Chapter 20
Genome Editing in Forest Trees

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Abstract Since the first CRISPR/Cas-mediated genome editing of poplar in 2015, an increasing number of tree species are being genome-edited. Although the availability of genome sequences, tissue culture and transformation systems are limiting factors, research is ongoing on advanced methods such as DNA-free genome editing and gene targeting approaches in addition to the optimisation of single gene knockouts. These can be used to address ambitious issues and perform genome editing more accurately, which has implications for the legal assessment of edited trees. Once technically established, CRISPR/Cas can be used to circumvent specific challenges related to forest tree species, e.g., longevity and extended vegetative phases, and to modify traits relevant for breeding, whether for direct application or to elucidate the genetic basis of individual traits. Not least due to climate change, adaptation to abiotic stress such as drought stress as well as biotic stresses caused by pathogens are strongly in focus. For the use as a renewable resource and as a carbon sink, wood productivity in forest trees as well as wood properties are of interest. In biosafety assessments, tree-specific aspects have to be considered, which result, among other aspects, from the long lifespan.

1 Prerequisites to Use Genome Editing in Trees

After CRISPR/Cas was first used for genome editing in plants as published in 2013 [1, 2], the first genome editing of a tree species was published only a short time later. Fan et al. [3] described the knockout of the visual marker gene *PDS* (encoding for phytoene desaturase) in *Populus* × *tomentosa*, resulting in albino phenotypes. For this, a Cas9 expression vector including matching guide-RNAs (gRNAs) was used

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A. Ricroch et al. (eds.), *A Roadmap for Plant Genome Editing*,
https://doi.org/10.1007/978-3-031-46150-7_20

for stable *Agrobacterium*-mediated transformation of *in vitro* poplars. Even though innovative methods are being developed, in practice mostly functioning tissue culture systems remain an essential basis for successful editing in trees. This involves three steps with respective hurdles: (1) establishment of an *in vitro* culture system that is able to regenerate plant shoots, (2) establishment of a protocol for the transfer of the Cas/gRNA into living cells, i.e., by classical genetic transformation, and (3) establishment of a protocol for genome editing.

Many tree species are considered *in vitro*-recalcitrant. It is difficult to transfer them to the *in vitro* culture and, once this initial step is accomplished, to regenerate them in large quantities and within manageable time frames for biotechnological purposes. The term recalcitrance summarizes many problems, some of them are still unidentified because the physiological basis of recalcitrance is not fully understood to date [4]. A fundamental difficulty is the need for clean (often generalised as "sterile") cultivars, i.e., without overwhelming bacterial or fungal contamination. In a few tree species, the transfer from sterilised vegetative organs such as leaves into *in vitro* culture has worked, e.g., for poplars. Sterilised embryos or somatic meristems from shoots are more suitable for many tree species [4]. For this purpose, younger starting material seems to be more suitable than older trees if plant material is to be used for organogenesis by the cultivation of meristems (unpublished data, [5]). If organogenesis cannot be induced directly, somatic embryogenesis is often used for plant regeneration. Here, the development of complete embryos with radicle, shoot, and cotyledons is induced from somatic cells, e.g., callus. These somatic embryos are similar to zygotic embryos. They can be easily separated from the mother tissue, have all the necessary structures for regeneration into a whole plant [6].

The ability to regenerate single cells into complete plants is necessary for genetic transformation and the generation of genetically uniform regenerates. For many tree species, regeneration media with tuned hormone contents can be used to induce the totipotency of somatic cells and stimulate regenerating callus for organogenesis. Cytokinins, such as 6-Benzylaminopurine (BAP), and/or auxins, such as Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D), are usually used for this purpose. Their concentrations should be optimised for each species [7]. The use of protoplasts with appropriate regeneration medium is also suitable. At this point, brief attention should be drawn to somaclonal variation that can be induced by *in vitro* techniques [8]. Even though these mutations usually have no effect on the transformability or editability of the plants, it should be considered that, in practice, some genetic variability is unavoidable.

If an *in vitro* culture system is achievable, the basis for genetic transformation is given. However, establishing the transformation method is far from easy, as existing transformation protocols can only be utilised in tree species to a limited extent. If modifications are needed, an establishment process follows which, if at all, can lead to success over a certain amount of time. Three conventional methods of genetic transformation are commonly used, with ascending relevance for tree species: ballistic transformation by particle gun, polyethylene glycol (PEG)-mediated transformation of protoplasts, and *Agrobacterium*-mediated transformation. The use of a particle gun requires good mechanical tuning to determine the bombardment

parameters that produce many transformations and little damaged plant material [9]. In addition, special equipment is required, especially the particle gun itself. Provided protoplasts can be generated, PEG-mediated uptake of plasmid DNA works reliably. To give some examples, PEG-mediated transformation of protoplasts, partly combined with electroporation, works in *Eucalyptus* species [10, 11], a poplar hybrid (*Populus tremula* × *Populus alba* [12]) and rubber tree (*Hevea brasiliensis* [13]). The challenge of this method is the isolation of vital protoplasts and their regeneration via callus stages into whole plants. For *Agrobacterium*-mediated transformation, bacteria with a natural competence to transfer genetic material are used. Although the term *Agrobacterium*-mediated transformation continues to endure in the research community, the associated bacterial species have been renamed *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*) and *Rhizobium rhizogenes* (formerly known as *Agrobacterium rhizogenes*). This commonly used method works for many tree species including conifers such as *Abies koreana* [14], *Larix decidua* [15], *Picea abies* [16], *Picea glauca* and *Picea mariana* [17], *Pinus radiata* [18], *Pinus taeda* [16], *Pseudotsuga menziesii* [19], and broadleaf trees such as *Ailanthus altissima* [20], *Castanea dentata* [21], *Castanea sativa* [22], *Eucalyptus globulus* [23], *Fraxinus americana* [24], *Fraxinus excelsior* [25], *Ginkgo biloba* [26], Poplar and aspen hybrids (*Populus* spp.) [27–29], *Quercus robur* [30], *Quercus suber* [31], *Robinia pseudoacacia* [32], and *Ulmus americana* [33].

After the establishment of the transformation method, the genome editing technique needs to be established. For forest trees, only CRISPR/Cas actually plays a significant role. The three other genome editing mechanisms using transcription activator-like effector nucleases (TALEN), zinc finger nucleases (ZFN) and oligo-directed mutagenesis (ODM) are of minor importance in trees. TALEN and ODM have not been used to date. In poplars, ZFN was used experimentally before CRISPR/Cas technology was made accessible [34]. However, the results indicated that further technical improvements would be needed to increase the moderate mutation frequency.

For CRISPR/Cas-mediated genome editing, suitable and efficiently editable targets need to be identified based on gene sequencing, following the selection of a suitable Cas nuclease. Sequence information of the individual to be edited is required for accurate and reliable genome editing. Even though the number of genome-sequenced tree species is steadily increasing since the first tree genome of the poplar species *Populus trichocarpa* [35], reference genomes are still quite limited to tree species with a manageable genome size. In particular, sequencing the complex genomes of some conifers remains a hurdle [36]. The availability of a reference genome is a prerequisite for selecting an editing target and verifying the presence of the protospacer-adjacent motif (PAM), as well as for identifying loci in the genome that might be considered as off-target sites. Avoiding off-target editing is one of the determining factors for the reliability of genome editing and its safety assessment. Meanwhile, some online tools for predicting potential off-targets have emerged for plants such as Cas-OFFinder, available at http://www.rgenome.net/cas-offinder/ [37]. Furthermore, it should be remembered that trees are nearly

undomesticated plants and therefore have high genetic diversity. Thus, it cannot be assumed that the sequence of a target gene is identical in all individuals of a species. Although a reference genome can assist genome editing by providing evidence for targeting, the target sequence should be verified in the individual being targeted before the final design. This challenge is addressed by the concept of the pan-genome, but this is still in the distant future for trees [38].

Genome editing in forest trees, despite all the challenges described, is a promising technology that can bring benefits to plant molecular genetics research in particular. CRISPR/Cas and the other editing techniques are excellent for gene characterization studies. In these classical knockout approaches, genome editing serves as a tool to knock out genes as described by Fan et al. [3], Zhou et al. [39], and Bruegmann et al. [40]. With knockout approaches, the function of genes can simply be characterised which could subsequently be good starting points for conventional forest plant breeding. With corresponding genetic markers, individuals can be selected from natural populations and used as crossing partners to introgress favourable traits. If legal conditions permit, genome-edited trees could be used directly, too. The targeted genetic modifications can be regarded as optimised breeding. Although the breeding goal can be achieved with conventional breeding methods based on crossing and selection, genome editing can accelerate genetic adaptation because trees take up to several decades to flower and fruit, depending on the species. Accelerating the adaptation process offers the possibility of adapting trees to rapidly advancing climate change and associated environmental conditions in foreseeable time frames. Plants' natural adaptation mechanisms such as genetic adaptation by recombination, mutation, and selection, or migration are likely to be too slow for adaptation to the new environmental challenges associated with climate change [41, 42].

2 Genome-Edited Forest Tree Species

Forests have significant ecological and economic functions, so their preservation and vitality are of great importance. Thus, by any method, breeding forest trees is an important human mission. The term "tree" is indistinctly defined. In general, it refers to perennial plants that have wood formation and secondary thickening growth and - to distinguish them from shrubby woody plants – a single main stem. The tree forms a more or less definite crown [43]. Some definitions add the size: The stem grows to a height of at least six meters without external disturbance [44]. As previously indicated, TALEN and ODM have not been applied in trees to date. A ZFN was used experimentally in poplar hybrids (*P. tremula* × *P. alba* [referred to as *Populus* × *canescens*] and *P. tremula* × *P. tremuloides*) to mutagenize poplar orthologs of *LEAFY* and *AGAMOUS*. The editing rate in this *Agrobacterium*-based approach was among the lowest of all experiments with plants overall [34]. Due to the technical advantages offered by CRISPR/Cas and the boost of these techniques

in plant research throughout, from which woody plant research also benefited and still benefits, research work focused on this technique.

The first CRISPR/Cas-edited tree was a Chinese white poplar (*P.* × *tomentosa*) in which the phytoene desaturase gene (*PDS*) was knocked out by mutation [3]. In subsequent years, *PDS* continues to serve as a marker gene in different annual and perennial plant species, as *PDS*-deficient plants are albinos [45]. Table 20.1 lists the forest tree species genome-edited to date. In trees, even though alternative Cas nucleases such as Cas12a have been available in principle for several years, Cas9 is the tool of choice. To our knowledge, the first Cas12a editing of a tree species was performed in 2020 using the *PDS* knockout in the poplar hybrid *Populus alba* × *Populus glandulosa* [46].

3 Advanced Editing Technologies and Current Developments

Since the first genome editing experiments that resulted in Cas9-mediated knockouts, CRISPR-based methods have continuously improved and evolved, particularly in annual crop plants and model species. Due to the tree-specific bottlenecks described above, the development of novel editing techniques in trees is not progressing as rapidly as in other model plants.

3.1 Effecting CRISPR/Cas Editing During Transformation and Regeneration

Despite the expanding range of tree species that can undergo genome editing, most forest tree species still exhibit low transformation efficiencies [58, 59]. Much time can be spent obtaining a sufficient number of transgenic and genome-edited plants by scaling up transformation experiments or optimising the transformation method. Those optimisations include the transfer of the DNA as well as regeneration during tissue culture.

To enhance transformation efficiency in recalcitrant species by boosting regeneration, morphogenic regulator genes like *WUSCHEL* or *BABY BOOM* can be co-expressed with CRISPR/Cas components [60]. In poplar, Pan et al. [61] significantly increased callus and root initiation as well as shoot growth by co-activation of endogenous morphogenic genes *WUSCHEL* (*PtWUS*) or *WUSCHEL-RELATED HOMEOBOX 11* (*PtWOX11*). While morphogenic regulator genes are facing the bottleneck of regeneration, using nanoparticles can enhance the direct delivery of plasmid DNA into the target tissue, making transformation more efficient or even independent of expensive and complicated laboratory equipment [62]. In *Paulownia tomentosa*, the polysaccharide nanoparticle Chitosan was used as a carrier for direct plasmid transfection of nodular segments. Since Chitosan has a positive charge, a

Table 20.1 CRISPR/Cas-edited forest tree species. For poplar trees, only inventions are mentioned here. More editing events are described in the section on traits and breeding

Scientific species name	Common species name	Editing nuclease	Infiltration mechanism	Reference
Betula platyphylla	Asian white birch	Cas9 targeting unspecified regions	*Agrobacterium*-based	[47]
Castanea sativa	European chestnut	Cas9 targeting *PDS*	RNP editing	[45]
Cryptomeria japonica	Japanese cedar	Cas9 targeting *GFP* and *CjCHLI*	*Agrobacterium*-based	[48]
Eucalyptus grandis	Rose gum	Cas9 targeting *CCR1*	*Agrobacterium*-based	[49]
Hevea brasiliensis	Rubber tree	Cas9 targeting *FT* and *TFL1*	RNP editing	[50]
Juglans regia	English walnut	Cas9 targeting *PDS*	*Agrobacterium*-based	[51]
Larix gmelinii	Dahurian larch	SpRY targeting three genomic sites	PEG-mediated protoplast transformation	[52]
Parasponia andersonii	w/o common name	Cas9 targeting *PanHK4*, *PanEIN2*, *PanNSP1*, and *PanNSP2*	*Agrobacterium*-based	[53]
Picea glauca (*Picea engelmannii*)	Silver spruce / Engelmann spruce	Cas9 targeting *DXS1*	*Agrobacterium*-based	[54]
Pinus radiata	Monterey pine	Cas9 targeting *GUX1*	RNP editing	[55]
Populus alba × *Populus glandulosa*	Poplar hybrid	Cas12a targeting *PDS*	*Agrobacterium*-based	[46]
Populus alba × *Populus glandulosa*	Poplar hybrid	Cas9 targeting *SAP1*	RNP editing	[56]
Populus davidiana × *Populus bolleana*	Poplar hybrid	Cas9 targeting unspecified regions	*Agrobacterium*-based	[47]
Populus × *tomentosa*	Chinese white poplar	Cas9 targeting *PDS*	*Agrobacterium*-based	[3]
Populus tremula × *Populus alba (P.* × *canescens)*	Grey poplar (hybrid)	Cas9 targeting *4CL*	*Agrobacterium*-based	[39]
Populus trichocarpa	Black cottonwood	Cas9 targeting *PtrADA2b-3*	*Agrobacterium*-based	[57]

RNP — ribonucleoprotein

complex was formed with negatively charged DNA improving the uptake through the negatively charged cell membrane [63].

Because a shoot often develops from more than one single transformed cell, regenerated transgenic plants are often chimeric resulting in possibly non-uniformly

edited regenerates in CRISPR/Cas approaches. This can affect the resulting phenotype and experiments based on genetically uniform tissue. A second step of regeneration decreased those chimeras in poplar and increased the frequency of homozygous CRISPR/Cas-mediated mutations [64].

Additionally, the activity of Cas9 and Cas12a can be enhanced by including a heat treatment during regeneration after transformation. This way, the editing efficiencies in poplar as well as birch have been increased [46, 47].

3.2 Optimising CRISPR/Cas Vectors for Forest Tree Editing

Many aspects influence the efficiency of CRISPR/Cas editing. On the one hand, editing efficiency depends on the accessibility of the genomic target sequence to the Cas-enzyme, a factor depending on chromosome structure [65]. On the other hand, it depends on the expression of the Cas-gene as well as the activity of the Cas-enzyme and the transcription of gRNA, which can be influenced by proper vector design.

In reports of CRISPR/Cas experiments in non-model forest trees like birch, chestnut, or walnut, the 35S promoter is the promoter of choice to drive Cas expression [45, 47, 51]. Because of its broad host range and well-studied functionality, as in classical gene technique experiments, this promoter is often used for the establishment of methods. In CRISPR/Cas attempts with the model tree genus *Populus*, Cas expression under control of a synthetic 35S-MAS fusion promoter increased editing efficiency by 11% compared to the 35S promoter, which still is one of the standard promoters in poplars to date [66]. Driving the Cas expression under heterologous ubiquitin promoters resulted in editing efficiencies of up to 95% in poplar [61, 67]. Those examples show the potential of optimisation by promoter choice in non-model forest tree species.

To enhance the translation of the Cas endonuclease in forest trees, codon-optimised variants like the plant codon optimised and the *Arabidopsis thaliana* codon-optimised Cas9 are routinely used [3, 40, 51, 53]. The use of a poplar codon-optimised AsCas12a, the first report of target organism specific codon optimisation for forest trees, resulted in editing efficiencies of up to 70% [46]. For this kind of optimisation, knowledge about the codon usage of the target tree species must be available. In the Codon Usage Database, codon usage tables for different forest trees like poplar (*Populus* spp.), beech (*Fagus* spp.), chestnut (*Castanea* spp.), pine (*Pinus* spp.) or eucalyptus (*Eucalyptus* spp.) are available [68]. These tables can be used as a query for a codon usage analysis and optimisation of Cas genes. To predict Cas expression levels based on codon usage, the Codon Adaptation Index (CAI) can be calculated [69]. A value of one implements an optimal translation rate. If values are low, the online tool Optimizer can be used to create a sequence with a maximum CAI [70]. If only the rare codons that limit translation are to be identified, the graphical codon usage analyser can be used to predict relative adaptiveness of each codon [71].

The most used promoter to drive gRNA transcription in forest trees is the U6-26 promoter from *A. thaliana* (*At*U6-26) [40, 51, 53], which is known for expressing high levels of gRNA in various plant species [72, 73]. In pine, a U6 promoter from *Pseudotsuga menziesii,* another member of the conifer family, is used [55]. Contrastively, in rubber tree (*Hevea brasiliensis*) endogenous U6 promoters were used for gRNA transcription resulting in editing efficiencies of *HbPDS* of up to 67% [49], representing higher efficiency compared to reports of *PDS* editing with *A. thaliana* U6 promoters in forest trees [3, 51]. These results correspond with reports in other tree species and plants indicating that endogenous polymerase III promoters can enhance editing efficiency in general [74, 75].

To obtain a knockout mutant or to edit multiple genes at once, it is often required to target multiple sites in the genome. In consequence, the ability to express multiple gRNAs at once is desired. In chestnut, poplar or eucalyptus, individual transcription units were used to drive sgRNA transcription for Cas9 under the same or different polymerase III promoters [3, 49, 76]. To avoid usage of repetitive sequences, and therefore recombination, silencing and large vector size, multiple gRNAs separated by tRNAs can be combined in one transcription unit [77]. In this way, five crRNAs for Cas12a were transcribed by a single *At*U6-26 promoter in poplar [46]. Pan et al. [61] in turn, used a polymerase II promoter to drive gRNA transcription for Cas9 in poplar, which is recommended for more than two gRNAs and offers the opportunity of inducible gRNA transcription.

In plant species having a long regeneration time like forest trees, the proper choice of spacer sequence and, therefore, the functionality of the corresponding gRNA is essential due to time-consuming and ecological reasons. In poplar, spacer sequences and secondary structures of multiple gRNAs were associated with Cas9 editing efficiencies resulting in recommendations for favourable gRNA structure and spacer sequence. These recommendations can be used to design gRNA candidates for Cas9-mediated genome editing by *in silico* prediction of secondary structure to avoid non-functional sgRNAs [40]. For Cas12a, the effect of secondary structure and spacer sequence on editing efficiency has been analysed in human cell lines, *E. coli* and maize, but not yet specifically for forest tree species [78–80]. Potential gRNAs for different Cas variants and their efficiencies can also be predicted *in silico* using online tools. But it must be considered that most of them like CRISPOR or CHOPCHOP are based on editing efficiency data from mammalian cells or zebrafish [81]. Although some tools, e.g., CHOPCHOP, use reference genome data from tree species, they are not specifically designed for forest tree species [82].

To evaluate gRNAs in the target organism, transient expression systems can be used prior to stable transformations. In rubber tree, protoplast transfection and amplicon deep sequencing of the target region were combined to check editing efficiency of Cas9 before stable transformation [83]. In poplar and birch, *Agrobacterium* inoculation of whole *in vitro* plantlets and quantitative PCR of the target locus are used [47]. It is likely that these transient systems can easily be adapted to other Cas nucleases, such as Cas12a. However, especially when using protoplasts, the cell type dependence on editing efficiency must be considered [65].

If not being able to test in advance, a second (or even more) gRNA targeting the same genomic region can be used as backup to increase the probability of inducing at least one mutation [40, 67, 76]. Moreover, a 1.3 kb deletion at the target site was obtained using two sgRNAs for Cas9-mediated genome editing in Monterey pine (*P. radiata*, [55]). This large deletion is more likely to completely destroy the gene function.

3.3 DNA-Free Editing

In conventional CRISPR/Cas9 approaches, the Cas9 nuclease and gRNA are stably transferred into the target organism as genetic information. However, integration of CRISPR/Cas-related transgenes can be disadvantageous. Continuous cleavage activity of the Cas nuclease can increase formation of chimeric plants and off-target cleavage. In addition, transgenes are a limiting factor in terms of legal regulation.

Since the outcrossing of transgenes in tree species is not an option in practice due to long reproduction cycles, it could be promising to obtain transgene-free edited plants in the first generation by avoiding transgene integration. Therefore, recombinant Cas-ribonucleoproteins (RNPs) can be used instead of plasmid-encoded CRISPR components. Since RNPs do not depend on gene expression and effective promoters, vector optimisation is omitted. However, the use of RNPs has its own parameters to be optimised, such as RNP concentration, protein-gRNA ratio and incubation temperatures. For proof of principle, RNPs for CRISPR/Cas editing were introduced into protoplasts of poplar (*P. alba* × *P. glandulosa*), chestnut (*C. sativa*) and rubber tree (*H. brasiliensis*) using PEG [45, 50, 56]. A biolistic approach was used to co-deliver Cas-RNPs and a plasmid-encoded selection marker to somatic embryos of Monterey pine (*P. radiata*). Editing efficiencies of up to 33% were observed in selection marker resistant plantlets [55].

3.4 Gene Sequence Modification

CRISPR/Cas-knockout mutants are primarily based on random indels obtained by error-prone non-homologous end joining (NHEJ) during the repair of DNA double-strand breaks (DSBs) [84]. But if interested in specific insertions, deletions, or exchanges of DNA, a more precise and accurate way of DNA modification is needed. Such ambitious techniques are at the very beginning in forest trees.

For the conversion of single base pairs, CRISPR base editors (BEs) have been invented. BEs combine DNA binding domains with a nucleotide base deaminase that chemically modifies certain nucleotide bases. Using cytosine (CBEs) or adenine base editors (ABEs), conversions of C•G to T•A or A•T to G•C are possible, respectively [85]. Expression of Cas9 nickase-based BE in poplar (*P. tremula* × *P. alba*) led to the precise conversion efficiency of up to 100% for CBE and 95% for

ABE. Thereby indicating that efficiency depends on the target and can be improved by using a U3 instead of a U6 *A. thaliana* promoter for sgRNA transcription [67].

To enable insertion or replacement of sequences larger than single base pairs and up to several kilobases, gene targeting based on homology-directed repair (HDR) is applied. Here, a donor DNA containing the intended DNA modification as well as flanking sequences homologous to the target site can be co-delivered with the CRISPR/Cas components. With the simultaneous inhibition of NHEJ by knocking *XRCC4* out and the enhancement of HDR by overexpressing *CtlP* and *MRE11* in poplar (*P. trichocarpa*), a bleomycin resistance gene was seamlessly integrated in frame of an endogenous promoter by Cas9 with knock-in efficiency of up to 48% [86].

3.5 CRISPR Activation

CRISPR/Cas can be used for activation of target genes by recruiting transcription activators, independent of CRISPR/Cas-mediated changes to the DNA sequence [87]. Because a DSB is not required, CRISPR activation is achieved by nuclease-inactive deadCas9 (dCas9). For gene activation in poplar (*P. alba* × *P. tremula*), a CRISPR-Combo system based on CRISPR-Act3.0 was used to enable gene editing and activation at the same time. Therefore, Cas9 endonuclease activity was deactivated by using short protospacer sequences of 14 to 16 nucleotides. Activators were acquired by gRNA using MS2-SunTags. Editing efficiencies of 100% and gene activation of up to 100-fold expression were achieved [61].

4 Forest Tree Relevant Traits as Breeding Objectives

Forest trees are important sequesters of CO_2 into biomass and components of terrestrial biodiversity. As sessile organisms with prolonged growth, forest trees are frequently exposed to diverse stresses derived from the abiotic and biotic environment. Climate change-related weather conditions contribute to novel and increasingly severe environmental stresses for forest trees, such as drought periods or increased soil and water salinisation in certain climate zones. Although trees are evolutionarily adapted to local environments, fast-changing fluctuations of local climate conditions strongly affect their viability. Moreover, the establishment of novel tree pathogens caused by climate fluctuations and the increased vulnerability of already stressed forest trees to domestic pathogens also put forest trees under stress [88]. Trees are increasingly required to withstand specific stresses and to remain upright, providing, in part, irreplaceable both ecological and economic value for countries and their people. The ability of trees to assimilate CO_2 in great quantities displays a natural mechanism to mitigate global warming effects. However, climate change-derived stresses may reduce carbon fixation due to reduced photosynthesis rates when water is scarce or temperatures are too high [89].

Forest tree tolerances, resistances, and the refined breeding of commercial traits display the present-day breeding objectives. Improved and especially accelerated breeding strategies and genetic research on traits need more attention if species or products are to be sustained in the near future. Even though implementing genome editing in forest trees to improve and accelerate breeding purposes is still young, some research has displayed this mechanism's fast potential, especially on functional analyses of single genes and their correlation with desired traits.

4.1 Abiotic Stress Tolerances

Abiotic stress tolerance-related research deals with elucidating and improving traits for tolerance of abiotic environmental factors, especially drought or salinity, that trees are increasingly confronted with. The continuing incorporation of genome editing mechanisms (particularly CRISPR/Cas) in this research field drastically improves the understanding of single gene functions and their impact on tolerance traits by subsequently isolated phenotype analyses. Even though, to date, the research on abiotic stress tolerance-related traits is still limited in trees, an increase is observable and will gain more attention in future. However, some research has been done regarding single or multiple stresses and their higher-ordered adaption mechanisms.

4.1.1 Drought Stress

Drought stress describes the stress caused by the absence of water supply, which can reduce biomass production and the energy-providing mechanism of photosynthesis. While the research on annual model plants already revealed important mechanisms and genes involved in drought stress tolerance, the research on forest trees (especially under the application of genome editing) is in its infancy. The research of recent years mainly applied CRISPR/Cas-mediated knockouts in the model genus *Populus* to verify observable phenotypes derived from overexpression of putative drought tolerance-related genes. Even though CRISPR/Cas-mediated knockout mutants did not improve traits of drought stress tolerance, the precise knockouts of candidate genes helped insights into gene functions and their further use for tree tolerance breeding purposes. As a trait of putative drought stress tolerance, Zhou et al. [90] analysed the mechanism of root growth under drought stress. Overexpression and CRISPR/Cas9 knockouts of the root-specific *NUCLEAR FACTOR-Y* transcription factor (TF) *NF-YB21* were analysed in the poplar hybrid 84 K (*P. alba* × *P. glandulosa*). Comparative analyses of one-month-old *nf-yb21* mutants and WT poplars revealed a reduced drought stress tolerance of the mutants by significantly reduced overall root growth and biomass, as well as thinner xylem vessels with tyloses and lower lignin contents, which reduced the hydraulic conductivity, an important indicator of water transport from soils [90].

As stomata regulate the flow of gases and thereby a plants water status, Shen et al. [91] analysed the impact of the TF *Pd*GNC on stomatal aperture in *P.* × *canescens*. 60-days-old CRISPR/Cas9-mediated *gnc* mutants exhibited increased stomatal aperture and water loss with reduced drought stress tolerance under drought stressed experimental conditions of 75 days. Analyses explained the drought-susceptible phenotype with lower nitric oxide (NO) levels and hydrogen peroxide (H_2O_2) production in guard cells, increasing the stomatal aperture and, thereby water loss [91]. Similarly, Bai et al. [92] studied the function of the TF gene *OSMOTIC STRESS INDUCED C2H2 1* (*OSIC1*) in *P. alba* var. *pyramidalis* that is likewise involved in the pathway of stomatal aperture. CRISPR/Cas9-mediated knockout mutants showed significantly reduced performance under drought stress by greater stomatal aperture and water loss. This was explained by the involvement of *OSIC1* in a newly discovered transcriptional regulatory mechanism of H_2O_2 production in guard cells. The results enrich the understanding of how perennial woody plants respond to drought-induced osmotic stress, which can be further used for refined breeding approaches [92].

4.1.2 Salt Stress

The salinisation of forest soils describes the excessive accumulation of water-soluble salts in upper soil horizons. It mainly derives from extreme weather conditions, with dry climates and low precipitation. If prolonged over a long time or at high concentrations, the salinity of the soil results in reduced water and increased salt uptake of trees, resulting in stress through ionic, osmotic, oxidative, and other secondary changes. Salt stress can, when exposed for a long time, end in plant die-offs [93]. Here, damage severity depends on salt concentrations, the growth stage of trees or the tree species, with *Populus euphratica* known to tolerate specific salt concentrations and growing in saline semi-arid areas [94].

Efforts have been made to study the impact of single gene modifications on salt stress tolerance by using genome editing in *Populus*. CRISPR/Cas9-mediated knockout of the TF gene *WRKY77* in *P. alba* var. *pyramidalis* significantly improved the salt tolerance of poplars under *in vitro* salt stress conditions [95]. After growing for one month in liquid medium with 150 mM NaCl, wild-type (WT) poplars showed leaf chlorosis symptoms, while *palwrky77* mutant leaves remained green with little discolourations. Further, significantly higher electrolyte leakage measurements suggested a higher cell disruption in WT compared to mutant poplars. *In vivo* and *in vitro* assays revealed the differences in salt stress tolerance by the *Pal*WRKY77-induced repression of abscisic acid-related genes. Therefore, *Pal*WRKY77 was found to be a negative regulator of salt stress response in poplars, providing a potential basis for genetic modification to generate salt-tolerant poplars in saline habitats [95].

To further elucidate salt stress tolerance in trees, candidate genes could be selected by the orientation of promising genes in annual plants such as *A. thaliana*, *Oryza sativa,* or *Solanum lycopersicum*. Shelake et al. [96] illustrate the potential of

genome editing in crop plants to increase salinity tolerance. Here, CRISPR/Cas-mediated knockouts of *AtAITR* genes involved in abscisic acid signalling in *A. thaliana* [97], the *OsPQT3* gene, an E3 ubiquitin ligase involved in the regulation of oxidative stress in *O. sativa* [98], or the *SlARF4* gene, an auxin response factor in *S. lycopersicum* [99], resulted in improved salinity tolerance and may be promising candidate genes for genetic modifications in forest trees if sequence information and genetic transformability are given.

4.2 Biotic Stress Resistance

Plant pathogens, including viruses, fungi, bacteria, or oomycetes, are part of every functional ecosystem. However, globalisation or changing environmental factors contribute to the spread and mutation of pathogens, leading to pathogens harmful to single species or whole plant ecosystems. Resulting emerging infectious diseases can cause landscape-level mortality and, subsequently, ecosystem-wide changes [100]. The importance of tree pathogen control can be exemplified in the interference of the fungus *Hymenoscyphus fraxineus*, causing ash dieback with severe mortality of common ash trees (*F. excelsior*) in most parts of the ash distribution range in Europe [101]. Other prominent examples of forest tree pathogens are chestnut blight, Dutch elm disease, myrtle rust, white pine blister rust, poplar leaf rust, and sudden oak death [100].

To date, the research on forest pathogen resistance by genome editing mechanisms is in its infancy. In addition to technical limitations in working with forest trees, the lack of knowledge of tree-pathogen interactions may restrict genome editing applications, as it denotes the basis for advanced research. Wang et al. [102] analysed the involvement of the TF MYB115 on the production of proanthocyanidins (PAs), a class of defence phenolic compounds in the leaves of poplars (*Populus* spp.) in response to abiotic and biotic stresses. CRISPR/Cas9 knockouts of *MYB115* in *P.* × *tomentosa* revealed significantly reduced levels of PAs and decreased expression of PA biosynthesis genes, suggesting the positive contribution of MYB115 to PA biosynthesis. After infection with *Dothiorella gregaria,* leaves from the poplar mutant *myb115* showed significantly higher damage [102].

Widespread biotrophic rust fungi of the genus *Melampsora* can reduce the economic value of trees, such as for *Populus* in natural stands and plantations, by reducing significant amounts of biomass [103, 104]. To elucidate genes involved in *Melampsora* resistance, Jiang et al. [105] focused on the TFs WRKY18 and WRKY35. The WRKY group is well known for being involved in abiotic and biotic stress responses in plants. Constitutive overexpression of *WRKY18* and *WRKY35* in *P.* × *tomentosa* led to increased resistance to *Melampsora* by elevated expression levels of downstream genes and lower H_2O_2 accumulation. In contrast, CRISPR/Cas9-mediated knockout mutants did not differ from WT poplars regarding the expression levels of downstream genes and H_2O_2 accumulation, concluding an unaltered *Melampsora* resistance [105].

4.3 Commercial Traits: Productivity and Wood Properties

Trees provide biomaterials with benefits for human life. Especially wood production and wood composition are interesting traits for refined breeding purposes due to the broad range of applications, ranging from the area of building and other industries up to energy supply. Economically important tree species include *P. abies*, *Tectona grandis*, or *Cedrus deodara* for timber production or *Picea rubens*, *Abies balsamea* or *Populus tremuloides* for the production of pulp and paper [106]. Prominent representatives of trees for biofuel production are species of the genus *Populus* or *Salix* due to their fast growth, allowing the production of significant amounts of biomass [107, 108].

4.3.1 Wood Productivity

The productivity of a forest is defined by the standing forest volume at a specific time and referred to as yield, expressed by the accumulation of aboveground stem wood in standing trees. This biomass formation and correlated wood production exhibit an essential trait to improve due to the increasing wood utilisation demands, especially under increasing climate change-related disturbances on wood supply [109].

Genome editing mechanisms have been used to investigate the growth and development of woody plants. Thereby, conclusions could be made of genes involved in productivity, mainly in wood formation, to meet the demands of future wood production. CRISPR/Cas9-mediated knockouts of the *A. thaliana BRANCHED* orthologs *BRANCHED1-1* (*PcBRC1-1*) and *BRANCHED2-1* (*PcBRC2-1*) genes in *P.* × *canescens* strongly enhanced bud outgrowth [110]. *Pcbrc2-1* mutants revealed a significantly higher number of branches, whereas mutants of *Pcbrc1-1* revealed significantly higher shoots. As poplar trees are used for bioenergy production on short rotation coppices (SRCs), the enhanced sylleptic branching of the mutants may be an improved trait regarding the critical plantation establishment phase in the first year. It may increase the biomass yield through the early closure of the canopy and, subsequently, the reduction of competing weeds by shading [110]. However, long-term biomass evaluation is still needed.

Fladung [111] generated CRISPR/Cas9-mediated knockouts of the rice ortholog *TILLER ANGLE CONTROL 1* (*TAC1*) in *P.* × *canescens* to investigate the function of the gene on the pyramidal plant growth, as could be seen by reduced expression levels of *TAC1* in *Prunus* species [112]. After a growth period of 3 years in the greenhouse, mutated poplars showed an altered phenotype compared to WT trees, with leaves of a narrower angle and an upright growth of shoots [111]. Even though no increased biomass production was detectable, upright-grown poplar trees may be interesting for SRCs, as the erect leaf or shoot growth allows more trees per area and, therefore, higher yield per area.

Further experiments of CRISPR/Cas knockouts of poplars were conducted to investigate the function of genes on wood productivity and growth [113]. However, knockouts were not correlated with improved biomass production.

4.3.2 Wood Composition

Wood mainly comprises the polymers lignin and the polysaccharidic cellulose and hemicelluloses, enriched in secondary-thickened cell walls. Polysaccharides are the desired substances of industries producing paper and pulp. In contrast, even though increasingly used for aromatic building blocks in the chemical industry, lignin impairs the extraction of cellulose and hemicelluloses, therefore being declared as a factor of biomass recalcitrance [114].

To reduce the amount of lignin in the wood composition, several genome editing attempts were conducted targeting lignin biosynthesis, ranging from involved transcription factors and oxidative enzymes up to the lignin biosynthesis genes themselves. Early genome editing via the CRISPR/Cas9 system in the *P.* × *canescens* produced biallelic knockouts of the *4-COUMARATE:COA LIGASE 1* (*4CL1*) gene, which was shown to be involved in the lignin biosynthesis [39]. The poplar mutants revealed 23% less lignin in stem wood. Xu et al. [115] genetically modified the TF gene *PtoMYB170* in *P.* × *tomentosa* by creating knockout mutants generated by three target sites for CRISPR/Cas9 endonuclease. Knockout mutants of *PtoMYB170* displayed inability for upright growth, resulting in a pendant phenotype due to significantly reduced lignin deposition in the stem's secondary xylem growth. Expression analyses of lignin biosynthesis genes indicated strongly reduced expression levels in the knockout mutants, demonstrating that *Pto*MYB170 is strongly influential on the downstream genes and lignin deposition in *P.* × *tomentosa* [115].

Other strategies involved the manipulation of genes involved in the direct biosynthesis of lignin. Vries et al. [116] conducted CRISPR/Cas9 knockouts of the *CAFFEOYL SHIKIMATE ESTERASE 1* and *2* (*CSE1, CSE2*) genes in *P.* × *canescens*. After 4 months of growth in the greenhouse, double mutants *cse1cse2* showed a height reduction of 35%, with further reduced stem diameter by 14%, stem fresh weight (not debarked) by 52% and stem dry weight (debarked) by 69% as compared to WT trees [116]. However, lignin contents were decreased by 35%, which translated into a fourfold increase in cellulose-to-glucose conversion upon limited saccharification. That indicates that the saccharification efficiency (hydrolysis from polysaccharides to monosaccharides), positively affects the fermentation of monosaccharides to ethanol, a favourable trait of biofuel production [117]. Jang et al. [118] conducted a comparable CRISPR/Cas9 knockout approach of *CSE1* and *CSE2* in the closely related poplar hybrid 84 K (*P. alba* × *P. glandulosa*). In contrast to Vries et al. [116], they found a reduction of lignin deposition of up to 29.1% in either *cse1* or *cse2* single mutants, along with reduced expression levels of lignin biosynthesis genes. Simultaneously, the genome-edited lines showed no growth retardation and a morphologically indistinguishable phenotype to WT trees in a

long-term living modified organism field test covering four seasons [118]. In addition, mutant poplars showed up to 25% higher saccharification efficiency than the WT control. The difference between both conducted studies from Vries et al. [116] and Jang et al. [118] may rely on the different species (*P.* × *canescens; P. alba* × *P. glandulosa*, respectively) or the amount of lignin reduction (35%, 29.1%, respectively) and thereby a specific threshold, under which no phenotypic changes are observable [118].

Within several years, genome editing positively affected the understanding of lignin-related genes in the model tree genus *Populus* and successfully established poplar trees with limited amounts of lignin and no growth retardation, providing essential insights into the future breeding of lignin-reduced wood composition in trees.

Apart from the genetic modification of wood composition-related genes, parameters of wood anatomy, particularly xylem fibre and vessel length were analysed by genome editing of Fasciclin-like arabinogalactan proteins (FLAs) in *P. trichocarpa* [119]. Out of 50 *in vivo* characterised poplar FLAs, two genes (*PtrFLA40* and *PtrFLA45*) were selected due to their high expression in the developing xylem as well as their high similarity in amino acids of 95.2%. Selected *FLA* genes were knocked out by CRISPR/Cas9. Double mutants *ptrfla40ptrfla45* revealed significantly increased stem length and diameter and enlarged cell sizes of xylem fibres and vessels of 4-month-old grown greenhouse poplars compared to unmodified WT trees [119]. These findings may be relevant for the paper and pulp industry, as the fibre length is an important quality trait due to its positive effect on sheet strength [120].

5 Biosafety of Genome-Edited Trees

Trees differ from most agricultural crop plants in many characteristics, such as long lifespan and long generation cycles, complex habitat, and low degree of domestication. As with genetically modified (GM) trees, biosafety has to be considered before their deployment [121, 122], but well-documented knowledge on specific biosafety aspects is rare for genome-edited trees. Thus, information on biosafety protocols for genome-edited trees is required which provide a scientific basis for future European Union regulations on environmental risk assessment to ensure the safe development and use of genome-edited trees.

Biosafety-relevant aspects comprise four main technological issues that need to be discussed for genome-edited trees [123]: (i) Are the gene-edited and naturally emerged modifications in fact identical? (ii) If not, are the differences potentially hazardous? (iii) Are efficient containment strategies required to avoid possible adverse outcomes from vertical and horizontal gene transfer? (iv) Are off-target effects probable, and if yes, is the selective inclusion of "omics"-technologies needed to study cellular effects following the expression of the gene-edited gene(s)?

Like other plant species, genome-edited trees have the potential for gene flow when they flower. Thus, edited gene(s) could be spread to wild relatives through gene flow (vertical gene transfer). Many tree species are wind pollinators releasing pollen into the environment, which can sometimes be transported over very long distances. In case that the edited gene(s) have a developmental/evolutionary advantage, this could pose an invasive potential of these trees. Unintended ecological consequences could occur, such as the unintended spread of invasive genes or of the whole tree (by vegetative propagation, e.g., root suckers [124]) into natural populations. Thus, at least theoretically, the necessity of establishing containment strategies has to be considered. Therefore, the establishment of confinement systems, i.e., by making trees sterile by suppressing either pollen production (in male stamens) or female ovule development, is considered to prevent the uncontrolled spread of the edited genes [125].

In addition, possible unintended effects of genome-edited trees on non-target organisms must be considered. For example, if a tree is modified in a gene involved in the secondary metabolism and, as consequence, produces a new ingredient, this could act as a toxin to the tree-interacting organisms. Trees fulfil numerous ecosystem services, such as carbon sequestration, soil conservation, and water regulation. Again, genome-edited trees producing a new ingredient, could impact these services. For example, if a tree is modified to grow faster, it may sequester more carbon, but it could also deplete soil nutrients faster.

If a mutation has been detected in the plant genome without knowing whether it was natural or induced, to date, there are no detection methods to distinguish between gene-edited and natural mutations. In addition, if a cultivar carrying a natural mutation has been assessed as being safe in biosafety testing, there is no reason to assume a hazard if the cultivar has an induced mutation similar to a natural one. However, the mechanisms leading to the mutations are different, thus, because of the longevity of trees, long-term effects of genome-edited trees have to be considered, at least theoretically. This includes, for example, the long-term stability of the gene-edited modification or the epigenetics of the whole edited DNA region. To study the stability of the edited gene, the establishment of field trials under natural conditions are necessary. Such field trials could (i) deliver results about phenotypic effects resulting from expression of the gene edited genes, (ii) validate observations made under greenhouse conditions, and (iii) unravel putative non-target effects when the trees are grown within the range of natural variation. However, similar to GM trees, field trials with gene-edited trees will be the exception rather than the rule in Europe. Reasons for this are manifold. Firstly, a high level of public concern exists against genetic engineering technologies. Thus, many consumers are reluctant to accept products made from genetically modified organisms. In addition, regulatory hurdles which are based on the precautionary principle are high in Europe, making the regulatory process for field trials extremely long and making it difficult to obtain approval for a field trial with gene-edited trees. And finally, similar to GM plants in general, many companies and researchers fear that anti-GMO activists will destroy field trials with gene-edited trees.

Off-target effects are unintended changes in the DNA resulting from gene editing in "wrong" genes. This could occur, for example, when gene editing is aimed at duplicated genomes and the target genes are present in duplicated or even in multiple copies and are highly similar in their sequence. As a consequence, it could be that the gRNA was not specifically designed for the one gene to be edited and that also sequence-homologous genomic regions (e.g., paralogous genes) are targeted by the gRNA. This could lead to mutations in other parts of the genome, potentially causing gene knockouts or activation or silencing of genetic regulatory elements. However, such off-target modifications could simply be avoided through improvement in the gRNA design [40], based on reliable genome sequences of the tree species to be edited.

It is common silvicultural practice to perform a formal evaluation of the behaviour of new tree varieties under natural field conditions. Accordingly, a number of field trials have to be set up to assess the safety of gene-edited trees modified for different genes, similarly as it was performed with GM trees [126]. However, in contrast to classical GM-technology, genome editing modified genomic information is targeted and precise, thus, organisms (microbes, plants and animals) harbouring mutations created by genome editing are indistinguishable from organisms carrying an identical but naturally emerged mutation. This could lead to the question whether the biosafety of gene-edited trees needs to be tested in the field at all.

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