Poplar as the Woody Plant Model Species for Science and Industrial Applications: Overviews, Problems and Perspectives

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
8-oxoG	7,8-dihydro-8-oxoguanine
BAP	6-benzylaminopurine
bp	base pairs
Cas9	CRISPR-associated protein 9
CRISPR	clustered regularly interspaced short palindromic repeats
DDBJ	DNA Data Bank of Japan
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EST	expressed sequence tag
FRET	fluorescent resonance energy transfer
GFP	green fluorescent protein
GUS	β-glucuronidase
Gy	gray (the SI unit of absorbed dose)
ha	hectare (although not an SI unit, the commonly used metric unit of an area of land)
LEA	late embryogenesis abundant
LMO	living modified organism
MES	2-(*N*-morpholino)ethanesulfonic acid
MSBS	Murashige and Skoog basal salts

MSV	Murashige and Skoog vitamin
NCBI	National Center for Biotechnology Information
NPBT	new plant breeding techniques
NPTII	neomycin phosphotransferase II
PAR	photosynthetically active radiation
PCR	polymerase chain reaction
PHB	poly-3-hydroxybutyrate
RIM	root-induction medium
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
RT-qPCR	reverse transcription quantitative real-time PCR
SAM	shoot apical meristem
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	shoot-induction medium
TALEN	transcription activator-like effector nuclease
ZFN	zinc finger nuclease

Abstract

Poplar trees (from the genus *Populus*) have been utilized for industrial and environmental applications such as wood production, biomass feedstock, and environmental conservation and restoration throughout the ages. The practical importance and the biological properties of poplars have enhanced many fields of study such as physiology, ecology, molecular biology, genetic engineering, forestry, chemistry, wood science and environmental science. Thereby, poplars have been recognized as a woody plant model species. For the advancement of science and applications of poplars as the woody plant model species, it was thought to be important to pioneer new fields of study on poplars and develop new technologies for the study. Thus, the objectives of this study are to elucidate the biological response to ionizing radiation as an abiotic stress in *Populus nigra* and improve the transformation system for *P. nigra*.

Ionizing radiation is an environmental stress; however, little is known about its effects on poplars or the tolerance mechanism of woody plants to ionizing radiation. Therefore, in this study, we investigated the biological effects of γ-rays and the tolerance mechanism of *P. nigra* as a model species. Poplar plantlets irradiated with 50–100 gray (Gy) of γ-rays were able to survive, although growth inhibition or morphological abnormalities were observed. A total dose of 200–300 Gy of γ-rays killed almost all poplar samples. A high dose of γ-rays also inhibited the regeneration of the shoots and roots. Comet assays demonstrated that the γ-rays damaged the nuclear DNA of the irradiated cells. To characterize the tolerance mechanism to ionizing radiation stress, six DNA repair-related cDNAs, *PnLIG4*, *PnKU70*, *PnXRCC4*,

PnRAD51, *PnPCNA*, and *PnOGG1*, were isolated and structurally analyzed. The expression of *PnLIG4*, *PnKU70*, *PnXRCC4*, *PnRAD51*, and *PnPCNA* was upregulated by γ-irradiation in a dose-dependent manner, while *PnOGG1* was downregulated. The expression of *PnLIG4*, *PnKU70*, and *PnRAD51* was also upregulated by the treatment of a DNA cleavage agent. Accordingly, it is concluded that the gene expression of *PnLIG4*, *PnKU70*, and *PnRAD51* was directly induced by DNA strand breaks.

A highly efficient transformation system for *P. nigra* was required to further study the DNA repair-related genes that were regulated in response to ionizing radiation stress. To improve the transformation system for *P. nigra*, it was aimed to construct a new binary vector, to shorten the time required and increase the efficiency of transformation. The newly designed binary vector has 11 restriction enzyme sites for DNA cloning and demonstrated higher resistance to selective antibiotics. *P. nigra* was transformed by *Agrobacterium* harboring the new vector fused to the enhanced green fluorescent protein gene. Successful transformation was confirmed by polymerase chain reaction (PCR), fluorescence microscopy, immunoblotting, Southern blotting, and resistance to kanamycin and G418. The period of transformation was shortened to a minimum of approximately 4 months by the direct regeneration of transgenic shoots from the *Agrobacterium*-infected stems. In the co-cultivation of poplars stems and *Agrobacterium*, the addition of dithiothreitol to the medium increased the transformation efficiency by approximately 20%.

In the present study, the response and tolerance mechanisms of *P. nigra* to ionizing radiation stress were elucidated for the first time. In addition, the transformation system for *P. nigra* was effectively improved. In the future, these results may lead to the generation of useful transgenic poplars, the study of gene function,

applications in environmental conservation, or mutation breeding. Furthermore, the value of *P. nigra* as the woody plant model species has been enhanced by understanding radiation stress physiology as a new field of study and by the development of improved tools for genetic engineering. Poplars can thus play the key role as the model species for studying the science and technology of woody plants. In turn, it is hoped that the science and applications of poplar can contribute to the research of woody plant species, the supply of forest resources, and the conservation of the global environment.

要旨

ポプラは、木材生産やバイオマス原料、環境保護や環境修復といった産業分野や環境分野で、長い間利用されてきた。ポプラの産業的な重要性と生物学的な特徴が、生理学や生態学、分子生物学、遺伝子工学、林学、化学、木材科学、環境科学など多くの研究を発展させてきた。それゆえ、ポプラは木本植物のモデル生物種として認識されている。モデル生物種としてポプラの科学と応用を進展させるためには、新しい研究分野を開拓すること、およびその研究に必要な技術開発を進めることが重要と考えられた。そのため、放射線による *Populus nigra*（クロポプラ）の生物学的応答の解明と形質転換法の改良を研究の目的とした。

電離放射線ストレスは環境ストレスの一種である。しかし、ポプラにおける電離放射線の影響、および木本植物における放射線ストレスの耐性機構はほとんど分かっていない。そのため、モデル生物種として *P. nigra* に対するガンマ線の生物学的影響とその耐性機構を調べた。*P. nigra* の苗木は、50 グレイ（Gy）から 100Gy の高線量ガンマ線を急照射しても生き残ることができた。しかし、一部の苗木には、成長の阻害や停止、形態的な異常が生じた。200Gy から 300Gy のガンマ線は、ほとんどの *P. nigra* を枯死させた。また、高線量のガンマ線は、シュートや根の形成を阻害した。コメットアッセイ法により、ガンマ線を照射した細胞では細胞核の損傷が生じていることが示された。電離放射線ストレスに対する耐性機構を解明するため、DNA 修復系タンパク質の 6 種の遺伝子、*PnLIG4*、*PnKU70*、*PnXRCC4*、*PnRAD51*、*PnPCNA*、*PnOGG1* の各 cDNA を *P. nigra* から単離し、構造を明らかにした。ガンマ線量に依存して、*PnLIG4*、*PnKU70*、*PnXRCC4*、*PnRAD51*、*PnPCNA* の遺伝子発現が増加する一方で、*PnOGG1* の発現は減少することが分かった。*PnLIG4*、*PnKU70*、*PnRAD51* の発現は、DNA の化学的切断処理によっても上昇することから、これらの遺伝子は DNA の切断損傷が引き金となって発現が誘導されると結論された。

上記の放射線ストレスの研究を進めるため、遺伝子工学技術の進展が必要とされた。*P. nigra* の形質転換法を改良するため、新しいベクターの構築、形質転換にかかる期間の短縮、および形質転換効率の上昇を目指した。構築した新しいベクターは、遺伝子クローニングのための 11 の便利な制限酵素切断部位を有し、選抜薬剤耐性を向上させることができた。この新しいベクターに緑色蛍光タンパク質遺伝子を組み込み、アグロバクテリウム法を用いて *P. nigra* を形質転換した。形質転換の成功は、PCR 法、蛍光顕微鏡観察、免疫ブロット法、サザンブロット法、およびカナマイシンと G418 に対する耐性で確認した。アグロバクテリウムに感染した茎切片から、直接、遺伝子組換えシュートを再生させることにより、遺伝子組換えに必要な時間は最短で 4 か月に短縮できた。また、アグロバクテリウム感染時に還元剤であるジチオスレイトールを添加することにより、形質転換効率を約 20%に上昇させることができた。

本研究により、*P. nigra* の電離放射線ストレスに対する応答と耐性機構が初めて解明された。また、形質転換法が効率的に改善された。将来的に、これらの結果は有用な遺伝子組換えポプラの作出や、遺伝子機能の研究、環境保全への応用、突然変異育種につなげることができる。さらに、新しい研究分野である放射線ストレス生理学の開拓と遺伝子工学技術の進展は、木本植物のモデル生物の一種として *P. nigra* の価値を高めたと考えられた。ポプラは木本植物の科学と技術のためのモデル生物種として、鍵となる役割を果たすことができる。ポプラの科学と応用が、木本植物の研究や森林資源の供給、地球環境の保全に貢献することが望まれる。

Chapter 1 Introduction

Poplars (also known as aspens or cottonwoods) are deciduous trees in the genus *Populus* (family Salicaceae), which consists of approximately 30 species. Poplar trees are familiar to humans and have been utilized since ancient times, appearing in Greek mythology and the Bible. They are important in industrial applications as a source of wood materials, paper, fuel, and forage. In addition, poplar trees have been utilized for the provision of preferable landscapes and farmland and in environmental conservation and restoration. These industrial, agricultural, and environmental uses of poplars have driven the investigation of their science; and improved scientific knowledge of these trees has led to them being recognized as model species for woody plants (Bradshaw et al. 2000; Taylor 2002; Jansson and Douglas 2007).

This chapter provides an overview of the science and applications of poplars. It includes a wide range of information about these trees, demonstrating why they are considered woody plant model species and are the subject of this thesis.

1.1 Taxonomy and distribution

It has been proposed that the genus *Populus* consists of either 29 species (Eckenwalder 1996) or 32 species (Dickmann and Kuzovkina 2014), which are further classified into six sections (Table 1). Various hybrids of poplar exist in nature, because they have the ability to hybridize with other species from the same section or from closely related sections (Willing and Pryor 1976; Vanden Broeck et al. 2005). Artificial hybrids have also been produced for the improvement of their agronomic and industrial

traits. The International Poplar Commission (IPC) holds the International Cultivar Registration Authority (ICRA) for poplars and maintains the *Populus* Cultivar Register (Kuzovkina and Vietto 2014). A database of poplar cultivars is open to the public and available at http://www.populus.it/.

Poplar trees mostly inhabit the Northern Hemisphere, including Asia, Europe, North Africa, Russia, and North America (Dickmann and Kuzovkina 2014) (Table 1). However, poplars are planted in both the Northern and Southern Hemispheres. In 2011, the reported total area of poplars worldwide was estimated at 87.3 million ha (FAO 2012), which compares with a total forest area on Earth of 4.03 billion ha in 2010 (FAO 2010). Poplar forests mainly consist of indigenous forests, planted forests, and agroforests (Table 2). Indigenous poplar forests cover approximately 75.5 million ha, and are mostly found in Canada (30.3 million ha), the Russian Federation (24.8 million ha), the USA (17.7 million ha in 2008), and China (2.5 million ha). Planted poplar forests cover approximately 8.6 million ha and are mostly found in China (7.6 million ha), France (236,000 ha), Iran (150,000 ha), Turkey (125,000 ha), Spain (105,000 ha), and Italy (101,430 ha). The area of poplar plantations in China is increasing rapidly, because these plantations only covered 4.3 million ha in 2008 and 3.9 million ha in 2004. Planted poplar forests also occur in the Southern Hemisphere, being found in Argentina and Chile. In New Zealand, poplars are planted alongside willows (*Salix* spp.). In agroforestry systems, poplar trees are cultivated with herbaceous crops or livestock.

1.2 Industrial applications

Poplars are used for fuel, timber, wood products, and environmental

conservation (Fig. 1). In 2011, the total amount of harvested poplar wood across the world (with the exception of Russia, China, and the USA) was 20.3 million m^3 (FAO 2012). The majority of this wood (17.4 million m^3) originated from planted poplar forests in India (5.1 million m^3), Iran (3.7 million m^3), Turkey (3.5 million m^3), Belgium (3.2 million m^3), and Argentina (1.67 million m^3), while the remaining 2.9 million m^3 originated from indigenous forests, mainly in Canada (2.8 million m^3). In contrast, the global amount of forest products from poplar in 2011 was estimated to be at least 70.4 million m^3 (FAO 2012) (Table 3). Although China did not release figures for the amount of harvested poplar wood, it consumed 50 million m^3 of logs for the production of plywood and wood pulp. Russia and the USA have vast areas of poplar forest; however, their consumption of poplar wood has not been made publicly available.

Poplar has a number of uses in the industrial sector, including timber, veneer, plywood, wood composites, pulp, paper, chemicals, fuel, and energy. In 2011, 44 million m^3 of veneer and plywood, 16.6 million m^3 of pulp, 5.7 million m^3 of timber, 2.2 million m^3 of wood composite panels, and 1.7 million m^3 of fuel wood and chips were produced globally (FAO 2012) (Table 4). Poplar wood is important in the manufacture of forest products, including pulp and paper, timber, veneer and plywood, composite panels, structural composite timber, pallets, furniture components, fruit baskets, containers, and chopsticks (Balatinecz and Kretschmann 2001). Wood-based composites include various products, such as veneer, plywood, fiberboard (categorized as insulation board, medium-density fiberboard, and hardboard), particle board, oriented strandboard, wood-cement composites, wood-plastic composites, and glued timber (categorized as laminated strand lumber, paraller strand lumber, and laminated veneer lumber) (Balatinecz et al. 2014).

1.3 New industrial applications

As new technologies are developed, new uses for poplar are being investigated. When used as a fuel source, poplars have traditionally been used in the form of firewood and branches. However, alternatives to fossil fuels are desired to meet increasing human demands and to mitigate global warming. One solution for this is the conversion of plant biomass to biofuels, and poplar is expected to be used as a feedstock for biofuels (Sannigrahi et al. 2010). An early experiment showed that when acid-treated hardwood of *Populus eugenii* DN34 (a hybrid of *P. deltoides* × *P. nigra*) was saccharified and fermented with thermotolerant yeast species, ethanol was produced at a concentration of 10–14 g l^{-1} of medium (Kadam and Schmidt 1997). Since this experiment, the production of ethanol from poplar wood has continued to be refined (Negro et al. 2003; Wang et al. 2012c) and bioethanol has also been produced from the leaves of *P. nigra* (Gupta et al. 2014). Poplar trees have also been genetically modified to increase the efficiency of ethanol production. For example, transgenic *P. alba* that expressed *Aspergillus aculeatus* xyloglucanase had 10–15% higher ethanol productivity than the wild type (Kaida et al. 2009). Bioethanol productivity was also examined in the knockdown poplars of *p*-coumarate 3-hydroxylase by RNAi (reduction in lignin content) and in poplars that overexpressed ferulate 5-hydroxylase (reduction in guaiacyl lignin) (Mansfield et al. 2012). A reduction in the lignin content in *P. tremula* × *P. alba* by the downregulation of cinnamoyl-CoA reductase improved the ethanol yield (Van Acker et al. 2014).

It is also expected that poplar will be able to be used as an ingredient for the production of bioplastics. Poly-3-hydroxybutyrate (PHB) is a biodegradable

thermoplastic material that is produced by bacteria such as *Pseudomonas oleovorans* (Anderson and Dawes 1990). PHB was successfully produced by the bacterial fermentation of a hydrolysate that was prepared from poplar wood (Dai et al. 2015). The hydrolysate, which included simple sugars, was prepared by soaking the wood in hot water at 200°C followed by treatment with lignocellulolytic enzymes. Mixed bacteria were then cultured in a bioreactor, using the hydrolysate as a carbon source. This resulted in the bacterial cells accumulating a maximum of 0.32 g of PHB per gram of bacteria (dry weight). An attempt has also been made to directly produce PHB in poplar plants by developing transgenic hybrid poplars (*P. tremula* × *P. alba*) that expressed three bacterial genes for PHB synthesis (Dalton et al. 2011). These transgenic poplars contained 1–2% (dry weight) PHB in their leaves, with the PHB granules being detected in the chloroplasts. The direct production of PHB in poplars could be less expensive than bacterial PHB production, which requires additional energy.

Phytochemicals from poplars are also expected to be utilized and commercialized in the future (Devappa et al. 2015). Poplars contain more than 160 types of chemicals, which have a number of useful functions, including pesticidal, antimicrobial, antioxidant, and anticancer properties. Although the wood of poplars is well utilized, the other parts are not. However, the value of bark, leaves, branches, and other residues including phytochemicals may be appreciated in the near future.

1.4 Environmental applications

Poplars are planted not only for wood production but also for environmental purposes (Fig. 1). For example, poplars are used as windbreaks and shelterbelts in Europe, North America, Russia, Chile, Argentina, northern India, and northern China

(Isebrands et al. 2014). In China, the Three North Shelterbelt Program (the Green Great Wall), which started in 1978, has established a putative 20 million ha of tree plantations, including *P. simonii* in 1991 (Carle and Ma 2005), although this poplar planting had low genetic diversity and was attacked by insect pests and diseases. Since then, the improvement of poplar has been actively advanced to enhance its vigor, disease resistance, and stress tolerance.

Poplars are often utilized for the prevention of soil erosion by water or wind, as they are able to stabilize large amounts of soil due to their extensive root systems. For example, in New Zealand, which is a mountainous country that experiences heavy rainfall over short periods of time leading to soil erosion, over 2 million poplars were planted for erosion control during the 1960s and 1970s (Wilkinson 1999); and the Three North Shelterbelt Program in China aims to prevent soil erosion as a result of strong wind.

Native poplar forests are thought to be valuable for the original landscape in their natural habitats. Furthermore, both native and planted poplar forests contribute to the maintenance of biological diversity, carbon sequestration, and the supply of ecosystem services (Rotach 2004; Kuhn et al. 2011; Isebrands et al. 2014).

Poplars are also considered to be suitable for phytoremediation (defined as the use of green plants to remove, contain, or render harmless environmental contaminants (Cunningham and Berti 1993)) because of their rapid growth, perennial habit, and deeper root systems than herbaceous plants, and therefore, this has been the subject of several studies (Yadav et al. 2010; Marmiroli et al. 2011). It has also been recently suggested that parasitism by endophytes are involved in the tolerance of poplars to pollutants. Novel endophytes were isolated from poplar trees and were able to degrade

organic pollutants such as benzene, toluene, ethylbenzene, xylene, and trichloroethylene (Moore et al. 2006; Taghavi et al. 2011; Van Aken et al. 2011; Kang et al. 2012). Furthermore, *P. trichocarpa* × *P. deltoides* trees that were inoculated with the endophyte *Burkholderia cepacia*, which normally parasitizes herbaceous yellow lupin (*Lupinus luteus*), exhibited a higher tolerance to toluene than non-inoculated poplars (Taghavi et al. 2005).

It is also intended that poplars will be used as biomonitors. The concentrations of trace elements in the leaves of *P. alba* are correlated with the levels of Cd, Zn, Mn, and Cu in surface soils and Cd, Zn, As, and Cu in deep soils (Madejon et al. 2004); and the concentrations of heavy metals in the bark of *P. nigra* have been investigated using neutron activation analysis to monitor the levels of air pollution (Berlizov et al. 2007). Chromosomal aberrations at the anaphase stage and the sterility of pollen in *P. simonii* were also attempted to be used as an indicator for assessing the levels of pollution in an urban area (Sluchyk et al. 2014). A technique that combines genetic engineering and a new technology, namely fluorescent resonance energy transfer (FRET), was recently invented for monitoring the levels of Zn in poplar (Adams et al. 2012). FRET is a fluorescent indicator that can be used to monitor the concentration of chemicals in a living cell. A FRET construct that was fused to the Zn transporter from *P. trichocarpa* was introduced into *P. tremula* × *P. alba*, following which the transgenic poplar leaves were treated with 1–10 mM Zn. The intracellular level of Zn was then detected as a change in the FRET fluorescence spectrum.

1.5 Biology of poplars

The biological properties of poplars are not only important for industry but are

also of interest as a model for other woody plant species (Table 5). The fast growth of poplars is one of the reasons they are frequently used in plantation forests. Poplars have very high growth rates in industrial plantations (Stanturf and Oosten 2014). For instance, an experiment with *P. trichocarpa* × *P. deltoides* yielded 15.6–27.8 Mg dry weight ha^{-1} $year^{-1}$ (Heilman and Stettler 1985). The rotation length in plantation forests varies depending on the purpose of production and the environmental conditions. For example, the rotation length is 12–15 years for log and veneer production in the western USA but a minimum of 2–3 years for a bioenergy system (Stanturf and Oosten 2014). The growth of poplar trees directly contributes to the production of woody biomass; therefore, poplar cultivars with higher growth potential have been produced through selective breeding and crossbreeding.

The ability of poplars to propagate vegetatively is useful in plantations. Vegetative (asexual) reproduction occurs naturally from crown breakage and treefall (Braatne et al. 1996). The broken branches become buried in the soil and then develop roots and shoots after a certain period of time. Any remaining tree stumps and roots are also able to produce adventitious shoots, and root suckers are formed under natural conditions. It is particularly easy to root cuttings from poplars belonging to sections *Aigeiros* and *Tacamahaca*; therefore, plantations of poplars outside these sections are less common globally (Dickmann and Kuzovkina 2014).

The major species of poplars can be artificially crossed (sexual reproduction) for breeding. Most poplar species are dioecious, but *P. lasiocarpa* is usually monoecious (Boes and Strauss 1994). The length of the juvenile stage, from germination to flowering, varies from 5 years to >10 years between species. In the reproductive stage, poplars bloom in early spring and have catkins (inflorescences) with many solitary

flowers. Poplars are wind-pollinated plants; therefore, their pollen can travel long distances. Following pollination and fructification, small seeds with hair-like cotton or silk are dispersed by the wind. The seeds of poplars are short lived and rapidly lose their germination ability (Popova et al. 2013). Intraspecific or interspecific crossing can be performed in some poplar species; however, self-fertilization is impossible due to the dioecism.

There is some controversy regarding sex determination in poplars. Chromosome 19 is responsible for sex determination, which occurs via a ZW system whereby the female is the heterogametic gender (Yin et al. 2008). In *P. tremula* × *P. tremuloides*, the sex trait was mapped on a non-terminal position of the linkage group 19 on the male map (Pakull et al. 2009). Three putative transcription factor genes and four genes that were potentially involved in flower development were identified in this region in *P. trichocarpa* (Kersten et al. 2014), and a male-specific gene from the DNA pools of *P. tremula* and *P. tremuloides* accorded with one of these candidate genes (Pakull et al. 2015). This candidate gene corresponded to Potri.019G047300 (*TOZ19*) in *P. trichocarpa* and was homologous to the *Arabidopsis thaliana* gene *TORMOZEMBRYO DEFECTIVE* (*TOZ*), which is involved in early embryo development and in meristem transition from the vegetative to reproductive phase in the early stages of flower development. Potri.019G047300 and Potri.014G155300 showed higher levels of expression in male *P. tremula* than in females (Robinson et al. 2014).

1.6 Biodiversity and conservation

Some native populations of poplar are threatened with extinction. One of the reasons for this is habitat destruction as a result of human activities such as agriculture,

deforestation, and urbanization. In addition, hybrid poplars compete with native poplars. Poplars have a high ability to cross with other *Populus* species to generate interspecific hybrids both naturally and artificially. Many hybrid poplars grow faster than native species. For example, the productivity of an indigenous *P. nigra* was lower than that of an interspecific hybrid (*P. maximowiczii* × *P. trichocarpa*) under unfavorable soil and climate conditions but statistically no different under marginal or favorable conditions in the Czech Republic (Benetka et al. 2014).

Genetically, pure *P. nigra* trees have been in danger of extinction in Europe (Lefèvre et al. 1998; Smulders et al. 2008; Vanden Broeck et al. 2012); therefore, activities for their conservation and management are being continued by the European Forest Genetic Resources Programme (EUFORGEN) *Populus nigra* Network among European countries (Lefèvre et al. 2001; Koskela et al. 2004). To preserve the diversity of natural *Populus* species, the short- and long-term storage of seeds is being attempted. Seeds of *P. alba* × *Populus glandulosa* (*P. tremula* var. *glandulosa*) exhibited a high germination rate and the normal proportion of seedlings when stored at 5°C for 7–10 weeks (Popova et al. 2013), and the same seeds were also successfully cryopreserved in liquid nitrogen.

1.7 Stress physiology

Higher plants such as poplars generally cannot move freely. These plants are exposed to a number of environmental stresses in their habitat through their lives; however, most are able to avoid the effects of these via stress response and tolerance mechanisms. Environmental stresses can mostly be divided into two groups: abiotic and biotic. Abiotic stresses includes physical, chemical, and meteorological stresses, such as

light (intensity, spectrum, and time), temperature, humidity, hypoxia, gravity, ionizing radiation, gases, chemicals, salinity, nutrients and metals in the soil, and wind. In contrast, biotic stresses originate from living organisms and ecosystems in the plant's surroundings, including pests, herbivores, fungal and bacterial pathogens, parasites, and allelopathic organisms. The harmful effects of both abiotic and biotic stresses on woody plants can result in growth retardation, low productivity of timber, unsuitable quality of the wood, increased forestry costs, deterioration of biodiversity, and forest degradation. Therefore, it is extremely important that the various effects of environmental stresses and woody plant defense mechanisms are elucidated, as increased knowledge about stress physiology in woody plants will enable their defense mechanisms to be enhanced or utilized to better control any stress effects.

Many physiological studies of stress responses have been conducted in poplars. For instance, salinity stress as a result of a 0.5% NaCl solution affects growth, photosynthesis, stomatal resistance, leaf water potential, and leaf area in various poplar clones (Fung et al. 1998), while treatment with 100 mM NaCl reduced the height growth, photosynthetic rate, and stomatal conductance in *P. nigra* and *P. alba* (Mao et al. 2010). It has also been shown that salinity stress changes the cell wall composition through the induction of the genes involved in tension wood formation in *P.* × *canescens* (a hybrid clone of *P. alba* × *P. tremula*) and *P. euphratica* (Janz et al. 2012). The concentration of Na^+, K^+, and other ions and solutes has also been comprehensively investigated in *P. euphratica* under saline conditions (Zeng et al. 2009).

Different genotypes of *P.* × *euramericana* (*P. deltoides* × *P. nigra*) were also found to have different levels of productivity under drought conditions (Monclus et al. 2006), and chilling stress altered the soluble carbohydrate content and protein

expression in *P. tremula* × *P. tremuloides* (Renaut et al. 2004). In addition, elevated CO_2 enhanced growth in *P. tremula* × *P. alba*, while ozone exposure decreased it (Richet et al. 2012). Low nitrogen levels have also been shown to increase the growth of roots and the number of lateral roots in poplars (Wei et al. 2013), while cadmium caused leaf chlorosis, inhibited growth, and changed the expression of proteins (Marmiroli et al. 2013). For a more detailed review of the effects of abiotic stresses and the tolerance of poplars to these stresses, see Chen and Polle (2010), Harfouche et al. (2014), and Marron et al. (2014).

Frequently, poplars are eaten by herbivores or affected by disease. The main insects that harm poplars and their distributions, types of damage, and control methods are listed in de Tillesse et al. (2007) and Charles et al. (2014). Several studies have investigated the defense systems that poplars use to prevent these insects from causing serious damage. One such study showed that the expression of the *Win3* gene from *P. trichocarpa* × *P. deltoides* was systemically induced by wounding (Bradshaw et al. 1990). The predicted Win3 protein is similar to Kunitz-type trypsin inhibitors, which have insecticidal and antifungal activity (Haq et al. 2004; Huang et al. 2010). Furthermore, wounding in *P. tremuloides* increased the number of transcripts of a dihydroflavonol reductase and thus, the concentration of condensed tannins, which are important for defense against herbivory (Peters and Constabel 2002). It has also been reported that *P. nigra* trees that were attacked by a herbivorous gypsy moth produced volatile compounds that attracted parasitic bees as parasitoids (Clavijo McCormick et al. 2014).

Poplars also become infected by many pathogenic fungi and bacteria (Anselmi 2009; Ostry et al. 2014), and specific elicitors derived from these pathogens are known

to initiate their defense system. For example, transcripts that code for 14-3-3 proteins in *P. trichocarpa* × *P. deltoides* were induced by chitosan, which is a constituent of fungal cell walls (Lapointe et al. 2001). Several elicitors from pathogenic fungi and bacteria also activated mitogen-activated protein kinases (MAPKs) in *P. trichocarpa* × *P. deltoides* (Hamel et al. 2005). Furthermore, rust disease, which is caused by pathogenic fungi and is the most important disease in poplars, induces oxidative stress, and has been shown to activate antioxidant enzymes (Zhang et al. 2010), as well as affecting carbohydrate metabolism, photosynthesis, and respiration (Major et al. 2010).

Comprehensive genetic resources are useful for investigating the stress physiology of poplars. Full-length cDNA libraries from poplars have been constructed for annotation and isolation of the genes involved in responses to abiotic stresses (Nanjo et al. 2004; Nanjo et al. 2007) and to insect herbivory (Ralph et al. 2006; Ralph et al. 2008). Stress-related small non-coding RNAs (ncRNAs), or microRNAs (miRNAs), have also been identified. The expression of small RNAs is regulated by mechanical, cold, heat, salt, and dehydration stresses in *P. trichocarpa* (Lu et al. 2005; Lu et al. 2008; Shuai et al. 2013), by UV-B stress in *P. tremula* (Jia et al. 2009), by a fungal pathogen stress in *P. cathayan*a × *P. nigra* (Chen et al. 2012), and by salt stress in *P. euphratica* (Li et al. 2013a; Si et al. 2014). In addition, long intergenic non-coding RNAs (lincRNAs) of more than 200 base pairs (bp) in length were newly isolated from *P. trichocarpa* under drought stress, while six lincRNAs were upregulated following a drought treatment (Shuai et al. 2014). Therefore, in addition to the mRNAs that encode stress-related proteins, these non-coding RNAs probably play important roles in stress responses in poplars.

Advances in comprehensive analysis methods such as DNA microarray

analysis and next-generation sequencing technologies have allowed genome-wide transcriptome analysis to be performed to elucidate the stress response mechanisms in poplars. The first such study was conducted using a DNA microarray that consisted of 315 cDNAs to investigate salt stress and recovery in *P. euphratica* (Gu et al. 2004). Once the *P. trichocarpa* genome had been sequenced (Tuskan et al. 2006), a whole-genome array could be used to elucidate various stress responses in poplars, including defense mechanisms against a rust fungus in *P. trichocarpa* × *P. deltoides* (Rinaldi et al. 2007), changes in gene expression in response to aluminum stress in *P. tremula* roots (Grisel et al. 2010), the comparison of two hybrid genotypes (*P. deltoides* × *P. nigra*) with different drought tolerances (Cohen et al. 2010), gene regulation in the leaves of *P.* × *euramericana* that were subjected to excess zinc (Di Baccio et al. 2011), and drought and salt stress in *P. alba* × *P. glandulos*a (Yoon et al. 2014). More recently, RNA-Seq by next-generation sequencing was used to investigate the salt stress response (Qiu et al. 2011), cold and chilling stress (Chen et al. 2014b), heat shock stress (Chen et al. 2014c), and rust infection response (Petre et al. 2012) in poplars. Genome-wide analysis can be used to examine the changes in several hundred to thousand genes as a result of environmental stress at a given time. All of this information is extremely valuable for elucidating the stress response and tolerance mechanisms in poplars.

1.8 Genetics and genomics

Genetics and genomics have accelerated the development of model organisms for scientific studies. Poplars usually have two sets of 19 chromosomes ($2n = 38$) in the nucleus. However, triploid or tetraploid plants have occasionally been found (Smith 1943; Mock et al. 2012), and polyploidy can be artificially induced by crossbreeding

(Bradshaw and Stettler 1993), colchicine treatment (Cai and Kang 2011), and high temperatures (Wang et al. 2012a). The segregation of many restriction fragment length polymorphism (RFLP), sequence-tagged site (STS), and random amplified polymorphic DNA (RAPD) markers was used to develop a genetic map for *P. trichocarpa* × *P. deltoides*, which resulted in the identification of 19 linkage groups (Bradshaw et al. 1994), indicating that the poplar genome consisted of 19 pairs of chromosomes. The genome size of poplars was also estimated to be approximately 1.2 pg (= 2C) in *P. deltoides* and 1.4 pg in *P. trichocarpa* (Bradshaw and Stettler 1993), which were relatively small in plants (Garcia et al. 2014). Recently, the 2C values of *P. trichocarpa* and *P. nigra* were reported to be 1.0 pg and 1.1 pg, respectively (Bennett and Leitch 2011).

The elucidation of the gene function in poplars began with a study on two wound-responsive genes from *P. trichocarpa* × *P. deltoides* in the late 1980s (Parsons et al. 1989). Since then, many genes involved in wood and cell wall formation, biotic and abiotic stress responses, flowering, metabolism, and transcription have been isolated from various poplars using protein sequences or homologous genes from other species of plants, animals, and microorganisms. Analysis of the isolated genes was accelerated by the development of genetic engineering for *Populus* species. Furthermore, progress in automatic DNA sequencing made the high-speed reading of many genes possible. For instance, the cDNA from the cambium of *P. tremula* × *P. tremuloides* and from the xylem of *P. trichocarpa* were analyzed, resulting in 5,692 expressed sequence tags (ESTs) being obtained (Sterky et al. 1998). Additional ESTs were then obtained to investigate leaf senescence (Bhalerao et al. 2003), the root system (Kohler et al. 2003), organs and tissues (Déjardin et al. 2004; Sterky et al. 2004), wound defense

(Christopher et al. 2004), and stress tolerance (Brosché et al. 2005). The full-length cDNA library was utilized not only for the collection of ESTs but also for the annotation of genes (Nanjo et al. 2004; Ralph et al. 2006; Nanjo et al. 2007; Ralph et al. 2008). The number of poplar ESTs registered in public databases has continued to increase, with the number of EST sequences in the National Center for Biotechnology Information (NCBI) increasing from 376,565 in August 2006 (Jansson and Douglas 2007) to 422,517 in August 2015.

In 2006, the genome of *P. trichocarpa* was revealed for the first time in woody plants (Tuskan et al. 2006). This was the third analysis of a genome in Spermatophyta, continuing on from work on *A. thaliana* (Arabidopsis Genome Initiative 2000) and rice (*Oryza sativa*) (Goff et al. 2002; Yu et al. 2002). The genome size of *P. trichocarpa* was initially estimated to be approximately 485 Mbp, and 45,555 protein-coding loci were identified. In addition, the chloroplast genome was estimated to be 157 kbp in length, including 101 genes, and the mitochondrial genome was estimated to be 803 kbp including 52 genes. The accuracy of genome sequencing and gene annotation have been gradually improved, and the latest version of the *P. trichocarpa* genome is open to the public through the Phytozome project (http://phytozome.jgi.doe.g.,ov/pz/portal.html). The assembled genome is approximately 423 Mbp, and 41,335 protein-coding loci and 73,013 protein-coding RNAs, including splicing variants, have been identified. The genome sequence of *P. euphratica* was also recently reported (Ma et al. 2013) and 34,279 protein-coding genes were predicted in this genome. Approximately 94% of these could be annotated using public databases.

The genome sequencing of *P. trichocarpa* enabled genome-wide comparative analyses to be conducted. Computational analyses and transcriptomics have been

vigorously conducted for the following specific gene families in *P. trichocarpa*, *A. thaliana*, *O. sativa*, as well as other plant species: protease family (García-Lorenzo et al. 2006), major intrinsic proteins including aquaporins (Gupta and Sankararamakrishnan 2009), CCCH zinc finger proteins (Chai et al. 2012), homeodomain-leucine zipper proteins (Hu et al. 2012), late embryogenesis abundant (LEA) proteins (Lan et al. 2013), leucine-rich repeat receptor-like protein kinases (Zan et al. 2013), WRKY transcription factors (Jiang et al. 2014a), and C2H2 zinc-finger transcription factors (Liu et al. 2015).

The genomics of poplars is progressing together with other areas in the omics field of study, including transcriptomics, proteomics, and metabolomics. It is expected that the knowledge obtained from these studies will help provide us with a better understanding of the biology of poplars, which can then be applied to their breeding, conservation of their biological diversity, and the development of new industrial applications. Two commendable books have recently been published on the genomics of poplars (Jansson et al. 2010; Joshi et al. 2011).

1.9 Genetic engineering

Poplar was the first woody plant to be successfully genetically transformed. Stems of the hybrid *P. trichocarpa* × *P. deltoides* that had been infected with *Agrobacterium tumefaciens* were demonstrated to form tumors producing *Agrobacterium* strain-specific opines and containing T-DNA sequences (Parsons et al. 1986). Over the next year, transgenic shoots were successfully regenerated using *Agrobacterium rhizogenes* in *P. trichocarpa* × *P. deltoides* (Pythoud et al. 1987) and *A. tumefaciens* in *P. alba* × *P. grandidentata* (Fillatti et al. 1987). Since then, *Agrobacterium*-mediated transformation has been applied to and advanced in other

Populus species and hybrids, including *P. nigra* (Confalonieri et al. 1994), *P. tomentosa* (*P. alba* × *P. adenopoda*) (Wang et al. 1990), and *P. tremula* × *P. tremuloides* (Nilsson et al. 1992). The neomycin phosphotransferase II (*NPTII*) gene and kanamycin were initially used as the selectable marker and antibiotic, respectively, for the selection of transgenic poplar plants. Subsequently, several combinations of the phosphinothricin acetyltransferase (*bar*) gene and phosphinothricin (glufosinate) (De Block 1990), the hygromycin phosphotransferase (*HPT*) gene and hygromycin (Nilsson et al. 1992), and the mutant acetolactate synthase (*crsl-1*) gene and chlorsulfuron (Brasileiro et al. 1992) have also been utilized. Furthermore, marker-free transgenic *P. sieboldii* × *P. grandidentata* were generated using the isopentenyl transferase (*ipt*) gene and the *Ac* transposable element (Ebinuma et al. 1997).

A direct gene transfer method that does not use *Agrobacterium* has also been studied. Electric discharge particle acceleration was used to introduce a Cry toxin from *Bacillus thuringiensis* into a hybrid poplar (*P. alba* × *P. grandidentata*), conferring pest resistance on the transgenic plants (McCown et al. 1991). Microprojectile DNA delivery by helium gas was also used to transform suspension-cultured cells of *P. nigra* × *P. maximowiczii* (Devantier et al. 1993). Electroporation of the protoplast of *P. tremula* × *P. alba* produced stable transgenic trees (Chupeau et al. 1994), and was also used for the transient expression of a foreign gene in *P. alba* protoplasts (Qiao et al. 1997). The microprojectile DNA delivery system was also used for the transformation of *P. alba* plastids, enabling the spectinomycin resistance gene and green fluorescent protein (*GFP*) gene to be integrated into the plastid genome (Okumura et al. 2006). However, these direct gene transfer systems are no longer greatly used due to the development of the *Agrobacterium*-mediated transformation system.

There are two main purposes of genetic engineering in poplar: to elucidate the function of the genes that are involved in various biological processes and to develop new, improved trees with excellent properties. Gene-specific overexpression (sense) and suppression (antisense and RNAi) techniques are often used to investigate gene functions in poplar. The modification of one particular gene sometimes confers multiple new traits, and in some cases, the genes from other plant species, animals or microorganisms are exploited in the development of useful transgenic poplars with new traits. Many types of transgenic poplar plants have been generated till date, information about which can be obtained from a number of comprehensive reviews (Busov et al. 2005; Ye et al. 2011; Dubouzet et al. 2013; Polle et al. 2013) and books (Klopfenstein et al. 1997; Fladung and Ewald 2006).

Wood formation is a typical property of woody plants, and therefore, the modification of wood properties is one of the main topics of interest for genetic engineering. Wood consists of cellulose, hemicellulose, and lignin, the synthesis and degradation of which are regulated by a number of enzymes. The pulp and paper industries desire a high cellulose content and low hemicellulose and lignin contents. One study showed that the downregulation of 4-coumarate:CoA ligase in *P. tremuloides* not only led to a 45% reduction in lignin but also a 9–15% increase in the cellulose content and enhanced growth (Hu et al. 1999). However, other studies reported that the reduction of 4-coumarate:CoA ligase by another distinct promoter did not lead to growth enhancement in transgenic poplars (Li et al. 2003; Voelker et al. 2010). When the *Arabidopsis* ferulate 5-hydroxylase (*F5H*) gene was introduced into *P. tremula* × *P. alba*, the resulting transgenic poplars showed a remarkable reduction in guaiacyl lignin (Franke et al. 2000). It has also been shown that transgenic *P. alba* expressing *A.*

aculeatus xyloglucanase contained 10% more cellulose than the wild type (Park et al. 2004) and that the overexpression of cotton (*Gossypium hirsutum*) sucrose synthase in *P. alba* × *P. grandidentata* increased the cellulose content by 2–6% (Coleman et al. 2009).

It is convenient to be able to artificially control morphogenesis and flowering in trees for forestry. For example, an increased number of branches is desirable to increase the leaf area and thus biomass production. It has been shown that *P. tremula* × *P. alba* that overexpress the sweet chestnut (*Castanea sativa*) *RAV1* gene exhibit the early formation of sylleptic branches (Moreno-Cortés et al. 2012). Some flowering-regulated genes have also been found in *A. thaliana*, mediating the analysis of many flowering mutants, and homologs of these flowering genes exist in poplars. The constitutive expression of the *Arabidopsis LEAFY* (*LFY*) gene in *P. tremula* × *P. tremuloides* produced solitary lateral and terminal flowers on 5-month-old shoots (Weigel and Nilsson 1995), and male *P. trichocarpa* that overexpressed *PtFT1* (*P. trichocarpa FLOWERING LOCUS T* homolog) produced normal flowers (inflorescences) with normal pollen within a year (Böhlenius et al. 2006). However, the overexpression of *PTLF* (*P. trichocarpa LFY* homolog) in *P. tremula* × *P. tremuloides* was not particularly successful in promoting early flowering (Rottmann et al. 2000). Recently, an early flowering male *P. tremula* that had been transformed with the *Arabidopsis FT* (*AtFT*) gene was successfully crossed with a wild type female *P. tremula*, and approximately half of the F1 seedlings possessed *AtFT* (Hoenicka et al. 2014). This demonstrates that the juvenile phase of poplar can be shortened through genetic engineering and that the introduced foreign gene can be eliminated by crossing.

Abiotic and biotic stresses reduce the biomass of trees and, in extreme cases, may destroy the forest. Therefore, trees that are tolerant to a range of stresses have been

bred, and genetic engineering provided the opportunity for the rapid breeding of such stress-tolerant trees. For example, the overexpression of a *TaLEA* gene from *Tamarix androssowii* in *P. simonii* × *P. nigra* enhanced salt and drought tolerance (Gao et al. 2013); and *Populus davidiana* (*P. tremula* var. *davidiana*) × *Populus bolleana* (*P. alba* var. *pyramidalis*) that overexpressed the type I proton pyrophosphatase gene produced 18–27% more fresh shoot mass than the wild type in the presence of 150 mM NaCl and showed a superior survival rate after the salt treatment (Yang et al. 2015). Transgenic *P. tomentosa* with introduced *Arabidopsis* homeodomain-START transcription factor, *AtEDT1*/*HDG11* (*ENHANCED DROUGHT TOLERANCE1/HOMEODOMAIN GLABROUS11*), also exhibited significantly enhanced salt and drought stress tolerance (Yu et al. 2015). In addition, a synthetic antimicrobial peptide, D4E1, was expressed in *P. tremula* × *P. alba*, conferring resistance to two bacterial pathogens, *A. tumefaciens* and *Xanthomonas populi* (Mentag et al. 2003).

Transgenic poplars that are tolerant to organic pollutants or heavy metals are believed to be more useful for phytoremediation. The hybrid *P. tremula* × *P. alba* with overexpressed γ-glutamylcysteine synthetase from *Escherichia coli* produced higher levels of glutathione, exhibited enhanced tolerance to chloroacetanilide herbicides, and accumulated more Cd in the root tissue than wild type plants (Gullner et al. 2001; Koprivova et al. 2002). In addition, transgenic *P. deltoides* expressing a modified mercuric ion reductase gene (*merA9* or *merA18*) were able to grow in the soil containing 400 ppm Hg^{2+}, whereas the wild-type shoots died (Che et al. 2003); and *P. deltoides* that expressed both *merA9* and *merA18* were able to detoxify phenylmercury acetate (Lyyra et al. 2007). Furthermore, yeast cadmium factor 1 from *Saccharomyces cerevisiae* conferred tolerance to high levels of As, Zn, Pb, and Cd in *P. alba* × *P.*

tremula, and the transgenic poplars had increased levels of Cd in their upper shoots and Cd, Zn, and Pb in their roots (Shim et al. 2013).

Genetic transformation has been successfully used to produce systematically tagged lines of poplars, similar to those produced for *A. thaliana*. Using the CaMV 35S enhancer, 627 activation-tagged transgenic *P. tremula* × *P. alba* were generated, with nine lines exhibiting an obvious morphological change. Among these nine lines, a dwarf mutant was discovered in which the endogenous GA2-oxidase had been activated (Busov et al. 2003). Several phenotypic variants were also obtained from the transposon and T-DNA tagged lines of *P. tremula* and *P. tremula* × *P. tremuloides*, and the tagged sites were sequenced (Fladung et al. 2004). To determine which genes were involved in the vascular system, 708 gene trap and 674 enhancer trap tagged trees of *P. tremula* × *P. alba* were established, and the expression of β-glucuronidase (GUS) was detected in 55 and 455 lines, respectively (Groover et al. 2004). A total of 12,083 activation-tagged trees of *P. tremula* × *P. tremuloides* have also been generated using the activation tagging Ds system with a heat-inducible *Ac*-transposase, which resulted in various types of phenotypic variation, including growth deficiency, chlorophyll abnormalities, and alterations in leaf form and shape (Fladung and Polak 2012). These tagged poplars are useful for clarifying the relationship between mutant phenotypes and the functions of unknown genes.

When transforming poplars, various factors need to be considered: suitable poplar clones or varieties; the type of initial explant (leaves, shoots or calli) for transformation; a plant regeneration system that is highly efficient and fast; a convenient vector for DNA manipulation; a method for selecting transgenic poplars; the selection of introduced genes controlling the target traits; and a regulation system for gene

expression, such as a promoter and enhancer. There is currently no common solution for each of these; therefore, further development is required to help address these issues and to contribute to the utilization of poplar trees. Attention also needs to be paid to the inherent problems of transgenic poplars as living modified organisms (LMOs).

1.10 *Populus nigra*

Populus nigra (black poplar) is one of the most important poplar species, having a wide range of applications. This species has a large distribution area throughout Europe, and is also found in North Africa and Central and West Asia (Vanden Broeck 2003). It is used for wood production, windbreaks, and landscaping all over the world. It has also often been used as a parent species for crossbreeding because of its excellent characteristics, such as wide adaptability to many environments, ease of rooting stem cuttings, and resistance to pests and pathogens (Cagelli and Lefèvre 1995), and the hybrid clones of *P. nigra* are planted widely worldwide. Native *P. nigra* is a typical pioneer tree that occurs in riparian areas. However, as described in section 1.6, native *P. nigra* forests are at a risk of extinction in Europe due to the reduction in riparian habitats and intercrossing with hybrid poplars. Therefore, measures to conserve native *P. nigra* have been taken by the EUFORGEN *Populus nigra* Network and individual country (Lefèvre et al. 2001; Koskela et al. 2004). Cryopreservation of the seeds has also been attempted to preserve the intraspecific diversity of this species (Suszka et al. 2014; Michalak et al. 2015).

Populus nigra was first imported to Japan in the middle Meiji era. Following World War II, *P. nigra* and improved hybrid poplars were imported once again, and planting tests were conducted. Poplar trees are familiar to Japanese people and are often

admired in streets and parks, including the well-known rows of *P. nigra* trees in Hokkaido.

Similar to other poplar species, *P. nigra* is considered to be a suitable model species for woody plants (Fig. 2). This species can be grown quickly and is also easily propagated from stem cuttings, allowing the plantlets of *P. nigra* to be used as homogenous experimental materials. Furthermore, sterile cell- and tissue-culturing systems are available, and *in vitro* organogenesis, such as shoot regeneration and rooting, can be controlled. There are also rich genomic resources on *P. nigra*. For example, a total of 54,152 genes and ESTs of *P. nigra* were registered in the DNA Data Bank of Japan (DDBJ) as at December 2015. Studies on *P. nigra* have progressed around the world, including in Japan (Mohri et al. 1996; Mohri et al. 1999; Kato et al. 2001; Nishiguchi et al. 2002; Nanjo et al. 2004; Nanjo et al. 2007; Igasaki et al. 2008; Mao et al. 2010).

Populus nigra can be transformed using an *Agrobacterium*-mediated method (Confalonieri et al. 1994; Mohri et al. 1996). In this method, *A. tumefaciens* harboring a binary vector that includes the intended target genes and selection marker genes is inoculated into poplar explants, such as leaf discs or stem segments. The *Agrobacterium*-infected explants are then cultured for several days to transfer a T-DNA region from the *Agrobacterium* into the poplar cells. Subsequently, the explants are transferred into a selection medium containing antibiotics that will kill non-transgenic cells. The explants are then subcultured for several weeks, during which time they generate transgenic calli or shoots (the transgenic calli are able to regenerate into transgenic shoots at a later date). Finally, regenerated transgenic shoots are excised and transferred into a root-induction medium (RIM).

Various transgenic trees of *P. nigra* have been generated, including those that contain the *GUS* gene (Confalonieri et al. 1994; Confalonieri et al. 1995; Mohri et al. 1996), a *B. thuringiensis* toxin gene that confers insect resistance (Wang et al. 1996), a soybean (*Glycine max*) Kunitz trypsin proteinase inhibitor gene (Confalonieri et al. 1998), and a rice homeodomain protein (*OSH1*) gene (Mohri et al. 1999). Hybrid clones from *P. nigra* have also been utilized for transformation (Han et al. 2000; Davis et al. 2006; Yevtushenko and Misra 2010; Gao et al. 2013).

As outlined in section 1.7, environmental stress often severely impacts the survival and growth of poplars. Various stress physiological studies have been conducted for *P. nigra* till date. For example, it was found that the expression of a lectin-like receptor protein kinase (*PnLPK*) gene from *P. nigra* was increased by wounding young leaves, suggesting that PnLPK is involved in the plant response to wounding (Nishiguchi et al. 2002). It was also shown that water limitation reduced the growth and photosynthetic rate of *P. nigra* and activated starch-degrading enzymes and peroxidase depending on the genotype (Regier et al. 2009). High temperatures have been linked to reduced photosynthesis, which is affected by water stress (Centritto et al. 2011); and treatment with high levels of nickel also resulted in reduced photosynthesis (Velikova et al. 2011). To elucidate the response mechanism to environmental stress, full-length cDNA libraries were constructed from various stress-treated *P. nigra* and approximately 20,000 ESTs were analyzed (Nanjo et al. 2004; Nanjo et al. 2007). In addition, the expression of two transcription factors, *PnDREB68* and *PnDREB69*, was elevated by cold, salinity, and osmotic stress in the leaves and stems of *P. nigra* (Chu et al. 2014).

1.11 Objectives

Many studies have been performed on poplars to date. Therefore, the question is what should be studied next to better understand the science of poplars as woody plant model species? One option is to use poplars to pioneer new fields of research on woody plants. A wide range of research topics have already been studied on woody plants, but unresolved issues and untouched areas of research remain. Therefore, such topics could be addressed using poplars, with their advanced technologies and research resources, and then applied to other woody plants. Another option is to further develop the technologies for poplars, including cultivation, biotechnology, genetic engineering, and other biological techniques, to support the research and application of poplars as model species. In this thesis, both of these options are explored by investigating the biological response of *P. nigra* to ionizing radiation as an abiotic stress and by improving the transformation system for this species (Fig. 3).

The effects of ionizing radiation on woody plants, including poplars, and their tolerance mechanisms for this remain to be understood in detail. Ionizing radiation is considered to be an inescapable abiotic stress. Almost all living organisms on Earth are exposed to natural ionizing radiation, such as cosmic radiation, terrestrial radiation, and internal radiation. The leaves of trees are estimated to generally be exposed to 0.6–7 mGy per year of natural radiation (UNSCEAR 1996) (Fig. 4). Because most woody plants are perennial and long lived, they are exposed to natural radiation over a long period of time. Therefore, it is assumed that woody plants must possess resistance mechanisms against ionizing radiation to avoid its harmful effects. In the 1960s and 1970s, the radiosensitivity of herbaceous and woody plants including poplar species were investigated (Scandalios 1964; Ohba and Murai 1966; Sparrow et al. 1968).

However, since then, the basic radiation biology of woody plants has not been advanced, with the exception of radiation breeding of fruit trees. Following the Chernobyl accident in 1986, the effects of ionizing radiation on local forest trees such as Scots pine (*Pinus sylvestris*) were studied with respect to the protection of the environment from ionizing radiation. However, the response and tolerance mechanisms of poplars to ionizing radiation have not been studied.

We currently have some scientific knowledge about the effects of environmental stresses on poplars and also have many genomic resources. Both of these will be very useful for analyzing the molecular mechanism of the response of poplars to exposure to ionizing radiation. In addition, the genetic engineering techniques that have been developed for poplars can help to clarify the functions of genes that are involved in radiation stress. Elucidation of the response and tolerance mechanisms of poplars as model trees will be very valuable for understanding radiation stress in woody plants. Therefore, in this thesis, the various biological effects of γ-irradiation on *P. nigra* were examined, and the DNA-repair related genes were newly isolated and characterized (Fig. 3). The obtained results may be used for the radiological protection of woody plants, environmental application, mutation breeding, or the generation of stress-tolerant trees.

Genetic engineering is potentially a valuable tool for the elucidation of stress tolerance mechanisms. Transformation systems for *P. nigra* have been reported previously (Confalonieri et al. 1994; Confalonieri et al. 1995; Mohri et al. 1996), but these have some practical issues (Fig. 5). Firstly, the transformation efficiency of *P. nigra* is not sufficiently high, making it difficult to produce a large number of transgenic poplar plants. Secondly, the previous transgenic system, which uses callus regeneration, takes a long time, because callus-, shoot-, and root-induction require 1–2 months each,

meaning that it takes more than 6 months for the generation of transgenic *P. nigra*. A more convenient binary vector is also desirable for the transformation of plant species including poplars, which would have characteristics such as many cloning sites that are recognized by restriction enzymes; selection marker genes that are suitable for plant species and have high selectivity; timely and spatially regulated promoters; and stability in *E. coli* and *Agrobacterium*. Therefore, one of the goals of this thesis was to improve the transformation system for *P. nigra*, to give a higher transformation efficiency and a shorter regeneration time and to simultaneously design and construct a new binary vector for transformation (Fig. 3). It was considered that the improved transformation system and the advanced new vector would contribute to the development of genetic engineering of not only *P. nigra* but also other poplar and plant species.

As mentioned in section 1.10, *P. nigra* is considered to be one of the most suitable poplar species for use as an experimental model of woody plants. Therefore, the information obtained from studies on *P. nigra* can be generalized to other species of poplars or plants using comparative studies. Furthermore, although it may be difficult to directly apply a specific technology such as transformation to other plant species, the information that is obtained may be valuable as a reference for similar studies.

Chapter 2 Elucidation of biological response to ionizing radiation in *Populus nigra*

2.1 Introduction

Ionizing radiation has been shown to deleteriously affect the growth and reproduction of herbaceous and woody plants. Early studies made detailed examinations of the radiosensitivity of various herbaceous and woody plants to gamma (γ) rays (Sparrow and Sparrow 1965; Capella and Conger 1967; Sparrow et al. 1968; Sparrow et al. 1970). These studies showed that woody gymnosperms are generally more sensitive to ionizing radiation than woody angiosperms and that the radiosensitivity of plant species is affected by the interphase chromosome volume. Following the Chernobyl Nuclear Power Plant accident in 1986, many important studies on the harmful effects of ionizing radiation and fallout on forest trees were performed (Arkhipov et al. 1994; Kaľchenko and Fedotov 2001; Kovalchuk et al. 2003; Tulik and Rusin 2005). Conversely, ionizing radiation has also been used as a tool for plant breeding. A total of 2,252 mutant varieties including woody plant species were recorded in the FAO/IAEA Mutant Varieties Database by the end of 2000, with 1,411 of these having been obtained using ionizing radiation as the mutagen (Maluszynski et al. 2000).

Ionizing radiation is considered an environmental stress alongside light, temperature, and water, with X- and γ-rays having been reported to cause DNA damage in plants such as *Vicia faba* (Koppen and Angelis 1998) and *Nicotiana tabacum* (Gichner et al. 2000). Ionizing radiation not only arises from human activities but also exists as natural background radiation, including cosmic and terrestrial radiation.

Almost all terrestrial plants are continuously exposed to natural background radiation during their growth and dormant periods. Although this natural background radiation is usually negligible, it is sufficient to ionize water and biological molecules such as DNA and proteins in cells; and if the free radicals and abnormal molecules that are generated by ionization increase and accumulate in such cells, they may be damaged and die. Perennial woody plants are particularly likely to be subjected to natural background radiation for an extended period due to their longevity and consequently, are likely to have developed adaptation and tolerance mechanisms to enable them to survive.

Versatile DNA repair systems have been reported as one of the mechanisms of radiation resistance. A wide range of DNA repair-related proteins for the detection and elimination of DNA damage and for DNA synthesis are believed to play an important role in maintaining genome stability. Consequently, activation of the genes that encode each of these proteins is considered to be a key event in the mechanism for tolerating ionizing radiation. The genes that encode DNA repair-related proteins have been isolated from plants such as *A. thaliana*. (Bleuyard et al. 2006), rice (Kimura and Sakaguchi 2006), and moss (*Physcomitrella patens*) (Ayora et al. 2002), and it has been shown that the knockout of these DNA repair-related genes partially reduces the tolerance of the knockout plants to γ-irradiation (Riha et al. 2002; Osakabe et al. 2005). However, to the best of our knowledge, DNA repair-related proteins in woody plants have not been studied in detail.

In the present study, *P. nigra* was acutely exposed to γ-rays to examine the adaptation and tolerance mechanisms to ionizing radiation in woody plants. *Populus nigra* is considered a good model for woody plant biology because of its rapid growth, the ease of clonal propagation, the ability to use *in vitro* cultured-plants and cells, the

advanced level of genetic engineering, and the abundance of molecular biological information about this species, as outlined in Chapter 1. Although various stress responses of this species have been elucidated, there has been very little study on the biological effects of ionizing radiation on *P. nigra* (Scandalios 1964) or other species of poplar (Ohba and Murai 1966; Stettler and Guries 1976). In this study, it was found that exposure of *P. nigra* to various doses of acute γ-irradiation caused morphological changes, delayed growth, withering, and nuclear DNA breaks, and that γ-rays or a DNA cleavage agent affected the gene expression of several DNA repair-related proteins, the cDNAs of which were newly isolated from this species.

2.2 Materials and methods

2.2.1 Plant materials

Young branches of *P. nigra* that were approximately 10 cm in length were excised and rooted in moist vermiculite. The rooted branches were then grown in a growth chamber (phytotron) at 25°C and 70% relative humidity under metal halide lamps (500 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), 16:8 h light:dark photoperiod). An aseptic tissue culture of the *P. nigra* plants was also maintained in RIM containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (pH 5.8), 0.5 × Murashige and Skoog Basal Salts (MSBS) (Murashige and Skoog 1962), 1 × Murashige and Skoog vitamin (MSV), 3% sucrose, 0.5 mg l^{-1} indole-3-butyric acid, 0.02 mg l^{-1} 1-naphthaleneacetic acid, and 0.6% (w/v) Phytagar (Invitrogen, Carlsbad, CA, USA).

2.2.2 Gamma irradiation

Poplar plantlets that were approximately 15 cm in height (6 weeks after excision) were used for the γ-irradiation experiments. The plantlets were transplanted into a Wagner pot and concomitantly exposed to γ-rays at doses of 0 (control), 10, 20, 50, 100, 200, and 300 Gy (dose rates ranged from 0.5 to 15 Gy h^{-1}) for 20 h in the cobalt-60 (^{60}Co) gamma room at the Institute of Radiation Breeding (Hitachiohmiya, Japan). Following γ-exposure, the irradiated plants were returned to the phytotron. For RNA isolation, shoots were harvested from each plantlet 1, 6, and 24 h after the end of γ-irradiation and frozen in liquid nitrogen. The other irradiated plants were allowed to continue to grow in the phytotron to observe and measure their growth. Each γ-irradiation treatment was replicated twice.

For *in vitro* organogenesis, the stems, petioles, and shoots of the tissue-cultured poplar plants were excised aseptically and exposed to γ-rays. The γ-irradiated stems and petioles were then transferred onto a new shoot-induction medium (SIM) containing 20 mM MES-KOH (pH 5.8), 1 × MSBS, 1 × MSV, 3% sucrose, 0.5 mg l^{-1} *trans*-zeatin, 0.1 mg l^{-1} 6-benzylaminopurine (BAP), and 0.8% (w/v) Bacto™ Agar (BD Diagnostic Systems, Sparks, MD, USA). The γ-irradiated-shoots were planted in a new RIM for the regeneration of roots. They were cultured at 25°C under cool white fluorescent light.

2.2.3 Measurement of growth

The plant height and stem diameter at ground level of the control (non-irradiated) and γ-irradiated poplar plants were measured immediately after irradiation and at weekly intervals for 10 weeks. The dry mass of the roots, stems, and leaves was also measured by harvesting these parts 10 weeks after γ-irradiation or when

the whole plant had turned brown and drying each sample at 60°C to a constant weight.

2.2.4 Treatment of suspension-cultured poplar cells with γ-rays and a DNA cleavage agent

Suspension-cultured poplar cells were derived from sterile calli (Nishiguchi et al. 2002) of *P. nigra* in a liquid medium containing 20 mM MES-KOH (pH 5.8), 1 × MSBS, 1 × MSV, 3% (w/v) sucrose, and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The suspension-cultured cells were cultivated in 100-ml Erlenmeyer flasks on orbital shakers (120 rpm) in the dark, and 1 ml of the cell suspension was subcultured every 14 days in 25 ml of the same fresh medium. For the γ-irradiation experiments, the cells were transferred into a thin-walled plastic flask 7 days after subculturing and exposed to γ-rays for 20 h. The irradiated cells were collected 1 h after the end of γ-irradiation and cooled on ice for a Comet assay. To treat the cells with a DNA cleavage agent, Zeocin™ (Invitrogen) was added to the medium at a concentration of 10, 50, or 250 μg ml^{-1} 7 days after subculturing. The cells were collected after being cultivated with Zeocin for 1, 6, and 24 h, and were either cooled on ice for the Comet assay or frozen in liquid nitrogen for RNA preparation.

2.2.5 Comet assay

Nuclei were isolated from the suspension-cultured poplar cells using a previously reported method with modifications (Ptáček et al. 2001). The cells were harvested using a cell strainer with 40-μm pores (BD Falcon, Bedford, MA, USA), and sliced with a razor blade in 50 mM sodium phosphate buffer (pH 6.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% (v/v) dimethyl sulfoxide in a Petri

dish kept on ice. The released nuclei were separated from the cell debris using a cell strainer. A Comet assay was performed using a CometAssay Kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Following alkaline electrophoresis, the nuclei were stained with SYBR® Green I and observed by fluorescence microscopy. Images of the nuclear DNA were taken with a digital camera and analyzed using the CASP Program (Comet Assay Software Project, http://casp.sourceforge.net/) for semi-quantification of the DNA damage based on the amount of DNA in the tail.

2.2.6 RNA preparation

Total RNA was prepared from each frozen sample as described by Shinohara and Murakami (1996), with the following modifications. The samples were ground in liquid nitrogen and subsequently mixed with 10 volumes (v/w) of a lysis solution of 100 mM Tris-HCl (pH 9.5), 20 mM EDTA, 1.4 M NaCl, 2% (w/v) hexadecyltrimethylammonium bromide, and 2% (v/v) 2-mercaptoethanol. This mixture was then heated at 65°C for 10 min. Following extraction with chloroform:isoamyl alcohol (24:1) and centrifugation, the supernatant was mixed with one-quarter of the volume of ice-cold 10 M LiCl and stored at −20°C for 2 h or overnight. The precipitated RNA was recovered by centrifugation and dissolved in an SV RNA lysis buffer from the SV Total RNA Isolation System (Promega, Madison, WI, USA). The RNA was then further purified to remove contaminated DNA and polysaccharides according to the manufacturer's instructions. Quantification of RNA was performed using a Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen).

2.2.7 cDNA cloning of DNA repair-related genes

PnRAD51, *PnKU70*, and *PnLIG4* cDNAs were cloned by reverse transcription PCR (RT-PCR) using the total RNA from the apical buds of *P. nigra*. Reverse transcription was performed using Transcriptor Reverse Transcriptase (Roche Applied Science, Penzberg, Germany) and oligo$(dT)_{18}$ primers. The genome sequence of *P. trichocarpa* (Tuskan et al. 2006) was used to design specific primer sets for PCR amplification of each target cDNA (Table 6). PCR was performed with the first-strand cDNA using KOD Plus DNA Polymerase (Toyobo, Osaka, Japan). The amplified DNA fragments were then cloned into pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) and sequenced. The 5′ and 3′-end sequences of each cDNA were obtained using a 5′/3′ RACE Kit (Roche Applied Science). *PnXRCC4* and *PnOGG1* cDNAs were identified by screening the full-length cDNA library of *P. nigra* (Nanjo et al. 2007) using the BLAST program. *PnPCNA* cDNA was obtained from our previous cDNA library from poplar buds (Nishiguchi et al. 2002).

The DNA sequences were analyzed using GENETYX (Genetyx, Tokyo, Japan), EMBOSS (Rice et al. 2000), and InterProScan (Quevillon et al. 2005). Multiple alignments of protein sequences were conducted using MAFFT (Katoh and Toh 2008). The cloned DNA sequences were submitted to DDBJ, and their accession numbers are shown in Table 7.

2.2.8 Semi-quantitative low-cycle RT-PCR and Southern blot assay

RT-PCR was performed with 1 μg RNA and gene-specific primers (Table 6), using the AccessQuick™ RT-PCR System (Promega) and 13 PCR cycles, to avoid reaching the plateau phase. The amplified DNA (which was not visible on ethidium

bromide-stained gel) was separated by 1% agarose gel electrophoresis and transferred onto a positively charged nylon membrane. Gene-specific DIG-labeled probes were synthesized with the same primers that were used for each RT-PCR from each cDNA using a PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridization and chemiluminescent detection were performed using CDP-Star® according to the manufacturer's instructions.

2.2.9 Reverse transcription quantitative real time PCR (RT-qPCR)

First-strand cDNA was synthesized from 2 µg of total RNA using a AffinityScript QPCR cDNA Synthesis Kit (Stratagene), and a mixture of oligo(dT) and random primers according to the manufacturer's instructions. RT-qPCR was performed using the Mx3000P Real-Time PCR System (Stratagene) and the Brilliant II SYBR Green QPCR Master Mix (Stratagene). The sequences of the primers used for RT-qPCR are shown in Table 6. The RT-qPCR reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 30 s. Three biological replicates were conducted. The specificity of each amplified DNA fragment was checked with a heat dissociation curve (55–95°C), and by agarose gel electrophoresis and DNA sequencing. The relative mRNA level was calculated by the standard curve method using serial dilution of the cDNA and was normalized to the mRNA level of the poplar ubiquitin gene homolog (GenBank ID: DB883027 and DB901131) of the *Arabidopsis UBQ5* gene (TAIR ID: AT3G62250).

2.3 Results

2.3.1 Morphological change in the γ-irradiated poplars

Populus nigra plantlets were first irradiated with a dose of 10 or 20 Gy of ^{60}Co γ-rays for 20 h. However, this had no visible effects on tree growth. Therefore, the poplar plantlets were subsequently exposed to 50–300 Gy of γ-rays, which led to changes in their growth and development (Fig. 6).

The growth of poplar plantlets was markedly suppressed by high doses of γ-irradiation (Figs. 6 and 7). There were no significant differences in height, stem diameter, or biomass between the non-irradiated control poplars and the 50 Gy γ-irradiated poplars. However, the 100 Gy γ-irradiated poplars fell into two groups. In the first group (shown as 100-A in Fig. 7), tree growth was arrested for approximately 3 weeks after γ-irradiation, following which the apical bud and axillary buds slowly developed, and new leaves were formed. Consequently, these plants had lower plant heights, stem diameters, and biomasses than the control trees 10 weeks after irradiation. In the second group (shown as 100-B in Fig. 7), tree growth was completely aborted but the leaves remained green for 10 weeks after γ-irradiation. None of the trees in this group died; however, because new shoots sprouted from the underground stems of all plants 4–10 weeks after γ-irradiation. All plantlets that received doses of 200 or 300 Gy of γ-rays stopped growing, and 87.5% (7/8) and 100% of these, respectively, turned brown and withered 4–10 weeks after γ-irradiation.

Morphological changes were also detected in the leaves of some of the poplar plants that were irradiated with 50 or 100 Gy of γ-rays, including the production of oblanceolate, lobed, or cleft leaves (Fig. 8B), whereas non-irradiated control plants produced normal deltoid-shaped leaves (Fig. 8A). Moreover, two types of unusual leaves that had a petiole with two leaf blades developed in the γ-irradiated plants. The

first type had adaxial surfaces that were in the same direction (Fig. 8C), while the second had abaxial surfaces that were opposite to each other and partially coalesced (Fig. 8D). Asymmetric and curved leaves with severely bent petioles (Fig. 8E) and a mosaic pale green leaf (Fig. 8F) were also observed.

Morphological changes in organs other than the leaves also occurred. In some cases, the internodes were partly shortened, as if there was an opposite or whorled phyllotaxis (Fig. 8G), whereas *P. nigra* leaves are usually arranged in a spiral pattern. Branching at an internode was also occasionally observed (Fig. 8H), the lower part of which fused and was somewhat fasciated. The time of occurrence of these morphological changes was restricted to around 4–6 weeks after 50 Gy of γ-irradiation, following which normal new leaves and stems developed. In some poplar plants that were irradiated with 100 Gy of γ-rays, apical growth was arrested, resulting in axillary buds developing and elongating (Fig. 8I). No change in plant shape was detected in plants that were irradiated with 200 or 300 Gy of γ-rays because all of these plants stopped growing, and therefore, no new stems and leaves developed (Fig. 6).

2.3.2 Inhibitory effect of γ-rays on organogenesis of the shoots and roots

γ-irradiation affected the growth and morphogenesis of poplar plantlets, as outlined in section 2.3.1. In the next experiments, it was investigated whether γ-rays affected the *in vitro* organogenesis of the shoots or roots. The stems, petioles, and shoots from tissue-cultured poplar plants were aseptically exposed to γ-rays. The formation of new shoots was inhibited in a dose-dependent manner in γ-irradiated stems and petioles (Fig. 9). A total of 200 Gy of γ-rays resulted in the complete arrest of regeneration and elongation of new shoots 4 weeks after irradiation. By contrast, 100 or 200 Gy of γ-rays

suppressed the regeneration of new roots from the shoots (Fig. 10), while new roots were formed following 50 Gy of γ-rays. These results demonstrate that high doses of γ-rays have harmful effects on the organogenesis of shoots and roots in *P. nigra*.

2.3.3 Nuclear DNA damage induced by γ-irradiation

It has previously been reported that ionizing radiation causes DNA damage in some plant species (Koppen and Angelis 1998; Gichner et al. 2000). To investigate whether γ-rays cause nuclear DNA damage in poplar cells, a Comet assay (alkaline single-cell gel electrophoresis assay) was performed, a test that is used to investigate the genotoxicity of physical and chemical factors (Hartmann et al. 2003). The suspension-cultured cells of *P. nigra* were used as the experimental materials for the Comet assay due to the difficulty in isolating intact nuclei from the leaves of poplar plantlets. DNA damage was evaluated based on the percentage of DNA in the tail (%T) as compared with the total DNA in the head and the tail.

The Comet assay showed that the nuclear DNA in the suspension-cultured poplar cells was broken by γ-irradiation (Fig. 11A). Approximately 36% of the total nuclei examined in the non-irradiated control cells were within the range of 0–20%T, indicating that these nuclei were undamaged (Fig. 11B and Table 8). Similarly, in the 50 Gy γ-irradiated poplar cells, approximately 49% of the nuclei ranged from 0 to 20%T. However, only 6.6% of the 100 Gy, 7.7% of the 200 Gy, and 4.9% of the 300 Gy γ-irradiated cells fell within the same range of 0–20%T. When compared with the total nuclei examined, the mean values of %T were 27.2 ± 14.6 for the control group and 24.3 ± 16.4 for the 50 Gy γ-irradiated poplar cells, which were not significantly different (non-parametric Steel-Dwass test, $p = 0.088$). By contrast, the mean values

of %T for the other treatment groups were 43.0 ± 15.7 for 100 Gy, 38.6 ± 14.9 for 200 Gy, and 36.7 ± 12.0 for 300 Gy γ-irradiated cells, with the 100 Gy group being significantly higher than the 50 Gy group (p = 9.7E-07). These results indicate that the nuclear DNA in the poplar cells was severely damaged by exposure to 100–300 Gy of γ-rays.

2.3.4 Cloning and sequence analysis of cDNAs encoding DNA repair-related proteins

There were obvious harmful effects of γ-irradiation on the growth, morphology, and DNA structure of poplar trees, as outlined in sections 2.3.1–2.3.3. Therefore, it was predicted that the γ-irradiated poplar plants would change the pattern of gene expression to protect themselves from the harmful effects of γ-rays. The expression of genes encoding DNA repair-related proteins was particularly focused on because DNA double-strand breaks induced by ionizing radiation are one of the most serious threats to cells and lead to cell death (Kobayashi et al. 2008), and the observed abnormalities in the γ-irradiated poplar plants were likely caused by DNA double-strand breaks induced by the γ-rays (Fig. 11). The repair of DNA damage is mediated by various DNA repair systems in living organisms and is vital for maintaining cellular function and ensuring cell survival (Fleck and Nielsen 2004) (Fig. 12). However, no information concerning the genes for DNA repair-related proteins in woody plants has been reported to date.

To investigate the expression of genes encoding DNA-repair related proteins in the γ-irradiated poplars, six cDNAs from *P. nigra* were newly cloned and sequenced (Table 7 and Fig. 13). The *PnRAD51* cDNA encoded the RAD51-homologous protein, and in the budding yeast (*S. cerevisiae*), the *RAD51* gene is involved in mitotic

recombination and DNA repair, as well as in meiosis (Shinohara et al. 1992). The *PnLIG4*, *PnKU70*, and *PnXRCC4* cDNAs encoded DNA ligase IV, the Ku70 protein, and the XRCC4 protein, respectively, all three of which are involved in the repair of DNA double-strand breaks by mediating non-homologous end-joining (Sancar et al. 2004). The *PnOGG1* cDNA encoded an 8-oxoguanine DNA glycosylase; this enzyme removes 7,8-dihydro-8-oxoguanine (8-oxoG) (Van Der Kemp et al. 1996), which is an oxidative DNA damage and is generated by ionizing radiation (Cadet et al. 2004). The *PnPCNA* cDNA encoded the proliferating cell nuclear antigen (PCNA) protein, which is a member of the DNA sliding clamp family; this protein binds to the DNA and is essential for providing DNA polymerase with high processivity in DNA synthesis (Maga and Hübscher 2003).

The predicted amino acid sequences of the DNA repair-related proteins from *P. nigra* were compared with those from *P. trichocarpa, A. thaliana*, humans, and budding yeast (Table 7 and Figs. 14–19). The degree of homology of each protein varied widely between species. For example, the PnRAD51 protein showed high identity with the RAD51 proteins from *P. trichocarpa* (91.9%), *A. thaliana* (90.4%), humans (68.9%), and the yeast (53.5%), whereas the PnXRCC4 protein showed a much lower degree of identity with the XRCC4 proteins from humans (19.9%) and budding yeast (10.9%) but correlated well with the XRCC4 proteins from *P. trichocarpa* (84.0%) and *A. thaliana* (58.2%). Each DNA repair-related protein was well conserved across the three plant species, however, with 58–99% identity.

2.3.5 Expression of the DNA repair-related genes in *P. nigra*

To explore the expression patterns of the cloned DNA repair-related genes

under normal growth conditions in *P. nigra*, low-cycle RT-PCR followed by Southern blotting was conducted for semi-quantitative evaluation. Each gene exhibited a diverse expression pattern across various organs and cells (Fig. 20). *PnLIG4*, *PnKU70*, *PnXRCC4*, *PnPCNA*, and *PnOGG1* were detected in all of the sampled organs and cells, i.e., the roots, stems, leaves, apical buds, calli, and suspension-cultured cells. The expression levels of *PnRAD51*, *PnKU70*, *PnXRCC4*, and *PnPCNA* were highest in the apical buds, with little to no mRNA of *PnRAD51* in the leaves.

2.3.6 Effect of γ-irradiation on the expression of DNA repair-related genes

To investigate the effect of γ-irradiation on the expression of genes encoding the DNA repair-related proteins, RNA was prepared from non-irradiated and γ-irradiated poplar shoots that included a stem, an apical bud, and three or four leaves. The mRNA levels of each DNA repair-related protein were then quantified using RT-qPCR.

The expression of the *PnKU70* and *PnLIG4* genes was found to have increased markedly 1 h after the shoots had been exposed to γ-irradiation for 20 h (Fig. 21). For example, the expression of *PnKU70* in the 200 Gy γ-irradiated shoots was approximately 48-fold higher than that in the non-irradiated poplars, and the expression of *PnLIG4* was also approximately 26-fold higher in the 200 Gy group. These increased levels of mRNA of *PnKU70* and *PnLIG4* were dependent on an increase in the dose of γ-rays up to 200 Gy. The elevated expression levels of both *PnKU70* and *PnLIG4* were found to have decreased 6 h after the end of γ-irradiation, but even after 24 h, their expression remained significantly higher in the γ-irradiated poplars than in the non-irradiated poplars.

The expression of *PnXRCC4*, *PnPCNA*, and *PnRAD51* was also upregulated by γ-irradiation, although to a lesser extent than that of *PnKU70* and *PnLIG4*. One hour after the end of γ-irradiation, their expression had increased by 1.9–2.9-fold for *PnXRCC4*, 2.1–4.0-fold for *PnPCNA*, and 1.6–2.4-fold for *PnRAD51*, with the increase largely depending on the radiation dose. The expression levels of *PnXRCC4* and *PnPCNA* remained high 24 h after the end of γ-irradiation, whereas that of *PnRAD51* returned to the basal level.

In contrast, the expression of *PnOGG1* decreased to approximately 30% of the non-irradiated control 1 h after the end of γ-irradiation. The downregulation of *PnOGG1* was observed across all doses of γ-rays (50–300 Gy). These reduced expression levels then gradually elevated and had almost returned to the basal level after 24 h.

2.3.7 Effect of treatment with Zeocin on the expression of DNA repair-related genes

To determine whether the change in gene expression caused by γ-irradiation was due to DNA strand breaks, the effect of the DNA cleavage agent Zeocin (phleomycin D1) on the gene expression was examined. Zeocin is a glycopeptide antibiotic of the bleomycin/phleomycin family, which causes single- and double-strand breaks in DNA (Huang et al. 1981). The addition of Zeocin to the medium fragmented the nuclei of suspension-cultured cells of *P. nigra* (Fig. 22A). Thus, it was inferred that Zeocin cleaved nuclear DNA in poplar cells, although the type of DNA strand breaks caused by Zeocin may not be exactly the same as that by γ-rays.

Treatment of the suspension-cultured cells with Zeocin significantly affected the expression of the genes encoding DNA repair-related proteins (Fig. 22B). One hour

after the addition of Zeocin, the expression levels of *PnKU70* and *PnLIG4* in Zeocin-treated cells were the same as that in the control cells with the addition of sterile water. However, 6 hours after the addition of Zeocin, the mRNA level of *PnKU70* increased by approximately 1.2-, 1.5-, and 3.5-fold in 10, 50, and 250 μg/ml of Zeocin-treated cells, respectively, in comparison to the control cells; and similarly, the expression of *PnLIG4* increased by approximately 1.3-, 1.4-, and 3.8-fold in 10, 50, and 250 μg/ml of Zeocin-treated cells, respectively. The expression levels of both genes remained substantially elevated 24 h after the addition of Zeocin.

The transcription of *PnRAD51* was also induced by Zeocin treatment, with poplar cells that were exposed to 250 μg/ml of Zeocin for 6 h showing an approximately 1.8-fold increase in expression compared with the control cells, which was a statistically significant change (two-way ANOVA, p = 1.61E-3) In contrast, the expression of *PnPCNA*, *PnXRCC4*, and *PnOGG1* appeared to be unaffected by Zeocin (two-way ANOVA, $p > 0.05$). Therefore, it was concluded that the expression of *PnKU70*, *PnLIG4*, and *PnRAD51* was induced by DNA strand breaks, whereas the expression of *PnPCNA*, *PnXRCC4*, and *PnOGG1* was not.

2.4 Discussion

The effects of ionizing radiation on the growth and morphology of many plant species have previously been reported and reviewed (Gunckel 1957; Sparrow et al. 1971; Holst and Nagel 1997; De Micco et al. 2011). In the present study, the biological effects of γ-rays on *P. nigra* plants were investigated in detail (Table 8), because despite the genus *Populus* having been extensively studied as a model organism for woody plant biology, very few studies have investigated the effects of γ-rays on these trees.

^{60}Co-gamma irradiation of *P. nigra* plantlets induced various morphological abnormalities, such as reduced growth, abnormally shaped leaves, distorted venation, fused leaf blades, chlorosis, stunted shoots, and fasciation (Fig. 8). These malformations were roughly similar to those observed in other plant species exposed to γ- or X-rays.

The poplar plantlets that had been exposed to 100 Gy (dose rate, 5 Gy h^{-1}) of γ-rays exhibited two types of growth: transient growth arrest (group 100-A in Fig. 7) and complete growth arrest (group 100-B in Fig. 7). However, all of these plants survived, and even in the completely growth-arrested 100-B group, new shoots sprouted on the lower parts of the γ-irradiated stems 4–10 weeks after γ-irradiation. These two different types of abnormal growth are believed to have arisen due to the differences in the viability of γ-irradiated cells in the shoot apical meristem (SAM). As in other higher plants, the SAM of poplars consists of many proliferating cells, the nuclear DNA of which is damaged by γ-rays. Although slight DNA damage is completely repaired, severe DNA damage is not, and any remaining DNA damage triggers cell death. If the number of cells in the SAM is greatly reduced by γ-rays, it will take a significant length of time for them to recover, resulting in an apparent cessation in shoot growth. It is likely that the surviving cells proliferated to reform the SAM, resulting in the γ-irradiated shoots starting to grow. However, if all of the cells in the SAM are killed by γ-rays, the SAM will surely lose its function, preventing the shoot from elongating.

Almost all of the poplar plantlets that were exposed to 200 Gy (10 Gy h^{-1}) or 300 Gy (15 Gy h^{-1}) of γ-rays stopped growing and then withered within 10 weeks of γ-irradiation. Sparrow et al. (1968) determined the LD_{50} (dose at which 50% of subjects will die) of γ-rays for many woody plant species based on the relationship between γ-ray survival curves and interphase chromosome volumes for each species. For

quaking aspen (*P. tremuloides*), which belongs to the same genus as *P. nigra*, the LD_{50} was predicted to be 4,800 roentgen for acute ^{60}Co-γ-irradiation (16 h), which corresponds to approximately 46.8 Gy (2.93 Gy h^{-1}) in terms of the absorbed dose to water. In the present experiment, however, 50 Gy (2.5 Gy h^{-1}) of γ-irradiation did not cause any withering in *P. nigra*. Although this result did not necessarily match the prediction of Sparrow et al. (1968), the biological effects of ionizing radiation have been shown to vary depending on the biological, radiological, and environmental factors (Sparrow et al. 1971). Therefore, the value of the lethal dose probably varies even within the genus *Populus* due to the differences between species, individual plants (e.g., size and age differences), and abiotic factors.

A Comet assay indicated that poplar cells that were exposed to γ-rays at doses of 100–300 Gy exhibited increased levels of DNA damage, while non-irradiated control cells and 50 Gy-irradiated cells were hardly damaged (Fig. 11). These results suggest that γ-rays have a dose-dependent biological effect on the growth and development of poplar plantlets. However, it was difficult to clearly quantify the degree of DNA damage in a dose-dependent manner in the γ-irradiated poplar cells. One possible explanation for this is that the DNA damage caused by γ-rays may be rapidly repaired during and after γ-irradiation. It has previously been reported that the time taken to repair 50% of the DNA damage after γ-irradiation is approximately 50 min in *N. tabacum* seedlings (Ptáček et al. 2001) and approximately 100 min in *Calamagrostis epigejos* leaves (Ptáček et al. 2002). In the present experiment, the γ-irradiation occurred for 20 h, following which the irradiated cells were harvested and analyzed using a Comet assay. Because the upregulation of the expression of the genes encoding DNA repair-related proteins was detected 1 h after the end of γ-irradiation (Fig. 21), it

seems that these genes were already activated during γ-irradiation, and thus it is likely that the DNA damage had started to be repaired.

Six cDNAs of DNA repair-related proteins were isolated from *P. nigra* and their gene expression patterns were investigated. It was found that the *PnLIG4*, *PnKU70*, *PnXRCC4*, *PnPCNA*, and *PnRAD51* genes were upregulated by γ-irradiation, whereas the *PnOGG1* gene was downregulated (Fig. 21). The five upregulated genes are likely to be involved in adaptation and tolerance to γ-rays based on protein function prediction, although the degree of increase in gene expression varied considerably for each of these genes. The expression of *PnLIG4* and *PnKU70* peaked 1 h after the end of γ-irradiation for each dose and then gradually declined, although the expression levels were still significantly higher than in the non-irradiated poplars 24 h after irradiation. In contrast, the highest level of mRNA expression of *PnRAD51* also occurred 1 h after the end of γ-irradiation, but this had almost returned to the original expression level 6–24 h after irradiation; and the high level of expression observed in *PnXRCC4* and *PnPCNA* remained constant even 24 h after the end of γ-irradiation. Expression of the *AtRAD51*, *AtLIG4*, and *AtXRCC4* genes in *A. thaliana* have previously been reported to increase in response to γ-irradiation (Doutriaux et al. 1998; West et al. 2000); however, it was unclear whether the upregulated gene expression was sustained at a high level, because gene expression was only investigated 1–6 h after the start of γ-irradiation. In the present study, *PnLIG4*, *PnKU70*, *PnXRCC4*, and *PnPCNA* remained activated until 24 h after the end of γ-irradiation, suggesting that DNA damage by γ-rays continues during this time.

The regulation of gene expression by γ-irradiation did not necessarily match that by Zeocin, a DNA breaking agent that is similar to bleomycin. Comet assay

analysis revealed that treatment with Zeocin caused nuclear DNA breaks in the poplar cells, as observed with γ-rays (Fig. 22A). Furthermore, Zeocin treatment led to an increase in the expression of the three genes *PnLIG4*, *PnKU70*, and *PnRAD51* (Fig. 22B), suggesting that either Zeocin itself or the DNA strand breaks and triggers the expression of these genes. It has previously been reported that bleomycin treatment results in an increase in the expression of *AtKu70* in *A. thaliana* (Tamura et al. 2002); however, this is the first study to demonstrate that a DNA breaking agent induces the expression of the genes encoding DNA ligase IV and RAD51 from plant species. Based on the findings of this study, it was concluded that the expression of *PnLIG4*, *PnKU70*, and *PnRAD51* are directly induced by DNA strand breaks, because all three of these genes are upregulated by both γ-rays and Zeocin. In contrast, the expression of *PnXRCC4* and *PnPCNA* was not elevated by treatment with Zeocin, despite both genes being upregulated by γ-rays, suggesting that neither of these genes are directly induced by DNA strand breaks.

The expression pattern of *PnOGG1* was completely different from the five γ-ray-induced genes. The OGG1 protein is an important DNA glycosylase that removes 8-oxoG (Van Der Kemp et al. 1996), which is generated by hydroxyl radicals produced in the γ-radiolysis of water, and is an oxidative DNA damage (Cadet et al. 2004). *PnOGG1* was predicted to encode an 8-oxoguanine DNA glycosylase and was expressed in *P. nigra* under normal conditions (Figs. 20 and 21). However, γ-irradiation reduced the expression level of *PnOGG1* and the subsequent disappearance of γ-rays increased the mRNA of *PnOGG1*. These observations imply that the *PnOGG1* gene is not required for the repair of oxidative DNA damage by γ-rays. In *A. thaliana*, AtOGG1 is capable of nicking an oligonucleotide duplex containing a single 8-oxoG, but its

expression is not influenced by either γ-rays or hydrogen peroxide (Dany and Tissier 2001). However, *A. thaliana* also possesses the *AtMMH*/*AtFPG* gene, which encodes a formamidopyrimidine-DNA glycosylase (Gao and Murphy 2001; Ohtsubo et al. 1998). AtMMH/AtFPG is not homologous to AtOGG1 but is able to nick double-stranded oligonucleotides containing 8-oxoG. Therefore, although it is presently unclear why *P. nigra* that were exposed to γ-irradiation had reduced the levels of *PnOGG1* mRNA, it is possible that an as yet unknown poplar homolog of AtMMH/AtFPG is involved in the cellular response to oxidative DNA damage by γ-rays.

It was initially speculated that any induced gene expression would reflect part of the defense mechanisms of *P. nigra* against γ-irradiation. However, the obtained data are insufficient to explain the response mechanism to ionizing radiation stress in poplars because more than 45,000 protein-coding genes have been predicted to exist in the *Populus* nuclear genome (Tuskan et al. 2006). In the future, it will be possible to use gene expression profiling by DNA microarray analysis or transgenic technology to identify the genes that are involved in the adaptation to ionizing radiation stress.

2.5 Conclusion

In this study, the biological effects of γ-rays on *P. nigra* were investigated to elucidate the mechanisms of adaptation and tolerance to ionizing radiation in woody plants. The γ-irradiated poplar plants exhibited abnormal leaf shapes and colors, fusion, distorted venation, shortened internodes, fasciation of the stems, and the induction of axillary shoots. In addition, acute γ-irradiation at a dose of 100 Gy greatly reduced the height, stem diameter, and biomass of poplar plantlets; and after receiving doses of 200 and 300 Gy, all of the plantlets stopped growing, and most withered 4–10 weeks after

γ-irradiation. Comet assays showed that the nuclear DNA in suspension-cultured poplar cells was damaged by γ-rays. To determine whether DNA repair-related proteins are involved in the response to γ-rays in *P. nigra*, the cDNAs of *PnRAD51*, *PnLIG4*, *PnKU70*, *PnXRCC4*, *PnPCNA*, and *PnOGG1* were cloned and their mRNA expressions were investigated. The *PnRAD51*, *PnLIG4*, *PnKU70*, *PnXRCC4*, and *PnPCNA* mRNAs were increased by γ-rays, but the *PnOGG1* mRNA was decreased. Moreover, the expression of *PnLIG4*, *PnKU70*, and *PnRAD51* was upregulated by Zeocin, which is a known DNA cleavage agent. These observations suggest that morphogenesis, growth, and protective gene expression are severely affected by DNA damage and unknown cellular events that are caused by γ-irradiation in *P. nigra*.

Chapter 3 An improved transformation system for *Populus nigra*

3.1 Introduction

Genetic engineering can allow the selective improvement of individual traits in forest trees without losing any of the desirable traits of the parental line and is thought to be useful for overcoming the difficulties associated with breeding long-lived perennials, which take a long time to produce progeny. *Agrobacterium*-mediated transformation has traditionally been the preferred method for introducing foreign genes into plants. Numerous plant species, including a wide range of woody species, are susceptible to infection by the genus *Agrobacterium* (Gelvin 2003). However, many difficulties have been encountered when attempting to regenerate transgenic woody plants and, in many cases, appropriate regeneration systems have not yet been established. Efficient and reproducible transformation systems have been reported for a limited number of broadleaved trees, however, including poplars.

Efforts to develop a model tree have focused on the genus *Populus* (Bradshaw et al. 2000; Nanjo et al. 2004). This genus consists of six sections: *Abaso, Turanga*, *Leucoides, Aigeiros* (cottonwoods), *Tacamahaca* (balsam poplars), and *Populus* (aspens and white poplars) (Eckenwalder 1996). Because *Aigeiros* and *Tacamahaca* can hybridize with each other, both are termed cottonwoods in some instances. The frequency of successful transformation varies between *Populus* species. In general, aspens have a much higher transformation frequency than cottonwoods; for example, the transformation frequency in aspens is 25–75% in *P. tremula* × *P. tremuloides*

(Nilsson et al. 1992), 80% in *P. tremula* (Tzfira et al. 1997a), and 40% in *P. sieboldii* × *P. grandidentata* (Ebinuma et al. 1997), while that in cottonwoods is 10% in *P. trichocarpa* × *P. deltoids* (De Block 1990), 2% in *P.* × *euramericana* (Heuchelin et al. 1997), and 2% in a wide variety of other cottonwood genotypes (*P. deltoides*, *P. deltoides* × *P. nigra*, and *P. trichocarpa* × *P. deltoides*) (Han et al. 2000).

Populus nigra is one of the important trees for forestry and is also an appropriate species for scientific research, as described in Chapter 1. Some transformation systems for *P. nigra* have previously been reported for leaves (Confalonieri et al. 1994; Confalonieri et al. 1995) or stems (Mohri et al. 1996). In these systems, however, a long time is required for the regeneration of the transgenic plants because calli need to form in the *Agrobacterium*-infected leaves and stems (Fig. 5). Furthermore, the efficiency of the transformation is not sufficiently high and non-transgenic poplar plants often appear. To facilitate the development of molecular biology and molecular breeding of *P. nigra*, a highly efficient and reproducible transformation system is required for this species. Therefore, in this study, a novel binary vector was designed and constructed, and an improved procedure for *A. tumefaciens*-mediated transformation of *P. nigra* was developed, which used the direct regeneration of adventitious shoots rather than relying on the formation of calli.

3.2 Materials and methods

3.2.1 Plant Material

Populus nigra plants were propagated by excising young branches and rooting these in moist vermiculite. Rooted branches were then grown in a phytotron at 25°C and 70% relative humidity under metal halide lamps (500 μmol m^{-2} s^{-1} of PAR, 16:8 h

light:dark photoperiod). Young stems that were approximately 2 weeks old were used as the explants in the transformation experiment.

3.2.2 Construction of binary vectors

The binary vector pBI121 (Jefferson et al. 1987) was digested with *Sma*I and *Sac*I, and then ligated to a DNA linker that had been prepared by annealing two synthetic oligonucleotides, BF-1 (5′-GGGTCGACGGTACCTAGGCGTACGACTAGT CTCGAGTATACGAGCT-3′) and BF-2 (5′-CGTATACTCGAGACTAGTCGTACGCCT AGGTACCGTCGACCC-3′). The resulting binary vector, which was designated pBF1, had lost the *GUS* gene and instead carried 11 unique recognition sites for restriction enzymes.

Following the digestion of pBF1 with *Apa*I and *Not*I, the excised DNA fragment (4 kbp) that contained the *NPTII* gene was inserted into the *Apa*I and *Not*I sites of pBluescript II KS(+) (Stratagene). The resulting plasmid, pBS2NPT, was then digested with *Apa*I and *Pst*I, and the excised DNA fragment (0.9 kbp) was newly inserted into the *Apa*I and *Pst*I sites of another pBluescript II KS(+). This plasmid was digested with *Nco*I and *Sph*I for the removal of the mutation site, and then ligated to a DNA linker that had been prepared by annealing two oligonucleotides, TTOG1 (5′-CCCGACGGCGAGGATCTCGTCGTGACC-3′) and TTOG2 (5′-CATGGGTCACG ACGAGATCCTCGCCGTCGGGCATG-3′). The resulting plasmid was digested with *Apa*I and *Pst*I, and the excised DNA fragment (0.9 kbp) was inserted again into *Apa*I and *Pst*I predigested pBS2NPT. Following the digestion of this plasmid with *Apa*I and *Not*I, the resulting fragment (4 kbp) was reinserted into the first *Apa*I and *Not*I digested pBF1. The resulting binary vector was designated pBF2.

To use the enhanced green fluorescent protein (*EGFP*) gene as a reporter gene, pBF2::EGFP was constructed. A fragment of the *EGFP* gene was amplified by means of PCR using pEGFP (Clontech, Palo Alto, CA, USA), and the oligonucleotide primers GFP1 (5′-GGATCCCCGGGTACC-3′) and GFP2 (5′-ACGAGCTCAGTTGGTAATGG TAGCGA-3′). Following digestion of the amplified DNA fragment with *Bam*HI and *Sac*I, the excised fragment was inserted into the *Bam*HI and *Sac*I sites of pBF2. The sequence of each constructed plasmid was confirmed by DNA sequencing.

3.2.3 Transformation of *P. nigra*

Agrobacterium tumefaciens EHA105 (Hood et al. 1993) harboring either pBF2::EGFP or pIG121Hm (Ohta et al. 1990) was grown in 2 × YT liquid medium (Sambrook and Russell 2001) containing 20 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin at 28°C for 24 h. The cells were harvested by centrifugation and suspended to an optical density of 600 nm (OD_{600}) = 0.5 in 20 mM MES-KOH (pH 5.8), 200 mM glucose, 1 × MSBS, and 1 × MSV.

Stems of *P. nigra* were sterilized with sodium hypochlorite solution (approximately 1% active chlorine) for 10 min, and then washed with sterile water. Following this, 1-cm-long segments were excised from the stems (avoiding the nodes) and divided in half vertically. The stem segments were soaked for 30 min in the bacterial suspension. The inoculated explants were then cultured on a cocultivation medium containing 20 mM MES-KOH (pH 5.8), 1 × MSBS, 1 × MSV, 3% sucrose, 50 mM glucose, 0.5 mg l^{-1} 2,4-D, 1 mg l^{-1} BAP, 5 mM dithiothreitol (DTT), 40 mg l^{-1} acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone), and 0.8% Bacto Agar at 25°C in the dark for 3 days. Next, they were washed three times with sterilized water and

once with a mixture of 20 mM MES-KOH (pH 5.8), 1 × MSBS, 1 × MSV, and 500 mg l^{-1} cefotaxime. The washed explants were transferred into a bactericidal medium containing 20 mM MES-KOH (pH 5.8), 1 × MSBS, 1 × MSV, 3% sucrose, 0.5 mg l^{-1} 2,4-D, 1 mg l^{-1} BAP, 5 mM DTT, 500 mg l^{-1} cefotaxime, and 0.8% Bacto Agar, and were then incubated at 25°C in the dark for a further 3 days.

For regeneration of the transgenic shoots, the explants were subcultured every 2 weeks on SIM (Table 9) containing 20 mM MES-KOH (pH 5.8), 1 × MSBS, 1 × MSV, 3% sucrose, 0.5 mg l^{-1} *trans*-zeatin, 0.1 mg l^{-1} BAP, and 0.8% Bacto Agar with the appropriate antibiotics, as follows: 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime at 25°C under cool white fluorescent light (approximately 6 μmol m^{-2} s^{-1} of PAR, 16:8 h light:dark photoperiod) in weeks 1 and 2; 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime under the same temperature and light conditions in weeks 3 and 4; and 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime under the same temperature but stronger light (approximately 60 μmol m^{-2} s^{-1} of PAR, 16:8 h light:dark photoperiod) from week 5 onward. Regenerated shoots were excised and transferred into RIM (Table 10) containing 20 mM MES-KOH (pH 5.8), 0.5 × MSBS, 1 × MSV, 3% sucrose, 0.5 mg l^{-1} indole-3-butyric acid, 0.02 mg l^{-1} 1-naphthaleneacetic acid, and 0.6% Phytagar with 20 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime in a Petri dish. After 4 weeks, each rooted shoot was transferred into RIM containing 10 mg l^{-1} kanamycin in a plastic plant culture jar, in which the shoots continued to be incubated.

3.2.4 Fluorescent microscopy

Expression of the *EGFP* gene in the transgenic poplar plants was detected using an SZX12 stereomicroscope (Olympus, Tokyo, Japan) equipped with an

epifluorescence attachment. A GFP filter set (a 460–490 nm excitation filter and a 510-nm long-pass emission filter) was used for detection.

3.2.5 Protein analysis

A crude protein solution was prepared from the leaves of the transgenic poplar plants as follows. The leaves were frozen in liquid nitrogen and then ground using a cell disruptor (Yasui Kikai, Osaka, Japan). The disrupted leaves were mixed with an extraction buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, 2 mM DTT, and cOmplete™, EDTA-free Protease Inhibitors (Roche Diagnostics, Mannheim, Germany) at 4°C for 15 min. Following centrifugation at 18,000 *g* for 10 min, the crude protein solution was obtained. The protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA), with bovine serum albumin as the standard. The crude protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The fractionated proteins were then stained with Coomassie blue R-350 or blotted onto polyvinylidene fluoride (PVDF) membranes. Immunoblotting was performed using either the Living Colors® A.v. Peptide antibody (Clontech) against EGFP or immunoglobulin G (IgG) (Molecular Probes, Eugene, OR, USA) against GUS. The signal was detected using the ECL Plus Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ, USA).

3.2.6 DNA analysis

Genomic DNA was isolated from leaves of the transgenic poplar plants using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The foreign *NPTII* gene was

detected by PCR using two primers, the NPT2U primer (5′-GCTATTCGGCTATGACT GG-3′) and the NPT2R primer (5′-ATAGAAGGCGATGCGCTG-3′). Southern hybridization was performed using ^{32}P-labeled *NPTII*, *GUS*, and *EGFP* genes as the probes (Nishiguchi et al. 2002).

3.3 Results and discussion

3.3.1 Construction of a new binary vector

A new binary vector, pBF2, was designed and constructed to enable versatile DNA cloning and to enhance the resistance of transformed plants to antibiotics (Fig. 23). The pBI121 vector, which has generally been used for the transformation of plants including *P. nigra*, contains only four restriction enzyme sites (*Xba*I, *Bam*HI, *Sma*I, and *Sac*I) for DNA cloning (Fig. 23). By substituting the DNA cloning site of pBI121, the recognition sites of seven restriction enzymes (*Sal*I, *Kpn*I, *Bln*I, *Bsi*WI, *Spe*I, *Xho*I, and *Bst*1107I) were added to the pBF2 vector, allowing 11 unique restriction enzyme sites to be utilized for DNA cloning. Among these restriction enzymes, *Xba*I, *Bln*I, and *Spe*I produce the common cleaved ends, which can ligate to each other. *Sal*I and *Xho*I generate other common cleaved ends, while *Sma*I and *Bst*1107I produce blunt ends. The arrangement of these restriction enzyme sites will likely facilitate the cloning of various DNA fragments into pBF2 (Fig. 24).

The *NPTII* gene in pBI121 is known to contain a mutation. The resulting mutant NPTII enzyme exhibits lower levels of kanamycin phosphorylation than the normal NPTII enzyme (Yenofsky et al. 1990). Since this reduced activity decreases resistance to antibiotics, it may explain the low transformation efficiency in *Brassica napus* (Datla et al. 1992). To improve the activity of the mutant NPTII, the aspartic acid

residue at position 182 in the mutant NPTII enzyme was replaced with glutamic acid during the construction of the pBF2 vector. The normal NPTII enzyme can inactivate not only kanamycin but also G418 (Geneticin®), which is an aminoglycoside antibiotic similar to kanamycin. It was demonstrated that *E. coli* JM109 harboring pBF2 could survive in the presence of 50 mg l^{-1} kanamycin or 20 mg l^{-1} G418, while *E. coli* harboring pBI121 could not grow on G418 (Fig. 25), demonstrating that the reverse mutation of NPTII succeeded and that the activity of NPTII from pBF2 is higher than that from pBI121.

3.3.2 Improvement of the transformation process

It usually takes 1–2 months to induce the formation of calli and increase their size, and a similar period to regenerate and elongate shoots in the transformation of *P. nigra* (Mohri et al. 1996). In addition, the appearance of non-transformed cells and somaclonal variation has been detected in the regeneration of organs from calli (Tzfira et al. 1997b). To reduce the time required for transformation and to avoid these risks, the present study attempted to directly induce adventitious shoots from poplar explants rather than from calli (Fig. 26).

Populus nigra explants were infected with *A. tumefaciens* EHA105 harboring either pBF2::EGFP or pIG121Hm (Fig. 27) as the control, and were then subcultured in SIM containing kanamycin along with *trans*-zeatin and BAP. This mixture induced the production of adventitious shoots within 1–2 months (Fig. 28A). These shoots were then excised and transferred into RIM supplemented with kanamycin, which resulted in approximately 20% of the shoots rooting within 4 weeks (Fig. 28B and Table 11). PCR showed that almost all of the rooted plants contained the *NPTII* gene (Fig. 29).

3.3.3 Characterization of the transgenic poplars

All of the regenerated transgenic poplar plants exhibited normal growth, without any obvious morphological changes (Fig. 28C–D). The expression of EGFP in the pBF2::EGFP-transformed poplars was mainly detected in the roots and at the edges of the leaves (Fig. 30). The fluorescent segments along the leaf edges were considered to be hydathodes (Curtis and Lersten 1974; Wilson et al. 1991). The GFP fluorescence was not observed in the stems or the mesophyll cells in the leaves likely due to the strong intrinsic fluorescence of the chlorophylls interfering with the detection of the emitted EGFP signal in these parts.

To examine the protein levels in the transgenic poplars, immunoblotting was performed with antibodies against EGFP and GUS. Nine of 18 transgenic poplars harboring the *GUS* gene expressed GUS (Fig. 31A), and 12 of 18 randomly selected transgenic poplars harboring the *EGFP* gene expressed EGFP (Fig. 31B). This demonstrates that the levels of protein expression varied among the transgenic lines, with the GUS or EGFP proteins not being detected in several transgenic plants. To estimate the copy number of the introduced foreign genes, Southern blotting was performed using the probes of the *EGFP*, *NPTII*, or *GUS* genes (Fig. 32). Based on the number of signals detected, it was estimated that one to multiple copies of these genes had been inserted in the transgenic plants. Thus, not all of the transgenic poplars harboring the foreign gene were able to produce the foreign proteins.

It was found that most of the transgenic poplars carrying the multicopy transgenes did not express GUS (lines 3-1, 3-6, 3-30, and 3-33) or EGFP (lines 4-9, 4-13, 4-20, 4-26, and 4-31) (Figs. 31 and 32). One possible explanation for this may be epigenetic

gene silencing by multicopy integration of the transgenes (Rajeevkumar et al. 2015). However, some of the transgenic poplars that only contained one copy of the transgene also did not express the gene (lines 3-4 and 3-14). The reason for this is unknown, but the findings infer that there may be a position effect on expression, as the upstream or downstream region of integrated foreign genes affects their expression (Gallie 1998; Matzke and Matzke 1998). Although the dispersion of the expression levels of transgenes cannot be completely controlled at present, the high productivity of transgenic plants contributes to the acquisition of suitable transformants. As an alternate approach, heterogeneity in protein expression may be eliminated by using a more potent promoter or several matrix attachment regions (Han et al. 1997).

3.3.4 Increased efficiency of transformation of *P. nigra*

Each transgenic poplar was regarded as a different genotype based on the differences in the signal patterns in the Southern blots (Fig. 32). The transformation frequency was calculated by dividing the number of genetically independent transformants by the number of infected explants (Table 11), which provided values of 11.7% with the pBF2::EGFP vector and 6.6% with the pIG121Hm vector, in the absence of DTT. The elevated transformation frequency with pBF2::EGFP may have resulted from the use of the improved *NPTII* gene. These results are consistent with transformation frequency values for *B. napus* (Datla et al. 1992).

To further increase the transformation efficiency of *P. nigra*, the use of reducing agents was investigated. Several antioxidants have been shown to increase the efficiency of *Agrobacterium*-mediated transformation of soybean (Olhoft et al. 2003) and maize (*Zea mays*) (Frame et al. 2002). The presence of DTT in the cocultivation and

bactericidal media increased the transformation frequency to 19.4% with pBF2::EGFP and 21.4% with pIG121Hm (Table 11). The positive effect of antioxidants on the transformation of grape (*Vitis vinifera*) has been explained by the reduction in tissue necrosis caused by a hypersensitive response and increased peroxidase activity (Perl et al. 1996). Thus, DTT may also prevent the necrosis of *Agrobacterium*-infected poplar cells, increasing the number of viable cells.

3.3.5 pBF2-conferred resistance to G418

As described in section 3.3.1, *E. coli* harboring pBF2 exhibited resistance to G418 (Fig. 25). G418 is an antibiotic that is similar to kanamycin or gentamycin, and which inhibits cell proliferation in tobacco (*N. tabacum*) (Ursic et al. 1981), *A. thaliana* (Sheikholeslam and Weeks 1987), and rice (Dekeyser et al. 1989). Normal NPTII can inactivate G418 and therefore, has sometimes been used for plant transformation. To reconfirm the antibiotic resistance of the regenerated transgenic poplars, the effects of kanamycin and G418 on callus formation and organogenesis were examined (Fig. 33). The cell growth of non-transgenic *P. nigra* was inhibited by 20 mg l^{-1} G418 (data not shown). The petioles and stems of transgenic poplars with the pBF2::EGFP vector could produce calli and adventitious shoots, respectively, in the presence of 50 mg l^{-1} G418 or 100 mg l^{-1} kanamycin. In contrast, the segments of transformants with the pIG121Hm vector were unable to produce calli or shoots in the presence of G418 but could grow in kanamycin. These results indicate that the pBF2 binary vector provided resistance to both G418 and kanamycin in the transgenic poplars, maybe allowing G418 to be used as a selection agent in the transformation of *P. nigra*.

3.4 Conclusion

In the present study, an improved transformation system was developed for *P. nigra*. A new binary vector, pBF2, was constructed, which contained 11 unique restriction enzyme sites and the normal *NPTII* gene. Stem segments were cocultivated with *A. tumefaciens* EHA105 harboring pBF2 inserted in the *EGFP* gene. Genetically transformed adventitious shoots were directly regenerated from the stem segments and rooted. Successful transformation was confirmed by demonstrating resistance to kanamycin and G418 and performing PCR, fluorescence microscopy, immunoblotting, and Southern blotting analyses. Furthermore, when explants were incubated in a medium containing DTT, the transformation frequency increased to approximately 20%. This improved transformation system requires less time for the regeneration of transgenic shoots and thus is valuable for efficiently generating transgenic poplar plants in a short time. This, in turn, will facilitate the genetic improvement of traits through the introduction of useful genes involved in growth, wood formation, flowering, tolerance to environmental stress, and resistance to pests and disease.

Chapter 4 Conclusion

4.1 Conclusions and perspectives

As described in Chapter 1, research into the biology and utilization of poplars is a topic of great interest. Poplar research includes a wide range of studies in areas such as physiology, ecology, molecular biology, genetic engineering, forestry, chemistry, wood science, and environmental science. This wide range of research is due to the fact that poplars are not only important in industrial applications but are also key model organisms representing woody plants. To promote the usage of poplars as a model species, it is important to pioneer new fields of poplar research and develop new technologies for their study. Therefore, the elucidation of its biological response to ionizing radiation and the improvement of the transformation system for *P. nigra* were performed in this thesis (Fig. 3).

In Chapter 2, ionizing radiation was used as an abiotic stress to study the biological effects on *P. nigra*. Various abiotic and biotic stresses have been examined in *Populus* species; however, the effects of ionizing radiation have not been well studied. All living organisms on Earth are exposed to ionizing radiation and should be influenced by it. Many organisms are equipped with response and tolerance mechanisms to deal with the harmful effects of ionizing radiation. Understanding the biological response to ionizing radiation in *P. nigra* is important to understand its adaptive strategy not only under ionizing radiation stress, but also under various other environmental stresses. Moreover, γ-irradiated *P. nigra* trees showed growth retardation, withering, morphological changes and the suppression of organ regeneration in a dose-dependent

manner (Table 8 and Figs. 6–10). Nuclear DNA was damaged by the γ-rays (Table 8 and Fig. 11). Six cDNAs of the DNA repair-related proteins, PnRAD51, PnLIG4, PnKU70, PnXRCC4, PnOGG1, and PnPCNA were isolated and structurally analyzed (Figs. 13–19). *PnRAD51*, *PnLIG4*, *PnKU70*, *PnXRCC4*, and *PnPCNA* were upregulated by γ-irradiation, whereas *PnOGG1* was downregulated (Fig. 21). The expression of *PnLIG4*, *PnKU70*, and *PnRAD51* was also increased in cells with DNA damage induced by Zeocin, therefore, it was concluded that these three genes were induced by DNA cleavage (Fig. 22).

To the best of our knowledge, for the first time, these results demonstrated that γ-rays are a genotoxic stress for *P. nigra* and that *P. nigra* probably has a defense mechanism against them (Fig. 34). *P. nigra* was able to survive in 50–100 Gy of γ-irradiation. Considering the dose of natural radiation that wild woody plants are exposed to (Fig. 4), *P. nigra* has a high and excessive tolerance to γ-rays. Furthermore, in comparison to other woody plant species, *P. nigra* is thought to possess a higher tolerance to ionizing radiation (Table 12). As the nuclear DNA content of a cell becomes larger, the radiation sensitivity is generally considered to increase (Sparrow and Miksche 1961). Therefore, one of the reasons for the high tolerance of *P. nigra* to ionizing radiation may be due to the smaller genome size of poplars as compared to other woody plant species, which are more sensitive to ionizing radiation.

On the other hand, almost all the poplar plantlets died under a higher dose (200–300 Gy) of γ-irradiation. However, the Comet assay showed that the DNA damage suffered by poplar cells under this amount of γ-irradiation was the same as that under 100 Gy (Table 8 and Fig. 11). Although the Comet assay is able to determine DNA cleavage, it cannot usually find any qualitative DNA damage such as DNA oxidation by

γ-rays-induced reactive oxygen species (ROS). In addition, the lethal effect of γ-rays probably depends not only on DNA damage but also on the synergistic effect of damage and other adverse reactions induced by radiation. For example, the higher dose of γ-rays may directly destroy not only DNA but also other intracellular molecules or organelles. A larger amount of ROS, which is generated by high doses of γ-rays, may more intensely damage lipids or proteins in poplar cells.

Although the isolated DNA-repair related proteins have been thought to be involved in the tolerance response to genotoxic stress via DNA repair, the function and relationships of these proteins need to be clarified to better understand the poplar defense mechanisms against genotoxic stress. In addition to DNA repair-related proteins, many other proteins perhaps are involved in the tolerance response to genotoxic stress. *P. nigra*, as well as *P. trichocarpa*, is expected to have many genes encoding proteins (Tuskan et al. 2006; Nanjo et al. 2007). For instance, enzymes that remove ROS are potential candidates for mediators of radiation tolerance. The relationship between such candidate proteins and tolerance to genotoxic stress should be investigated to better understand stress tolerance mechanisms.

Genetic engineering has been crucial for investigating tolerance mechanisms including assaying DNA repair-related proteins. However, further development was required to make these studies possible in *P. nigra* (Fig. 5), although the transformation of *P. nigra* has been reported previously (Confalonieri et al. 1994; Confalonieri et al. 1995; Mohri et al. 1996). In Chapter 3, the transformation system for *P. nigra* was originally improved, via the creation of a new binary vector, and the transformation process was examined (Fig. 35). The transformation efficiency improved to approximately 20% of the infected explants using DTT (Table 11). The raised efficiency

was estimated to be 1.6- to 3.2-fold higher compared with experiments without DTT. It was difficult to directly compare the improved method to previous methods, because the clone of *P. nigra* utilized was different from those in previously reported studies. Due to the omission of callus induction, the regeneration time of transgenic poplar plants was shortened from approximately 6 months to 4 months, thus, it is reported to be an approximately 1.5-fold rise in transgenic efficiency. Consequently, the productivity of transgenic *P. nigra* was computationally estimated to increase to 2.4- to 4.8-fold per year by the improved method (Fig. 35).

The newly constructed vector, pBF2, has many restriction enzyme sites (Fig. 23). Some restriction enzymes give compatible ends in pBF2 (Fig. 24). For example, *Xba*I, *Bln*I and *Spe*I produce the same cleaved ends, which can ligate to each other. *Sal*I and *Xho*I also generate common cleaved ends. On the other hand, *Sma*I and *Bst*1107I create blunt ends. The design of pBF2 is convenient for ligating foreign DNA fragments. Thus, pBF2 should be useful for the transformation of not only *P. nigra* but also other plant species. The pBF2 vector also conferred G418 resistance in addition to kanamycin resistance on the transformants (Figs. 25 and 33). These results were probably caused by the reverse mutation of the *NPTII* gene on pBF2. G418 has not been previously reported as a selection reagent for transgenic poplars. Although G418 is assumed to be used as a selection reagent for the poplar transformation, the appropriate concentration of G418 for the selection of transgenic poplars remains to be examined.

By improving the transformation system, *P. nigra* is easier to transform than that reported previously, enhancing the value of *P. nigra* as a woody plant model species. The improved system is available for the study of gene function and for the generation of transgenic poplars. However, some limitations remain. The established

transformation system may require modification for other clones or hybrids of *P. nigra*. Unlike general herbaceous model plants such as *A. thaliana* and rice, poplar plants are heterozygous genotypically. Poplars with different genotypes may show different responses during regeneration and antibiotic selection; thus, their transformation efficiency may be reduced. Moreover, in comparison with herbaceous model plants, few promoters and enhancers that regulate gene expression have been isolated and characterized from poplars. To control gene expression and avoid adverse effects, various promoters or enhancers with distinct expression patterns are needed. On the other hand, although the traits of transgenic poplars are controlled by introduced transgenes, reliable methods for the selection of useful transgenes have not been established. These difficult problems must be resolved to optimally utilize not only *P. nigra* but also other *Populus* species as woody plant models.

In terms of practical applications, the tolerance of poplars to ionizing radiation is higher than that of humans, a trait that may prove beneficial. *P. nigra* trees can survive under >50 Gy of γ-irradiation, although the radiation doses at which 10%, 50% and 90% of humans die after whole body exposure are estimated to be 1–2 Gy, 4 Gy and 5–7 Gy, respectively (ICRP 2007). Thereby, poplar trees are able to grow where humans cannot continuously live due to ionizing radiation above the natural background, such as at uranium ore mines or atomic accident locations that are cited as radioactively-contaminated areas. The phytoremediation of radionuclides in the soil has been studied (Dushenkov 2003; Gupta and Walther 2014; Sharma et al. 2015). As described in Chapter 1, poplars are suitable plants for phytoremediation because they are perennials with rapid growth and deep root systems. Therefore, if poplar trees are planted in such places, they may enhance the accumulation and elimination of

radionuclides. Moreover, using genetic engineering, transgenic poplars with higher tolerance to ionizing radiation may enhance environmental restoration in higher-dose regions polluted by radionuclides.

To biologically monitor radiation dose, chromosomal aberrations in living organisms have often been employed. After the Chernobyl accident in 1986, transgenic plants were tested for their use in radiation monitoring. To analyze the influence of chronic irradiation from radioactive-contaminated soil, the transgenic *A. thaliana* plant harboring a chimeric *GUS* gene was generated as a bioindicator (Kovalchuk et al. 1998). In this transgenic line, the chimeric *GUS* gene was repaired by homologous recombinational repair, stimulated by ionizing radiation. Radioactive pollution could be significantly detected from 3,700 Bq m^{-2} to 33 MBq m^{-2}. On the other hand, a γ-irradiation hypersensitive mutant of *A. thaliana* was used for the detection of γ-rays (Peng et al. 2014). AtATM is a DNA repair-related protein, and an *AtATM*-disrupted mutant does not have a functioning DNA repair system. Subsequently, the *GFP* gene was introduced into the *AtATM* mutant. In this transgenic *A. thaliana* line, the radiation-damage *GFP* was not adequately repaired, and thus, the quenching of GFP was utilized to monitor γ-ray exposure.

As described in Chapter 1, poplar trees have been utilized as environmental monitors. Genes encoding DNA repair-related proteins from *P. nigra* may also be utilized for radiation monitoring. The expression of *PnLIG4* and *PnKU70* increased under exposure to γ-rays or Zeocin (Figs. 21 and 22). These results show that the promoters of *PnLIG4* and *PnKU70* are activated by genotoxic stress. If the promoters of these genes are fused to a suitable reporter gene, and the fused genes are introduced into poplars, it may become possible to generate transgenic genotoxic-monitoring trees that

recognize γ-rays or genotoxic agents and express the reporter gene of interest. By combining genetic engineering technology and DNA repair-related genes, the monitoring of genotoxic environmental pollution by radioactive substances or chemical carcinogens may be possible by transgenic poplars.

The control and utilization of DNA repair mechanisms may make mutation breeding advantageous. Although ionizing radiation has often been utilized for mutation breeding, the probability of a mutation occurring in a target gene is low, because radiation randomly damages DNA, and the induced DNA damage is mostly repaired. A higher dose of ionizing radiation raises the efficiency of generating mutations; however, it also increases harmful effects such as protein and membrane lipid damage, excessive ROS production and death of the irradiated cells in the extreme case. Therefore, a high frequency of mutation should be balanced with the the mitigation of cell damage by ionizing radiation. If the DNA repair system is temporarily weakened by genetic engineering during irradiation, a lower radiation level may be sufficiently able to cause the desired mutation.

Consequently, the present study increased the value of *P. nigra* as a woody plant model species by pioneering a new field of study, radiation stress physiology, and the development of better genetic engineering technologies (Fig. 36). In addition, synergy between a greater understanding of stress physiology and improved genetic engineering has the potential to spark the creation of new technology and applications for poplars. However, transgenic poplars, which are generated by modern genetic engineering techniques, have intrinsic and difficult limitations that are related to biosafety. For the practical utilization of transgenic poplars, these problems must be resolved. In the later sections, new technology beyond traditional genetic engineering

methods and the assessment of risk in the use of transgenic poplars will be addressed.

4.2 New plant breeding techniques (NPBT)

NPBT are expected to substitute for conventional mutation breeding or transgenic technology, because some NPBT allow site-specific and targeted changes in the genome with no traces of the foreign gene (Lusser et al. 2011). Zinc finger nuclease (ZFN) technology is an NPBT that introduces site-specific mutations into the plant genome or allows the site-specific integration of genes (Lloyd et al. 2005). In addition, TAL effector nuclease (TALEN) technology (Cermak et al. 2011) and the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system (Li et al. 2013b) were recently developed as tools for genome editing in plants.

The CRISPR/Cas9 system was used to independently disrupt two 4-coumarate:CoA ligase genes (*4CL1* and *4CL2*) in *P. tremula* × *P. alba* (Zhou et al. 2015), resulting in the lignin content being reduced in the *4CL1* mutants but remaining the same as control plants in the *4CL2* mutants. The CRISPR/Cas9 system was also used to disrupt the phytoene desaturase (*PDS*) gene in *P. tomentosa*, leading to albino shoots being formed that had lost their green color (Fan et al. 2015).

Virus-induced gene silencing or gene expression is also an NPBT, which uses a virus that cannot be integrated into the host genome as the vector. Gene silencing based on the tobacco rattle virus led to a reduction in the phytoene desaturase in *P. tomentosa* (Jiang et al. 2014b), and in *P. euphratica* and *P.* × *canescens* (Shen et al. 2015). Another NPBT is the grafting of a non-transgenic scion on a transgenic rootstock. Here, the scion does not contain the foreign gene. However, one study showed that the Bt toxin

protein that was expressed in transgenic rootstock poplars was translocated within the phloem and detected in the grafted scion, resulting in the leaves of the grafted scion having a lethal effect on herbivorous larvae (Wang et al. 2012b).

Although NPBT are very useful for gene targeting or for the introduction of new traits, there is controversy over whether NPBT products lack foreign genes such as *NPTII* are LMOs (Podevin et al. 2012; Heap 2013; Hartung and Schiemann 2014). Each country independently regulates LMOs through process- or product-based regulatory frameworks; however, the regulation of NPBT remains under consideration (Araki and Ishii 2015). If the new annual crops that are produced by NPBT possess no foreign genes, they may not be treated as LMOs for agriculture and commerce in the future. However, the improved trees produced by NPBT may need to be assessed in terms of safety and risk due to their perennial longevity.

4.3 Risk assessment for transgenic poplars as living modified organisms

The planted area of living modified crops for commercialization reached 181.5 million ha across 28 countries in 2014 (James 2014). However, genetically engineered forest trees, including transgenic poplars, are hardly used for practical purposes. For example, the only country that has commercially planted transgenic poplars is China, where trees expressing *B. thuringiensis* toxin genes were first planted in 2002. The plantation area of the transgenic poplars has since increased, reaching 490 ha in 2011 (Hu et al. 2014); and in 2015, the commercial cultivation of transgenic eucalyptus (*Eucalyptus* spp.) was approved in Brazil (ISAAA 2015). One of the reasons why transgenic trees are less popular than transgenic herbaceous crops is likely due to the difficulties in assessing their risks as LMOs to the environment.

The issue of LMOs was first addressed at the Convention on Biological Diversity at the Rio Earth Summit in 1992 (UN 1992). Following this, the Cartagena Protocol on Biosafety defined an LMO as any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology such as *in vitro* nucleic acid techniques (SCBD 2000). The original aim of the Cartagena Protocol was to prevent LMOs from having an impact on biological diversity. Because transgenic poplars are LMOs, their generation, utilization, and management should be conducted with a consideration for biological diversity and according to their regulatory framework. Therefore, the risks and environmental impacts of transgenic poplars need to be assessed in the laboratory, greenhouse, and field, according to the laws of each country.

Gene flow and transfer from LMOs may affect other plants, animals, and microorganisms. Recombinant DNA is discharged from transgenic poplars into the soil through their elongated roots, and fallen leaves and branches; for example, the DNA sequences of the introduced T-DNA were detected in the soil of cultivated transgenic *P. alba* using PCR/Southern blot hybridization (Bonadei et al. 2009). However, any DNA that is present in the soil is not consistently transferred into other organisms. For example, in the case of transgenic *P. tomentosa* containing the introduced *Atriplex hortensis DREB1* (*AhDREB1*) gene and the *NPTII* gene, 37 species of microorganisms in the planted soil showed a tolerance to kanamycin, but none contained the introduced *NPTII* gene (Lu et al. 2014). The transfer of the *bar* gene from transgenic poplars to *Amanita muscaria*, an ectomycorrhizal fungus, was also examined, which showed that 35,000 ectomycorrhizas did not show phosphinothricin resistance, and 102 of these did not possess the *bar* gene (Zhang et al. 2005). Horizontal gene transfer between

transgenic poplars and other organisms is crucial for biosafety; therefore, this should be tested and challenged continuously.

The pollen of poplars is wind-dispersed and can be spread widely, with estimates including 7.6 km (Slavov et al. 2009) and 8.2 km (Rathmacher et al. 2010). The seeds of poplars are also very small and have light hair, allowing them to be transferred a great distance from the mother tree. The seeds of *P. nigra* have been reported to move more than 100 km along an entire river system (Imbert and Lefèvre 2003). Moreover, vegetative reproduction by root suckers (root sprouts) is well known in some poplar species. Therefore, to avoid hybridization between transgenic and non-transgenic poplars, transgenic poplars should be cultivated in a confined field that is a sufficient distance from natural poplar trees and that is surrounded by an impermeable barrier. Moreover, any disused transgenic poplars should be cut down and completely withered by a herbicide. Transgenic sterility has also been studied to prevent the dispersal of pollen and seed. For example, male sterile transgenic poplars harboring tapetum-specific promoter-driven barnase (ribonulease) were produced, and the barnase-expressed poplars did not produce pollen for 4 years in the field (Elorriaga et al. 2014).

Investigation of the effects of transgenic poplars on biological diversity is essential. The environmental impact of transgenic *P. alba* × *P. glandulosa* that expressed insecticidal proteins, Bt-Cry3A, and oryzacystatin I was investigated in field studies over 3 years, which showed that they had no effect on non-target pests and other arthropods (Zhang et al. 2011a; Zhang et al. 2011b). Similarly, transgenic *P. tremula* × *P. alba* with modified lignin metabolism had no effect on the fungal communities in the roots or soil (Danielsen et al. 2012). However, the fallen leaves of the Bt-Cry3A

expressed *P. tremula* ×*P. tremuloides* were reported to affect aquatic insect communities (Axelsson et al. 2011). Because different transgenic poplars are likely to have different impacts on biological diversity, these should be investigated carefully over long time periods.

Poplar is likely to continue to be planted for many years both for commercial cultivation and environmental conservation. Therefore, when utilizing transgenic poplars, it is essential that the stability of the transgenes is investigated. Transgenic *P. tremula* × *P. alba* expressing cytosolic pine glutamine synthetase exhibited a higher growth rate than wild type trees for 3 years in a field test, and the amount of glutamine synthetase also remained at a higher level (Jing et al. 2004); and 3 years later, the fiber length of the transgenic poplars was still longer than that of the wild type (Coleman et al. 2012). Similarly, 2,256 transgenic *P. tremula* × *P. tremuloides* and *P. tremula* × *P. alba* that were planted and grown in a greenhouse and in the field expressed the *rbcS* promoter-driven *bar* gene and the CaMV 35S promoter-driven *GFP* gene for 3 years (Li et al. 2009); expression of the *Agrobacterium rolC* gene in transgenic *P. tremula* × *P. tremuloides* was detected in 19-year-old tissue cultures and more than 18-year-old trees in a glasshouse (Fladung et al. 2013); and the *AhDREB* gene in transgenic *P. tomentosa* was expressed 7 years after cultivation in the field (Lu et al. 2014). These results suggest that transgenes are stably maintained and expressed in transgenic poplars over a long time period.

The results obtained in the laboratory do not always match those in the greenhouse or field. For instance, transgenic *P. alba* overexpressing *Aspergillus* xyloglucanase exhibited faster growth than non-transgenic poplars in a growth chamber (Park et al. 2004); however, both types exhibited similar growth in a greenhouse

(Taniguchi et al. 2008), and the transgenic poplars exhibited slower growth than the non-transgenic poplars in the field (Taniguchi et al. 2012). It was also found that although transgenic *P. trichocarpa* had a lower lignin content than the wild type in a greenhouse, this increased in field trials (Stout et al. 2014). These differences between laboratory and field results may be attributed to differences in the environmental conditions, but are not yet fully understood.

There are many things that we do not yet understand about the risks of living modified trees to the environment and to humans. Therefore, assessment of the environmental impacts of transgenic poplars is important and related research into the practical utilization of transgenic poplars should continue. The risk assessment of transgenic poplars is also considered to be of great importance and value for the assessment of transgenic trees of other woody plant species.

4.4 The role of the poplar in science and its applications

In 1990, the total forested area in the world was 4.17 billion ha. Due to deforestation, the forested area decreased to 4.03 billion ha in 2010 (FAO 2010). A total of 135 million ha of forest disappeared in 1990–2010. The deforested area is equal to 5.4-fold of the total forested area in Japan (approximately 25 million ha). Deforestation was particularly prevalent in South America, Africa, and Oceania. The world's forests accumulate enormous biomass and contribute to carbon sequestration. In addition, the total biomass in the world's forests has been estimated to be 600 gigatons (Gt) in dry weight (FAO 2010). The carbon stock pooled in biomass, soil, and other factors in forests have further been calculated as 289, 292, and 72 Gt, respectively. Accordingly, the decrease in the world's forests led to the reduction of available woody biomass and

the progress of global warming by the emission of carbon. Furthermore, an increasing human population requires the products and services of forests more than ever.

Under these circumstances, the advancement of the science of woody plants is significantly important for resource and environmental conservation. The study of woody plants is required for optimizing the high productivity of tree biomass, the quality of wood, accessible resources, economic benefits, greening, environmental protection and restoration, and sustainable ecosystem services. However, most forest tree species remain underrepresented in biological research, with less scientific knowledge about them than poplar trees. For instance, adequate genomic resources such as molecular markers, ESTs, and genomic sequences are unavailable, and experimental techniques such as cell engineering or genetic engineering remain underdeveloped for many tree species. Therefore, it is often difficult to study various problems directly using such forest tree species.

Poplars can play a key role as the woody plant model species for developing the science and technology of these forest trees. Various studies on poplars have been widely performed and can provide not only poplar biologists but also other many scientists with useful information. For example, the number of studies including the term "*Populus*" per year in NCBI Medline increased nearly tenfold from 1998 to 2006 (Jansson and Douglas 2007). In 2014, this number was approximately 2-fold more than that in 2006, representing over 400 publications/year. Moreover, poplar research has incorporated innovative concepts and technologies such as genomics, other omics, NPBT, and systems biology approaches (Street et al. 2011; Chen et al. 2014a; Dash et al. 2015). As in this thesis that focuses on studying ionizing radiation stress, a large number of genomic resources for poplar research is also available for research into various other

fields. Genetic engineering of poplars can be used to develop experimental tools for the investigation of genes not only from poplars but also from other woody plants. Thus, the knowledge gained from studying poplars should be applied to research other woody plants.

However, poplars also have limitations as a model species. Bradshaw et al. (2000) explained that because of the long juvenile period, it takes long time to select homozygous mutants by mutagenesis and crossing. In addition, self-fertilization cannot occur due to dioecism; and thus, backcrosses must be performed for the production of inbred lines. The experimental cultivation of poplars requires large facilities or fields. From the viewpoint of industrial application, poplars are regarded as less important than eucalyptus for paper production or softwoods such as pines and cedars for wood materials. In addition to these factors, poplars are often difficult to use as model plants as they do not always have the characteristic properties of the target woody plant such as chemical composition or specific stress tolerance. Considering these problems, it is better to consider poplars as experimental materials.

On the other hand, in industrial applications using poplars, various issues of forestry and economy remain, such as productivity and wood quality, control of pests and disease, stressful environmental conditions, labor, costs, industrial demand and the environmental impact of poplar plantations. The developing science and technology of poplars as a model species are also likely to be useful for the solution of these practical problems. Further development of the science and application of poplars will likely contribute to the sustainable supply of forest resources and the conservation of the global environment.

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Publication lists

This thesis is based on the following papers.

1. Mitsuru Nishiguchi, Kazumasa Yoshida, Takeshi Mohri, Tomohiro Igasaki and Kenji Shinohara (2006) An improved transformation system for Lombardy poplar (*Populus nigra* var. *italica*). Journal of Forest Research, 11: 175-180.

2. Mitsuru Nishiguchi, Tokihiko Nanjo and Kazumasa Yoshida (2012) The effects of gamma irradiation on growth and expression of genes encoding DNA repair-related proteins in Lombardy poplar (*Populus nigra* var. *italica*). Journal of Environmental Radioactivity, 109: 19-28.

Tables

Table 1. Taxonomy and distribution of 32 species of the genus *Populus*.

Section	Species	Distribution
Abaso	*P. mexicana* Wesmael	Mexico
Turanga	*P. euphratica* Olivier	North Africa and Asia
	P. ilicifolia (Engler) Rouleau	Kenya
	P. pruinosa Schrenk	China and Central Asia
Leucoides	*P. glauca* Haines	India and China
	P. heterophylla Linnaeus	Eastern North America
	P. lasiocarpa Oliver	China
Aigeiros	*P. deltoides* Marshall	North America
	P. fremontii S. Watson	North America
	P. nigra Linnaeus	Europe, North Africa and West Asia
Tacamahaca	*P. angustifolia* James	North America
	P. balsamifera Linnaeus	North America
	P. cathayana Rehder	China
	P. ciliate Royle	Central Asia
	P. koreana Rehder	East Asia
	P. laurifolia Ledebour	Asia
	P. maximowiczii Henry	East Asia
	P. simonii Carrière	Asia
	P. suaveolens Fischer	Asia
	P. szechuanica Schneider	China
	P. trichocarpa Torrey & Gray	North America
	P. yunnanensis Dode	China
Populus	*P. adenopoda* Maximowicz	China
	P. alba Linnaeus	Europe and North Africa
	P. gamblei Haines	Himalaya
	P. grandidentata Michaux	Eastern North America
	P. guzmanantlensis Vazquez & Cuevas	Mexico
	P. monticola Brandegee	Mexico
	P. sieboldii Miquel	Japan
	P. simaroa Rzedowski	Mexico
	P. tremula Linnaeus	Eurasia
	P. tremuloides Michaux	North America

(Eckenwalder 1996; Dickmann and Kuzovkina 2014)

Table 2. Area of poplar forests in 2011.

Category	Country	Area (thousand ha)
Indigenous forests	Canada	30,296
	Russian Federation	24,757
	USA	17,653[a]
	China	2,530
	India	47
	Italy	42
	France	40[a]
	Other countries	99
	Subtotal	75,464
Planted forests	China	7,570
	France	236[a]
	Iran	150
	Turkey	125
	Spain	105
	Italy	101
	Sweden	49
	Romania	48
	USA	45[a]
	Canada	44
	Argentina	41
	Other countries	132
	Subtotal	8,646
Agroforestry	China	2,800
	India	315
	Other countries	57
	Subtotal	3,172
Total		87,282

[a] The data was reported in 2008.
(FAO 2012)

Table 3. Poplar wood production in 2011.

Country	Product (thousand m^3)
China	50,000[a]
India	5,290
Canada	4,450
Turkey	3,501
Argentina	1,639
Iran	1,460
Italy	973
Belgium	780
Spain	675
Other countries	1,584
Russian Federation	NR
USA	NR
Total	70,352

NR, not reported.
[a] The data was estimated by the consumption.
(FAO 2012)

Table 4. Uses for poplar wood in 2011.

Use	Product (thousand m^3)
Round timber (for logs or pulp)	3,415
Sawn timber	2,307
Veneer sheets	1,162
Plywood	42,919
Composites	2,190
Pulp	16,626
Fuelwood	1,734
Total	70,352

(FAO 2012)

Table 5. Relationship between biological properties and applications of poplars.

Event/field of study	Biological property	Related issue
Growth	Fast growth	Productivity Costs of silviculture
	Wood production (lignification)	Suitable quality of wood for applications
	Susceptibility and tolerance to abiotic and biotic stresses	Productivity Conservation Improvement of stress tolerance
Ecology	Threat of extinction of some native species such as *P. nigra*	Conservation of genetic resources and biological diversity
Asexual (vegetative) reproduction	Root suckering Easy to root from cuttings (in mainly *Aigeiros* and *Tacamahaca*)	Natural regeneration Plantation Ecological invasion
Sexual reproduction	Dioecism	Difficulty of inbreeding
	Interspecific crossability	Crossbreeding
	Wind dispersal of pollen and seeds	Ecological invasion Gene flow from transgenic poplars Male or female sterility
	Long juvenile period of 5–10+ years	Long breeding period Flowering control
Genetics and genomics	Small genome size (485 MBp, 2C = 1.0 pg) Richest genetic and genomic information in woody plants (Genes, genome sequences, ESTs and molecular markers)	Extension of genetic and genomic resources Utilization for breeding and conservation
Biotechnology and genetic engineering	*In vitro* culture system Earliest and advanced transgenic technology in woody plants	Technological development Generation of excellent transgenic poplars Safety management of transgenic poplars

Table 6. PCR primers used for cDNA cloning, semi-quantitative RT-PCR/Southern blot assay and RT-qPCR analysis.

Gene	Primer sets	
cDNA cloning		
PnRAD51	5′-GAGCTCAGTAGATCTCTGGAN-3′	5′-CAGGAGATGATAACTAAAATAGN-3′
PnKU70	5′-ATCAACAAAGAAAGAAAGAAATG-3′	5′-CTCATGCCCTTGTGAAAACA-3′
PnLIG4	5′-GAAAAAAAAAGCCCCCGAAAATG-3′	5′-GCAACAAAATACACTTAAGTTGCTA-3′
Semi-quantitative RT-PCR/Southern blot assay		
PnRAD51	5′-TGGTCAGAGGCAAGAGATAA-3′	5′-ACACCGAACTCATCTGCTAA-3′
PnKU70	5′-TAGTGGTGGTGGTCAGGTTG-3′	5′-AGCTGCATTTTCGACAGCTG-3′
PnLIG4	5′-CTAAATTCCGCAAATTCATCG-3′	5′-ATTCAGCTCCTCAATCGTCA-3′
PnXRCC4	5′-AAGGCACTTGGTTTCCCTTT-3′	5′-TGTGCCAAACACTTCTCAGC-3′
PnOGG1	5′-CTGCCAAATCAGCTCTCCTC-3′	5′-CAAGCCATTTCACACCTCCT-3′
PnPCNA	5′-TCAAATCGGAGGGTTTTGAG-3′	5′-TTCTGGCTTGTCAACAGTGG-3′
PnPGK	5′-TGCTCAGAGGCAAGAGATAA-3′	5′-AGGTCAACCTTCTCCAAGAG-3′
RT-qPCR analysis		
PnRAD51	5′-CTGGACCTCAAATCAAGCCTA-3′	5′-GATCTGGAACCGTGCTTCA-3′
PnKU70	5′-CCCTGGTTTGCAGAGACACTA-3′	5′-CTGCTTTAACAACTCCTGGTCTG-3′
PnLIG4	5′-GGACATGCCGAGTGCTTC-3′	5′-TCTCCGTCCGATTACTTCTCC-3′
PnXRCC4	5′-ACAGAGAGGCATGGTGAAGTG-3′	5′-CCTCGGGAGAAACTTGCAG-3′
PnOGG1	5′-TGGCCGTAAATCTTCAAACTG-3′	5′-CTTCCAGACCAGAAAATGGTG-3′
PnPCNA	5′-ACAAGCCAGAAGATGCAACAG-3′	5′-CAACCACAACAGGCAGGTC-3′
PnUBQ5	5′-ACCTACACGAAGCCCAAGAAG-3′	5′-CATGAAAGTACCAGCACCACA-3′

Table 7. Properties of the predicted DNA repair-related proteins from *P. nigra*.

cDNA	Accession number	Length of the predicted protein (Amino acid residues)	Identity[a] (%) of amino acid residues to homologous proteins[b]			
			P. trichocarpa	*A. thaliana*	humans	yeast
PnRAD51	AB269815	342	91.9 POPTR_0006s13750	90.4 U43528	68.9 D14134	53.5 D10023
PnLIG4	AB269887	1319	85.7 POPTR_0018s13870	58.7 AF233527	22.1 BC037491	17.2 Z74913
PnKU70	AB270699	627	98.7 POPTR_0011s10870	71.0 AF283759	31.7 AK055786	17.4 D15052
PnXRCC4	AB270700	255	84.0 POPTR_0010s08650	58.2 AF233528	19.9 U40622	10.9 Z72612
PnOGG1	AB270701	378	95.5 POPTR_0005s20290	58.7 AJ302082	32.4 U96710	27.4 U44855
PnPCNA	AB041506	264	99.6 POPTR_0009s04560	86.9 AF083220	64.8 M15796	35.2 X16676

[a] The sequence identity was observed from the global alignment of the predicted amino acid sequence of the DNA repair protein between *P. nigra* and other organisms using the Needleman-Wunsch algorithm.

[b] Locus name of gene model in the *P. trichocarpa* genome assembly v2 (http://www.phytozome.net/poplar) and GenBank accession number of the homologous protein are represented under each amino acid sequence identity.

Table 8. Biological effects of γ-rays on *P. nigra*.

Dose of γ-rays (Gy)	Growth of plantlets	Morphogenesis	*In vitro* regeneration of shoot and root	Damage of nuclear DNA	
				Average tail DNA%[a]	% of a few damaged cells[b]
0	NE	NE	NE	27.2 ± 14.6	35.8
50	NE	Some transient mutations in leaves and stems	NE	24.3 ± 16.4	48.6
100	Transient or completely growth arrest but no withering, and sprouting	Transient mutations in leaves and stems	Suppression of shoot regeneration, No roots	43.0 ± 15.7	6.6
200	Completely growth arrest and withering after 4–10 weeks	Stopped	No shoots or roots	38.6 ± 14.9	7.7
300	Completely growth arrest and withering after 4–10 weeks	Stopped	ND	36.7 ± 12.0	4.9

NE, no effect. ND, not determined.
[a] Average percentage of DNA in the tail compared to the total DNA in the Comet assay.
[b] The rate of cells of which tail DNA% was in the range of 0 to 20%.

Table 9. Composition of shoot-induction medium (SIM) for transformation of *P. nigra*.

Medium composition
1 × Murashige and Skoog basal salts
1 × Murashige and Skoog vitamin
3% sucrose
20 mM MES-KOH (pH 5.8)
0.5 mg l^{-1} *trans*-zeatin
0.1 mg l^{-1} 6-benzylaminopurine
50–100 mg l^{-1} kanamycin
500 mg l^{-1} cefotaxime
0.8 % Bacto Agar

Table 10. Composition of root-induction medium (RIM) for transformation of *P. nigra*.

Medium composition
0.5 × Murashige and Skoog basal salts
1 × Murashige and Skoog vitamin
3% sucrose
20 mM MES-KOH (pH 5.8)
0.5 mg l^{-1} indole-3-butyric acid
0.02 mg l^{-1} 1-naphthaleneacetic acid
20 mg l^{-1} kanamycin
250 mg l^{-1} cefotaxime
0.6 % Phytagar

Table 11. Frequency of the transformation of *P. nigra* with two types of binary vectors in the presence and absence of dithiothreitol (DTT).

Vector	DTT	Number					Frequency of trans-formation (%)[a]
		Explants infected with *Agrobacterium*	Shoots transferred to RIM	Shoots rooted on RIM	*NPTII* positive shoots	Genetically independent transformants	
pBF2::EGFP	–	111	94	20	18	13	11.7
pIG121Hm	–	106	61	12	8	7	6.6
pBF2::EGFP	+	93	115	28	25	18	19.4
pIG121Hm	+	98	151	41	39	21	21.4

[a] Transformation frequency was calculated from the number of genetically independent transgenic poplar plants divided by the number of *Agrobacterium*-infected explants.

Table 12. Relationship between the radiation sensitivity and the nuclear DNA content of woody plant species.

Species	LD_{100} (Gy)[a]	Nuclear DNA C-value (pg)[b]
Angiosperm		
Populus nigra	>100	0.54
Fraxinus americana	97	1.37[c]
Acer saccharum	78	0.7[c]
Prunus laurocerasus	58	3.65
Clematis virginiana	26	11.96[d]
Sambucus canadensis	19	14.3[c]
Paeonia suffruticosa	10	19.99[d]
Gymnosperm		
Taxus canadensis	16	11.60
Abies balsamea	15	16.40
Sequoiadendron giganteum	15	9.93
Picea abies	13	20.01
Larix laricina	11	9.50
Tsuga canadensis	10	18.60
Pinus strobus	7	25.65

[a] Radiation sensitivity of each woody plant species is shown as the dose of γ-rays that caused 100% mortality (LD_{100}) (Sparrow et al. 1968). Although the original data had been measured in the unit of roentgen, they were converted to the unit of gray for comparison with *P. nigra*. One kiloroentgen was calculated as 9.747 Gy (based on absorbed dose to water).

[b] C-values of plant species are cited from the Plant DNA C-value Database (release 6.0, December 2012) (http://data.kew.org/cvalues/).

[c] Cited from Bai et al. (2012).

[d] C-value has not been reported. The mean C-value of the same genus species is shown on the Plant DNA C-value Database.

Figures

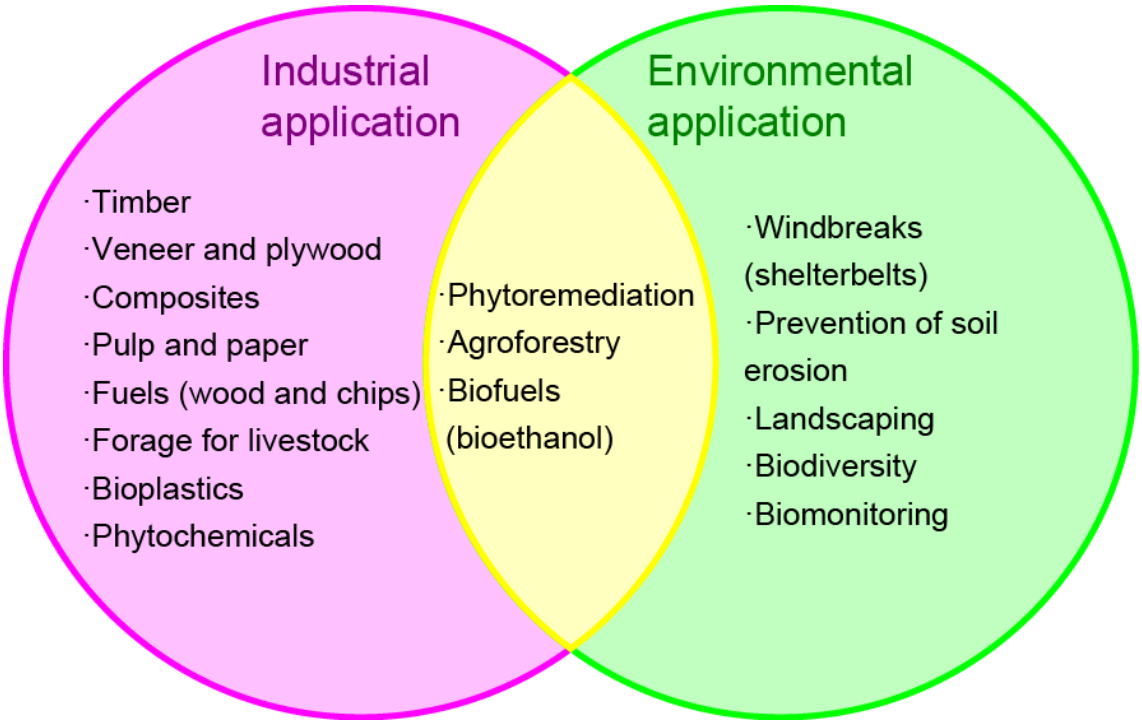


Figure 1. Utilization and applications of poplars. Poplars are versatile and their usage is mainly divided into three groups, industrial application (pink), environmental application (green), and the overlap between them (yellow). In addition to traditional applications such as timber and landscaping, new fields of use have recently been exploited, including, for example, bioplastics, phytochemicals, biofuels and biomonitoring.

Properties

- Fast growth
- Simple clonal propagation by cutting
- Frequent use as a parent for crossbreeding
- Ecological importance as a native riparian pioneer tree and genetic resources
- Various biological information about physiology, ecology and molecular biology
- Availability of *in vitro* cell culture, organization (totipotency) and genetic engineering
- Rich genomic resources such as genes and ESTs

17-year-old *P. nigra*
(Photograph on June 1, 2015 in Forestry and Forest Products Research Institute)

Figure 2. Biological properties of *Populus nigra*. As well as some other *Populus* species, *P. nigra* (black poplar) is considered to be one of the important woody plant model species for scientific research and applications.

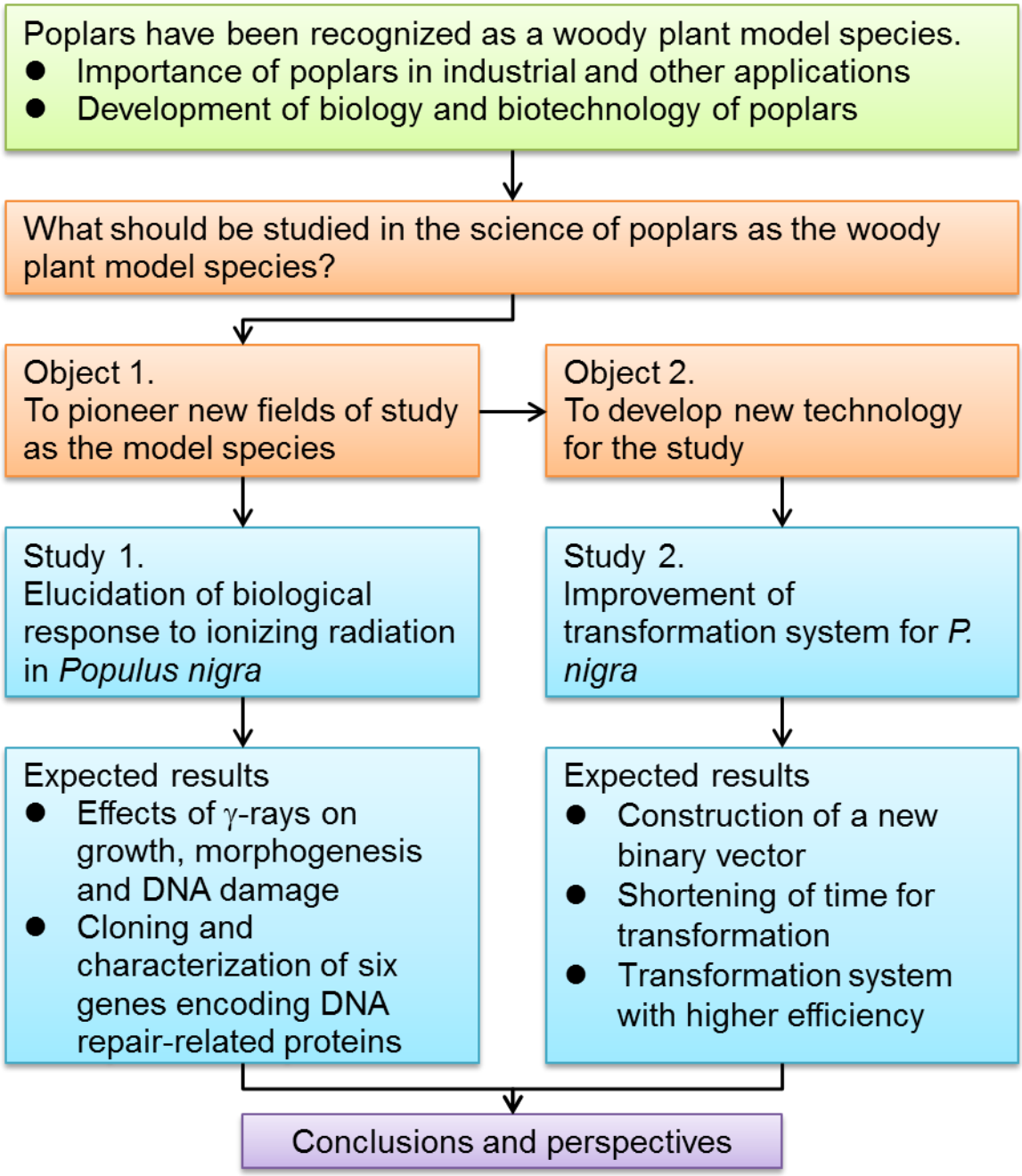


Figure 3. Flow of the research in this thesis. The pioneering of new fields of study and the development of technology are important in poplars as a woody plant model species. Therefore, this study aimed to elucidate the biological response to ionizing radiation in *Populus nigra* and improve its genetic transformation system.

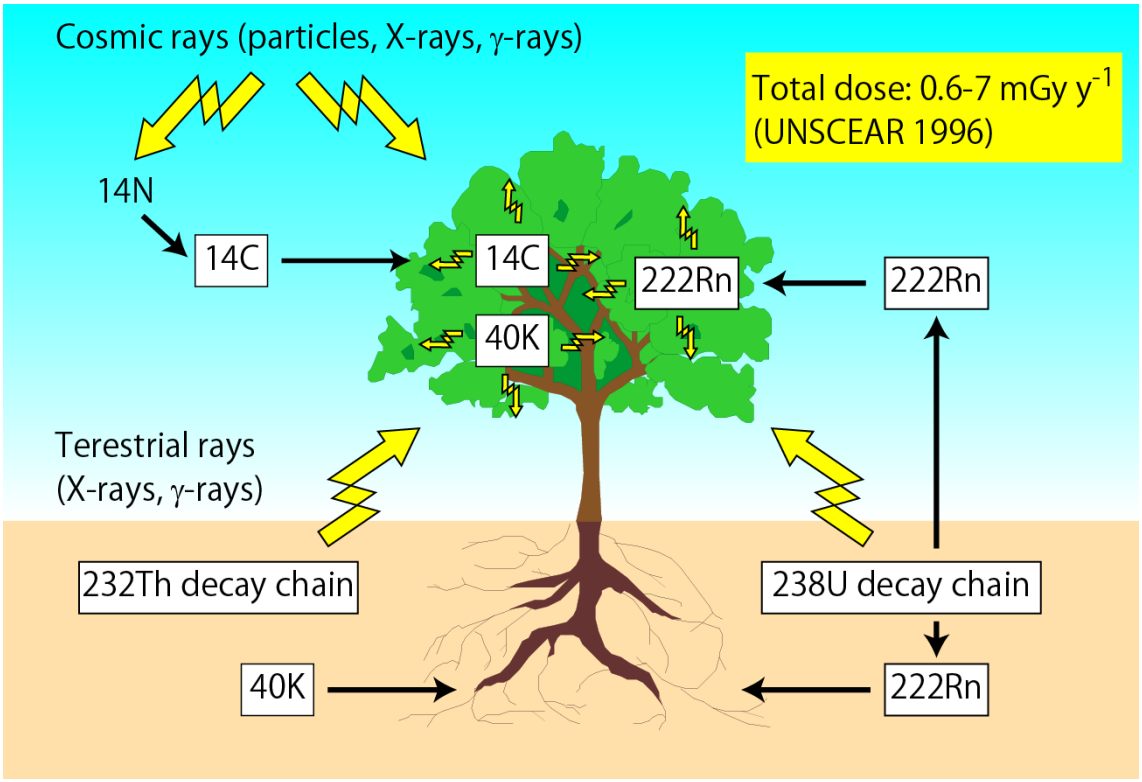


Figure 4. Exposure of trees to natural background radiation. External radiation consists of cosmic rays from space and terrestrial rays from radionuclides in the soil. Internal radiation is emitted from absorbed radionuclides. The total dose to the leaves of trees are estimated to be 0.6–7 mGy per year.

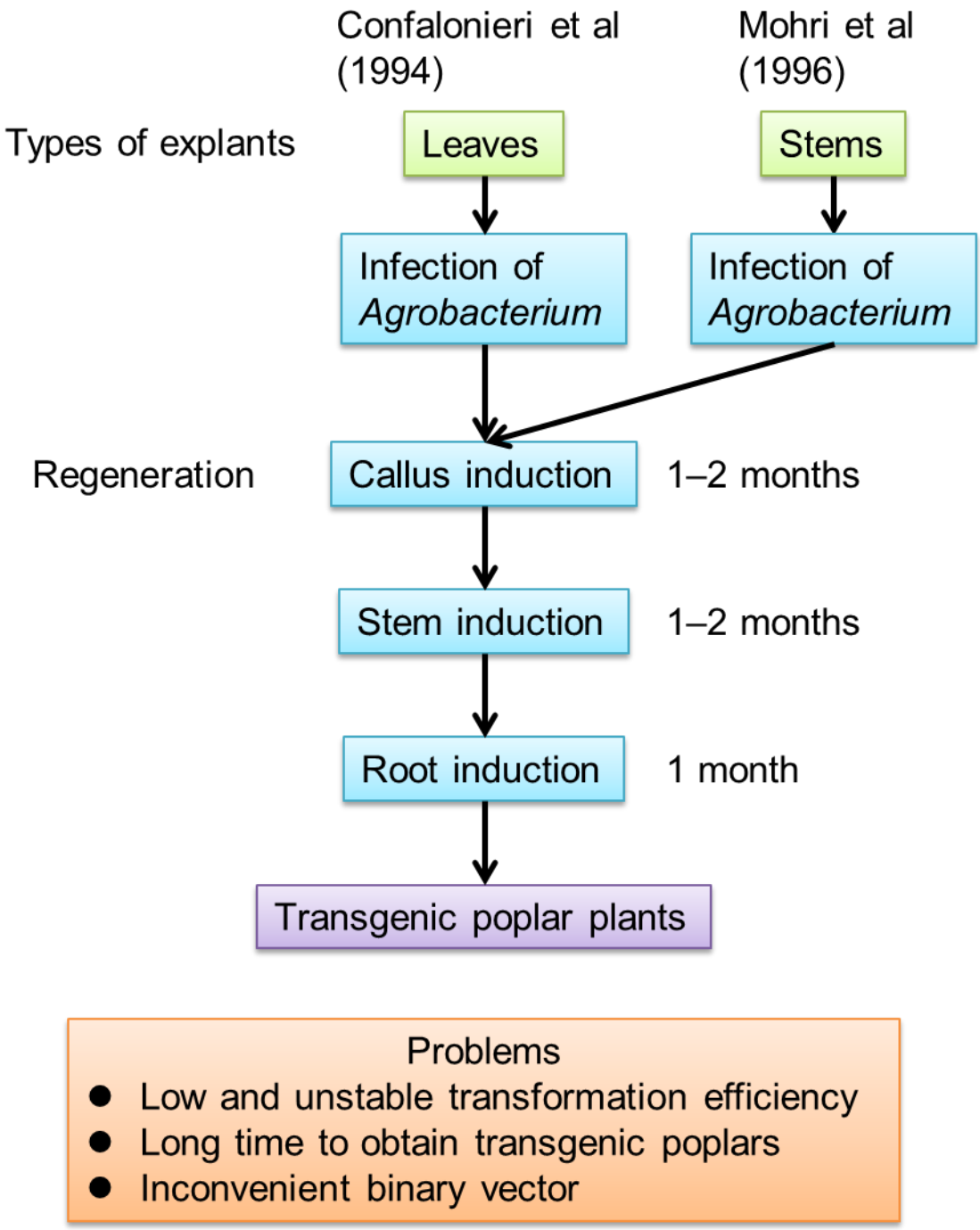


Figure 5. The transformation process of *P. nigra* as reported previously. Using *Agrobacterium*, *P. nigra* can be transformed. The *Agrobacterium*-infected explants (leaves or stems) are regenerated to produce transgenic poplar plants under antibiotic selective pressure. However, the transformation of *P. nigra* was not optimal.

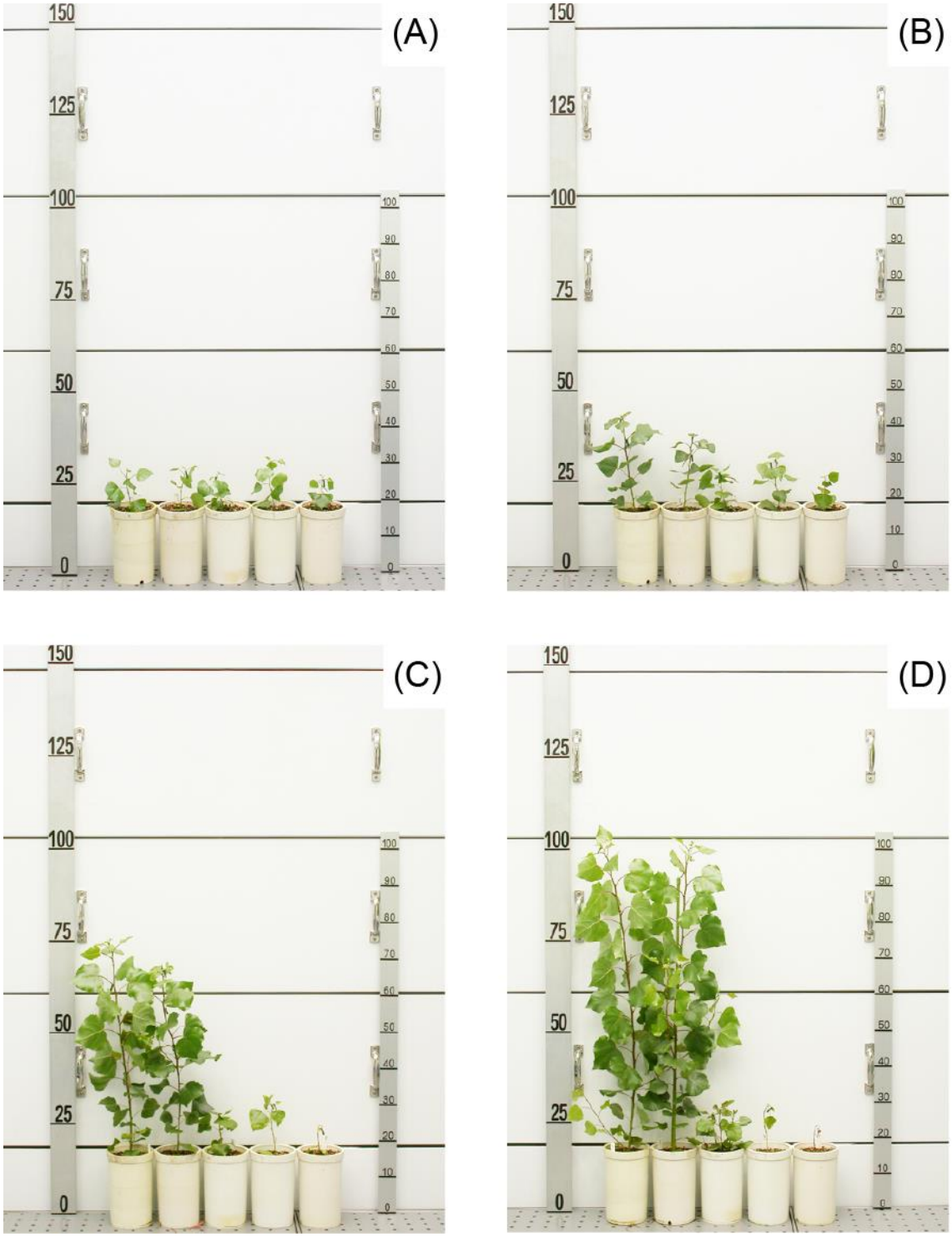
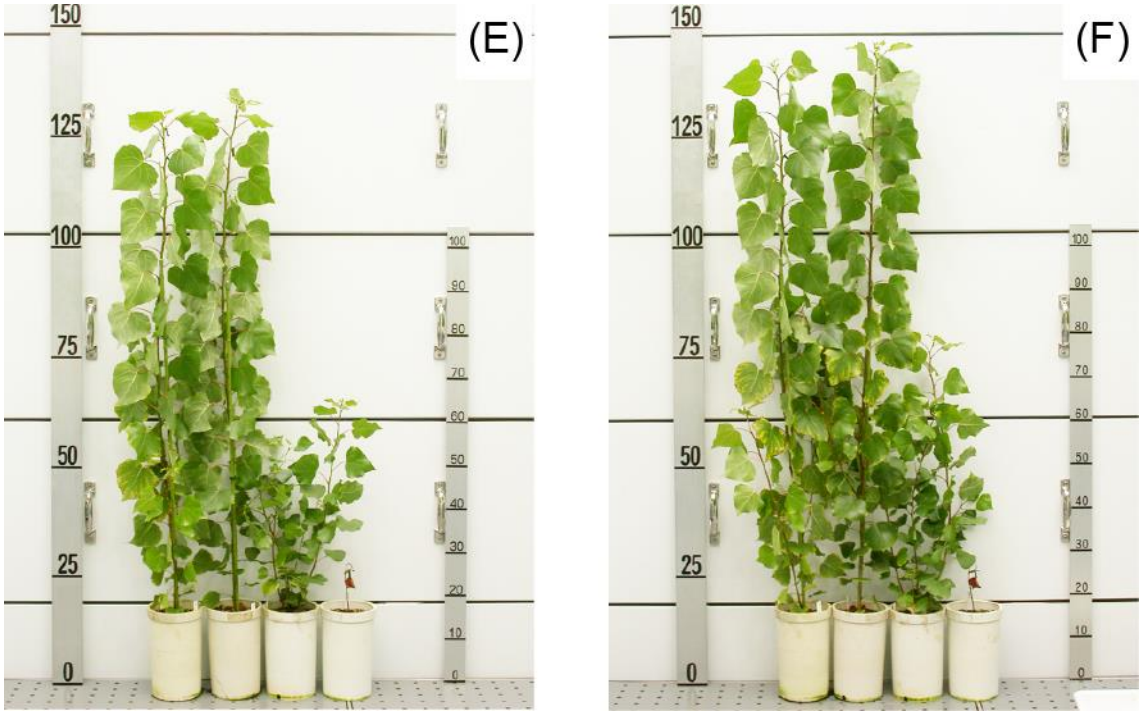


Figure 6. The effect of γ-irradiation on the growth of *P. nigra*. The poplar plantlets were exposed to 0, 50, 100, 200, and 300 Gy of γ-rays (left to right) for 20 h and were then grown under normal conditions. Photographs were taken immediately (A), and 2 weeks (B), 4 weeks (C), 6 weeks (D), 8 weeks (E), and 10 weeks (F) after γ-irradiation. The pot of the 300 Gy of γ-irradiated poplar plant was moved after 8 weeks (E and F). (Continued on next page)



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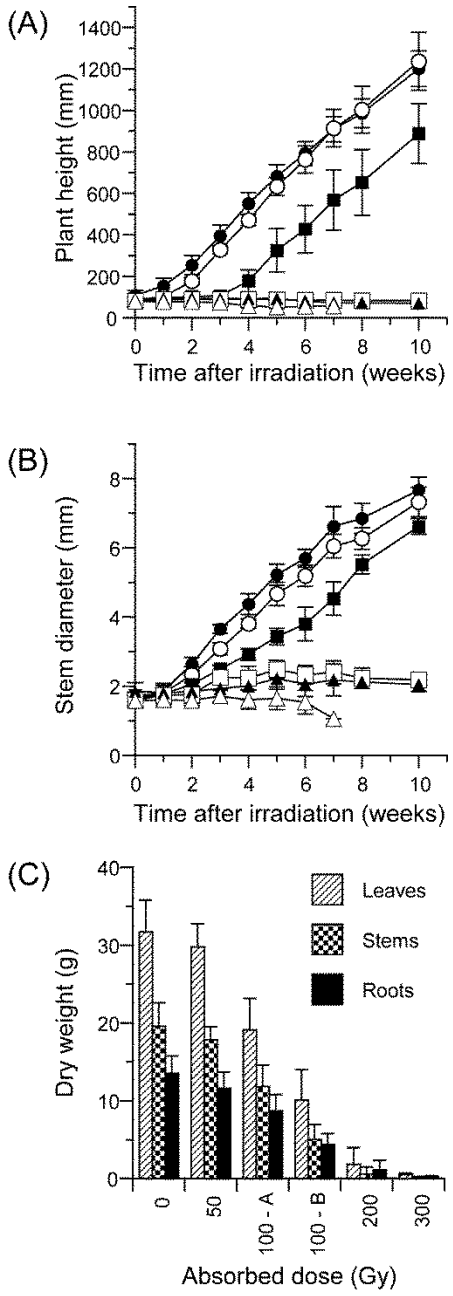


Figure 7. The effect of γ-irradiation on the tree growth of *P. nigra*. Plant height (A) and stem diameter at the ground level (B) of each main stem directly exposed to γ-rays were measured. Closed circles (●), 0 Gy; open circles (○), 50 Gy; closed squares (■), 100 Gy-A group; open squares (□), 100 Gy-B group; closed triangles (▲), 200 Gy; open triangles (Δ), 300 Gy. (C) Dry weight 10 weeks after γ-irradiation or at the time when the entire plant turned brown. Dry weight of leaves and stems includes not only each main stem directly exposed to γ-rays but also axillary branches that sprouted and elongated after γ-irradiation. The number of plantlets was initially eight (n = 8) in each γ-rays group. The plantlets exposed to 100 Gy of γ-rays were separated into two groups, the 100 Gy-A group (n = 3) and the 100 Gy-B group (n = 5) according to their growth. At 200 and 300 Gy, the number of plantlets gradually decreased for 10 weeks ($0 \leq n \leq 8$). The data are expressed as mean ± standard deviation (SD) of the indicated measurements.

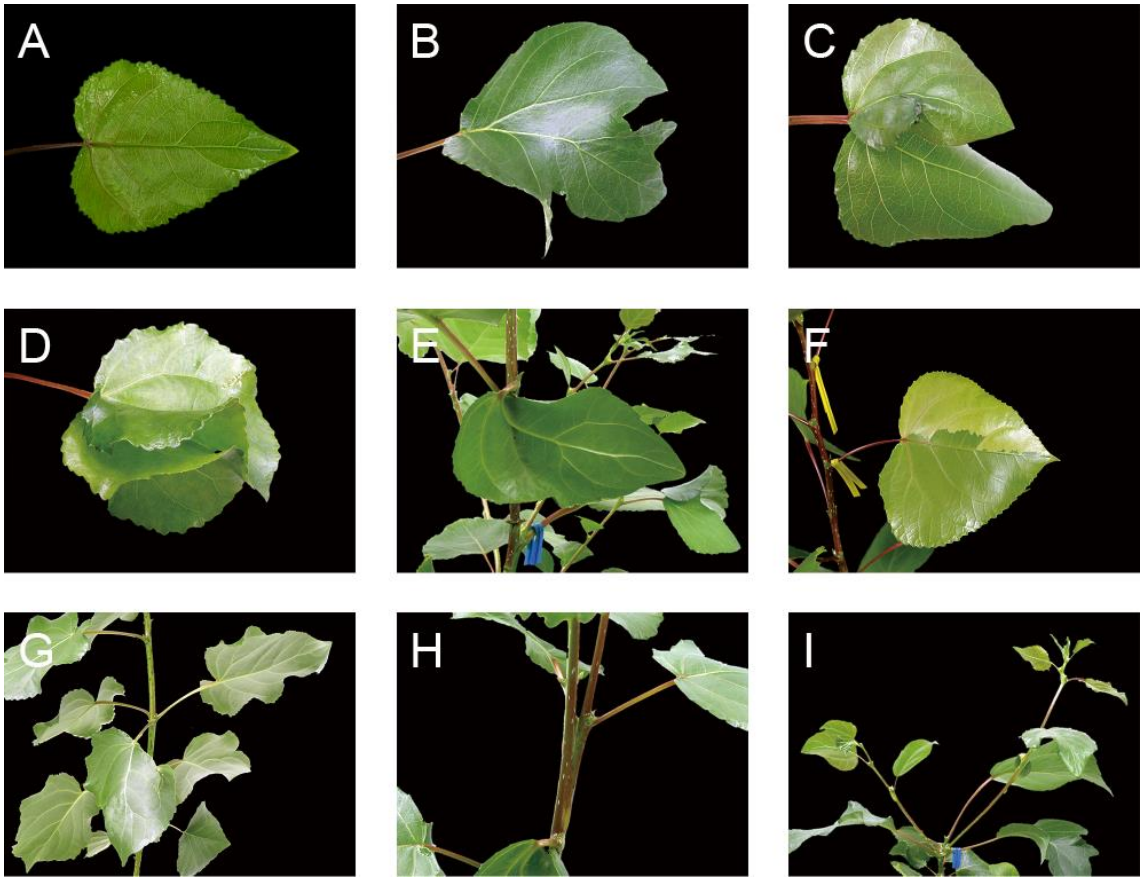


Figure 8. Morphological effects of γ-irradiation on *P. nigra*. (A) A normal deltoid leaf of a non-irradiated poplar plantlet. (B) An oblanceolate and cleft leaf of a γ-irradiated poplar plantlet. (C–D) Two leaf blades with a petiole. (E) An asymmetric leaf with a bent petiole. (F) A mosaic pale green leaf. (G) Severely shortened internodes. (H) A stem branched off and partly fascinated in an internode. (I) Arrest of apical growth and elongated lateral shoots. (B–D) and (H–I) were exposed to 100 Gy of γ-rays. (E–G) were exposed to 50 Gy of γ-rays.

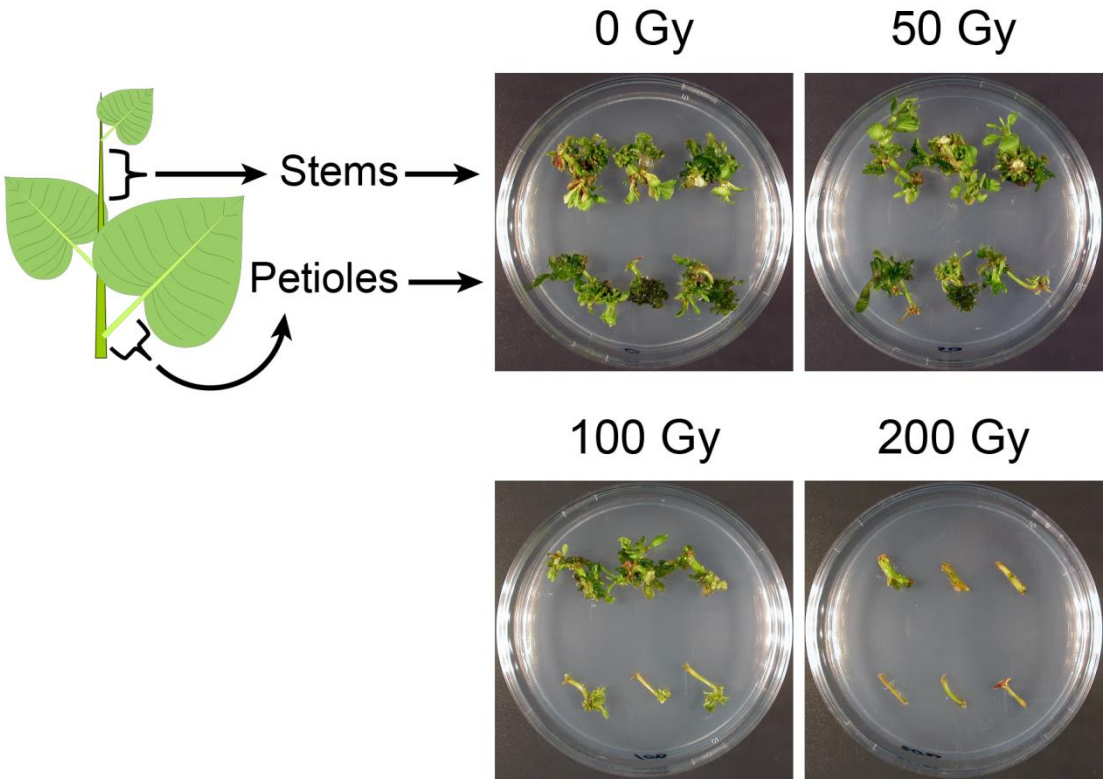


Figure 9. Inhibition of shoot regeneration by γ-irradiation. Stems and petioles were cut off from aseptic poplar plants and exposed to the indicated dose of γ-rays on the shoot-induction medium (SIM). After exposure, they were transferred onto a new SIM and cultivated. Photographs were taken 4 weeks after γ-irradiation.

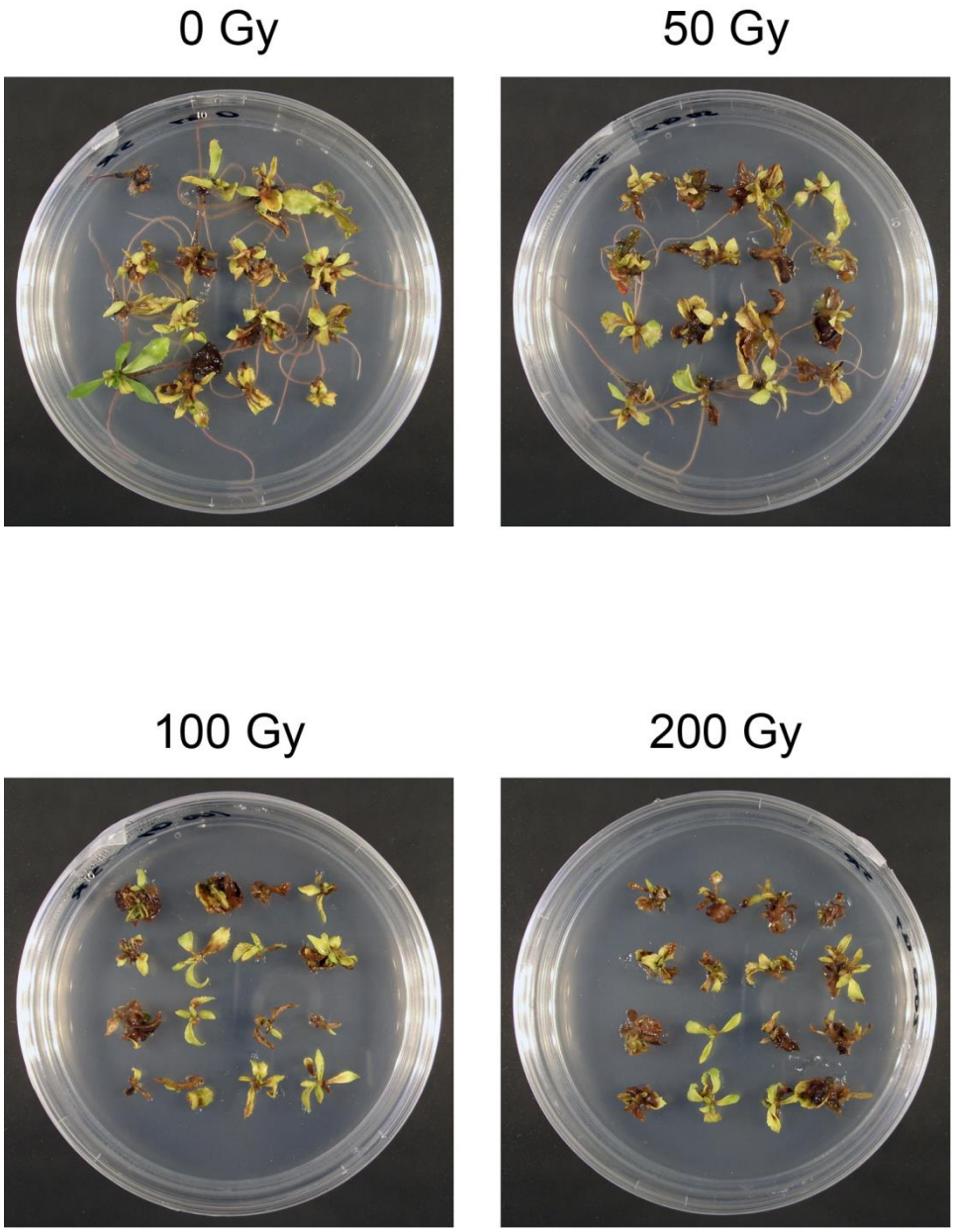


Figure 10. Inhibition of root regeneration by γ-irradiation. Apical stems of aseptic poplar plants (approximately 1 cm in length) were put into root-induction medium (RIM) and exposed to the indicated dose of γ-rays. After exposure, the stems were transferred into a new RIM and cultivated. Photographs were taken 4 weeks after γ-irradiation.

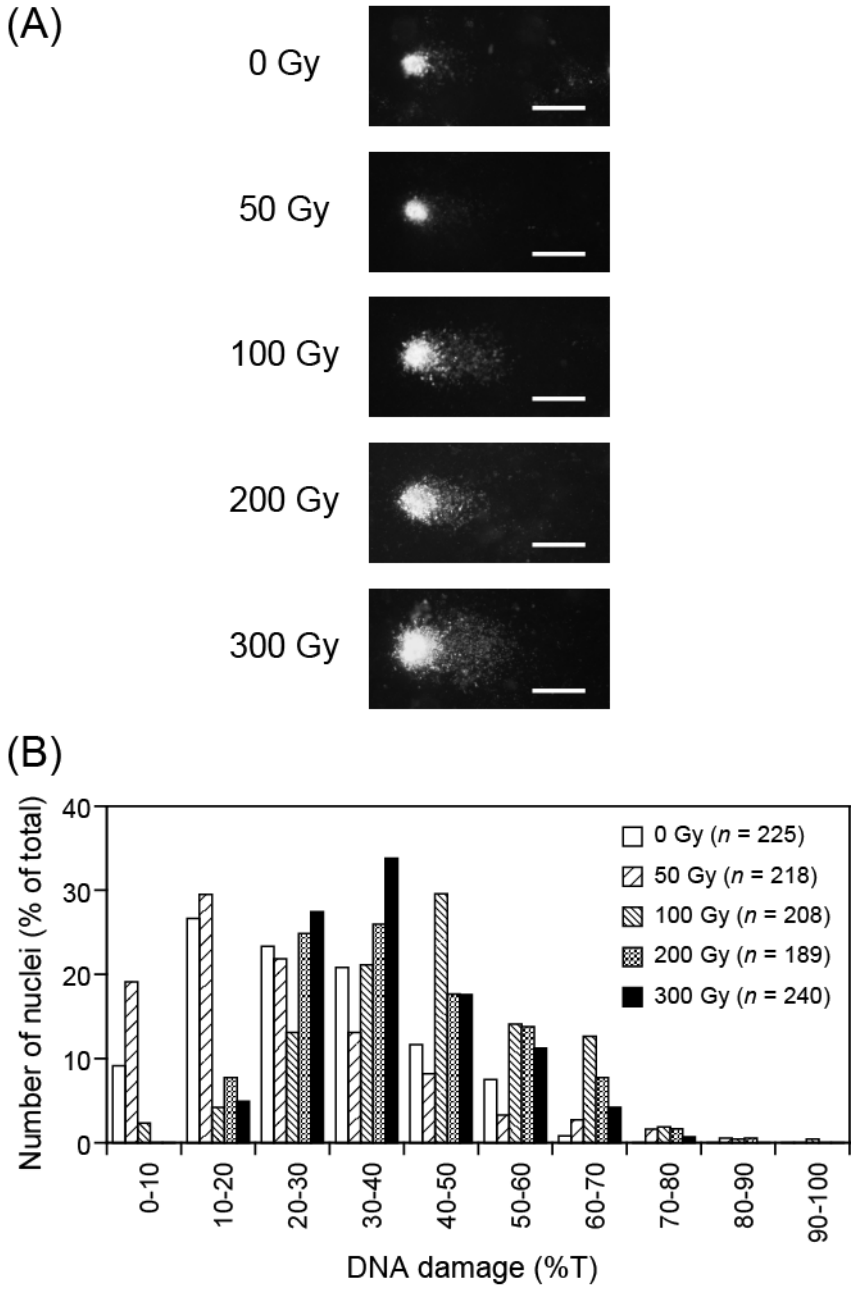


Figure 11. Nuclear DNA damage caused by γ-irradiation in suspension-cultured poplar cells. (A) Photographs of the electrophoresis of nuclear DNA damaged by γ-irradiation. Mid-log phase suspension-cultured cells were exposed to γ-rays at the indicated dose and were subjected to a Comet assay. Scale bar = 50 μm. (B) Comparison of the degree of DNA damage among cells irradiated with γ-rays at doses from 0 to 300 Gy. DNA damage is expressed as %T, which is a percentage of the amount of DNA in the tail divided by the total amount of DNA in the head and the tail. *n* represents the number of nuclei examined by the Comet assay.

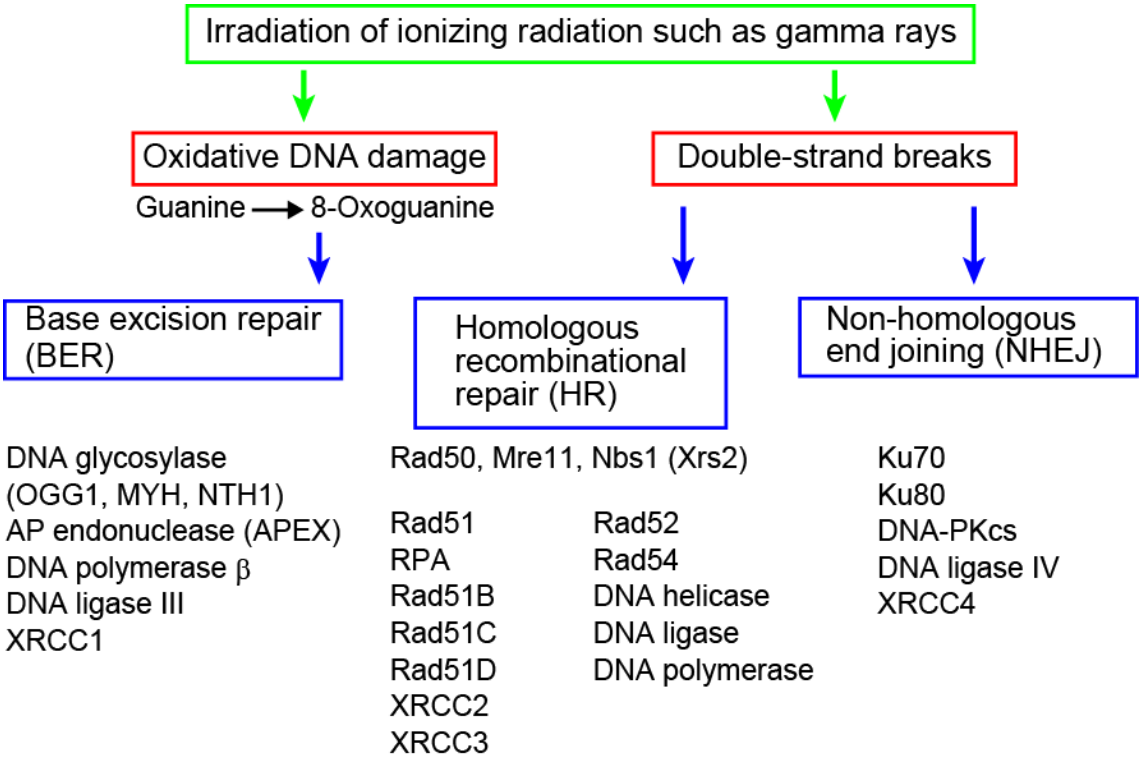


Figure 12. DNA damage and repair system in eukaryotes. Ionizing radiation (IR) induces oxidative DNA damage via reactive oxygen species. IR also directly cleaves DNA strands. Oxidative DNA damage is repaired by the base excision repair system (BER). DNA double-strand breaks are repaired by homologous recombinational repair (HR) or non-homologous end joining (NHEJ). Each repair system consists of various DNA-repair related proteins as shown in the lower part of the figure.

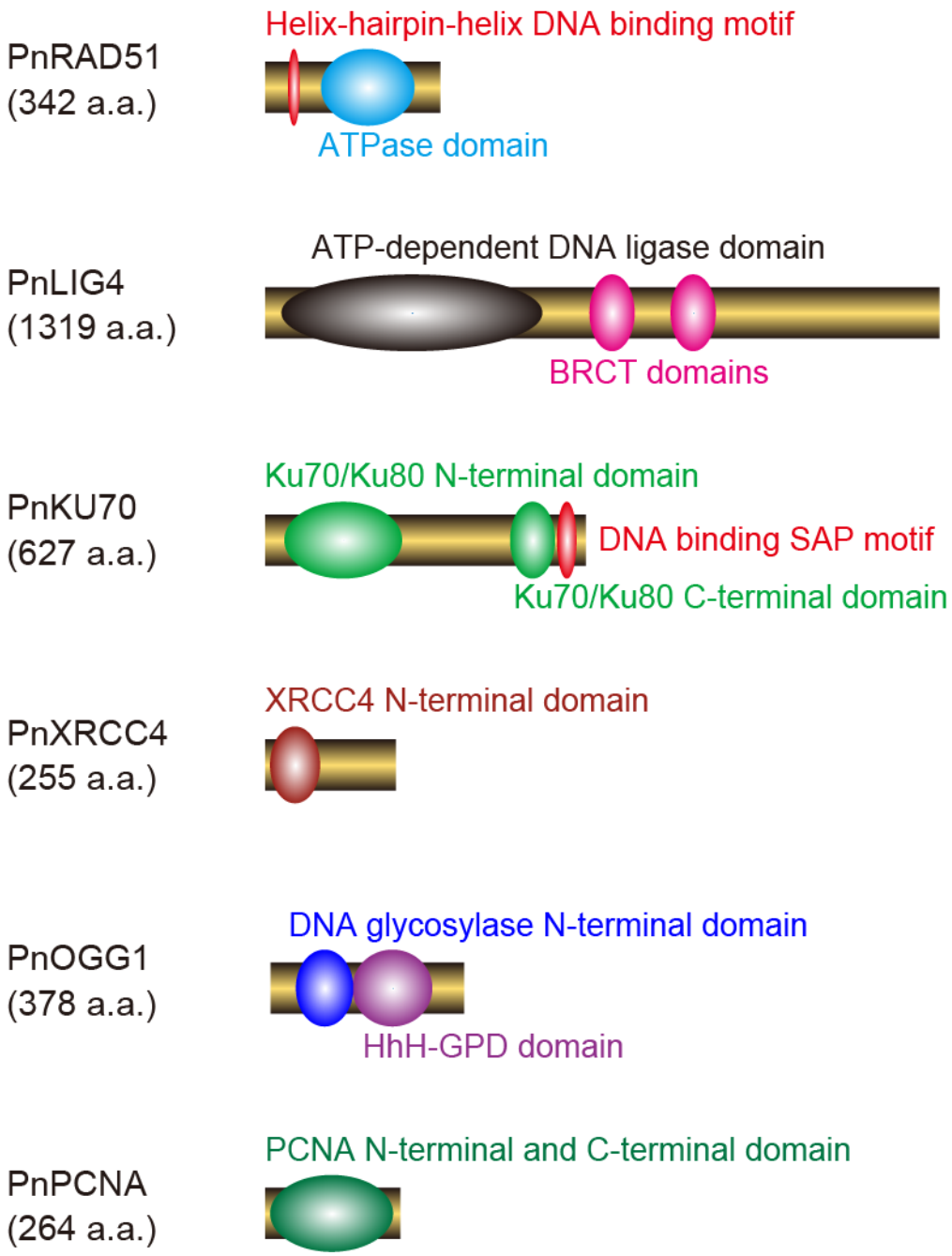


Figure 13. Schematic models of the DNA repair-related proteins predicted from the cDNA of *P. nigra*. The number of amino acid residues of each protein is indicated under the gene/protein name. The predicted functional domains and motifs are shown in each protein model.

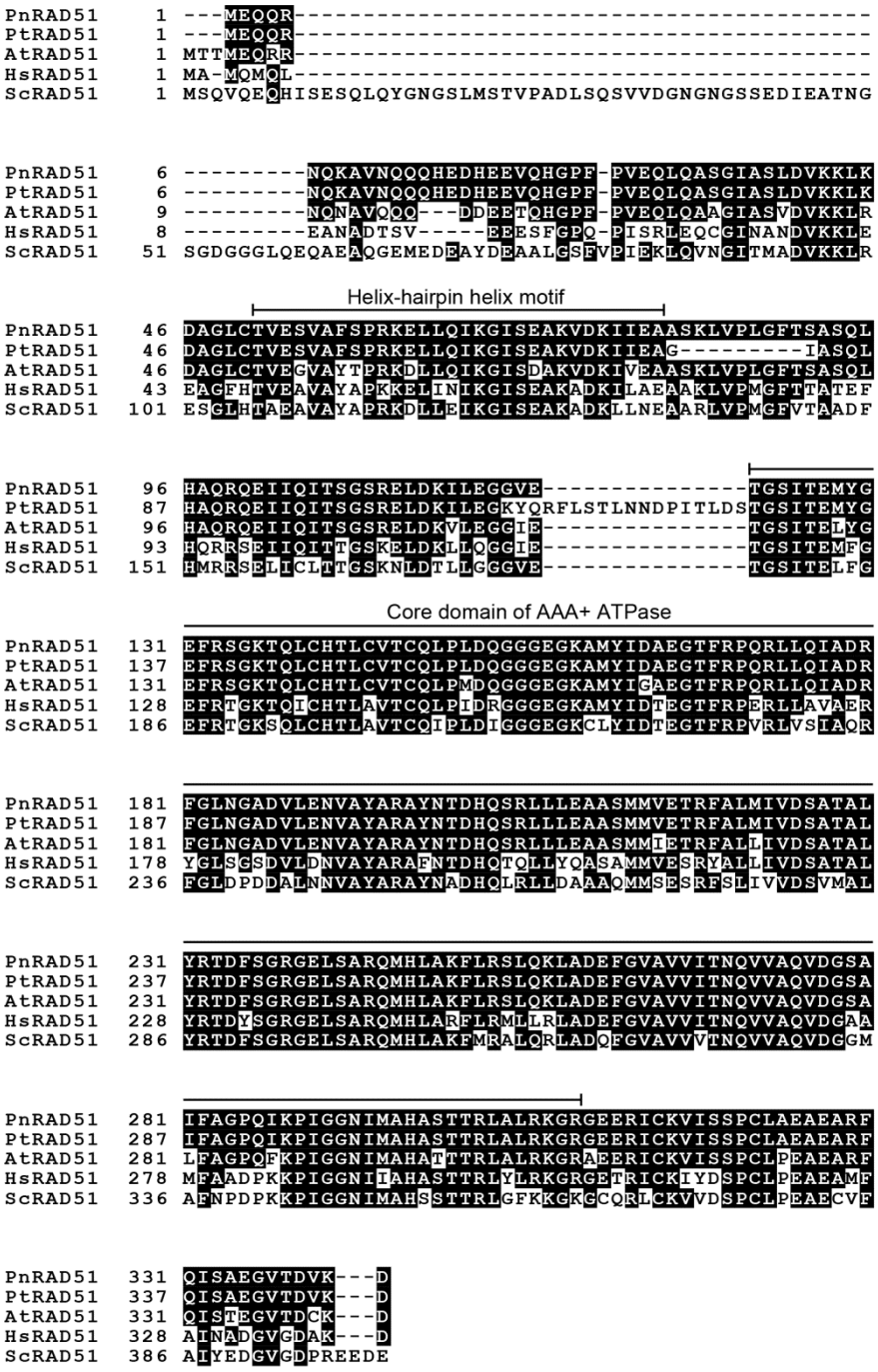


Figure 14. Comparison of the deduced amino acid sequence of PnRAD51 with related sequences. The amino acid identities to PnRAD51 are boxed in black, and gaps in the alignment are denoted by dashes. PtRAD51, a hypothetical protein from *P. trichocarpa* (JGI v2 gene model name: POPTR_0006s13750); AtRAD51, *A. thaliana* RAD51 homolog (GenBank ID: U43528); HsRAD51, human RAD51 homolog (D14134); ScRAD51, budding yeast RAD51 (D10023). The lines indicate the helix-hairpin-helix motif (InterPro ID: IPR000445) and the core domain of AAA+ ATPase (IPR003593).

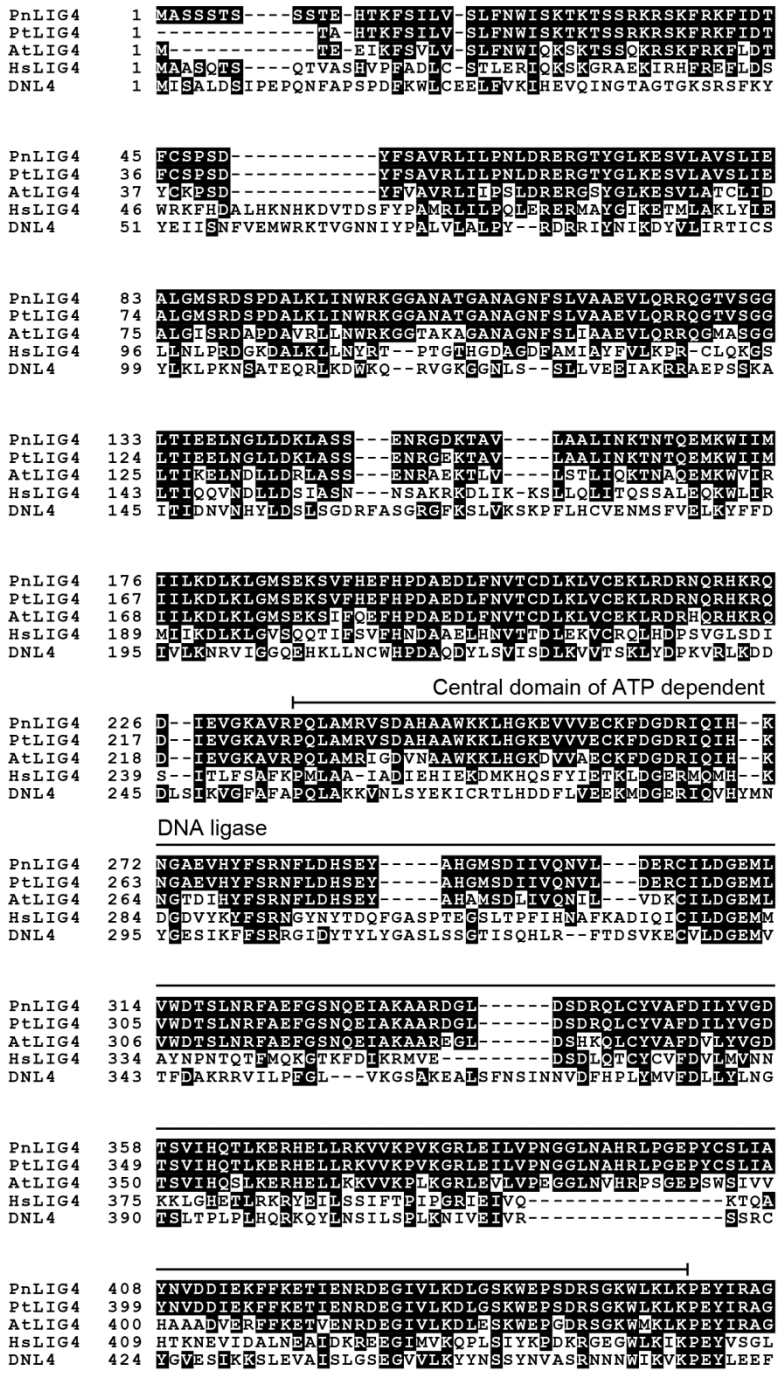
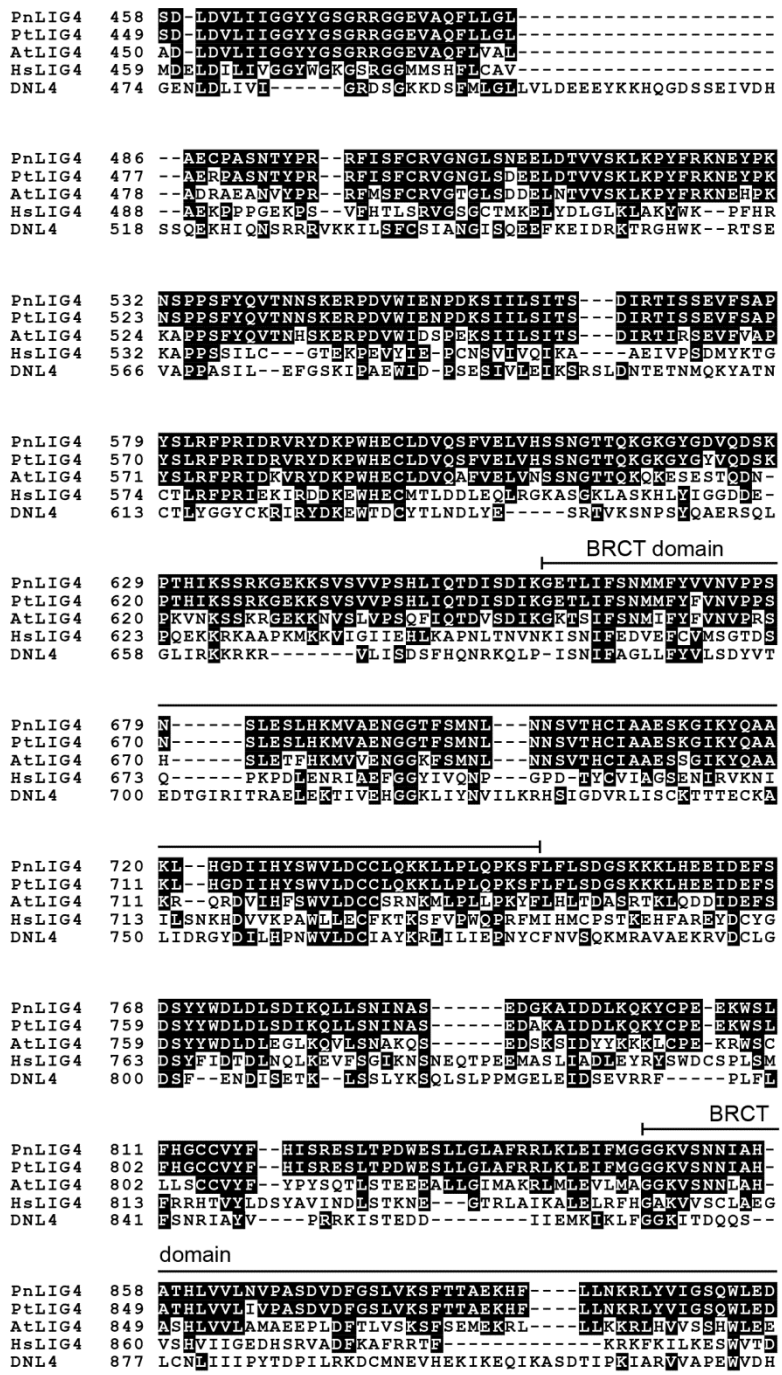


Figure 15. Comparison of the deduced amino acid sequence of PnLIG4 with related sequences. The amino acid identities to PnLIG4 are boxed in black, and gaps in the alignment are denoted by dashes. PtLIG4, a hypothetical protein from *P. trichocarpa* (JGI v2 gene model name: POPTR_0018s13870); AtLIG4, *A. thaliana* DNA ligase IV homolog (GenBank ID: AF233527); HsLIG4, human DNA ligase IV (BC037491); DNL4, budding yeast DNA ligase IV (Z74913). The lines indicate the central domain of ATP dependent DNA ligase (InterPro ID: IPR012310) and two BRCT domains (IPR001357). (Continued on next page)



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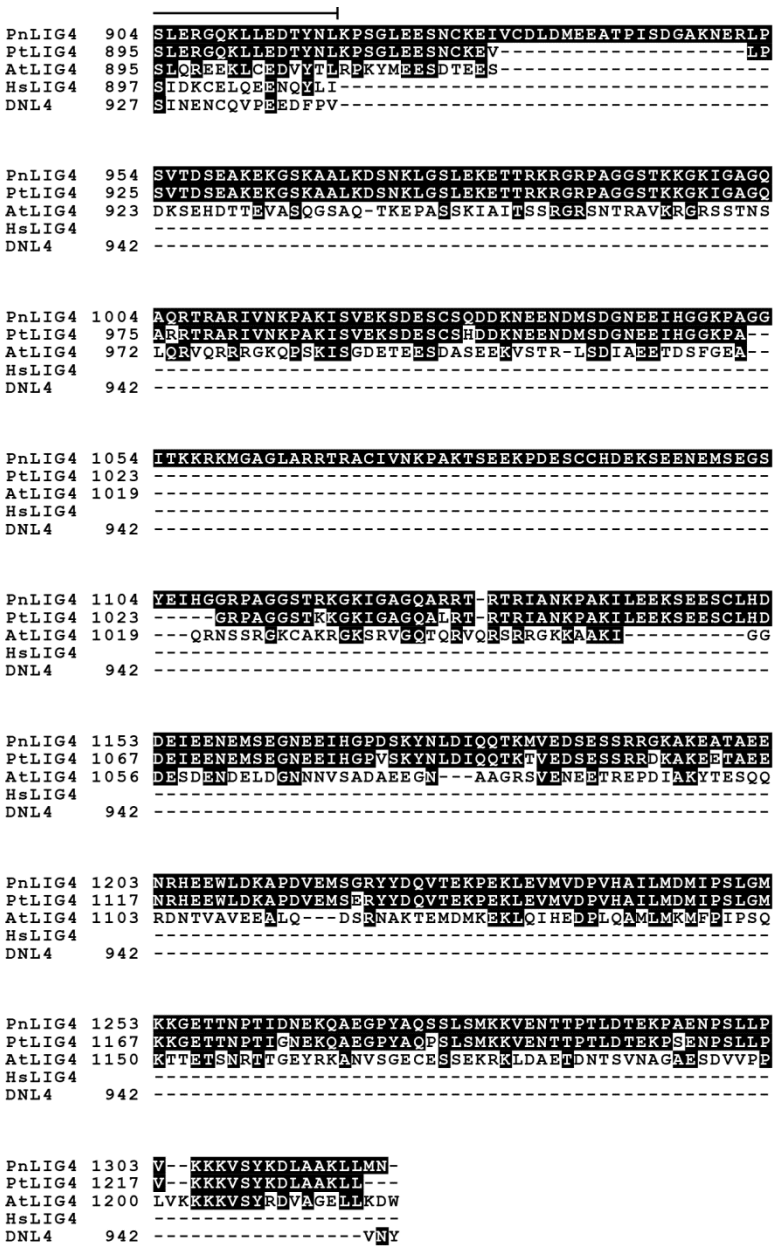

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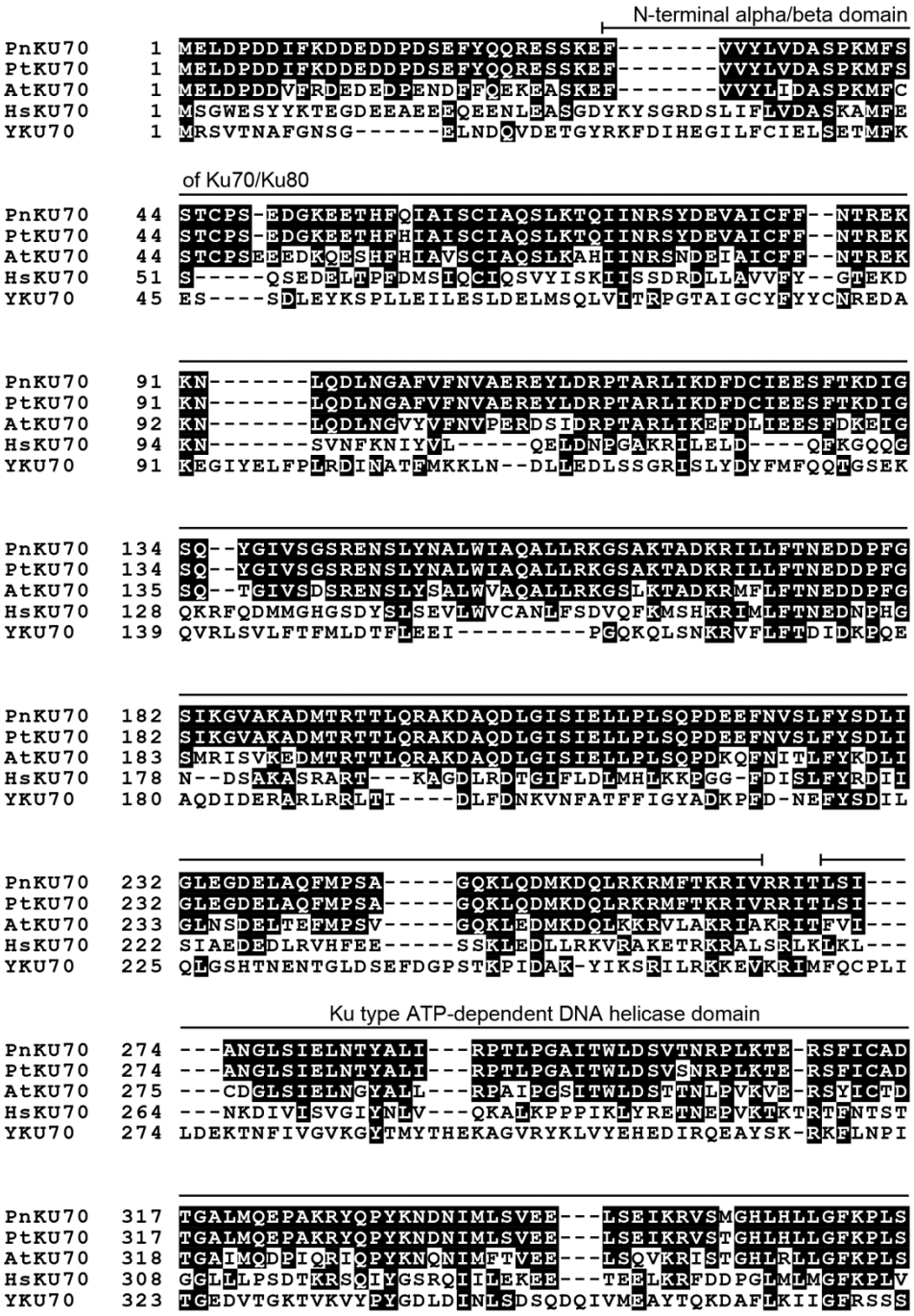
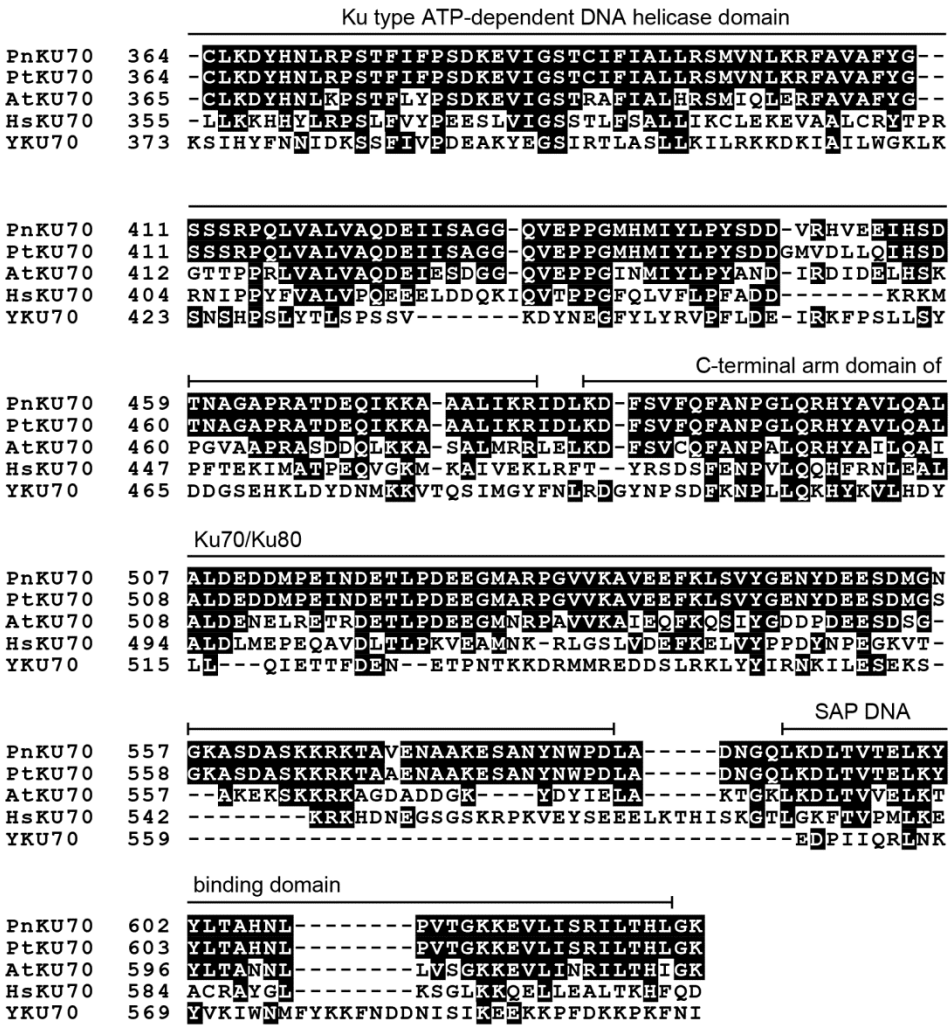


Figure 16. Comparison of the deduced amino acid sequence of PnKU70 with related sequences. The amino acid identities to PnKU70 are boxed in black, and gaps in the alignment are denoted by dashes. PtKU70, a hypothetical protein from *P. trichocarpa* (JGI v2 gene model name, POPTR_0011s10870); AtKU70, *A. thaliana* KU70 homolog (GenBank ID: AF283759); HsKU70, human KU70 subunit (XRCC6) (AK055786); YKU70, YKU70 Subunit in budding yeast (D15052). The lines indicate the N-terminal alpha/beta domain of Ku70/Ku80 (InterPro ID: IPR005161), the Ku type ATP-dependent DNA helicase domain (IPR006164), the C-terminal arm domain of Ku70/Ku80 (IPR005160) and the SAP DNA binding domain (IPR003034). (Continued on next page)



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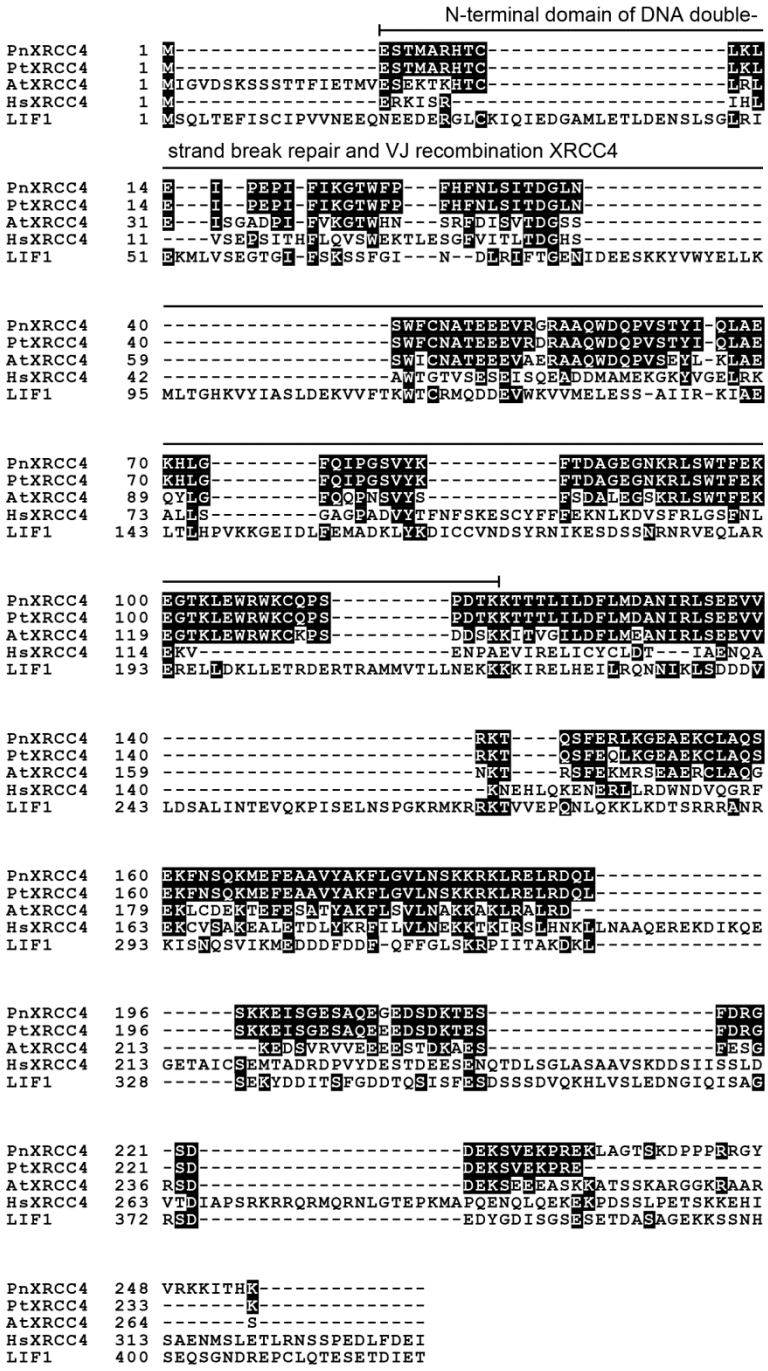

Figure 17. Comparison of the deduced amino acid sequence of PnXRCC4 with related sequences. The amino acid identities to PnXRCC4 are boxed in black, and gaps in the alignment are denoted by dashes. PtXRCC4, a hypothetical protein from *P. trichocarpa* (JGI v2 gene model name, POPTR_0010s08650); AtXRCC4, *A. thaliana* XRCC4 homolog (GenBank ID: AF233528); HsXRCC4, human XRCC4 (U40622); LIF1, budding yeast XRCC4 homolog (Z72612). The line indicates the N-terminal domain of DNA double-strand break repair and VJ recombination XRCC4 (InterPro ID: IPR009089).

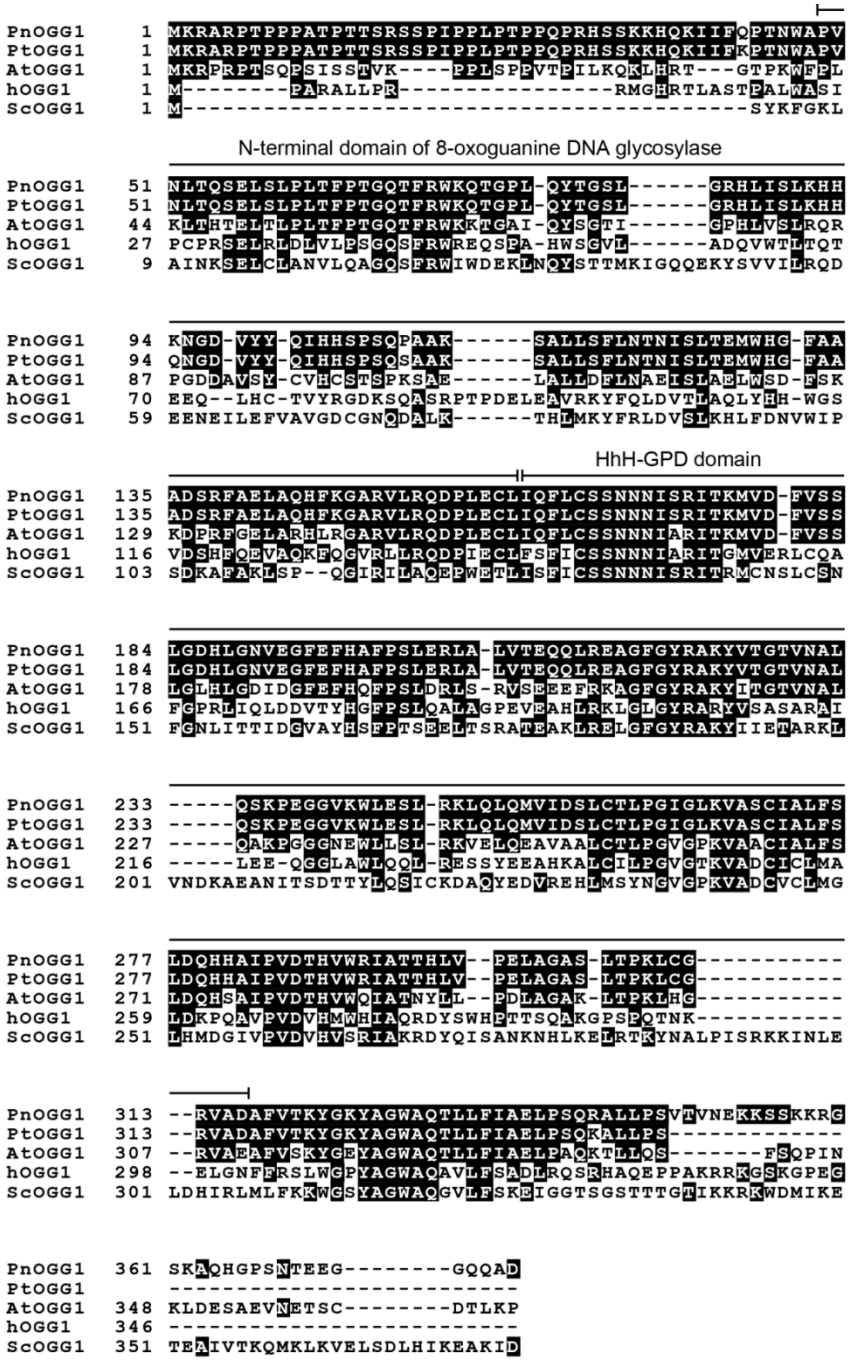

Figure 18. Comparison of the deduced amino acid sequence of PnOGG1 with related sequences. The amino acid identities to PnOGG1 are boxed in black, and gaps in the alignment are denoted by dashes. PtOGG1, a hypothetical protein from *P. trichocarpa* (JGI v2 gene model name, POPTR_0005s20290); AtOGG1, *A. thaliana* OGG1 homolog (GenBank ID: AJ302082); hOGG1, human OGG1 (U96710); ScOGG1, budding yeast OGG1 (U44855). The lines indicate the N-terminal domain of 8-oxoguanine DNA glycosylase (InterPro ID: IPR012904) and the HhH-GPD domain (IPR003265).

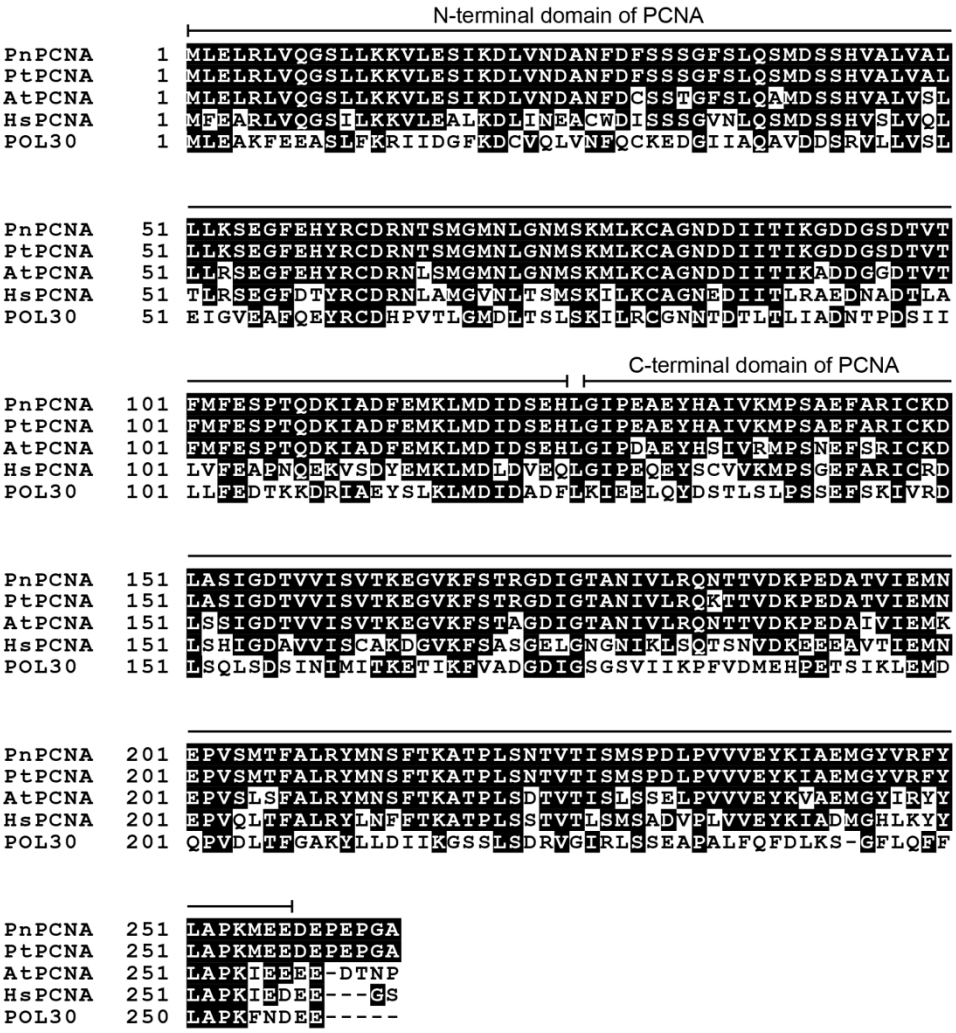

Figure 19. Comparison of the deduced amino acid sequence of PnPCNA with related sequences. The amino acid identities to PnPCNA are boxed in black, and gaps in the alignment are denoted by dashes. PtPCNA, a hypothetical protein from *P. trichocarpa* (JGI v2 gene model name, POPTR_0009s04560); AtPCNA, *A. thaliana* PCNA homolog (GenBank ID: AF083220); HsPCNA, human PCNA (M15796); POL30, budding yeast POL30 protein homologous to PCNA (X16676). The lines indicate the typical N-terminal and C-terminal domains which exist in the proliferating cell nuclear antigen family (InterPro ID: IPR000730).

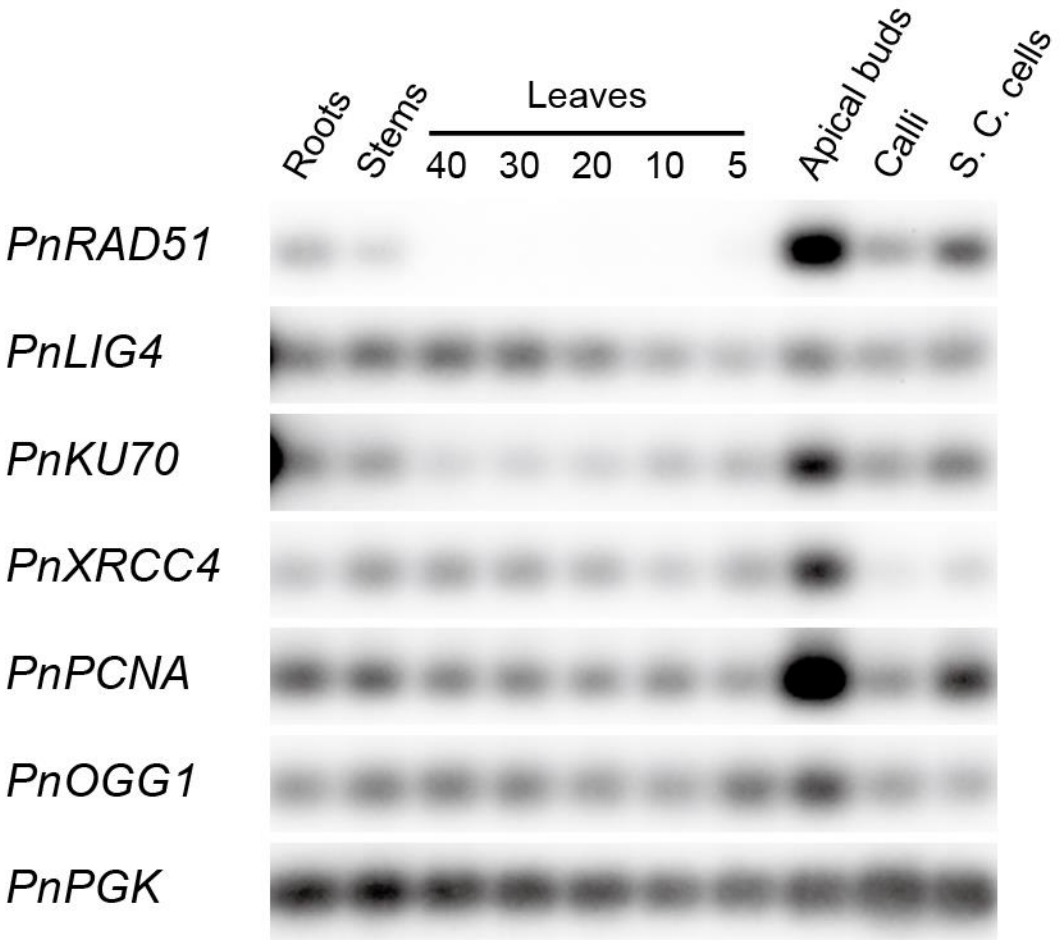

Figure 20. Expression of DNA repair-related genes in different organs and cells of *P. nigra*. The leaves are numbered from the most apical unfolded but not yet expanded leaf to the bottom. S.C.cells, suspension-cultured cells. One microgram of RNA was used for RT-PCR/Southern blotting. The gene-specific RT-PCR products were separated by a 1% agarose gel, blotted, and hybridized with each gene-specific DIG-labeled probe. The *PnPGK* (cytosolic phosphoglycerate kinase 1) gene was used as a control (Nishiguchi et al. 2002).

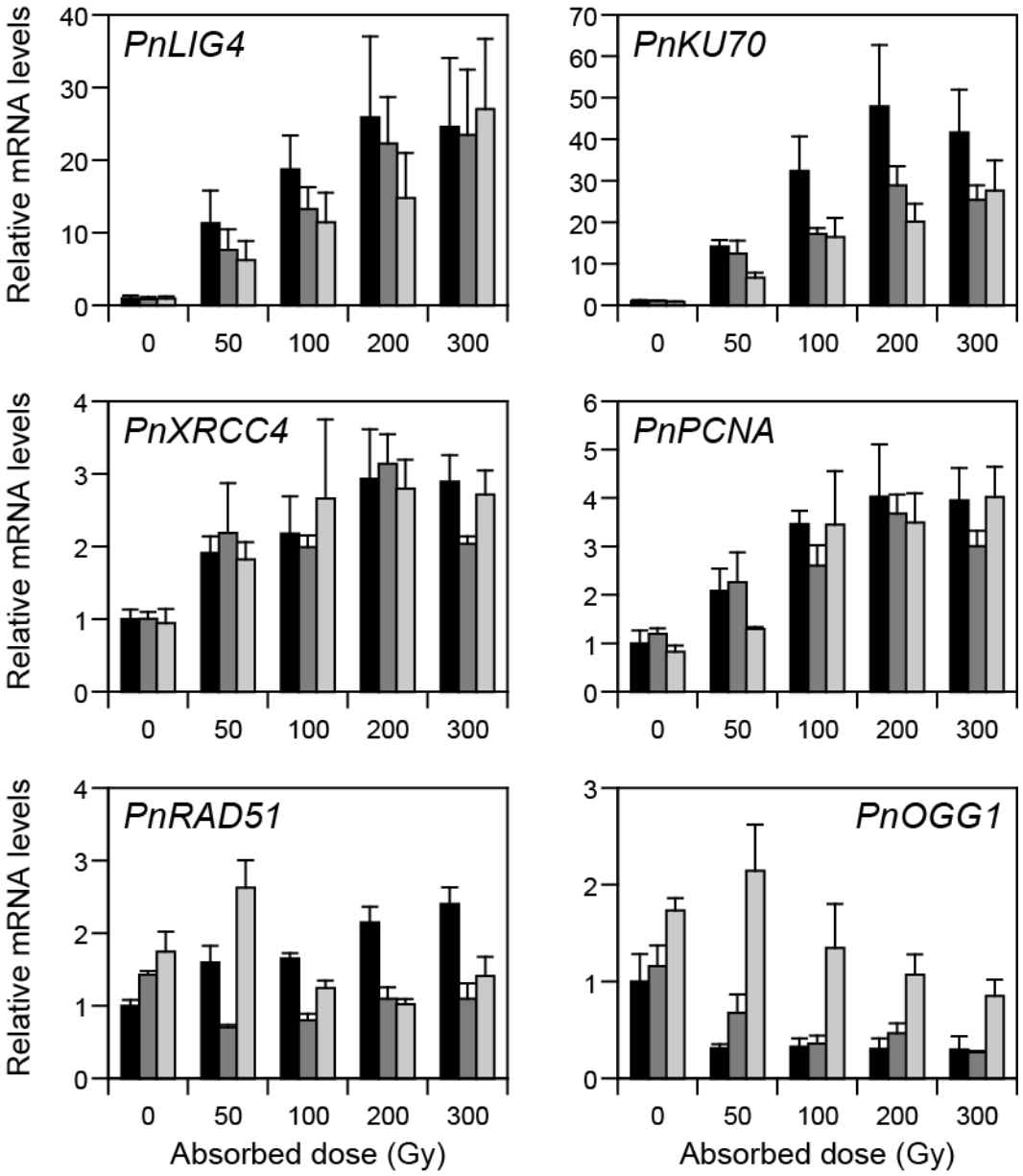

Figure 21. The effect of γ-irradiation on the gene expression of DNA repair-related proteins. *P. nigra* plantlets were exposed to the indicated doses of γ-rays for 20 h. For RNA preparation, the irradiated shoots were collected 1 h (black), 6 h (dark gray), and 24 h (light gray) after the end of γ-exposure. Total RNA was used for RT-qPCR as described in the Materials and Methods. Relative mRNA levels were normalized to the ubiquitin mRNA. The mRNA level of each gene in the non-irradiated plantlets (0 Gy) sampled at 1 h was defined as 1.0. Error bars represent ± SD (n = 3).

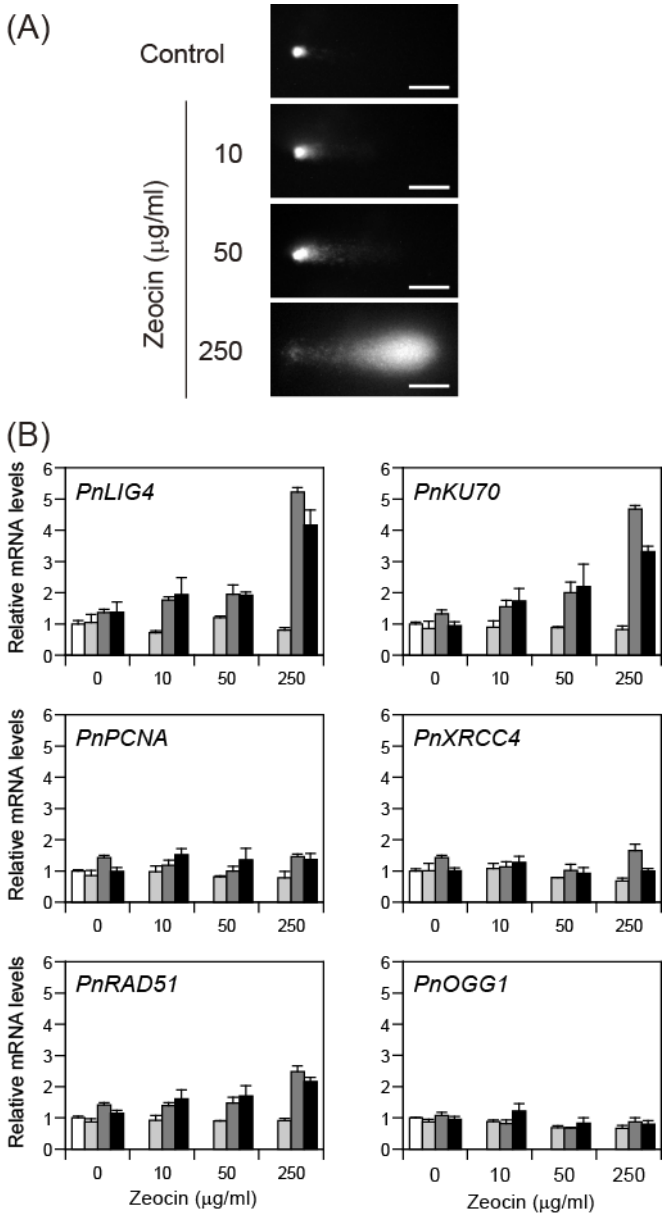

Figure 22. Effects of Zeocin treatment on DNA damage and gene expression in suspension-cultured poplar cells. (A) DNA damage of poplar nuclei induced by Zeocin treatment. The indicated concentration of Zeocin was added to the medium of mid-log phase suspension-cultured cells. A Comet assay was performed 6 h after the addition of Zeocin. As the control, sterile water was added to the medium. Scale bar = 50 μm. (B) The gene expression of DNA repair-related proteins in the Zeocin-treated cells. Sterile water or Zeocin solution was added to the suspension-cultured cells, which were incubated at 25ºC with rotary shaking. For RNA preparation, the cells were collected before (white) and after incubation for 1 h (light gray), 6 h (dark gray), and 24 h (black). RT-qPCR were performed as described in Fig. 21. The mRNA level of each gene in the cells before the treatment was defined as 1.0. Error bars represent ± SD (n = 3).

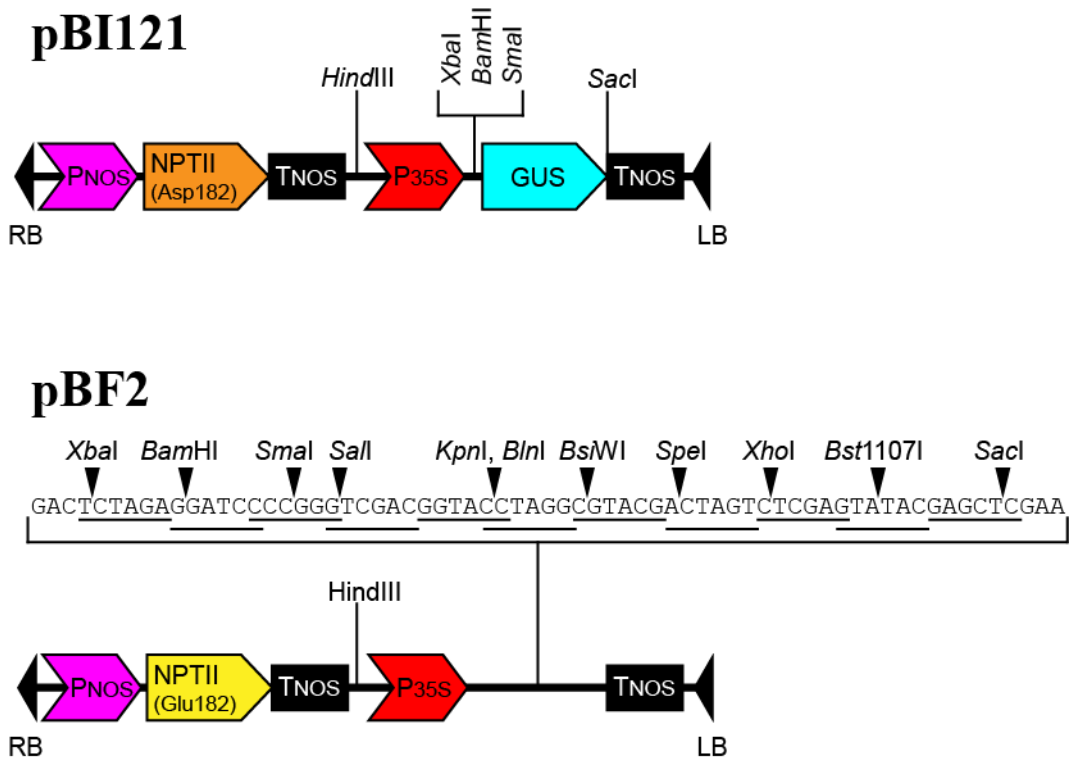

Figure 23. Schematic representation of the T-DNA regions of pBI121 (upper) and pBF2 (lower) binary vectors. Restriction enzyme sites are indicated by lines and arrow heads. The amino acid residue at position 182 of NPTII in pBI121 is aspartic acid, while the aspartic acid is replaced with glutamic acid in pBF2. Abbreviations: RB, right border of T-DNA; LB, left border of T-DNA; PNOS, promoter of the gene for nopaline synthase; NPTII, neomycin phosphotransferase II; TNOS, terminator of the gene for nopaline synthase; P35S, promoter of the gene for the 35S RNA of cauliflower mosaic virus; GUS, β-glucuronidase.

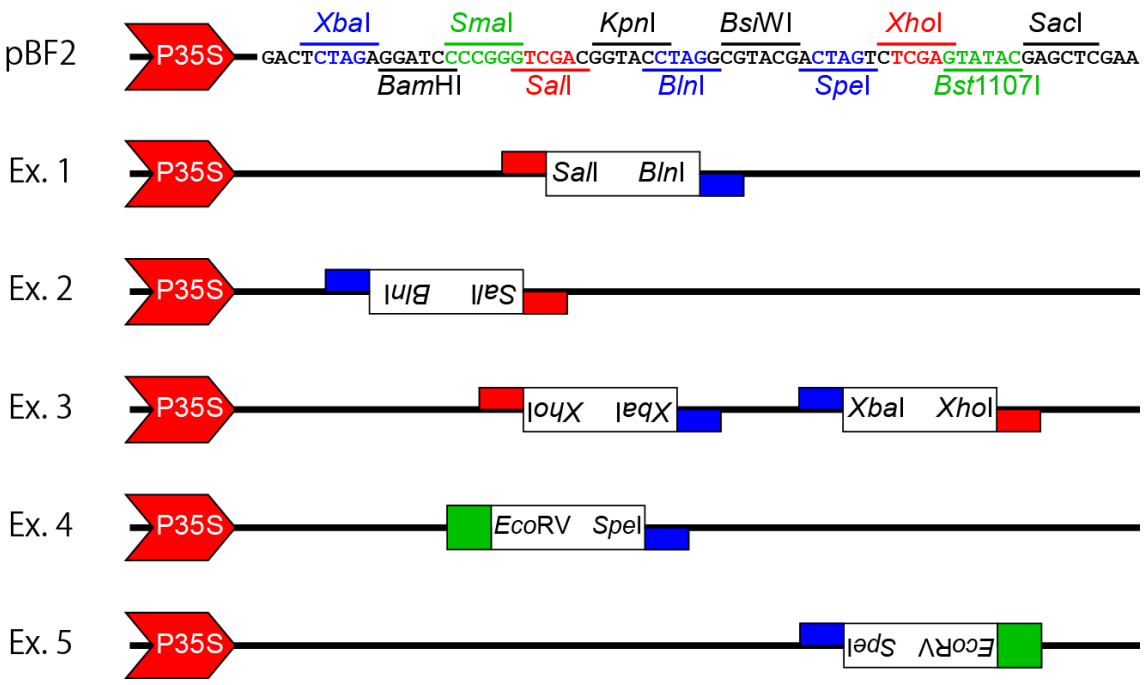

Figure 24. Examples of the cloning of foreign DNAs into pBF2. pBF2 possesses 11 restriction enzyme sites downstream of the promoter (P35S) of the gene for the 35S RNA of cauliflower mosaic virus. (Ex. 1) Insertion of the *Sal*I-*Bln*I fragment into *Sal*I-*Bln*I sites. (Ex. 2) Opposite insertion of the same *Sal*I-*Bln*I fragment as Ex. 1 into *Xba*I-*Sal*I sites. (Ex. 3) Opposite and right insertions of two *Xba*I-*Xho*I fragments into different sites. (Ex.4) Insertion of the *Eco*RV (blunt end)-*Spe*I fragment into *Sma*I-*Bln*I sites. (Ex. 5) Opposite insertion of the same *Eco*RV-*Spe*I fragment as Ex. 4 into *Spe*I-*Bst*1107I sites.

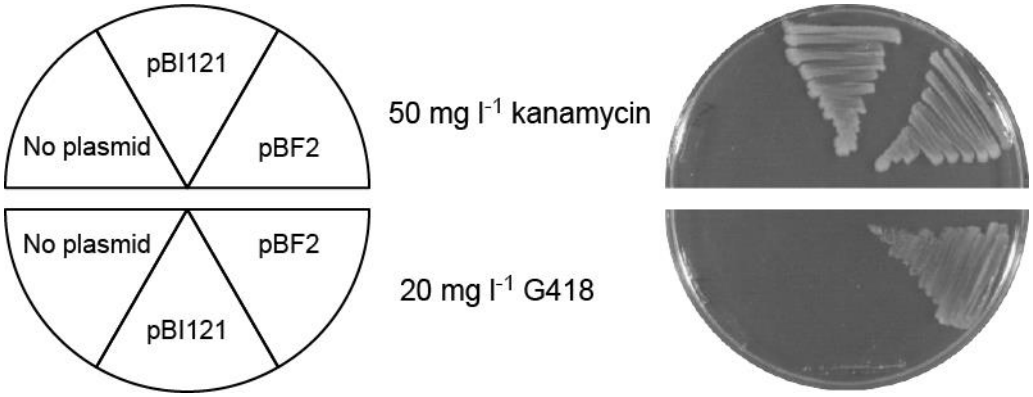

Figure 25. Improved resistance to antibiotics by a reverse mutation of the *NPTII* gene. *Escherichia coli* JM109 was transformed with pBI121 or pBF2. Bacteria harboring the indicated plasmid were streaked onto LB agar medium containing 50 mg l^{-1} kanamycin (upper) or 20 mg l^{-1} G418 (lower). The culture media were incubated at 37°C overnight.

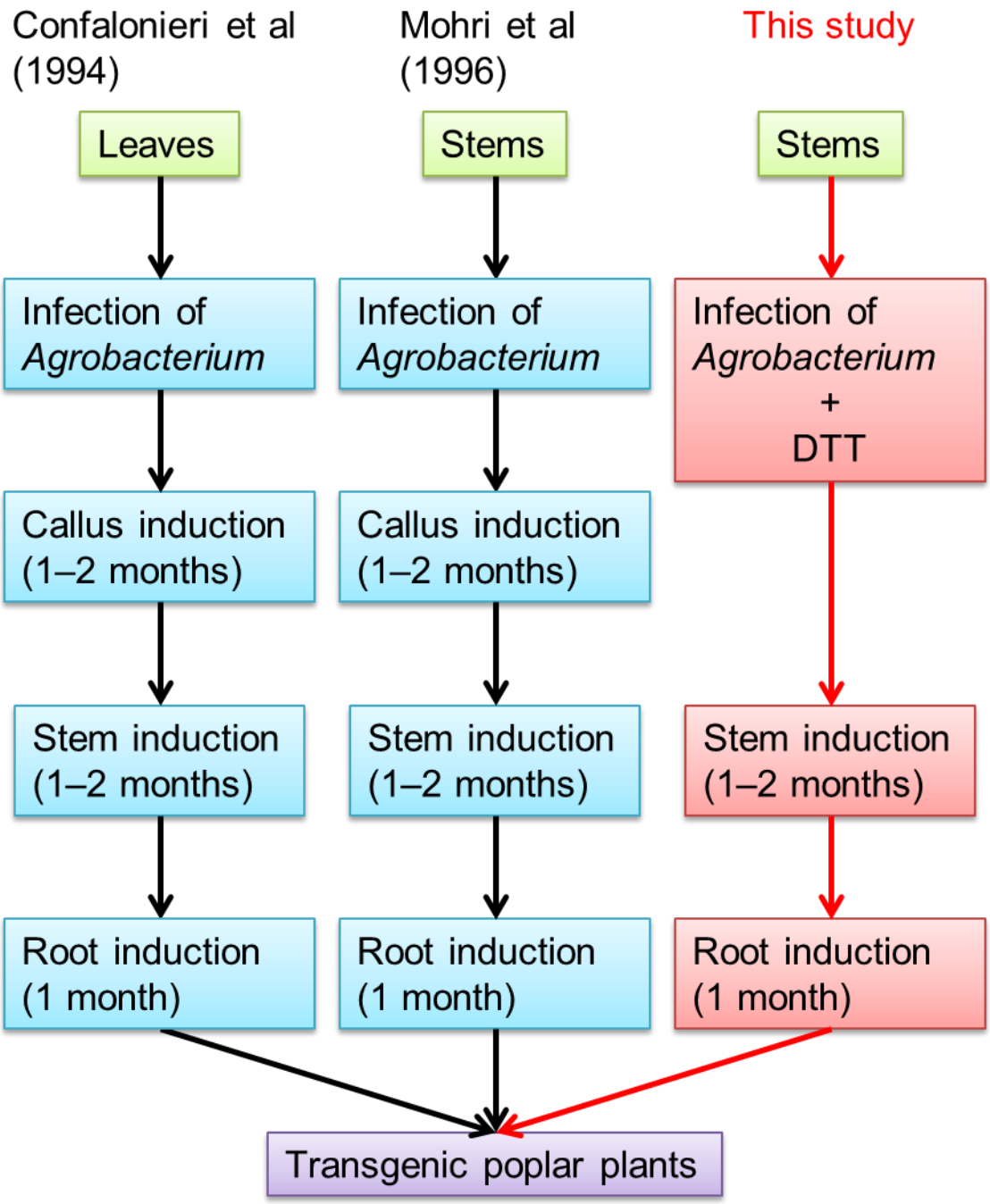

Figure 26. Strategy for the improvement of the transformation process of *P. nigra*. To shorten the time for transformation, the transgenic shoots were directly regenerated from the *Agrobacterium*-infected stems and not via calli. Moreover, the use of dithiothreitol (DTT), a reducing agent, at the infection and bactericidal steps of *Agrobacterium* was expected to increase the transformation efficiency.

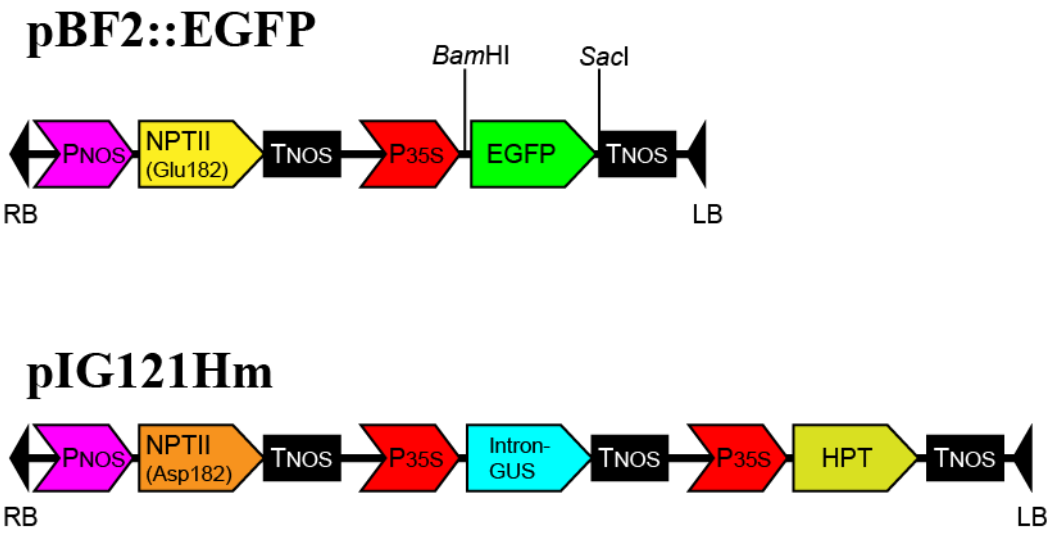

Figure 27. Schematic representation of the T-DNA regions of pBF2::EGFP (upper) and pIG121Hm (lower) binary vectors for the transformation experiments. Restriction enzyme sites are indicated by lines and arrow heads. RB, right border of T-DNA; LB, left border of T-DNA; PNOS, promoter of the gene for nopaline synthase; TNOS, terminator of the gene for nopaline synthase; NPTII, neomycin phosphotransferase II; P35S, promoter of the gene for the 35S RNA of cauliflower mosaic virus; EGFP, enhanced green fluorescent protein; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase.

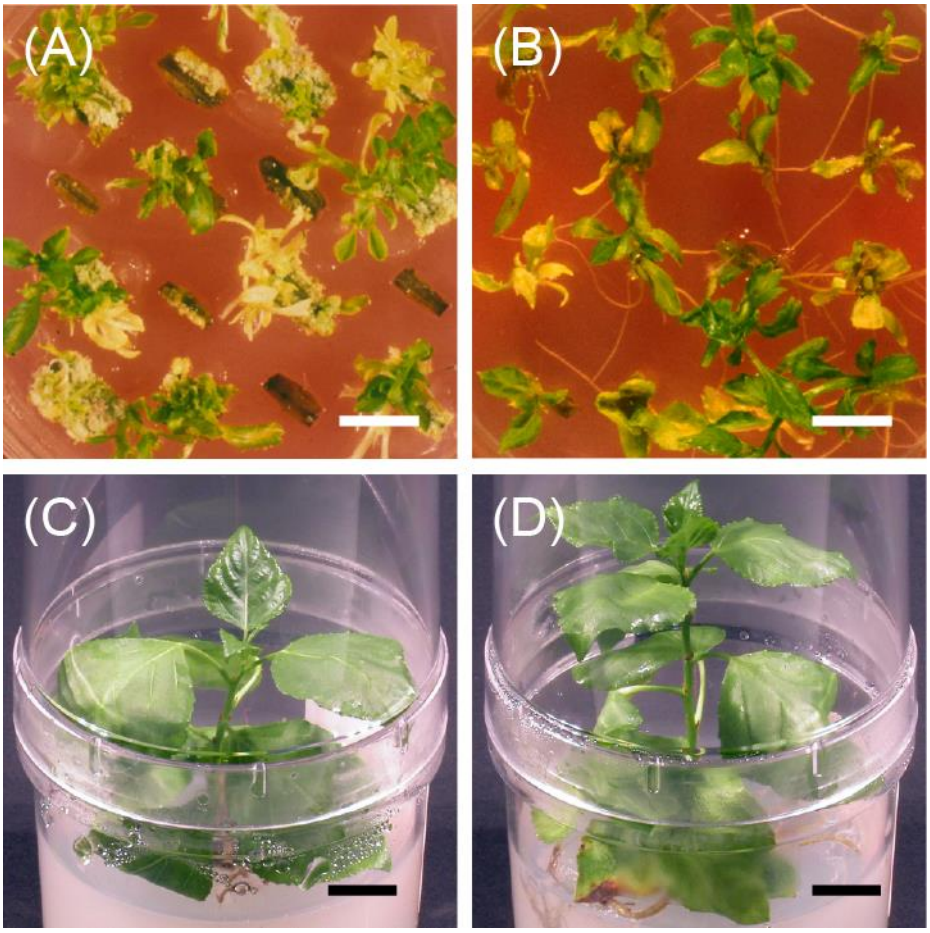

Figure 28. The regeneration process for transgenic *P. nigra*. (A) Regeneration of adventitious shoots 8 weeks after transfer into SIM containing kanamycin. (B) Rooting of the transgenic shoots 4 weeks after transfer into RIM containing kanamycin. (C) An EGFP-expressed transgenic plantlet. (D) A control non-transgenic plantlet. All scale bars represent 1 cm.

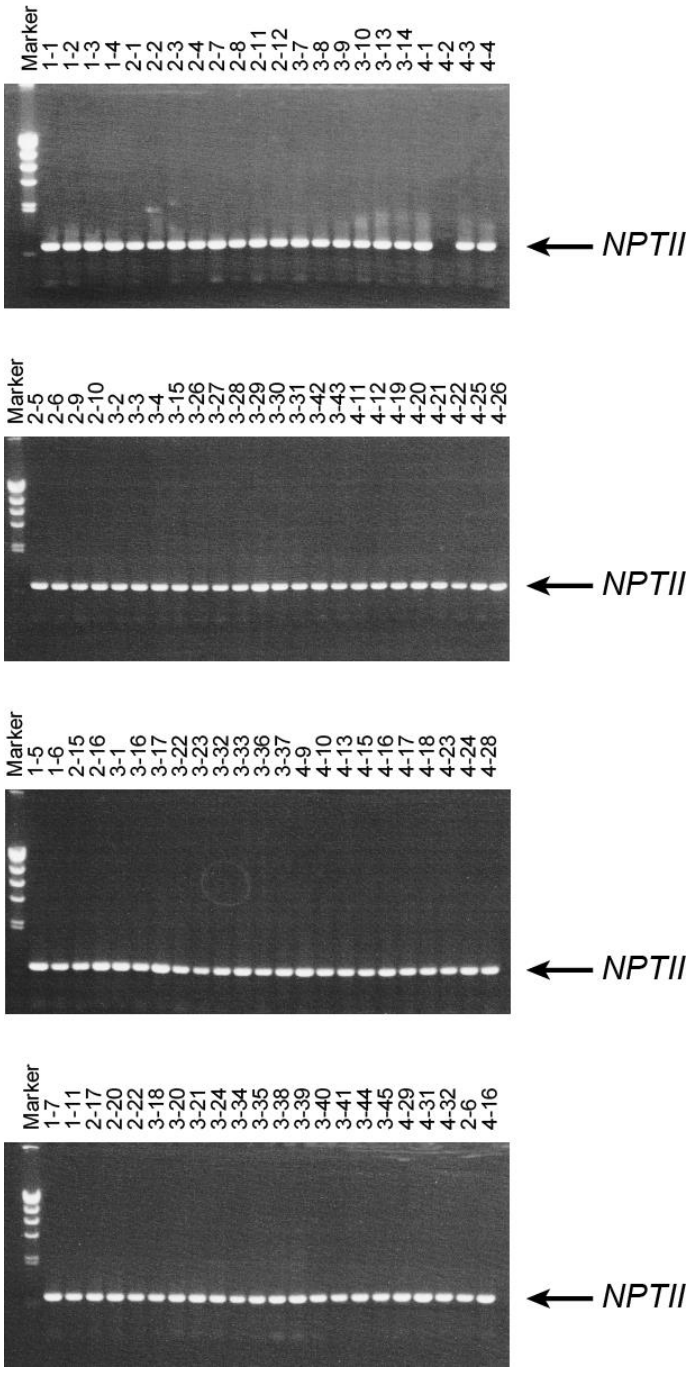

Figure 29. Confirmation of the introduced *NPTII* gene in the transgenic poplar plants. The PCR-amplified DNA fragments of the *NPTII* gene were separated by electrophoresis on 0.7% agarose gels (arrow). The lines of the transgenic poplar plants are indicated above. Marker, *Hin*dIII-digested λDNA.

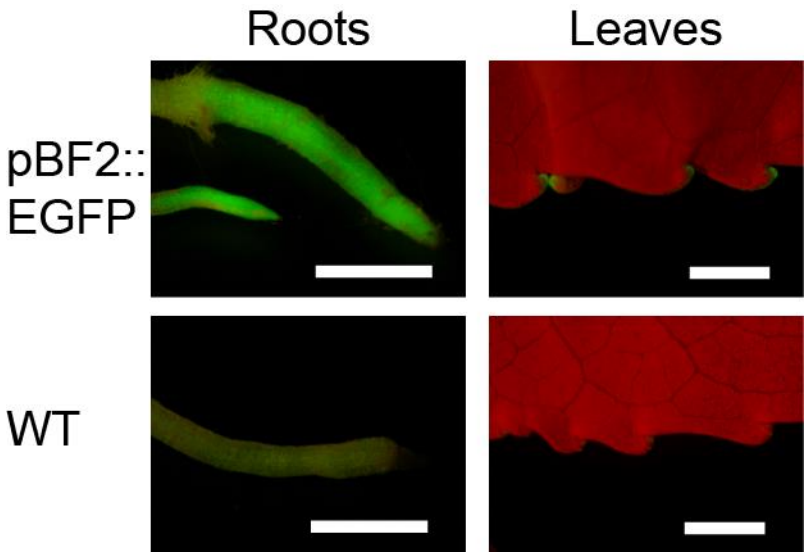

Figure 30. Fluorescent microscopy of the EGFP-expressed transgenic (pBF2::EGFP) and non-transgenic (WT) poplar plants. All scale bars represent 1 mm.

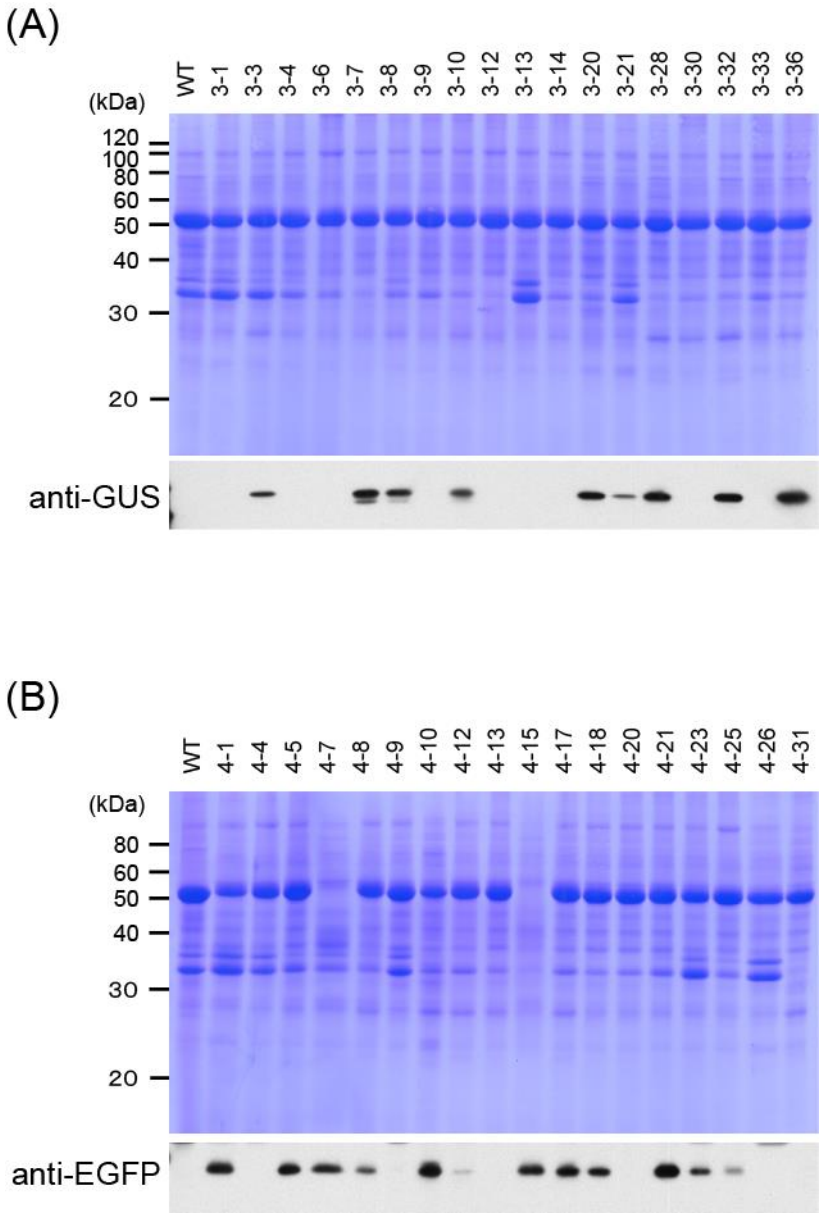

Figure 31. Analysis of the foreign proteins in the transgenic poplars by SDS-PAGE. The crude proteins (50 µg) extracted from non-transgenic (WT) and transgenic poplar plants were subjected to SDS-PAGE and analyzed. The lines of the transgenic poplar plants are indicated above. (A) The poplars transformed with pIG121Hm. The gel was stained with Coomassie blue R-350 (upper). Immunoblotting was performed using rabbit IgG against GUS (lower). (B) The poplars transformed with pBF2::EGFP. Coomassie blue staining (upper) and immunoblot analysis using rabbit antibody against synthetic peptides of GFP (lower) are shown.

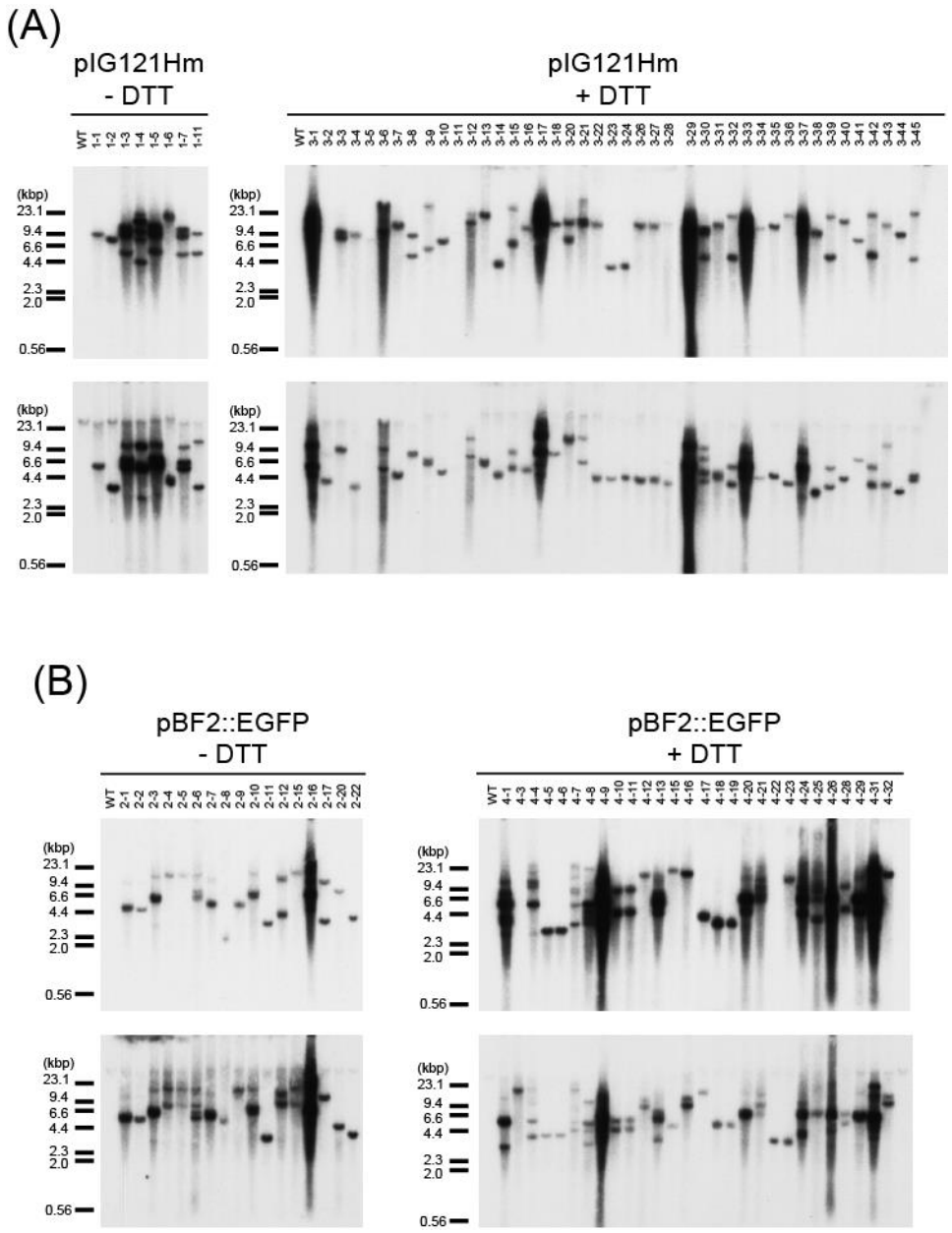

Figure 32. Southern analysis of the transgenic poplar plants. The genomic DNAs from the transgenic and non-transgenic (WT) poplars were digested with *Hin*dIII, electrophoresed, and blotted on Hybond-N+. The lines of the transgenic poplar plants are indicated above. (A) The poplars transformed with pIG121Hm. The DNA-bearing membranes were hybridized with the ^{32}P-labeled *GUS* gene, washed, and autoradiographed (upper). After the *GUS* probe had been removed, the same membranes were rehybridized with the ^{32}P-labeled *NPTII* gene (lower). (B) The poplars transformed with pBF2::EGFP. The membranes were hybridized with the ^{32}P-labeled *EGFP* gene, washed, and autoradiographed (upper). After the *EGFP* probe had been removed, the same membranes were rehybridized with the ^{32}P-labeled *NPTII* gene (lower).

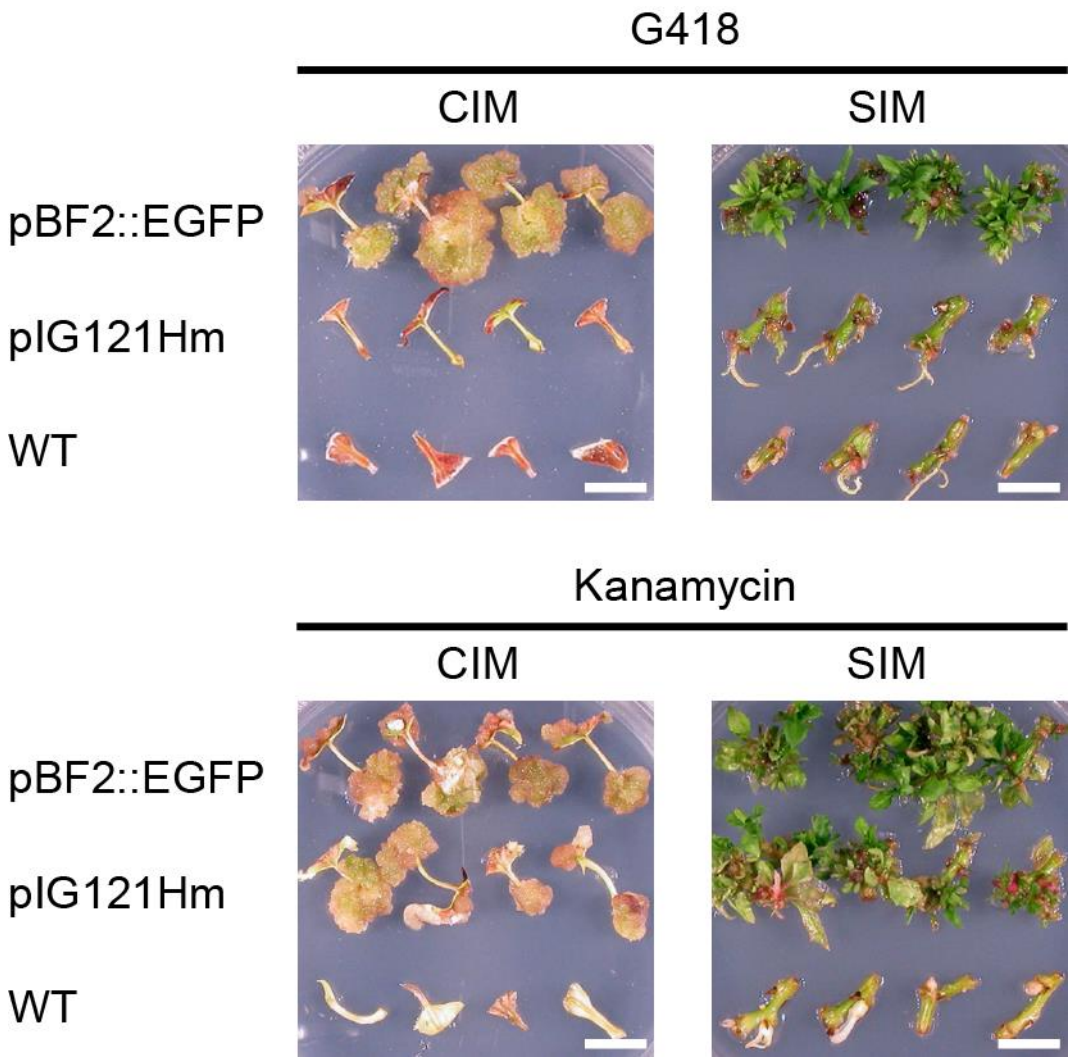

Figure 33. Antibiotic resistance of the transgenic *P. nigra* plants. Explants of the transgenic poplars with the pBF2::EGFP (top) and pIG121Hm (middle) vectors, and of the control non-transgenic poplar (WT, bottom) were placed on the growth medium in a petri dish. Callus-induction medium (CIM) containing 20 mM MES-KOH (pH 5.8), 1 × Murashige and Skoog basal salts (MSBS), 1 × Murashige and Skoog vitamin (MSV), 3% sucrose, 2 mg l^{-1} 2,4-D, and 0.8% Bacto Agar was used for callus formation from the petioles (left). SIM was used for the regeneration of adventitious shoots from the stem segments (right). Each medium contained 50 mg l^{-1} G418 (upper) or 100 mg l^{-1} kanamycin (lower). Photographs were taken 4 weeks after the start of this experiment. All scale bars represent 1 cm.

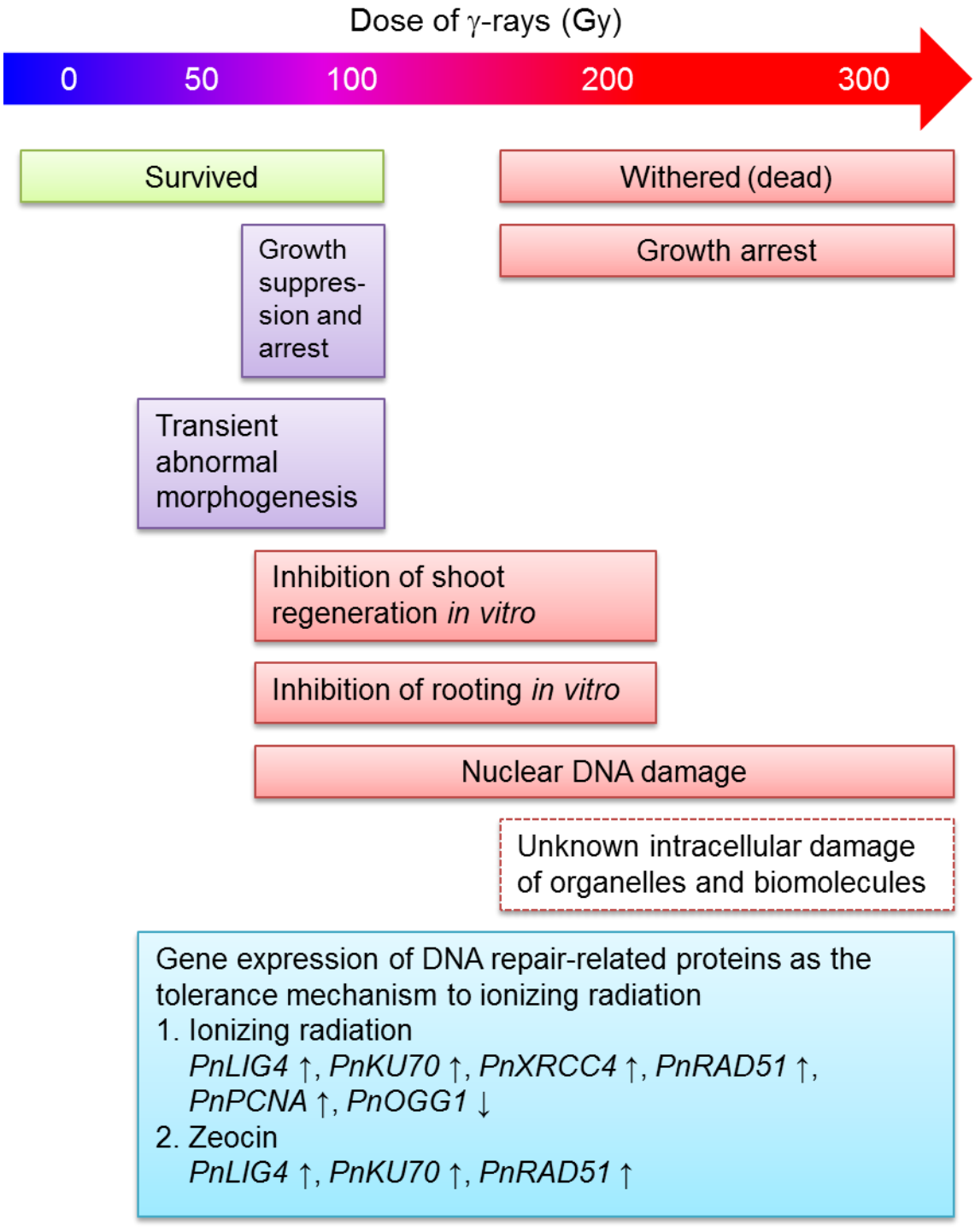

Figure 34. Biological effects of γ-rays on *P. nigra*. The plantlets of *P. nigra* were able to survive in 50–100 Gy of γ-irradiation, though a growth abnormality was caused. The genes of DNA-repair related proteins were upregulated or downregulated by γ-rays and were probably involved in the repair of the damaged DNA. At the higher dose of γ-irradiation, however, almost all poplars died. This result may be due to unknown harmful effects of γ-rays in addition to DNA damage.

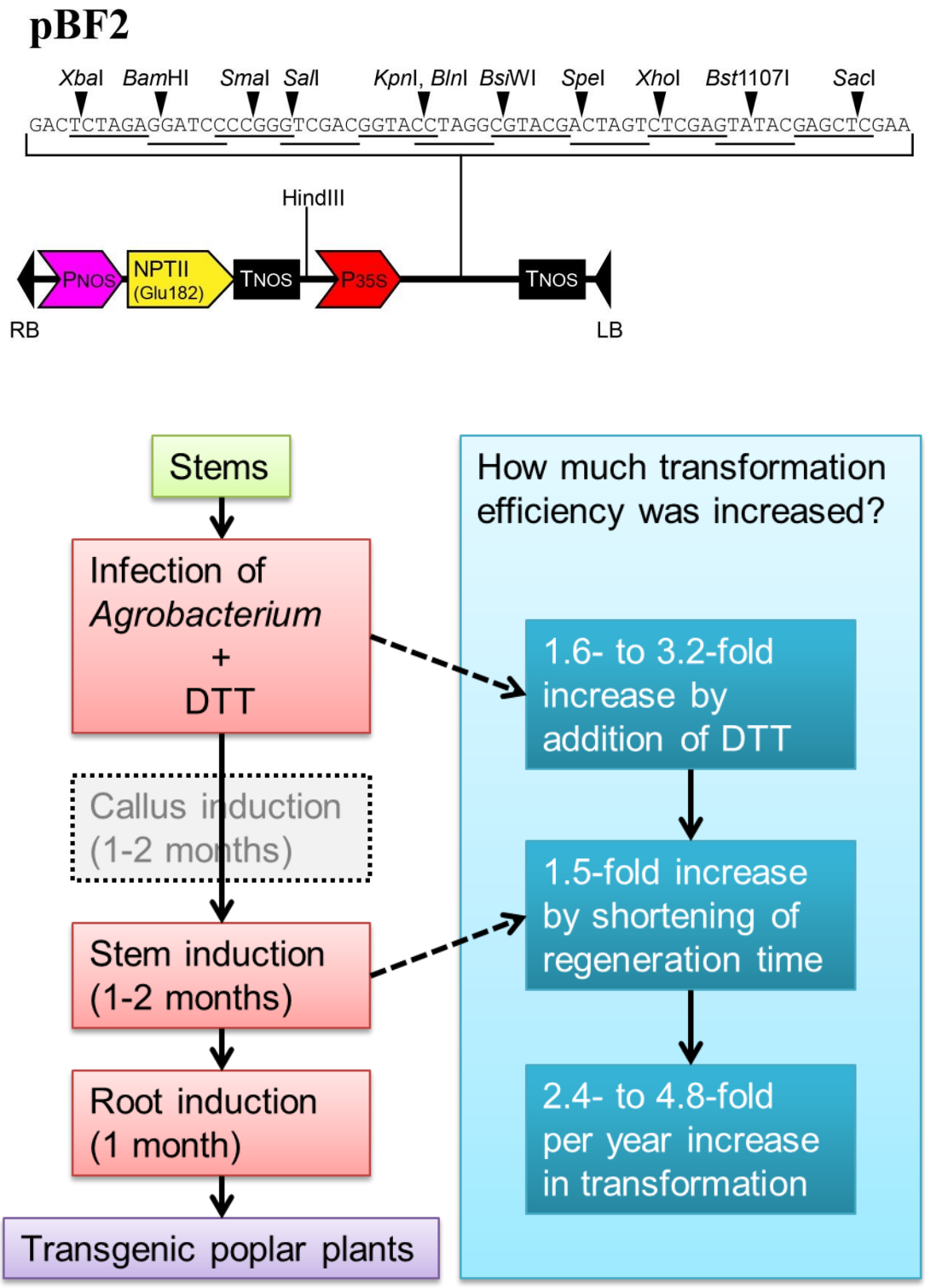

Figure 35. Improved transformation system for *P. nigra*. A new binary vector, pBF2, was constructed (upper). The transformation process was examined, and consequently the transformation efficiency was increased (lower).

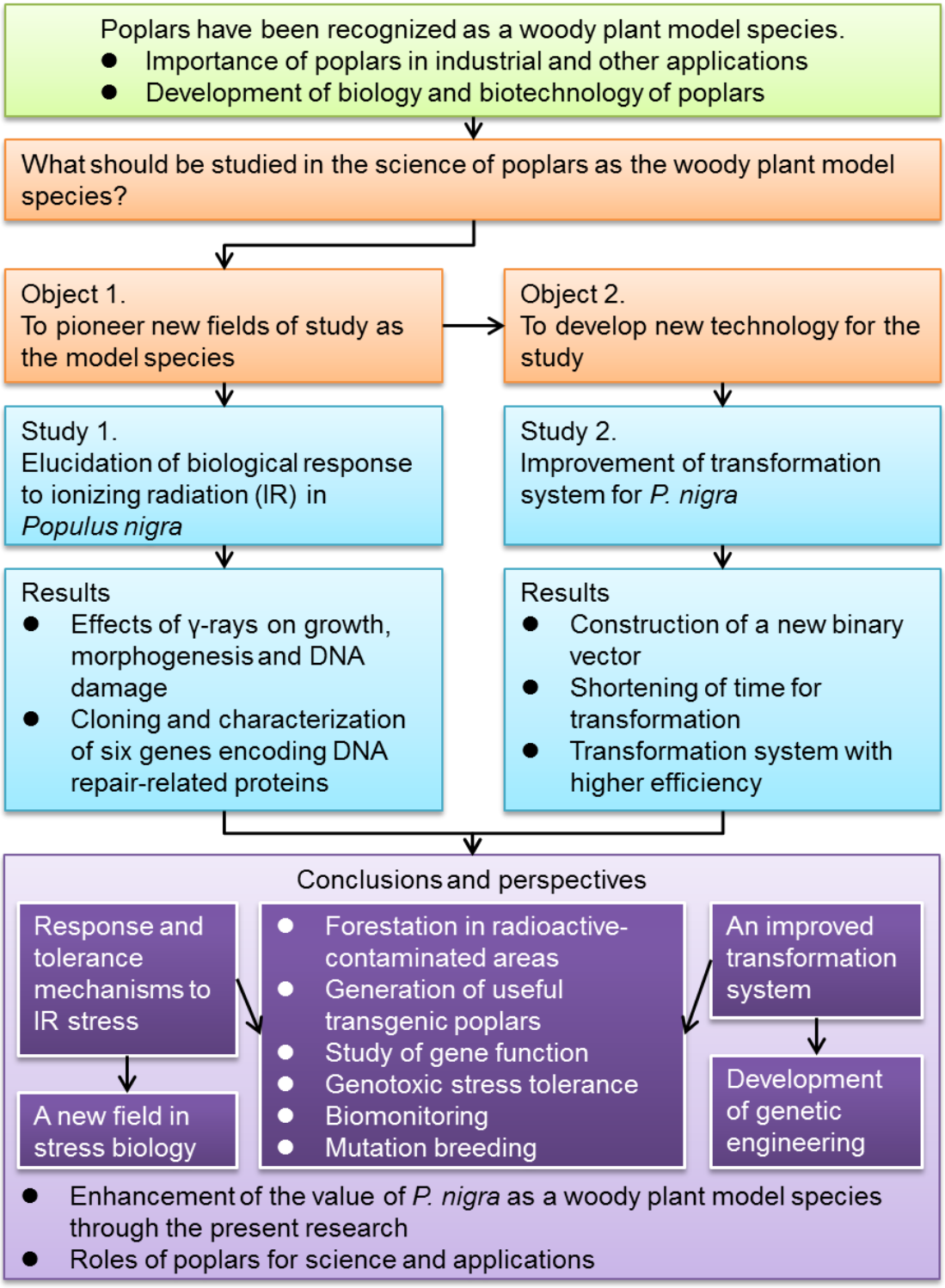

Figure 36. Summary of the research in this thesis. The elucidation of biological responses to ionizing radiation (IR) and the improvement of the transformation system for *P. nigra* enhance the value of *P. nigra* as a woody plant model species. A greater understandings of IR stress physiology and improved genetic engineering techniques increase the possibility of developing new applications for poplars.