

Recent Persimmon Research in Japan

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ABSTRACT

This review attempts to introduce the results of recent findings in the areas of genetics and new released cultivars, tree and fruit physiology, new cultural practices with the use of a horizontal trellis or a central leader training grown in container, applications of plant growth regulators for labor-saving and high quality fruit production, heating plastic houses for advanced shipping, humidity control and chemical treatment for shelf-life elongation after harvest of Japanese persimmon (*Diospyros kaki* Thunb.) in Japan. Other research trends focus on methods related to biotechnology and molecular biology for the classification of persimmon cultivars and their relatives, for breeding programs including ploidy manipulation through tissue culture, genetic engineering, DNA markers linked to the trait of fruit astringency, for understanding physiological changes including ethylene production, fruit softening, tannin accumulation and deastringency mechanism in persimmon fruit after harvest. In addition, we will discuss the potential use of health-promoting benefits such as tannins for preventing hangovers or the presence of vitamins.

Keywords: astringency, biotechnology, breeding, *Diospyros kaki*, genetics, postharvest physiology, production technique, tannin

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ADH, alcohol dehydrogenase; AFLP, amplified fragment length polymorphism; BAP, benzylaminopurine; CTSD, constant temperature short duration; DAFB, days after full bloom; DVR-DVI, development rate and development index; EP, expected proportion; F, inbreeding coefficient; FISH, fluorescence *in situ* hybridization; FW, fruit weight; GA, gibberellic acid; GISH, genomic *in situ* hybridization; GPRS, Grape and Persimmon Research Station; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; JA, jasmonic acid; LAI, leaf area index; LTE, low-temperature exotherm; MCP, 1-methylcyclopropene; MP, mid-parental; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; NIFTS, National Institute of Fruit Tree Science; PCA, pollination constant astringency; PCNA, pollination constant non-astringency; PDJ, *n*-propyl dihydrojasmonate; PG, polygalacturonase; PVA, pollination variant astringency; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SAAS, Shaanxi Academy of Agricultural Sciences; SCPL1, serine carboxypeptidase-like protein 1; SSC, soluble solids concentration; TA, thermal analysis

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INTRODUCTION

Japanese persimmon (*Diospyros kaki* Thunb.) is a deciduous fruit tree that is believed to have originated in China, and has been developed in China, Japan, and Korea. Japanese persimmon is an attractive fruit with yellow to orange to deep red skin color, and the intense red of its autumn leaves makes it an attractive ornamental. Persimmons are a good source of β -carotene, potassium, and vitamin C, and are eaten fresh or dried, raw or cooked. They have been introduced into a number of temperate countries such as the USA (California), Italy, Israel, Brazil, Australia, and New Zealand (Yonemori *et al.* 2000).

Japanese persimmon has been grown in Japan since ancient times, and superior selections have been recognized and propagated for hundreds of years. In 2005, it ranked fourth in fruit production at 285,400 t and third in production area at 24,800 ha, but the area has gradually fallen from a peak of 27,500 ha in 1996. Production has been declining for a number of reasons (Japan Fruit Growers' Cooperative Association 2006). Young orchardists have been leaving rural areas. More than 90% of persimmon orchards are smaller than 1 ha. The small orchards tend to make production uneconomical and cause the decrease of income by the price slump in recent years. For example, the average retail market price of 'Fuyu' Japanese persimmon was ¥397 per kg in 1996, ¥201 per kg in 2000, and ¥164 per kg in 2005 (Japan Fruit Growers' Cooperative Association 2006). The use of tall ladders is necessary for most management practices, including pruning, flower and fruit thinning, and harvesting. And younger people do not eat persimmons as much. Therefore, scientists and growers are looking for new production techniques such as a horizontal trellis training system (Hayashi *et al.* 2004) to reduce labor and to halt the decline in production and consumption, releasing new cultivars that produce high-quality fruit which can be sold at a high price (Yamada 2005).

The physiology of Japanese persimmon has been comprehensively studied. The roles of calyx lobes and fruit respiration in fruit development have been elucidated (Yonemori *et al.* 1996). Tolerance of some cultivars and related species to low temperature has been evaluated (Leng *et al.* 1993; Kang *et al.* 1998a). Many studies have examined early fruit drop in relation to plant hormones, and the competition for photosynthates between seeded fruits and parthenocarpic fruits (Kitajima *et al.* 1992). Moreover, the roles of ethylene metabolism and cell wall polysaccharides in the mechanism of fruit softening and the inhibition of softening during loss of astringency have been examined (Nakano *et al.* 2002, 2003; Xu *et al.* 2004).

Useful genes have been isolated and analyzed in recent years. In particular, the genes involved in astringency of Japanese persimmon at the molecular level have been isolated (Ikegami *et al.* 2005a, 2005c). DNA markers for the identification of pollination-constant non-astringent (PCNA)

characteristics have been developed for early selection among offspring (Kanzaki *et al.* 2000b, 2001; Yonemori *et al.* 2003b). Randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analyses have been used for cultivar identification and classification of *Diospyros* species (Nakamura and Kobayashi 1994; Yonemori *et al.* 1998; Yamagishi *et al.* 2005). In addition, regeneration of plants from protoplast cultures (Tao *et al.* 1991; Tamura *et al.* 1993) and protoplast fusion (Tamura *et al.* 1995) and gene transfer via *Agrobacterium*-mediated transformation (Tao *et al.* 1997a) has been successful.

In this review, we summarize the results of research on Japanese persimmon in Japan in the fields of breeding, tree and fruit physiology, training systems, production techniques, control of fruit maturation and ripening, postharvest physiology, and advances in biotechnology and molecular biology since the 1990s.

BREEDING PROGRAM AND NEW CULTIVARS

Natural loss of astringency in fruit in Japanese PCNA cultivars

Tannin in Japanese persimmons becomes highly localized and is stored in large cells named "tannin cells" during the process of ripening. The developing fruit of Japanese persimmon are extremely astringent owing to soluble tannins in the vacuoles of tannin cells. Non-astringent cultivars lose astringency naturally during fruit development, whereas astringent cultivars retain astringency until the soft over-ripened stage. Furthermore, the fruit fresh of pollination variant (PV) types become dark around the seed, while the flesh color of pollination constant (PC) types is not influenced by the presence of seeds. Therefore, persimmons are horticulturally categorized into four types: pollination-constant non-astringent (PCNA), pollination-variant non-astringent (PVNA), pollination-variant astringent (PVA), and pollination-constant astringent (PCA). In PVNA groups, the production of a large amount of volatile compounds (acetaldehyde: 0.02 to 0.06 mg/g FW in the seed) by the seeds during the middle stages of fruit development causes coagulation of tannins in the large tannin cells of the flesh in association with the loss of astringency and thus flesh darken (Sugiura *et al.* 1979). Since the seeds of PVA cultivars produce low amounts of volatile compounds, the coagulation of tannins is restricted around the seeds, so that the astringency remains in the flesh. In PCA cultivars, seeds produce no volatile compounds, and fruit remain astringent even if they have many seeds (Sugiura and Tomana 1983). In PCNA cultivars of Japanese origin, the dilution of tannins by fruit growth is the main cause of natural astringency loss because of cessation of the development of tannin cells in the early stage of fruit growth (at the end of June), whereas the development of tannin cells continue in non-PCNA

cultivars until late stages of fruit development (Yonemori and Matsushima 1985; Yonemori *et al.* 2003a). However, the mechanism of the cessation of the tannin cells in PCNA cultivars fruit has remained to be elucidated. Tannin cells of 15 Japanese PCNA cultivars tested were much smaller than those of non-PCNA cultivars (Yonemori *et al.* 2003a). The tannins of PCNA fruit do not coagulate by ethanol treatment to fruit on trees at the immature stage, but those of the other three types do so readily. For this reason, Sugiura (1984) proposed another classification, dividing cultivars between the volatile-independent group (VIG), including the PCNA types, and the volatile-dependent group (VDG), including the non-PCNA types.

Inheritance of astringency type and breeding of PCNA

PCNA fruit are the most desirable for fresh consumption because the post harvest treatment to removal astringency is not necessary. Hence, the breeding of new PCNA cultivars is the most important breeding objective in Japan. The inheritance of PCNA versus non-PCNA is qualitative, and PCNA are recessive to non-PCNA (Ikeda *et al.* 1985). That means that PCNA-type F_1 offspring can be obtained only by inter-crossing of PCNA genotypes. Backcrossing of non-PCNA F_1 offspring derived from PCNA \times non-PCNA to PCNA yielded only about 15% PCNA offspring (Ikeda *et al.* 1985; Yamada and Sato 2002). It is presumed that two or three allele pairs regulate the inheritance of PCNA traits, because PCNA Japanese persimmons are hexaploid ($2n = 90$) (Zhuang *et al.* 1990), and PCNA is recessive.

So far only eighteen native PCNA cultivars of Japanese origin (excluding bud sports) are known, originating in central Japan (Yamada 1993). All the 18 cultivars always bear female flowers but not always male flowers. Principal-components analysis for various fruit morphological characters separated PCNA cultivars from the other types along the first principal component axis (Yamada *et al.* 1993a). PCNA cultivars of Japanese origin show narrow diversity, having flat fruit, a depressed calyx, and crinkles at the calyx end (Yamada *et al.* 1993a; Yamada 2005), late ripening (Yamada 1993; Yamada *et al.* 1994a), and fruit cracking at the calyx or stylar end (Yamada *et al.* 1988). Cross-breeding among the limited number of PCNA parents resulted in a considerable reduction of the fruit weight of offspring and weak tree vigor due to inbreeding depression (Yamada 1993; Yamada *et al.* 1994b). Further, some offspring have whitish leaves and late fruit drop. To avoid problems of inbreeding, researchers at the Grape and Persimmon Research Station (GPRS) of the National Institute of Fruit Tree Science (NIFTS) began new approaches in the 1990s of crossing PCNA cultivars of Chinese and Japanese origin and obtaining PCNA offspring from the backcross of (PCNA \times non-PCNA) \times PCNA (Yamada 2005).

Inheritance of other important fruit characters

Since most native PCNA cultivars of Japanese origin ripen in late autumn, releasing early-ripening PCNA cultivars had been one of the most important breeding objectives in Japan. The inheritance of fruit ripening time (FRT) is a quantitative character with a high broad-sense heritability: 0.84 when evaluated with five fruits on a single tree without yearly repetition, and 0.92 when evaluated the same way over 3 years (Yamada *et al.* 1993b, 1995c). The within-family genetic variance was fairly small, whereas the genetic differences among families in progeny were explained solely by mid-parental (MP) value (Yamada *et al.* 1995c). In addition, the within-family genetic variance was fairly small. Therefore, it is difficult to yield early-ripening offspring among native PCNA cultivars. However, parental genotypes gradually shifted toward earlier ripening after several cycles of selection over 50 years (Yamada 1993, 2005). Further, a new PCNA cultivar, 'Soshu', released in 2000, ripens very early (late September to early October)

(Yamada *et al.* 2004).

Fruit cracking at the calyx end and at the stylar end are independently and quantitatively inherited (Yamada *et al.* 1988). As the magnitude of cracking is influenced by environment and cracking at the fruit apex is generally caused by seed formation (Yamada *et al.* 1991), it is not easy to precisely evaluate the genetic property of a cultivar or offspring. Yamada *et al.* (2002) indicated that the largest component of the fluctuation is the genotype \times year interaction. Because most PCNA cultivars are susceptible to cracking, it is not easy to obtain offspring with no cracking habit by crossing among PCNA parents. However, the proportion of PCNA offspring that do not crack at either ends have been increasing in the last two decades by the effort to use PCNA cultivars/selections that have little cracking habits as cross-parents (Yamada 2005). Recently released early-ripening PCNA cultivars, 'Shoshu', 'Kanshu' and 'Kishu', have rare fruit cracking at the calyx ends (Yamada *et al.* 2004; Yamada 2005; Yamada *et al.* 2006).

Breeding for larger size fruit is also one of the most important breeding objectives in Japan. Fruit weight (FW) is a quantitative character with a high broad-sense heritability (Yamada *et al.* 1993b). Yamada *et al.* (1994b) reported that genetic differences between offspring among crosses could be explained solely by the inbreeding coefficient (F) and the MP value, and that F had the larger effect. The expected proportion (EP) of offspring with large fruit decreased as the MP value decreased or as F increased. This means that fruit weight is greatly reduced by inbreeding. The observed distribution of the genotypic values of offspring largely coincided with that predicted (Yamada and Yamane 1997).

Soluble solids concentration (SSC), an important factor in fruit quality, is a quantitative character and fluctuates easily with environmental conditions. Its broad-sense heritability is lower than those of FRT and FW (Yamada *et al.* 1993b). The year effect is the largest component causing yearly fluctuation. The yearly variability of SSC could be reduced to less than a fifth by subtracting the yearly deviation (Yamada *et al.* 1994c). Adjusting for the year effect revealed that the EP of SSC depended mainly on the within-family genetic variance. The predicted distribution of SSC in offspring coincided with that observed (Yamada *et al.* 1997).

Chinese PCNA cultivars

Although PCNA-type persimmons had been believed to have originated only in Japan, the existence of a non-astringent cultivar of Chinese origin, 'Luo Tian Tian Shi', was reported by Wang (1983). It originates in Loutian county in Hubei province (Wang 1983), and was identified as a PCNA type after it had been introduced into Japan (Yamada *et al.* 1993a). Interestingly, the genetic behavior of the PCNA properties was different from that of PCNA genotypes of Japanese origin (Ikegami *et al.* 2004, 2006): Whereas all offspring of Japanese PCNA cultivars contain only small tannin cells showing non-astringency, hybrids between 'Luo Tian Tian Shi' and a Japanese PCNA cultivar, 'Taishu', segregated into small cells that do not show astringency and large tannin cells showing astringency. This indicates that 'Luo Tian Tian Shi' is likely to be heterozygous for astringency and that its alleles are dominant. Recent surveys in China found several native PCNA cultivars in Hubei, Henan, and Anhui provinces (Wang *et al.* 2005; Yonemori *et al.* 2005). The use of Chinese PCNA cultivars offers a new strategy for persimmon breeding by overcoming inbreeding depression, and has the potential for progressing the breeding of new PCNA cultivars.

Comparison of genetic resources between Japanese and Chinese origins

Wang *et al.* (1997) reported that there are over 900 local persimmon cultivars in China. Their group collected a large number of domestic cultivars and conserved them at the

Table 1 Fruit characteristics of PCNA cultivars released by NIFTS, Japan since 1990.

Cultivar	Ripening time ^z	Fruit weight (g)	Skin color	SSC (%)	Flesh firmness	Flesh juiciness	Fruit cracking at calyx end	Stained fruit skin
Yoho	Early Nov.	280	Red-orange	16	Firm	Medium	Few	Little
Shinshu	Late Oct.	250	Orange	17	Soft	Much	Few	Much
Taishu	Early Nov.	400	Orange	17	Soft	Much	Few-medium	Medium
Yubeni	Late Nov.	280	Deep red-orange	17	Medium	Medium	Rare	Rare
Soshu	Early Oct.	250	Red-orange	15	Medium	Much	Rare	Little
Kanshu	Late Oct.	230	Orange	18	Medium	Medium	Rare	Much
Kishu	Late Oct.	350	Red-orange	15	Firm	Much	Rare	Little

^zThe cultivars were evaluated in Akitsu, Hiroshima in Japan.

National Persimmon Germplasm Repository, North West Science Technology University, formerly the Experimental Farm of the Pomology Institute, Shaanxi Academy of Agricultural Sciences (SAAS), Meixian, Shaanxi Province, China. GPRS and SAAS researchers analyzed the influences of climate and cultural practices at different sites and investigated means of comparing genetic variations among persimmon cultivars of Chinese and Japanese origin.

Fifteen identical cultivars were evaluated for FRT, FW, and SSC at both SAAS in China and GPRS in Japan (Yamada *et al.* 1995a). Two-way analysis of variance with factors of location and cultivar was made. FRT and FW were greatly affected by the effect of location and cultivar ($P < 0.01$). Coefficients of correlation between the two locations were 0.79** for FRT, 0.89** for FW, and 0.52* for SSC. Therefore, FRT and FW of cultivars in one location can be estimated from those in the other location by adjusting data using the mean differences between locations. Yamada *et al.* (1995b) evaluated FRT, FW, and SSC of 83 PCA cultivars of Japanese origin at GPRS and 132 PCA cultivars of Chinese origin at SAAS. After FRT and FW were adjusted for the effect of location, they found little difference in the mean and variation of FRT and FW between origins. The mean of SSC also showed little difference. These results indicate that the Chinese and Japanese cultivars had similar variations in FRT and FW, suggesting that selection pressures for FRT and FW were nearly the same in both countries.

Recently released cultivars

The oldest national horticultural research station in Japan was established at Okitsu, Shizuoka prefecture in 1902.

Fruit of more than 1,000 cultivars were collected and identified from all over the country. The persimmon breeding program began in 1938 at the Horticultural Experimental Station (presently the Citrus Research Station of NIFTS at Okitsu). Thereafter, the breeding work was passed on to the Akitsu Branch of Fruit Tree Research Station, whose present name is GPRS of NIFTS, in 1968. The program has focused on the breeding of fruits with PCNA, early ripening, large size, high quality and absence of physiological disorders such as cracking (Yamada 1993, 2005).

NIFTS has released seven PCNA persimmon cultivars for the production of fruit for fresh use since 1990 (Table 1). ‘Shinshu’, released in 1990, is a semi-early-maturing cultivar and is suitable for plastic house growing with high-eating-quality, moderately fruit size (about 250 g) (Yamane *et al.* 1991b). The fruit frequently show skin blackening and softening around the apex in the open field, so ‘Shinshu’ is recommended for greenhouse growing. ‘Yoho’, released in 1990, is a mid-ripening (early November), high productivity having high parthenocarp with moderate fruit size (Yamane *et al.* 1991a) (Fig. 1A). ‘Taishu’, released in 1995, is a mid-ripening type with very large fruit (about 400 g) with very soft, juicy flesh (Fig. 1B). Fruit can develop shallow, concentric cracking (Yamane *et al.* 2001), which lead to high sugar content in the flesh just under the cracks (Iwanami *et al.* 2002). ‘Yubeni’, released in 1997, is a late-ripening type (late November) with flat fruit shape with a deeply reddish-orange skin (Yamada *et al.* 2003). ‘Soshu’, released in 2000, ripens very early (early October) (Yamada *et al.* 2004) (Fig. 1C). ‘Kanshu’, released in 2002, is a semi-early-ripening with excellent eating quality and rare fruit cracking habit (Yamada *et al.* 2006), but skin darkening is likely to occur. ‘Kishu’, released in 2003, is a semi-

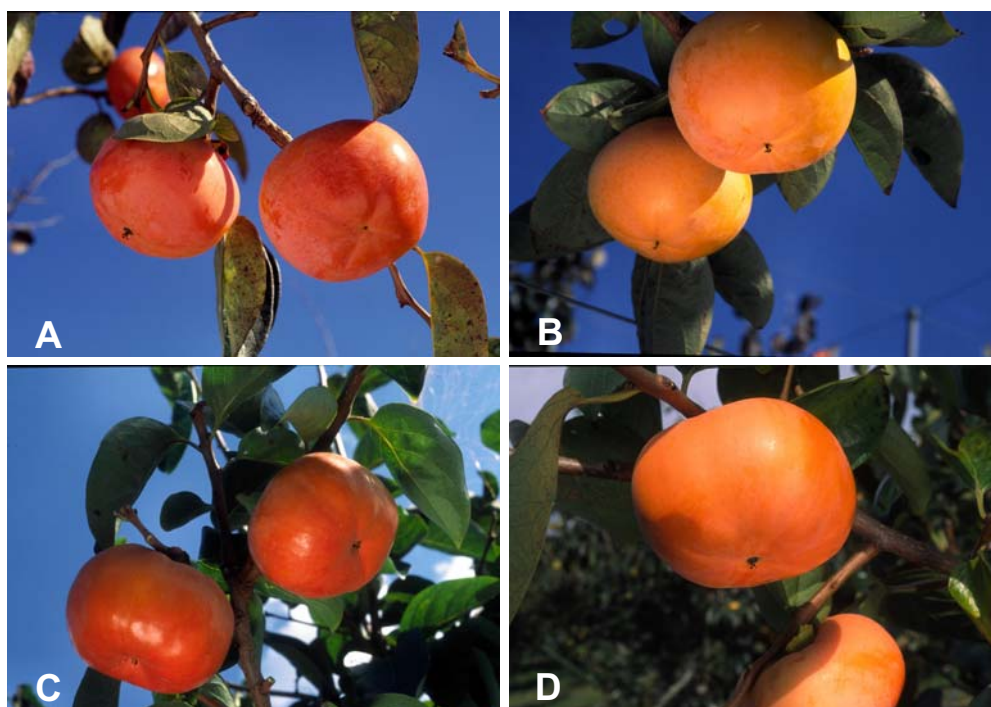


Fig. 1 New released PCNA cultivars of Japanese persimmon. (A) ‘Yoho’, (B) ‘Taishu’, (C) ‘Soshu’ and (D) ‘Kishu’.

early-ripening (late October) with big fruit (about 350 g) (Yamada 2005) (**Fig. 1D**). Among these new cultivars, 'Taishu' orchards covered more than 100 ha in 2003. 'Soshu' production is increasing (Yamada 2006). 'Tanrei' and 'Kinshu' were released in 1993 for ornamental use of red colored leaves at defoliation (Yamane *et al.* 1998). Although they are PCNA cultivars, neither is suitable for commercial fruit production owing to poor fruit quality. However, they produce conspicuously bright, deep red leaves only during the leaf-fall season (from early December to the middle of December at Akitsu, Hiroshima in Japan). These leaves are very attractive as garnishes on dishes and for ornamental planting. The leaves of 'Tonewase' for the same usage can be preserved for up to 3.5 months by immersion in 1% ascorbic acid and 30% NaCl in a refrigerator (Hamazaki and Nishida 2002).

Although these new cultivars have some advantageous fruit characteristics, they also have a few physiological disorders (Yamada and Sato 2003). The main problem is the skin blackening, which is a physiological disorder in which lines, dots or irregular shapes of the fruit skin is stained. It does not affect the eating quality, but it reduces attractiveness and marketability (Yamada 2006). However, skin blackening can be reduced by lowering the humidity within the canopy by summer pruning and excluding water from the fruit surface by bagging fruit or growing trees under plastic covers.

TREE AND FRUIT PHYSIOLOGY

Role of calyx lobes

Japanese persimmon fruit has relatively large calyx lobes compared with other fruit trees. The calyx at flowering time occupies more than 50% of the fruit weight (Kitagawa and Glucina 1984). The calyx has a major influence on fruit growth, early fruit drop, and fruit development. Removal of the lobes of the calyx at growth Stages I and II inhibited fruit development in 44% and 76% of the control, respectively (Yonemori *et al.* 1995). Simultaneously, removal of the calyx lobe at Stages I and II inhibited the accumulation of hexoses (glucose and fructose), while sucrose content of treated fruit was higher than in controls fruit (Hirano *et al.* 1995; Yonemori *et al.* 1995). However, removal at Stage III had no effect on not only fruit growth but also sugar content (Hirano *et al.* 1995; Yonemori *et al.* 1995; Itai *et al.* 1997). Sucrose is the main translocated sugar from leaves in Japanese persimmon fruit (Zimmermann and Ziegler 1975), and acid invertase is responsible for the hydrolysis of imported sucrose to hexoses in the fruit (Zheng and Sugiura 1990). The calyx lobes showed the greatest photosynthetic ability among the fruit parts, and accounted for almost all of fruit photosynthesis (Nakano *et al.* 1997a). However, the calyx lobes are not a significant source of photosynthates during early fruit development by $^{13}\text{CO}_2$ feeding experiments (Nakano *et al.* 1997a). Persimmon fruits whose calyx lobes were removed at Stage I accumulated more sucrose and less hexoses due to a decline of acid invertase in the flesh (Hirano *et al.* 1995). The changes in sugar composition by calyx lobe removal induced higher water potential and higher osmotic potential than in untreated fruit (Itai *et al.* 1997). These results indicated that inadequate sucrose gradient in the fruit by removing calyx lobes reduced further assimilate import by the fruit from leaves, and resulted in inhibited fruit growth.

Calyx lobe removal at growth Stage I or II led to a significant decrease in abscisic acid (ABA) content of fruit but no change in endogenous gibberellic acid (GA)-like substances (Yonemori *et al.* 1995). In addition, the rise in respiration of fruit was retarded by five applications of 100 ppm GA₃ to whole branches during the growth stage II treatment and advanced by 100 or 250 ppm ABA treatment to individual fruit before entering growth stage III (Nakano *et al.* 1997b). Unlike fruit skin, calyx lobes have many stomata on their surface. Since removing calyx lobes at an early

growth stage decreased respiration, a possible relationship between growth potential and gas exchange was suggested (Yonemori *et al.* 1996). Therefore, the growth inhibition by calyx lobe removal in Japanese persimmon is related to changes in sugar metabolism and respiration. Since the calyx lobes have important roles in fruit development and fruit quality, other ways in which calyx lobe removal could inhibit growth need to be investigated in more detail.

Floral ontogeny and sex determination

The flowers of Japanese persimmon are polygamo-dioecious with three types of sex expression: pistillate only, monoecious (both pistillate and staminate flowers), and polygamo-monoecious (hermaphroditic, pistillate, and staminate flowers) (**Fig. 2**). No cultivars bearing only staminate flowers have been reported. Flowers are borne laterally on current-season shoots. Some PCNA cultivars produce monoecious flowers, including 'Hanagosho', 'Hazegosho', and 'Okugosho', making them favorable for cross-breeding (Yamada 1993). However, superior cultivars do not always bear staminate flowers. Although the type of sex expression is genetically determined, bud-sports producing staminate flowers on pistillate cultivars are found on rare occasions (Yakushiji *et al.* 1995). After such shoots of 'Fuyu' and 'Jiro' were top-grafted onto rootstocks, the plants bore staminate flowers bearing viable pollen for several years. Since there were no differences in leaf isozymes and morphology, the shoots with staminate flowers are enzymatically and morphologically identical to 'Fuyu' and 'Jiro' except for the staminate flowers (Yakushiji *et al.* 1995). Such

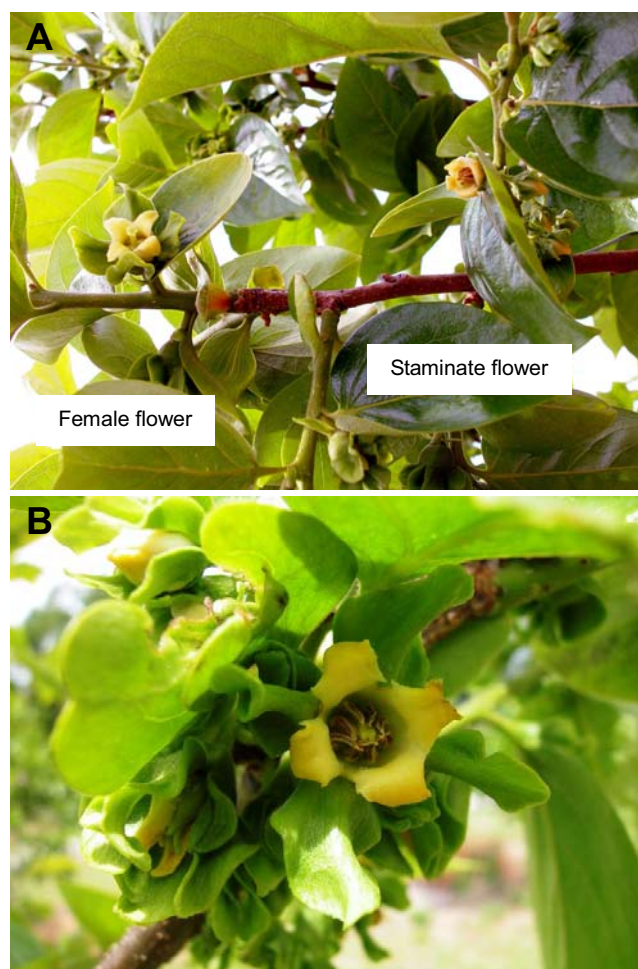


Fig. 2 The female flower (A-left), staminate flower (A-right) and hermaphrodite (perfect) flower (B) in Japanese persimmon ('Taishu'). The stamens of female flowers are sterile, and the ovary of staminate flowers are non-functional. Staminate flowers occur in clusters and are much smaller than the female flowers.

staminate flowers of leading PCNA cultivars should be very useful for developing improved PCNA cultivars. In fact, pistillate flowers of 'Fuyu' pollinated from staminate flowers found on a 'Jiro' shoot resulted in a new cultivar, 'Yoho' (Yamane *et al.* 1991a).

Information on floral ontogeny and the differentiation of pistillate and staminate flowers can be used to control sex expression of flowers. Scanning electron microscopy of two monoecious cultivars, 'Hanagosho' and 'Kakiyama-gaki', revealed that the pistillate and staminate floral primordia in the buds of current shoots started to differentiate in early June, and continued to differentiate until August. The buds became quiescent and then overwintered (Yonemori *et al.* 1993). Thus, the sex differentiation of monoecious persimmons is determined at a relatively early stage of floral development.

Although sex expression of monoecious Japanese persimmons is believed to be affected by the nutrient status of shoots and branch size (Nishida and Ikeda 1961), Yonemori *et al.* (1992) reported that there was no obvious correlation between the branch length and the flower type that differentiated on the new shoots. In addition, they indicated that staminate flowers were predominantly formed on the shoots from a branch that had borne staminate flowers, whereas female flowers differentiated on shoots from a branch that had borne female flowers. These means that the previous year's flower type and bud position on new shoots were more influential in determining sex expression than in branch size. Similarly, the 'Fuyu' bud-sport bearing staminate flowers produced staminate flowers only on shoots that bore staminate flowers during the previous year (Yakushiji *et al.* 1995). On the other hand, Yonemori *et al.* (1990) demonstrated that six applications of 1,000 ppm benzylaminopurine (BAP) on the branches before and during flower initiation (early June to early July) were able to induce about 15-30% higher ratio of pistillate flowerers than that of control branches in monoecious cultivars in next spring. Also, 1,000 ppm BAP applications in April were effective to convert staminate flowers to hermaphroditic flowers due to develop pistil in the same year. Therefore, the balance between cytokinins and auxins, which determine apical dominance, plays an important role in sex expression in monoecious cultivars.

Low temperature response

The cold hardiness of some persimmon cultivars and related species has been evaluated (Leng *et al.* 1993; Kang *et al.* 1998a). The critical low temperatures during the coldest period were -10°C for *D. taitoensis*, -15°C for *D. kaki*, and -20°C for *D. lotus*. The $\text{LT}_{50\text{s}}$ (lethal temperatures to 50% of a population) of twigs were -12 , -20 , and -25°C , respectively (Leng *et al.* 1993). Leng *et al.* (1993) found a close relation between the freezing resistances evaluated based on electrolyte leakage and anthocyanin content in the cortical tissues. Kang *et al.* (1997, 1998b) used computer-assisted thermal analysis (TA) to determine the cold hardiness of dormant mixed buds of Japanese persimmon. A typical profile of a TA of bud without nodal tissue showed only one exotherm whose temperature coincided with bud death. On the other hand, the exotherm of nodal tissue or buds with nodal tissue showed two distinct exotherms: low-temperature exotherm (LTE) and high-temperature exotherm. LTE of bud with nodal tissue was derived from nodal tissue and the LTE was much lower than the killing temperature of the bud. Therefore, Kang *et al.* (1998b) reported that a bud without its nodal tissue can be used to evaluate cold hardiness in persimmons by TA. The LT_{50} of the buds almost coincided with their exotherm temperatures, and naked primordia of 'Hiratanenashi' were killed between -20 and -25°C . The bud cold hardiness during the deep dormant period (from the middle of October to early November) was affected by neither the low nor the high temperatures in ambient (Kang *et al.* 1998b). After release from bud dormancy, the high ambient temperature decreased the

bud cold hardiness. At the budburst stage (Fig. 3) many cultivars and species, including *D. lotus*, *D. oleifera*, *D. rhombifolia*, and *D. virginiana*, were the most susceptible to freezing injury (Kang *et al.* 1998a). However, as the buds grew, regardless of cultivars or species, some except for 'Fuyu', *D. lotus*, and *D. virginiana* became less susceptible to freezing injury. Thus, Kang *et al.* (1998a) pointed out that frost injury of persimmons in spring is influenced by the time of budburst and the cultivar- or species-specific hardiness is expressed by the hardiness of young shoots developed after budburst.

Physiological fruit drop

Physiological fruit drop is a major problem in the production of Japanese persimmons. Early fruit drop occurs in virtually all cultivars after flowering to July in Japan (Fig. 3), and late drop occurs in some particular cultivars from August to harvest time. The higher the ability of parthenocarpy or the larger the number of seeds formed even in cultivars with a low parthenocarpic ability, the less the early fruit drop. Another factor causing early fruit drop is insufficient sunlight (Suzuki *et al.* 1989; Kitajima *et al.* 1990; Yakushiji and Hase 1991; Yano *et al.* 1999a). However, pollinated 'Fuyu' fruit containing three or more seeds hardly dropped even when the light intensity was reduced to 18% (Yakushiji and Hase 1991). In addition, girdling at 10 days after full bloom (DAFB) DAFB of trees grown under shading was more effective at inhibiting early fruit drop than GA_3 or 1-(2-chloro-4-pyridyl)-3-phenylurea (forchlorfenuron) application in pollinated 'Tonewase', which produces few seeds (Yano *et al.* 1999a, 1999b). Application of 1-naphthaleneacetic acid (NAA) to the apex reduced fruit abscission, but application to the calyx induced abscission on trees with or without shading (Suzuki *et al.* 1989). The endogenous indole-3-acetic acid (IAA) level of the calyx in the fruit that stayed on the tree was higher than that of the flesh during early fruit drop (Suzuki *et al.* 1989; Yakushiji and Hase 1991; Kojima *et al.* 1999; Yano *et al.* 2002). Also, the IAA (5.5 ng/g FW) content in the flesh of non-pollinated 'Fuyu' fruit was lower than that of pollinated fruit (10.1 ng/g FW) after 15 DAFB (Yakushiji and Hase 1991). Endogenous IAA levels in the flesh of fruits on 'Hiratanenashi' trees with shading decreased from 10.1 to 2.2 ng/g FW remarkably, to much lower than the levels in the calyx (17.5 to 20.0 ng/g FW) (Suzuki *et al.* 1989). Thus, Suzuki *et al.* (1989) proposed that early fruit drop is induced by an imbalance of the IAA levels between flesh and calyx a few days before abscission.

On the other hand, Kitajima *et al.* (1990) reported that fruit abscission was induced by competition for photosynthates between fruit and vegetative organs, because the leaves and shoots were the largest sinks until 3 weeks after bloom, and the rate of accumulation of dry matter in fruits destined to abscise decreased about 1 week before abscission. Moreover, they revealed that a major factor in fruit set was the competition between parthenocarpic fruit and seeded fruit on the same tree (Kitajima *et al.* 1992, 1993a). Although the absence of seeds generally induced early fruit drop, it is possible that many parthenocarpic fruits set, and their quality is as good as that of seeded fruit, when all flowers of a tree are prevented from being pollinated (Kitajima *et al.* 1993b). Since the application of GA_3 can induce parthenocarpy in Japanese persimmon (Yakushiji and Hase 1991; Kitajima *et al.* 1992) and the level of GA-like substances in the flesh is high (0.25 to 0.34 pmol/g FW) during the early fruit developing stage (Kojima *et al.* 1999), endogenous GAs play a role in regulating parthenocarpy and promoting cell division in fruit in relation to the fruit's sink ability. However, the relationship between endogenous GA and early fruit drop remains unknown.

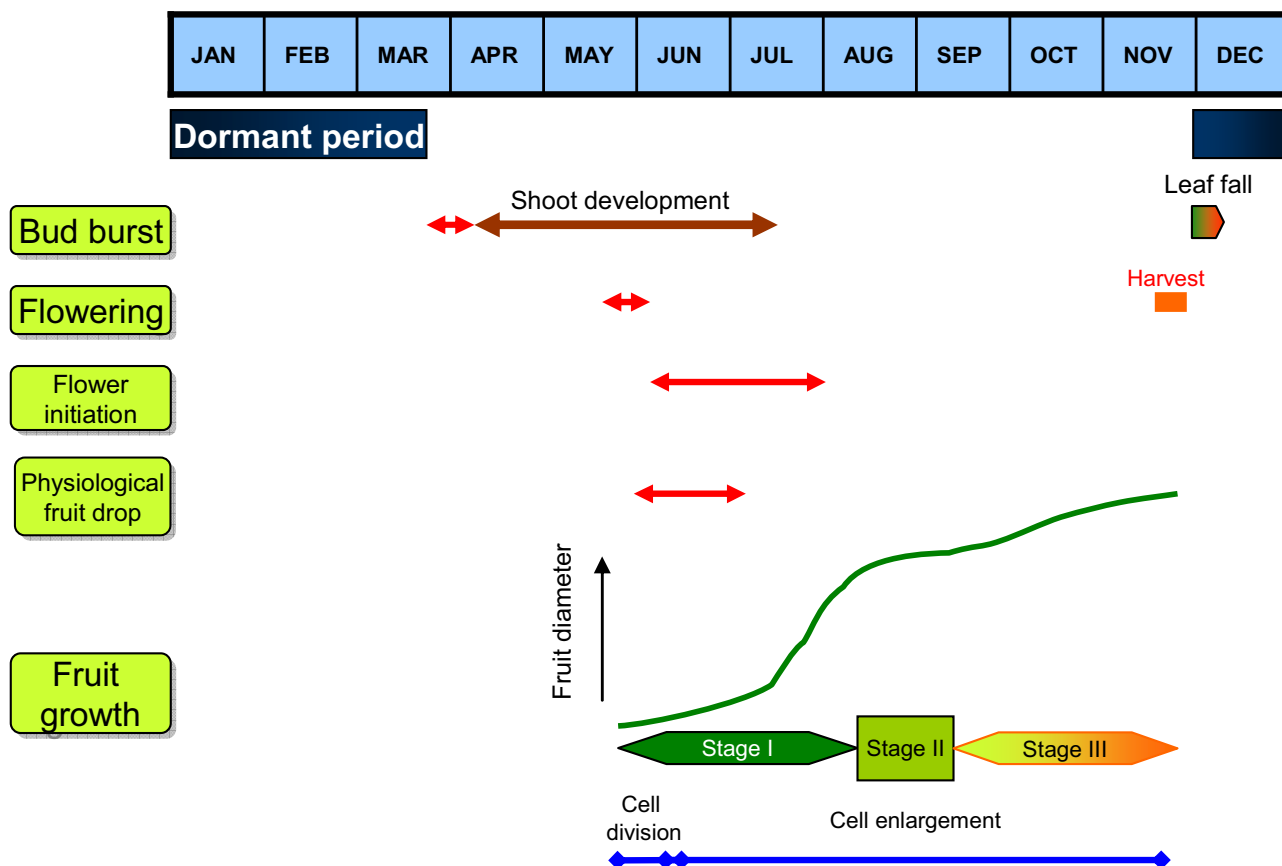


Fig. 3 Phenological growth stages of Japanese persimmon trees ('Fuyu') in Japan.

ROOT RESTRICTION AND BRANCH TRAINING SYSTEMS

Root restriction cultivation

By reducing canopy size through root restriction, container cultivation allows effective production of high-quality fruit at a high plant density with reduced labor. Matsumura and Ozeki (1998) reported that 'Maekawajiro', which grew rapidly to about 2.4 m within 6 years, is suitable for cultivation in a 40-L container. They recommended sand cultivation because of its lower cost than that of mixed soil and superior to control the growth. Even though trees planted in bigger containers grew more rapidly, the researchers recommended 40 L because it balanced ease of container handling with high fruit quality, a fruit weight of 340 g, an SSC of 18.3%, and a low occurrence of fruit cracking at the calyx end. 'Maekawajiro' yielded about 4 kg per tree in 40-L containers. The highest fruit quality was achieved by irrigating each container with 3 to 5 L/day and by supplying it with 10 g nitrogen per year (Matsumura 1999). In addition to the hard containers, soft root-through containers made of non-woven fabric proved ideal, improving fruit quality without excessively restricting root systems (Gemma and Toyonaga 2003).

In other work, persimmon trees were grown at high density in beds with limited rooting zones. Three cultivars trained in central-leader style planted in beds made of wooden frame of 40 m long \times 60 cm wide \times 30 cm high spaced 2.2 m \times 1 m apart (4,550 trees/ha) cropped early with high yield from the third year after planting (Fumuro *et al.* 1997). In this system, trees were trained to a central leader along a 1.8 m high trellis with lateral branches being trained horizontally. Then, all management practices can be done by hand without using ladders. Standard persimmon trees without a limited rooting zone usually begin to manifest their full performance in 14-15 years after planting. Therefore, this culture system could shorten the time re-

quired to obtain high performance by one fourth to one fifth compared to an ordinary culture system. Pruning these trees back to about 2 m high made for lighter work than did the conventional central-leader training. Four-year-old 'Shinshu' trees planted at a density of 4,550 trees per ha, growing in beds with 180 L soil, produced an annual yield of about 40 t/ha (Gamo and Fumuro 2001). That indicated that early cropping with a considerable amount of yield can be obtained by this high density planting grown under root zone restriction from the fourth year after planting.

Thus, root zone restriction is effective at shortening the time to reach high performance, and saves labor with no need to use ladders for management practices relative to traditional culture systems. However, it remains to be investigated how long Japanese persimmon trees can maintain high productivity and fruit quality under root restriction.

Y-shaped training

Y-shaped training was developed to improve the productivity of Japanese persimmon. This system increases the yield per tree and per unit of trunk cross-sectional area and reduces fluctuations in annual yields compared with the traditional open-center training system (Himeno *et al.* 1991). The total number of current shoots and their vertical distribution in the crown are similar to those in the traditional method, but the length of the current shoots and growth of the trunk are decreased in Y-shaped training. In addition, Y-shaped training gave a higher LAI (3.15) than open-center training (2.54) in July. There were no differences in yield or harvest time of 'Tonewase' between horizontal trellis and Y-shaped training (Kawao *et al.* 2005). This method seemed to have little effect on fruit weight and SSC (Himeno *et al.* 1991).

Horizontal trellis

A horizontal-trellis training system developed at the Fuku-

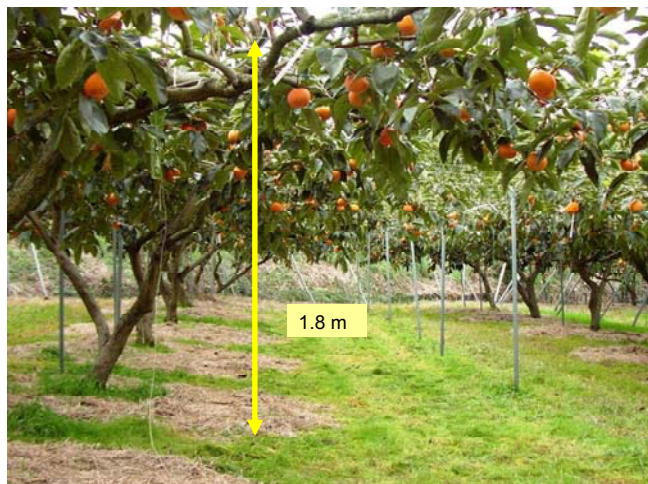


Fig. 4 The horizontal trellis training system of 'Fuyu' Japanese persimmon trees. Photograph by courtesy of Mr. H. Chijiwa.

oka Agricultural Research Center has been gaining popularity as a way to reduce labor in the cultivation of Japanese persimmon cultivars, which tend to be too vigorous. Hayashi *et al.* (2004) altered the framework of open-center, 3.6-m-high 'Matsumotowase-Fuyu' trees to a horizontal frame at 1.8 m height by cutting back secondary scaffold branches at 1.5 to 1.6 m above ground level (Fig. 4). The canopy area of horizontally trellised trees expanded vigorously, and the number of shoots per unit of canopy area was greater than that of the open-center free standing trees 2 years after retraining (Hayashi *et al.* 2004). However, the numbers of shoots on lateral branches and mean shoot length were not significantly different. Kawao *et al.* (2005) retrained 12-year-old 'Tonewase' trees grown with Y-shaped training onto a horizontal trellis. Even though horizontal training was developed for young trees, retraining allows farmers to switch mature trees to a horizontal trellis in a couple of years.

Although the capital investment required to establish horizontal trellises was estimated to be more ¥4,000,000 to 5,000,000/ha than the conventional stands, the trellises allow shorter working hours in disbudding, fruit thinning, harvesting, and pruning (though not in training shoots to the trellis), because it reduces the use of stepladders (Kawao *et al.* 2005). In addition, the volume of chemical application from a speed sprayer was about 20% less than in Y-shaped training. The fruit weight and SSC of fruit grown on horizontal trellises were higher than those of trees grown with open centers ('Matsumotowase-Fuyu'; Hayashi *et al.* 2004) or Y-shaped training ('Tonewase'; Kawao *et al.* 2005). Additionally, the fruits of 'Matsumotowase-Fuyu' grown on horizontal trellises matured earlier (Hayashi *et al.* 2004). On the other hand, horizontal trellising had no effects on fruit quality of 'Taishu' (Fujishima *et al.* 2006). These differences in fruit quality might be varietal characteristics.

As the current shoots are horizontally bent and evenly distributed, growers can easily maintain the optimum LAI. The horizontal bending could cause strong suppression of apical dominance, because horizontally trellised trees produced more flower buds than did both open-center (Hayashi *et al.* 2004) and Y-shaped trees (Kawao *et al.* 2005). Both 'Taishu' and 'Matsumotowase-Fuyu' yielded more than the open-center trees (Hayashi *et al.* 2004; Fujishima *et al.* 2006). Horizontally trellised trees had a higher ratio of assimilate partitioning to fruit on account of less dry matter production in the current shoots and older wood than in the open-center trees (Hayashi *et al.* 2002).

PRODUCTION TECHNIQUE

Plant growth regulators

Some plant growth regulators have been tested in Japanese persimmon for regulation of tree growth, control of flowering and fruiting, and improvement of fruit quality by controlling maturation.

Forchlorfenuron is a cytokinin-like plant growth regulator that increases berry size in grapes and kiwifruit. Application of 10 to 20 ppm to trees from flowering to young fruit 10 DAFB increased fruit set and fruit size in some cultivars (Hasegawa *et al.* 1991; Yano *et al.* 1999a). Treatment at 20 DAFB or with a higher dosage delayed harvest time, and the following year, the number of flower buds was reduced (Yano *et al.* 1999a). Although Hasegawa *et al.* (1991) indicated that the application of 10 ppm at 10 DAFB was a practical procedure, the use of this chemical on Japanese persimmon is not registered in Japan.

Pacllobutrazol (2RS, 3RS)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1H-1, 2, 4-triazol-1-yl) pentan-3-ol) is a triazole-type plant growth retardant which blocks gibberellin biosynthesis, and causes dwarfing in a wide range of plants. Application of 1,000 to 4,000 ppm to bearing shoots during late April to early May inhibited shoot elongation (Fumuro and Murata 1990; Murai 1992) and increased early fruit drop (Murai 1992). Application in late May significantly inhibited secondary shoot growth (Murai 1992). Application to 'Saijo' fruits promoted peel coloration and higher SSC content but reduced fruit size (Murai 1992). Pacllobutrazol enhanced the number of flower buds per shoot in the following year (Murai 1992). Soil application at 2.5 to 10 g per tree shortened 'Fuyu' fruit ripening by 1 to 2 weeks and increased fruit size during the year of treatment and the following year (Fumuro and Murata 1990). Shoot elongation was inhibited in the year following soil treatment. However, pacllobutrazol is not registered for commercial use on Japanese persimmon in Japan.

Ethylchlorzate (ethyl 5-chloro-1H-3-indazolyl-acetate) is an auxin. Although it is registered for various purposes and is the main fruit-thinning agent for citrus in Japan, it has been tested mainly as a maturity accelerator in Japanese persimmon. Chijiwa *et al.* (2003) recommended that spraying of 40 ppm at 74 and 100 DAFB enhanced the peel color of 'Nishimurawase' by inhibiting leaf fall because 80 ppm was more effective than 40 ppm but resulted in severe leaf fall. No difference was observed in fruit size, firmness, or SSC, and shelf life was slightly extended. The use of two applications of 40 ppm at 70 to 80 DAFB is registered in Japan for the purpose of enhancing peel coloration in some cultivars of Japanese persimmon.

Jasmonic acid (JA) and methyl jasmonate occur widely in the plant kingdom, and have various effects on plant growth. For practical use, *n*-propyl dihydrojasmonate (PDJ) is effective and is more stable than natural JA. PDJ application (50-250 ppm) in early October to middle October seems to promote both peel coloring and fruit maturation (Fujisawa *et al.* 1997; Gemma *et al.* 1997). Gemma *et al.* (1997) reported that the level of endogenous ABA in fruits treated with PDJ was generally lower than in the control at harvest. JA and its derivatives are also well known to affect ethylene synthesis. It is necessary to investigate further the functions of PDJ and how it affects maturation.

Girdling and strapping

Girdling of trees or stems is used to control tree growth, prevent early fruit drop, and improve fruit quality in Japanese persimmon (Figs. 5A, 5B). Trunk girdling (2-3 cm wide) in the middle of June produced larger fruit with higher SSC in 'Hiratanenashi' (Fujimoto and Maesaka 1998). Similarly, the strapping of lateral branches (Fig. 5C) with wires in early June enhanced fruit size and peel coloration in 'Saijo' and 'Maekawajiro' (Hasegawa and Nakajima 1991). Hasegawa *et al.* (2003) reported that the best strap-

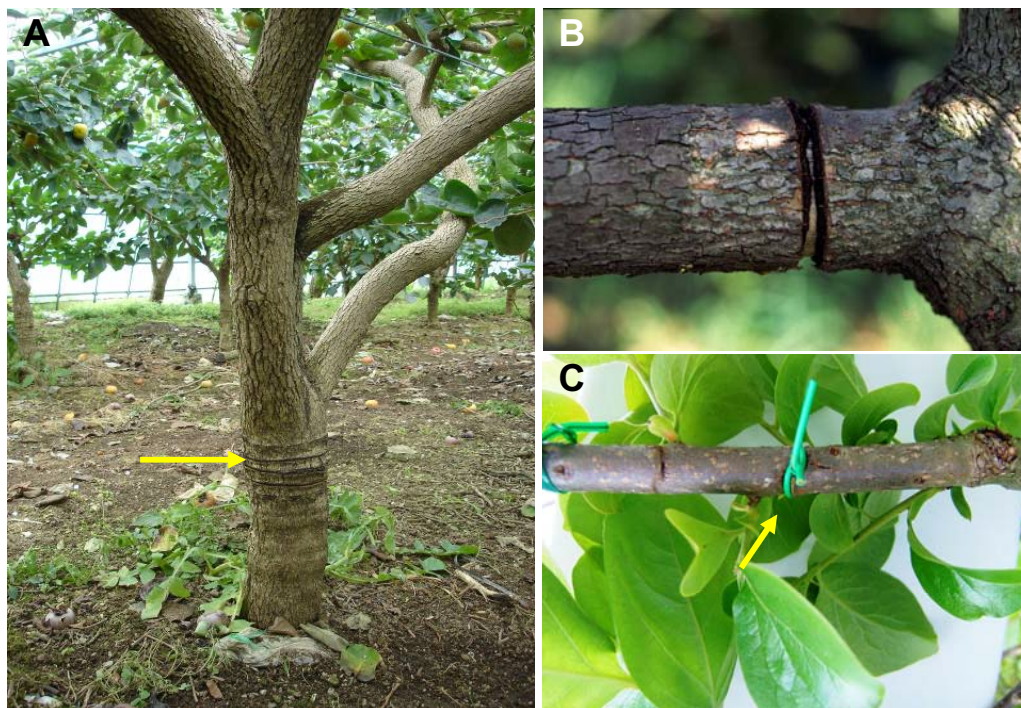


Fig. 5 Trunk girdling (A), secondary scaffold branch girdling (B), and strapping of lateral branch with a wire (C) of Japanese persimmon trees. Photograph (B) and (C) courtesy of Dr. T. Yano, and Dr. K. Hasegawa, respectively.

ping time for fruit set and quality was 2 weeks before full bloom. Both girdling and strapping prevented early fruit drop (Hasegawa and Nakajima 1991; Fujimoto and Maesaka 1998; Yano *et al.* 1999a, 1999b; Hasegawa *et al.* 2003). One year after strapping of lateral branches, flower formation increased in ‘Saijo’, ‘Maekawajiro’, and ‘Tonewase’ (Hasegawa and Nakajima 1991; Yano *et al.* 1999a). The increase of fruit set and fruit growth in trees treated by girdling was thought to be connected with an increase in carbohydrate accumulation in leaves and shoots during Stage I, because girdling limits the downward transport of photosynthates (Hasegawa *et al.* 2002, 2003). The accumulation of carbohydrates by trunk strapping before full bloom was highest in leaves (Hasegawa *et al.* 2003), and girdled trees showed a higher top-to-root ratio than non-girdled trees (Fumuro 1997).

On the other hand, the earlier the trunk was girdled, the greater was the inhibition of shoot and trunk growth (Fumuro 1998). Trunk girdling during the early shoot elongation period can dwarf closely planted Japanese persimmon trees. The growth inhibition by girdling is caused by a reduction of dry matter production attributable to the smaller number and area of leaves and by inhibition of root functions (Fumuro 1998). In contrast, vegetative growth was not inhibited strongly by trunk strapping owing to the complete healing of the strapping position by callus formation a month after removal of the wire (Hasegawa *et al.* 2003). Therefore, the inhibition of vegetative growth or improvement of fruit quality depends on suitable timing or duration of girdling in Japanese persimmon trees.

Forcing culture

The cultivation of persimmon trees in heated plastic houses in some regions allows fruit to be marketed 3 months early and sold at much higher prices. Heating can be divided into two main types: early heating and ordinary heating. Early heating in Nara prefecture, which starts in late December, allows mainly ‘Tonewase’ fruit to be harvested from late June. Ordinary heating, beginning in late January, advances harvesting from mid-August to mid-September. However, such forcing culture needs plenty of fuel during winter. As the price of fuel remains high, farmers and researchers are seeking ways to reduce costs.

A new prediction method, called the development rate and development index (DVR-DVI) method, has recently

been developed for predicting budburst of Japanese persimmon trees (Nakamura 2001; Sugimura *et al.* 2006), and has proved to be more practical and accurate than the traditional thermal unit method in many cases. Temperatures at 5 to 8°C are most effective for breaking bud endodormancy in ‘Tonewase’ (Sugimura *et al.* 2006). The endodormancy of ‘Tonewase’ was completed in mid- to late December in Nara prefecture, and the heating start time could be estimated from the DVR-DVI for bud burst when the minimum air temperature in a greenhouse remained above 15°C. The DVR model correctly predicted budburst within 3 days in forced culture (Sugimura *et al.* 2006). Thus, the DVR-DVI method can be used to precisely identify the optimal time for beginning early heating; fuel consumption will still increase compared with late heating, but the increase will be smaller than if the date of early heating is not based on physiological indicators such as DVR-DVI.

Early heating of ‘Tonewase’ decreased the number of flowers and fruit weight in the next year (Imagawa and Urasaki 2001), perhaps through reduced photoassimilation during differentiation of flower buds in April. Two techniques were established to counteract that effect (Imagawa and Urasaki 2001). In one, fruit was thinned to a ratio of 8 to 11 leaves per fruit, potentially increasing the number of flowers in the following year and fruit weight and SSC in the current year. In the second, 1,500 ppm of CO₂ enrichment from 2 weeks before full bloom to the end of heating (just before fruit coloring) enhanced the number of flowers and fruit weight in the next year, although it did not affect SSC. CO₂ application increases operating costs, although it achieves high fruit quality and stable fruit production in early-heating culture. CO₂ enrichment (1,500 ppm) from full bloom to the end of heating applied for 5 h early every morning with compressed gaseous CO₂ (liquefied carbon dioxide) or all day long with oil-burner exhaust cut down the cost of CO₂ application to one-half to one-third of the cost of conventional treatment using compressed gaseous CO₂ all day, and improved flower bud formation, fruit weight, and yield per tree compared to conventional treatment (Imagawa *et al.* 2005).

When the temperature is not low enough to break endodormancy and allow heating of deciduous fruit trees to start, it is necessary to break endodormancy artificially. In early November, a leaching solution of 20% calcium cyanamide (CaCN₂) was the most effective at inducing bud break in Japanese persimmon, followed by 50% Merit Blue™ (foliar

fertilizer containing 7% nitrogen, 5% phosphate and minor minerals), whereas GA₃ slightly promoted bud break in early November but not in early December (Itamura *et al.* 1994). Application of garlic oil, which induces bud break of grapevines, was not only ineffective but also injured buds. However, the use of calcium cyanamide on Japanese persimmon is not registered in Japan, and Merit Blue is not widely used on Japanese persimmon.

Delaying harvest

When the shipping of fruit to the market peaks, market prices often reach their lowest value. Thus, harvesting later than usual and improving the fruit quality is a useful cultural technique to enhance profitability. Higher temperature during growth stage III delayed the onset of fruit expansion and ripening (Sugiura *et al.* 1991; Isobe and Kamada 2001). The peel coloration of 'Maekawajiro' fruits grown in a vinyl house controlled at 30°C or higher was delayed by 10 to 20 days (Isobe and Kamada 2001). The coverage of trees or branches with non-woven cheesecloth after 100 to 120 DAFB delayed harvest by 7 to 14 days compared with open culture (Nishikawa *et al.* 2002). The use of cheesecloth delayed maturation not by reducing the light intensity but by increasing the daytime temperature. Kurahashi *et al.* (2002) found that a long-day photoperiod (about 17 h) from late August to early December delayed 'Saijo' fruit maturation by 20 to 30 days and enhanced vegetative growth.

FRUIT MATURATION AND RIPENING

Ethylene is related to ripening and fruit softening

Persimmon fruit has a short shelf-life due to fruit softening. This process is accelerated after the removal of astringency, and significantly influences the acceptability of the fruit. Ethylene plays an important role in fruit softening (Itamura *et al.* 1991). Unlike typical climacteric fruit, persimmons have some unique characteristics: 1) the younger the stage at which the fruit is detached, the greater the amount of ethylene produced; and 2) ethylene biosynthesis is only induced when the fruit is detached from the parent tree (Takata 1983; Itamura 1986).

Treatment with 1-methylcyclopropene (MCP), an inhibitor of ethylene action, inhibits fruit softening without any de-astringency treatment, confirming that the softening process in persimmon fruit is directly dependent on ethylene (Nakano *et al.* 2002, 2003). Three cDNAs encoding the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene (*DK-ACS1*, *DK-ACS2* and *DK-ACS3*) and two cDNAs encoding the ACC oxidase gene (*DK-ACO1* and *DK-ACO2*), which are involved in ethylene biosynthesis (Fig. 6), were isolated from 'Hiratanenashi' fruit and their expression levels were extensively investigated (Nakano *et al.* 2002, 2003). In plastic-house 'Tonewase' fruit, rapid fruit softening

can only be caused by the action of ethylene, which is produced in the calyx in direct response to water stress. Initially, the primary water stress signal induces ethylene production in the calyx by activating *DK-ACS2* expression, and this ethylene then induces autocatalytic ethylene production in the pulp by activating expression of the *DK-ACS1*, *DK-ACS2* and *DK-ACO1* genes (Nakano *et al.* 2002). Young 'Hiratanenashi' fruit also produced ethylene in response to water stress followed by rapid fruit softening (Nakano *et al.* 2003). In 'Rendaiji' persimmon, treatment with ethanol to remove the astringency and then MCP delayed fruit softening, extended the shelf-life, and inhibited the accumulation of ACC and the ACC synthase and ACC oxidase activities (Ortiz *et al.* 2005a, 2005b). Ethylene biosynthesis associated with rapid ripening was accompanied by expression of the *DK-ACS1*, *DK-ACS2*, *DK-ACO1* and *DK-ACO2* genes (Ortiz *et al.* 2006), whereas *DK-ACS3* expression was not induced.

Fruit softening

Plant cell walls consist of cellulose microfibrils, lignin and matrix substances (pectins, hemicelluloses, proteins and phenolics). The degradation processes of both xyloglucans and polyuronides are cooperatively involved in fruit softening processes (Wakabayashi 2000).

In 'Fuyu' persimmon fruit under low humidity (60% relative humidity), decreases in the amounts of arabinose and galactose in the pectin and the cellulose content in the mesocarp lead to fruit softening. Fruit firmness of the mesocarp is not influenced by changes in a single cell wall fraction but rather by changes in all the cell wall fractions. For example, the overall amount of cell wall polysaccharides plays a greater role in determining fruit firmness than their individual molecular weights (Tsuchida *et al.* 2003, 2004). Itamura *et al.* (1995) showed that decreased levels of arabinose and galactose in cell walls had close relationships with fruit softening in 'Tonewase' persimmon after constant temperature short duration (CTSD) treatment to remove astringency.

Ishimaru *et al.* (2001, 2002) also showed that fruit softening of 'Hiratanenashi' and 'Fuyu' persimmons was attributable to increased water-soluble polyuronide contents and decreased water-insoluble polyuronide contents. MCP suppressed the softening and polygalacturonase (PG) activity in 'Matsumotowase-Fuyu' persimmon fruit, which is an early-maturing strain selected from 'Fuyu' persimmon (Niikawa *et al.* 2005). At the molecular level, the ethylene-dependency of *DK-PG1* gene expression is closely involved in 'Hiratanenashi' fruit softening (Kubo *et al.* 2003).

Ripening of 'Saijo' persimmon was induced by solubilization of pectic polysaccharides via the actions of endo-type glycanases in immature green or yellow fruits and digestion by exo-type glycosidases, in particular β-galactosidase, in mature red fruits (Nakamura *et al.* 2003). Rapid

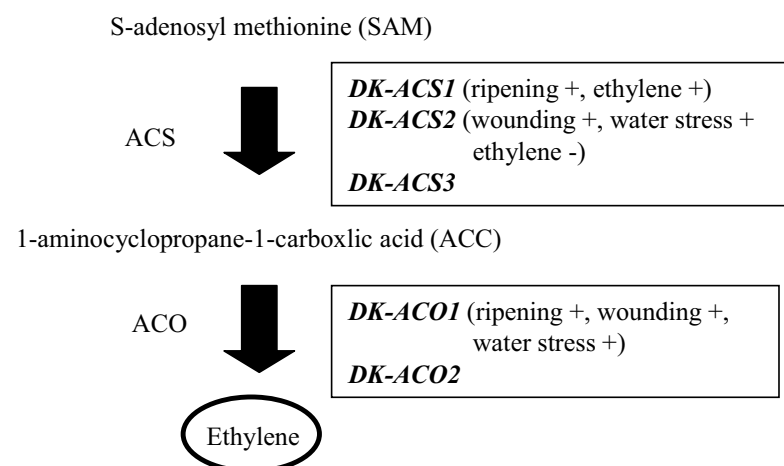


Fig. 6 Ethylene biosynthesis pathway and ACS and ACO gene regulation by internal and external stimuli in persimmon. ACS, ACC synthase; ACO, ACC oxidase. ACS catalyzes SAM to ACC and ACO converts ACC to ethylene. + positively, - negatively.

fruit softening and increased α -arabinofuranosidase activity occurred in young 'Saijo' persimmon without a de-astringency treatment (Xu *et al.* 2003), and MCP suppressed the rapid flesh softening and α -arabinofuranosidase activity after removal of the astringency with dry ice in mature 'Saijo' persimmon (Xu *et al.* 2004). β -galactosidase gene expression coincided with the progress of fruit softening and was suppressed by MCP (Nakatsuka *et al.* unpublished). β -galactosidase activities and fruit softening were significantly inhibited by MCP in 'Rendaiji' persimmon treated with ethanol to remove the astringency (Ortiz *et al.* 2005b).

In persimmon fruit, the level and molecular mass of xyloglucans during excessive softening decreased to about 70% and 30% of the corresponding values in pre-ripe fruit, respectively (Cutillas-Iturralde *et al.* 1993, 1994). Nakatsuka *et al.* (2004, 2005) reported that two xyloglucan endotransglucosylase/hydrolase genes (*DkXTH1* and *DkXTH2*) were isolated from 'Saijo' persimmon and that fruit softening was closely related to ethylene-dependent *DkXTH1* expression in 'Saijo' persimmon following both dry-ice and alcohol treatments for de-astringency.

As described above, the diversity in softening behavior and textural changes among persimmon cultivars could be related to the solubilization and depolymerization of pectic and hemicellulosic polymers.

Ethylene production after wounding

Mechanical wounding (e.g., cutting or bruising) also induces ethylene production via the induction of ACC synthase and ACC oxidase gene expressions in some plants (Kende 1993). However, the mechanism for this wound-induced ethylene production is currently unclear at the enzymatic and molecular levels. Zheng *et al.* (2005a) successfully extracted ACC synthase and ACC oxidase enzymes from wounded persimmon fruit using a polyethylene glycol acetone method to remove the high tannin content. The optimal reaction conditions for these enzymes were thoroughly investigated, and subsequent studies determined their activities (Ortiz *et al.* 2005b; Zheng *et al.* 2005b, 2006). ACC synthase is the rate-limiting factor in ethylene biosynthesis in mature wounded 'Hiratanenashi' fruit as well as in other

plants. *DK-ACS2* and *DK-ACO1* are the dominant genes in wounded mature persimmon fruit (Zheng *et al.* 2005b). Following the application of MCP pretreatment, wound-induced ethylene biosynthesis was auto-inhibited in mature 'Saijo' persimmon fruit tissues. Wound-induced ACC synthase activity and *DK-ACS2* gene expression were negatively regulated by ethylene, whereas *DK-ACO1* gene expression was independent of ethylene feedback control (Zheng *et al.* 2006).

Proanthocyanidin synthesis

The astringency of persimmon fruit is caused by proanthocyanidin (condensed tannins) located in the vacuoles of tannin cells, which are specialized cells for tannin accumulation within the fruit. In persimmons, partial DNA sequences of nine structural genes involved in tannin accumulation via the flavonoid biosynthetic pathway (Fig. 7), namely phenylalanine ammonia lyase, cinnamate-4-hydroxylase, 4-coumarate:coenzyme A ligase, chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, flavonoid-3'-hydroxylase, flavonoid-3',5'-hydroxylase and dihydroflavonol reductase, were isolated by PCR using degenerate primers and their expression levels were analyzed (Ikegami *et al.* 2005a). A comparison of PCNA ('Hanagosyo' and 'Suruga') and non-PCNA ('Kuramitsu' and 'Yokono') cultivars revealed that the sudden termination of tannin accumulation in PCNA-type cultivars is due to termination of the transcription of all nine structural genes involved in condensed tannin biosynthesis at an early stage of persimmon fruit development (Ikegami *et al.* 2005a).

In addition, anthocyanidin reductase, which is a key enzyme in proanthocyanidin biosynthesis) and *D. kaki* serine carboxypeptidase-like protein 1 (*DkSCPL1*) were isolated by suppression subtractive hybridization from artificially astringency-removed fruit on the tree (Ikegami *et al.* 2005c). The Chinese PCNA cultivar 'Luo Tian Tian Shi' behaves in a similar way to the PCA cultivar 'Kuramitsu' with regard to expression of the phenylalanine ammonia lyase, chalcone synthase, flavanone-3-hydroxylase, dihydroflavonol reductase and anthocyanidin reductase genes, but differs in its *DkSCPL1* gene expression. Specifically, expression of the

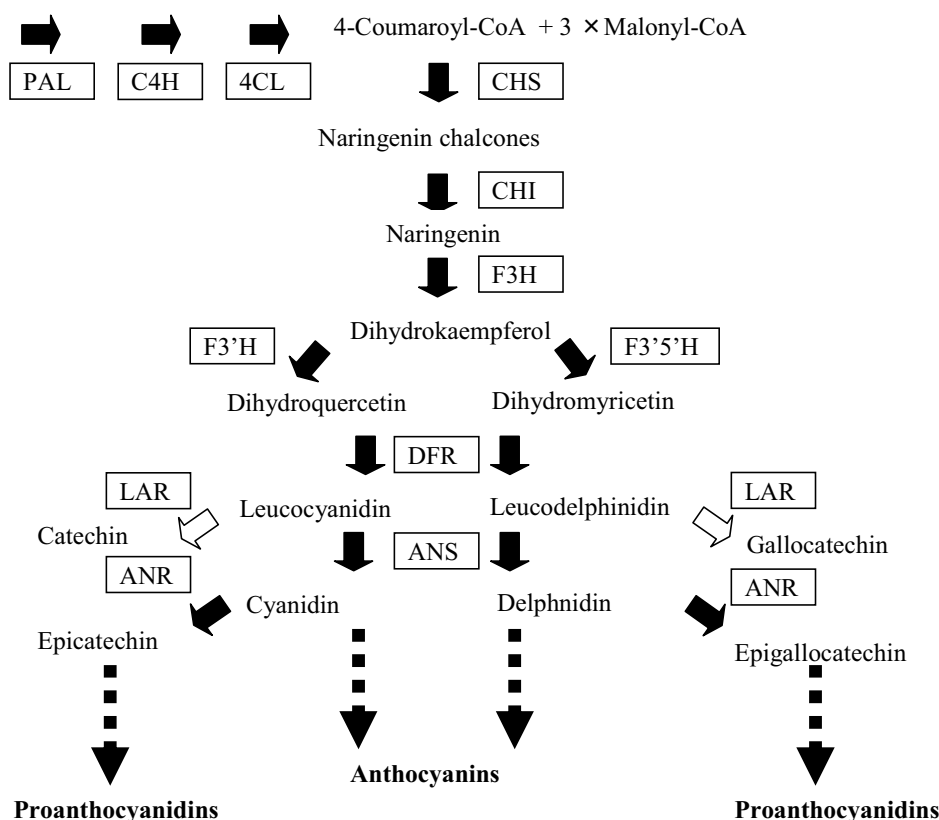


Fig. 7 Proanthocyanidin biosynthesis pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. Closed arrows show gene encoded enzymes are isolated from Japanese persimmon. (Ikegami *et al.* 2005a, 2005c)

DkSCPL1 gene declined at an earlier stage than those of other flavonoid biosynthesis genes, concomitant with the termination of tannin cell development (Ikegami *et al.* 2005c). On the other hand, a phylogenetic tree constructed on the basis of the similarity indices of amplified fragment length polymorphism (AFLP) markers suggested close relationships among Japanese PCNA cultivars, but more distant relationships with the Chinese PCNA cultivar 'Luo Tian Tian Shi' (Kanzaki *et al.* 2000a). The chemical properties of tannin, especially the size distribution of tannin molecules after size-exclusion chromatography, and expression analyses of proanthocyanidin biosynthesis-related genes also support the conclusion that this Chinese PCNA cultivar is different from Japanese PCNA cultivars (Ikegami *et al.* 2005b).

As described above, the early steps of the proanthocyanidin synthesis pathway are well understood, whereas the late steps, such as polymerization, remain unclear. We conducted Expressed Sequence Tag analyses using young and mature 'Saijo' persimmon fruit and obtained many genes related to the proanthocyanidin pathway. This DNA information will be investigated by DNA arrays to further our understanding of the late steps in the pathway.

POSTHARVEST PHYSIOLOGY AND HANDLING

Extended shelf-life

Inhibition of ethylene production

A number of persimmon storage techniques have recently been developed, particularly the development of 'Tonewase' fruit. 'Tonewase' is an early-maturing bud sport of 'Hiratanenashi' persimmon. Postharvest fruit softening after removal of astringency was investigated in detail in force-cultured 'Tonewase' persimmon, and often resulted in rapid softening during postharvest distribution.

'Tonewase' fruits exposed to air and 100% CO₂ softened within 6 days, whereas those exposed to 100% CO₂ and then gradually reduced to 0% CO₂ for 6, 12 or 20 hours maintained their firmness for 2 weeks, with inhibited respiration and ethylene production (Ishimaru *et al.* 1998). The inhibition of ethylene production in 'Tonewase' fruit following exposure to 100% CO₂ and gradual reduction to 0% CO₂ for 7 hours to remove their astringency may be attributed to the ACC synthase activity (Ishimaru *et al.* 1999).

The fruit softening mechanism was revealed by researchers of the Okayama University and Wakayama Fruit Tree Experiment Station. Harima *et al.* (2001a) reported that postharvest fruit softening was closely dependent on fruit maturity at harvest and that exposure to high temperatures during the maturation stage affects the maturity indices and physiological activities in the fruit, resulting in rapid fruit softening. Nakano *et al.* (2001) reported that the softening was caused by water stress-induced ethylene production, which can be suppressed by reducing the water loss by using perforated polyethylene bags. The use of perforated polyethylene packaging is a useful technique for extending the shelf-life of 'Tonewase' persimmon fruit, even those marketed during the summer season (Harima *et al.* 2001b). These researchers established a method for prolonging the postharvest life of this persimmon cultivar by using perforated polyethylene film or a corrugated cardboard container coated with water-impervious material (Harima *et al.* 2002a, 2002b). Persimmon fruit packaged in perforated polyethylene bags evolved trace levels of ethylene without any significant change in softening for 10 days. A 6-hour delay in packaging the fruit into the perforated polyethylene bags after harvest eliminated the beneficial effects of the packaging. Packaging the fruit in improved carbon boxes maintained the relative humidity at about 70%, effectively reduced weight loss, delayed ethylene induction and suppressed fruit softening under commercial conditions.

Fumuro and Gamo (2002) reported that CO₂-treated 'Hiratanenashi' persimmon could be stored in 0.05-mm

thick polyethylene bags at 0°C for no more than 4 months to maintain their flavor and texture, and suggested that fruit softening was effectively delayed by individual sealing due to inhibition of water loss from the fruit, consistent with the findings of Nakano *et al.* (2001).

Block of ethylene action

For astringent-type persimmons, it is necessary to remove the astringency with CO₂ gas and alcohol (ethanol) vapor treatments after harvest. A serious problem in persimmon handling is that mature fruits soften quite rapidly, accompanied by increased ethylene production after treatments to remove astringency. The ethylene action inhibitor MCP has been shown to successfully prolong the shelf-lives of 'Tonewase', 'Saijo' and 'Rendaiji' persimmons in combination with different de-astringency methods (Harima *et al.* 2003; Kurahashi *et al.* 2005; Ortiz *et al.* 2005b). In 'Tonewase' and 'Saijo' fruit with CTSD de-astringency treatment, MCP treatment inhibited postharvest fruit softening at room temperature for 12 and 16 days, respectively, while non-MCP-treated fruit softened within 5 days after harvest (Harima *et al.* 2003). When 'Saijo' persimmon was treated with MCP followed by dry ice for de-astringency, the shelf-life was prolonged by about 6 days, compared with non-MCP-treated fruit (Kurahashi *et al.* 2005). Treatment of 'Rendaiji' fruit with ethanol vapor to remove astringency and then MCP delayed fruit softening and extended the shelf-life by 8 days, compared with non-MCP-treated fruit (Ortiz *et al.* 2005b).

A combination of 50% CO₂ and ethanol for 10 hours at 35°C was used to treat 'Tonewase' persimmon (Imagawa *et al.* 2003). This technique inhibited fruit softening but did not inhibit ethylene production, suggesting that the shelf-life extension may be attributed to a reduction in ethylene sensitivity induced by the high temperature. In total, 80% of greenhouse-grown 'Tonewase' persimmons in Nara prefecture were treated by this de-astringent method in 2001 and the ratio of fruit softening was reduced by about 3%.

De-astringency mechanism

The astringency of astringent persimmon fruit can be removed by insolubilizing the soluble tannins that give rise to the astringent taste. These tannins were polymerized by acetaldehyde that can accumulate in the flesh during exposure to either ethanol vapor or high levels of CO₂ gas (Taira 1996). Recently, tannin was found to be insolubilized by other new factors in 'Hiratanenashi' persimmon without acetaldehyde accumulation (Taira and Ono 1997; Taira *et al.* 1997). Specifically, the tannins adhered to cell wall fragments, thereby becoming insoluble, and the astringency of the flesh decreased without any accumulation of acetaldehyde (Taira and Ono 1997). Furthermore, Taira *et al.* (1997) reported that the formation of complexes between soluble pectins released from the flesh of cell walls and tannins released from the vacuoles in tannin cells can result in reduced persimmon astringency during postharvest fruit softening, with no relationship to acetaldehyde accumulation.

Acetaldehyde is believed to be synthesized from alcohol by the action of alcohol dehydrogenase (ADH). However, Kawakami *et al.* (2005, 2006) doubt that ethanol is converted to acetaldehyde, leading to astringency removal by ethanol treatment, and proposed a new theory. Briefly, the cultivars associated with easy astringency removal ('Tonewase' and 'Saijo') have low *DKADH1* expression; whereas those associated with difficult astringency removal ('Yokono' and 'Atago') have very high *DKADH1* expression. These findings suggest that ADH is regulated by positive feedback in ethanol biosynthesis, thereby causing ethanol accumulation. In addition, ethanol and CO₂ treatments both enhance the expression of the pyruvate decarboxylase gene (*DKPDC1*), with higher *DKPDC1* expression observed after CO₂ treatment than after ethanol treatment. Varietal differences in the ease of astringency removal may be dependent on the ratio

of *DKPDC1/DKADH1* expression. This concept may lead to the development of suitable de-astringency methods for individual cultivars.

Functional components

Functional materials, such as ascorbic acid (vitamin C) and polyphenols, are present in persimmons. For example, kakinins contained in persimmon fruit have traditionally been used as a medicine for hangovers. Isoquercitrin (quercetin 3-glucoside) and astragaloside (kaempferol 3-glucoside) as main flavonoids in persimmon are antioxidative. Itamura *et al.* (2004) reported the possibility that ingestion of persimmon fruit before drinking alcohol would alleviate the deconditioning of hyper-drunkness in humans because ingestion of persimmon fruit (both mature fruit after astringency removal and semi-dried fruit containing 32% of the moisture of the fresh fruit) would inhibit uptake of ethanol into the blood. On the other hand, suitable manufacturing processes for maintaining high levels of functionality (Tsurunaga *et al.* 2004, 2005) and suitable materials as sources (Tsurunaga *et al.* 2006a, 2006b) were investigated in 'Saijo' persimmons. Steam treatment before drying is very effective for inhibiting decreases in the total ascorbic acid content in persimmon leaves during the manufacture and storage of persimmon tea. Using young leaves harvested in May, steam treatment for 5 min without roasting provided the highest total ascorbic acid content after 170 days of storage (Tsurunaga *et al.* 2005). On the other hand, in 'Saijo' persimmon leaves, the concentrations of total ascorbic acid and polyphenol were at their highest levels from June to July (3,700 mg/100 g DW and 16,100 mg astragaloside eq/100 g DW, respectively), while the concentrations of isoquercitrin and astragaloside were at their highest levels in May (Tsurunaga *et al.* 2006a). In June, shoots and leaves are suitable materials for health foods as sources of total ascorbic acid (4,690 mg/100 g DW), while foliating leaves are suitable sources of functional compounds (polyphenols) (Tsurunaga *et al.* 2006b).

ADVANCES IN BIOTECHNOLOGY AND MOLECULAR BIOLOGY

Classification of *Diospyros* species

Phylogenetic studies are required to discover novel breeding materials and further our understanding of the relationships among wild species and cultivated hexaploids.

Nakamura and Kobayashi (1994) observed polymorphisms among *Diospyros* species from temperate origins by RFLP analyses, using chloroplast DNA and mitochondrial DNA probes for hybridization. *D. kaki*, *D. oleifera*, *D. kurowaii*, *D. virginiana* and *D. lotus* were found to be highly related to each other, but less related to *D. rhombifolia*. RFLP analyses using a chloroplast DNA probe revealed that *D. lotus*, *D. virginiana* and *D. kaki* have the same banding pattern (Yonemori *et al.* 1998). Yamagishi *et al.* (2005) reported that *D. kaki* is more closely related to *D. lotus* than to *D. oleifera* and *D. rhombifolia* using RAPD markers, consistent with RFLP analyses using the cpDNA probe (Yonemori *et al.* 1998).

Multi-color genomic *in situ* hybridization (GISH) could distinguish the parental chromosomes of somatic hybrids between *D. kaki* and *D. glandulosa* (Choi *et al.* 2002). *D. kaki* and its wild relatives were also investigated by GISH (Choi *et al.* 2003a). The genome of *D. kaki* is most closely related to that of *D. glandulosa* among the species tested (*D. oleifera*, *D. lotus*, *D. ehretoides*, *D. rhodocalyx*, *D. mespiliformis*, *D. rhombifolia* and *D. virginiana*). This supports the argument of Ng (1978), who suggested that *D. glandulosa* could be involved in the speciation of *D. kaki* on the basis of morphological similarities and geographical distributions.

On the other hand, three repetitive DNAs from *D. kaki* and two other species, *D. oleifera* and *D. ehretoides*, were isolated and used for genomic Southern and fluorescence *in*

situ hybridization (FISH) analyses (Choi *et al.* 2003b). Three diploid species (*D. glandulosa*, *D. oleifera* and *D. lotus*) and *D. kaki* contained *EcoRV* and *HincII*-repetitive DNA from *D. kaki* and *D. oleifera*, respectively. However, *D. virginiana* contained *EcoRV*-repetitive DNA, but not *HincII*-repetitive DNA. The copy numbers of *Ty1*-copia group retrotransposons also differed between *D. virginiana* and *D. kaki* (Nakatsuka *et al.* 2002), consistent with the idea that *D. virginiana* is unlikely to be related to *D. kaki*.

In addition, FISH using 5S and 45S rDNA probes successfully visualized their sites on the chromosomes of *Diospyros* species (Choi *et al.* 2003c, 2003d). Chromosomes of an Asian diploid, *D. lotus*, more closely resembled those of *D. kaki* among the Asian diploid species examined based on the numbers and chromosomal distributions of 45S rDNA sites (Choi *et al.* 2003c). Within the Asian species, the numbers of 5S and 45S rDNA sites seemed to increase depending on the ploidy levels of the species. The phylogenetic relationship and speciation of polyploidy species of *Diospyros* require further investigation.

In vitro propagation

Improvement of rooting and acclimatization

The methods of bud explant establishment, shoot proliferation, and rooting have been summarized by Tao and Sugiura (1992a). However, one standard set of tissue culture conditions do not give the same results in every persimmon cultivar. For example, shoot tip cultures (Fukui *et al.* 1990) and rooting potential of the shoot (Fukui *et al.* 1992) are largely influenced by genotype. Tao and Sugiura (1992a) suggested that more research is needed to develop a commercially feasible micropropagation method for Japanese persimmon.

The optimum rooting conditions that have been reported for persimmon are Murashige and Skoog (MS) (1962) medium as the basal medium with nitrate reduced to half-strength (MS (1/2 N)), containing 1.25 mM indole-3-butyric acid (IBA). Explants are grown at 28°C under a 16 h/day photoperiod at 60 $\mu\text{mol}/\text{m}^2/\text{s}$, after an initial dark incubation for 10 days (Tao and Sugiura 1992a). The effects of different plant growth regulators (Tetsumura 1997) and sugars (Kagami 1999) were investigated to improve rooting of micropropagules. Tetsumura (1997) reported effects of different cytokinins on shoot proliferation and rooting of 'Fuyu' and 'Hana Goshō' persimmons. After 74 to 76 subcultures, the shoots subcultured with 20 μM BAP showed a substantially higher rooting percentage (63-65%) than those subcultured with 5 μM zeatin (less than 30%). Tetsumura (1997) recommended that BAP should be substituted for zeatin in the proliferation medium because zeatin is the most expensive cytokinin, and shoots can effectively proliferate on the medium with BAP. Kagami (1999) cultured shoots of Japanese persimmon strain No. 3 on MS (1/2 N) medium with 250 mg/l IBA and 0.2 M fructose, in growth conditions of 25°C, and a 16 h/day photoperiod at 60 $\mu\text{mol}/\text{m}^2/\text{s}$. In that study rooting rate was 94%, but it was only 30% in cultures where fructose was substituted with glucose or sucrose. Interestingly, filter-sterilized fructose had no effect on rooting improvement.

Tetsumura *et al.* (1993) investigated suitable acclimatization conditions to obtain micropropagules using 'Nishimurawase' persimmon. During acclimatization, optimum plantlet survival (85%) was obtained at 28°C under continuous lighting (60 or 120 $\mu\text{mol}/\text{m}^2/\text{s}$) using 15 mm length shoots with three leaves. They suggested that light intensity is important for survival rate and growth of micropropagules. When the micropropagules were transplanted in the field, more than 90% survived. Matsumoto and Yamada (1993) reported acclimatization conditions used for seedlings of 'Saijo'. Seedlings with three or more leaves were grown using vermiculite as the soil substrate and sub-irrigated at 15 to 35°C in a greenhouse, resulting in 90% survival. We attempted to acclimatize micropropagated 'Saijo' in growth conditions of 16 h/day photoperiod at 60

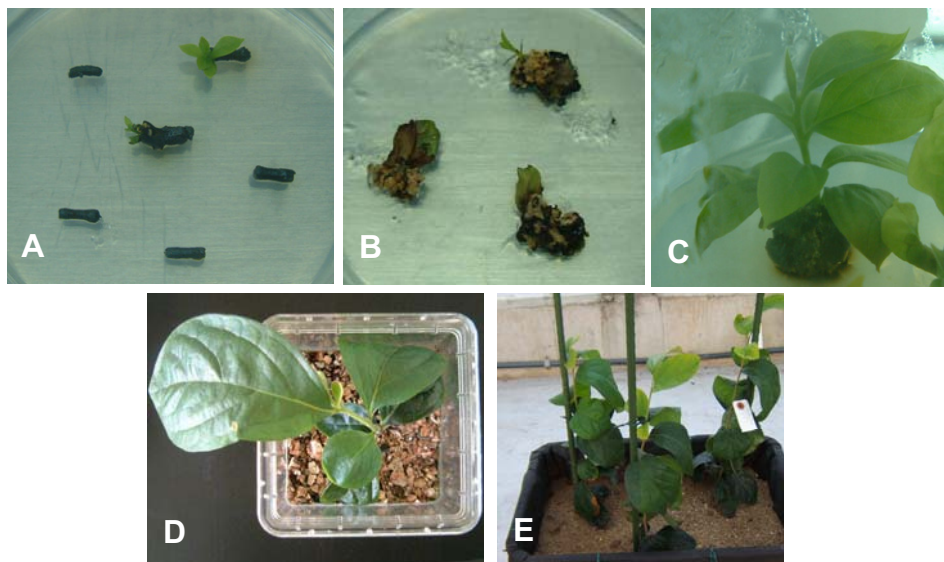


Fig. 8 Regeneration from ‘Saijo’ persimmon subcultured for 7 years after shoot tip culture. (A) Adventitious bud formation from root segments after ~1 month of culture. (B, C) Callus and adventitious bud formation, and adventitious shoot from leaf segments after approximately 1 and 3 months of culture, respectively. (D, E) Acclimatization of micro-propagated persimmon plantlets after 3 and 7 months of rooting in culture vessels and grown in a greenhouse, respectively.

$\mu\text{mol}/\text{m}^2/\text{s}$, 28°C , and vermiculite as the soil substrate, and obtained more than 90% survival (unpublished data, **Figs. 8D, 8E**).

Regeneration from callus, root, protoplast and endosperm

In micropropagated ‘Jiro’ and ‘Saijo’ persimmon, the leaf primordia were successfully regenerated from callus on MS (1/2 N) medium supplemented with $10 \mu\text{M}$ zeatin and $0.1 \mu\text{M}$ IAA, in growth conditions of 28°C , and 12 h/day photoperiod at $60 \mu\text{mol}/\text{m}^2/\text{s}$. The rates of adventitious buds formation from the two cultivars were 42 and 72%, respectively (Tao and Sugiura 1992b; **Figs. 8B, 8C**). Organogenetic capacity of the calli increased by more than 65% after subculturing, and a high frequency of adventitious bud formation (>50%) was obtained from callus cultures maintained for more than 4 years (Tamura *et al.* 1992). Adventitious bud formation from leaf segments of micropropagated ‘Saijo’ and ‘Fuyu’ persimmon was investigated in different culture conditions (Nishimura and Yamada 1992). Adventitious buds formed directly from the leaf tissue of both cultivars on MS (1/2 N) medium containing the cytokinin forchlorfenuron at 2 mg/l, and 0.002-0.02 mg/l of the auxin NAA. The growth conditions were 25°C under a 16 h/day photoperiod with a light intensity of $37.5 \mu\text{mol}/\text{m}^2/\text{s}$. In addition, adventitious shoots formed directly from micropropagated roots of ‘Jiro’ persimmon when they were cultured on MS medium containing $10 \mu\text{M}$ zeatin and $0.01 \mu\text{M}$ IAA, in growth conditions of 28°C and a 16 h/day photoperiod with a light intensity of $60 \mu\text{mol}/\text{m}^2/\text{s}$. The rate of adventitious shoot formation from root was 85% (Tetsumura and Yukinaga 1996). The calli of ‘Fuyu’, ‘Hana Goshō’ and ‘Hiratanenashi’ did not form any adventitious buds (Tao and Sugiura 1992b), but root segments of these cultivars formed adventitious buds on the shoot induction medium (Tetsumura and Yukinaga 2000). Roots formed adventitious buds on micropropagated ‘Saijo’ when it was cultured on MS (1/2 N) medium containing $9.1 \mu\text{M}$ (2 mg/l) zeatin and $0.01 \mu\text{M}$ IAA, grown at 26°C under a 16 hr/day photoperiod with a light intensity of $45 \mu\text{mol}/\text{m}^2/\text{s}$ (unpublished, **Fig. 8A**).

Callus cultures from protoplast and endosperm were attempted for ‘Jiro’ persimmon for polyploidy breeding (Tao *et al.* 1991; Tamura *et al.* 1993; Tao *et al.* 1997b). PKM8p and KM8 media for protoplast culture were developed by Kao and Michayluk (1975). Protoplasts were embedded in revised KM8p agarose medium containing $1 \mu\text{M}$ zeatin, $10 \mu\text{M}$ NAA, 150 mg/l ammonium nitrate, 0.5 M glucose and 2 mM glutamine (Tamura *et al.* 1993). After agarose-bead culture, the microcalli were released from the agarose blocks and transferred to modified KM8 agar me-

diu containing $1 \mu\text{M}$ zeatin, $1 \mu\text{M}$ NAA and 150 mg/l ammonium nitrate. On that medium, most microcalli grew to more than 5 mm in diameter in 6 weeks. Also, endosperm was cultured on the basal medium MS (1/2 N) containing $10 \mu\text{M}$ zeatin, $10 \mu\text{M}$ IAA and 500 mg/l casein hydrolysate in the dark at 28°C , and 24% of endosperms formed calli (Tao *et al.* 1997b). After 5 to 7 subcultures on MS (1/2 N) containing $10 \mu\text{M}$ zeatin and $1 \mu\text{M}$ IAA, calli were cultured in the same conditions as described in Tao and Sugiura (1992b), except that the continuous lighting was $20 \mu\text{mol}/\text{m}^2/\text{s}$. In those conditions adventitious buds formed at a rate of 10 to 37%.

These regeneration systems are applied for new methods of breeding of persimmon through somatic hybridization and genetic transformation (see below; “polyploidy breeding” and “*Agrobacterium*-mediated genetic transformation”).

Cutting of micropropagules

Japanese persimmons are considered to be difficult to propagate via cuttings (Tao and Sugiura 1992a). Therefore, it is necessary to try and develop efficient cutting propagation methods for persimmons. Micropropagated Japanese ‘Nishimurawase’ persimmon trees became better established without transplanting shock and grew more vigorously than grafted-on seedlings (Tetsumura *et al.* 1998, 1999), similar to the case for micropropagated ‘Jiro’ persimmon trees (Tao *et al.* 1994b). Moreover, for micropropagated ‘Jiro’ and ‘Nishimurawase’ persimmon trees, hardwood and softwood cuttings from the root suckers can be successfully propagated (Tetsumura *et al.* 2001a, 2001b). Tetsumura *et al.* (2004) reported that micropropagated ‘Nishimurawase’ trees maintained their vigor even 11 years after being planted. Micropropagated trees showed uniform growth, flowering and fruiting of mature persimmons from 8 to 11 years after planting (Tetsumura *et al.* 2004). There were no significant differences in the total yield and fruit quality between micropropagated trees and normal trees (Tetsumura *et al.* 1999, 2003, 2004). These results suggest that micropropagation causes reinvigoration rather than true rejuvenation in micropropagated Japanese persimmon trees. However, hardwood cuttings can not be directly propagated without micropropagation or the root suckers.

Polyploidy breeding

Ploidy manipulation techniques through protoplast cultures were developed for breeding of Japanese persimmons (Tamura *et al.* 1993; Tamura 1997).

Dodecaploid ($2n=12x=180$) plants were obtained from ‘Jiro’ persimmon by colchicine treatment of protoplasts (Ta-

Table 2 *Agrobacterium tumefaciens* mediated transformation in persimmon.

Plant materials	Basal medium	Plant growth regulator	Basal medium	Plant growth regulator	Efficiency (%) ^a	Reference
	Callus induction and subculture		Shoot induction and proliferation			
leaf	MS (1/2)	10µM zeatin + 10µM NAA 10µM zeatin + 1µM NAA 10µM zeatin + 1µM IAA	MS	10µM zeatin, 0.1µM IAA	1.3-3.5	Tao <i>et al.</i> 1997; Gao <i>et al.</i> 2001
	MS (1/2)	1µM zeatin + 1µM NAA 10µM zeatin + 1µM IAA	MS	10µM zeatin, 0.1µM IAA	10.0-18.0	Gao <i>et al.</i> 2000
	MS (1/2)	10µM zeatin + 10µM IAA	MS	10µM zeatin, 0.1µM IAA 5µM zeatin	4.8-11.0	Gao <i>et al.</i> 2004
hypocotyl	-	-	MS	2mg/l zeatin	11.1	Nakamura <i>et al.</i> 1998

^aEfficiency showed rate of kanamycin resistance shoot formation. Transformed plants were confirmed β-glucuronidase activity.

mura *et al.* 1996). Intraspecific somatic hybrids between 'Jiro' and 'Suruga' persimmons (Tamura *et al.* 1995) and interspecific somatic hybrids between *D. glandulosa* (2n=2x=30) and *D. kaki* 'Jiro' persimmons (Tamura *et al.* 1998) were produced by electrofusion of protoplasts.

It is known that almost cultivars of *D. kaki* are hexaploid (2n=6x=90) but seedless cultivars 'Hiratanenashi' and 'Tonewase' are nonaploid (2n=9x=135) because chromosome number of *Diospyros* plants is 15 (Zhuang *et al.* 1990, 1992). To obtain seedless nonaploid PCNA cultivars, endosperm culture was attempted for 'Jiro' persimmon. However, nonaploid plants were not regenerated from endosperm culture-derived calli, and only dodecaploid (2n=12x=180) and hexaploid (2n=6x=90) plants were observed (Tao *et al.* 1997b).

The novel method of giant pollen sorting in pollination combined with *in vitro* culture is a useful technique for breeding seedless nonaploid persimmons. Unreduced male gametes or unreduced pollen are produced in several persimmon cultivars, and both genetic and environmental factors affect their formation (Sugiura *et al.* 2000; Yamada *et al.* 2005). Yamada *et al.* (2005) demonstrated enhancement of unreduced pollen formation by low temperatures and suggested the potential use of low-temperature treatments to enhance unreduced pollen formation in Japanese persimmons. Sugiura *et al.* (2000) successfully obtained nonaploid plants by pollinating hexaploid cultivars with unreduced (2n) giant pollen. In addition, the possible use of 'Fujiwaragoshō' persimmon as a mother plant for sexual polyploidization of persimmons was investigated (Tao *et al.* 2003; Yamada and Tao 2006). 'Fujiwaragoshō' persimmon produced not only hexaploid, but also nonaploid and dodecaploid, seedlings (Tao *et al.* 2003). 'Fujiwaragoshō' persimmon specifically produced seedlings with higher ploidy levels than hexaploid at high frequencies.

Agrobacterium-mediated genetic transformation

'Jiro', 'Fuyu' and 'Nishimurawase' cultivars were investigated via molecular approaches to improve the rooting ability and alter the growth behavior using *Agrobacterium rhizogenes*-mediated genetic transformation (Tao *et al.* 1994a). The rates of crown gall formation were 40-60% for 'Fuyu', and 10-30% for 'Jiro' and 'Nishimurawase'. The tumors were cultured on MS (1/2 N) supplemented with 10 µM zeatin and 10 µM IAA for callus induction, and when the calli were cultured on MS containing 10 µM zeatin and 0.1 µM IAA under a 16 h/day photoperiod, the rate of adventitious shoot formation from tumor-derived calli formed more than 60%. The transformants showed alterations such as dwarfism and decreased rooting ability but those of horticultural importance have not yet been obtained.

To identify suitable strains for transformation of persimmons, the relative virulence of five wild-type strains of *Agrobacterium tumefaciens* were investigated (Tao *et al.* 1995). Since strain A281 was found to be the most virulent, a disarmed strain of A281, EHA101, was subsequently used as a host strain to introduce binary plasmid vectors for

transformation. The leaf discs of 'Jiro' persimmon were successfully transformed with foreign genes using *A. tumefaciens* EHA101 at the culture medium, as shown in **Table 2**, containing 100 µM acetosyringone. In the case of 'Jiro' persimmon, only the transgenic plants have the *cryIA* gene of *Bacillus thuringiensis* incorporated for insect resistance (Tao *et al.* 1997a), the *codA* gene encoding *Arthrobacter globiformis* choline oxidase (Gao *et al.* 2000), the gene encoding apple NADP-dependent sorbitol-6-phosphate dehydrogenase for salt stress tolerance (Gao *et al.* 2001) and the gene encoding pear polygalacturonase inhibiting protein for disease tolerance (Tamura *et al.* 2004) in combination with the β-glucuronidase gene. The obtained transgenic lines caused significant mortality of some insects, enhanced salt stress tolerance and inhibited fungal PG activity, respectively. Gao *et al.* (2000) suggested that the reduction of the concentration of zeatin from 10 µM to 1 µM during the initial period of transformation might have increased the efficiency of transformation (2.3 to more than 20% of transformed callus). It is necessary to establish an optimal regeneration medium for each cultivar for callus culture, as well as shooting and rooting.

On the other hand, the hypocotyl segments of 'Saijo' persimmon seedlings were transformed with the β-glucuronidase gene (Nakamura *et al.* 1998) and the *rolC* gene of *A. rhizogenes* for dwarf rootstock (Koshita *et al.* 2002) using *A. tumefaciens* EHA101 in the culture medium, as shown in **Table 2**, containing 100 µM acetosyringone. The *rolC* transgene caused dwarfism of 'Saijo' persimmon seedling.

Marker-assisted selection

Molecular markers were developed for selecting PCNA cultivars from breeding populations, and effectively distinguished non-PCNA and PCNA persimmons at the juvenile stage (Kanzaki *et al.* 2000b, 2001; Yonemori *et al.* 2003b). AFLP markers were obtained using bulked segregant analysis for the trait of natural astringency loss of PCNA-type fruit (Kanzaki *et al.* 2001; Yonemori *et al.* 2003b). One of these markers (EACC/MCTA-400) was absent from PCNA parents and all individuals in the bulk PCNA cultivars, but present in non-PCNA parents and some individuals in the bulk non-PCNA cultivars. Furthermore, RFLP markers using EACC/MCTA-400 as a probe linked to the dominant alleles controlling the PCNA/non-PCNA trait. In addition, this RFLP marker distinguished PCNA cultivars of Japanese origin from non-PCNA cultivars (**Fig. 9**; Kanzaki *et al.* 2000b; Yonemori *et al.* 2003b). In the case of other backcrossings using 'Aizumishirazu' as the seed parent, PCNA candidates were obtained and some of them were confirmed for their PCNA/non-PCNA trait (Yonemori *et al.* 2003b). This technique using RFLP analysis is an effective genetic tool for selecting PCNA-type cultivars from backcrossed populations. At present, detection of RFLP markers by PCR is under development to establish easier selection.

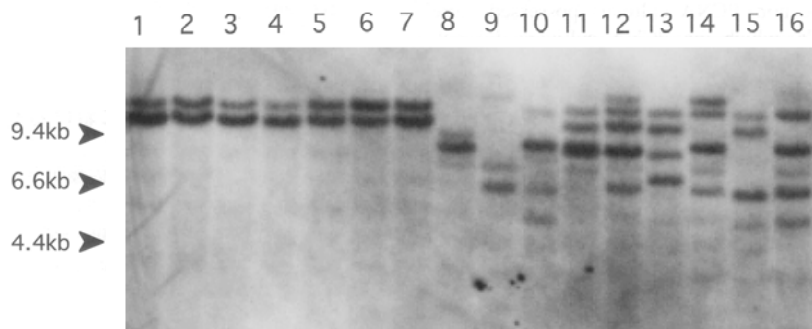


Fig. 9 Banding pattern derived from RFLP analysis of some Japanese persimmon cultivars. Lanes 1-8 are PCNA cultivars; 1: Fuyu, 2: Jiro, 3: Hana-gosho, 4: Oku-gosho, 6: Fujiwara-gosho, 7: Yamato-gosho, 8: Luo Tian Tian Shi. Lanes 9-16 are non-PCNA cultivars; 9: Shou-gatsu, 10: Saijo, 11: Aizumishirazu, 12: Atago, 13: Tenryubou, 14: Kikuhira, 15: Yokono, 16: Amayotsu-mizo. (Kanzaki *et al.* 2000b)

CONCLUDING REMARKS

The persimmon industry is currently facing some problems, such as decreased production. Many researchers and growers are making efforts to reduce labor, produce higher quality fruit, decrease production loss, and develop new uses and so on.

Many PCNA persimmon cultivars have been released by crossing a narrow genetic resource, in order to force early ripening, large size fruit, high quality and absence of physiological disorders such as cracking in NIFTS. In addition, in order to avoid inbreeding depression, other approaches for PCNA type persimmon breeding, such as searching for new PCNA resources and developing DNA marker selection, have been investigated. The PCNA cultivar 'Luo Tian Tian Shi' was discovered in China. Interestingly, the astringency loss trait is dominant in Chinese PCNA cultivars and recessive in Japanese PCNA cultivars. Chinese PCNA cultivars have the potential for use in PCNA breeding. PCNA type selection by the RFLP method has been developed and this technique can effectively distinguish PCNA persimmons from non-PCNA persimmons. Furthermore, the PCNA type 'Jiro' persimmon has been improved by a new approach involving biotechnology methods, such as polyploidy manipulation by chemical materials or somatic hybridization and the introduction of transgenes for culture benefits, such as environmental stress tolerance. On the other hand, chromosomal markers identified by FISH and GISH confirmed close relationships among wild species (*D. glandulosa* and *D. lotus*) and the cultivated hexaploid *D. kaki*. These phylogenetic analyses may lead to the discovery of novel breeding materials.

Some culture practices (root restriction culture or horizontal-trellis training system) have been developed to alleviate labor, which shortens the working hours involved in disbudding, fruit thinning, harvesting and pruning, improves the fruit quality and advances fruit maturation. Moreover, girdling of Japanese persimmon trees can inhibit vegetative growth and improve fruit quality. Forced culture with heaters shortens the harvest time but is expensive. To reduce fuel consumption, the most effective method for breaking bud endodormancy has been investigated in 'Tonewase' persimmon on the basis of the DVR-DVI, leading to the identification of suitable heating conditions. Furthermore, chemical treatments or light and temperature conditions for cultivation have been applied to Japanese persimmons to improve the fruit quality. Ethychlozate, a kind of auxin, can accelerate peel coloration, and has already been registered for practical use on persimmons in Japan. A higher temperature (30°C) or long day photoperiod (17 hours) under culture conditions can delay fruit maturation. On the other hand, efficient cutting propagation methods have been established via micropropagation. Micropropagated 'Nishimurawase' persimmon trees grew more vigorously than grafted-on seedlings without transplanting shock.

Ripening and postharvest physiology have found new focuses on tannin synthesis, fruit softening and ethylene production at the molecular level. However, the last step of tannin synthesis, the cell wall changes for fruit texture and

the ethylene signal transduction pathway remain unclear. In addition, other mechanisms for de-astringency in PCNA type fruit during natural ripening on the tree and sex determination, physiological fruit drop are unknown. These unsolved questions require further investigation by molecular approach.

Regarding postharvest issues, understanding of the fruit physiology and storage techniques have been much advanced in 'Tonewase' persimmon. Ethylene production during rapid softening of persimmon is caused by water stress, and effective prolongation of persimmon shelf-life by methods based on inhibition of ethylene induction via humidity control has been achieved. Furthermore, application of MCP delays rapid softening in some cultivars. MCP is now being registered for practical use on persimmon in Japan. Furthermore, persimmons are a valuable source of useful molecules, such as polyphenols, high molecular weight tannins, low molecular weight catechins and ascorbic acid. The potential use of the health-promoting benefits of persimmons may be attributed to increasing persimmon consumption.

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JAPANESE ABSTRACT

本総説では、主に 1990 年以降の日本におけるカキの研究成果について概説する。育種・植物生理・栽培・貯蔵に関する分野では、果実形質の遺伝解析、新品種の育成、ヘタおよび花性の生理、低温耐性、生理的落果機構、新栽培技術による省力・低コスト・高品質化、植物生長調節剤の作用、休眠覚醒推定による加温栽培の低コスト化ならびに湿度制御やエチレン作用阻害剤による貯蔵性向上に関する研究を議論した。バイオテクノロジー分野では、DNA あるいは染色体解析に基づくカキの品種・近縁野生種間の類縁性解析、プロトプラストおよび細胞融合由来の植物体再生技術、遺伝子組換え体の作出、倍数体操作を取り上げた。さらに、甘渋性に連鎖した DNA マーカーの開発、収穫後のエチレン生成と果実軟化、タンニン蓄積と脱渋機構など分子生物学的手法を用いた研究の進展について言及した。最後に、タンニンやビタミンなどの健康増進物質による機能性研究について議論した。