Growth Characteristics of a Small-fruit Dwarf Mutant Arising from Bud Sport Mutation in Japanese Persimmon (Diospyros kaki Thunb.)

Hisayo Yamane¹, Megumi Ichiki, Ryutaro Tao, Tomoya Esumi, and Keizo Yonemori
Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
Takeshi Niikawa
Gifu Prefectural Agricultural Technology Center, Gifu 501-1152, Japan
Hino Motosugi
University Farm, Kyoto Prefectural University, Seika-cho, Kyoto 619-0244, Japan

Additional index words. dwarf rootstock, fruit size, sugar composition, tannin, tissue culture

Abstract. Fruit size is one of the most important traits that affect the economic value of fruit. In persimmon (Diospyros kaki Thunb.), somatic and bud-sport mutations that affect the fruit traits are frequently observed. Recently, a small-fruit mutant, Totsutannenashi (TTN), was discovered in Japan as a bud-sport mutant of the leading cultivar Hiratanenashi (HTN). In this study, we investigated the morphological and physiological characteristics of TTN and HTN focusing on the tree architecture, fruit size, and the fruit flesh chemical composition. The objectives of the study were to evaluate the potential horticultural use of TTN and to characterize the differences between HTN and TTN. Both TTN and HTN are nonaploid plants, indicating that a difference in ploidy is not the cause of the small-fruit mutation. The vegetative growth of trees and tissue-cultured shoots of TTN was more compact than that of HTN. The floral organs of TTN appeared similar to those of HTN before flowering, but the TTN flowers opened earlier, resulting in smaller ovaries than in HTN flowers. The fruit size of TTN was consistently lower than that of HTN at all fruit developmental stages. TTN fruit had a higher sugar content and a higher proportion of sucrose to total sugars than HTN fruit. TTN fruits contained lower levels of secondary metabolites such as soluble tannins and ascorbate than HTN fruits. These results suggest that the fruit size mutation also affects the fruit biochemistry, leading to alterations in the fruit flesh composition. TTN may be a valuable genetic resource because compact trees require less labor and maintenance, and small, sweeter fruits may meet the various needs of consumers. The use of TTN in studies of the genetic control of fruit size is also discussed.

Fruit size is one of the most important traits that affect the economic value of fruit. Although fruit size is controlled by both environmental and genetic factors, the latter have the greatest impact, because small genetic changes can dramatically affect the fruit size. Thus, improvements in fruit size are usually achieved by the introduction of new traits. However, little is known about the key genetic factors that regulate fruit size in most fruit tree species. Our goal is to isolate these factors that control fruit size and to elucidate the physiological changes caused by changes in the fruit size.

The weight of persimmon (Diospyros kaki Thunb.) fruit ranges from less than 60 g to more than 300 g depending on the cultivar.

Received for publication 18 Apr. 2008. Accepted for publication 4 July 2008.
We thank Mr. M. Kondo and Dr. H. Kunii, Miyazaki University for providing us the plant materials.
¹To whom reprint requests should be addressed; e-mail hyamane@kais.kyoto-u.ac.jp

Materials and Methods

Plant materials and characterization of vegetative growth. Six trees of each of the two Japanese persimmon cultivars, HTN and its bud-sport mutant, TTN, both of which were grafted onto seedling rootstocks, were used. All trees were grafted at the same time and grown in 60-L containers for 3 years at the Kyoto University experimental farm, Kyoto, Japan (34°N, 135°E). In July 2005, the shoot and internode lengths were measured. Three of six pot-grown trees were then planted in an orchard at the Kyoto University experimental farm in Mar. 2006 and cultivated using commercial management methods, including a normal pruning system. In Oct. 2007, the shoot and internode lengths of the five uppermost shoots from the longest shoots on each plant were investigated.

The weight of persimmon (Diospyros kaki Thunb.) fruit ranges from less than 60 g to more than 300 g depending on the cultivar. In July 2005, zeatin and indoleacetic acid (IAA) were used as the basal medium. Thunb.), somatic and bud-sport mutations that are usually achieved by the introduction of new traits. However, little is known about the key genetic factors that regulate fruit size in most fruit tree species. Our goal is to isolate these factors that control fruit size and to elucidate the physiological changes caused by changes in the fruit size.

The weight of persimmon (Diospyros kaki Thunb.) fruit ranges from less than 60 g to more than 300 g depending on the cultivar.

Received for publication 18 Apr. 2008. Accepted for publication 4 July 2008.
We thank Mr. M. Kondo and Dr. H. Kunii, Miyazaki University for providing us the plant materials.
¹To whom reprint requests should be addressed; e-mail hyamane@kais.kyoto-u.ac.jp

Materials and Methods

Plant materials and characterization of vegetative growth. Six trees of each of the two Japanese persimmon cultivars, HTN and its bud-sport mutant, TTN, both of which were grafted onto seedling rootstocks, were used. All trees were grafted at the same time and grown in 60-L containers for 3 years at the Kyoto University experimental farm, Kyoto, Japan (34°N, 135°E). In July 2005, the shoot and internode lengths were measured. Three of six pot-grown trees were then planted in an orchard at the Kyoto University experimental farm in Mar. 2006 and cultivated using commercial management methods, including a normal pruning system. In Oct. 2007, the shoot and internode lengths of the five uppermost shoots from the longest shoots on each plant were investigated.
Three flower buds from the largest one in each branch were collected. After dehydration through a butanol series, the buds were embedded in paraffin wax and sliced to 10-μm thickness on a rotary microtome. The sliced tissues were stained with toluidine blue and observed under an optical microscope (BX50; Olympus, Tokyo, Japan). Flowering dates of pot-grown trees were recorded in 2006. Fruit diameters of triplicates of three pot-grown trees of each strain were measured periodically during fruit growth and development in 2005.

**Determination of sugar, soluble tannin, and ascorbate contents of fruits.** Sugar contents were measured as described by Hirano et al. (1995) and Gao et al. (2001). In brief, 10 g of fruit flesh was excised and immediately heated for a short time in a microwave oven to inactivate the invertase in the flesh. The tissues were then homogenized in 30 mL of 100% methanol and centrifuged. The supernatant was collected and made up to 100 mL with 80% methanol. Aliquots of 100 μL of the supernatant were combined with 50 μL of 2% xylitol as an internal control and dried at 60 °C. The resulting residue was resuspended in 100 μL of TMS-HT (Tokyo Kasei Kogyo, Tokyo, Japan) and incubated for 30 min at room temperature. An aliquot of 1 μL was injected into a gas chromatograph (GC-2014; Shimadzu, Kyoto, Japan) fitted with a DB-1701 capillary column (J&W Scientific, Folsom, CA) with helium as the carrier gas. The peaks corresponding to glucose, fructose, and sucrose were plotted and measured.

Tannin contents were measured as described by Oshida et al. (1996). In brief, mesocarp samples were homogenized in 80% methanol and centrifuged. Folin-Ciocalteau reagent (Wako, Tokyo, Japan) was added to the supernatant, and the soluble tannin concentration was analyzed with an ultraviolet/visible spectrophotometer (725 nm) (Shimadzu; ultraviolet-1600). The tannin concentration was expressed as (+)-catechin equivalents.

For ascorbate (vitamin C) analysis, samples were homogenized with 5% metaphosphoric acid and centrifuged. Ascorbate and dehydroascorbate concentrations in the supernatant were measured using a reverse-phase high-performance liquid chromatography-ultraviolet/visible system fitted with an ODS-3 column (Nihon Bunko, Tokyo, Japan) as described by Niikawa et al. (2007). Measurements were made in triplicate on three replicate fruits.

### Table 1. Growth properties of vegetative organs of pot-grown (3-year-old) and field-grown (5-year-old) HTN and TTN trees.

| Pot-grown trees | Field-grown trees | Shoot length (cm) | Node no. (per shoot) | Node no. (per cm of shoot) | Internode length (cm) |
|-----------------|-------------------|-------------------|----------------------|---------------------------|----------------------|
| TTN             |                   | 20.9 ± 0.2        | 111.5 ± 20.6         | 47.7 ± 14.0               | 0.42 ± 0.06          |
| HTN             |                   | 38 ± 0.2          | 77.1 ± 5.8           | 19.4 ± 6.9                | 0.25 ± 0.08          |

*Branches longer than the average length of HTN branches (14.3 cm) were categorized as long stem.

*Mean ± so.

**Significant at P < 0.05 and 0.01 by t test, respectively.

HTN = ‘Hiratanenashi’; TTN = ‘Totsutanenashi’.

### Table 2. Growth properties of vegetative organs of micropropagated HTN and TTN shoots in flask.

| Zeatin 5 μM | Zeatin 10 μM |
|-------------|-------------|
| Number of shoots | Shoot length (mm) | Number of shoots | Shoot length (mm) |
| TTN         | 3.08 ± 0.95 | 9.65 ± 5.88 | 3.55 ± 0.88 | 9.52 ± 3.84 |
| HTN         | 2.00 ± 0.92 | 16.20 ± 9.90 | 1.75 ± 0.46 | 17.55 ± 9.56 |

*Murashige and Skoog medium containing either 5 or 10 μM zeatin was used for shoot culture.

*Mean ± so.

**Significant at P < 0.05 and 0.01 by t test, respectively.

HTN = ‘Hiratanenashi’; TTN = ‘Totsutanenashi’.
showed dwarf growth characteristics in all growth environments examined in this study. A dwarf phenotype in fruit trees is favored by farmers because of their ease of management (Faust and Zagaja, 1984). Indeed, dwarfing rootstocks have been bred and introduced into many fruit tree species such as apple (Koike and Tsukahara, 1993), Citrus (Phillips and Castle, 1977), and sweet cherry (Giorgio and Standardi, 1993). At present, no practical dwarfing rootstock or dwarfing culture is available for Japanese persimmon, and there are efforts to create dwarfing rootstocks (Koshita et al., 2007). The dwarf trait in TTN could be used as a valuable material for breeding dwarf Japanese persimmon cultivars.

Growth characteristics of ’Totsutanenashi’ reproductive organs. Floral organ development in flower buds of TTN and HTN was examined on 25 Apr. and 2 May 2006. The average flowering date was 10 May 2006 in TTN and 20 May 2006 in HTN. On 25 Apr., initiation of stamens (later aborted because both TTN and HTN only bear female flowers) and carpels were observed in both TTN and HTN followed by ovule formation and the completion of floral organ development on 2 May, suggesting that floral organs develop similarly in TTN and HTN (Fig. 2B). Considering that TTN flowers opened ≈10 d earlier than HTN flowers, it appears that TTN flowers open before their floral organs are fully expanded (Fig. 2B–C).

In Japanese persimmon, fruit growth and development is typically divided into three phases: 1) maximal growth corresponding to the first sigmoidal growth phase; 2) cessation of growth corresponding to a lag between the first and second sigmoidal phases; and 3) maximal growth corresponding to the second sigmoidal phase. Although changes in the diameters of HTN fruit followed a double-sigmoidal curve during the fruit growth and development period, no similar rapid growth was observed in TTN fruits (Fig. 3). At the time of commercial harvesting, the average diameter of TTN fruit was less than one-third that of HTN fruit (Figs. 2A, D). Considering that TTN flowers opened ≈10 d earlier than HTN flowers, it appears that TTN flowers open before their floral organs are fully expanded (Fig. 2B–C).

Flesh composition in ’Totsutanenashi’ and ’Hiratanenashi’ fruits. The major soluble sugars in persimmon (glucose, fructose, and sucrose) were examined. The sum of these three sugars is referred to as the total sugar content. During both developmental stages examined, the sucrose content was higher in TTN than HTN, resulting in a higher total sugar content in TTN fruit (Table 3). Zheng and Sugiura (1990) found that the amount of sucrose as a percentage of the total sugars was lower in HTN fruit (41% when the fruit reached 8.0 on the color chart) than in other persimmon varieties. TTN fruit, however, contained more sucrose (49% when the fruit reached 8.0 on the color chart) and had a higher total sugar content (more than 20% of fresh weight) than HTN fruit. Hence, the higher amount of sugar in TTN fruit is an advantageous agronomic characteristic. It is thought that the lower level of sucrose in HTN fruit than TTN is caused by the higher invertase activity in HTN fruits, because the leaf photosynthate is translocated to the sink organs (fruits) as sucrose, which is stored in fruit or converted to monosaccharides such as glucose and fructose by mainly invertase and other metabolic enzymes in persimmon. Thus, the higher percentage of sucrose in TTN may be related to the lower invertase activity in the fruits. However, because lower invertase activity often causes lower amounts of fructose and glucose but appeared not occur in TTN fruits, further studies are required to examine this idea.

We also determined the soluble tannin and ascorbate contents, because relatively high amounts of these compounds are unique to persimmon fruits. Pollination-variant astringent-type persimmon cultivars, including HTN, have high levels of soluble tannins in the tannin cells of fruits during their development and maturation (Yonemori et al., 1983). An increase in the soluble tannins at the beginning of fruit development and a subsequent decrease resulting from tannin polymerization was observed in TTN fruits (Fig. 4), similar to the pattern of HTN and other non-pollination-constant non-astringent cultivars (Ikegami et al., 2004; Oshida et al., 1996). The soluble tannin content per gram fresh weight in TTN fruits
was lower than that in HTN fruits throughout fruit development (Fig. 4), suggesting that tannin biosynthesis is lower in TTN fruits than in HTN fruits. In both developmental stages, the ascorbate and dehydroascorbate contents in TTN fruits were lower than in HTN fruits (Table 3). The lower amounts of soluble tannins and ascorbates suggest that the secondary metabolic activity of TTN is lower than that of HTN. The higher sucrose content as a percentage of total sugars, probably caused by the lower invertase activity in TTN, also supports this suggestion. Similar results were also found in a grape mutant. Fernandez et al. (2006) reported that the fleshless berry mutant of grape, which has a low pericarp weight, contains fewer phenolic compounds and a higher proportion of sucrose in relation to total sugars during berry development. This finding suggested that a mutation in the fruit size affects the biochemical properties of the fruit. On the other hand, however, Klann et al. (1996) reported that the changes in sugar composition in tomato fruit contribute to alterations in fruit size. This finding suggested that smaller fruit size could be caused by alterations of biochemical properties of the fruits. In this study, dwarf growth character, reduced fruit size, and alterations of fruit composition were observed in TTN. Although we could not rule out the possibility that its reduced fruit size is caused by the alterations of fruit biochemical compounds, the changes in both vegetative and reproductive organs of TTN suggested that the mutation affecting the whole plant growth could contribute to the alterations in fruit biochemical properties. Further biochemical studies would be required for characterization of the mutation in TTN.

Many bud-sport mutants have been identified in HTN (Yonemori et al., 2000). For example, ‘Otanenashi’ is a bud-sport mutant bearing bigger fruits (Hamada et al., 2004). ‘Tonewase’, an early-maturing mutant derived from HTN, is widely cultivated in Wakayama Prefecture, one of the main persimmon-producing areas in Japan. The increased frequency of transpositions and other chromosomal rearrangements is thought to be associated with recent polyploidy (Zhao et al., 1998). Thus, polyploidy in Japanese persimmons, in which hexaploid or nonaploid plants are common, would enhance the occurrence of mutations, resulting in the emergence of many valuable bud sports such as TTN. Little is known about the genetic control of fruit size in Japanese persimmons, despite the diverse fruit sizes of the cultivars. Hence, TTN may prove useful for studying genes that control fruit size in Japanese persimmon as recently elucidated for the fleshless berry grape mutant (Fernandez et al., 2007). To date, because relatively small genetic changes might be associated with arising bud-sport mutation from original plant, bud-sport mutants were successfully used for the discovery of the genes related to important agronomic traits in fruit tree species such as skin color in grape (Kobayashi et al., 2004) and self-incompatibility in Rosaceae (Sassa et al., 1997; Tao et al., 1997). In addition, bud-sport deletion mutant was successfully used for complementation experiment by introducing intact functional genes, resulting in the alteration of phenotype (Kobayashi et al., 2004). Further genomic studies by searching for the genomic differences despite the nearly same genetic background between TTN and HTN would lead to finding the factors causing the morphological and physiological differences between TTN and HTN.

This study has clearly demonstrated that a ploidy mutation is not associated with the morphology of TTN. The growth characteristics of TTN, which forms compact trees with small fruits, may provide a valuable genetic resource that requires a less labor-intensive cultural system while providing small fruit to meet the various needs of consumers. In addition, the higher sugar content of TTN fruits is an attractive commercial characteristic. We are currently undertaking an analysis of the basis of the fruit size differences between TTN and HTN.

Table 3. Soluble sugar and ascorbates contents in mature fruits of HTN and TTN.

| Soluble sugar contents (6.5%) | Soluble sugar contents (8.0%) |
|-----------------------------|-----------------------------|
| Fructose (%) | Glucose (%) | Sucrose (%) | Total sugar (%) | Fructose (%) | Glucose (%) | Sucrose (%) | Total sugar (%) |
| TTN | 2.93 ± 0.90* | 4.57 ± 0.21 | 9.33 ± 1.01 | 16.83 ± 0.51 | 4.90 ± 0.71 | 5.78 ± 0.32 | 9.54 ± 0.68 | 20.21 ± 0.95 |
| HTN | 2.60 ± 0.70 | 4.77 ± 0.60 | 8.02 ± 0.92 | 15.39 ± 0.64 | 3.42 ± 0.92 | 5.77 ± 0.75 | 6.14 ± 1.00 | 15.33 ± 2.57 |
| Statistical significance | NS | NS | NS | NS | NS | NS | NS | NS |

| Ascorbate (mg/g FW) | Dehydroascorbate (mg/g FW) | Ascorbate (mg/g FW) | Dehydroascorbate (mg/g FW) |
|---------------------|---------------------------|---------------------|---------------------------|
| TTN | 0.24 ± 0.03 | 0.068 ± 0.004 | 0.20 ± 0.04 | 0.109 ± 0.021 |
| HTN | 0.35 ± 0.06 | 0.100 ± 0.014 | 0.33 ± 0.07 | 0.073 ± 0.007 |
| Statistical significance | * | * | * | * |

*Fruit maturation stages were designated by color chart index.

*Color chart index values developed for HTN (National Agricultural Research Station, Japan).

*Mean ± se.

NS*: **NS: Nonsignificant or significant at P < 0.05 and 0.01 by t test, respectively.

HTN = ‘Hiratanenashi’; TTN = ‘Totsutanenashi’; FW = fresh weight.
HTN with an ultimate goal of identifying the genetic factors that regulate fruit size.

**Literature Cited**

Faust, M. and S.W. Zagaja. 1984. Prospects for developing low vigor fruit tree cultivars. Acta Hort. 146:21–27.

Fernandez, L., C. Romieu, A. Moing, A. Bouquet, M. Maucourt, M.R. Thomas, and L. Torregrosa. 2006. The grapevine fleshy berry mutation. A unique genotype to investigate differences between fleshy and nonfleshy fruit. Plant Physiol. 140:537–547.

Fernandez, L., L. Torregrosa, N. Terrier, L. Sreekantan, J. Grimmelt, C. Davies, M.R. Thomas, C. Romieu, and A. Ageorges. 2007. Identification of genes associated with flesh morphogenesis during grapevine fruit development. Plant Mol. Biol. 63:307–323.

Gao, M., R. Tao, K. Miura, A.M. Dandekar, and A. Sugiuira. 2001. Transformation of Japanese persimmon (Diospyros kaki Thunb.) with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. Plant Sci. 160:837–845.

Giorgio, V. and A. Standardi. 1993. Growth and Production of two sweet cherry cultivars grafted on 60 ecotypes of Prunus mahaleb. Acta Hort. 410:471–476.

Hamada, K., K. Hasegawa, A. Kitajima, and T. Ogata. 2004. Relationship of fruit size and mesocarp cell size and cell number in ‘Otanenashi’, ‘Hiratanenashi’ and ‘Saijo’. J. Jpn. Soc. Hort. Sci. 73:(Suppl. 2):344 (abstr. in Japanese).

Hirano, K., K. Yonemori, and A. Sugiuira. 1995. Involvement of sugar metabolism in persimmon growth inhibition by calyx lobe removal. J. Amer. Soc. Hort. Sci. 120:75–77.

Ikegami, A., K. Yonemori, A. Sugiuira, A. Sato, and M. Yamada. 2004. Segregation of astringency in F1 progenies derived from crosses between pollination-constant, nonastringent persimmon cultivars. HorticScience 39:371–374.

Klann, E.M., B. Hall, and A.B. Bennett. 1996. Antisense acid invertase (TIV1) gene alters soluble sugar composition and size in transgenic tomato fruit. Plant Physiol. 112:1321–1330.

Kobayashi, S., N. Goto-Yamamoto, and H. Hirochika. 2004. Retrotransposon-induced mutations in grape skin color. Science 304:982.

Koike, H. and K. Tsukahara. 1993. Studies on root system and growth of ‘Fujis’ apple trees on dwarfing interstock and rootstocks. J. Jpn. Soc. Hort. Sci. 62:49–54.

Koshita, Y., K. Morinaga, Y. Tsuchida, T. Asakura, H. Yakushiji, and A. Azuma. 2007. Selection of interstocks for dwarfing Japanese persimmon (Diospyros kaki Thunb.) trees. J. Jpn. Soc. Hort. Sci. 76:288–293.

Namikawa, I. and M. Higashi. 1928. On the chromosomes in Diospyros kaki L. F. and Diospyros lotus L. Bot. Magazine 42:436–438.

Niikawa, T., T. Ozeki, N. Miyake, and T. Kurata. 2007. Effect of variety, maturation and cultiva-tion method on vitamin C contents in Japanese persimmon. Hort. Res. 6:(Suppl. 2):114. [in Japanese].

Oshida, M., K. Yonemori, and A. Sugiuira. 1996. On the nature of coagulated tannins in astringent-type persimmon fruit after artificial treatment of astringency removal. Postharvest Biol. Tech. 8:317–327.

Phillips, R.L. and W.S. Castle. 1977. Evaluation of the twelve rootstocks for dwarising citrus. J. Amer. Soc. Hort. Sci. 102:526–528.

Sassa, H., H. Hirano, T. Nishino, and T. Koba. 1997. Style-specific self-compatible mutation caused by deletion of the S-RNase gene in Japanese pear (Pyrus serotina). Plant J. 12:223–227.

Soriano, J.M., S. Pecchioly, C. Romero, S. Vilanova, G. Llacer, E. Giordani, and M.L. Badenes. 2006. Development of microsatellite markers in polyploidy persimmon (Diospyros kaki Thunb.) from an enriched genomic library. Mol. Ecol. Notes 6:368–370.

Sugiura, A., T. Ohkuma, Y.A. Choi, R. Tao, and M. Yamada. 2000. Production of nonaploid (2n=9x) Japanese persimmons (Diospyros kaki) by pollination with unreduced (2n=6x) pollen and embryo rescue culture. J. Amer. Soc. Hort. Sci. 125:609–614.

Sugiura, A., R. Tao, H. Murayama, and T. Tomana. 1986. In vitro propagation of Japanese persimmon. HortScience 21:1205–1207.

Tao, R., H. Murayama, K. Moriguchi, and A. Sugiuira. 1988. Plant-regeneration from callus cultures derived from primordial leaves of adult Japanese persimmon. HorticScience 23:1055–1056.

Tao, R., H. Yamane, H. Sassa, H. Mori, T.M. Gradziel, A.M. Dandekar, and K. Yonemori. 1997. Identification of stylar RNases associated with gametophytic self-incompatibility in almond (Prunus dulcis). Plant Cell Physiol. 38:304–311.

Yonemori, K., J. Matsushima, and A. Sugiuira. 1983. Differences in tannins of non-astringent and astringent type fruits of Japanese persimmon (Diospyros kaki Thunb.). J. Jpn. Soc. Hort. Sci. 52:135–144.

Yonemori, K., A. Sugiuira, and M. Yamada. 2000. Persimmon genetics and breeding. p. 191–225. In: Janick, J. (ed.). Plant breeding reviews. New York, NY.

Zhao, X., Y. Si, R.E. Hanson, C.F. Crane, H.J. Price, D.M. Stelly, J.F. Wendel, and A.H. Paterson. 1998. Dispersed repetitive DNA has spread to new genomes since polyploidy formation in cotton. Genome Res. 8:479–492.

Zheng, G.H. and A. Sugiuira. 1990. Changes in sugar composition in relation to invertase activity in the growth and ripening of persimmon (Diospyros kaki) fruits. J. Jpn. Soc. Hort. Sci. 59:281–287.

Zhuang, D., A. Kitajima, M. Ishida, and Y. Sobajima. 1996. Propagation of two sweet cherry cultivars grafted on 60 ecotypes of Prunus mahaleb. Acta Hort. 410:471–476.