The origin and distribution of ‘Kokubu’-type splice-site mutations of the MLO genes in tobacco varieties

Masao Arai, Tomoyuki Komatsu, Hisashi Udagawa, Tomoyuki Tajima and Seiki Sato*

Leaf Tobacco Research Center, Japan Tobacco Inc., 1900 Idei, Oyama, Tochigi 323-0808, Japan

The Japanese domestic tobacco (Nicotiana tabacum L.) cultivar ‘Kokubu’ shows high powdery mildew resistance that is controlled by splice-site mutations of two MILDEW LOCUS O genes, NtMLO1 and NtMLO2. We investigated the existence of the same NtMLO1/2 splice mutations in the genomes of various tobacco varieties cultivated in Japan and other countries. In total, 14 Japanese domestic cultivars, which were mainly distributed in Kagoshima, had splice-site mutations in both NtMLO1 and NtMLO2. In addition, tobacco cultivars containing only the NtMLO1 splice-site mutation were found in various tobacco production areas in Japan, but no cultivars with only the NtMLO2 splice-site mutation were detected. Moreover, the NtMLO1 splice-site mutation was detected in native Asian, Oriental and cigar tobacco varieties. Consequently, we speculate that these powdery mildew-resistant tobacco cultivars were generated relative recently in the Kagoshima area when a spontaneous mutation occurred at the NtMLO2 splice site in a cultivar already containing the NtMLO1 splice-site mutation and that the NtMLO1 splice-site mutation occurred during the early period of tobacco seed dissemination from the Americas to Asia and Japan.

Key Words: cultivar ‘Kokubu’, Japanese domestic tobacco, leaf shape, MILDEW LOCUS O, powdery mildew resistance, splice-site mutations.

Introduction

Tobacco (Nicotiana tabacum L.) is a Solanaceae plant that originated from tropical or subtropical America but is now commercially cultivated in more than 120 countries. It is thought that tobacco seeds were initially introduced into Japan by the Portuguese or Spaniards in the 16th–17th centuries, but this has not been confirmed. Tobacco plant cultivation and smoking tobacco quickly spread in Japan (Handa 2009). The Edo Shogunate of Japan banned or restricted tobacco cultivation several times to secure annual tributes of rice. These policies led to the cultivation of tobacco in isolated mountains and remote areas that were not fit for rice farming, which in turn led to the development and accumulation of technologies unique to each habitat. The plant type and leaf shape became differentiated by the selection of tobacco suitable for local weather and climatic conditions. Additionally, the taste and aroma were diversified by the development of drying methods, and so-called domestic cultivars were gradually established in each region (Ohashi 1984). According to a survey by the Leaf Tobacco Division, Ministry of Finance conducted in 1898, there were more than 170 domestic tobacco cultivars in Japan, and these cultivars have been divided into six groups (‘Daruma’, ‘Nakano’, ‘Usuha’, ‘Hatano’, ‘Suifu’ and ‘Kokubu’) on the basis of leaf shape (Ohashi 1984).

The Japanese domestic tobacco cultivar ‘Kokubu’ was isolated as a new variety from the fields of the Kokubu area of Kagoshima (Satsuma) during the Bunka Period (1804–1818) (Eyama 1992). Kagoshima is a famous tobacco cultivation area of Japan, and several other domestic cultivars, such as ‘Maru’, ‘Izumi’, ‘Tarumizu’ and ‘Ibusuki’, were cultivated in the past. Because the cured leaves of cultivar ‘Kokubu’ have superior characteristics for kiseru smoking, Kokubu became a famous tobacco production area of Japan.

The cultivar ‘Kokubu’ is resistant to the plant fungal disease powdery mildew owing to recessive alleles at two loci. This resistance was identified by Wan (1962), and Fujimura et al. (2016) revealed that this resistance results from splice-site mutations of two MILDEW LOCUS O genes, NtMLO1 and NtMLO2. The MLO genes encode a plant-specific seven-transmembrane domain protein that localizes to the plasma membrane (Devoto et al. 2003). The N. tabacum genome contains 15 MLO genes forming seven clades, and the two MLO genes of clade V, NtMLO1 and NtMLO2, are related to powdery mildew susceptibility (Appiano et al. 2015). Splice-site mutations in the NtMLO1 and NtMLO2 genes of cultivar ‘Kokubu’ confer resistance to powdery mildew owing to the resulting deletions or insertions in the
transcripts of both genes that lead to the production of incorrect transcripts (Fujimura et al. 2016). Powdery mildew resistance as a result of loss-of-function MLO alleles has been observed in various dicots and monocots, such as pea (Pavan et al. 2011), Arabidopsis (Consonni et al. 2006), tomato (Bai et al. 2008), cucumber (Nie et al. 2015) and barley (Büschges et al. 1997). The powdery mildew resistance trait of ‘Kokubu’ has been introduced into the various modern tobacco varieties (Hamamura et al. 1981).

Furthermore, some other domestic tobacco cultivars of Japan are powdery mildew resistant (Ohashi 1984), and many, such as ‘Maru’ and ‘Izumi’, originated in Kagoshima. Thus, we hypothesized that the powdery mildew resistant loci of such domestic cultivars may have been introduced from ‘Kokubu’. Because this resistance is caused by the two natural recessive mutations in the NtMLO1/2 genes, it is reasonable to assume that ‘Kokubu’ was either generated by crossing two varieties each having a splice-site mutation in either the NtMLO1 or NtMLO2 gene, or by a spontaneous mutation of the NtMLO gene in a variety already having a splice-site mutation in the other NtMLO gene. If this hypothesis is correct, then there should be varieties containing a single mutant of NtMLO1 or NtMLO2 among Japanese domestic tobacco cultivars. However, it is unclear when and how these two natural mutations formed, and existence of cultivars harboring a single of the NtMLO gene mutation has not been confirmed.

To investigate these hypotheses, we determined the presence of NtMLO1/2 splice-site mutations in 84 Japanese domestic tobacco cultivars and 94 foreign tobacco varieties using an allele-specific PCR method (Komatsu et al. 2020). We identified 14 Japanese domestic cultivars with NtMLO1/2 splice-site mutations, and we also identified some Japanese domestic cultivars and foreign varieties containing only the NtMLO1 splice-site mutation. Consequently, we developed a hypothesis for the origins of the two splice-site mutations.

**Materials and Methods**

**Materials**

Tobacco seeds were obtained from the genetic resources of the Leaf Tobacco Research Center, Japan Tobacco Inc. In total, 84 Japanese domestic cultivars from various tobacco production areas were used for the analysis. In addition, 30 native tobacco varieties of Asia (Philippines, Indonesia, Thailand, India and Nepal), 6 native tobacco varieties of the Americas (Brazil, Colombia and Canada), 35 Oriental tobacco varieties (Turkey, Greece and the former Yugoslavia) and 23 cigar tobacco varieties from various countries (Canada, USA, Colombia, Puerto Rico and the Philippines) were used for the analysis. Many of these varieties have been selected in their respective regions and cultivated for many years. The origin and leaf shape of each variety were provided in the Leaf Tobacco Research Center genetic resources database.

**Splice-site mutation analysis**

Genomic DNA was isolated from young leaf tissues using a Gentra Puregene Cell kit (Qiagen) according to the manufacturer’s instructions. The splice-site mutations in the NtMLO1/2 genes were analyzed using the duplex allele-specific PCR method described in Komatsu et al. (2020). The obtained PCR fragments were separated using a QIAxcel Advanced System (Qiagen), and the presence of splice-site mutations was determined by the amplification or non-amplification of DNA fragments with the primer pairs specific for the mutant or wild type of each NtMLO gene.

**Phylogenetic analysis**

Genotyping data for each Japanese domestic cultivar and modern tobacco varieties K326 (flue-cured variety) and TN90 (burley tobacco) as an outgroup were obtained from 30 simple sequence repeat (SSR) markers and 31 loci. The sequences of SSR markers used are provided in Table 1. Each forward primer was labeled with a FAM, VIC, TET or NED fluorescent dye (Applied Biosystems) at the 5’ end, and a tail sequence (5’-GTGTTCTT-3’) was added to each reverse primer. PCR amplification was performed using a QIAGEN Multiplex kit, modified to three set for multiplex reactions in accordance with Komatsu et al. (2020). The PCR products were diluted 50-fold, and 1 μl of Hi-Di formamide (Thermo Fisher Scientific) was added to 10 μl of diluted solution. The mixture was heated for 5 min at 96°C, cooled rapidly on ice, electrophoresed using a 3730xl DNA Analyzer (Life Technology, Applied Biosystems) and analyzed using GeneMapper ver. 4.0 software (Applied Biosystems). A dendrogram was constructed by the neighbor-joining method (Nei et al. 1983) using Populations 1.2.3 (Langella 1999), and a phylogenetic tree was constructed using MEGA X software (megasoftware.net). The reliability of each node was evaluated by 1000 trials of the bootstrap method.

**Powdery mildew susceptibility analysis**

The susceptibility of tobacco plants to powdery mildew was confirmed using the method described in Fujimura et al. (2016). The tobacco plants were grown in clay pots in a greenhouse maintained at 25°C. The powdery mildew spore solution (approximately 2.5 × 10⁴ spore/ml) was splayed on the tobacco plants, and they were checked for disease symptoms at 3 weeks after inoculation.

**Results**

**NtMLO1/2 splice-site mutations in Japanese domestic cultivars**

To determine the presence of NtMLO1/2 splice-site mutations, 84 Japanese domestic cultivars originating from various tobacco production areas were analyzed (Table 2). Among them, 14 cultivars had splice-site mutations in both NtMLO1 and NtMLO2 genes and showed high resistance...
against powdery mildew. Interestingly, 13 of 14 cultivars originated from Kagoshima and the leaf shape of each cultivar is ‘Kokubu’ type. The remaining cultivar, ‘Bingo (Ito)’, was cultivated in Hiroshima and has the ‘Suifu’-type leaf shape.

In total, 18 cultivars had a mutation only in the NtMLO1 splice site. These varieties were from various tobacco production areas, ranging from Iwate to Okinawa, and had four splice-site mutations, including ‘Kokubu’ and ‘Bingo (Ito)’, which have only the NtMLO1 splice-site mutation, but the ‘Kokubu’-type leaf shape. The cultivars with ‘Suifu’- and ‘Daruma’-type leaf shapes were roughly divided into two major groups within the phylogenetic tree. The cultivars having the NtMLO1 splice-site mutation and the ‘Suifu’-type leaf shape were distributed throughout the tree branches. However, the cultivars having the NtMLO1 splice-site mutation and the ‘Daruma’-type leaf shape, which were five ‘Daruma’ group cultivars and cultivar ‘Katsuyama’, formed one group.

### Phylogenetic analysis of the Japanese domestic cultivars

We generated a phylogenetic tree of 84 Japanese domestic cultivars (Fig. 2). The cultivars with the NtMLO1 or NtMLO2 splice-site mutations, including ‘Kokubu’ and ‘Bingo (Ito)’, formed one group. Interestingly, this group contained cultivars ‘Tarumizu’ and ‘Miyazaki’, which have only the NtMLO1 splice-site mutation, but the ‘Kokubu’-type leaf shape. The cultivars with ‘Suifu’- and ‘Daruma’-type leaf shapes were roughly divided into two major groups within the phylogenetic tree. The cultivars having the NtMLO1 splice-site mutation and the ‘Suifu’-type leaf shape were distributed throughout the tree branches. However, the cultivars having the NtMLO1 splice-site mutation and the ‘Daruma’-type leaf shape, which were five ‘Daruma’ group cultivars and cultivar ‘Katsuyama’, formed one group.
| Cultivar                        | NtMLO1 | NtMLO2 | Leaf type | Origin        |
|--------------------------------|--------|--------|-----------|---------------|
| Aizu                           | W      | W      | Suifu     | Fukushima     |
| Akatsuka (Kataikari)           | M      | W      | Suifu     | Niigata       |
| Akatsuka (Matsuyama-Edo)       | W      | W      | Suifu     | Niigata       |
| Awa (Chisha)                   | W      | W      | Daruma    | Tokushima     |
| Awa (Senmai)                   | W      | W      | Daruma    | Tokushima     |
| Awa (Tayou)                    | W      | W      | Daruma    | Tokushima     |
| Awa (Yamashiro)                | W      | W      | Daruma    | Tokushima     |
| Awa (Sadamitsu)                | W      | W      | Daruma    | Tokushima     |
| Bichu                          | W      | W      | Suifu     | Okayama       |
| Bichu (Nocchi)                 | W      | W      | Suifu     | Okayama       |
| Bingo (Ito)                    | M      | M      | Suifu     | Hiroshima     |
| Bingo (Shinsaka)               | W      | W      | Suifu     | Hiroshima     |
| Bungo                          | W      | W      | Daruma    | Ohita         |
| Daruma (Chu-daruma)            | M      | W      | Daruma    | Tochigi       |
| Daruma (Oh-daruma)             | M      | W      | Daruma    | Tochigi       |
| Daruma (Takeda-daruma)         | M      | W      | Daruma    | Ohita         |
| Daruma (Fuku-daruma)           | M      | W      | Daruma    | Ibaraki       |
| Daruma (Batou 12)              | M      | W      | Daruma    | Ibaraki       |
| Ensyu                          | W      | W      | Daruma    | Shizuoka      |
| Hatano                         | W      | W      | Hatano    | Kanagawa      |
| Hatano (Kankou)                | W      | W      | Kanagawa  | Kanagawa      |
| Higashine                      | W      | W      | Suifu     | Yamagata      |
| Higashiyama                    | W      | W      | Suifu     | Iwate         |
| Higo (Takachiho)               | W      | W      | Suifu     | Kumamoto      |
| Hino (Oh-ha)                   | W      | W      | Daruma    | Tottori       |
| Hino (Shou-ha)                 | W      | W      | Daruma    | Tottori       |
| Hino (Tachi-ha)                | W      | W      | Suifu     | Tottori       |
| Hiraie tabako                  | W      | W      | Suifu     | Kagoshima     |
| Ibusuki (Kensaki)              | W      | W      | Suifu     | Kagoshima     |
| Ibusuki (Kensakitamarishouha)  | W      | W      | Suifu     | Kagoshima     |
| Ibusuki (Oh-muhei)             | W      | W      | Suifu     | Kagoshima     |
| Ibusuki (Shou-muhei)           | W      | W      | Suifu     | Kagoshima     |
| Ibusuki (Bulgaria)             | W      | W      | Suifu     | Kagoshima     |
| Ibusuki primitive 1            | W      | W      | Suifu     | Kagoshima     |
| Ikusaka                        | W      | W      | Suifu     | Nagano        |
| Izumi                          | M      | M      | Kokubu    | Kagoshima     |
| Izumi (Komatsu)                | M      | M      | Kokubu    | Kagoshima     |
| Izumi (Kamimasumi)             | M      | M      | Kokubu    | Kagoshima     |
| Izumi (Tanoue)                 | M      | M      | Kokubu    | Kagoshima     |
| Joza                           | W      | W      | Daruma    | Fukuoka       |
| Kangaku                        | M      | M      | Kokubu    | Kagoshima     |
| Katsuyama                      | M      | W      | Daruma    | Fuku          |
| Kirigasakü                     | W      | W      | Daruma    | Chiba         |
| Kibi                           | M      | W      | Suifu     | Okayama       |
| Kokubu (Oh-horo)               | M      | M      | Kokubu    | Kagoshima     |
| Kokubu (Ko-horo)               | M      | M      | Kokubu    | Kagoshima     |
| Kuro (No. 1)                   | M      | W      | Suifu     | Kumamoto      |
| Kuro (No. 2)                   | M      | W      | Suifu     | Kumamoto      |
| Kurokamiyama                   | W      | W      |           |               |
| Maru                           | M      | M      | Kokubu    | Kagoshima     |
| Maru (Kagoshima)               | M      | M      | Kokubu    | Kagoshima     |
| Maru (Kagomaru)                | M      | M      | Kokubu    | Kagoshima     |
| Maru (Ryuou)                   | M      | M      | Kokubu    | Kagoshima     |
| Maru (Tabuse 3)                | M      | M      | Kokubu    | Kagoshima     |
| Matsukawa                      | W      | W      | Suifu     | Fukushima     |
**Table 2.** (continued)

| Cultivar                        | NiMLO1 | NiMLO2 | Leaf type | Origin       |
|---------------------------------|--------|--------|-----------|--------------|
| Matsukawa (Kanto)               | W      | W      | Suifu     | Fukushima    |
| Matsukawa (Ohkoshichikanari)    | W      | W      | Suifu     | Fukushima    |
| Mihiara                         | W      | W      | Harima     | Hiroshima    |
| Mihiara (Awa)                   | W      | W      | Harima     | Kanagawa     |
| Miyazaki                        | M      | W      | Kokubu     | Miyazaki     |
| Nakano                          | M      | W      | Nakano     | Shiga        |
| Nanbu                           | M      | W      | Suifu      | Iwate        |
| Ogasawara primitive             | W      | W      | Ogasawara-jima |            |
| Ohkusa (Hanken)                 | W      | W      | Suifu     | Aichi        |
| Ohkusa (Ishiken)                | W      | W      | Suifu     | Aichi        |
| Okinawa                         | M      | W      | Suifu      | Okinawa      |
| Renge (shirobana)               | W      | W      | Harima     | Saitama      |
| Saga primitive                  | W      | W      | Suifu     | Saga         |
| Sakusyu (Aka-arifuku)           | W      | W      | Suifu     | Okayama      |
| Sakusyu (Nawashiro-arifuku)     | W      | W      | Suifu     | Okayama      |
| Seinaiji                        | M      | W      | Suifu     | Nagano       |
| Suifu (Kataikari)               | M      | W      | Suifu     | Ibaraki      |
| Suifu (Oh-ha)                   | W      | W      | Hatano    | Ibaraki      |
| Suifu (Mineshima)               | W      | W      | Ibaraki    |             |
| Tarumizu                        | M      | W      | Kokubu    | Kagoshima    |
| Tarumizu (Ishiodori)            | M      | M      | Kokubu    | Kagoshima    |
| Tsurugi                         | W      | W      | Suifu     | Ishikawa     |
| Ueji                            | M      | W      | Suifu     | Mie          |
| Usuha                           | W      | W      | Usuha     | Niigata      |
| Yoshino                         | W      | W      | Daruma    | Nara         |
| Yonezawa                        | W      | W      | Suifu     | Yamagata     |
| Yonezawa (Kanto)                | W      | W      | Suifu     | Yamagata     |

This table shows the existence of splice-site mutations in NiMLO1/2 genes (W: wild type, M: mutant), leaf type (‘Suifu’, ‘Kokubu’, ‘Daruma’, ‘Nakano’, ‘Hatano’ or ‘Usuha’ type), and original cultivation area (indicated by Prefecture name) of each cultivar. Leaf types and origins of some cultivars are unknown (vacant cells).

**Fig. 1.** Leaf types of Japanese domestic tobacco cultivars harboring only the NiMLO1 splice-site mutation. Each cultivar was selected and cultivated in the individual tobacco production area (‘Origin’) of Japan. ‘Daruma’- and ‘Nakano’-type leaves are sessile, and ‘Suifu’- and ‘Kokubu’-type leaves have petioles. All these cultivars are susceptible to powdery mildew. The leaves in the upper part of the figure show the typical shape of each leaf type.
Phylogenetic tree of 84 Japanese domestic tobacco cultivars based on 30 markers. Cultivars in red and blue indicate the \( \text{NtMLO1}/2 \) double and \( \text{NtMLO1} \) single mutants, respectively. The symbol at the head of the cultivar name indicates the type of leaf shape; ●: ‘Kokubu’ type, ○: ‘Suifu’ type, ■: ‘Daruma’ type, □: ‘Nakano’ type, ▲: ‘Hatano’ type, and ◇: ‘Usuha’ type. Some cultivars do not have their leaf types listed in the Leaf Tobacco Research Center genetic resource database. The numbers in phylogenetic tree indicate the exceeded bootstrap value by 50. The scale bar at the bottom indicates the genetic distance. Modern tobacco varieties K326 (flue-cured variety) and TN90 (burley tobacco) were added as an outgroup.

### Discussion

We surveyed the splice-site mutations of the \( \text{NtMLO1} \) and \( \text{NtMLO2} \) genes in various Japanese domestic tobacco cultivars and found that 14 cultivars harbor \( \text{NtMLO1}/2 \) splice-site mutations. They formed a distinct group in the phylogenetic tree. Among them, 13 cultivars had the ‘Kokubu’-type leaf shape and originated from Kagoshima. However, tobacco cultivars containing only the \( \text{NtMLO1} \) splice-site mutation were observed from various tobacco production areas of Japan, and their leaf shapes varied. Additionally, tobacco cultivar containing only the \( \text{NtMLO2} \) splice-site mutation was not found. Consequently, we speculated that powdery mildew-resistant tobacco cultivar ‘Kokubu’ was generated relatively recently in the Kokubu area by the occurrence of a spontaneous \( \text{NtMLO2} \) splice site mutation the ‘Kokubu’-type ancestral tobacco already harboring the \( \text{NtMLO1} \) splice-site mutation. Then, this \( \text{NtMLO1}/2 \) double-mutant tobacco spread to several production areas in Kagoshima and was established as a local
Table 3. *NtMLO1*/*2* splice-site mutations in 94 foreign tobacco varieties

| Variety                        | *NtMLO1* | *NtMLO2* | Class     | Origin       |
|--------------------------------|----------|----------|-----------|--------------|
| Dork Dang                      | W        | W        | Native    | Thailand     |
| Hu Chang                       | W        | W        | Native    | Thailand     |
| Hu Lahm                        | W        | W        | Native    | Thailand     |
| Sle                           | M        | W        | Native    | Thailand     |
| Sukhothai                      | M        | W        | Native    | Thailand     |
| Bansud                         | M        | W        | Native    | Philippines  |
| Romero                         | M        | W        | Native    | Philippines  |
| Sinai                          | W        | W        | Native    | Philippines  |
| Orinoco                        | W        | W        | Native    | Indonesia    |
| Done Vittanam (Nallapati)      | M        | W        | Native    | India        |
| Karravittanam                  | W        | W        | Native    | India        |
| Medarametlanuta                | M        | W        | Native    | India        |
| Naru (Nellore)                | W        | W        | Native    | India        |
| Rayala                         | M        | W        | Native    | India        |
| Toka-Aku                       | W        | W        | Native    | India        |
| Rayala (No. 1)                 | M        | W        | Native    | India        |
| RPK                            | W        | W        | Native    | India        |
| Sazi                           | W        | W        | Native    | India        |
| Shah-Kot                       | W        | W        | Native    | India        |
| Sivapuri                       | W        | W        | Native    | India        |
| Snuff (Eluru)                  | W        | W        | Native    | India        |
| Thakeri Ramperr                | W        | W        | Native    | India        |
| Thatayan                       | W        | W        | Native    | India        |
| Tholan                         | W        | W        | Native    | India        |
| Vadamugam                      | M        | W        | Native    | India        |
| Vazhai Kappal                  | W        | W        | Native    | India        |
| Nepal 1288                     | W        | W        | Native    | Nepal        |
| Nepal 8039                     | M        | W        | Native    | Nepal        |
| Nepal 6184 (Damre Kacho)       | M        | W        | Native    | Nepal        |
| Nepal KY                       | M        | W        | Native    | Nepal        |
| Galpao                         | W        | W        | Native    | Brasil       |
| Brazilian domestic variety     | W        | W        | Native    | Brasil       |
| Carotte                        | W        | W        | Native    | Brasil       |
| Garcia                         | W        | W        | Native    | Colombia     |
| Ambalema                       | W        | W        | Native    | Colombia     |
| Petit Havana                   | W        | W        | Native    | Canada       |
| Agrinion Djebel                | W        | W        | Oriental  | Greece       |
| Agrinion Myrodata              | W        | W        | Oriental  | Greece       |
| Agrinion Smyrna Seed           | W        | W        | Oriental  | Greece       |
| Basma                          | W        | W        | Oriental  | Greece       |
| Basma Boukia Paranestion       | W        | W        | Oriental  | Greece       |
| Basma Drama Drama              | W        | W        | Oriental  | Greece       |
| Basma Drama Ferai              | W        | W        | Oriental  | Greece       |
| Basma Kavala Amisiana          | W        | W        | Oriental  | Greece       |
| Basma (5A) Kilikis Lakhanas    | W        | W        | Oriental  | Greece       |
| Basma Komotini-1 Miskon        | W        | W        | Oriental  | Greece       |
| Basma Komotini Ova Kallisti    | W        | W        | Oriental  | Greece       |
| Basma Xanthi Ova Bafika        | W        | W        | Oriental  | Greece       |
| Basma II Zichna Mesorachi      | W        | W        | Oriental  | Greece       |
| Kabakoulak Gumenitsa Gumenitsa | W        | W        | Oriental  | Greece       |
| Kawalla                        | W        | W        | Oriental  | Greece       |
| Kilikis                        | W        | W        | Oriental  | Greece       |
| Kozani                         | W        | W        | Oriental  | Greece       |
| Mahala                         | W        | W        | Oriental  | Greece       |
| Nigrita                        | W        | W        | Oriental  | Greece       |
domestic cultivar. This may have led to the large number of cultivars with the \textit{NtMLO1/2} splice-site mutations in Kagoshima. Cultivar ‘Bingo (Ito)’ has the \textit{NtMLO1/2} splice-site mutations, but the leaf shape is ‘Suifu’ type and it originates from Hiroshima. The phylogenetic analysis included cultivar ‘Bingo (Ito)’ in the same group as the other cultivars with the \textit{NtMLO1/2} splice-site mutations. Cultivar ‘Bingo (Ito)’ was isolated in 1901 (Ohashi 1984). Therefore, this cultivar is a new variety compared with other ‘Kokubu’ type \textit{NtMLO1/2} double-mutant cultivars, and this cultivar might have been generated by breeding in Hiroshima using the \textit{NtMLO1/2} double-mutant tobacco introduced from Kagoshima.

Cultivars ‘Tarumizu’ and ‘Miyazaki’, which originated in Kagoshima and Miyazaki, respectively, only have a mutation in the \textit{NtMLO1} splice site and have the ‘Kokubu’-type leaf shape. They belong to the group on the phylogenetic tree that contained the \textit{NtMLO1/2} double-mutant cultivars although the bootstrap values are lower than 50 in Fig. 2. Consequently, we speculate that both cultivars may have originated from the \textit{NtMLO1} single-mutant tobacco that is the common ancestor of the \textit{NtMLO1/2} double-mutant

| Variety             | \textit{NtMLO1} | \textit{NtMLO2} | Class       | Origin          |
|---------------------|-----------------|-----------------|-------------|-----------------|
| Sochos 1            | W               | W               | Oriental    | Greece          |
| Dzebel              | W               | W               | Oriental    | Ex-Yugoslavia   |
| Othija              | M               | W               | Oriental    | Ex-Yugoslavia   |
| Prilep              | W               | W               | Oriental    | Ex-Yugoslavia   |
| Ravnjak             | M               | W               | Oriental    | Ex-Yugoslavia   |
| Yaka Bolsunov       | W               | W               | Oriental    | Ex-Yugoslavia   |
| Akhissar-1          | W               | W               | Oriental    | Turkey          |
| Akhissar-1          | W               | W               | Oriental    | Turkey          |
| Bafra (Black sea)   | M               | W               | Oriental    | Turkey          |
| Bursa               | M               | W               | Oriental    | Turkey          |
| Izmir               | W               | W               | Oriental    | Turkey          |
| Samsun              | M               | W               | Oriental    | Turkey          |
| Samsun Holmes       | M               | W               | Oriental    | Turkey          |
| Smyrna              | W               | W               | Oriental    | Turkey          |
| Welwendo            | W               | W               | Oriental    | Turkey          |
| Zihina              | W               | W               | Oriental    |                |
| Beinhart1000-1      | W               | W               | Cigar       | USA             |
| Connecticut         | W               | W               | Cigar       | USA             |
| Florida301          | M               | W               | Cigar       | USA             |
| Havana              | W               | W               | Cigar       | USA             |
| Warne               | W               | W               | Cigar       | USA             |
| Ottawa705           | W               | W               | Cigar       | Canada          |
| Cubita              | W               | W               | Cigar       | Colombia        |
| Olor                | W               | W               | Cigar       | Puerto Rico     |
| Espado              | M               | W               | Cigar       | Philippines     |
| Manilla             | M               | W               | Cigar       | Philippines     |
| Manilla (Cagayan)   | M               | W               | Cigar       | Philippines     |
| Oxsinsim            | M               | W               | Cigar       | Philippines     |
| Oxviz               | M               | W               | Cigar       | Philippines     |
| Pampano             | M               | W               | Cigar       | Philippines     |
| Repollo             | M               | W               | Cigar       | Philippines     |
| Simmaba             | M               | W               | Cigar       | Philippines     |
| Simox               | W               | W               | Cigar       | Philippines     |
| Vizcaya             | M               | W               | Cigar       | Philippines     |
| Vizoxvizz           | M               | W               | Cigar       | Philippines     |
| Java                | M               | W               | Cigar       |                |
| Penn Leaf1          | W               | W               | Cigar       |                |
| Sumatra             | W               | W               | Cigar       |                |
| Tuta                | W               | W               | Cigar       |                |

This table shows the existence of splice-site mutations in \textit{NtMLO1/2} genes (W: wild type, M: mutant), as well as the class (Native: native variety, Oriental: oriental tobacco variety and Cigar: cigar tobacco variety) and origin (indicated by country name) of the varieties examined. The origins of some varieties are not listed in the Leaf Tobacco Research Center genetic resource database. Ex-Yugoslavia: the former Yugoslavia.
cultivars or from progeny of the NtMLO1/2 double-mutant tobacco in which the NtMLO2 splice-site mutation was lost.

Our survey of foreign tobacco varieties showed that the NtMLO1 splice-site mutation occurred not only in Japanese domestic cultivars but also in some of Asian native, Oriental and cigar tobacco varieties. This result indicates that the NtMLO1 splice-site mutation did not occur in Japan. It is unclear when and where the NtMLO1 splice-site mutation occurred during the dissemination of tobacco seeds worldwide, but because it was not detected in the tested native varieties of the Americas, this mutation may have occurred early during the spread of tobacco seeds from the Americas to Asia and Japan via Europe. Japanese domestic tobacco cultivars are thought to have been established from a few ancestral varieties introduced into Japan by the Portuguese and/or Spaniards (Ohashi 1984). Therefore, we speculate that two types of tobacco varieties with and without the NtMLO1 splice-site mutation were included in the tobacco seeds imported into Japan and that both types of seeds were spread through Japan during the national isolation period.

The splice-site mutation in the NtMLO2 gene was not found in foreign tobacco varieties. This result emphasizes that the NtMLO2 splice-site mutation is a spontaneous mutation that occurred in a tobacco plant harboring the NtMLO1 splice-site mutation in Japan.

At present, the cultivation of Japanese domestic tobacco cultivars is decreasing owing to the reduced demand for Japanese domestic tobacco leaves as a tobacco product in cigarettes. However, Japanese domestic tobacco cultivars have diverse agronomic traits suitable for each cultivation area. They are useful materials for plant research owing to their diverse genetic traits and have the potential to be good materials for future tobacco breeding.

Author Contribution Statement

MA, TT and SS designed this study. MA, TK and SS contributed the splice-site mutation analysis. SS and HU contributed the phylogenetic analysis. MA contributed the powdery mildew susceptibility analysis and drafted the manuscript. All authors read and approved the final manuscript.

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