse abiotic stresses. Transcriptomics can track gene expression adjustments that result from exposure to abiotic and biotic stresses, enabling valuable insights into stress adaptation regulation by regulatory networks (Kumar *et al.*, 2019) [18]. The knowledge will be critical to decipher the induction and repression of stress-associated genes to permit tailored breeding practices that boost resilience.

For instance, transcriptomic analysis has shown that genes for oxidative stress response, water transport, and heat shock proteins are highly expressed in drought-resistant apple genotypes. Likewise, secondary metabolite and antimicrobial compound-related genes are overexpressed in disease-resistant cultivars. This information helps in the selection of genotypes with better adaptability and productivity. By combining WGS with transcriptomics, breeders can create genomic selection (GS) models that are able to predict the performance of novel apple varieties under stress conditions. This accelerates breeding cycles and enhances the efficiency of the selection of elite genotypes with improved drought tolerance, better fruit quality, and increased disease resistance.

### CRISPR-Cas9 in Apple Breeding

CRISPR-Cas9 technology has proved to be an effective tool for making accurate genome modifications in apple breeding. Genome editing through this method allows specific modification of genes responding to stress, including aquaporin genes (MdPIP1;3), which have been linked with drought tolerance (Zhang *et al.*, 2019) [49]. Through such gene editing, scientists can improve the tolerance of apple varieties to water deficit conditions without compromising productivity.

(Malnoy *et al.*, 2016) [22] were able to use CRISPR-Cas9 to create fire blight and powdery mildew-resistant apples through the targeting of susceptibility genes (S-genes) that are responsible for pathogen recognition and infection. The scientists knocked out these genes to produce apples that are more resistant to fire blight and powdery mildew. CRISPR-Cas9 also enables precise editing of flowering time, fruit quality characteristics, and stress-response pathways, which opens the door to future breeding programs involving less agrochemical inputs. The incorporation of CRISPR in apple breeding offers great promise for the adaptation of cultivars to precise environmental conditions, lowering production expenses, and enhancing sustainability in orchard management.

### Flow Cytometry Method

Flow cytometry is a technique widely used in apple breeding to manage stress by analyzing the characteristics of individual cells. It helps identify plants with enhanced tolerance to both biotic (like pests and diseases) and abiotic (such as drought and temperature) stress factors. This method works by measuring cellular traits such as size, granularity, and the fluorescence emitted by markers that indicate stress responses. In apples, flow cytometry is particularly useful for examining DNA content, ploidy levels, and gene expression linked to stress resistance. For example, stress-response proteins or reactive oxygen species (ROS) can be tagged with fluorescent markers to observe how apple cells react under various stress conditions. Nuclear DNA content, determined by the flow cytometric method and molecular markers, is an effective approach for determining hybrid/apomictic state in seedlings that result from intergeneric, intra-specific, intraploid crosses. Hybrids of parents having different genome sizes are likely to have intermediate nuclear DNA content (Kamiński *et al.*, 2016) [15]. Identification of genome size is important to identify the ploidy levels of parents and offspring. Using triploids and tetraploids within crosses to other genotypes, independent of ploidy level, may end up in a recurrence of aneuploids within the progeny. Similarly, when diploids are hybridised into tetraploids, triploids are bound to emerge in the offspring.

This approach allows breeders to select varieties that are more resilient and require fewer chemical treatments. One area where flow cytometry has proven valuable is in identifying polyploid apple varieties like triploids and tetraploids, which tend to exhibit greater resistance to biotic stress due to their genetic diversity. In tetraploid apple varieties like 'Red Chief', flow cytometry has been used to confirm the ploidy level and assess genetic stability under stress conditions. With higher gene dosage, tetraploids often express disease-resistance genes more effectively, making them more resilient. This technique thus supports the development of robust, stress-tolerant apple cultivars, reducing reliance on chemicals and promoting sustainable farming practices.

### Conclusion

These methods allow for the discovery of stress-tolerant genotypes by analyzing chromosomal abnormalities such as polyploidization. Understanding the effects of polyploidization on both abiotic and biotic stress tolerance allows researchers to create techniques to improve stress resilience among orchard crops. Furthermore, nanobiotechnology, when combined with cytogenetic methods, has the potential to improve crop stress management by enabling the delivery of stress-relieving medicines at the nanoscale. Overall, incorporating cytogenetic techniques into orchard management strategies provides a comprehensive strategy for improving stress tolerance, which leads to increased crop output and sustainability. Taxonomists and breeders rely heavily on karyotype traits in their investigations. To avoid apomictic progeny, seedlings from intergeneric & intrageneric hybridizations must be evaluated for hybrid status. This can be done through interspecific or intraspecific crossings. Traditional cytogenetic methods for studying the apple genome have been well-documented. However, limited research has explored the use of in situ hybridization techniques, including FISH (fluorescence in situ hybridization), GISH (genomic in situ hybridization), and chromosome painting with BACs (bacterial artificial chromosomes), to analyze genome evolution and identify hybrids in *Malus* species. The physical and genetic changes in

newly formed polyploids, whether autopolyploid or allopolyploid, remain poorly understood. While polyploid breeding significantly enhances genetic diversity in apples, it is often overlooked. Polyploidization can lead to structural, genetic, and physiological changes, influencing tolerance to environmental stresses, disease resistance, and secondary metabolite production, which are crucial for crop improvement.

## References

- Abdolinejad R, Shekafandeh A. Tetraploidy confers superior *in vitro* water-stress tolerance to the fig tree (*Ficus carica*) by reinforcing hormonal, physiological, and biochemical defensive systems. *Front Plant Sci.* 2022;12:796215. doi: 10.3389/fpls.2022.796215.
- Ballester AR, Norelli J, Burchard E, Abdelfattah A, Levin E, González-Candelas L, *et al.* Transcriptomic response of resistant (PI613981-*Malus sieversii*) and susceptible ('Royal Gala') genotypes of apple to blue mold (*Penicillium expansum*) infection. *Front Plant Sci.* 2017;8:1981. doi: 10.3389/fpls.2017.01981.
- Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114-2120. Doi: 10.1093/bioinformatics/btu170.
- Considine MJ, Wan Y, D'Antuono MF, Zhou Q, Han M, Gao H, *et al.* Molecular genetic features of polyploidization and aneuploidization reveal unique patterns for genome duplication in diploid *Malus*. *PLoS ONE.* 2012;7(1):e29449. Doi: 10.1371/journal.pone.0029449.
- Cornille A, *et al.* A multifaceted overview of apple tree domestication. *Trends Plant Sci.* 2019. Doi: 10.1016/j.tplants.2019.05.007.
- Esumi T, Tao R, Yonemori K. Isolation of LEAFY and TERMINAL FLOWER1 homologues from six fruit tree species in the subfamily Maloideae of the family Rosaceae. *Sex Plant Reprod.* 2005;17:277-287. doi: 10.1007/s00497-004-0234-3.
- Foster TM, Ledger SE, Janssen BJ, Luo Z, Drummond RSM, Tomes S, *et al.* Expression of MdCCD7 in the scion determines the extent of sylleptic branching and the primary shoot growth rate of apple trees. *J Exp Bot.* 2018;69(10):2379-2390. Doi: 10.1093/jxb/ery090.
- Green AF, Ramsey TS, Ramsey J. Polyploidy and invasion of English ivy (*Hedera* spp., Araliaceae) in North American forests. *Biol Invasions.* 2013;15(10):2219-2241. Doi: 10.1007/s10530-013-0445-4.
- Greilhuber J, Doležel J, Lysak MA, Bennett MD. The origin, evolution, and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Ann Bot.* 2005;95(1):255-260. doi: 10.1093/aob/mci019.
- Hias N, Leus L, Davey MW, Vanderzande S, Van Huylenbroeck J, Keulemans J. Effect of polyploidization on morphology in two apple (*Malus × domestica*) genotypes. *Hortic Sci.* 2017;44(2):55-63. Doi: 10.17221/119/2015-HORTSCI.
- Höfer M, Meister A. Genome size variation in *Malus* species. *J Bot.* 2010;2010:1-9. Doi: 10.1155/2010/160603.
- Jędrzejczyk I, Śliwińska E. Leaves and seeds as materials for flow cytometric estimation of the genome size of 11 Rosaceae woody species containing DNA-staining inhibitors. *J Bot.* 2010;2010:1-7. Doi: 10.1155/2010/527357.
- Jia S. *In vitro* induction and identification of tetraploid in *Malus zumi* and its evaluation of stress tolerance. Master's Thesis, Agricultural University of Hebei, China; 2009. Available from: <https://www.dissertationtopic.net/doc/517919>.
- Jiang GX, Li ZW, Song YB, Zhu H, Lin S, Huang RM, *et al.* LcNAC13 physically interacts with LcR1MYB1 to coregulate anthocyanin biosynthesis-related genes during litchi fruit ripening. *Biomolecules.* 2019;9(4):135. Doi: 10.3390/biom904013.
- Kamiński P, Podwyszyńska M, Starzycki M, Starzycka-Korbas E. Interspecific hybridisation of cytoplasmic male-sterile rapeseed with Ogura cytoplasm and *Brassica rapa* var. *pekinensis* as a method to obtain male-sterile Chinese cabbage inbred lines. *Euphytica.* 2016;208(3):519-534. Doi: 10.1007/s10681-015-1598-9.
- Khan MA, Han Y, Zhao YF, Korban SS. A high-throughput apple SNP genotyping platform using the GoldenGate™ assay. *Gene.* 2012;494(2):196-201. Doi: 10.1016/j.gene.2011.12.007.
- Khan MN, Zhang J, Luo T, Liu J, Ni F, Rizwan M, *et al.* Morpho-physiological and biochemical responses of tolerant and sensitive rapeseed cultivars to drought stress during early seedling growth stage. *Acta Physiol Plant.* 2019;41(2):31. Doi: 10.1007/s11738-019-2812-2.
- Kumar S, *et al.* Genomic selection and marker-assisted selection in apple breeding: Advances and future perspectives. *Horticult Plant J.* 2019;5(4):231-239. Doi: 10.1016/j.hpj.2019.08.004.
- Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25(14):1754-1760. Doi: 10.1093/bioinformatics/btp324.
- Limera C, Sabbadini S, Sweet JB, Mezzetti B. New biotechnological tools for the genetic improvement of major woody fruit species. *Front Plant Sci.* 2017;8:1418. Doi: 10.3389/fpls.2017.01418.
- Lloyd A, Bomblies K. Meiosis in autopolyploid and allopolyploid *Arabidopsis*. *Curr Opin Plant Biol.* 2016;30:116-122. Doi: 10.1016/j.pbi.2016.02.004.
- Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS, *et al.* DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front Plant Sci.* 2016;7:1904. doi: 10.3389/fpls.2016.01904.
- Minamikawa M, Kakui H, Wang S, Kotoda N, Kikuchi S, Koba T, *et al.* Apple S locus region represents a large cluster of related, polymorphic, and pollen-specific F-box genes. *Plant Mol Biol.* 2010;74(1-2):143-154. Doi: 10.1007/s11103-010-9664-0.
- Noormohammadi Z, Fazeli S, Sheidai M, Farahani F. Molecular and genome size analyses of somaclonal variation in apple rootstocks Malling 7 and Malling 9. *Acta Biol Szeged.* 2015;59(2):139-149.
- Osakabe Y, Osakabe K. Genome editing with engineered nucleases in plants. *Plant Cell Physiol.* 2015;56(3):389-400. Doi: 10.1093/pcp/pcu170.
- Ou S, Su W, Liao Y, Chougule KR, Agda JRA, Hellings AJ, *et al.* Benchmarking transposable element annotation methods for the creation of a streamlined, comprehensive pipeline. *Genome Biol.* 2019;20:275. Doi: 10.1186/s13059-019-1905-y.
- Parisod C, Holderegger R, Brochmann C. Evolutionary consequences of autopolyploidy. *New Phytol.* 2010;186(1):5-17. Doi: 10.1111/j.1469-8137.2009.03142.x.
- Peil A, Emeriewen OF, Khan A, Kostick S, Malnoy M.

- Status of fire blight resistance breeding in *Malus*. *J Plant Pathol*. 2020. <https://doi.org/10.1007/s42161-020-00581-8>.
29. Podwyszyńska M, Kruczyńska D, Machlańska A, Dyki B, Sowik I. Nuclear DNA content and ploidy level of apple cultivars including Polish ones in relation to some morphological traits. *Acta Biol Cracov Ser Bot*. 2016;58(1):81-93. <https://doi.org/10.1515/abcsb-2016-0005>.
30. Proost S, Pattyn P, Gerats T, Van de Peer Y. Journey through the past: 150 million years of plant genome evolution. *Plant J*. 2011;66(1):58-65. <https://doi.org/10.1111/j.1365-313X.2011.04521.x>.
31. Ramsey J, Ramsey TS. Ecological studies of polyploidy in the 100 years following its discovery. *Philos Trans R Soc B Biol Sci*. 2014;369(1648):20130352. <https://doi.org/10.1098/rstb.2013.0352>.
32. Ramzan F, Younis A, Lim K. Application of genomic in situ hybridization in horticultural science. *Int J Genomics*. 2017;2017:1-12. <https://doi.org/10.1155/2017/7561909>.
33. Ru S, Main D, Evans K, Peace C. Current applications, challenges, and perspectives of marker-assisted seedling selection in Rosaceae tree fruit breeding. *Tree Genet Genomes*. 2015;11:8. <https://doi.org/10.1007/s11295-015-0834-5>.
34. Rubuluza T, Nikolova R, Smith M, Hannweg K. *In vitro* induction of tetraploids in *Colophospermum mopane* by colchicine. *S Afr J Bot*. 2007;73(2):259-261. <https://doi.org/10.1016/j.sajb.2006.11.001>.
35. Schuster M, Fuchs J, Schubert I. Cytogenetics in fruit breeding: Localization of ribosomal RNA genes on chromosomes of apple. *Theor Appl Genet*. 1997;94(3-4):322-324. <https://doi.org/10.1007/s001220050410>.
36. Sedov EN, Sedysheva GA, Serova ZM, Gorbacheva NG, Melnik SA. Breeding assessment of heteroploid crosses in the development of triploid apple varieties. *Russ J Genet Appl Res*. 2014;4(1):52-59. <https://doi.org/10.1134/S2079059714010093>.
37. Selmecki AM, Dulmage K, Cowen LE, Anderson JB, Berman J. Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. *PLoS Genet*. 2009;5(10):e1000705. <https://doi.org/10.1371/journal.pgen.1000705>.
38. Sharma AK, Sharma A. Chromosome techniques: A manual. 3<sup>rd</sup> ed. Harwood Academic Publishers; 1994.
39. Silva KJP, Singh J, Bednarek R, Fei Z, Khan MA. Differential gene regulatory responses to fire blight infection in two apple cultivars (*Malus domestica*). *Hortic Res*. 2019;6:35. <https://doi.org/10.1038/s41438-019-0120-z>.
40. Spornberger A, Schueller E, Noll D. The influence of Geneva rootstocks on the vegetative and generative characteristics of the apple cultivar 'Topaz' in an organically managed replanted orchard. *Int J Fruit Sci*. 2020;20(S3):S1436-S1444. <https://doi.org/10.1080/15538362.2020.1799475>.
41. Sun Y, Wang C, Chen HYH, Ruan H. Response of plants to water stress: A meta-analysis. *Front Plant Sci*. 2020;11:978. <https://doi.org/10.3389/fpls.2020.00978>.
42. Velasco R, *et al*. The genome of the domesticated apple (*Malus × domestica*). *Nat Genet*. 2010;42(10):833-839. <https://doi.org/10.1038/ng.654>.
43. Wada M, Nishitani C, Komori S. Stable and efficient transformation of apple. *Plant Biotechnol*. 2020;37(2):163-170. <https://doi.org/10.5511/plantbiotechnology.20.0602a>.
44. Wang D, Gu J. Integrative clustering methods of multi-omics data for molecule-based cancer classifications. *Quant Biol*. 2016;4:58-67. <https://doi.org/10.1007/s40484-016-0063-4>.
45. Wang H, Zhao S, Mao K, Dong Q, Liang B, Li C, *et al*. Mapping QTLs for water-use efficiency reveals the potential candidate genes involved in regulating the trait in apple under drought stress. *BMC Plant Biol*. 2018;18:136. <https://doi.org/10.1186/s12870-018-1308-3>.
46. Wendel JF, Jackson SA, Meyers BC, Wing RA. Evolution of plant genome architecture. *Genome Biol*. 2016;17:37. <https://doi.org/10.1186/s13059-016-0908>.
47. Yamamoto M, Shigeki M, Yamamoto T. Detection of ribosomal RNA genes in apple (*Malus domestica*) using fluorescence in situ hybridization. *Chromosome Sci*. 2016;19:33-36.
48. Yang J, Gao M, Huang L, Wang Y, van Nocker S, Wan R, *et al*. Identification and expression analysis of the apple (*Malus × domestica*) basic helix-loop-helix transcription factor family. *Sci Rep*. 2017;7:17316. <https://doi.org/10.1038/s41598-017-17733-5>.
49. Zhang K, Wang X, Cheng F. Plant polyploidy: Origin, evolution, and its influence on crop domestication. *Hortic Plant J*. 2019;5(5):231-239. <https://doi.org/10.1016/j.hpj.2019.09.001>.