34
Breeding for Resistance to Pine Wilt Disease

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34.1 Introduction

To cope with pine wilt disease the first resistance breeding program started in western Japan in 1978. In this program, resistant Japanese black pines (*Pinus thunbergii*) and Japanese red pines (*P. densiflora*) were selected. Subsequently, their progenies have come into wide use as resistant seedlings. Breeding programs are also carried out in the other parts of Japan and Anhui Province in China, and resistant pines, including Ryukyu pine (*P. luchuensis*) and Masson pine (*P. massoniana*), are also being selected. The demand for more resistant pines is increasing. In Kyushu region, second-generation breeding that creates more stable resistant cuttings has been carried out since 2004. Since resistance to the pine wood nematode (PWN) seems to be a polygenic trait, gathering genes in a single cultivar by crossing resistant clones would make resistance higher.

The studies and strategies of the breeding plans for pines' resistance to pine wilt disease will be explained in this chapter. This knowledge can be used to plan more efficient programs for other regions or other tree species in the future.

34.2 Strategies of Resistance Breeding

34.2.1 Breeding for Resistance in Forest Trees

In forest trees, breeding for resistance is one of the important methods to prevent damage from organisms such as pathogens and insect pests, and environmental stresses such as cold temperatures and drought. The resistant individuals are

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selected (selection breeding) or new cultivars are created by making crossings with resistant species or individuals (cross breeding). Those individuals or populations are examined for their resistance and then put to practical use. A key feature of tree breeding is that it has no direct effect on existing forests but contributes to future forests (Fujimoto 1991). Because of their long life cycle and large stature, time and effort are necessary to provide resistant trees for practical application. For these reasons, though referring to breeding strategies of short life agricultural crops is necessary, it is more important to work out specific strategies for tree breeding. Also, we need to keep in mind that while agricultural breeders have been working essentially at the single genotype level for both hosts and pathogens, tree breeders have been working at the population level (Carson and Carson 1989).

34.2.2 Breeding for Disease Resistance

Intraspecific crossing with resistant individuals and interspecific crossing with resistant allied species are effective strategies of breeding for resistance. Selecting resistant candidates from natural forests or plantations where the disease has been severe are also basic components of resistance breeding. As there may be resistant populations or individuals within a species, the surviving populations or individuals can be free of disease symptoms or less impacted by the disease. Clonal propagules or seedling progenies of selections are often tested by artificial inoculation to ascertain the level of resistance and the type of resistance responses. The inoculum may involve a mixture of pathogen genotypes or specific isolates, and the length of testing depends on the nature of the disease (Sniezko 2006). Field-testing is also a key component of resistance breeding since resistance to a given disease mainly depends upon both genotype and environmental interactions (Carson and Carson 1989). The selections are then evaluated as to whether or not resistance is expressed under a variety of field conditions and the stability of the resistance.

Accumulating not only a single resistance gene, but also different kinds of genes in a population or an individual may provide long-term utility (Namkoong 1991; Burdon 2001; Winter et al. 2005). In the case of plants in which QTL (quantitative trait loci) analysis has already made progress, MAS (marker-assisted selection) can be an effective tool (Lande and Thompson 1990; Xie and Xu 1998). We need to know that inbreeding and loss of genetic diversity within a population may reduce not only productive fitness and evolutionary potential, but also resistance to the disease. In pines, the number of seed parents in an open-pollinated orchard cannot be reduced to less than about 15 without risking an unacceptable amount of inbreeding and decline of genetic diversity (Carson and Carson 1989).

Some examples of successful resistance breeding programs include: larch (*Larix kaempferi*) to needle cast (*Mycosphaerella larici-leptolepis*), chestnut (*Castanea crenata*) to chestnut gall wasp (*Dryocosmus kuriphilus*), Japanese cedar (*Cryptomeria japonica*) to criptomeria bark midge (*Reeseliella odai*), Japanese black pine to pine needle gall midge (*Thecodiplosis japonensis*), Western white pine (*P. monticola*) to white pine blister rust (*Cronartium ribicola*), Port-Orford-ceder (*Chamaecyparis lawsoniana*) to root rot (*Phytophthora lateralis*), American elm (*Ulmus americana*) to Dutch elm disease (*Ophiostoma ulmi* & *O. novo-ulmi*), American chestnut (*Castanea dentata*) and to chestnut blight (*Cryphonectria parasitica*).

34.3 Pine Wood Nematode Resistance Breeding

In Asia, pines provide wood and pulp and as well pine species also play important roles in spiritual and cultural aspects. Since the PWN invaded Japan from North America in the early twentieth century (de Guiran and Bruguier 1989), natural forests and plantations of Japanese black and red pines have been severely damaged by the disease. The damage has expanded during the last five decades, and the volume of damaged trees surpassed 2 million m^3 in 1978. According to the record of the Japanese Forestry Agency, its peak was in 1979 when wood stock of 2.43 million m^3 was damaged. To address this situation, a PWN-resistant breeding plan has been carried out. Two methods of resistance breeding have been used: selecting highly resistant individuals within the species, and creating resistant trees by making crossings within and between species.

34.3.1 Selection Breeding

Three mechanisms, avoidance of the vector, antibiosis and tolerance to the pathogen, are important components in resistance to plant pathogens. For pine wilt disease, since there were no differences in the preference of vector beetles (*Monochamus alternatus*) to pine species, avoidance of the vector was not of necessity considered in the breeding strategy (Toda 2004). Surviving trees were selected from stands heavily damaged by the disease, and grafts were used to evaluate their resistance by artificial inoculation (Fig. VI.8). Before starting the plans, three fundamental technical studies on resistance breeding for the pine wilt disease were made in the western part of Japan.

34.3.1.1 Fundamental Research

1. Survey of PWN Isolates for Use in the Artificial Inoculation Testing

For the resistance breeding program, it was necessary to survey PWN isolates to determine their pathogenicity for use in the artificial inoculation tests. The PWN used for inoculum must have stable virulence, high reproducibility, and low host

Fig. VI.8 Inoculation of Japanese black pine to determine resistance to the pine wood nematode (see Color Plates)

preference. Twenty-two PWN isolates and an isolate of a closely related nematode (*B. mucronatus*) were collected from damaged pine trees in various areas of Japan. These isolates had different reproduction rates. They were injected into pine seedlings and to evaluate their virulence. The results showed that the virulence of the isolates varied, causing 0–97.3% pine seedling mortality (Ibaraki et al. 1978). It was also found that there was no host preference in PWNs. As a result, a highly virulent "Shimabara" isolate with a high reproduction rate was chosen for use in the inoculations. Although Kiyohara (1976) reported a decrease in virulence due to in vitro subculture, this did not occur for the "Shimabara" isolate (Toda 2004).

2. Pine Seedlings for the Artificial Inoculation Tests

In some pine species, because cutting propagation is difficult, plantlets have been raised from seeds or grafts; however, there are some problems with using seedlings for inoculation tests. Many seedlings are needed for accurate examination since their genotypes vary due to high heterozygosis in pine species, which is an allogamous (reproducing by cross-fertilization) plant. The selected resistant candidate is required to have high flowering ability. For these reasons, grafts were used in the selecting breeding program. By using graftings, inoculation tests can be done with fewer plantlets since they have the same genotype as their mother trees. The level of resistance to pine wilt disease varies greatly among pine species (Kiyohara and

Tokushige 1971). Japanese black pine is often used as the root stock because of its good rooting ability and growth. Though the scion and stock of a graft consist of different species, Toda (2004) reported that the resistance level of the scion was not influenced by that of the stock. Inoculation of 2- to 60-year-old trees with PWNs showed that the trees were damaged by pine wilt disease regardless of their age (Toda 1997). However, other reports (Mamiya 1975d, 1980) have suggested nematode invasion might change with the growth of pine trees. The PWN migrates mainly via cortical resin canals in the branches. Since the canals are generally not present in stem tissue of trees over 4 years old, nematode invasion in mature pine trees might differ from that in young seedlings (Ichihara et al. 2000b).

3. A PWN Artificial Inoculation Method (see also the column in Chap. 21)

Development of an inoculation test to evaluate the resistance level of pine trees made it possible to carry out resistance breeding for pine wilt disease. A very simple and accurate inoculation method with low damage to pine tree vigor was essential for treating mass numbers of samples (Nishimura et al. 1977; Tsuda et al. 1978). Many different methods were tried, and "a modified peeling bark method" was chosen (Fujimoto et al. 1989). In this method, the bark on a 1–1.5 cm (width) by 3–5 cm (length) section on the main, current year shoot is cut and peeled back and the slit is scratched with a saw to make it rough, and a PWN suspension is pipetted into the slit using a micropipette (Fig. VI.9). The number of nematodes in the inoculum affects the survival rate of the pine seedlings. The more PWNs, the higher the pine wilt rate (Kiyohara et al. 1973). At least 6,000 nematodes are required for uniform dispersal of PWNs inside a tree (Hashimoto et al. 1976). However, pine

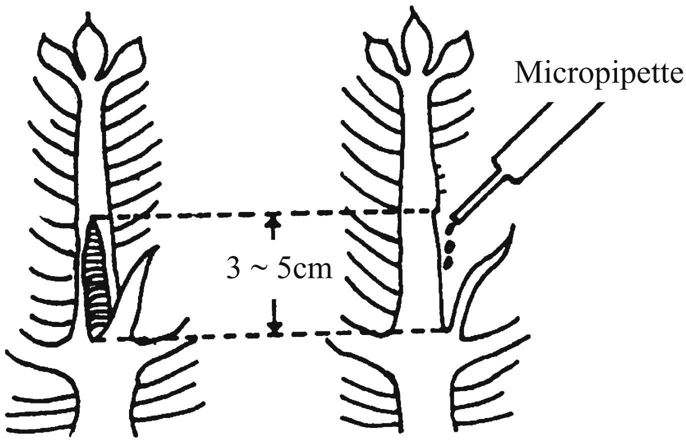

Fig. VI.9 Bark peeling inoculation method for pine wood nematode inoculations (from Fujimoto et al. 1989, with permission) mortality is not influenced by the number of inoculation sites (Yamate and Okubo 1977). According to these results and considering the loss of nematodes prior to host invasion because of resin exudation and drying, 10,000 nematodes are injected into an inoculation site on a tree for most of resistance breeding projects.

34.3.1.2 Three Selection Breeding Plans in Japan

The first resistance breeding program against the PWN was carried out from 1978 onward in western Japan. Later, a similar breeding plan started in 1992, since major pine wilt damage increased also in the Tohoku region, the northern part of Japan. Many pine forests have been damaged in China since the 1980s (Sun 1982; Enda and Taketani 1992), and a resistance breeding program against the PWN has been carried out in Anhui Province in cooperation with Japan since 2001. The strategy based on selection breeding was adopted in these three plans (Fig. VI.10).

1. Breeding Plan for Resistance to PWN in Western Japan

This work was started in 1978 in cooperation with the national forest tree breeding institute and 14 prefectural research organizations. Firstly, resistant candidate trees were selected. Generally, disease-resistant individuals are estimated to exist in 1×10^{-3} to 1×10^{-4}% in natural pine forests (Ohba 1976). Many resistant candidates were selected from severely damaged stands since resistant individuals may exist more frequently there than in non- or only slightly damaged stands. After further consideration of tree growth and stem straightness, survivors older than 30 years in the stands, where more than 90% of trees were damaged, were selected as candidates. Japanese black pines (14,620) and Japanese red pines (11,446) were selected and their ramets were propagated by grafting (Toda 2004).

The first inoculation test to Japanese black and red pine was done from 1980 to 1982. Ten grafts of each candidate were inoculated with 10,000 “Shimabara” isolate PWNs. The candidates which showed survival (the total of non and partial damaged trees) and sound (no damage) rates equal to or higher than those for Loblolly pine (*P. taeda*) seedlings, which were used as resistant controls, were examined to reconfirm their resistivity in the second round of the tests done from 1982 to 1984. For Japanese red pine, the acceptable survival rate for the second inoculation test was the same as that of the first. For Japanese black pine, however, 60% of the Loblolly pine survival rate was adapted as the lower acceptable limit since the resistant level of Japanese black pine was lower than that for Japanese red pine. Base on the results of these two inoculation tests, 16 Japanese black pines and 92 Japanese red pines were finally selected for further use (Research and Extension Division, Forestry Agency 1994b).

After the first breeding plan, an additional selection of resistant Japanese black pine was carried out from 1995 onward in the Kyushu region. Surviving mother trees were surveyed in heavily damaged forests and open pollinated seeds were

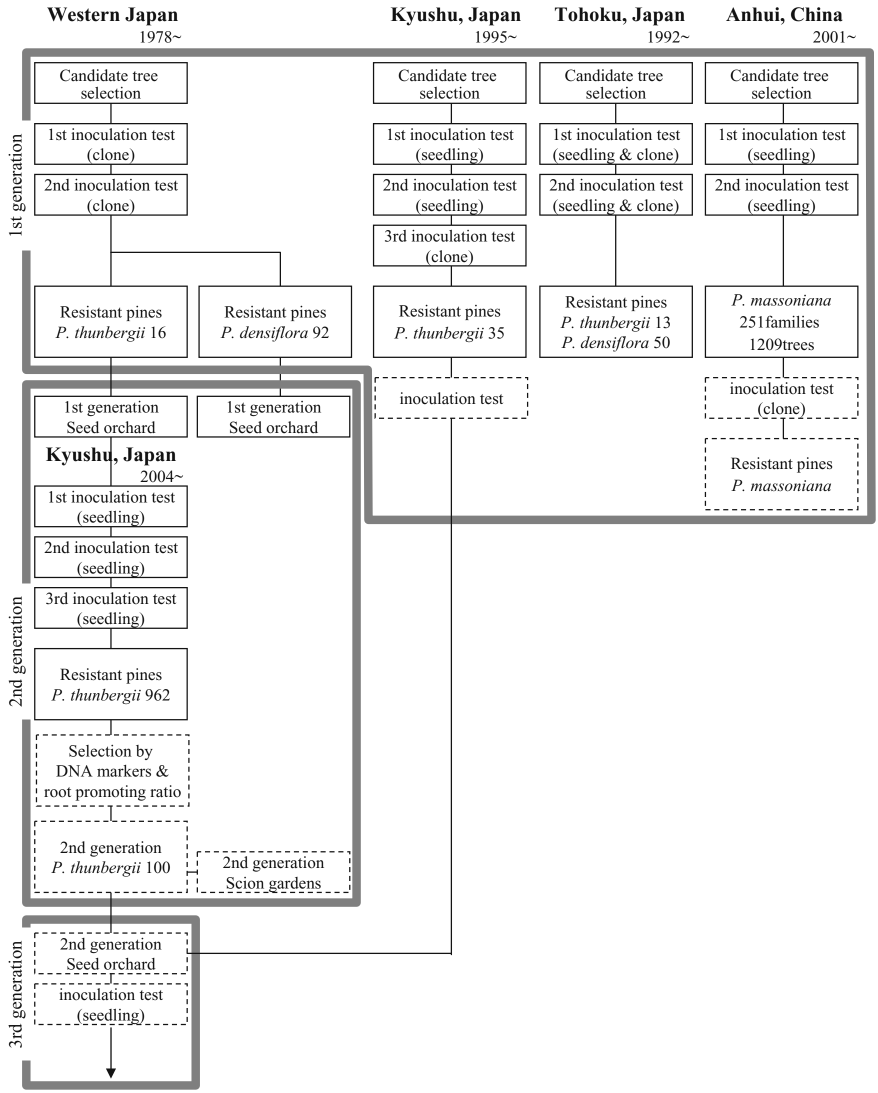

Fig. VI.10 Breeding plans to obtain pine wood nematode resistant pines in Japan and China. The actions with the solid line quadrilateral have been completed, the *dotted line* indicates that the plan put in practice or has not yet started. The figure in the quadrilateral shows the number of plus trees or candidates that have been selected for these plans collected from the stands. The seedlings originating from the seeds were screened twice by artificial inoculation with the “Shimabara” PWN isolate (Okamura 2004). Thirty-five clones were registered as being resistant to the PWN.

2. Another Resistance Breeding Plan in Japan

As PWN damage expanded to northeastern Japan, a resistant breeding plan was started in 1992 in the Tohoku and other regions, cooperating with the national tree breeding institution and nine prefectures. Grafts or seedlings from candidate trees were used for the inoculation tests. The advantages of selections originating from seedlings are the short growing period, high seed production rate of selected seedlings (Toda 2006), and the possibility of a higher resistance level than that of the mother tree (Higashihara 2004).

In this program “the cutting-main-stem method (cut the main stem, crush it and inoculate the nematodes on the crushed area)” was used as an inoculation method in addition to “the peeling-bark method”. Inoculation tests were done from mid-June to the beginning of July, since trees inoculated after the mid-July do not show pine wilt symptoms during the current year in cold districts such as the Tohoku region. The “Shimabara” and more virulent PWN isolates were used as the inoculum, and 10,000 nematodes per tree were used as the inoculum dose. Symptom development was observed for 10 weeks following inoculation (Research and Extension Division, Forestry Agency 1994a).

Instead of Loblolly pine, which is difficult to grow in cold areas, plus trees of Japanese red pine with resistance to PWN, selected in the Tohoku breeding region, were used as a reference of resistance. Survivors of two inoculation tests were assessed for resistance. As pine trees are needed which possess both resistance to pine wilt disease and are cold hearty in these districts, additional resistance breeding will be carried out during the 2006–2010 period. As of April 2007, there were 13 and 50 resistant clones of Japanese black pine and Japanese red pine, respectively, in the northern Kanto and districts along the Sea of Japan. However, the number of resistant plus trees is still not enough.

3. The Resistance Breeding Plan for PWN in Anhui Province, China

In China, the first damage from pine wilt disease was recognized at Nanjing in 1982 (Sun 1982), and the disease had spread to 14 provinces by 2004 (Toda 2006). Initially, only Japanese black pine was damaged, but by now Masson pine (*P. massoniana*) has been damaged. The forest area covers 3.32 million hectares in Anhui Province, and 42% is mostly Masson pine. The “Resistance Breeding plan for Pine Wood Nematode in Anhui Province” was carried out by Japan-China technical cooperation from 2001 to 2006. A native PWN isolate with stable virulence and high pathogenicity was used for the inoculation tests. Nineteen PWN isolates were collected from 11 forest stands in Anhui Province and their virulence was evaluated by inoculating pine seedlings. The "KS3B" PWN isolate was used for the inoculation tests, since it met the above conditions the best (Cai et al. 2003; Gao et al. 2003).

The goal of the plan is to select 200 resistant trees. Two hundred and eighty-eight mother trees were chosen from survivors in 31 heavily damaged stands. Seedlings from those trees were evaluated for their resistance by the first inoculation test and 20% of them remained healthy. The second inoculation test was carried out on the surviving seedlings the next year, and 1,209 seedlings of 251 families were finally selected (Toda 2006). To confirm that they show enough resistance for practical use, they will be propagated by grafting and used for in further inoculation tests (Cai et al. 2006).

34.3.2 Cross Breeding

Cross breeding is a method to create new varieties with desirable characteristics by repeated crossing and backcrossing. In crop breeding, inbred lines are usually created by repeatedly selfing and inbreeding, and heterosis (hybrid vigour) is created by crossing between those inbred lines. In forest tree breeding, however, since it is difficult to create inbred lines, F_1 generations are created by interspecific or intraspecific crossing and put to practical use directly, so the selection of parent species/genotypes must be paid the closest attention (Ohba 1991).

There are some reports on cross breeding for resistance to pine wilt disease. The resistance level varies greatly among and within pine species. Some exotic pine species, such as Loblolly pine, Jack pine (*P. banksiana*), Eastern White pine (*P. strobus*), and Table Mountain pine (*P. pungens*) show high resistance to pine wilt disease (Kiyohara and Tokushige 1971). Interspecific crossing with those resistant species, or intraspecific crossing among resistant individuals may create higher resistance. The high cross-affinity between species, the ability to improve, and adaptability to the growing environment are required for successful cross breeding.

34.3.2.1 Interspecific Hybrid

In Japan, two major pine species, Japanese black pine and Japanese red pine, because of their economic and cultural significance, were selected for use in the national program. There are 19 species within subsection *Sylvestres.* in the world including the two species mentioned above. Crossing the two Japanese pines with other 14 *Sylvestres* species was tried, and all 28 combinations resulted in hybrids (Furukoshi and Sasaki 1979). These F_1 hybrids were used for inoculation tests, and it became clear that the resistance level varied within *Sylvestres* species. Japanese red pine × Dwarf mountain pine (*P. mugo*), Japanese red pine × Corsican pine (*P. nigra* var. *corsicana*), Japanese black pine × Masson pine, Japanese black pine ×

Chinese pine (*P. tabulaeformis*), and Japanese black pine × Corsican pine showed higher resistance than the hybrid between Loblolly pine and Pitch pine (*P. rigida*), which is highly resistant. Moreover, feeding preference tests indicated no significant difference in the preference of the vector among the hybrids (Furukoshi and Sasaki 1979, 1983, 1985; Sasaki et al. 1982a,b).

1. Japanese Black Pine × Masson Pine

The hybrid between Japanese black pine and Masson pine showed higher resistance, higher fertility and vigorous growth among the above mentioned 28 hybrids. For these reasons, this hybrid was practically supplied to western Japan in the "Breeding plan on resistance to the pine-wood nematode" from 1983 to 1988 (Sasaki et al. 1982b). According to reports on interspecific hybrids, it is estimated that Japanese black pine has a single or a few recessive susceptible genes and Masson pine has a dominant resistance gene almost in homozygosis (Sasaki et al. 1983a,b).

2. Loblolly Pine × Pitch Pine

The hybrid between Loblolly pine and Pitch pine showed higher resistance than Japanese black pine and Japanese red pine (Ohba et al. 1977), and it is well known for its greater cold tolerance and higher ability to sprout than that of Loblolly pine (Fukuda and Iwakawa 1979); however, this hybrid was not used for plantations, because its adaptability to Japanese environmental conditions are still unknown.

3. Japanese Black Pine × Japanese Red Pine

In crossing Japanese black pines as the mother tree with Japanese red pine as the father (pollen) tree, the following observations were noted. When both parents are resistant, the resistance level of their hybrid lines was improved. Also, when either the mother tree or the pollen tree is resistant, the resistance level of the progeny was improved except for combinations of some mother trees. Moreover, selfing reduces the resistance level, and there seems to be selfing depression in resistance to the PWN (Toda 1996, 1997; Toda et al. 1997).

34.3.2.2 Intraspecific Crossing

1. Japanese Red Pine

According to the crossing test using resistant and non-resistant Japanese red pines, the survival rate of the hybrid between resistant × resistant was high in every pair (survival rate: 82.5–100%), while resistant × non-resistant varied from 25.0 to 95.0%. The specific combining ability was reported by Handa et al. (1995).

2. Japanese Black Pine

In resistance breeding in the Tohoku region, the pollen of resistant Japanese black pine selected in Western Japan was used for crossings made with pines native to Tohoku, and resistance candidates were selected from these seedlings (Toda and Terada 2001). In Kyushu, the resistance of seedlings of seven clonal seed orchards is influenced by the clone layout, and the progenies of mother trees surrounded with highly resistant clones show stable resistance (Miyahara et al. 2005). As a result of the statistical genetic analysis of three Japanese black pine families created by controlled pollination, the heritability of broad sense (the proportion of total genetic variance to phenotypic variance) and narrow sense (the proportion of additive genetic variance to phenotypic variance) were 0.985 and 0.623, respectively (Kuramoto et al. 2007). This high heritability suggests that accumulating resistant genes by repetitive crossing is an effective breeding strategy to improve the resistance to the PWN.

34.3.3 Characteristics of Resistant Pine Clones

Sixteen, Japanese black pine clones and 92 Japanese red pine clones were selected in the previous resistance breeding plan in western Japan. Thirty-four resistant seed orchards have been established using these resistant clones in 21 prefectures, mainly in Western Japan. For effective management of the clonal orchards, their various characteristics such as flowering and seed production were surveyed. Japanese black pines with genetically improved PWN resistance grow better than non-improved pines. This may be because growth and stem straightness were also taken into adequate consideration when the selections were taken from heavily damaged pine populations (Toda et al. 1993).

Resistant pines begin to flower when they are 2 years old, and all clones flower until 4 years old in Japanese red pine, while 80% of the Japanese black pine clones flower by 6 years old, but no flowering was observed in two clones until 1992. The number of seeds per cone, 14.7 in Japanese red pine and 5.8 in Japanese black pine, is lower than in general pine seed orchards (Toda et al. 1993). A shortage of pollen in the resistance seed orchards is a problem in Japanese black pine. In seed orchards consisting of resistant clones, pollen contamination from outside of orchards was investigated by using DNA molecular markers (Goto et al. 2002a,b), and its influence on resistance depression determined (Handa et al. 1995).

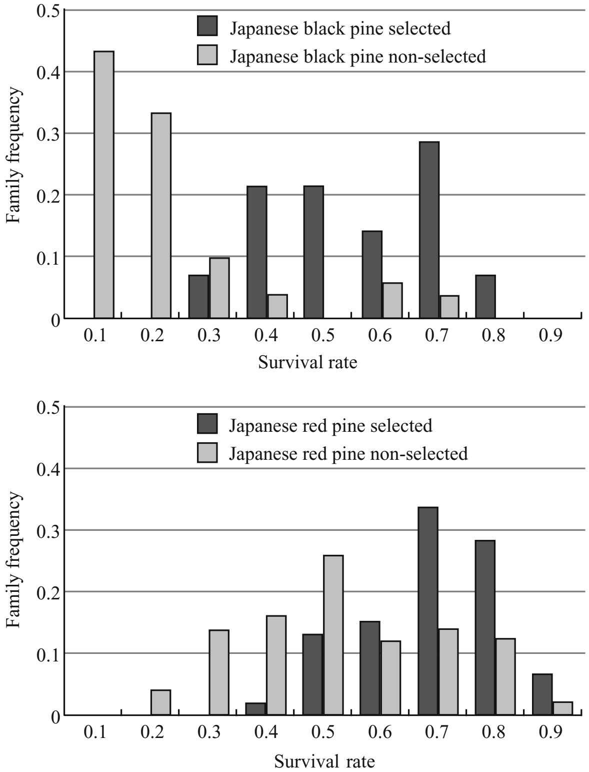

Fig. VI.11 Nursery survival rate of Japanese black pine and Japanese red pine families three months after inoculation with the pine wood nematode (from Toda and Kurinobu 2002, with permission)

Genetic gains in the resistance breeding program in Western Japan were estimated by analyzing the 10-year data of the artificial inoculation, PWN tests. The survival rates of the improved population were higher than the non-improved population (Fig. VI.11). In the improved population the rates were 0.514 in Japanese black pine and 0.650 in Japanese red pine, while in the non-improved population they were 0.166 and 0.473, respectively. The realized genetic gain was 0.348 and 0.177, respectively. The larger value in Japanese black pine may be because the susceptibility of the non-improved population is higher in this species (Toda and Kurinobu 2002). To confirm the improvement in resistance in the field, progeny test plantations were established in two places. Feeding marks made by pine sawyers were detected after 3–4 years, and about 50% of trees were attacked by sawyers by the fourth year. Although these pines were inoculated with the PWN by the pine sawyers, their survival rates were still high. Ninety percent of pines survived in both plantations for 7 years. This indicates that resistant pines selected by artificial inoculation test would also show high resistance in the field (Toda 2004).

34.4 Progress Toward Greater Resistance

34.4.1 Present Resistant Seedling Production System

Japanese black pine has played an important role in protection against wind and to control soil erosion, since it is well adapted to conditions along the coast and in the low mountains running along the coastline (Toda and Terada 2001). For these reasons, many resistant Japanese black pines have been planted in the Kyushu region, which has a long coastline. Since the resistance level differs within seedlings (second-generation) obtained from first-generation seed orchards, artificial inoculation tests are carried out to eliminate susceptible individuals before planting them as resistant seedlings. As pine wilt disease still damages pine forests, about 100,000 seedlings are produced every year in the Kyushu region.

There are some problems in the present resistant seedling supply system. Firstly in this decade, only 34% of the seedlings on average passed the inoculation test. This means that more than 60% of the cost and labor to raise the seedlings is useless. Human errors are likely to occur since the inoculation tests are done in mid-summer under the scorching sun. Also, since the inoculation test using the peeling bark method makes the main stem of seedlings thin, it is easily fractured by strong winds such as typhoons. The results of the test are thought to be influenced by weather, and the pass rate in inoculation tests goes down in high temperature and low precipitation summers (Tobase 2003; Toda 1997). Furthermore, the production of seeds changes annually. For these reasons, the costly production system that requires so much time and labor makes resistant seedlings expensive. While a normal 2-year-old pine seedling costs 50–60 yen, a resistant seedling costs about 670 yen, or about 10 times more.

34.4.2 A New Production System for Second-Generation Pine Cuttings Resistant to Pine Wilt

To solve the problems mentioned above, the development of a new production system of second-generation pines resistant to PWN started in Kyushu region in 2004. The goals of this program are to improve the resistance of Japanese black pine by gathering resistance genes, and to stabilize the supply of clonal plantlets, which contain the same genes using cutting techniques.

The number of resistance genes, resistance factors and mechanisms have not been clarified yet. Since there is variation in the progenies' resistance level, it is assumed that several genes take part in resistance. Since the multiplication of the PWN in pine branches was much different among the 16 resistant clones, it seems there are some resistance factors among them (Nose et al., data not shown).

34.4.2.1 Fundamental Research

In the new breeding plan, the most virulent PWN isolate was inoculated to select highly resistant trees from seedlings obtained from first-generation seed orchards. Of these 962 individuals survived, and about 100 clones will be selected, considering their genetic diversity and rooting ability. Plantlets of these clones, which possess highly resistance genes, will be propagated by cutting and supplied to foresters for planting. To carry out this work, three fundamental research areas were identified.

1. Isolation of the PWN from Kyushu and Preparation of the PWN Population for Inoculation Tests

In advanced breeding, the creation of new PWN populations is needed for the inoculation tests. This must be a highly virulent population with high genetic diversity and low host preference. Considering those requirements, a "Highly virulent PWN population" was made by mixing six virulent isolates chosen from different strains.

2. A New Technique to Propagate Cuttings

It is said that Japanese pine species are difficult to propagate by cutting because of their poor rooting ability, but using juvenile stools and optimizing growth conditions such as the composition of cutting beds and auxin treatment, more than 60% of the cuttings successfully rooted. By pruning the scion trees, the number of branches increased. Consequently, about 40% of the cost can be reduced compared to the present resistant seedling production system. By using this new technique, there is no decline in the resistance level by contaminant pollen coming from outside the seed orchards and no annual changes in the seed yield; therefore, it will be possible to stabilize the supply of high resistant plantlets (Ohira et al. 2007).

3. Pedigree Management of Resistant Trees Using DNA Markers

By using genetic markers to determine the genetic relationship among resistant trees, the genetic bias in the breeding population might be reduced. Especially in tree species of large stature or with a long generation time, reducing the breeding period and labor by introducing a family management system using DNA markers is extremely needed (Paterson et al. 1991). Actually, DNA markers are used in hybrid Eucalyptus (*Eucalyptus grandis* × *E. urophylla*) seed orchard management. Parents with a low reproduction rate were culled from orchards, and management procedures were adopted to minimize external pollen contamination (Grattapaglia et al. 2004).

There are two roles in genetic management with DNA markers: One is to select the next generation without reducing the genetic diversity and creating the genetic bias in the breeding population. Every clone of a seed orchard has to contribute equally to the selected next generation. For this purpose, parentage diagnosis is done using DNA markers. Another role is the clonal management of planted trees to estimate the actual in situ adaptability of each clone. The most suitable clone for each situation could be revealed by measuring the growth rate and pattern, and so on. Usually, in progeny test plantations all the planted trees are labeled to keep track of their clone or pedigree, but this is not necessary owing to DNA markers (Shiraishi 1996, 1997).

Two analysis systems, a multiplex SSR (simple sequence repeat, microsatellite) for parentage diagnosis and MuPS markers [multiplex-PCR of SCAR (sequence characterized amplified region) markers; Hisaeda et al. 2003] for clone identification, were developed in this program. SSR has been detected universally in the genomes of every organism analyzed to date (Tautz 1989; Tautz and Renz 1984), and is suitable for parentage diagnosis, which is a powerful family management tool, while MuPS is an effective tool for retrospective analysis of clones in progeny tests and clonal management of scion gardens and seed orchards.

34.5 Future of Resistance Breeding Against Pine Wilt Disease

34.5.1 Durability of Resistance

It is always unclear whether the resistance gained by breeding provides long-term usefulness. The effect of resistance breeding might often break down because of genetic shifts in the pathogen population (Burdon 2001). In rice, for example, the virulence of a brown plant hopper (*Nilaparvata lugens*) strain has continued to become stronger between 1988 and 1990, and the insect has attacked *indica* rice varieties carrying the resistant *Bph1* gene (Tanaka and Matsumura 2000). In Sugar pine (*P. lambertiana*), resistance to white blister rust that is attributed to a single dominant gene has also been overcome (Kinloch and Comstock 1981). This resulted from breeding practices based on ignorance of the resistance mechanism and the genetic variation of hosts (Burdon 2001). For long-lived organisms such as pines, it is desirable to maintain genetically diverse populations and to combine different types of resistance (resistance mechanisms) for long-lasting or durable resistance.

It is important for resistance breeding to investigate the strain of regional PWNs and their virulence. It is reported that the virulence level of the PWN varies (Kiyohara and Bolla 1990), but presently it is said that there are no geographical differences in their pathogenicity in Japan (Akiba 2006). Based on studies done in Kyushu, it has become clear that PWN strains differ among regions. There are some reports that pathogenicity of the PWN varies in North America and shows different host preference (Wingfield et al. 1983; Bolla et al. 1986, 1988).

Damage from pine wilt disease is not serious in North America, where the PWN originated, because the host and pathogen have coevolved for a long time. Since resistance breeding is an action that promotes co-evolution, it might allow the PWN and pines to coexist in countries where the PWN have invaded. Resistant trees will be expected to serve as the sources of future generations of trees and to co-evolve with the pathogen (Sniezko 2006).

34.5.2 Resistance Breeding in Future

Selection of the first generation of PWN-resistant pines is underway in the Tohoku region, Japan and Anhui province, China, and selection of the second generation and development of a resistant cutting supply system is ongoing in the Kyushu region, Japan. Greater resistance is expected by accumulating several types of resistance genes. In future generations, that is, the third and fourth generations and beyond, the accumulation of resistance genes will develop, and the level and endurance of resistance will improve greatly. To assure great improvement in future generations, the genetic diversity of the original population is extremely important. In Japan, the Seed and Seedling Law limits free movement of seeds and seedlings from the point of view of environmental adaptation. There are, by law, two appropriate seed and seedling zones for Japanese black pine and three for Japanese red pine (Fig. VI.12). For this reason, resistant pine trees can only be planted in limited areas based on their place of origin. Therefore, the number of resistant trees is not enough to maintain genetic diversity in some areas. Further efforts are required for the additional selection of resistant pines and their occasional inclusion into the breeding population to minimize inbreeding and loss of genetic diversity. The concentration and diversity of PWN-resistant genes in the pine populations are key ingredients for stabilizing pine forests against the disease.

Selection-assisted DNA markers, which are valuable for the reduction of scale and time of breeding, are essential tool in the breeding of most tree species (Peterson et al. 1991). Recently, DNA markers for Japanese black pine and Japanese red pine have been developed (Lian et al. 2000; Watanabe et al. 2006b) and used for estimating the outcross rate and paternity analysis (Lian et al. 2001). Moreover, QTL analysis of the resistance to the PWN will be made by using a linkage map constructed with DNA markers (Isoda et al. 2007). Attempts are also being made to identify resistance genes by gene-expression profiling such as EST (expressed sequence tag) (Watanabe et al. 2006a, 2007). PWN-resistant breeding will make great progress by using highly efficient selection using MAS. There are some reports that efficiency of artificial selection can be increased substantially using MAS or multiple-trait MAS (Lande and Thompson 1990; Xie and Xu 1998).

Although resistance of a selected population is certainly higher than that of a non-selected population, we must recognize that it is not absolute. The resistant trees might still be damaged by pine wilt disease when the environmental conditions are less than ideal for the pines. The resistance of trees is influenced by many

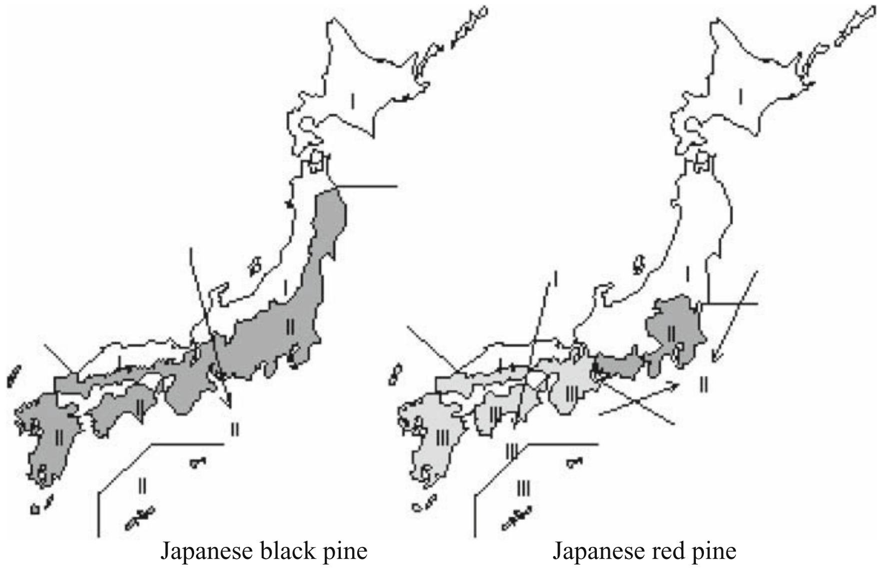

Fig. VI.12 Distributable area of seeds and plantlets of for Japanese black pine and Japanese red pine. *I–III* indicate distributable area; arrows indicate direction permitted for genetic material transfer

factors, such as environmental conditions, tree age, virulence and pathogenicity of nematodes, and the population density of the vector beetles. To maintain pine forests, it is essential to keep the forests healthy by integrated PWN control such as removal of damaged trees and aerial insecticide spraying plus the use of resistant trees (Taoda 1996; Yoshida 2005, 2006).