VYTAUTAS MAGNUS UNIVERSITY

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IDENTIFICATION OF NOVEL ALLELES FOR WAXY WHEAT IN WINTER WHEAT TILLING POPULATION

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ABSTRACT

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Mutations in hexaploid wheat (*Triticum aestivum* L.) are often masked due to the presence of the wild-type allele from one or both the remaining loci. Consequently, valuable mutations are not easily incorporated in the genome through traditional plant breeding techniques. TILLING is a reverse genetic approach that makes use of some chemical mutagens such as ethyl methanesulfonate (EMS) in order to induce a high density of randomly induced mutations in the DNA. The mutations obtained can be identified with different methods such as PCR markers and the combination of DNA sequencing with some bioinformatics tools. The aim of this paper is to identify novel alleles of *waxy* genes for waxy wheat in a winter wheat TILLING population.

The aims of the study are:

1. To identify novel alleles in *waxy* genes *Wx-D1* and *Wx-B1* in two winter wheat TILLING populations named "T-WW-Gaja" and "T-WW-Kena" developed at the Lithuanian Research Centre for Agriculture and Forestry (LAMMC).
2. To verify if the mutations occur in the DNA coding region.
3. To verify if the discovered mutations change the translated sequences.

Methods – DNA extraction of the TILLING populations → Amplification of *waxy* genes *Wx-B1* and *Wx-D1* → PCR products purification → Sequencing → Mutation identification (BLAST).

Results – A point mutation was found in the mutant MG532 (*Wx-B1* gene – C1771T); a second point mutation was found in the mutant MK283 (*Wx-D1* gene – G1805A). Both the mutations were found in the coding sequences and they both altered the translated amino acids sequences (missense mutations).

ABSTRACT

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Le mutazioni nel grano (*Triticum aestivum* L.) esaploide sono spesso mascherate a causa della presenza dell'allele "wild type" presente in uno o in entrambi i loci rimanenti, di conseguenza mutazioni importanti non sono facilmente incorporabili nel genoma attraverso le tradizionali tecniche di selezione. TILLING è un approccio genetico inverso che fa uso di alcuni agenti chimici mutageni, come l'etilmetansolfonato, per indurre un'alta densità di mutationi nel DNA. Le mutazioni ottenute possono essere identificate con diversi modi come i PCR markers e/o la combinazione del sequenziamento del DNA con alcuni strumenti di bioinformatica. Lo scopo di questa tesi è di identificare nuovi alleli "*waxy*" in una popolazione di frumento che è stata modificata chimicamente.

Obiettivi della tesi:

1. Identificare nuovi alleli nei geni "*waxy*" *Wx-D1* e *Wx-B1* in due popolazioni TILLING di frumento invernale denominate "T-WW-Kena" e "T-WW-Gaja" sviluppate presso il Centro di Ricerca Lituano per l'Agricoltura e la Silvicoltura (LAMMC).
2. Verificare se le mutazioni si sono verificare nella regione codificante del DNA.
3. Verificare se le mutazioni scoperte alterano le sequenze tradotte.

Metodi – Estrazione del DNA delle popolazioni TILLING → Amplificazione dei geni "*waxy*" *Wx-B1* e *Wx-D1* → Purificazione dei prodotti della PCR → Sequenziamento → Identificazione delle mutazioni (BLAST).

Risultati – È stata rilevata una mutazione puntiforme nel campione mutante MG532 (gene *Wx-B1* – C1771T); una seconda mutazione puntiforme è stata trovata nel campione mutante MK283 (gene *Wx-D1* – G1805A). Entrambe le mutazioni sono state trovate nelle sequenze codificanti ed entrambe hanno alterato le sequenze tradotte degli amminoacidi (mutazioni missenso).

SANTRAUKA

Magistrinio darbo autorius:	Salvatore Paradiso
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Mutacijos heksaploidiniuose kviečiuose (*Triticum aestivum* L.) dažnai yra užmaskuotos dėl laukinių alelių buvimo viename ar dviejuose lokusuose. Dėl šios priežasties vertingas mutacijas yra sudėtinga įtraukti į genomą, taikant tradicinius selekcijos metodus. TILLING yra atvirkštinės genetikos metodas, kuris pasitelkia cheminius mutagenus, tokius kaip etilio metasulfonatą (EMS) tam, kad paskatintų didelį, atsitiktinai sukeltų mutacijų dažnį DNR grandinėse. Gautos mutacijos gali būti identifikuojamos naudojant įvairius metodus: PGR žymenis ir DNR sekoskaitą derinant su įvairiais bioinformatikos įrankiais. Šio darbo tikslas yra identifikuoti naujus *Waxy* genų alelius mutagenizuotoje žieminių kviečių populiacijoje.

Tyrimo tikslai:

1. Identifikuoti naujus alelius Waxy genuose *Wx-D1* ir *Wx-B1* dvejuose žieminių kviečių mutagenizuotose populiacijose “T-WW-Gaja” ir “T-WW-Kena”, sukurtose Lietuvos agrarinių ir miškų mokslų centre (LAMMC).
2. Patikrinti, ar mutacijos yra DNR koduojančiame regione.
3. Patikrinti, ar aptiktos mutacijos keičia trancliuojamas sekas.

Metodai - mutagenizuotų populiacijų DNR išskyrimas → Waxy genų *Wx-B1* ir *Wx-D1* amplifikavimas → PGR produktų gryninimas → DNR sekoskaita → Mutacijos identifikavimas (BLAST).

Rezultatai – taškinė mutacija buvo nustatyta mutante MG532 (*Wx-B1* genas – C1771T); antra taškinė mutacija buvo nustatyta mutante MK283 (Wx-D1 genas - G1805A). Abi mutacijos buvo rastos koduojančiose sekose, ir jos abi pakeitė transliuotas aminorūgščių sekas (klaidingos prasmės mutacijos).

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the oldest crops cultivated in many countries around the world, and it is the third most produced crop in the world after maize and rice (Wang *et al.*, 2015).

Wheat is consumed by over a third of the global population in different ways such as bread, noodles and other steamed or baked products, and China, is the largest wheat consumer in the world, besides being also the biggest wheat producer (Wang *et al.*, 2015). Although wheat grows betterer in those areas where the daily maximum temperature is about 15°C, it can be cultivated in many different areas around the world, from cold regions (such as the regions in the Arctic Circle) to regions with hot and dry climate (Uthayakumaran and Wrigley, 2017). According to the FAO (Food and Agriculture Organization) in 2017 the global wheat production was of 771.7 million tonnes and the top three producers were China (134.3 million tonnes), India (98.5 million tonnes) and Russian Federation (85.9 million tonnes). In Europe, wheat production was of 270.1 million tonnes (around 35% of the total global wheat production) (*FAOSTAT*, 2017). *T. aestivum* is the most grown wheat worldwide (over 90% of the total wheat production) (Uthayakumaran and Wrigley, 2017).

Due to the global importance of wheat production, it is beneficial to study spontaneous or induced mutations in wheat for understanding the genetic defects that can influence gene expression. Mutations in hexaploid wheat are often masked due to the presence of the wild-type gene from one or both the remaining loci however, the production of waxy wheat lines has been possible (Vrinten *et al.*, 1999). Waxy wheat (amylose-free wheat) have been developed in Japan (1995) through classical breeding techniques (Nakamura *et al.*, 1995) and since that also Australia, USA and China have produced waxy wheat lines (Wang *et al.*, 2015). In 2008, Shun-he Cheng, a famous Chinese wheat breeding expert, produced a new waxy wheat variety by fixing the *waxy* gene to the "Yangmai 158" wheat variety and a planting base for this new waxy wheat variety was established by Jiangsu Chulong Flour Co., Ltd, a Chinese company settled in Xinghua City, China (Zhang *et al.*, 2014).

The interest of waxy wheat lines in the food industry is increasing due to their many valuable properties that have been reported in the literature. For instance, the higher capacity of water-binding in waxy wheat has been reported to extend the shelf-life of baked, refrigerated and frozen wheat-based products (Graybosch, 1998). Partial waxy wheat lines have been reported to increase the quality of Japanese Udon (a type of noodle similar to Italian pasta, but thicker) which in general decreases with the increase of amylose in the flour (Miura *et al.*, 1994). The rapid gelatinisation at a lower temperature of waxy starch gives it a potential application in the instant food products (Yasui *et al.*, 1996); another potential use of waxy wheat starch is its use as a gelling agent for soups, sauces and confectionary products (Chakraborty *et al.*, 2004).

The aim of this paper is to identify novel *waxy* alleles from two winter wheat cultivars TILLING populations "T-WW-Kena" and "T-WW-Gaja", developed at the Lithuanian Research Centre for Agriculture and Forestry (LAMMC). The first part of this work consists in a literature analysis that starts with a general overview about wheat, to continue in more details about wheat grain and wheat starch with its composition and functionality. The second part of the literature analysis focuses on *waxy* genes and waxy wheat lines with details about their characteristics, applications and production.

The analytical part of this work consists of screening the "T-WW-Gaja" and "T-WW-Kena" TILLING population to identify new alleles in the *waxy* genes *Wx-D1* and *Wx-B1*. The analytical part consists of a series of methods that can be schematically summarised in:

1. DNA extraction of the TILLING populations;
2. Amplification of the *Wx-B1* and *Wx-D1* genes;
3. PCR products purification;
4. Sequencing;
5. Mutation identification (BLAST).

In the final part of this paper, all the results obtained are analysed and discussed. This paper ultimately aims to show that genetic variation in the hexaploid wheat genome (that is often masked in nature by the wild type genes) can be obtained by using TILLING, a reverse genetic approach, combined with PCR markers and some bioinformatics tools, such as BLAST, for the identification of the mutations obtained.

The aims of the study are:

1. To identify novel alleles in *waxy* genes *Wx-D1* and *Wx-B1* in two winter wheat TILLING populations named "T-WW-Gaja" and "T-WW-Kena" developed at the Lithuanian Research Centre for Agriculture and Forestry.
2. To verify if the mutations occur in the DNA coding region.
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1. WHEAT OVERVIEW

1.1 Botanical and genetic characteristics

Wheat is an annual monocotyledonous plant that belongs to the Gramineae (Poaceae) family that can reach a height of 60 to 150 cm (but it may also have a height of ~ 30 cm). The leaves are long and narrow with or without hair on the surface. The grain is botanically classified as caryopsis or kernel (dry fruit), and it has an oval shape with a colour that ranges from white to red (Amessis-Ouchemoukh *et al.*, 2017). Genetically, *T. aestivum* L. is hexaploid (it has six copies of each of its seven chromosomes: 2n = 6x = 42, genetically characterised as AABBDD) originated presumably around 6000 years BC in the Iranian highlands (or in the neighbouring areas known as 'Fertile Crescent') by natural hybridisation and chromosome doubling between *Triticum turgidum* L. *ssp. durum* Desf. MacKey (tetraploid: 2n = 4x = 28, genetically characterised as AABB) and *Aegilops tauschii* Coss. (diploid: 2n = 2x = 14, genetically characterised as DD) (Arendt and Zannini, 2013; Kishii *et al.*, 2018). The D genome has been indicated as the one that gives most of the good attributes in bread (Arendt and Zannini, 2013).

1.2 Wheat Grain

Compared to most of the plants, where the fruit contains one or more seeds easily detachable from the rest of the fruit, the seed of the wheat is itself the whole fruit; the pericarp (fruit wall) and the seed coat are fused, and consequently, they are undetachable. This particular type of fruit is botanically classified as "caryopsis" (Belderok *et al.*, 2013). The grain develops within the glumes ("lemma and "palea", Figure 1), a floral envelop that is constituted by modified leaves (Grundas and Wrigley, 2015). The caryopsis has a thickness of ~ 2.5 – 3 mm, a width of ~ 3 – 3.5 mm and a length of ~6 to 7 mm; its weight is ~30 – 40 mg (Arendt and Zannini, 2013). The longitudinal and cross sections of wheat grain is shown in Figure 2 (Arendt and Zannini, 2013). Depending to the wheat variety the grain may have an elongated, elliptical, truncated, ovate or oval shape (Grundas and Wrigley, 2015).

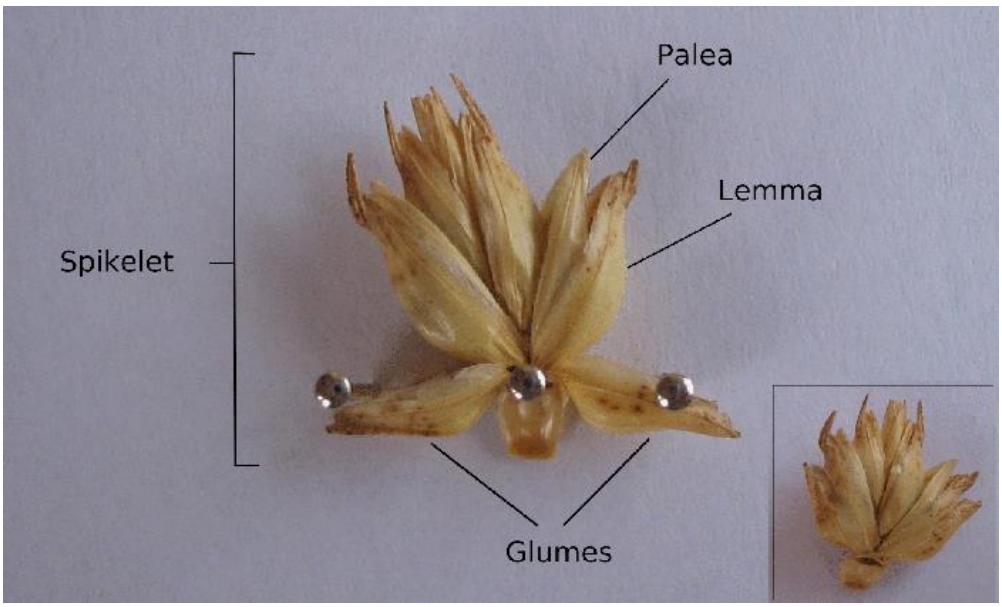


Figure 1 – Wheat spikelet (*Triticum aestivum* L.) – License under: Creative Commons CC BY-SA.

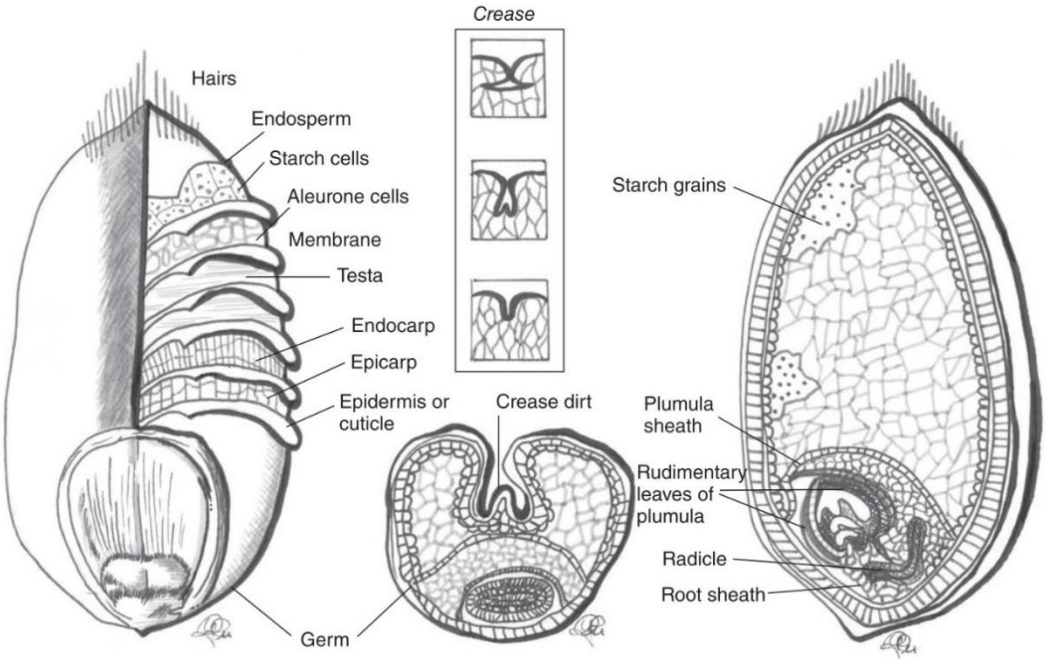


Figure 2 – Wheat grain longitudinal and cross sections (Arendt and Zannini, 2013).

The external layer of the grain is called “epidermis”, and it has the function to cover and protect other cell layers that together form a ~50 μm tick “pericarp” (Cornell, 2012). The grain is mainly constituted by the bran, the endosperm and the germ (Arendt and Zannini, 2013; Solah *et al.*, 2015).

The bran that includes the pericarp and the aleurone layer has the function to protect the grain, and it is composed by several layers of water-insoluble fibre made of cellulose and pentosans (Cornell, 2012; Solah *et al.*, 2015). The bran, together with the aleurone layer, is around 14% of the total grain weight and it contains most of the grain vitamins and minerals (Corke, 2015; Solah *et al.*, 2015). The aleurone layer encloses the endosperm and part of the germ, and it is composed by cells that are starch free and cube shaped with a width of around 30 to 70 µm and a thick wall of around 6 - 8 µm (Cornell, 2012; Grundas and Wrigley, 2015).

The starchy endosperm makes up around 80 - 85% of the whole grain weight and it contains carbohydrates, proteins (from 7 to 15%), vitamins, minerals and lipids (Solah *et al.*, 2015). All these nutrients are used by the seedling to grow during the germination phase (Liu *et al.*, 2018). The starchy endosperm is composed of three types of cells: peripheral cells, prismatic cells and central cells. The peripheral cells have a diameter of 60 µm, a cell wall of 8 µm and they are the smallest of the three endosperm cells. Furthermore, they have the lowest starch content but the highest protein percentage. The prismatic cells have a length of 150 to 200 µm and a width of 40 to 60 µm. The central cells have a more variable size and shape compared to the other two cells with a thin wall of 2 µm, a length of 72 - 144 µm and a width of 70 - 120 µm (Arendt and Zannini, 2013; Grundas and Wrigley, 2015).

The germ, also known as "embryo", is around 2 - 3% of the whole grain weight and it contains the highest concentration of lipids (~16%) (Cornell, 2012; Belderok *et al.*, 2013; Uthayakumaran and Wrigley, 2017). The germ is located at the lower end of the grain and, together with the endosperm, it has a crucial role in the vegetative growth of the new plant (Liu *et al.*, 2018). The germ consists of a plumule, a scutellum and a stem. The plumule is that part of the embryo that develops in a shoot when the seed germinates; the scutellum is a small structure that has the shape of a shield and that absorbs nutrients from the endosperm during the germination phase; the stem is attached to a protective sheath known as coleoptile (Cornell, 2012).

1.3 Starch composition

Starch is formed and stored in a particular plasmid called "amyloplasts" through the photosynthesis process (Arendt and Zannini, 2013). Starch is composed of two classes of porous and semi-crystalline granules that can be distinguished in type A and B (Figure 3). Type A granules have a diameter that ranges from 15 to 40 µm and have a lenticular shape; type B granules have a diameter that is between 5 to 10 µm and have a spherical shape (Copeland *et al.*, 2009). By weight, starch is composed from 70 to 80% by type A granules (despite being ~20% by number) (Uthayakumaran and Wrigley, 2017). Different wheat cultivars have different dimensions of type A and B starch granules,

and this difference may be useful for distinguishing soft wheat varieties from hard wheat varieties (Seib, 1994). Furthermore, type A and B starch granules have a different chemical composition such as the concentration of amylose and amylopectin, but also the concentration of proteins and lipids (Seib, 1994).

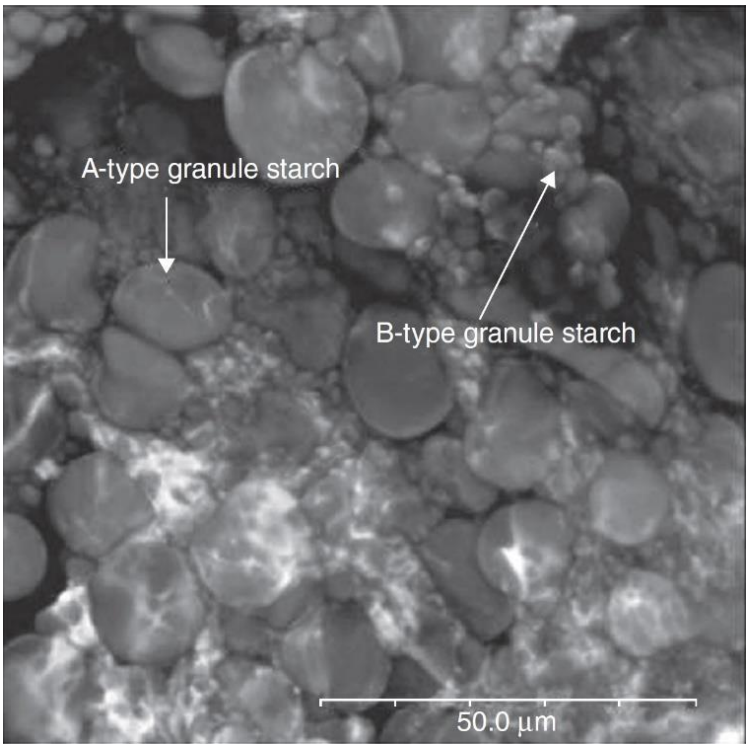


Figure 3 - Type A and B starch granules process (Arendt and Zannini, 2013).

Regular starch in *T. aestivum* L. is composed of ~25% of amylose and ~75% of amylopectin (Garimella Purna *et al.*, 2015). Amylose is a linear (1,4)-α-D-glucan (although in some amyloses there is evidence of few 1,6 branches); amylopectin is highly branched containing (1,4)-α-D linear linkage between D-glucose residues and (1,6)-α-D branch points (Šárka and Dvořáček, 2017). Figure 4 shows the structure of amylose and amylopectin and the two different types of chain linkages (Herrero-Martínez *et al.*, 2004).

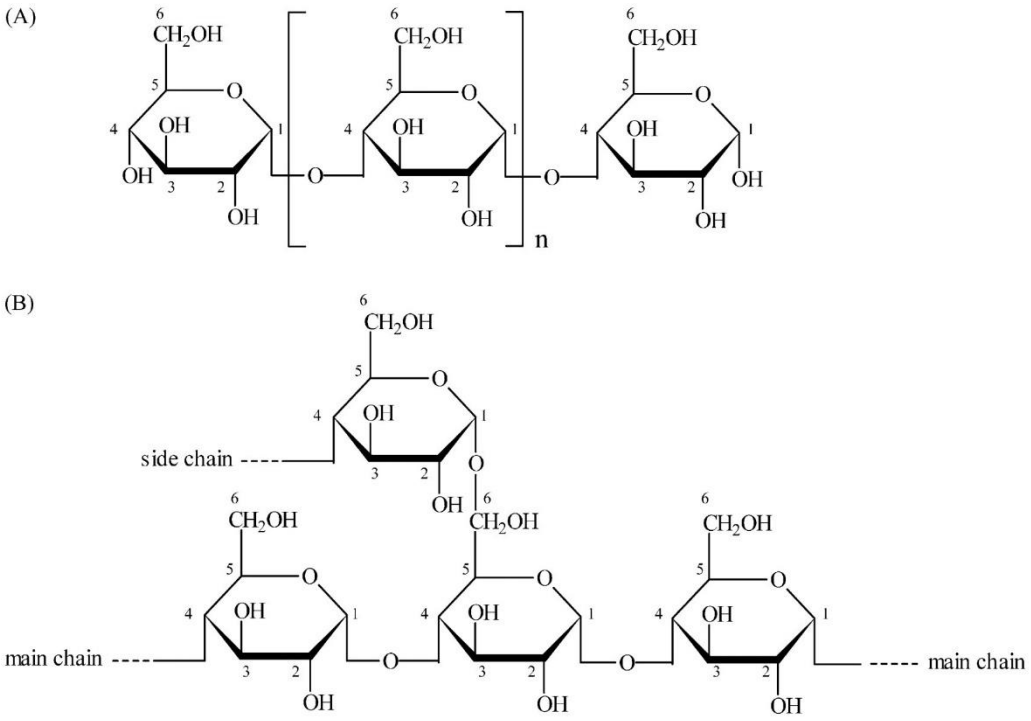


Figure 4 - Structures of amylose (A) and amylopectin (B) showing the two different types of chain linkages (Herrero-Martinez *et al.*, 2004).

The proportion of amylose and amylopectin is determinant for the end-uses of the product because of their influence of many starch properties such as gelatinisation, gelation and pasting (Guzmán *et al.*, 2012). The helical and linear structure of amylose gives it a tendency to retrograde and therefore, after cooked and cooled, it produces a solid gel; on the contrary, amylopectin has a branched structure that impedes retrogradation, and as a consequence, after cooking and cooling, the formation of starch gel is slowed and produces a paste that is cohesive and sticky (Chakraborty *et al.*, 2004).

Iodine-binding procedures (amperometric, potentiometric or spectrophotometric) have been traditionally used for the determination of the amylose-amylopectin ratio in cereal starch. However these methods do not give an exact measurement leading to an overestimation of the amylose content (Herrero-Martínez *et al.*, 2004). Gibson *et al.* in "A Procedure to Measure Amylose in Cereal Starches and Flours with Concanavalin A" used an alternative procedure for the determination of amylose content. In that work, the determination of the amylose content was obtained by the measurement of the amount of carbohydrates in the supernatant after selective precipitation of amylopectin with concanavalin A (Gibson *et al.*, 1997). However, this alternative procedure is more complex than the traditional methods and it has not been fully defined (Herrero-Martínez *et al.*, 2004). Other alternative methods for the determination of the amylose-amylopectin ratio (with their pros and cons) are the size-exclusion chromatography (native or debranched starch), capillary electrophoresis and iodine-

affinity capillary electrophoresis (Herrero-Martínez *et al.*, 2004). Many new wheat lines with different proportion of amylose and amylopectin have been studied and used in breeding programmes to establish new wheat lines with different proportion of these two components (Guzmán *et al.*, 2012).

1.4 Functionality of wheat starch

The importance of starch in the food industry takes place due to its unique properties such as gelatinisation, retrogradation, pasting, etc. (Rosicka-Kaczmarek *et al.*, 2017; Agama-Acevedo *et al.*, 2018). Because products derived from starch are cooked or heated before consumption, all the significant properties of starch are determined in the presence of water, while cooking or heating (Agama-Acevedo *et al.*, 2018).

Starch granules that are not damaged are insoluble in cold water, but they can slightly absorb it. This absorption is reversible: the starch granules return to their original size while drying (BeMiller, 2019). This situation is different when starch granules are heated to a temperature higher than the "initial gelatinisation temperature". The traditional gelatinisation process consists in slowly heating (while stirring) the starch granule with water. This process promotes the swelling and the later the breaking of the starch granules with the release of the molecular components (including amylose and amylopectin) in the water affecting the viscosity of the medium. During gelatinisation countless of hydrogen bonds are formed with water, forming a thick solution that has many culinary applications (Schmiele *et al.*, 2019).

When starch after gelatinisation is stored and left to cool down (for hours or days) becomes gradually less soluble, making the solution difficult to re-dissolve by heating. This turning back to an insoluble state is called retrogradation (BeMiller, 2019). During retrogradation, the molecules obtain a new organisation with an increment of the medium viscosity and water release (Schmiele *et al.*, 2019). Many factors influence the rate and the degree of retrogradation such as the ratio of amylose and amylopectin, starch concentration and temperature (BeMiller, 2019).

Pasting is a process that follows gelatinisation. This process consists of the formation of a paste by continuing to heat and stir starch granules in excess of water (BeMiller, 2019). With constant heating and stirring starch granules break and starch molecules disperse in the medium increasing its viscosity (Ai and Jane, 2018). During the pasting process, starch goes under some structural changes, and these properties have been studied using the Brabender amylo/viscograph (Eliasson, 2012; Chen *et al.*, 2018). The paste that is formed is composed of a continuous phase of starch polymers, and a discontinuous phase of swollen granules and granule fragments in a solution consisted of mostly amylose molecules (BeMiller, 2019).

2. WAXY WHEAT

2.1 Characterization of *waxy* genes

Amylose is synthesised by granule-bound starch synthase (GBSS), coded by the *waxy* genes which names are: *Wx-A1* (located on chromosome 7AS), *Wx-B1* (chromosome 4AL translocated from the original 7BS) and *Wx-D1* (chromosome 7DS) (Debiton *et al.*, 2010). Figure 5 (Graybosch, 1998) shows the location of the *waxy* loci in wheat chromosomes. The association of GBSS to the biosynthesis of amylose in cereals has been known since the 1960s (Seib *et al.*, 2009). GBSSI is active in the starchy endosperm and in the embryo of the seed while GBSSII (a starch synthase isoform) is found in tissues such as the stem, pericarp, leaf and other non-storage tissues (Seib *et al.*, 2009).

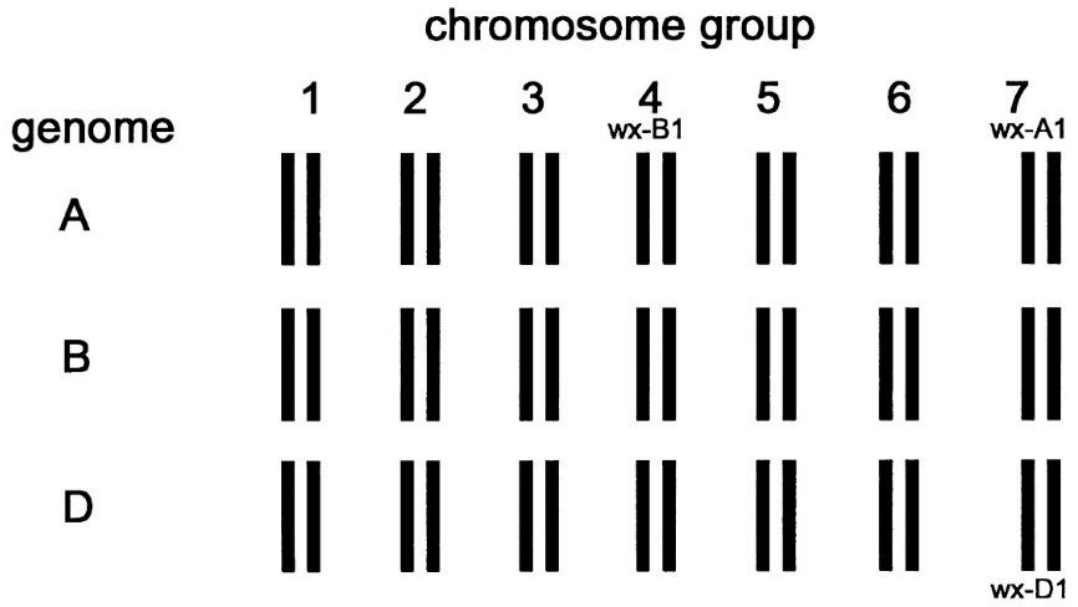


Figure 5 - Location of *waxy* loci in wheat chromosomes (Graybosch, 1998).

Murai *et al.* after sequencing the three genes showed that each *waxy* gene consists of eleven exons and ten introns coding for three peptides of 604 amino acid residues in the *Wx-A1* gene, 605 amino acids residues in the *Wx-B1* gene and 604 amino acid residues in the *Wx-D1* gene (Murai *et al.*, 1999). The following picture (Figure 6) shows the amino acid sequences alignment of the three homoeologous *waxy* genes in hexaploid wheat (Murai *et al.*, 1999).

Figure 6 - Amino acid sequences alignment of the three homoeologous *waxy* genes in hexaploid wheat (Murai *et al.*, 1999).

Because the exon sequences of the three *waxy* genes have minor differences, the molecular weight of the three proteins is very similar; this characteristic has made very challenging the identification of allelic variants between them; nevertheless, the studies performed on durum wheat and common wheat have permitted the detection of different alleles for these genes (Guzmán *et al.*, 2012). The structure of the downstream regions of waxy genes is shown in the next figure (Figure 7) (adapted from Saito *et al.*, 2008).

Figure 7 – Structure of the downstream regions of waxy genes (adapted from Saito *et al.*, 2008).

In wildtype lines of wheat, the three structural genes that encode GBSS isoforms are active and functional; when one or two genes are not functional the wheat lines are called partially waxy; when no one of the three structural genes that encode GBSS are functional (triple null), the starch has very low or zero amylose (Ma *et al.*, 2013). Depending on the null *Wx* allele (or alleles) there are differences in the content of amylose and other functional properties (Ma *et al.*, 2013).

A natural mutation that leads to the formation of waxy lines is unlikely to happen in wheat because of its hexaploid genome, for this reason, the waxy hexaploid wheat lines have been produced by crossing partial waxy mutants such as Kanto 107 and Bai-Huo (Sang-Ho and Jay-Lin, 2002). The partially waxy wheat Kanto 107 is an amylose reduced wheat that carries null alleles at the *Wx-A1* and *Wx-B1* loci while the Bai-Huo at the Wx-D1 locus (Sang-Ho and Jay-Lin, 2002). In an experiment conducted by Vrinten *et al.* tetraploid waxy wheat were obtained by crossing the tetraploid wheat Adura with the partial waxy mutant Kanto 107 and hexaploid waxy wheat was obtained by crossing the partial waxy mutant Kanto 107 with Bai-Huo (Vrinten *et al.*, 1999). The next figure (Figure 8) shows the endosperms stained with an iodine solution of partial waxy mutants Kanto 107 (type 7) and Bai-Huo (Type 4), and F2 generation waxy type (Nakamura *et al.*, 1995).

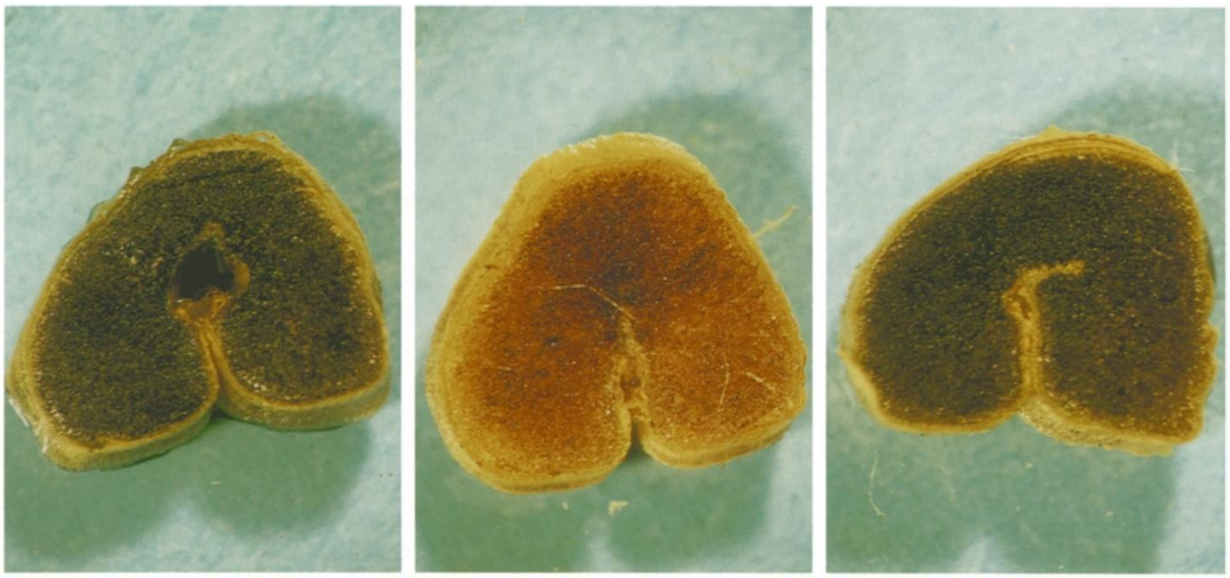


Figure 8 - Endosperms of Kanto 107 (type 7), Bai-Huo (Type 4) and F2 generation waxy type stained with iodine solution (Nakamura *et al.*, 1995).

Waxy wheat lines have many interesting physiochemical and functional properties. Chakraborty *et al*. reported that many important differences affect the physicochemical and functional properties of non-waxy, partial waxy and waxy starches (Chakraborty *et al.*, 2004). This study

reported that the increase of crystallinity and paste viscosity was related to a decrease in the amylose content in starch. The amylose content was associated with the number of null *waxy* alleles. In the study was also reported that the amylose content and degree of crystallinity of waxy hexaploid wheat starch did not differ significantly from the waxy tetraploid wheat starch (Table 1).

Table 2 - Physicochemical properties of non-waxy, partial waxy, and waxy wheat flours. Ben: tetraploid non-waxy durum control; Gunner: hexaploid hard red spring control; Parshall: partial waxy hard red spring control; HRS: hexaploid hard red spring; Waxy hexaploid hard red spring: 619362, 619368, 619374, 619375; Waxy durum: WD1, WD2, WD3. (Chakraborty et al., 2004).

Geno-types	Wheat type	Protein [%]	Starch [%]	Starch damage [%]	α-Amy-lase [CU/g]	Amylose [%]
Ben	durum	13.3	67.2	10.3	0.02	29.2
Gunner	HRS	13.2	67.9	6.4	0.06	26.0
Parshall	partial waxy HRS	12.2	68.3	6.9	0.08	22.3
619362	waxy HRS	13.8	64.3	5.8	0.14	2.3
619368		11.8	68.1	5.8	0.15	2.4
619374		15.5	64.0	6.4	0.11	2.4
619375		12.4	66.2	4.7	0.09	2.1
WD1	waxy durum	13.3	64.1	11.0	0.06	2.6
WD2		12.7	67.1	10.9	0.05	2.4
WD3		12.9	66.0	10.7	0.05	2.3

In the study, waxy tetraploid genotypes had a higher enthalpy of gelatinisation, and higher differential scanning calorimetry (DSC) compared to the hexaploid genotypes. On the contrary, it was reported that starches from waxy hexaploid genotypes had greater granule fragility.

Wang *et al.* analysed the properties of three Chinese wheat varieties (Ning Waxy1, Tian Waxy1, and Yang Waxy1). The study showed that the starch content of the three varieties of waxy wheat was about 54.1-55%; the protein content of waxy wheat grains ranged from 13.7% for the Yang Waxy1 variety to 15.2 for the Ning Waxy1 variety; the amylose content varied from 0.71% for the Tian Waxy1 variety starch to 1.63% for the Ning Waxy1 variety starch. This study also showed that the three different varieties of waxy wheat had no significant differences in the granular morphology and that the three waxy wheat starches had similar bimodal particle size distribution pattern (Wang *et al.*, 2015).

2.2 Applications of waxy wheat

In the recent years, waxy wheat lines have been the object of many studies because important starch properties such as gelatinisation, pasting and retrogradation highly depend on the amylose and amylopectin ratio (Guzmán *et al.*, 2012). Gelatinisation is the phenomena that cause the disorganisation of the starch granules order; pasting causes the swelling and rupture of the granules; retrogradation is the reorganisation of the disordered molecules (Matignon and Tecante, 2017). For this reason, partial waxy or full waxy wheat lines are currently under investigation among scientists, geneticists and wheat breeders for a better understanding of their potential use in the food industry (Chakraborty *et al.*, 2004). In the following paragraphs is given a literature review of the actual and potential use of waxy wheat lines in the food production industry.

Waxy flour acquires a soft and glutinous texture when is mixed with water to form a dough and later boiled. It also acquires an elastic texture that resembles the one of noodles (Fujita *et al.*, 2012). Waxy wheat flour starch has also been reported to have superior water absorption and has been used in the baking industry to improve processing quality and shelf-life stability (Jia *et al.*, 2017).

Fujita *et al*. has analysed the mechanical behaviours in the oral cavity during mastication and deglutition of *mochi* (a Japanese rice cake) prepared from waxy wheat and waxy rice. The study has shown that the *mochi* prepared with waxy wheat is less sticky and with improved texture compared to the one prepared with waxy rice. This property of waxy wheat has an important potential application for all those people with reduced oral functions (e.g. elderly or infants) (Fujita *et al.*, 2012).

Frozen dough is gaining a lot of interest in the food industry because it has many advantages such as time and energy saving for the companies, a more standardized quality of the final products and a decrease in financial loss caused by staling (a chemical and physical process that reduces the quality of the product) (Matuda *et al.*, 2005). However, some unwanted changes occur in the dough during and after frozen storage, such as the death of yeast cells, damage to the gluten network and increase in dough fermentation time while the specific volume of the final products decrease (Jia *et al.*, 2017). Waxy wheat flour has been reported to show less change of stickiness and extensibility after a frozen treatment. Furthermore, the dough is more stable after a frozen treatment when 10% of regular wheat flour is substituted with waxy wheat flour (Rayas-Duarte, 2012). The study of Rayas-Duarte showed that at the same frozen storage a dough with 10% of waxy wheat flow, compared to the regular wheat flour, decreased the content of dry gluten and glutenin macropolymer, increased gelatinisation temperature, enthalpy and relative crystallinity of starch. It also decreased the content of damaged starch and it increased the swelling power of starch (Rayas-Duarte, 2012).

In recent years, Asian noodles have become a popular food that is consumed all around the world. At the same time, the interest of wholegrain food is increasing among the consumers who prefer a healthier diet rich in fibre (Dykes and Rooney, 2007). For this reason, the development of noodles high in fibre and in bioactive substances is important for the consumer's health benefits (Chen *et al.*, 2011). However, the noodle texture produced with wholegrain is negatively affected by the weakening of the dough strength, and the presence of the wheat bran causes a rough surface caused by the larger particle size (Niu *et al.*, 2017). There are only a few scientific studies that focused on the influence of waxy wheat flour on the quality of whole wheat noodles. Niu *et al.* investigated the dough rheological properties and noodle-making performance of whole wheat flour with the substitution of partial waxy and full waxy wheat flour at levels of 0, 250, 500, 750 and 1000 g/kg (Niu *et al.*, 2017). This study showed that the different starch compositions of whole wheat flour with full waxy or partial waxy wheat influenced the starch pasting properties. The study reported that full waxy whole wheat flour interfered with gluten development and the whole wheat dough showed an adverse effect on cooked noodle texture and induced lower starch pasting viscosities. On the contrary partial waxy whole wheat flour increased the elasticity of the noodle and its integrity after cooking. The elasticity increased mainly due to the improved starch pasting properties and due to the increase of protein content. This study has shown that partially waxy whole wheat flour could be used for the production of whole wheat noodle, high in fibre content; nevertheless, further studies are required for a better understanding of the true potential of waxy wheat for the production of whole food (Niu *et al.*, 2017).

Hung *et al.* analysed the properties of dough, and qualities of bread made from 100% whole waxy wheat flour and compared it with the flour made with regular wheat (Hung *et al.*, 2007). The study showed that the bread was significantly softer when made from waxy whole wheat flour and this characteristic is fundamental in breadmaking (Figure 9).

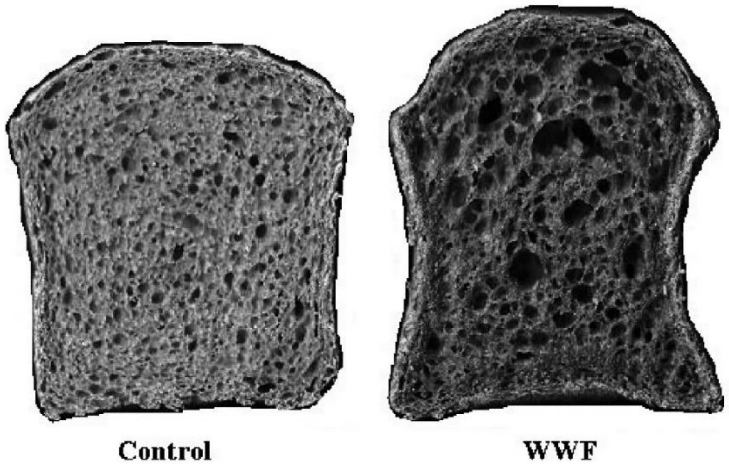


Figure 9 - Cross-sectional views of breads baked from regular wheat (left) and whole waxy wheat flour (right) (Hung et al., 2007).

The consumption of whole food is a good habit for a healthier diet, and the production of whole waxy wheat flour should be encouraged for both the improved bread quality and the nutritive value (Hung *et al.*, 2007).

2.3 New techniques for waxy wheat lines production

The genetic improvement of a crop is very important to ensure food security and nutrition in the future years (Ronald, 2011). Sufficient variation in the wheat genome cannot be found in nature because mutations are often masked by the presence of wild-type genes from the remaining loci (Vrinten *et al.*, 1999). Traditional plant breeding consists in the incorporation of a valuable mutation into agricultural genotypes by hybridisation, recombination and selection, but this approach is very long, and it takes many years to achieve it (Dong *et al.*, 2009). However, this variation can be created through random or targeted processes. The most common approach for generating variation in plant materials is the use of chemical or physical mutagens. The type and the dosage of the mutagens influence the results of the mutation (Jankowicz-Cieslak *et al.*, 2017). This approach can be considered random because it is not possible to predict in advance the location of the DNA lesions caused by the chemical or physical mutagens (Greene *et al.*, 2003).

Targeting induced local lesions IN genomes (TILLING) is a reverse genetic approach that makes use of some chemical mutagens such as ethyl methanesulfonate (EMS) in order to induce a high density of randomly induced mutations in the DNA (McCallum *et al.*, 2000). TILLING is mostly used in plants; however it is one rare example of a method that was developed firstly in plants and then adopted in animals (Wang *et al.*, 2012). This technique is combined with high-throughput screening to detect sequence variations in specific genes (Dong *et al.*, 2009).

Dong *et al*. used a modified TILLING procedure for breeding waxy wheat from a soft wheat variety, QAL2000, and a hard wheat variety, Ventura (Dong *et al.*, 2009). In this study, the seeds were soaked in the EMS solution for about 18 hours, and after this treatment the seeds were washed for about 2 hours under running tap water. The washed seeds were then sown in pots in a greenhouse at 16-18°C until spouting and later transferred outdoor for two weeks and then transplanted to the field. From each harvested spike one M_2 seed sown in a seedling tray and after about 20 days the leaves of the seedlings were collected and dried in a closed box with silica gel. After DNA extraction the samples were pooled for PCR and screening. The allele *Wx-B1* (chromosome 4A) was null in about 50% of the population, and thus the screening was performed only for *Wx-A1* and *Wx-D1*. The PCR products of the identified mutants were then sequenced to determine the mutation. In total were

screened 2348 samples, and for the *Wx-A1* and *Wx-D1* gene fragments were detected 121 mutants. Later, the M_3 generation was made by the truncation mutants of the *Wx-A1* and *Wx-D1* loci in order to identify homozygous lines. The homozygous lines were then selected and inter-crossed. After carrying out progeny tests, it was confirmed that waxy individuals were homozygous. With the method briefly described above, it took 18 months to produce a waxy wheat line, a very short time compared to classical breeding techniques (Dong *et al.*, 2009).

A technique for the identification of mutations (including mutations in the *waxy* genes) is the high-resolution melting (HRM) analysis. HRM is a technique that compared to other methods has many advantages including the relatively low cost, the uses of generic instrumentation easily available in many laboratories and the simplicity of the technique (Tong and Giffard, 2012). The HRM method requires standard PCR reagents that are supplemented only with new generation of fluorescent dsDNA dyes that interact only with dsDNA (Jankowicz-Cieslak *et al.*, 2017). At room temperature, the double-stranded DNA (dsDNA) is very stable, but the increasing of temperature brings the dsDNA to dissociate until the two strands of the DNA are completely separated (Druml and Cichna-Markl, 2014). The temperature at which 50% of the DNA is single-stranded is called melting temperature (Tm), and HRM detects small variation in the sequences based of their different melting temperature (Pereira *et al.*, 2018). The following figure (Figure 10) shows an example of a melt curve and a melting temperature. The Tm depends on the length of the DNA fragment and on its guanine-cytosine content (Druml and Cichna-Markl, 2014). DNA sequences with high guanine-cytosine content have higher melting temperature because GC base pairs are more stable than AT base pairs due to the three hydrogen bonds (Druml and Cichna-Markl, 2014).

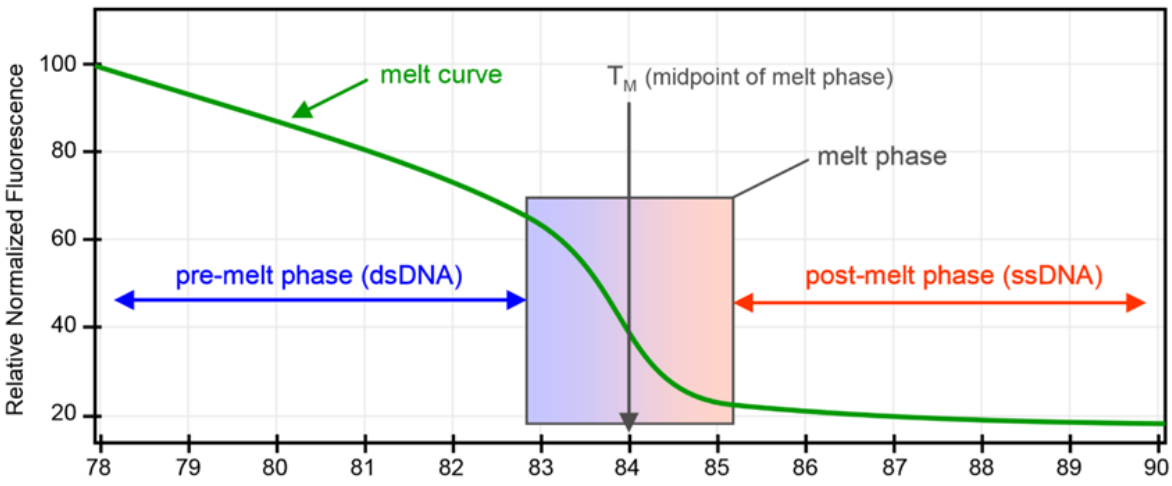


Figure 10 - Melt curve and melting temperature (Tm) (Source: Corbett Research HRM Assay Design and Analysis).

The identification and production of waxy wheat lines can be carried out also through Marker-assisted selection (MAS). MAS is a technique that can be used to identify a gene quickly and accurately through the detection of the target gene at the DNA level, reducing in this way the number of generations required for establishing a homozygous line (Yang *et al.*, 2013). The use of MAS is particularly important in wheat because, due to its hexaploid nature, many recessive mutations are difficult to detect (Saito *et al.*, 2010). Saito *et al.* used primer sets to identify mutations at the *Wx-A1*, *Wx-B1* and *Wx-D1* loci in a single reaction by using multiplex PCR (Saito *et al.*, 2010). In this study, the PCR markers were used to identify waxy mutations in 168 wheat lines from 20 countries. Most of the cultivar that lacked the *Wx-A1* protein had the same mutation that was found in Kanto 107, but among all the cultivar analysed a new mutation was identified in wheat lines from Turkey. In these lines, the fragment of the *Wx-A1* gene was found to be 173 bp longer than the wild-type fragment due to the insertion of a transposable element. Different mutations were found in the *Wx-B1* and *Wx-D1* alleles, but when in the lines the *Wx-B1* protein was absent, the *Wx-B1* mutation was the same as the one in Kanto 107. By using backcrossing combined with MAS, it is possible to accelerate wheat breeding programs. With MAS technique it is possible to combine multiple markers for the identification of genotypes at multiple loci from the same DNA template; furthermore, it is possible to extract DNA for MAS from any tissue at almost all plant growth stages (Saito *et al.*, 2010).

Another method for the identification of mutations in DNA is the combination of PCR and gene sequencing (Rosenberg, 2017). This combinations of PCR and DNA sequencing revolutionised the field of genetics making of it a powerful tool that is used every day in thousands of laboratories (Rosenberg, 2017). The most common sequencing method, developed in 1975, is the Sanger method (first generation of DNA sequencing methods) (Hoy, 2019). The Sanger method now is highly automated, and it makes use of bases labelled with fluorescent dyes and a detector that automatically reads the sequence (Hoy, 2019). The sequenced DNA can be then analysed via some bioinformatics tools such as BLAST. Basic local alignment search tool (BLAST) is a similarity search tool that finds regions of local similarity between sequences. This tool can be used for analysing a user's nucleotide or protein sequence to an online database of sequences (Ye *et al.*, 2006). The BLAST servers are kept online by the National Center for Biotechnology Information (NCBI), and the website can be reached online worldwide from any pc with an internet connection. Among all the alignment options, the "Pairwise with identities" alignment makes possible the identification of mutations such as a single nucleotide substitution (Ye *et al.*, 2006). The analytical part of this paper makes great use of BLAST and pairwise alignments for the identification of novel alleles in the *waxy* genes of a TILLING population.

3. MATERIALS AND METHODS

3.1 Winter wheat TILLING Populations: "T-WW-Kena" and "T-WW-Gaja"

The TILLING populations "T-WW-Kena" and "T-WW-Gaja" were developed from two new promising cultivars "Kena DS" and "Gaja DS" using EMS, a well-known mutagens with chemical formula $C_3H_8SO_3$ (Armonienė and Brazauskas, 2014). The following figure (Figure 11) shows the schematic process of the development of the "T-WW-Gaja" and "T-WW-Kena" TILLING population (Armoniene and Brazauskas, 2018). From the TILLING populations were chosen 88 samples (the whole population consists of 756 samples). In order to determine the amount of amylose present in the starch, all the 756 samples of the TILLING populations were stained with a iodine solution (0.025% I2–KI) and left to precipitate for 24 hours at room temperature (Armoniene and Brazauskas, 2018). The 88 samples chosen for the development of this work showed a lower or higher amount of stained amylose content compared to the wild type (Armoniene and Brazauskas, 2018). The wild type "Kena DS" and "Gaja DS" were used as control.

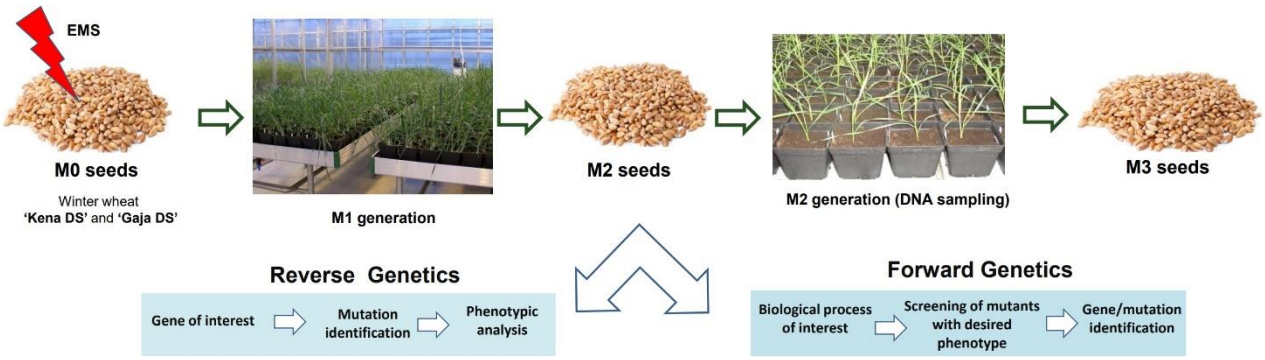


Figure 11 - Scheme of the development of the "T-WW-Gaja" and "T-WW-Kena" TILLING populations (Armoniene and Brazauskas, 2018).

3.2 DNA extraction of the TILLING Population

The protocol used for DNA extraction was based on the protocol of Lassner *et al., (1984)* . For the DNA extraction, 200 mg of fresh plant material was put inside a 2 ml Eppendorf tube with 3-4 metal balls. The tubes were then placed in liquid nitrogen, and the grinding was performed with a Laboratory Mixer Mill MM 400 (RETSCH, Germany). After grinding, 1 ml of DNA extraction buffer and 400 µl chloroform-isoamyl alcohol were added into each tube. The DNA extraction buffer was pre-warmed in water bath to 55°C and, after the grinding process, it was added to the tubes as fast as

possible in order to avoid DNA fragmentation. The samples were then incubated in water bath for 10 min at 55°C, and the tubes were shaken every 2 minutes. After the incubation in water bath, the samples were centrifuged for 5 min at 12.000 rpm. The supernatants were then transferred to new tubes, and equal volumes of chloroform were added. After mixing on a shaker, the tubes were once more centrifuged for 5 min at 12.000 rpm. The supernatants were transferred to a new 1.5 ml tube, and 20 μl RNazeA (10μg/μl) were added in each tube. The tubes were then kept at 37°C for 15 min. For the precipitation of the DNA, 1.2 volume of isopropanol were added in each tube. After mixing gently, the tubes were centrifuged for 10 min at 12.000 rpm. After decanting the supernatant, the samples were washed by adding 900 ml of 70% ethanol in the tubes. The tubes were gently shaken to detach the pellet from the tube wall. After centrifuging the tubes for 5 min at 12.000 rpm, the cleaning process with ethanol was repeated once more. After the second wash, the pellet was drained to remove the presence of ethanol, and it was dissolved in 50 μl of EB (elution buffer).

3.3 DNA quality assessment

In order to assess the quality of the DNA extracted some analysis were performed. The first analysis was performed with the NanoDrop™ 2000/c Spectrophotometers (Thermo Fisher Scientific, USA). For each analysis were used 1.5 μl of the extracted DNA. Prior analysis, the instrument was calibrated with a "blank" sample of EB. It is important that the blank has the same pH and similar ionic strength as the samples analysed. The sample concentrations are calculated by a modification of the Beer-Lambert equation (Thermo Fisher Scientific, 2010):

(1)

$$c = (A * \varepsilon)/b$$

- c is the nucleic acid concentration in ng/μL;
- A is the absorbance in AU;
- ε is the wavelength-dependent extinction coefficient in ng-cm/μL;
- b is the pathlength in cm.

The concentration of the nucleic acid samples is based on the absorbance at 260 nm. The next figure (Figure 12) shows a typical nucleic acid spectrum.

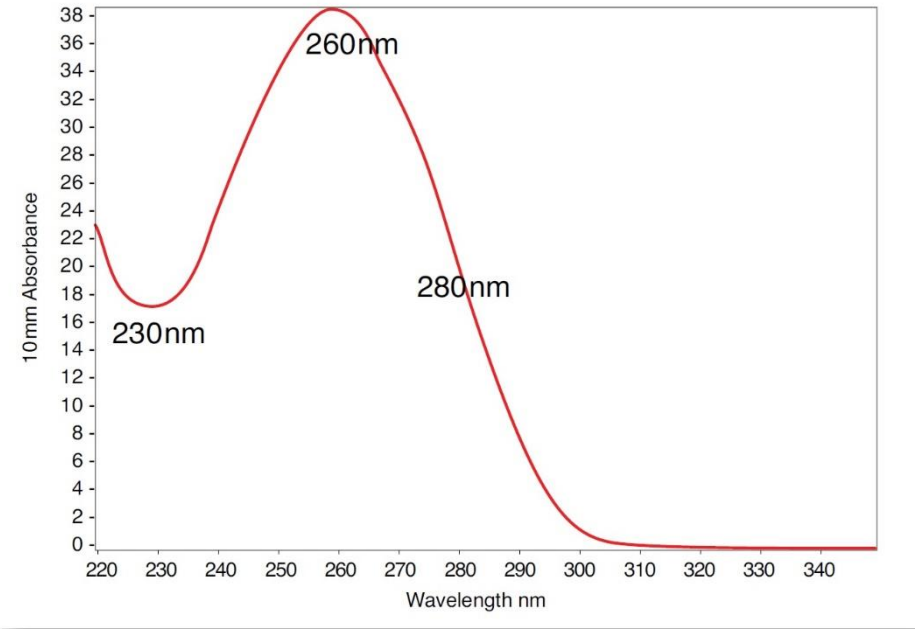


Figure 12 - Typical nucleic acid spectrum (Thermo Fisher Scientific, 2010).

It is generally accepted as "pure" DNA a value of ~ 1.8 for the 260/280 ratio, and a value in the range of 1.8 - 2.2 for the 260/230 ratio. The pH and the ionic strength of the blank and the samples analysed influence the 260/280 ratio and the 260/230 ratio, for this reason, it is very important that the blank has the same characteristics of the buffer used for diluting the DNA. If the 260/280 ratio and the 260/230 ratio and, as a consequence, the nucleic acid spectrum is different from the usual target, it may indicate that the blank or the samples do not have good quality for the presence of contaminants (such as proteins or phenols) (Thermo Fisher Scientific, 2010). The following table (Table 2) shows the results of the samples quality check with the NanoDrop™ 2000/c Spectrophotometers.

Table 2 – List of the samples analysed with their respective ID, concentrations (expressed in ng/µl) and their 260/280 – 260/230 ratios. The samples are two varieties of a winter wheat TILLING populations. The samples with the ID "MK" belong to the TILLING winter wheat variety "T-WW-Kena , while the samples with the ID "MG" belong to the TILLING winter wheat variety "T-WW-Gaja".

Sample ID	**Conc. ng/µl**	**260/280**	**260/230**	**Sample ID**	**Conc. ng/µl**	**260/280**	**260/230**
MK-4	243.1	1.8	2	MG-509	293.4	1.82	2.05
MK-13	224.5	1.82	2.08	MG-512	249.5	1.74	2.1
MK-30	359.6	1.81	2.01	MG-520	493.2	1.8	2.05
MK-38	316.4	1.82	2.02	MG-522	206.9	1.8	2.13
MK-44	428.3	1.79	1.83	MG-523	229.8	1.78	1.91
MK-59	286	1.81	2.02	MG-532	401.9	1.81	2.05
MK-77	291.3	1.83	2.17	MG-537	326.9	1.8	1.9
MK-82	164.6	1.83	2.14	MG-564	390.7	1.81	1.94
MK-83	308.8	1.81	2.11	MG-569	198.3	1.82	2.14
MK-87	261.4	1.82	2.12	MG-585	229.1	1.81	2.17
MK-102	231.9	1.8	1.86	MG-601	198.2	1.82	2.03
MK-114	367.3	1.82	2	MG-614	499.8	1.79	2.03
MK-125	240.4	1.81	2.11	MG-643	290.5	1.7	2.07
MK-181	281.4	1.81	1.99	MG-651	360.1	1.81	1.98
MK-183	466.7	1.8	2.05	MG-657	280.4	1.8	1.92
MK-207	251.1	1.81	2.05	MG-659	360.7	1.8	2.07
MK-216	227.4	1.79	2.02	MG-688	361.3	1.75	2.05
MK-222	251.3	1.83	2.11	MG-720	400.2	1.83	2.02
MK-225	315.4	1.82	2.06	MG-727	309.5	1.82	2.03
MK-244	420	1.81	2	MG-744	282.4	1.82	2.05
MK-249	160.6	1.81	2.09	MG-748	314.8	1.82	2.14
MK-260	296.2	1.79	2.12	MG-782	233.5	1.81	1.95
MK-264	324.2	1.81	2.04	MG-791	310.5	1.81	2.03
MK-276	212.1	1.73	1.95	MG-800	227.7	1.82	2.14
MK-283	468.6	1.81	2.02	MG-802	412.7	1.79	1.91
MK-289	250.8	1.74	2.12	MG-815	854.7	1.86	1.9

MK-302	353.9	1.82	2.06	MG-819	354	1.71	2.09
MK-306	241	1.82	2.11	MG-827	378.8	1.76	2.15
MK-323	346	1.81	2.14	MG-829	300.9	1.82	2.05
MK-335	257.8	1.81	2.11	MG-830	267.3	1.78	1.94
MK-344	285.6	1.81	2.16	MG-835	287.1	1.79	1.78
MK-345	291.3	1.81	2.08	MG-847	262.6	1.83	2.17
MK-368	312.1	1.81	2.13	MG-907	331.3	1.83	2.18
MK-395	289.7	1.83	2.14	MG-912	555.3	1.74	1.9
MK-396	215.6	1.78	1.82	MG-930	290.6	1.79	1.95
MK-402	318.2	1.79	1.94	MG-955	119.6	1.85	2.21
MK-426	272.9	1.81	1.95	MG-959	362.2	1.81	2.14
MK-464	486.7	1.77	2.09	MG-960	433.6	1.74	2.26
MK-465	231.6	1.82	2.03	MG-962	297.3	1.82	2.06
MK-476	180.1	1.81	2.07	MG-985	306.5	1.81	2.03
MK-480	393.3	1.83	2.17	MG-987	221.4	1.8	2.14
MK-500	562.9	1.77	1.93	MG-991	296	1.81	2.01
				MG-998	326.4	1.82	2.17
				MG-1000	207.8	1.81	2.13

Another quality test was given by the analysis of the DNA through gel electrophoresis. Before the analysis, 1 µl of the extracted DNA samples was diluted in 4 µl of nuclease-free water. For the preparation of 1.5% agarose gel for the electrophoresis analysis, 150 ml of 10x TAE buffer were added in a 250 ml glass flask with 2.25 g of agarose powder. The solution was mixed and heated in the microwave until the agarose powder was fully melted. The solution was left to cool down for a little while at room temperature, and a drop of 250 µg/ml ethidium bromide was added in the solution. Before pouring the gel, the electrophoresis plate was glued up with duct tape in order to prevent the spill of the gel itself, and the wells comb was added. The plate was then left to cool down at room temperature for 15 minutes, and after that, the wells comb was slowly removed. As DNA ladder were used 2 µl of 0.1µg/ml GeneRuler Mix DNA ladder (Thermo Fisher Scientific, USA). The 6X DNA Loading Dye (Thermo Fisher Scientific, USA) was used for loading on the agarose gel the DNA samples. In the rest of the wells were added 5 µl of the genomic DNA samples. After

electrophoresis, the gel was analysed with GelCapture software. The following figures (Figure 13 and Figure 14) show the results of two electrophoresis analyses.

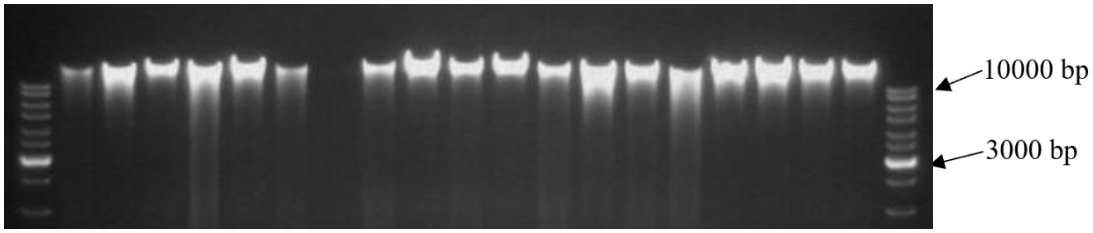


Figure 13 - Gel electrophoresis results of the following samples (from left to right): MK-38, MG-720, MG-564, MG-520, MG-962, MK-302, MK-183, MK-59, MG-727, MK-426, MG-744, MG, 835, MK-30, MK-283, MK-44, MK-114, MG-791, MG-829, MG-509.

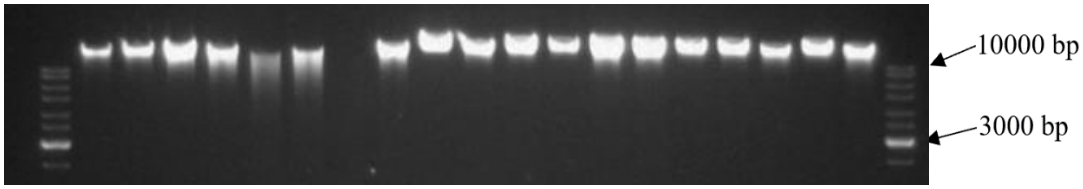


Figure 14 - Gel electrophoresis results of the following samples (from left to right): MG-782, MG-985, MG-537, MG-651, MG-532, MK-464, MG-688, W-1, MG-512, MG-827, MG-643, MK-276, MG-819, W-2, MK-289, MK-225. MK-102, MK-465, MK-264.

3.4 Amplification of *Wx-B1* and *Wx-D1* genes

In order to amplify the genes of interest (*Wx-B1* and *Wx-D1* genes), many PCR analyses were performed. For the amplification of the *Wx-B1* gene and the *Wx-D1* gene, 38 samples of the TILLING winter wheat varieties "T-WW-Kena" and "T-WW-Gaja" were chosen from the stock of the extracted DNA samples. Before the PCR analyses, all the extracted DNA samples were diluted to 50 ng/µl. The PCR mixture was prepared with 10 µl of diluted DNA, 2.5 µl of the forward primer (5 ng/µl), 2.5 µl of the reverse primer (5 ng/µl), 10 µl of nuclease-free water and 25 µl of DreamTaq PCR Master Mix (Thermo Fisher Scientific, USA). The following table (Table 3) show the sequences of the primers used for the identification of the *Wx-B1* and the *Wx-D1* genes.

Table 3 – Forward and Reverse primers used for the amplification of the *Wx-B1* and *Wx-D1* genes.

Primer (*Wx-B1*)	**Sequence (5'-3')**	**T_a**	**Size (bp)**
BDFL-F	CTGGCCTGCTACCTCAAGAGCAACT	63°C	778
BRC1-R	GGTTGCGGTTGGGGTCGATGAC		
Primer (Wx-D1)			
BDFL-F	CTGGCCTGCTACCTCAAGAGCAACT	63°C	2307
DRSL-R	CTGTTTCACCATGATCGCTCCCCTT		

The PCR was carried out using the following setting on an Eppendorf Mastercycler DNA Engine Thermal Cycler PCR (Germany):

- 95°C for 3 min,
- 95°C for 30 sec,
- 63°C for 30 sec,
- 72°C for 1 min;

x29

- 72°C for 5 min;
- 10°C holding temperature.

The set of primers for the amplification of the *Wx-B1* and *Wx-D1* work under the same PCR conditions.

The PCR products were then checked via gel electrophoresis on 1.5% (w/v) agaroseTAE gel. Before the analyses, 2 µl of PCR products were diluted in 3 µl nuclease-free water. The diluted PCR products were then loaded in each well, along with 1 µl of 6X DNA Loading Dye (Thermo Fisher Scientific, USA). As DNA ladder were used 2 µl of 0.1µg/ml GeneRuler Mix DNA ladder (Thermo Fisher Scientific, USA). The gel was then analysed with the GelCapture software.

3.5 PCR products purification with magnetic beads

In order to sequence the DNA, the PCR products have to be purified. The procedure for the DNA purification was based on the protocol of Mayjonade *et al.*, (2016). Before starting, the magnetic beads were left at room temperature for 30 min, and after to ensure the corrected resuspension of the beads, the solution was vortexed for 20 seconds. The PCR products were transferred into separate 1.5 ml tubes, and a volume of the Serapure beads (50 µl of working solution) was added into each tube.

The tubes were then inverted 20 times and after were left to incubate at room temperature for 10 minutes with a gentle agitation. The tubes were then centrifuged for 1 second. The 1.5 ml tubes were then placed in a magnetic rack (DynaMag™-2 Magnet, Thermo Fisher Scientific, USA) until the solution became clear. The supernatant was then removed as slowly as possible to ensure that the beads pellet was not resuspended. The beads pellet was then washed with a solution of 70% ethanol without removing the tubes from the magnetic rack. The washing procedure was carried out two times. The tubes were then removed from the magnetic rack, centrifuged for 1 second and re-placed in the magnetic rack to completely remove the washing solution. The tubes were left to air-dry for few seconds at 50°C (it is important to do not over dry the beads or the elution efficiency will decrease significantly, but it is also important to completely remove the washing solution). The beads were then resuspended with 40 µl of preheated (50°C) EB. The tubes were then centrifuged for one second and placed once more in the magnetic rack. The clear solution was then transferred in new 1.5 ml tubes.

The concentration and the quality of the purified DNA was checked with the NanoDrop™ 2000/c Spectrophotometers (Thermo Fisher Scientific, USA). The following table (Table 4) shows the results of the quality test of the purified PCR products of the *Wx-B1* and *Wx-D1* genes.

Table 4 – Nucleic acid concentrations (ng/µl) of the purified PCR products of wild types “Kena DS” and “Gaja DS” and of the mutant TILLING populations “T-WW-Kena” and “T-WW-Gaja”.

Sample ID Primer B	Conc. ng/µl	260/230	Sample ID Primer D	Conc. ng/µl	260/230
Kena DS	46	2.08	Kena DS	25.8	2.16
MK38	19.7	1.92	MK38	16.2	2.11
MK114	26.9	1.8	MK114	12.5	2.2
MK283	22.8	1.98	MK283	14.2	1.74
MK464	25.2	1.75	MK464	21	1.79
MK480	13.9	1.78			
MK44	38.9	1.99			
MK59	39.6	1.94	MK59	14.4	2.04
MK82	17.9	1.7			
MK87	38.7	1.88	MK87	10.3	2.05
MK102	18.6	1.93			
MK264	54.7	2.05			

MK276	38.9	2.01	MK276	25.8	1.81
MK335	21.5	1.94			
MK426	40	2	MK426	27.4	2.16
MK323	11.7	2.08			
Gaja DS	31.4	2.01	Gaja DS	20	2.02
MG512	42.5	2	MG512	25.4	1.95
MG520	32.1	2.24	MG520	13.6	1.88
MG522	32.5	1.83			
MG523	23.8	1.89	MG523	10.3	2.05
MG532	42.4	2.05	MG532	14	2.17
MG537	37.9	2.16	MG537	26.4	2.11
MG564	19.8	1.98	MG564	23.2	1.98
MG569	37.5	2.29			

3.6 PCR products sequencing and mutation identification

In order to identify mutations in the *Wx-B1* and *Wx-D1* genes, the PCR products were sent for sequencing. The sequencing process was carried out by Eurofins Genomics, Germany. The service provided was a Sanger sequencing – LightRun with barcodes. The sequencing method used was the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machines.

The samples sent for sequencing are listed in table 4. For sequencing the samples, 5 µl of PCR product were transferred into an Eppendorf safe-lock tube with 5 µl of the forward primer. Both for the *Wx-B1* and the *Wx-D1* samples was used the BDFL-F primer. Each sample was labelled with a unique barcode placed on the tube.

Nucleotides sequencing is a powerful tool for finding mutations in a gene. In order to find a mutation in the gene of interest, the nucleotide sequence of the wild type gene has to be compared to the nucleotide sequence of the suspect mutant gene. In the case of this paper, the wild type *waxy* genes *Wx-B1* and *Wx-D1* of the winter wheat cultivars “Kena DS” and “Gaja DS” were compared to the ones of the TILLING population “T-WW-Kena” and “T-WW-Gaja”. The wild type sequences of the two alleles were aligned to each mutant sample.

There are many tools for aligning two or more nucleotides sequences. In this paper was used mostly the NCBI (National Center for Biotechnology Information) BLAST tool. BLAST (Basic Local Alignment Search Tool) is a powerful tool that finds regions of similarity between biological sequences. BLAST can be accessed online through the NCBI website. Other softwares used for sequencing alignment were Mega X and Geneious Prime.

The mutation density of the screened population was calculated as follows (Botticella *et al.*, 2011):

(2)

$$Mutation\ density = \frac{(total\ size\ of\ amplicon) \times (total\ number\ of\ screened\ lines)}{(number\ of\ identified\ mutations)}$$

The mutation density is an estimation that gives some indications regarding the mutation frequency in a screened population.

4. RESULTS AND DISCUSSION

4.1 PCR results

In order to compare the "Gaja DS" and "Kena DS" wild type *Wx-B1* and *Wx-D1* genes with the TILLING population, the wild type genes had to be amplified. The primers for the amplification of the *waxy* genes *Wx-B1* and *Wx-D1* were designed based on the nucleotide sequences of the wild-type genes (Saito *et al.*, 2010). The following figures (Figure 15 and Figure 16) show the position and the orientation of the primers comparing the wild *waxy* alleles *Wx-B1* and *Wx-D1* with their respective null alleles (Saito *et al.*, 2008; Saito *et al.* 2010).

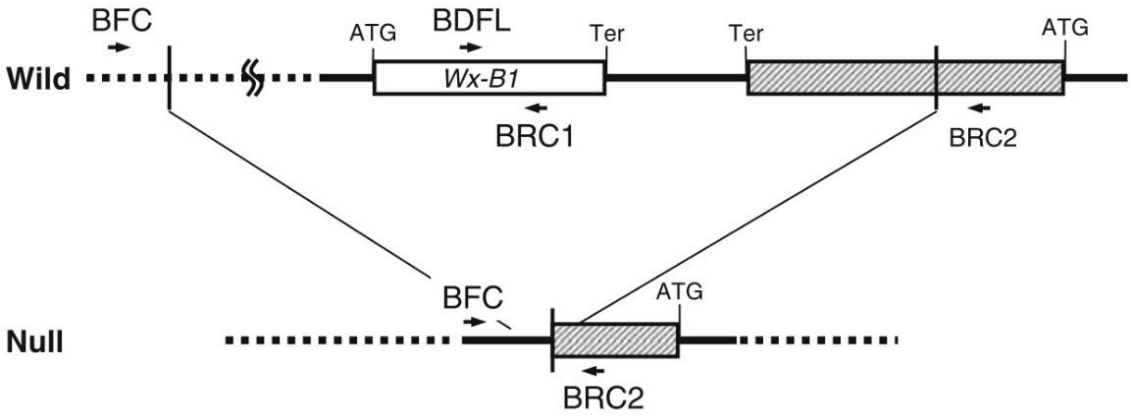


Figure 15 - Position and the orientation of the primer set Wx-B1 (Saito et al., 2008).

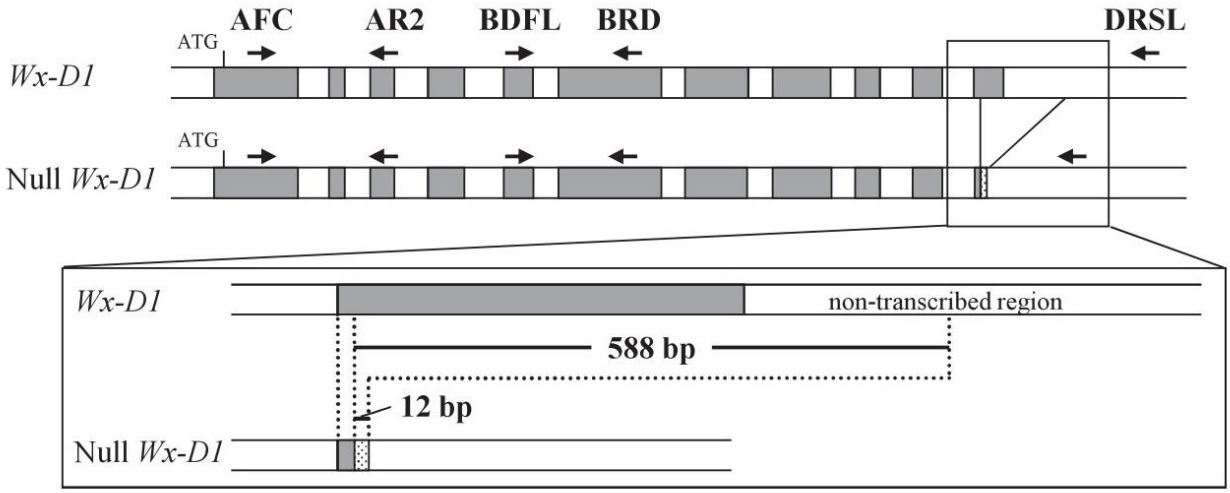


Figure 16 - Position and the orientation of the primer set Wx-D1 (Saito et al., 2010).

The PCR products of the wild type *waxy* genes of “Gaja DS” and “Kena DS” on 1.5% (w/v) agaroseTAE gel are shown in the next figure (Figure 17).

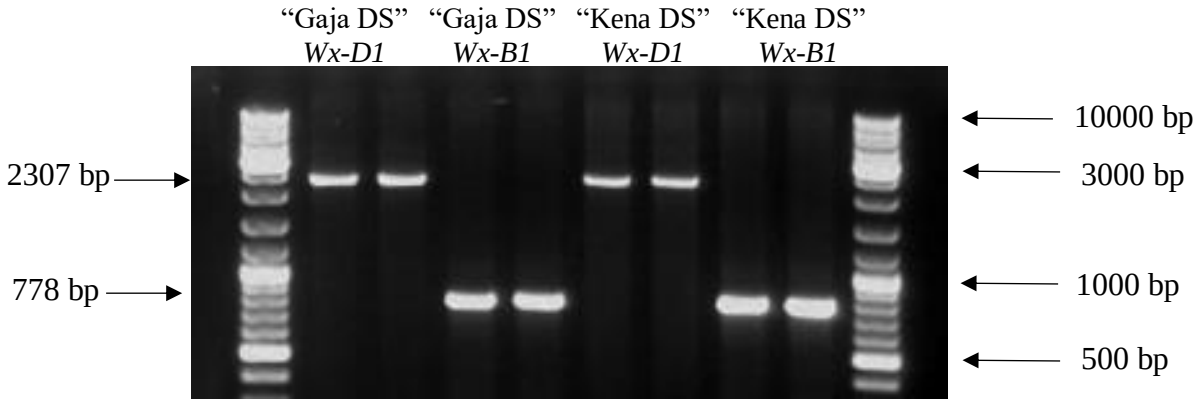


Figure 17 - From the left, "Gaja DS" and "Kena DS" wild type *Wx-D1* and *Wx-B1* genes.

The results of the *Wx-B1* PCR products on 1.5% (w/v) agaroseTAE gel of some “T-WW-Kena” mutant samples are shown in the next figure (Figure 18).

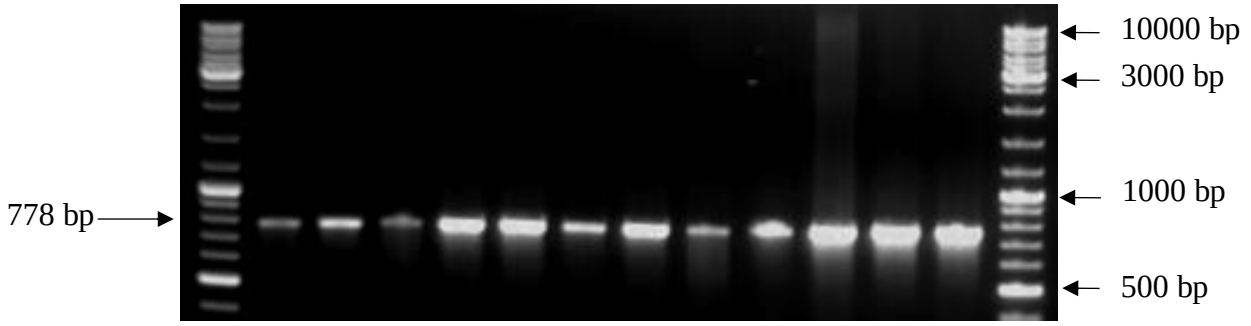


Figure 18 - PCR products of the “Kena DS” *Wx-B1* gene. Samples IDs (from left to right): MK-480, MK-335, MK-38, MK-44, MK-59, MK-82, MK-87, MK-102, MK-181, MK-264, MK-276, MK-426.

The following figure (Figure 19) shows the results of the *Wx-B1* PCR products on 1.5% (w/v) agaroseTAE gel of some "T-WW-Gaja" mutant samples.

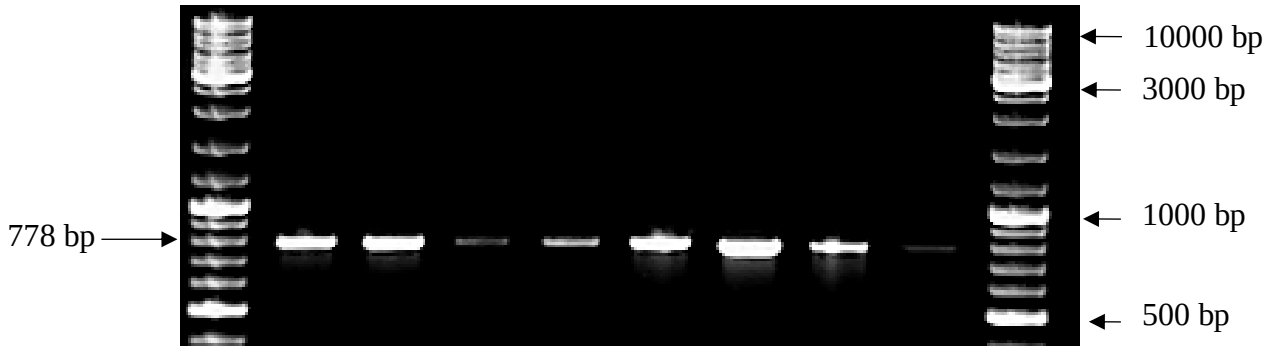


Figure 19 - PCR products of the "Gaja DS" *Wx-B1* gene. Samples IDs (from left to right): MG512, MG520, MG522, MG523, MG532, MG537, MG564, MG569.

A mutation in the DNA sequence could affect slightly the migration rate during gel electrophoresis. Molecules that have different sizes migrate at different rates as well as molecules that have the same size but different charges. The bands obtained through gel electrophoresis of the "T-WW-Kena" and "T-WW-Gaja" *Wx-B1* gene are equal to the wild type gene, and it seems that a waxy mutation has not occurred. Nevertheless, EMS may produce mutations such as single nucleotide substitutions, base-pair insertions or deletions, and these mutations are often not visible on agarose gel.

The results of the *Wx-D1* PCR products on 1.5% (w/v) agaroseTAE gel of some "Kena DS" mutant samples are shown in the next figure (Figure 20).

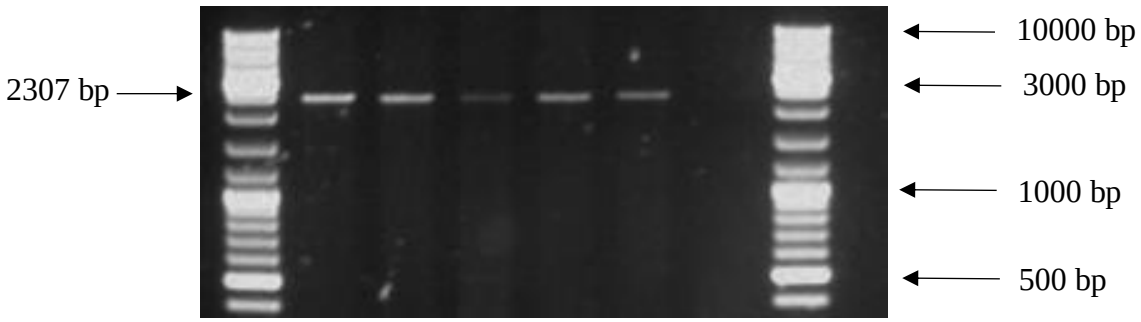


Figure 20 - PCR products of the "Kena DS" *Wx-D1* gene. Samples IDs (from left to right): MK38, MK44, MK283, MK464, MK480.

The *Wx-D1* PCR products on 1.5% (w/v) agaroseTAE gel of some "Gaja DS" mutant samples are shown in the next figure (Figure 21).

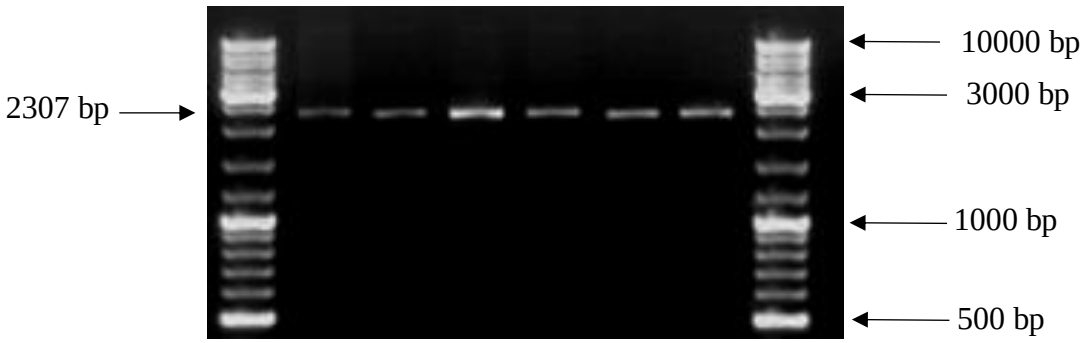


Figure 21 - PCR products of the "Gaja DS" *Wx-D1* gene. Samples IDs (from left to right): MG512, MG520, MG522, MG532, MG537, MG564.

Also in this case, the wild type gene *Wx-D1* of "Kena DS" and "Gaja DS" appears to be the same as the TILLING populations "T-WW-Kena" and "T-WW-Gaja". From the analysis of the gel bands, there is no difference in migration time between the wild type allele and the TILLING population. Therefore, the TILLING population appear to carry the wild type gene and it seems that a waxy mutation has not occurred. However, as explained above, EMS may produce mutations that are not visible on gel electrophoresis.

The primers set for the amplification of the *Wx-B1* gene worked every time without any problems. The amplification of the *Wx-D1* gene was challenging. Many times the amplification was not successful with no PCR product obtained and the formation of primer dimers (Figure 22).

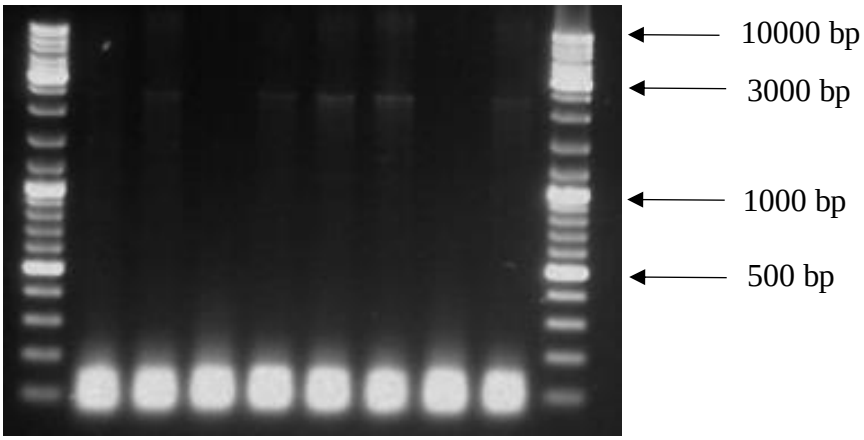


Figure 22 - Unsuccessful *Wx-D1* amplification with the formation of primer dimers.

After many attempts and investigations, it was concluded that the diluted set of primers (concentration 5 ng/μl) for the amplification of the *Wx-D1* gene was susceptible to the thawing and refreezing process. The next figure (Figure 23) shows the PCR products of a new diluted set of primers compared with a set of diluted primers that have been thawed and refreeze after each use.

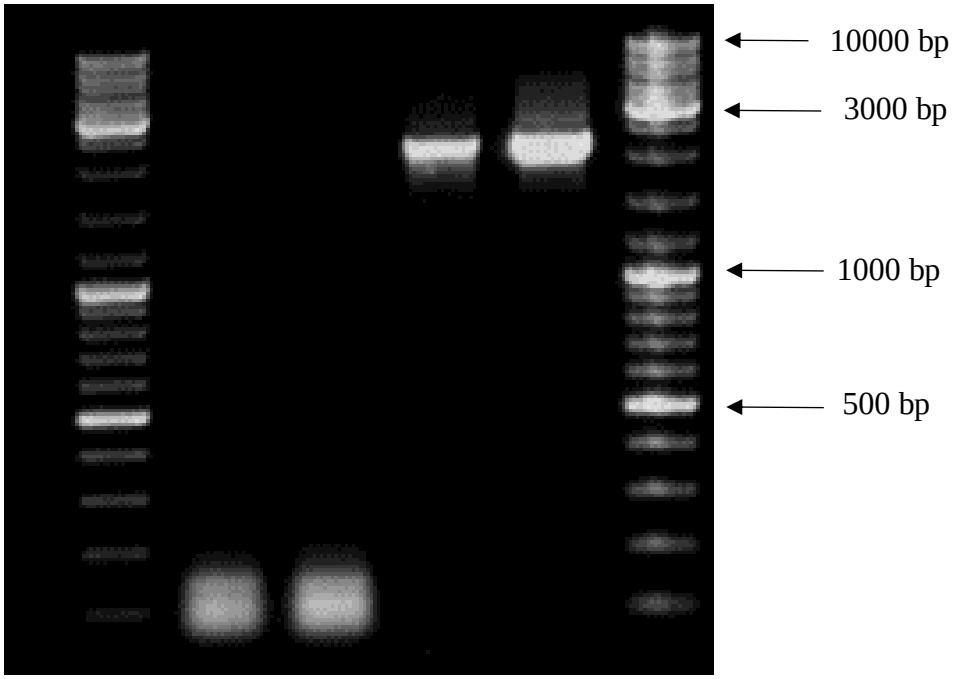


Figure 23 – New diluted set of primers (right) compared with a set of diluted primers that has been thawed and refreezed (left).

The reason for the behaviour of the BDFL-F and DRSL-R primers for the amplification of the *Wx-D1* gene is unknown because in general primers are very stable and they are not susceptible to the thawing and refreezing process.

4.1 Sequencing and BLAST alignment results

In total 37 mutant samples were sequenced of which 23 for the "T-WW-Kena" population (15 for *Wx-B1* and 8 for *Wx-D1*) and 14 for the "T-WW-Gaja" population (8 for *Wx-B1* and 6 for *Wx-D1*). In order to align the mutants with the wild type samples, the *Wx-B1* and *Wx-D1* genes of "Kena DS" and "Gaja DS" were sequenced. The next figure (Figure 24) shows the result of the electropherogram Sanger sequencing of the "Kena DS" wild type *Wx-B1* gene.

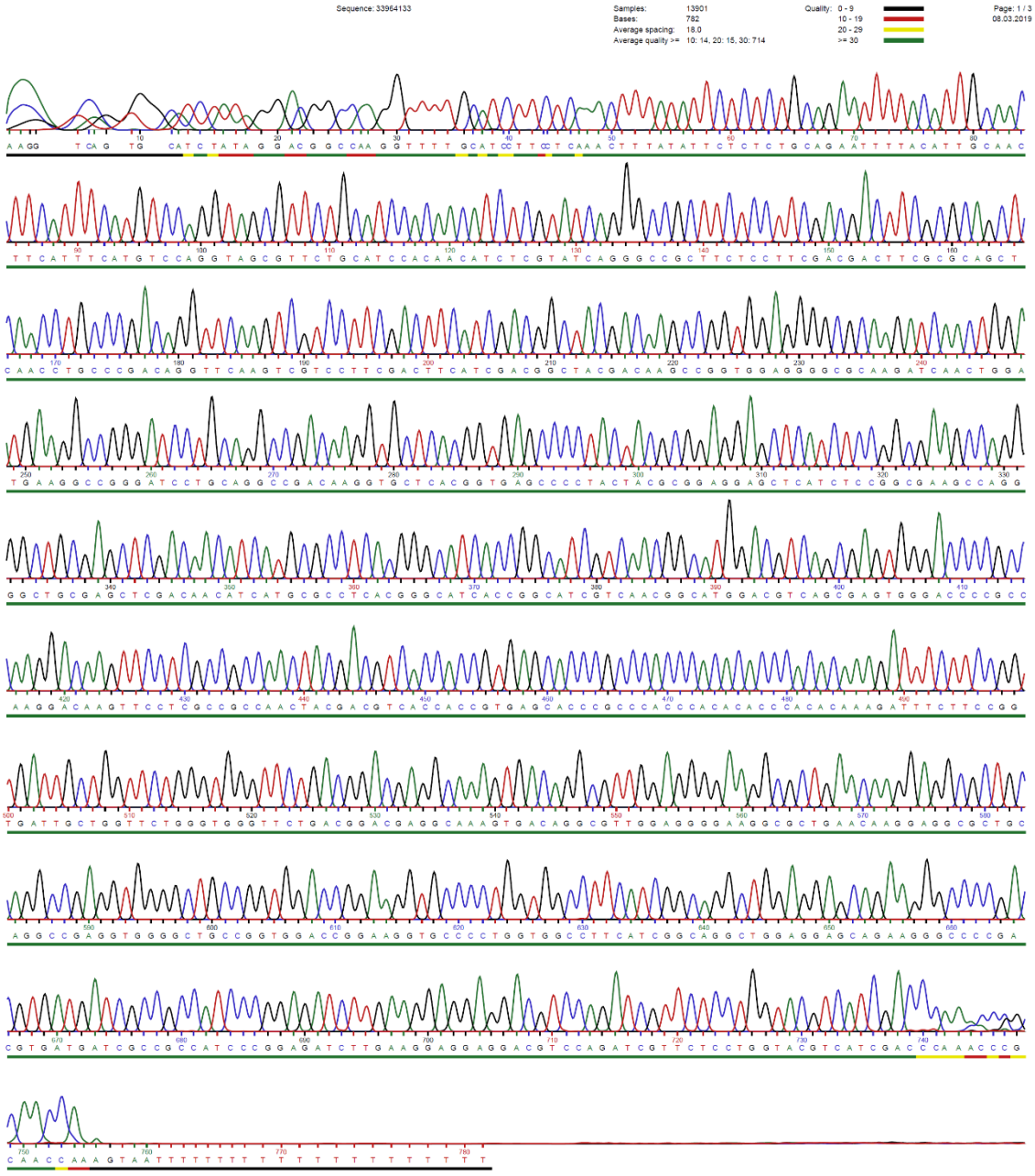


Figure 24 – Electropherogram of the "Kena DS" wild type *Wx-B1* gene.

The electropherogram gives some indication of the quality of the DNA sequenced. Each nucleotide is indicated with a different colour: green for adenine, red for thymine, black for guanine and blue for cytosine. The quality and the accuracy of the sequence is indicated with a coloured line placed under the nucleotides sequence. As it is shown in electropherogram (Figure 23), the beginning and the ending of the sequence appears to be not accurate. This is normal, and it depends on the sequencing process. All the wrong alignment obtained at the beginning and/or at the end of the BLAST sequence alignment are not mutations and therefore and not considered.

The BLAST alignment between the wild type *Wx-B1* "Kena DS" gene and the *T. aestivum* L. *Wx-B1* wild type gene (accession n°: LC379880) is shown in the next figure (Figure 25).

Triticum aestivum Wx gene for waxy protein, complete cds, allele: Wx-B1c#1

Sequence ID: gi|1377224452|LC379880.1 **Length:** 2817 **Number of Matches:** 1

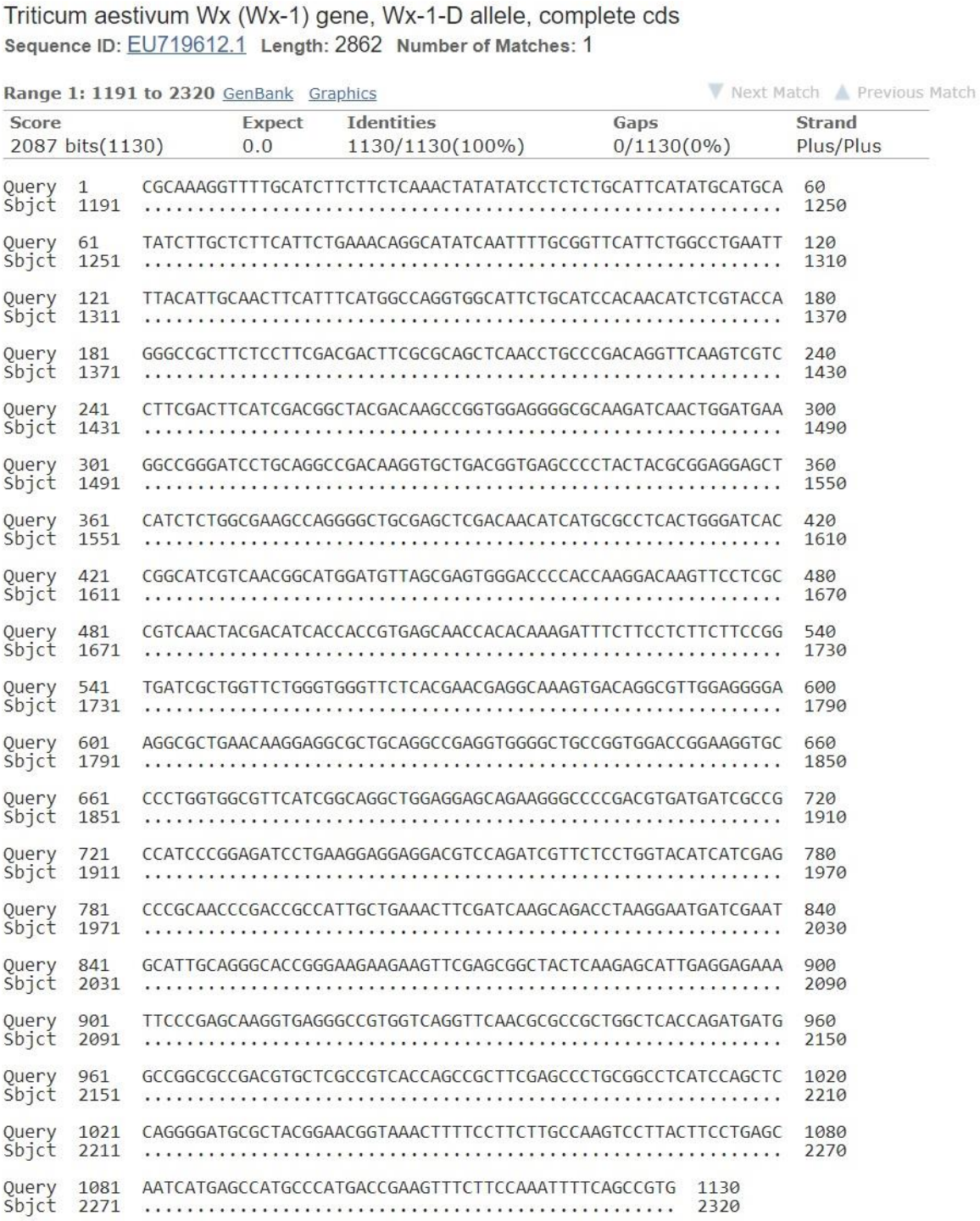
Range 1: 1186 to 1925 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1347 bits(729)	0.0	739/743(99%)	3/743(0%)	Plus/Plus

```
Query  1     CATCTATAGGACGGCCAAGGTTTTGCATCCTTCCTCAAACTTTATATTCTCTCTGCAGAA  60
Sbjct  1186  ............................-..-............................  1243

Query  61    TTTTACATTGCAACTTCATTTCATGTCCAGGTAGCGTTCTGCATCCACAACATCTCGTAT  120
Sbjct  1244  ............................................................  1303

Query  121   CAGGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCCGACAGGTTCAAGTCG  180
Sbjct  1304  ............................................................  1363

Query  181   TCCTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATG  240
Sbjct  1364  ............................................................  1423

Query  241   AAGGCCGGGATCCTGCAGGCCGACAAGGTGCTCACGGTGAGCCCCTACTACGCGGAGGAG  300
Sbjct  1424  ............................................................  1483

Query  301   CTCATCTCCGGCGAAGCCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCACGGGCATC  360
Sbjct  1484  ............................................................  1543

Query  361   ACCGGCATCGTCAACGGCATGGACGTCAGCGAGTGGGACCCCGCCAAGGACAAGTTCCTC  420
Sbjct  1544  ............................................................  1603

Query  421   GCCGCCAACTACGACGTCACCACCGTGAGCACCCGCCCACCCACACACCCACACAAAGAT  480
Sbjct  1604  ............................................................  1663

Query  481   TTCTTCCGGTGATTGCTGGTTCTGGGTGGGTTCTGACGGACGAGGCAAAGTGACAGGCGT  540
Sbjct  1664  ............................................................  1723

Query  541   TGGAGGGGAAGGCGCTGAACAAGGAGGCGCTGCAGGCCGAGGTGGGGCTGCCGGTGGACC  600
Sbjct  1724  ............................................................  1783

Query  601   GGAAGGTGCCCCTGGTGGCCTTCATCGGCAGGCTGGAGGAGCAGAAGGGCCCCGACGTGA  660
Sbjct  1784  ............................................................  1843

Query  661   TGATCGCCGCCATCCCGGAGATCTTGAAGGAGGAGGACGTCCAGATCGTTCTCCTGGTAC  720
Sbjct  1844  ............................................................  1903

Query  721   GTCATCGACCCAAACCCGCAACC  743
Sbjct  1904  ..........C....-......  1925
```

Figure 25 – BLAST alignment between the "Kena DS" *Wx-B1* wild type gene and the Triticum aestivum L. Wx-B1 wild type gene.

The BLAST alignment between the wild type *Wx-D1* "Kena DS" gene and the *T. aestivum* L. *Wx-D1* wild type gene (accession n°: EU719612) is shown in the next figure (Figure 26).



Triticum aestivum Wx (Wx-1) gene, Wx-1-D allele, complete cds

Sequence ID: EU719612.1 **Length:** 2862 **Number of Matches:** 1

Range 1: 1191 to 2320 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
2087 bits(1130)	0.0	1130/1130(100%)	0/1130(0%)	Plus/Plus

```
Query  1     CGCAAAGGTTTTGCATCTTCTTCTCAAACTATATATCCTCTCTGCATTCATATGCATGCA  60
Sbjct  1191  ............................................................  1250

Query  61    TATCTTGCTCTTCATTCTGAAACAGGCATATCAATTTTGCGGTTCATTCTGGCCTGAATT  120
Sbjct  1251  ............................................................  1310

Query  121   TTACATTGCAACTTCATTTCATGGCCAGGTGGCATTCTGCATCCACAACATCTCGTACCA  180
Sbjct  1311  ............................................................  1370

Query  181   GGGCCGCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCCGACAGGTTCAAGTCGTC  240
Sbjct  1371  ............................................................  1430

Query  241   CTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAA  300
Sbjct  1431  ............................................................  1490

Query  301   GGCCGGGATCCTGCAGGCCGACAAGGTGCTGACGGTGAGCCCCTACTACGCGGAGGAGCT  360
Sbjct  1491  ............................................................  1550

Query  361   CATCTCTGGCGAAGCCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCACTGGGATCAC  420
Sbjct  1551  ............................................................  1610

Query  421   CGGCATCGTCAACGGCATGGATGTTAGCGAGTGGGACCCCACCAAGGACAAGTTCCTCGC  480
Sbjct  1611  ............................................................  1670

Query  481   CGTCAACTACGACATCACCACCGTGAGCAACCACACAAAGATTTCTTCCTCTTCTTCCGG  540
Sbjct  1671  ............................................................  1730

Query  541   TGATCGCTGGTTCTGGGTGGGTTCTCACGAACGAGGCAAAGTGACAGGCGTTGGAGGGGA  600
Sbjct  1731  ............................................................  1790

Query  601   AGGCGCTGAACAAGGAGGCGCTGCAGGCCGAGGTGGGGCTGCCGGTGGACCGGAAGGTGC  660
Sbjct  1791  ............................................................  1850

Query  661   CCCTGGTGGCGTTCATCGGCAGGCTGGAGGAGCAGAAGGGCCCCGACGTGATGATCGCCG  720
Sbjct  1851  ............................................................  1910

Query  721   CCATCCCGGAGATCCTGAAGGAGGAGGACGTCCAGATCGTTCTCCTGGTACATCATCGAG  780
Sbjct  1911  ............................................................  1970

Query  781   CCCGCAACCCGACCGCCATTGCTGAAACTTCGATCAAGCAGACCTAAGGAATGATCGAAT  840
Sbjct  1971  ............................................................  2030

Query  841   GCATTGCAGGGCACCGGGAAGAAGAAGTTCGAGCGGCTACTCAAGAGCATTGAGGAGAAA  900
Sbjct  2031  ............................................................  2090

Query  901   TTCCCGAGCAAGGTGAGGGCCGTGGTCAGGTTCAACGCGCCGCTGGCTCACCAGATGATG  960
Sbjct  2091  ............................................................  2150

Query  961   GCCGGCGCCGACGTGCTCGCCGTCACCAGCCGCTTCGAGCCCTGCGGCCTCATCCAGCTC  1020
Sbjct  2151  ............................................................  2210

Query  1021  CAGGGGATGCGCTACGGAACGGTAAACTTTTCCTTCTTGCCAAGTCCTTACTTCCTGAGC  1080
Sbjct  2211  ............................................................  2270

Query  1081  AATCATGAGCCATGCCCATGACCGAAGTTTCTTCCAAATTTTCAGCCGTG  1130
Sbjct  2271  ..................................................  2320
```

Figure 26 - BLAST alignment between the "Kena DS" Wx-D1 wild type gene and the Triticum aestivum L. *Wx-D1* wild type gene.

From the results of the previous two BLAST alignments (Figures 24 and 25), it is possible to see that the wild type winter wheat cultivars "Kena DS" and "Gaja DS" carry the same *Wx-B1* and *Wx-D1* genes of *T. aestivum*. The alignment of the wild type "Kena DS" *Wx-D1* gene shows 100% identities with the *T. aestivum* L. gene. The alignment of the wild type "Kena DS" *Wx-B1* gene shows an alignment of 99% with the *T. aestivum* L. gene. As explained above, the gaps and the single nucleotide mutation at the beginning and the end of the sequence alignment are not considered because they are instrument errors.

The same BLAST alignment was carried out between the "Gaja DS" *Wx-B1* and *Wx-D1* genes and the *T. aestivum* L. gene. Also in this case, the wild type cultivar "Gaja DS" carries the same two *waxy* genes as *T. aestivum* L.

All the BLAST alignments that were carried out between the wild type "Gaja DS" and "Kena DS" *waxy* genes and the "T-WW-Gaja" and "T-WW-Kena" TILLING populations are reported in table 5. The table reports the alignments and gaps percentages.

Table 5 – Results of the alignments between the wild type genes *Wx-B1* and *Wx-D1* and the TILLING population genes. All the samples have 0% gaps and 99-100% identities. Most of the TILLING population carries the wild type gene *Wx-B1* and *Wx-D1*. In the two samples marked in red a mutation was found.

Sample ID *Wx-B1*	**Identities**	**Gaps**	**Sample ID *Wx-D1***	**Identities**	**Gaps**
MK38	100%	0%	MK38	100%	0%
MK114	100%	0%	MK114	100%	0%
MK283	100%	0%	MK283	99%	0%
MK464	100%	0%	MK464	100%	0%
MK480	100%	0%			
MK44	100%	0%			
MK59	100%	0%	MK59	100%	0%
MK82	100%	0%			
MK87	100%	0%	MK87	100%	0%
MK102	100%	0%			
MK264	100%	0%			
MK276	100%	0%	MK276	100%	0%
MK335	100%	0%			
MK426	100%	0%	MK426	100%	0%
MK323	100%	0%			
MG512	100%	0%	MG512	100%	0%
MG520	100%	0%	MG520	100%	0%
MG522	100%	0%			

MG523	100%	0%	MG523	100%	0%
MG532	99%	0%	MG532	100%	0%
MG537	100%	0%	MG537	100%	0%
MG564	100%	0%	MG564	100%	0%
MG569	100%	0%			

From the results of the alignments (Table 5), only two samples were identified to carry a mutation: the mutant MK283 (*Wx-D1* gene) and the mutant MG532 (*Wx-B1* gene). The alignment between the mutant sample MK283 and the wild type allele *Wx-D1* of the winter wheat cultivar "Kena DS" is shown in the next figure (Figure 27).

Figure 27 - Alignment of the sample MK283 with the wild type allele *Wx-D1* "Kena DS".

The area marked in red (Figure 27) shows that a single nucleotide substitution has occurred (G1805A). The alignment of the same but translated sequences are shown in the next figure (Figure 28).

Range 1: 1 to 291 Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
588 bits(1517)	0.0	Compositional matrix adjust.	288/291(99%)	289/291(99%)	1/291(0%)

```
Query  7    HILLFILRHINFAVHSGLNLHCNFIMAVAFCIHNISYQGRFSFDDFAQLNLPDRFKSSFD  66
Sbjct  1    ............................................................  60

Query  67   FIDGYDKPVEGRKINWMKAGILQADKVLTVSPYYAEELISGEARGCELDNIMRLTGITGI  126
Sbjct  61   ............................................................  120

Query  127  VNGMDVSEWDPTKDKFLAVNYDITTALEGKALNKEALQAEVGLPVDRKVPLVAFIGRLEE  186
Sbjct  121  ..............................K.............................  180

Query  187  QKGPDVMIAAIPEILKEEDVQIVLLGTGKKKFERLLKSIEEKFPSKVRAVVRFNAPLAHQ  246
Sbjct  181  ............................................................  240
```

Figure 28 – Alignment of the amino acids sequence between the mutant MK283 and the wild type allele *Wx-D1*.

The point mutation is a missense mutation that has changed the amino acids sequence of the mutant MK283 (E380K). The translated sequences show that in the area marked in red the wild type "Kena DS" carries the amino acid glutamic-acid (E) while the mutant MK283 carries the amino acid lysine (K). Glutamic-acid is an acidic polar amino acid while lysine is a basic polar amino acid. The next figure (Figure 29) shows the alignment between the complete *T. aestivum L. Wx-D1* CDS (coding sequence) (accession n°: EU719612), the wild type "Kena DS" *Wx-D1* allele and the mutant MK283 *Wx-D1* allele. It is very important to understand if the mutation occurs in the coding sequence or if it is part of the non-translated regions. In the former case the mutation may affect the final protein; in the latter, the chances that the mutation affects the final protein are very low.

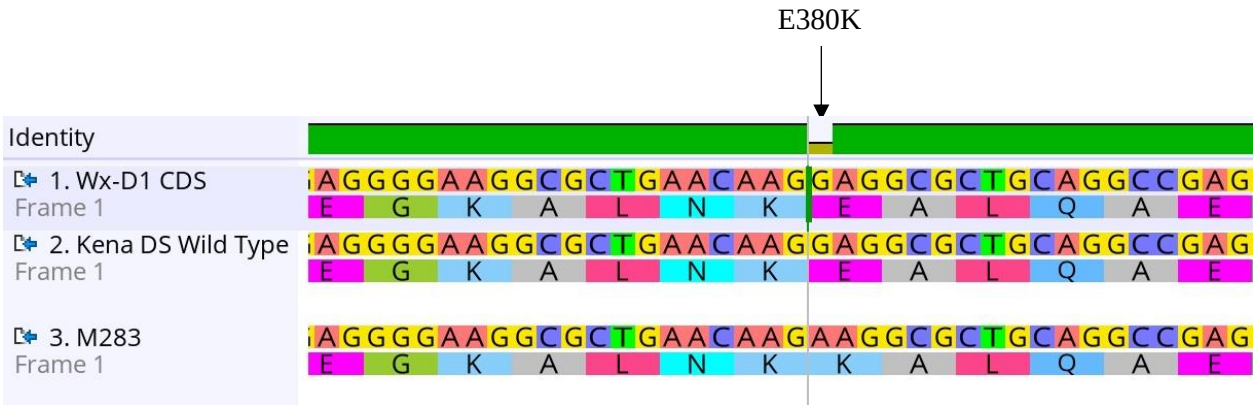


Figure 29 – Alignment between the *T. aestivum* L. *Wx-D1* CDS, the wild type "Kena DS" *Wx-D1* allele and the mutant MK283.

The alignment result (Figure 29) shows that the point mutation has occurred in the coding sequence. The next figure (Figure 30) highlight the mutation in the whole *Wx-D1* gene and it shows that the mutation has occurred in the 7th exon of the total 11. The *T. aestivum* L. gene and the wild type "Kena DS" gene share the same amino acids sequence, while in the mutant MK283 the single nucleotide substitution has changed the translated codon in a different amino acid (E380K). This mutation could affect the function of the GBSS protein and, as a consequence, it could affect the amylose content in wheat starch. In other to understand how and if the GBSS protein function has changed, further investigations need to be carried out.

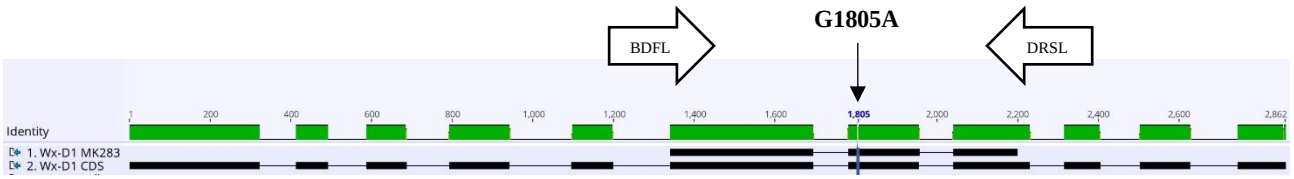


Figure 30 – *Wx-D1* gene. The single nucleotide substitution has occurred in the 7th exon.

The alignment between the mutant sample MG532 and the wild type gene *Wx-B1* of the winter wheat cultivar "Gaja DS" is shown in the following figure (Figure 31).

Range 1: 1 to 736 Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1349 bits(730)	0.0	735/737(99%)	1/737(0%)	Plus/Plus

```
Query  6    ATAGGACGGCCAAGGTTTTGCATCTTCTCAAACTTTATATTCTCTCTGCAGAATTTTACA  65
Sbjct  1    ............................................................  60

Query  66   TTGCAACTTCATTTCATGTCCAGGTAGCGTTCTGCATCCACAACATCTCGTATCAGGGCC  125
Sbjct  61   ............................................................  120

Query  126  GCTTCTCCTTCGACGACTTCGCGCAGCTCAACCTGCCCGACAGGTTCAAGTCGTCCTTCG  185
Sbjct  121  ............................................................  180

Query  186  ACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGCGCAAGATCAACTGGATGAAGGCCG  245
Sbjct  181  ............................................................  240

Query  246  GGATCCTGCAGGCCGACAAGGTGCTCACGGTGAGCCCCTACTACGCGGAGGAGCTCATCT  305
Sbjct  241  ............................................................  300

Query  306  CCGGCGAAGCCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCACGGGCATCACCGGCA  365
Sbjct  301  ............................................................  360

Query  366  TCGTCAACGGCATGGACGTCAGCGAGTGGGACCCCGCCAAGGACAAGTTCCTCGCCGCCA  425
Sbjct  361  ............................................................  420

Query  426  ACTACGACGTCACCACCGTGAGCACCCGCCCACCCACACACCCACACAAAGATTTCTTCC  485
Sbjct  421  ............................................................  480

Query  486  GGTGATTGCTGGTTCTGGGTGGGTTCTGACGGACGAGGCAAAGTGACAGGCGTTGGAGGG  545
Sbjct  481  ............................................................  540

Query  546  GAAGGCGCTGAACAAGGAGGCGCTGCAGGCCGAGGTGGGGCTGCCGGTGGACCGGAAGGT  605
Sbjct  541  ..........................................T.................  600

Query  606  GCCCCTGGTGGCCTTCATCGGCAGGCTGGAGGAGCAGAAGGGCCCCGACGTGATGATCGC  665
Sbjct  601  ............................................................  660

Query  666  CGCCATCCCGGAGATCTTGAAGGAGGAGGACGTCCAGATCGTTCTCCTGGTACGTCATCG  725
Sbjct  661  ............................................................  720

Query  726  ACCCAACCCCGCAACCA  742
Sbjct  721  .........-.......  736
```

Figure 31 - Alignment of the sample MG532 with the wild type allele *Wx-B1* "Gaja DS".

The area marked in red (Figure 31) shows that also in this case a single nucleotide substitution has occurred (C1771T). The same aligned but translated sequences are shown in the next figure (Figure 32).

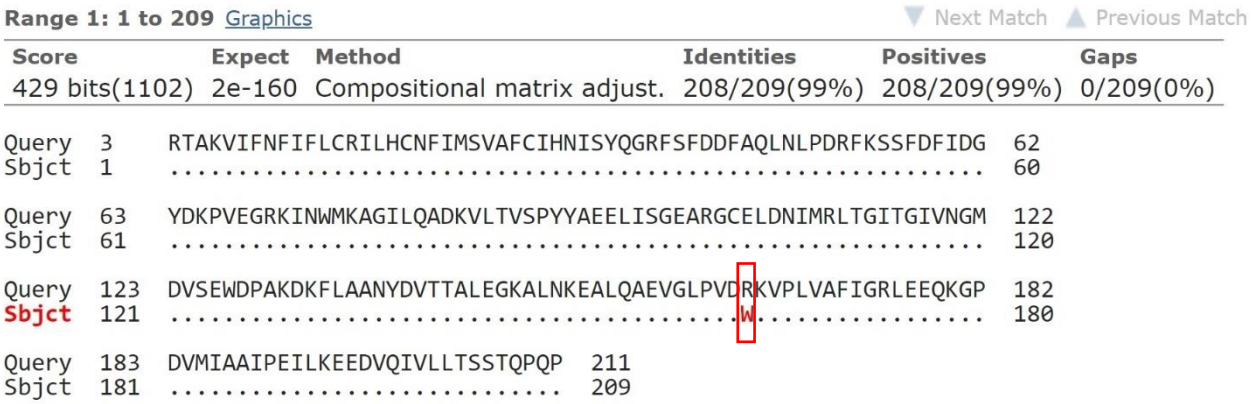


Figure 32 - Alignment of the amino acids sequence between the mutant MG-532 and the wild type allele *Wx-B1* of "Gaja DS".

Also in this case the point mutation is a missense mutation that has changed the amino acids sequence (R393W). The wild type "Gaja DS" *Wx-B1* allele carries the amino acid arginine (R) while the mutant MG532 carries the amino acid tryptophan (W). Arginine is a basic polar amino acid while tryptophan is an aromatic polar amino acid. Also in this case, it is very important to determine if the mutation occurs in the coding sequence or in the non-translated regions of the *waxy* gene. If the mutation occurs in the non-translated regions of the gene, there are low chances that the mutation affects the final protein. If the mutation occurs in the coding sequence, the final protein encoded by the gene may be affected. The alignment between the complete *T. aestivum* L. *Wx-B1* CDS (accession n°: BBD75660), the wild type "Gaja DS" *Wx-D1* allele and the mutant MG532 *Wx-D1* allele is showed in the next figure (Figure 33).

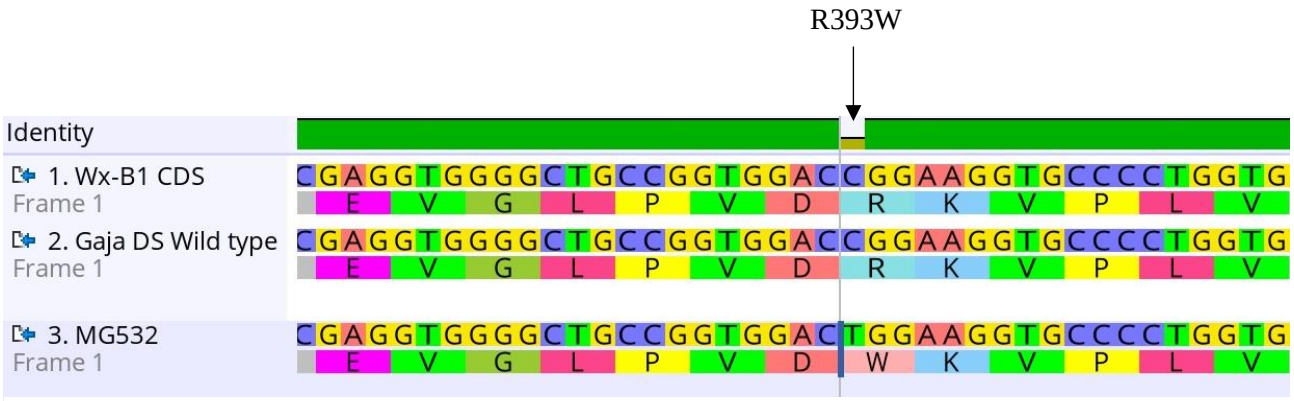


Figure 33 - Alignment between the *T. aestivum* L. *Wx-B1* CDS, the wild type "Kena DS" *Wx-B1* allele and the mutant MG532.

The alignments result (Figure 33) shows that the point mutation has also occurred in this case in the coding sequence. The mutation in the *Wx-B1* gene is pointed out in the next figure (Figure 34). Also in this case it has occurred in the 7th exon of the total 11. The *T. aestivum* L. gene and the wild type "Gaja DS" gene share the same amino acids sequence, while in the mutant MG532 the point mutation has changed the translated codon in a different amino acid (R393W). This mutation could also affect the function of the GBSS protein. In other to understand how and if the GBSS protein function has changed, further investigations need to be carried out.

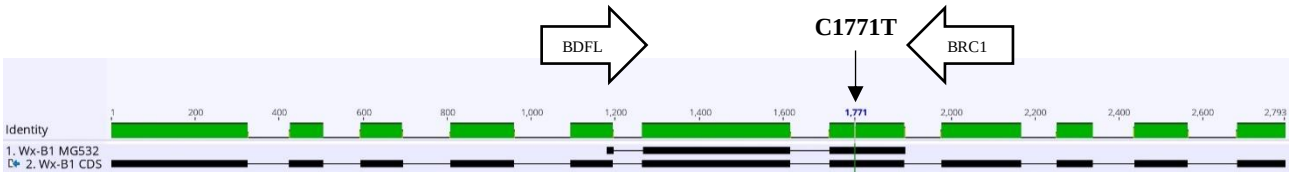


Figure 34 – *Wx-B1* gene. Also in this case the mutation has occurred in the 7th exon.

The mutation density of the different TILLING populations screened are reported in the following table (Table 6).

Table 6 – Mutation density of the TILLING populations in the *Wx-B1* and *Wx-D1* genes.

Amplicon	**Size (bp)**	**Total number of screened lines**	**Mutations**	**Mutations density (mutation per kb)**
Kena DS Mutants *Wx-B1*	748	15	No mutations found	/
Kena DS Mutants *Wx-D1*	1134	8	1	9.1
Gaja DS Mutants *Wx-B1*	748	8	1	6
Gaja DS Mutants *Wx-D1*	1134	6	No mutations found	/

The table gives some statistical indications of the identified mutations in the screened population. In the mutants "T-WW-Kena" *Wx-B1* gene and "T-WW-Gaja" *Wx-D1* gene, no mutations were found therefore, it is not possible to calculate the mutation density. In the mutants "T-WW-Kena" *Wx-D1* gene was found one mutation. The mutations density estimated in this population is 1 mutation per 9.1 kb screened. In the mutants "T-WW-Gaja" *Wx-B1* gene was found one mutation as well. The

mutation density estimated is 1 mutation per 6 kb screened. Both the mutations identified were missense mutations.

Li *et al.* (2017) identified novel alleles induced by EMS in key genes of wheat quality. In this work the authors treated the wheat variety Longfumai 17 was with EMS and they produced a M_2 population that included 1122 plants. The authors analysed a total length of 3906.80 kb and the mutation density was of 1/244.17 kb. The mutations obtained led to changes in amino acids and the 18.75% of the mutations were missense mutations (Li *et al.*, 2017).

Botticella *et al.* (2011) used high resolution melting analysis for the detection of mutations in wheat *SbeIIa* genes induced by EMS. In this work were found 53 novel alleles for *SBEIIa-A* of which 36 were missense, 14 in *SBEIIa-B* of which 10 were missense and 50 in *SBEIIa-D* of which 34 were missense. The overall estimated mutation density was of 1 mutation per 40 kb screened (Botticella *et al.*, 2011).

Dong *et al.* (2009) used a modified TILLING method on a soft wheat cultivar, QAL200, and a hard wheat cultivar, Ventura. Both the cultivars were treated with EMS. In 2348 plants were screened the waxy genes *Wx-A1* and *Wx-D1* and 119 mutations were found (plus two duplicated mutations). Among all the mutations identified 54.6% were nonsilent including truncation and missense mutations. The mutation density was 1 in 20-30 kb (Dong *et al.*, 2009).

This study and the studies reported above show that TILLING is a powerful technique for the creation of variations in the wheat genome.

CONCLUSIONS

The aim of this work was to identify novel waxy alleles in a winter wheat TILLING population. TILLING is a powerful tool for the creation of variations in the genome, and although this process is purely random and it is not possible to predict in advance the location of the DNA lesions caused by the mutagens, many new wheat lines have been produced with this approach.

1. The first objective of this study was to identify novel alleles in *waxy* genes *Wx-D1* and *Wx-B1* in two winter wheat TILLING populations named "T-WW-Gaja" and "T-WW-Kena". From the 37 mutant samples sequenced, two mutations were found in two different samples:
    - A mutation was found in the mutant MK283 (G1805A) in the *Wx-D1* gene;
    - Another mutation was found in the mutant MG532 (C1771T) in the *Wx-B1* gene.
2. The second objective of this work was to verify if the mutations occurred in the coding sequences. After aligning the mutant samples with their respective complete coding sequences (*T. aestivum* L. *Wx-D1* CDS accession n°: EU719612 and *T. aestivum* L. *Wx-B1* CDS accession n°: BBD75660) it was discovered that both the mutations are located in the coding sequence in the 7th exon of the total 11.
3. The third objective of this work was to verify if the mutations discovered change the translated sequences:
    - In the mutant MK283 the mutation obtained is a missense mutation that has changed the translated codon from glutamic-acid to lysine (E380K) in the *Wx-D1* gene;
    - In the mutant MG532 the mutation obtained is also a missense mutation that has changed the translated codon from arginine to tryptophan (R393W) in the *Wx-B1* gene.

The mutations pointed above could affect the GBSS. The GBSS is responsible for the production of amylose in the starchy endosperm of the wheat grain. The content of amylose in wheat starch is an essential characteristic in the food production industry because it affects many different starch properties such as gelatinisation, pasting and retrogradation. The mutations obtained in this work are not yet validated and further analyses need to be carried out in order to understand how and if the discovered mutations affect the GBSS and the amylose content in the mutants MK283 and MG532.

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