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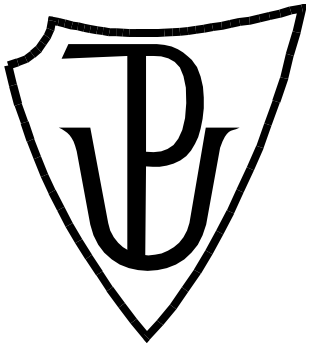

Pochopení úlohy TGW6 při určování velikosti zrn u ječmene (*Hordeum vulgare* L.)

DIPLOMOVÁ PRÁCE

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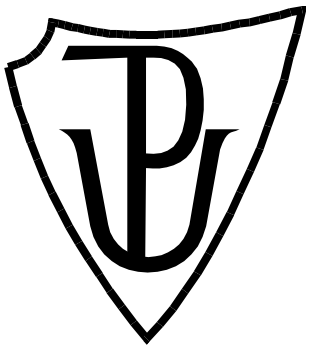

Understanding the role of TGW6 in the determination of grain size in barley (*Hordeum vulgare* L.)

MASTER THESIS

Author: Bc. Tereza Tomíčková

Study Programme: Biology

Field of study: Experimental Plant Biology

Form of Study: Full-time

Supervisor: Véronique Bergougnoux-Fojtík, Ph.D.

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Bibliographical identification

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Title of thesis	Understanding the role of TGW6 in the determination of grain size in barley (*Hordeum vulgare* L.)
Type of thesis	Master
Department	CATRIN-CRH "Plant genetics and engineering"
Supervisor	Véronique Bergougnoux-Fojtik, Ph.D.
The year of presentation	2021
Abstract	The aim of the thesis is to investigate genes with a potential role in controlling grain size in barley (*Hordeum vulgare* L.). In the theoretical part, the student will describe the potential function of THOUSAND GRAIN WEIGHT 6 (TGW6), the history and development of crop breeding, and the CRISPR-Cas9 technology. The experimental part of the thesis will focus on the barley Hv*TGW6* gene: its identification based on sequence homology, natural variation, gene expression and effect of knock-out using CRISPR-Cas9 technology on the grain size. Methods and tools: searching in barley database for the rice Os*TGW6* orthologue, building phylogenetic tree using MEGA-X, design and preparation of gRNA to produce barley knockout via CRISPR-Cas9 methodology in barley, study of polymorphism, and gene expression by qPCR
Keywords	Barley; CRISPR; grain size; Thousand grain weight6 (TGW6)
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Abstrakt	Cílem této diplomové práce je studovat geny s potenciální rolí při regulaci velikosti zrna u ječmene *(Hordeum vulgare* L.). V teoretické části je popsána předpokládaná funkce TGW6, historie a vývoj šlechtění plodin a CRISPR-Cas9 technologie. Experimentální část práce je zaměřena na *THOUSAND GRAIN WEIGHT 6* (*TGW6*): identifikace genu na základě sekvenční homologie, analýza genové exprese, příprava knock-out rostlin v tomto genu pomocí CRISPR-Cas9 technologie a identifikace přirozeně se vyskytujících polymorfismů TGW6. Metody: vyhledávání ortologů OsTGW6 v ječmeni pomocí databází, vytvoření kladogramu, navržení gRNA pro cílený knock-out potenciálních genů TGW6 v ječmeni, studium polymorfismů a analýza genové exprese pomocí qPCR.
Klíčová slova	Ječmen, CRISPR-Cas9, *THOUSAND GRAIN WEIGHT 6* (*TGW6*)
Počet stran	88
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Jazyk	Anglický

I hereby declare that this thesis has been prepared by myself during my Master degree under the leadership of my supervisor Véronique Bergougnoux-Fojtík, Ph. D. All the sources are listed in the References section.

In Olomouc

I would like to thank Véronique Bergougnoux-Fojtík, Ph.D. for a great opportunity to work in her research group, for her dedication, patience and all the experiences I have gained under her supervision. Further, I would like to thank all the members of the Group of Plant Genetic and Engineering, CATRIN-CRH for their willingness to help, especially Bc. Vendula Svobodová and Mgr. Mária Majeská Čudejková, Ph.D.

Contents

OBJECTIVES OF THE MASTER THESIS

The objectives of the experimental part of this master thesis were (1) to isolate and characterize *TGW6* orthologs in barley (*Hordeum vulgare* L.), (2) to analyse the expression of the candidate Hv*TGW6* genes in several plant organs of barley, (3) to identified natural variation in HvTGW6, (4) to perform a knock-out of the candidate Hv*TGW6* in barley using CRISP-Cas9 and *Agrobacterium*-mediated immature embryo transformation.

1 INTRODUCTION

Global agriculture is currently facing the difficult challenge to feed billions of people all around the world and at the same time to reduce negative impact on natural environment (Foley *et al*., 2011; Parfitt *et al*., 2010). Barley (*Hordeum vulgare* L.) belongs among the most abundant cereal in the world (Baik and Ullrich, 2008) which include maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum* spp.), sorghum (*Sorghum* sp.) and oat (*Avena sativa*). Altogether, they provide approximately two-thirds of the world food supply (FAO, 2020). Due to this fact, increasing grain yield has a potential in improving food security. The number of studies focusing on the discovery of new genes controlling yield in cereals and understanding their functions has increased in the last years *(*Garcia *et al., 2019;* Wang *et al., 2019;* Xu *et al.*, 2018; Zhang *et al.*, 2018).

Sharma *et al.* (2018) performed genome-wide association study on a nested association mapping population which contained 25 exotic barley genomes in cultivated barley genome background. They identified a hotspot located on chromosome 7 which showed association with almost all traits: grain area, grain length, grain width, grain roundness (GW/GL), grain number per ear and grain yield (seed weight per plot). Moreover, SNP markers associated with reduced thousand grain weight were found in the same spot. This region overlaps with two rice grain trait-related genes: a mitogen-activated protein kinase OsMAPK6 (Liu *et al*., 2015b) and an IAA-glucose hydrolase TGW6 (Ishimaru *et al.*, 2013).

Ishimaru *et al.* (2013) clarified that *tgw6* loss-of-function lead to the increase of yield due to longer endosperm and grain. The plants showed greater dry weight throughout grain development and significantly higher number of endosperm cell layers. The functional allele encodes a protein with an indole-3-acetic acid (IAA)-glucose hydrolase activity. The authors concluded that the functional TGW6 controls the endogenous concentration of IAA during grain development leading to the limited cell number and grain length.

In this thesis, several approaches were used in order to initiate the characterization of HvTGW6 in barley during grain development. Among other, CRISPR-Cas9 and *agrobacterium*-mediated immature embryo transformation was performed and T_0 knock-out plants in the candidates Hv*TGW6* were selected and will be used for further study.

2 LITERATURE REVIEW

2.1 Current situation

Global agriculture is currently facing the difficult challenge to feed billions of people all around the world, while simultaneously reducing negative impact on natural environment. People are dependent on agriculture not only as a source of human food (63 % of crop production) but also for animal feed (35 %) and bioenergy (3 %) (Foley *et al.*, 2011; Parfitt *et al.*, 2010). Human population expand and according to many predictions, the amount of food supply must be at least doubled in the next few years. The potential of the Green revolution based on finding crucial genes, expansion of cropland and, unbridled usage of pesticides and fertilizers of different kinds has already been exhausted (Foley *et al.*, 2011). There are many reasons resulting in stagnation of crop harvest and yield: climate change associated with abiotic stresses and biotic stresses (Raza *et al.*, 2019), "domestication bottleneck" (Dempewolf *et al.*, 2017), salinity caused by excessive fertilization (Foley *et al.*, 2005) or lack of cropland (Godfray *et al.*, 2010).

Human emissions of dangerous gases create the greenhouse effect leading to the rise of the average global temperatures. Increase in temperatures alone is stressful enough for crop plants due to negative effects on photosynthesis, but when it is associated with drought and water deficit plants are suffering. Furthermore, strong continuous fluctuations between drought and heavy rain and storm periods create flood and waterlogged land. However, global warming has also some positive effects such as longer growing season, possibility of growing new crops or increased productivity of plants from warmer areas. Nevertheless, crops which are more stress-resistant are necessary and plant scientists are working on their development (Raza *et al.*, 2019).

During domestication, plants were selected in order to become the most suitable crop for human use but during selection process, some potentially valuable traits have been lost. Therefore, the price for desirable characteristics is the "domestication bottleneck", i.e. the loss of genetic diversity. It means that nowadays plants have less allelic diversity compared to their wild progenitors or other wild

relatives. The interesting traits are still available in wild relatives and they are used for introduction to the modern cultivated varieties (Dempewolf *et al.*, 2017).

During the Green revolution, technologies have changed including global use of fertilizers which has been increased up to approximately 700 %. Our contemporary agriculture depends on overusing pesticides and fertilizers more than on anything else. Especially, nitrogen (N) is considered an inseparable part. Every year, around 1 403 106 MT of N is released to terrestrial ecosystem naturally and humans already release comparable number. But in 2050 it is believed that this amount will be almost doubled what could have significant environmental impact (Tilman *et al*., 2001).

Further, human activities change the hydrologic balance in the soil causing soil salinity. There are two types of salinity: natural salinity which results from the salt accumulation over long time and human-induced salinity which is caused by irrigation and land clearing. While original perennial vegetation is adapted to drought and due to its developed roots is able to reach water deep below surface, annual crops are dependent on irrigation. Irrigation water leaks below the roots and causes water tables to rise leading to the releasing of the salt previously stored in subsoil. The salt moves up and increase topsoil salinity. When saline water reaches the surface, water evaporates but salts accumulate on the surface and form salt scald (Munns, 2005). This scenario is already happening. Indeed, in many regions, water quality is affected and hectares of land have become heavily salinized every year (Foley *et al.*, 2005).

The production of food to ensure food security of billions of people can be increased by two main ways. The first one is to increase quantity through expanding cropland but this is becoming more complicated as the agricultural area on Earth is limited (Godfray *et al.*, 2010). According to the Food and Agriculture Organization (FAO), cropland in 2020 occupies 38 % of the global land surface. About one-third of this is used as cropland, while the remaining two-thirds consist of meadows and pastures what makes it the largest terrestrial biomes on Earth. The second one includes improvement in quality of crop plants on existing area. As mentioned, during the Green revolution people discovered the benefits of fertilization and interesting genes in connection with production. However, the potential of the Green revolution is not sufficient to feed the ever-growing worldwide population, expected to exceed 10 billion in the close future. Therefore, it is necessary to consider other options.

In the past decades, plant breeders and scientists have been already working on breeding crops for higher yield (Godfray *et al.*, 2010) . Better understanding of tolerance to biotic and abiotic stresses, molecular and cellular development pathways, identification of genes related to higher yields or molecular markers and quantitative trait loci are necessary to reach the desired progresses in agriculture. Plants introduced with improved agronomic traits in the last years proved that this is an effective strategy. New technologies such as sequencing, targeted mutation breeding, and especially genome editing seem to be a valuable long-term solution (Nawaz and Chung, 2020).

With all this said, there are many possibilities how to increase amount of food without any genetic changes to the crop plants and without using advanced genetic technologies. The first one is to stop wasting food. According to the FAO (2015), around one third of the produced food is wasted, representing 1,3 billion tonnes of food every year (Fig 1A). Specifically, it is 30 % of cereals, 20 % of dairy products, 35 % of fish and seafood, 45 % of fruit and vegetables, 20 % of meat, 20 % of oilseed and pulses and 45 % of roots and tubes. From this, one third is wasted at the production level, another 24 % is lost during handling and storage, 4 % during processing and packaging, 12 % is wasted at distribution and market level. Finally, 35% of the food is lost by consumers what is more significant in developed countries then in developing ones. Regarding cereals which cover around two-thirds of the world food supply, famers lose around 30 % of yield each year (Fig 1B).

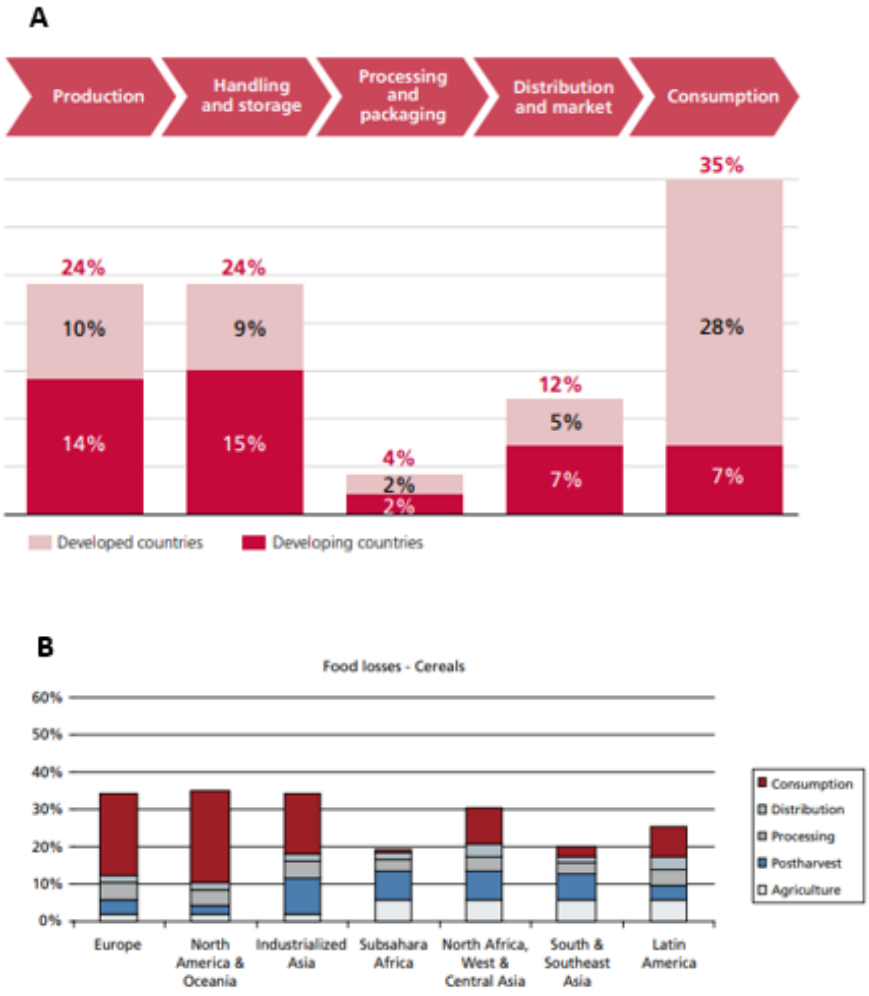

Figure 1. (A) Food wasting from production to consumption in developed and developing countries (source: copied from the Gender and food loss is sustainable food value chain, FAO, 2018). (B) Specific cereal losses from production to consumption in different world's regions. The data are expressed as percentage of food losses and waste of the edible parts of food products that were produced for human consumption. (source: copied from the Global food losses and food waste, FAO, 2011).

Altogether, wasting and improving food supply chain are possibilities how to slow down the ongoing problems. But this is just a short-term solution (Foley *et al.*, 2011). In connection to agriculture and feeding more and more people in the future, novel editing technologies, especially CRISPR-Cas, seem to be inevitable (Gao, 2018).

2.2 History of breeding technologies and strategies

Around 50 000 years ago, humans were hunter-gatherers and many years passed until they started to breed the first crop. This transition is called “domestication” and it is dated 10 000 years ago (Dockter and Hansson, 2015; Pankin and von Korff, 2017). Since then, humans kept trying to increase yield as much as possible. Before all else, humans used selection for desired traits based on phenotype of a plant, especially height, size of a spikes and grain yield. The disadvantage of this method is the reduction of both genetic variation and population size (Jonas and De Koning, 2013; Voltas *et al.*, 2002). The important milestones not only in agriculture were experiments done by Gregor Mendel (1856-1863) which resulted into laws of Mendelian inheritance, three decades after his original work, marking the modern age of genetics.

In the beginning of the 20th century, Thomas Morgan concluded that genes are carried on specific chromosomes. In the following years Mendelian-chromosome theory was accepted in scientific field (Vega and White, 2019). Since then, scientists started to realise that it is necessary to concentrate more on genotype, but most crop-breeding programs were still dependent on hybridization and selection from lines. However, except of hybridization there was an another way how to study genes called induced mutagenesis (Ohnoutková *et al.*, 2018). Concurrently, in physics, the electromagnetic radiation of wavelengths was described after the discovery of X-radiation in 1895 (Berejka *et al*., 2014). Around 1930 this two fields crossed in order to induce mutation in plants (Ohnoutková *et al.*,2018) and one decade later the first tests with chemical compounds on plants started. Both methods were used to obtain mutants and study genes related to higher yield productivity (Khlestkina and Shumny, 2016). However, they cause random modifications of the genome, affecting more than one gene at the same time. Therefore, the selection of a specific mutant is a long and laborious process (Khlestkina and Shumny, 2016).

As the population have been growing, in part due to progresses in medicine and longer life, the amount of food begun to be insufficient. In the 60’s, the interest of many organizations all around the world highlighted the question of food-population balance trying to deal with forthcoming food crisis. Fortunately, any

famine did not happen thanks to the significant increase in cereals yields due to development of dwarf wheat variety by Norman Borlaug. The entire period is known as “Green Revolution” dated from the 1940s–1960s (Borlaug, 1997). The rise in grain production was enormous. It took approximately 10 000 years to reach around 1 billion tons and in 2000, only after another 40 years, the production was doubled (Foley *et al*., 2005; Khush, 2001; Tilman *et al.*, 2001) due to the development of genetically modified varieties of cereals and improvement of agronomic technologies such as fertilization, irrigation and pesticides (Khush, 2001). Mutations which played the most important role were mostly single genes: for example, *semidwarf1* (*sdw1*) in barley or *reduced height-1* (*Rht*) in wheat changing gibberellin signalling. Premature stop codon in these alleles lead not only to the reduction of plant height but also caused an increase of grains number in spike and has an effect on disease resistance. Since then, the phenotype of cereals has changed: cereals are shorter, not so prone to stem lodging, able to tolerate nitrogen-based fertilizers and adapt to many environments. Moreover, knowledge about biology of plants in connection with productivity dramatically enlarged, as well as novel biotechnological tools which are supposed to increase production in the next years similarly to Green Revolution (Nadolska-Orczyk *et al.*, 2017).

Mutagenesis persisted until 1980s as the only tool for studying agricultural traits, before new strategies based on genome modification were developed. These novel, more sophisticated strategies included gene silencing or overexpression, molecular markers and marker-based genetics maps or molecular cloning. At the same time, genomics and transcriptomics enabled the identification of new genes and their functional analysis (Nadolska-Orczyk *et al.*, 2017). Selection of lines focused on traits such as resistance to abiotic and biotic stresses, short growth duration, adaptation to a wide range of environments, or grain quality. As said before, selection from so many lines is difficult and long-term (Khush, 2001). Therefore, application of molecular markers in 1983 was extremely important and have opened new avenues not only to plant breeding strategies. The tool was still usable mainly for monogenic or sometimes for oligogenic traits but most of the plant-breeding traits are polygenic (Khlestkina and Shumny, 2016). For those particular cases, another genomic-based selection method was developed by Meuwissen *et al.* (2001) which is based not on

phenotypic but genotypic selection leading to a prediction of complex traits. Thanks to molecular markers it is possible to define chromosome parts and then observe their effects on the phenotype. It is assumed that these effects should be the same in the whole population because they are closely bound to regions of the genome that affect such characters. These regions are termed quantitative trait loci (QTL) and their main characteristic is that they behave as major genes so it is easier to manipulate them (Nadolska-Orczyk *et al.*, 2017; Thomas, 2003). In other words, molecular markers are believed to be connected to specific QTL. It basically means that even if a sequence of desired polygenic trait is not known there is still a chance that there is a marker which appears always with the desired phenotype. Genomic selection works simultaneously with many markers whereas marker assisted selection uses only few of them. Genomic selection could be also used in order to choose the best lines to cross and to reduce heterozygosity in imbred lines. (Jonas and De Koning, 2013). In the 2000s, the new era of in-depth genome sequencing and the development of the next-generation sequencing have started. This tremendously empowered plant research (Reuter *et al.*, 2015) (Fig 2).

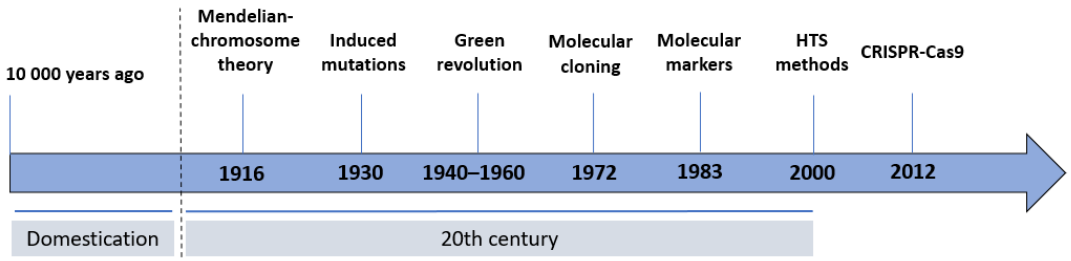

Figure 2. Timeline of important milestones leading to the modern plant breeding.

From the Green revolution till 2005 the production increased by 86% but nowadays it seems that its potential has been exhausted (Li *et al*., 2016; Tilman *et al.*, 2001). According to many recent publications, TALEN and CRISPR-Cas9 technologies have been successfully used in a plethora allopolyploid species including wheat, *Brassica oletacea* or barley, suggesting that modern editing technologies provide a huge opportunity for change in agriculture in the future (Nadolska-Orczyk *et al.*, 2017).

2.3 Barley (*Hordeum vulgare* L.)

Barley is a cereal from the family of *Poaceae* and the genus *Hordeum* (Ji *et al*., 2013). It belongs among the most abundant cereals in the world (Baik and Ullrich, 2008) which include maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum* spp.), sorghum (*Sorghum* sp.) and oat (*Avena sativa*). In numbers, barley represent almost 12 % of the cultivated cereal (Idehen *et al.*, 2017). Further, it is an interesting plant model for cereal research since it has a relatively small diploid genome compared to the tetraploid or hexaploid wheat genome. All known cultivated barleys are annuals, self-fertilizing and diploid (2*n* = 14) (MacGregor, 2003).

Barley has been one of the world´s earliest domesticated crop species, dated back thousand years BC (Baik and Ullrich, 2008; Idehen *et al.*, 2017). At that time, barley was an important source of human food worldwide. But in the 19th and 20th centuries, its popularity has decreased and it has been replaced by product of higher quality prepared from rice or wheat. Nowadays, barley harvested areas are spread basically all around the world, however, about three quarters of the total annual barley grain production is located in Europe (Friedt *et al.*, 2011) where it is mainly used for animal feeding (approximately 75 %), and malting (approximately 20 %). Just smaller quantities are used for direct human consumption (approximately 5 %) (Idehen *et al.*, 2017; Ji *et al.*, 2013; Sullivan *et al.*, 2013). However, for some cultures, especially in Asia and Africa, it has remained a major food source due to its wide adaptability to extreme environments, such as cold, drought, salinity or alkalinity, by which it outperforms other cereals (Idehen *et al.*, 2017; Ji *et al.*, 2013). Barley grain consists of about 10-20 % protein, 70 % starch, 2-3 % free lipids, 4-10 % β-glucan and 2,5% minerals (Czuchajowska *et al.*, 1998; Izydorczyk *et al.*, 2000; Quinde *et al.*, 2004). Further, barley has the highest concentration of polysaccharide β -glucans from all cereals (Shewry *et al.*, 2008; Sullivan *et al.*, 2013) and contains tocols as well. Both components are known to reduce LDL cholesterol through their antioxidant action (Baik and Ullrich, 2008) therefore, barley could soon catch more attention and become a cereal with health potential (Edelmann *et al.*, 2013).

Barley morphology is similar to all other cereals (Fig 3, Tab 1). It consists of stem which carry the head on its tip. The head is covered inside of the upper leaf

sheath and as the stem elongates, head appears. The last leaf which is closest to the head is called flag leaf. The head of barley, also called spike, consists of the spikelets and in each spikelet there is a floret. Barley can be either two-row or six-row type depending on number of spikelets. Barley has typical hair like structures called awns (Wrigley, 2017).

Table 1. Barley morphological terms (adapted from Lindsey *et al.*, 2017)

Term	Description
Anthern	Pollen-producing part of the flower
Awn	Hair like structures on the barleay spikes
Flag leaf	The last leaf to emerge before the head
Floret	Spikelets consist of florets which contain antherns and stigma
Spike	Barley head
Spikelet	Basic unit of barley flower
Tiller	A shoot originating from the main stem from th ecoleoptile node
1st, 2nd leaf	First and second leaf below the flag leaf

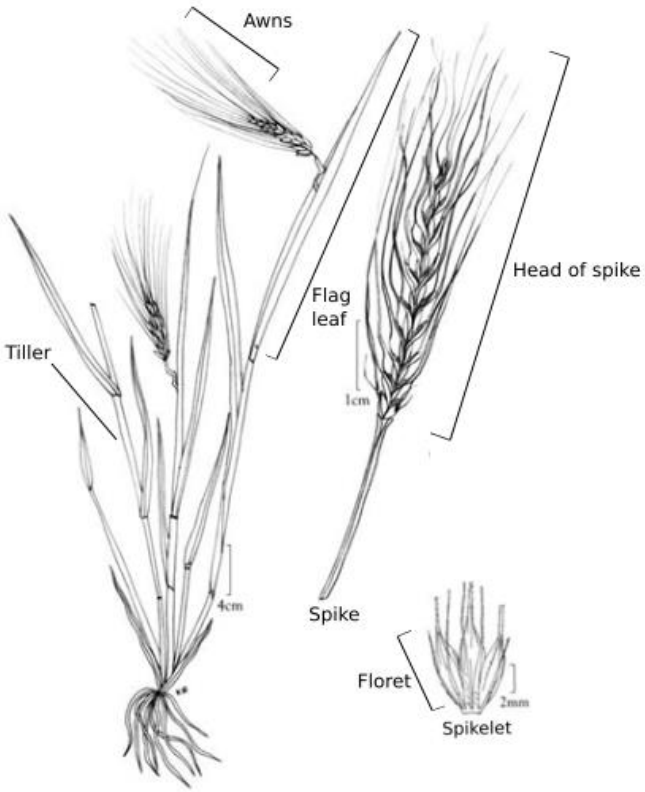

Figure 3. Barley (Hordeum vulgare L.) (adapted from Ibrahim and Peterson, 2015).

2.4 Grain yield

Crop yield is calculated from the number of harvested grains per square meter and individual grain weight both established at a similar timing, around the flowering period (Gambín and Borrás, 2010). However, grain yield is a complex genetic trait which is given by inflorescence number, grains per inflorescence, seed size or tiller number. Since the complexity of traits contributing to the grain yield is so wide, people are using parameters such as grain yield ($kg \cdot ha^{-1}$) or thousand grain weight (TGW) which represents weight (g) of 1 000 grains (Khyber, 2012). Study of these traits on genetic level is currently very difficult. The reason is that the traits are controlled by many genes which interact among each other and moreover are influenced by different environmental conditions (Sharma *et al.*, 2018). The number of seeds per plant is usually determined during the flowering period and it relies on the resource availability at that time. Moreover, weight of seeds is established during the early seed development corelating with the storage capacity of individual seed. Noticeably, development time of both components overlap. Seed size is heritable and conservative for a species and environment, and seed size affects fitness. The main storage organ of the seed is the endosperm: the bigger it is, the higher is the storage capacity (Berger, 2003). Importantly, seed number and weight are mostly negatively correlated, meaning that while one feature is increased, the second is decreased (Gambín and Borrás, 2010; Sadras, 2007).

It was found that during the domestication of barley from an ancestor (*Hordeum vulgare* ssp. *spontaneum*) many of the alleles have been lost. Therefore, backcrosses of modern barley cultivars with wild barley are an interesting source of new genetic diversity. Sharma *et al.* (2018) described fourteen QTL hotspots co-segregating with genes known to affect spike morphology or flowering time. According to their results, QTL hotspot on chromosome 7 seemed to be associated with traits related to grain development. In particular, this QTL contains two genes whose orthologues in rice were described to be involved in grain development. These are *OsTHOUSAND GRAIN WEIGHT6* (*OsTGW6*; (Ishimaru *et al.*, 2013) and *OsMAPK6* (Liu *et al.*, 2015b).

2.5 Endosperm development in cereals

Seed development (Fig 4) in higher flowering plants (including cereals) is initiated by double fertilisation for what two male gametes are needed. The first gamete fuses with the oosphere (egg) to produce the diploid zygote and the second gamete fuses with central cell nuclei in order to form the triploid nucleus which subsequently divides and form the endosperm (Lopes and Larkins, 1993). The central cell nuclei undergo a multiple synchronous mitotic division without cytokinesis creating syncytium, also called the endosperm coenocyte. The number of nuclei determines the number of endospermic cells and therefore defines the final grain size. Since the size of seed is primarily dependent on endosperm size, this stage is crucial for yield in monocots (Jameson and Song, 2016). The nuclei are at the final stage moving to the thin outside layer of the central cell where each nucleus is surrounded by microtubules which determine the localization of future phragmoplast and cell wall. This is considered as the starting point of cellularization; in barley it occurs 5 to 6 days after pollination (DAP). During this time, the vacuole starts to form, the cytoplasm is shaped and material for the cell wall accumulates (Doan *et al*., 1996). Factors involved in this process as well as the timing are still unknown and therefore the whole transition from syncytial to cellular endosperm remains undetermined not only in barley but also in cereals in general, or even dicot plant model *Arabidopsis* (Batista *et al.*, 2019). The completed cellularization represents the beginning of the transition from cell division to accumulation of starch and other storage products such as proteins. Around this time, peripheral cells differentiate into aleurone cells. Since this point (10 DAP), grain size is increasing mainly due to thickening of the starchy endosperm. Cells are growing rapidly but eventually undergo programmed cell death remaining only aleurone cells alive (Sreenivasulu *et al.*, 2010).

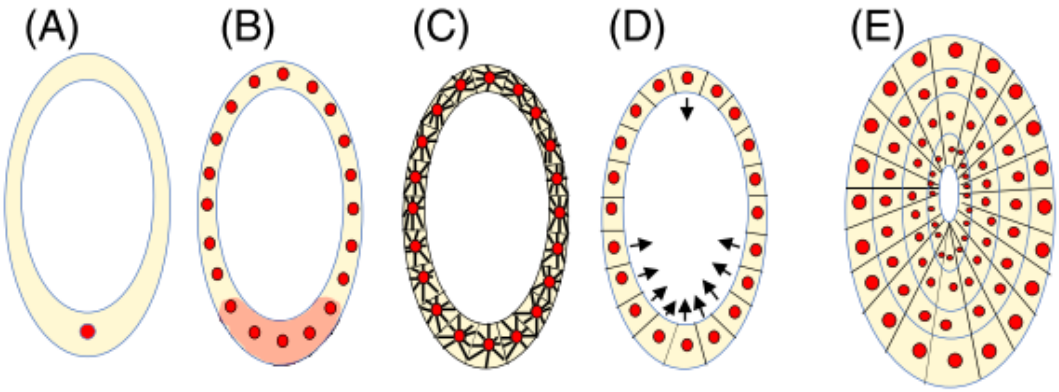

Figure 4. Endosperm development (A) Primary endosperm cell with a triploid nucleus within a thin cytoplasm layer with a big central vacuole. (B) Central cell nuclei undergo a multiple synchronous mitotic division without cytokinesis creating the endosperm coenocyte. (C) Each nucleus is surrounded by microtubules which determine the localization of future phragmoplast and cell wall. (D) Cell wall formation in the periphery of the developing endosperm. (E) Fully cellular endosperm after several repetitions of cell wall formation (Source: copied from Olsen, 2020).

2.6 Endosperm and phytohormones

Seed development of cereals is controlled by phytohormones (Cao *et al.*, 2020), essentially auxin and cytokinin which are predominantly naturally occurring as indole-3-acetic acid (IAA) and zeatin, respectively (Fengmei *et al.*, 2015).

Auxin regulates plethora of plant growth and developmental processes (Ludwig-Müller, 2011). However, current research is mainly focusing on *Arabidopsis thaliana* and only a little is known about its role in crop plants. Although, this research could unravel an interesting information, especially in connection to crop yield which could eventually lead to the enhancing of crop productivity (Cao *et al.*, 2020; L. Liu *et al.*, 2015a). *MADS29*, a rice auxin-inducible transcriptional factor whose inhibition lead to the abnormal seed development, belongs to such genes (Yin and Xue, 2012). *Big grain 1 (BG1*) is another rice gene encoding a plasma membrane localized protein involved in auxin transport. Overexpression of *BG1* induced high sensitivity to auxin and the production of big grains, whereas its suppression resulted in decreasing grain size (Liu *et al.,* 2015).

Cytokinins (CK) are also involved in many essential aspects of plant growth, development and physiological processes, such as cell division and differentiation, delay of senescence, seed and fruit development, and the response to biotic and abiotic stresses. Their regulation is complex, based on balance between biosynthesis, activation, inactivation, and degradation which is among others controlled by the cytokinin dehydrogenase (CKX) (Jameson and Song, 2016). Barley homolog *HvCKX1* gene is expressed especially in aleuronic layer and its mutation lead to the higher grain yield (Zalewski *et al.*, 2010).

2.6.1 IAA conjugates as a regulators of IAA content in plants

IAA exists in two forms either as a hormonally active free acid or as inactive conjugated forms for example IAA-amido conjugates or IAA esters (Xiao lu Yu *et al.*, 2019). While both forms are commonly occurring *in planta*, only a little is known about the specific role of IAA conjugates. It is currently widely accepted that IAA-conjugates control supplies of free IAA. Auxin responses are concentration-dependent in the way that high auxin concentration often have inhibitory effects. Some of them for example IAA–Ala, IAA–Leu, IAA–Phe could be hydrolysed back to free IAA by hydrolases; whereas others, such as IAA–Glu, are considered to be precursors of the degradation pathway (Ludwig-Müller, 2011). In line with this idea, IAA-Glu accumulated in *Arabidopsis* after treatment with high IAA concentration (Barratt *et al.*, 1999).

High concentrations of IAA-conjugates in plants organs presume their importance in regulating the availability of endogenous free IAA. For instance, in maize the conjugated forms (including IAA-glucose) represent 97-99 % of the total IAA in seed endosperm (Bandurski *et al.*, 1995). In rice, 62-70% of total IAA occur as IAA esters (Bandurski and Schulze, 1977). In tomato, ripening stage of the fruit depends on the ratio between IAA-glucose and amide-linked IAA (Jakubowska, 2004). In a deficiency of free IAA, plants have shorter panicles, smaller crown roots in rice (Du *et al.,* 2012) or *Arabidopsis* plants are dwarf in maturation and suffer from curly leaf blades (Jackson *et al.*, 2002).

In 2019, a protein called IAAGLU was found to catalyse the reaction of free IAA with glucose to generate IAA-glucose which could potentially regulate IAA

homeostasis in rice and maize. In rice, the overexpression of this gene lead to decrease of plant height and more importantly panicle length compared to wild type. Moreover, concentration of free IAA in leaves or root tips was lower than in wild type (Yu *et al*., 2019)

2.7 Thousand grain weight 6 (TGW6)

Ishimaru (2003) analysed QTL associated with the thousand grain weight (TGW) trait in rice (*Oryza sativa*) using ninety-eight BC1F5 lines which were developed from a backcross of rice (*Oryza sativa*) cvs Nipponbare/Kasalath//Nipponbare. A new locus responsible for improving TGW was identified. Among all studied QTLs, only *tgw6* had an allele from rice cv Kasalath which seemed to have a major effect in higher yield. Interestingly, different studies described QTL responsible for the grain weight in the similar region (Li *et al*., 1997; Xing *et al.,* 2002). In a Nipponbare NIL population where the chromosomal region corresponding to tgw6 was substituted by the Kasalath , the yield per plant was significantly higher compared to the high-yielding rice cv Nipponbare. Furthermore, *tgw6* locus did not affect other traits of plant type besides TGW so as a conclusion of the study he suggested that the introduction of *tgw6* into new cultivars can improve the yield in rice.

In a further studies, Ishimaru *et al.* (2013) focused on the function of this gene. The authors clarified that *tgw6* in Kasalath is mutated, whereas Nipponbare allele encodes a functional protein with an indole-3-acetic acid (IAA)-glucose hydrolase activity. TGW is a single exon gene and in Kasalath the 1-bp deletion at the nucleotide 313 causes a frameshift, leading to a premature stop codon and consequently to a non-functional protein.

According to their results, *TGW6* was expressed in sink and source organs, especially in panicles around endosperm. In the study, the authors compared Nipponbare with NIL(TGW6) and found that the expression of *TGW6* in leaves is similar in both genotypes. Regarding grain characteristics, NIL(TGW6) had longer endosperm and grain, whereas wideness or thickness of the grain remained unchanged. Most importantly, NIL(TGW6) showed greater dry weight throughout grain development, and significantly higher number of endosperm cell layers.

The rice OsTGW6 contains the hydrolase active site of the strictosidine synthase (STR1) and the catalytic calcium ion–binding residues of the diisopropyl fluorophosphatase (DFPase) (Ishimaru *et al.*, 2013). Due to its hydrolyse activity, TGW6 hydrolyses bond between indole-3-acetic acid (IAA) and glucose. It was previously described in maize that auxin analogues activate cellularization, whereas auxin inhibitors delay cellularization. Therefore, TGW6 is believed to control the endogenous amount of IAA during grain development leading to limited cell number and grain length. However, non-functional gene in the Kasalath does not control the supply of IAA leading to the higher number of cells and longer grain. In conclusion, TGW6 seems to control the timing of the transition from the syncytial to the cellular phase in sink organs and the absence of the TGW6 in cv. Kasalath results in desired effects in connection to the grain size and yield (Ishimaru *et al.*, 2013).

Consecutive to this study, Hanif *et al.* (2015) identified an ortholog of *OsTGW6* in bread wheat. Authors suggested that *TaTGW6* was already positively selected in wheat breading because they found 80% correlation between *TaTGW6* and higher TGW during their association analysis of 242 cultivars. Hu *at al.* (2016) observed that low expression of *TaTGW6* in the middle and late stages of grain filling is also connected to the higher TGW. Therefore, they suggested that TaTGW6 is a negative regulator of grain weight, similarly to OsTGW6. Moreover, according to their results IAA content of immature seed was associated with low relative expression of *TaTGW6* and with high TGW (Hu *et al.*, 2016). Ishimaru *et al.* (2013) tested *OsTGW6* expression in panicle, flag leaf, 1st leaf and 2nd leaf a week after heading and they observed the highest relative expression in panicle at 2 days after anthesis (DAA). On the other hand, Hu *et al.* (2016), reported that TaTGW6 has the highest expression at 20 DAA. Kabir *et al.* (2020) reinvestigated the expression of *TaTGW6* in wheat using inflorescence. They analysed several *TaTGW6*-like genes which they divided into three clades according to their phylogenetic analysis. Their data showed that *TaTGW6* and *TaTGW6*-like genes have low expression only in the early inflorescence development regarding clade I. A single gene from clade II showed detectable expression in grains at 20 DAA but this gene had the highest expression in the early inflorescence development. In clade III, they observed three different expression patterns: two genes were strongly expressed in spike including anthers; other two

genes were upregulated in leaf tissue and young grains (2 DAA) and one gene was expressed in the stem and flag leaf. Moreover, they also could not confirm *OsTGW6* expression.

Sharma *et al.* (2018) studied in barley grain phenotype of a population containing 25 diverse exotic barley genomes superimposed on an ~70% genetic background of cultivated barley. They made a scan of grains of this population and analysed grain yield component traits such as thousand grain weight (TGW), grain number per ear (GPE), grain area (GA), grain length (GL), grain width (GW), and ear length (EL). The authors identified a hotspot located on chromosome 7 which showed association with almost all these traits. Moreover, SNP markers associated with reduced thousand grain weight were found in the same spot. This region overlaps with two rice grain trait genes: a mitogen-activated protein kinase OsMAPK6 (Liu *et al.*, 2015b) and IAA-glucose hydrolase TGW6 (Ishimaru *et al.*, 2013).

2.8 CRISPR-Cas genome editing tool

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system is naturally occurring in many bacteria and archaea as an adaptable immune system. Scientists have been intrigued by this feature for many decades. However, the true changing point was the discovery of the caspase 9 (Cas9) protein and its ability to be programmed to cleave specific DNA sequences (Ishino *et al.*, 2018; Jinek *et al.*, 2012). Since then, CRISPR-Cas9 has been repeatedly used as DNA manipulation tool in animals, plants or humans (Sandhya *et al.*, 2020). Even though, editing tools such as zinc fingers nucleases (ZNFs) (Kim *et al.*, 1996) or transcription activator-like effector nucleases (TALENs) (Christian *et al.*, 2010) had existed years before CRISPR-Cas9 was discovered, the genome editing has never been as common as it is now (Bortesi and Fischer, 2015). In fact, they made the CRISPR-Cas9 technique inexpensive, universal and available.

2.8.1 CRISPR-Cas9 discovery

In the middle of 1980s, while studying a different topics, scientists noticed an unusual arrangement within an *E. coli* DNA which consisted of regularly repetitive sequences interspaced by other noncoding tandemly repeated sequences, representing around 1 % of the bacterial genome (Ishino *et al.*, 1987). Since then, the formation had been

described a few more times not only in *E. coli* but in other bacteria and archaea. However, at that time nobody knew its function. A few suggestions appeared such as that the sequences could be involved in the regulation of gene expression or in replicon partitioning. During 90s, sequencing methods have dramatically improved and scientists found out that these special repetitive sequences occurred in many bacteria and archaea genomes. In 2011, one or more such loci were identified in 310 out of 703 (44%) bacterial and archaeal genomes (Groenen *et al.*, 1993; Mojica *et al.*, 1995; Nakata *et al.*, 1989), but the function remained unrevealed and many research groups had been intrigued by this question (Yoshizumi *et al.*, 2018).

In 2002, Jansen *et al.* used the term "CRISPR" as an acronym for "clustered repetitive interspaced palindromic repeats" which became widely accepted by the scientific community. The comparison of a collection of CRISPR regions led to the identification 4 classes of CRISPR-associated genes (Cas) (Jansen *et al.*, 2002). However, similarly to CRISPR, their function remained unknown. In 2005, two different groups uncovered that the sequences located between repeats are homologous to the sequences of bacteriophages and that the corresponding bacteriophages do not infect these cells (Mojica *et al.*, 2005; Pourcel *et al.*, 2005). This observation led to the hypothesis that the sequences protect the cells from entry of foreign DNA and that the organisms are probably able to capture pieces of DNA in their genome what was proved by Barrangou *et al.* in 2007. A year later, it was demonstrated that RNA transcribed from CRISPR region cooperates with closely located Cas proteins (Brouns *et al.*, 2008).

In 2012, a unique Cas protein called Cas9 was identified. The characteristic which makes it special is that it is able to do the whole defence process on its own whereas other Cas proteins need cooperation (Gasiunas *et al.*, 2012). The Cas9 not only works as an immune system in bacteria but can also target eukaryotic DNA (Jinek *et al.*, 2012). One year later, genome editing using CRISPR-Cas9 was already reported for species across the whole plant land, including *Arabidopsis thaliana* and crops (Feng *et al*., 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013).

2.8.2 CRISPR Cas as immune system

To fight against viruses, bacteria and archaea have developed an ingenious immune system. It is based on small RNAs which navigate a protein called Cas to the complementary DNA. The Cas protein is an endonuclease responsible for the enzymatic cleavage of nucleic acid in a sequence-dependent manner, thus inactivating the foreign DNA. The process can be divided into three phases: adaptive, expression and interference (Fig 5). After a viral attack, 30 bp long piece DNA homologous to virus sequence is integrated into the leader part of bacteria CRISPR loci. The integration is accompanied by the duplication of DNA in bacteria CRISPR loci and therefore each spacer is interspaced by the same repeat DNA. Selection of a proto-spacer sequence from invading DNA is controlled by protospacer-adjacent motifs (PAMs). During expression, crRNA is generated. Finally, complexes of Cas proteins are navigated by crRNA to the complementary viral nucleic acid. Also, in this process PAM seems to be important (Jinek *et al.*, 2012).

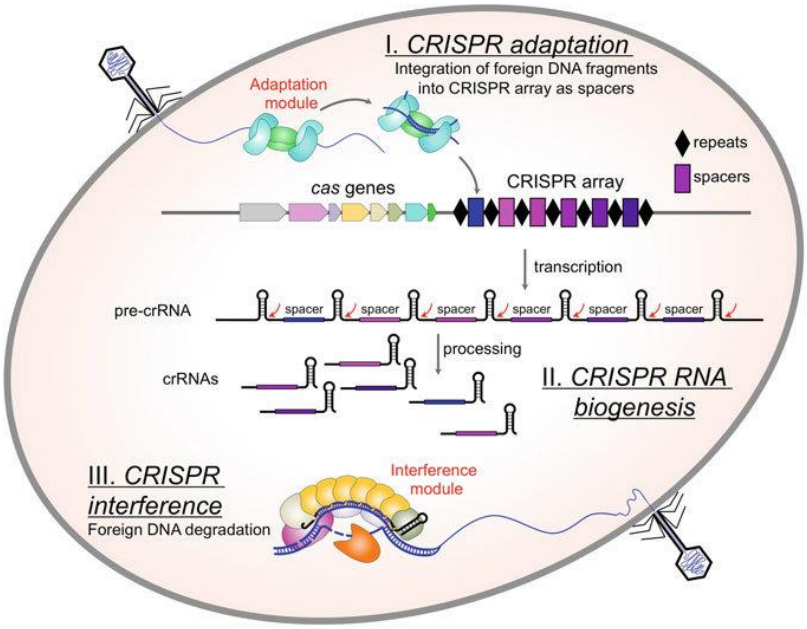

Figure 5. CRISPR-Cas as an immune system. Three phases of process: I-adaptive, when foreign DNA fragments are integrated into the leader part of bacteria CRISPR loci, II-expression during which crRNA is generated and III-interference when Cas proteins are navigated by crRNA in order to degrade foreign DNA (copied from Semenova and Severinov, 2017).

2.8.3 CRISPR-Cas9 as an editing technology in plants

Cas9 makes double-stranded breaks (DSBs) which are common in eukaryotes. Cells repair DNA damages either by homologous recombination (HR) or non-homologous end joining (NHEJ). Both repair systems trigger insertion or deletion (*indels*) of nucleotide(s) which can cause a frameshift if they occur in the coding region of the gene, resulting in mutations and potential knockout of the gene (Bortesi and Fischer, 2015). Cas9 is made up of two nuclease domains termed HNH and RuvC which are responsible for cleaving target DNA (Jinek *et al.*, 2012) and PAM interacting site. The protein also includes a nuclear localization signal (NLS) attached to N or/and C terminal of the protein enabling transport into nucleus. The transcription of Cas9 in most cases relies on inducible or constitutive promoters (Kumar *et al.*, 2019).

Two classes of CRISPR-Cas systems have been identified and each is subdivided into 2 to 3 types (Fig 6) (Makarova *et al.*, 2015). CRISPR-Cas systems belong to class I that typically consist of complexes of Cas proteins which are navigated by crRNAs to the target, causing degradation of targeted nucleic acids. CRISPR-Cas system from class II, currently including type II, consists of endonuclease Cas9 which enables cleaving of target DNA without ensembles of Cas proteins. For the system crRNA:tracrRNA duplex navigating Cas9 to target DNA sequence and PAM are required (Nishimasu *et al.*, 2014; Karvelis *et al.*, 2015).

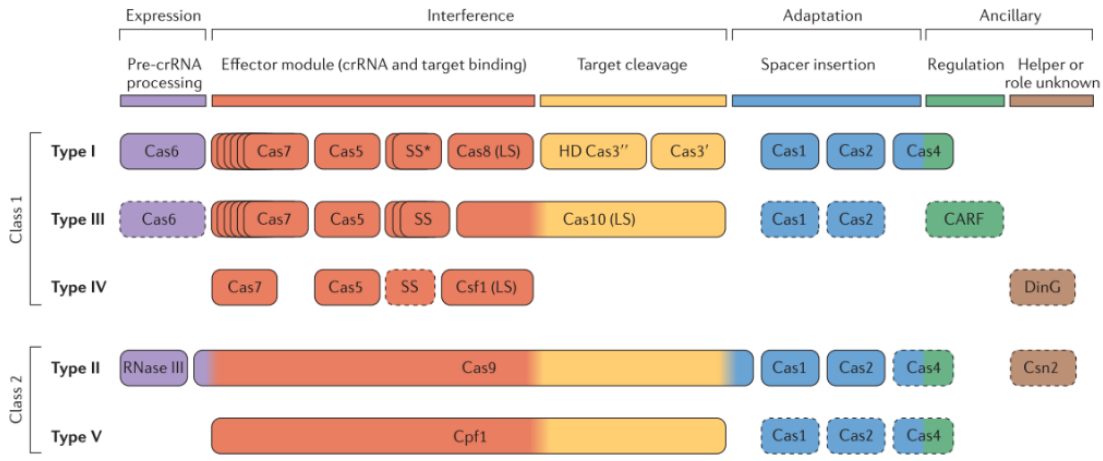

Figure 6. Classification of CRISPR-systems. Copied from Makarova *et al.*, 2015.

The sgRNA consists of two parts: CRISPR RNAs (crRNA) and transactivating CRISPR RNA (tracrRNA) (Fig 7). CRISPR RNAs are approximately 20-40 nt in length and define a cleavage site at the target DNA. Transactivating CRISPR RNA is a non-coding

DNA which is important for the type II because of two functions: it enables cutting of pre-crRNA by the enzyme RNase III and activates the cleavage of target DNA by Cas9. The cleavage always produces blunt ends (Kumar *et al.*, 2019). In 2012, Jinek *et al.* described that the sgRNA can be design and combined with tracrRNA by scientists what initiated genome editing in many organisms. Transcription of sgRNA is usually regulated by RNA polymerase III under control of promoters for instance U3 or U6 (Kumar *et al.*, 2019).

In order to design target specific sgRNA for knocking out of gene it is important to consider certain parameters: (1) sgRNA specific to target site should be at least 18-21 nucleotide long, (2) all isomers of the gene of interest and design sgRNA in the way that it targets most of them, (3) three nucleotide PAM site need to be included, (4) sgRNA should be the targeted to the first exon of the gene of interest, (5) in order to clone, sgRNA should contain restriction enzymes sites, (6) the less off targets is detected, the better. There are bioinformatics tools which help to design the best sgRNA according to the given parameters including GC content or secondary structure of sgRNA (Kumar *et al.*, 2019).

Prerequisite for cleavage are very short conserved sequences, later called protospacer-adjacent motifs (PAM). First observations of these sequences correlated with CRISPR were firstly observed by Bolotin *et al.* (2005) and Kunin *et al.* (2007). They are di- or trinucleotides long, starting immediately or one position after the protospacer (Mojica *et al.*, 2009). The PAM sequence in type III system is located downstream of the protospacer sequence and triggers cleavage of Cas9 three base pairs upstream of the PAM sequence in case of complementary pairing (Fischer *et al.*, 2012). Its sequence is usually 5'-NGG-3' (Jinek *et al.*, 2012) at 3' end (Mojica *et al.*, 2009).

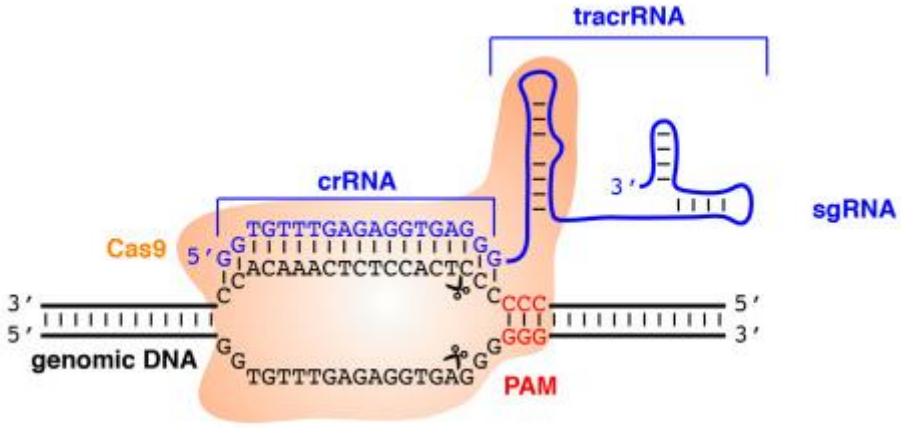

Figure 7. CRISPR-Cas9 technology: sgRNA consisting of crRNA and tracrRNA navigating Cas protein to the target site (protospacer) on DNA for what PAM is required and DNA is cut by protein (copied from Lin *et al*., 2019).

All the components of CRISPR construct listed above are cloned into an appropriate vector which is chosen according to the method of transformation. Among the most used methods for incorporation belongs enzyme restriction followed by ligation or Golden gate cloning strategy. The transformation is considered a major weakness of the whole strategy (Kumar *et al.*, 2019). Regarding plants, the construct is delivered using three main methods: PEG mediated transformation, *agrobacterium*-mediated transformation, and bombardment or biolistic transformation. In the future, there are coming novel approaches using vector-nanoparticle complex or pollen magnetofection-mediated delivery (Sandhya *et al.*, 2020).

3 EXPERIMENTAL PART

3.1 MATERIAL

3.1.1 Biological material

Two-raw spring barley (*Hordeum vulgare* L.), cultivar Golden Promise

Chemocompetent cells *Escherichia coli* TOP10 (NEB, USA)

Electrocompetent cells *Agrobacterium tumefaciens* strain AGL1, kindly provided by Dr. G. Hensel (CEPLAS, Dusseldorf)

3.1.2 Kits

QIAGEN PCR Cloning Kit (Qiagen, Germany)

QIAprep Spin Miniprep (Qiagen, Germany)

NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany)

NEB PCR Cloning Kit (NEB, USA)

RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA)

3.1.3 Vectors

Binary vector *p6i-d35S-TE9* (DNA-Cloning-Service, Hamburg, Germany)

pDRIVE cloning vector (Qiagen, Germany)

pMiniT 2.0 cloning vector (NEB, USA)

pSH91 expression vector (Budhagatapalli *et al.,* 2016)

3.1.4 Solutions

Luria-Bertani (LB) medium (1 l): 15,5 g LB broth; 9,5 g NaCl; Milli-Q H_2O; pH 7.0. For solid medium: 15 g agar was added.

MG/L medium (1 l): 250 mg KH_2PO_4; 100 mg NaCl; 100 mg $MgSO_4 \cdot 7\ H_2O$; 1 g L-glutamic acid; 5 g mannitol; 5 g tryptone; 2,5 g yeast extract. The pH was adjusted to 7.0. After autoclaving of the medium, 0,1 g biotin was added. For solid medium: 12 g agar was added.

DNA extraction buffer I: 1% N-lauryl-sarcosine, 100mmol·l^{-1}Tris-HCl (pH 8), 10 mmol·l^{-1} EDTA (pH 8), 100mmol·l^{-1} NaCl.

DNA extraction buffer II: 200mM TrisHCl (pH8), 250 mM NaCl, 20mM EDTA (pH8), 0,5% SNS.

SOC medium: 0,5% yeast extract; 2% trypton; 10 mmol·l^{-1} NaCl; 2,5 mmol·l^{-1} KCl; 10 mmol·l^{-1} $MgCl_2$; 10 mmol·l^{-1} $MgSO_4$; 20 mmol·l^{-1} glucose.

TE buffer (100x): 1mol·l^{-1} Tris-HCl, 0,1mol·l^{-1} EDTA, dH_2O.

RNA extraction buffer: 100mM Tris-HCl (pH 9.0), add 2% β-mercaptoethanol (v/v) just before use (prepared with autoclaved DEPC (diethyl pyrocarbonate)-treated MilliQ water), store at room temperature.

20% SDS (sodium dodecyl sulphate) (w/v) add 0.1% DEPC

Cocultivation medium (BCCM) (1 l): 4,3 g MS mineral salts; 1 g casein hydrolysate; 0,69 g proline; 0,25 mg myoinositol; 30 g maltose·H2O; 2,5 mg Dicamba; 1 mg thiamine-HCl. The pH was adjusted to 5.8. The solution was filter-sterilized in a laminar flow hood. 800 mg L-cysteine and 500 µl 1 mol·l-1 acetosyringone in DMSO were added.

Callus induction medium (BCIM) (1 l): 4,3 g MS mineral salts; 1 g casein hydrolysate; 0,69 g proline; 0,25 mg myoinositol; 30 g maltose·H2O; 2,5 mg Dicamba; 1 mg thiamine-HCl; 0,25 mg CuSO4·5 H2O. The pH was adjusted to 5.8; 50 mg hygromycin and 150 mg timentin were added. The solution was filter-sterilized in a laminar flow hood. One volume was mixed with three volumes of 0,4% Phytagel.

Regeneration medium (BRM) (1 l): 50 ml K4N macro mineral stock solution (above); 1 ml 75 mmol·l-1 NaFeEDTA; 1 ml K micro stock solution (above); 112 mg vitamin B5; 1 ml 1 mmol·l-1 6-BAP; 146,4 mg L-glutamine; 36 g maltose·H2O; 196 µl 25 mmol·l-1 CuSO4·5 H2O. The pH was adjusted to 5.8. The solution was filter-sterilized in a laminar flow hood. One volume was mixed with three volumes of 0,4% Phytagel.

3.1.5 Equipments

BTX ECM 399 Exponential decay wave electroporator (Thermo Fisher Scientific, USA)
Electrophoretic chamber for horizontal electrophoresis (Biometra, Germany)
Environmental chamber MLR 351 (Sanyo, Japan)
Gel Doc EZ Gel Imaging System (Bio-Rad, USA)
Mixer Mill MM 400 (Retsch, Germany)

NanoDrop One^{C} Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, USA)

Scanner Epson perfection V700 photo (Epson, Japan)

Zeiss Axio Zoom v16 Stereo Microscope (Zeiss, Germany)

and other laboratory equipment of the Group of Plant Genetic and Engineering, CATRIN-CRH.

3.1.6 Softwares

BioEdit (Hall, 1999)

GrainScan (Whan *et al*., 2014)

GraphPad (GraphPad Software, USA)

Image Lab 5.1 (Bio-Rad, USA)

MEGA X (Kumar *et al*., 2018)

SnapGene Viewer (GSL Biotech, USA)

ZEN software (Zeiss, Germany)

3.1.7 Chemicals

Agarose (Sigma-Aldrich, USA), Ampicillin (Sigma-Aldrich, USA), *BsaI-HF* (20 000 U·ml^{-1}) (NEB, USA), CutSmart (10x) (NEB, USA), Deoxynucleoside triphosphates (dNTPs, 10 mmol·l^{-1}) (Sigma-Aldrich, USA), DNase I (1 500 U·ml-$^{-1}$) (Qiagen, Germany), *EcoR*I-HF (20 000 U·ml^{-1}) (NEB, USA), Ethanol (70%) (Lach:ner s.r.o.), Ethidium Bromide (Ambion, USA), GoTaq G2 Flexi DNA polymerase (5 000 U·ml-1) and 5x buffer (Promega, USA), GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA), Gel Loading Dye Purple (6X) (NEB, USA), Isopropanol, Kanamycin (Duchefa, The Netherlands), Ligation Master Mix (2x) (Qiagen), Luna Universal qPCR Master Mix (NEB, USA), 10X NEBuffer 3 (NEB, USA), *NheI* (20 000 U·ml^{-1}) (NEB, USA), *NotI* (20 000 U·ml^{-1}) (NEB, USA), Nuclease-Free water (Qiagen, Germany), Oligo dT primer (100 µmol·l^{-1}) (Sigma-Aldrich, USA), Phusion DNA polymerase (2 U·µl^{-1}) and 5x buffer HF (Thermo Fisher Scientific, USA), Proteinase K (400 U·ml^{-1}) (Thermo Fisher Scientific, USA), Phenol – chloroform – isoamyl alcohol mixture (25:24:1) (Sigma-Aldrich), Ribonuclease A (Top-Bio s.r.o.), RNase (DNase free) (Top-Bio s.r.o.), *Sfi*I (20 000 U·ml^{-1}) (NEB, USA), Sodium acetate (3 mol·l^{-1}, ph 5,2) (Serva), Spectinomycin (Duchefa, The Netherlands), T4 DNA ligase (5 U·µl^{-1}) and 10x buffer (Thermo Fisher Scientific, USA), Tris ultrapure (Duchefa The Netherlands), TRIzol (Tiangen, Beijing,

China), Turbo DNase I (2000 U·ml^{-1}) and 10 X DNase Turbo Buffer (Thermo Fisher Scientific, USA).

And other chemicals of the Group of Plant Genetic and Engineering, CATRIN-CRH

3.2 METHODS

The experimental part of this master thesis was performed at the Group of Plant Genetic and Engineering, CATRIN-CRH (previously Department of Molecular Biology, Centre of the Region Haná for Biotechnological and Agricultural Research in Olomouc).

3.2.1 Identification of putative barley TGW6

The rice gene *OsTGW6* (*Os06g0623700*) was used as a query to search in the Barlex databases (Barley Draft Genome Explorer) and Ensembl Plants database for a homologous sequences in barley. Two sequences sharing high similarity were selected as a putative *TGW6* in barley: *HvTGW6a* (*HORVU7Hr1G028580*) and *HvTGW6b* (*HORVU5Hr1G091230*). Using Primer3Plus, primer pair specific to each of the two putative genes were designed. The two genes were amplified from genomic DNA of the barley cv. Golden Promise. This was possible as these two genes are predicted to be composed of a single exon.

Phylogenetic analysis of protein sequences was carried out in MEGA X. For this purpose, Ensembl Plants database (Hordeum vulgare) was searched in order to identified proteins containing strictosidine synthase-like domain. As a reference protein was used OsSTR1. Further, OsTGW6 from Nipponbare and Kasalath were included in order to see whether TGW6a and TGW6b are organized in close clusters. Phylogenetic tree was constructed using the Neighbor-joining statistical method. Bootstrap confidence levels were obtained using 500 replicates. Jones-Taylor-Thornton model was used as a substitution model. All ambiguous positions were removed for each sequence pair since pairwise deletion option was selected.

3.2.2 DNA isolation of genomic DNA (gDNA)

Isolation of gDNA was performed according to Palotta *et al.* (2000) using Golden Promise.

Two young leaves were transferred into 2 ml Eppendorf tube and immediately frozen in liquid nitrogen. The frozen plant material was homogenized in a homogenizer machine (Mixer Mill MM 400) with two glass beads (Sigma-Aldrich, 5 mm) for 1 minute with a frequency of 27 Hz. Then 800 µl of DNA extraction buffer I were added. After that the experiment continue under a fume hood. Proteins were removed by adding 800 µl of phenol:chloroform:isoamyl alcohol mixture (25:24:1; v:v:v). The sample was centrifuged at 3 000 x g for 3 minutes at room temperature. The supernatant was transferred to a new 1,5ml Eppendorf tube and precipitated with 80 µl of sodium acetate (3 $mol \cdot l^{-1}$) and 800 µl of isopropanol. The sample was centrifuged at 18 000 x g for 10 minutes at room temperature. The supernatant was discarded, and the pellet was washed with 70 % ethanol. The sample was centrifuged one more time at 18 000 x g for 5 minutes at room temperature. The supernatant was discarded, and pellet was air-dried. The pellet was dissolved in 100 µl of TE buffer with RNase (DNase free) of a final concentration 40 $\mu l \cdot ml^{-1}$. The concentration and quality of isolated gDNA was measured with NanoDrop One^{C} spectrophotometer and the samples were stored at -20 °C.

3.2.3 DNA extraction from agarose gel

For extraction from agarose gel NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany) was used.

DNA fragments were excised from agarose gel and gel slices were transferred into a 2 ml tube and weighed. For each 100 mg of agarose gel 200 µl Buffer NTI was added. The samples were incubated at 50 °C and vortexed every 3 min until the gel slices were completely dissolved. Subsequently 700 µl of each sample was loaded on a column followed by centrifugation for 1 min at 11 000 x g and discarding flow-through. Afterwards, that 700 µl of Buffer NT3 was added and centrifuged at the same conditions as before. Flowthroughs were discarded and washing step was repeated to minimized chaotropic salt. Centrifugation was rerun to remove Buffer NT3 completely. Columns were placed into a new 1.5 ml microcentrifuge tubes and 15 µl Buffer NE (heated to 70 °C) was added. After 1 min long incubation at room temperature the samples were centrifuged for 1 min at 11 000 x g.

3.2.4 PCR clean-up

For PCR clean-up NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany) was used.

One volume of each sample was mixed with two volumes of Buffer NTI and the whole volume was transferred to the column placed to the collection tube (provided by kit). Followed by centrifugation at room temperature for 30 sec at 11 000 x g. Flow-through was discarded and column was placed back to the collection tube. 700 µl of Buffer NT3 was added to the column and centrifuged for 30 sec at 11 000 x g. Flow-through was discarded and the column was placed back into the collection tube. Centrifugation was repeated at the same conditions and column was transferred into new 1,5 ml tube. 15 µl of Buffer NE was applied and incubated at room temperature for 1 min. Finally, samples were centrifuged for 1 min at 11 000 x g.

3.2.5 Cloning by QIAGEN PCR Cloning Kit

Firstly, A-tailing reaction was performed in order to enable cloning of blunt-ended products into *pDrive* cloning vector (Fig 8). Reaction mix was prepared according to Tab 2. and incubated in 70°C for 30 minutes in thermocycler.

Table 2. A-tailing reaction

Component	10µl reaction	Final concentration
DNA fragment	4 µl	100 ng· μl^{-1}
5X GoTaq Flexi reaction buffer	2 µl	1X
dATP (1 $mmol \cdot l^{-1}$)	2 µl	0,2 $mmol \cdot l^{-1}$
GoTag Flexi DNA polymerase (5 U/µl)	0,6 µl	0,23 $U \cdot \mu l^{-1}$
$MgCl_2$ (25 $mmol \cdot l^{-1}$)	1,4	3,5 $mmol \cdot l^{-1}$

The ligation-reaction mixture consisted of 2,5 µl ligation master mix, 2 µl PCR product and 1 µl *pDrive* Cloning Vector DNA was incubated for 2 hours at 16 °C in thermal cycling block. The mixture was placed on ice for 2 minutes and after that *E. coli* chemically competent cells were transformed by heat shock (Chapter 3.2.9).

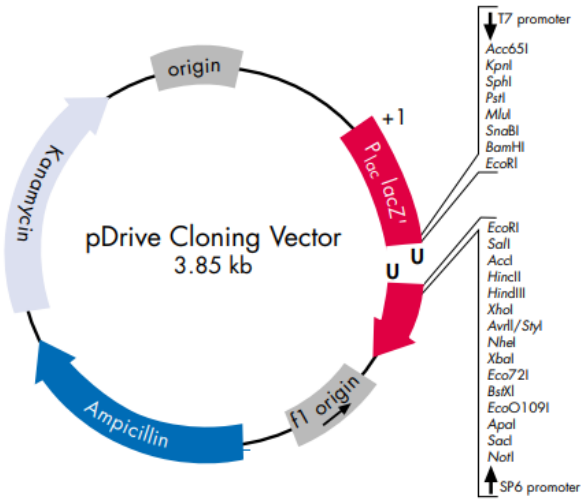

Figure 8. *pDrive* Cloning Vector contains ampicillin and kanamycin resistance genes, multiple cloning site, pUC origin of replication, M13 forward and reverse primer binding sides, *LacZ* gene for blue-white selection, phage f1 origin (enables preparation of single-stranded DNA). Source: www.qiagen.com.

3.2.6 Cloning by NEB PCR Cloning Kit

Ligation-reaction mixture consisted of 1 µl Linearized *pMiniT* 2.0 Vector (25 ng/µl) (Fig 9), 4 µl PCR product , 4 µl Cloning Mix 1 and 1 µl Cloning Mix 2 was incubated for 15 minutes at 25 °C. The mixture was placed on ice for 2 minutes and after that *E. coli* chemically competent cells were transformed by heat shock (Chapter 3.2.9).

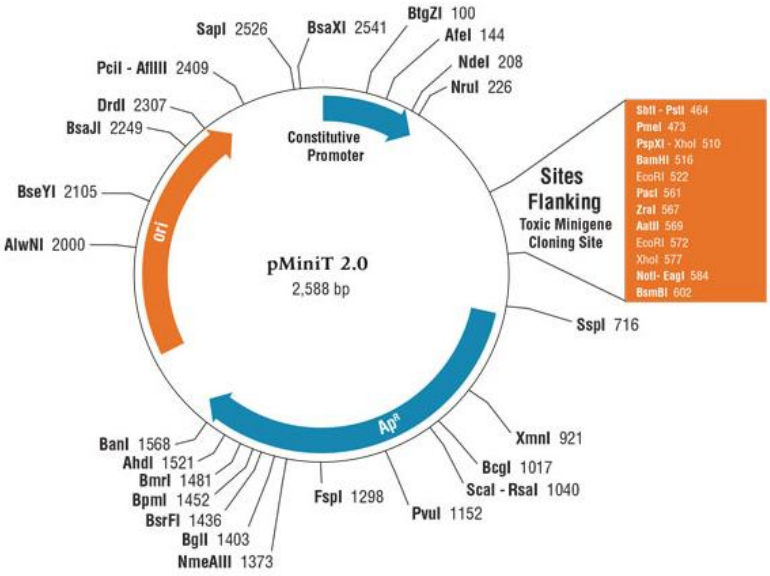

Figure 9. *pMiniT* 2.0 Vector contains ampicillin resistance gene, multiple cloning site, pUC origin of replication and constitutive promoter. Source: www.neb.com

3.2.7 Colony PCR and liquid culture

For each construct, few isolated colonies were chosen for PCR screening. For this purpose, the colony was picked up with a sterile tip, streaked to the marked place on LB-Agar medium containing the appropriate antibiotic and finally washed in the PCR mixture (Tab 15). The Petri dishes were incubated over night at 37°C. The PCR reactions were run on thermocycler according the following parameters. Initial denaturation: 95°C for 10 min; 35 cycles: 95°C for 30 sec, Tm for 30 sec, 1min/kb at 72°C; final extension in 72°C for 10 min. Tm for each primer pair is described in Tab 14. The PCR reaction was loaded on 1% agarose gel containing ethidium bromide and ran in 1X TAE at 90 volts for 30 min. Gels were visualized using Gel Doc EZ Gel Imaging System (Bio-Rad, USA). When a PCR product of the correct length was obtained, the corresponding bacteria colony was used for liquid culture preparation. The colony was picked up from a marked place on LB-Agar medium and placed in 3-5ml of liquid LB medium supplied with corresponding antibiotic. Bacteria cultures were incubated overnight at 37°C, shaking 180 rpm. Next day, plasmids were isolated using QIAprep Spin Miniprep Kit.

3.2.8 Purification of plasmid DNA

QIAprep Spin Miniprep Kit was used for purification of plasmid DNA (pDNA) from the bacterial cultures of *E. coli* and *A. tumefaciens*.

Firstly, 5 ml bacterial culture was centrifuged at 7000 x g for 3 min at room temperature. Bacterial pellet was resuspended in 250 µl of buffer P1 by vortexing at least 30 s, the mixture was then transferred to a new microcentrifuge tube and 250 µl of P2 buffer was added. Afterwards, the sample was mixed by inverting and incubated in room temperature for maximum 15 minutes. Then 350 µl of N3 buffer was added for neutralization immediately followed by inverting the tube 6 times. The mixture was centrifuged for 10 minutes, at room temperature at 17 000 x g and 800 µl supernatant was transferred to the column followed by 1 min centrifugation in the same conditions. Flow-through was discarded and the column was washed by adding 0,5 ml of PB buffer and centrifugation for 1 min. In the next step 0.75 ml of PE buffer was applied followed by centrifugation for 1 min. Flow-through was discarded and centrifugation was repeated in order to remove residual wash buffer, containing

ethanol. Subsequently the column was placed in a clean 1.5ml microcentrifuge tube, 50 µl EB buffer was added followed by centrifugation for 1 min. The extracted pDNA was measured with NanoDrop One^{C} spectrophotometer. The samples were sequenced by Sanger method by commercial services (SeqMe, Czech Republic).

3.2.9 Transformation of *E. coli* chemically competent cells by heat shock

1.5 µl of ligation product was added into volume of 50 µl chemically competent cells *E. Coli* TOP10. The mixture was gently mixed and chilled on ice for 30 min. Afterwards, the mixture was transformed to a heating machine prewarmed on 42 °C and heating shock was performed for 90 s. Immediately after that, the mixture was put on ice for 2 min. After cooling down, 450 µl of SOC medium was added following by incubation in a thermomixer for 60 min, at 37°C, shaking at 300 rpm. After incubation, the bacterial culture was spread over LB medium supplied with antibiotics for the selection of transformed cells. The bacterial culture was incubated at 37 °C overnight.

3.2.10 CRISPR/Cas9 mediated gene knock-out of barley Hv*TGW6*

3.2.10.1 Preparation of gRNA oligonucleotide duplex

For each gene, two guide RNAs (gRNA) targeting different regions of the corresponding gene were designed with CRISPOR (http://crispor.tefor.net/) (Concordet *et al.* 2018) (Fig 10). The gRNAs target close sequences upstream of the genes.

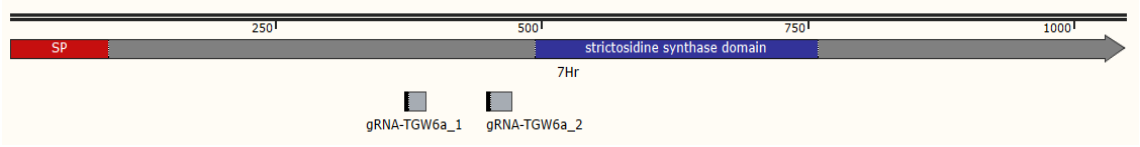

Figure 10. Schema of TGW6a gene with showing target sites of both gRNA (gRNA-TGW6a_1 and qRNA-TGW6a_2).

Since the transcription of the gRNA is under the control of OsU3 promoter, gRNA sequence should start with A or G. Moreover, it is recommended to have gRNA maximally 20 nucleotide long. The overall structure of the corresponding scaffold RNA was analysed using RNAfold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). The structure of final RNA molecule should be

represented by five loops and on one of them two structures like antennas are located (Fig 11).

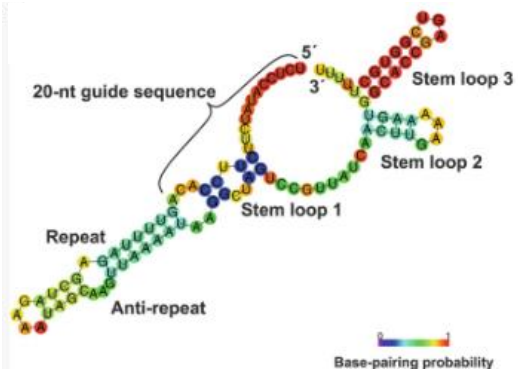

Figure 11. Structure of final RNA molecule (copied from Schindele *et al*., 2020).

For each gRNA, complementary primers pairs were designed (Tab 3). The primers contained overhangs allowing cloning of the gRNA into the *pSH91* vector at the *Bsa*I restriction site.

Table 3. List of oligonucleotides cloned into *pSH91* vector.

gRNA	Oligonucleotide	Length
TGW6b 5Hr1	Fw: 5´– GGCGAAAGGATTAGGAGCAGCA – 3´	18 bp
	Rv: 5´– AAACTGCTGCTGCTCCTAATCCTTTG – 3´	
TGW6b 5Hr2	Fw: 5´– GGCGAGGTGGAACCGCGACGCGCT – 3´	20 bp
	Rv: 5´– AAACAGCGCGTCGCCGTTGCACCT – 3´	
TGW6a 7Hr1	Fw: 5´– GGCGACAGGTACCCAGACTTT – 3´	17 bp
	Rv: 5´– AAACAAAGTCTGGGTAGCTGT – 3´	
TGW6a 7Hr2	Fw: 5´– GGCGATCAACCTCCGTGACCAACA – 3´	20 bp
	Rv: 5´– AAACTGTTGGTCACGGAGGTTGAT – 3´	

Annealing reaction was performed according to Tab 4 and program settings are described in Tab 5.

Table 4. Reaction mixture for gRNA oligonucleotide annealing.

Component	50µl reaction	Final concentration
10 X NEBuffer 3	5 µl	1x
Forward oligonucleotide (100 µmol·l^{-1})	1 µl	2 µmol·l^{-1}
Reverse oligonucleotide (100 µmol·l^{-1})	1 µl	2 µmol·l^{-1}
Nuclease-free water	43 µl	

Table 5. PCR program setting of gRNA oligonucleotide annealing.

Step	Temp [°C]	Time [min]
Initial Denaturation	95	4:00
Annealing	70	10:00
Annealing	From 69 to 4 (1°C/min)	66:00
Final incubation	4	∞

3.2.10.2 Cloning of gRNA into *pSH91* vector

The *pSH91* vector allows cloning the gRNA in frame with the gRNA scaffold whose expression is driven by the OsU3 promoter. This vector contains also the gene encoding the Caspase9 under the control of the ZmUbiquitin1 promoter. The *pSH91* vector (Fig 12) was cut with restriction enzyme *BsaI*-HF (Tab 6) in two positions which lead to a removal of a fragment containing spectinomycin resistance and to the subsequent linearization of the vector.

Table 6. Reaction mixture for restriction of *pSH91* vector by *BsaI*-HF.

Component	50µl reaction	Final concentration
pSH91	4 µl	~ 1µg/reaction
BsaI-HF (20 000 U·ml^{-1})	1 µl	400 U·ml^{-1}
10X CutSmart buffer	5 µl	1X
Nuclease-free water	40 µl	

The reaction mix was incubated overnight at 37°C. Afterwards, the plasmid was purified by NucleoSpin Gel and PCR Clean-up kit following the PCR Clean-up protocol as described in Chapter (3.2.4).

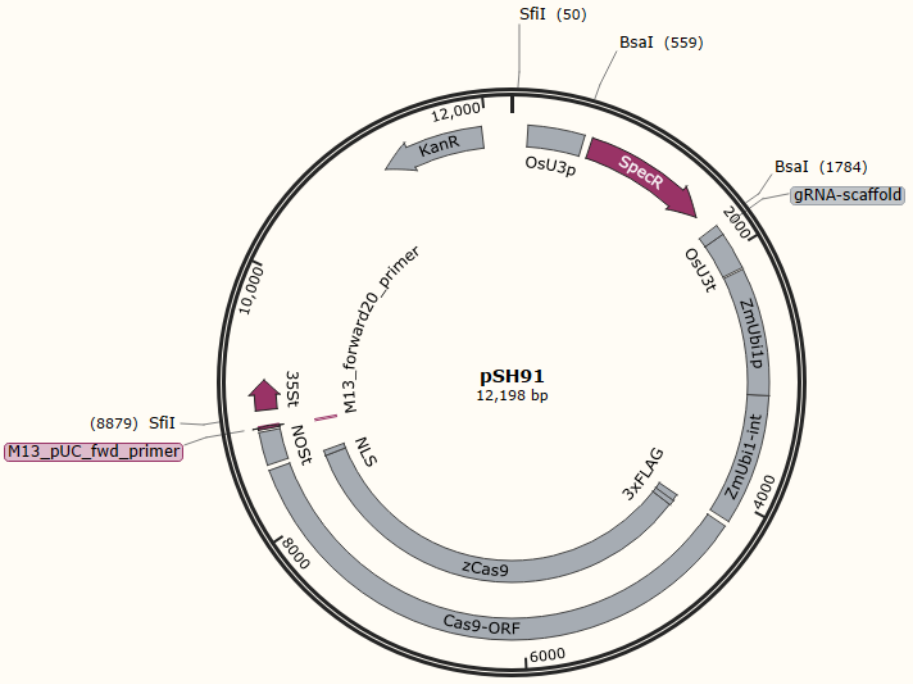

Figure 12. A map of *pSH91* vector. The vector contains *Spec*^{R}, spectinomycin resistance gene; gRNA scaffold, guide RNA scaffold for CRISPR-Cas9 system; OsU3t, rice snRNA U3 terminator; OsU3p, rice snRNA U3 promoter; ZmUbi1p and ZmUbi1-int, maize *Ubiquitin 1* promoter with first intron; zCas9, codon-optimized version of *Cas9*; NOSt, nopaline synthase terminator; 35St, 35S terminator; *Kan*^{R}, *Aminoglycoside Phosphotransferase* gene (kanamycin resistance gene).

3.2.10.3 Preparation of the binary vector for barley transformation

The *pSH91* vectors (*pSH91::gRNA-TGW6a-7Hr1*, *pSH91::gRNA-TGW6a-7Hr2*, *pSH91::gRNA- TGW6b-5Hr1*, *pSH91::gRNA-TGW6a-5Hr2*) and *p6i-d35S-TE9* vector were used for restriction with *SfiI* enzyme (Tab 7). The enzyme cuts at two positions what leads to removing fragments in both vectors.

Table 7. Mixture for restriction of *p6i-d35S-TE9* by *SfiI*

Component	20 µl reaction	Final concentration
p6i-d35S-TE9	4	~ 1 µg/reaction
10x CutSmart buffer	2	1X
SfiI (20 000 U· ml^{-1})	0,5	500 U·ml^{-1}
Nuclease-free water	13,5	

The mixtures were incubated at 50 °C overnight. Digested plasmids were purified by NucleoSpin Gel and PCR Clean-up kit (Chapter 3.2.4) and the length of the products was checked by electrophoresis. Purified *pSH91* vectors were cloned into the linearized *p6i-d35S-TE9* vector.

The fragments of *pSH91::gRNA-TGW6a-7Hr1*, *pSH91::gRNA-TGW6a-7Hr2*, *pSH91::gRNA- TGW6b-5Hr1*, *pSH91::gRNA-TGW6a-5Hr2* containing CRISPR-Cas9 cassette and the gRNA scaffold were cloned into the digested *p6i-d35S-TE9* vector (Tab 8).

Table 8. Reaction mixture of cloning *pSH91::gRNA* into *p6i-d35S-TE9* vector.

Component	10 µl reaction	Final concentration
Digested vector *pSH91::gRNA* (140 ng·µl^{-1})	6,5	~ 100 ng/reaction
Digested vector *p6i-d35S-TE9* (50 ng·µl^{-1})	1,5	~ 7,5 ng/reaction
10x T4 DNA ligase buffer	1	1X
T4 DNA ligase (120 U/µL)	1	12 U·µl^{-1}

The ligation mixture was incubated at 16 °C overnight. Next day, the ligation product was transformed by heat shock into *E. Coli* TOP10 (Chapter 3.2.9). The ligation product is spectinomycin resistant, therefore transformed bacteria were spread over solid LB agar medium supplied with 50 µg·ml^{-1} spectinomycin. The colonies were verified by colony PCR (Chapter 3.2.7).

3.2.10.4 Control restriction

Control restriction of *p6i-d35S-TE9::gRNA-TGW6a-7Hr1, p6i-d35S-TE9::gRNA-TGW6a-7Hr2, p6i-d35S-TE9::gRNA-TGW6a-5Hr1, p6i-d35S-TE9::gRNA-TGW6a-5Hr2* was done in order to check samples before sequencing (Tab 9) using *NheI*/*NotI* restriction enzymes.

Table 9. Composition of the enzymatic reaction used to control the integration of the "Cas9/gRNA" cassette inside the final *p6i-d35S-TE9::gRNA.* The *NheI* and *NotI* restriction enzymes were used.

Component	20 µl reaction	Final concentration
p6i-d35S-TE9::gRNA	0,5	~ 100 ng/reaction
10x CutSmart buffer	1	0,5X
NheI/*NOtI* (20 000 U· ml^{-1})	0,5	500 U·ml^{-1}
Nuclease-free water	8	

3.2.10.5 Preparation and culture of *Agrobacterium tumefaciens*

Verified constructs were transformed into *A. tumefaciens AGL1 strain* which were used for a transformation of barley immature embryo.

Plasmid DNA was added to 50 µl of the *A. tumefaciens* cells, gently mixed and kept on ice for 2 min. Then, the samples were transferred in to ice cold electroporation cuvettes and put into the electroporation chamber in BTX ECM 399 electroporator. High voltage was applied and, 200 µl of SOC media was added to the cells immediately after the pulse. The cells were gently mixed and transferred into a new 1.5 ml tube and incubated for 1 hour at 28°C, shaking 800 rpm. After the incubation, aliquots of the cell suspension were spread over MG/L agar medium containing 100 µg·ml^{-1} carbenicillin and 50 µg·ml^{-1} rifampicin. Bacterial cultures were incubated at 28°C for 48 hours. Afterwards, plasmids were isolated by QIAprep Spin Miniprep kit (Chapter 3.2.8) and purified by NucleoSpin Gel and PCR Clean-up kit (Chapter 3.2.4).

3.2.10.6 Barley immature embryo transformation

Barley immature embryos transformation was done by Bc. Vendula Svobodová according to the protocol by Marthe *et al.* (2015).

One day before the transformation, *A. tumefaciens* culture was prepared. The stock culture of *A. tumefaciens* in glycerol was dissolved in 10 ml of MG/L liquid medium in a 100 ml Erlenmeyer flask and the culture was incubated overnight at 28°C, shaking at 180 rpm. Firstly, the immature caryopses were collected in a 500 ml bottle in an ice bath. The caryopses were sterilized by 70% ethanol for 3 min and then with 4% sodium hypochlorite for 15 min. After this, caryopses had to be kept in sterile

conditions. In the laminar flow cabinet, the caryopses were washed with sterile water five times. The embryos were cut out from caryopses and transferred into a well of six-well plate filled with 3 ml of liquid cocultivation medium (BCCM). Manipulation with barley caryopses were done under a stereo zoom microscope. For the barley transformation, the OD600 of *A. tumefaciens* culture was measured and adjusted to OD600 = 2,5. The BCCM medium was removed from the wells and it was replaced by 600 µl of *A. tumefaciens* culture. Then the plate was transferred, and 500 mbar vacuum was applied for 1 min in a desiccator. Afterwards, the barley embryos were incubated at room temperature for 10 minutes followed by two times washing with 3 ml of BCCM medium for 15 min. Finally, the wells were filled with 3 ml of BCCM and the plate was sealed with parafilm and covered with aluminium foil where barley embryos were incubated for 2 days at 21°C. After incubation, the barley embryos were transferred with the scutellum side down to the Petri dish with a solid callus induction medium (BCIM). The Petri dish was incubated in the dark at 24°C for 2 weeks. Then, embryos were transferred to fresh BCIM and they were incubated for another three weeks. When embryos started to make callus, they were transferred to regeneration medium (BRM) and incubated in an environmental chamber with the photoperiod of 24°C/16 h/light (160 µmol·m^{-2}·s^{-1}) and 22°C/8 h/dark. Callusing embryos were transferred to fresh BRM every two weeks until shoots reached about 3 cm. Then, they were transferred to a plastic box containing BRM and incubated in the conditions of the environmental chamber until developing roots. Afterwards, they were transferred to soil and grown in high humidity conditions in a phytotron (16°C/16 h/light and 12°C/8 h/dark).

3.2.11 RNA isolation and DNaseI treatment

Plant material was collected from a plants of the modern barley cultivar Golden Promise which were grown in a phytotron with 16 hours photoperiod, 16°C/18°C night/day and 60% humidity. Three plants at three developmental stages were chosen: before heading (no heading visible), at the tipping stage and at the heading stage and from each plant tiller was taken in order to harvest ovules (Fig 13). Regarding the stage before tipping, the ovules were unable to remove therefore the florets were used for the experiment and moreover, middle part of flag leaf was

collected from the same plants as well. Samples taken randomly from all three tillers were mixed and sampling was done three times.

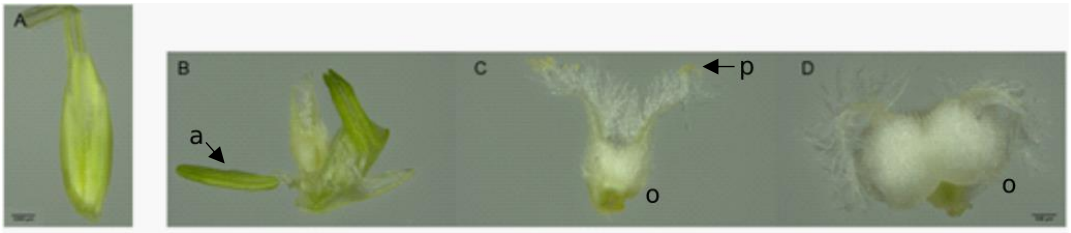

Figure 13. Pictures showing the different development stages of ovules chosen for expression analysis. (A) floret taken from a spike before tipping, (B) dissected ovule from a floret taken from a spike before tipping, a - anther (C) ovule (o) dissected from a floret in the middle part of the spike at the tipping stage, yellow dots are polled grains at the surface of the pistils (indicated by arrow), (D) ovule (o) dissected from a floret in the middle part of the spike at the heading stage. Bars represent in A: 1 cm and in B-D: 500 µm. Pictures were taken with a Zeiss Axio Zoom v16 Stereo Microscope and analyzed with ZEN software (Zeiss).

Samples were harvested directly in the tube with 5 zirconia beads (2 mm, BioSpec Products) and regularly freezed in liquid nitrogen. 1 ml of TRIZOL per 60/100 mg of sample was added and grid for 2 min using a homogenizer machine (Mixer Mill MM 400), 25 mHertz, followed by incubation at room temperature for 5 minutes. After that the samples were centrifuged at 12 000 x g for 10 minutes at 4 °C. Aqueous layer was transferred to a new Eppendorf tube and 200 µl of 100 % chloroform was added. After that, the samples were mixed by vortex until the solution was milky coloured and incubated at room temperature for 10 minutes. Then the samples were centrifuged at 12 000 x g for 15 minutes at 4 °C and aqueous layer was transferred to the new tube. By adding 500 µl of isopropanol, the nucleic acids were precipitated. The samples were incubated at room temperature for 10 minutes. After that, the samples were centrifuged at 12000 x g for 15 minutes at 4 °C and supernatant was removed. 500 µl f 75 % ethanol was added to the pellet and vortexed until the pellet was loose. Samples were centrifuged at 8900 x g for 5 minutes at 4 °C and ethanol was removed as preciously as possible. Centrifugation was repeated at the same conditions but for 2 minutes in order to collect remaining

ethanol and remove it. The pellet was allowed to dry at room temperature for 2 minutes and dissolved in 50 µl of RNase-free water by gently vortexing. RNA concentration was checked with NanoDrop One^{C} spectrophotometer.

DNaseI treatment was proceeded as described in Tab 10. Mixture was gently mixed and incubated in 37°C shaking on the orbiter inside the incubation oven for 30 minutes.

Table 10. DNase treatment

Component	56 µl reaction	Final concentration
Turbo DNase I (2000 U·ml^{-1})	1 µl	35 U·ml^{-1}
10x Turbo Reaction buffer	5 µl	0,8929 X
RNA	50 µl	< 200 ng·µl^{-1}

The aqueous phase was carefully transferred to a fresh tube, 1/10 volume of 3 M sodium acetate (pH 4.8, 40 µl) and two volumes of ice-cooled ethanol (0.8 mL) were added and mixed by inversion several times and precipitated at −20°C over the weekend. The sample was centrifuged at 12000 x g for 20 min at 4°C and the supernatant was removed using a pipette and 500 µl of precooled 70% ethanol were added. The sample was centrifuged at 12000 x g for 5 min at 4°C. The supernatant was discarded and the pellet was allowed to dry at room temperature for 10 min. 30 µl of DEPC-treated H2O (30 µl) was used to dissolve the pellet, and then stored at −80°C for further use. RNA concentration was checked with NanoDrop One^{C} spectrophotometer

3.2.12 cDNA synthesis

In order to validate the absence of gDNA contamination, a PCR using the primers *qTIP41* (Tab 14) was ran as described in table with the following conditions: Initial denaturation: 95°C for 2 min; 35 cycles: 95°C for 30 sec, 58 °C for 30 sec, 72°C for 30 sec; final extension in 72°C for 5 min.

cDNA synthesis was performed with 2 µg total RNA as estimated from nanodrop with RevertAidH minus reverse transcriptase following manufacturer instructions. First a volume of RNA corresponding to 2 µg was mixed with 1 µl of oligo(dT)18 (100 µM) and the volume was adjusted with water to 13 µl. In order to avoid any secondary structures, RNA was incubated for 5 min at 65°C (in

thermocycler), immediately chilled on ice, briefly centrifuged and placed back on ice. The mixture was prepared as described in Tab 11, added to each sample and mixed by pipetting.

Table 11. Reaction mixture for cDNA synthesis using RevertAid H Minus RTase.

Component	Final concentration
5X Rection Mix	1X
10 $mmol \cdot l^{-1}$ dNTP	100 $\mu mol \cdot l^{-1}$
200 $U \cdot \mu l^{-1}$ RevertAid H Minus RTase	10 $U \cdot \mu l^{-1}$
RNA	2 ng/reaction

Prepared synthesis reaction was incubated at 42 °C for one hour. After that, the enzyme was inactivated at 65 °C for 20 minutes. cDNA was stored in -20°C.

3.2.13 Probe based quantitative PCR

The qPCR mix was prepared for every sample according to the Tab 12.

Table 12. Reaction mixture for qPCR

Component	10,8 µl reaction	Final concentration
Luna buffer	5 µl	1X
Forward primer (10 $\mu mol \cdot l^{-1}$)	0,8 µl	0,4 $\mu mol \cdot l^{-1}$
Reverse primer (10 $\mu mol \cdot l^{-1}$)	0,8 µl	0,4 $\mu mol \cdot l^{-1}$
Probe (10 $\mu mol \cdot l^{-1}$)	0,4 µl	0,2 $\mu mol \cdot l^{-1}$
cDNA template	2 µl	<100 ng
Milli-Q H_2O	1 µl	

For cDNA level normalization, two reference genes were chosen – *actin* and *HORVU2Hr1G108490* (Serine/threonine-protein kinase) (Tab 13). The qPCR amplification was performed according the following parameters: 50°C for 2 min, 95°C 10 minutes; 40 cycles: 95°C for 15 sec, 60 °C for 1 min.

Table 13. List of primers used for qPCR.

Primer name	Sequence	Tm [°C]
qTGW6a	5´ AGAAGAATGAGCTCCCCTTTGG 3´	66,8
	3´ TCCCATGAACAATTTGCCGC 5´	70,3
Probe	5´ AGCGTGAGGCCGACCGAGGTGGTGGA 3´ [FAM/BHQ1]	83,7
qTGW6b	3´ ATACTGGATCAAGGGCTCCAAG 5´	65,2
	5´ TTTTCACGGTGTAGTGCCAC 3´	63,6
Probe	3´ GCGGGCACGATGGAGCTATTCGCCGACCT 5´ [FAM/TAMRA]	84,3
HORVU2Hr1G108490	5´ AGCACTCTTTGGACAACCAC 3´	61,8
	3´ AGCTCTGCCATATATCTGTGCC 5´	64,2
Probe	5´ TCACCACCATCGAAGCCGCCGGAACTGCT 3´ [FAM/TAMRA]	84,7
Actin	5´ TGTTGACCTCCAAAGGAAGCTATT 3´	66,0
	3´ GGTGCAAGACCTGCTGTTGA 5´	66,9
Probe	5´ TGTAGTATTCAGCTGGTTGGTGGCACAG 3´ [FAM/BHQ1]	73,0

3.2.14 Identification of natural variation in *HvTGW6* gene

3.2.14.1 Plant material

Landrace collection is collection of 1485 accessions at IPK Gatersleben (Germany) containing 2- and 6-raws type barley. From these, 648 accessions are considered core collection (308 2-raw type and 340 6-raw type). For our purpose, a smaller subset based on genetic diversity (including "Ethiopian group") was used. In this subset, there are 132 accessions referring to the barley cultivars collected in Europe, Africa and Asia.

3.2.14.2 Grain length measurements

To determine grain characteristics, landrace collection of spring barley containing 132 accessions was used. Grains were scanned using flattbed scanner Epson perfection V700 photo, using the following parameters: 24-bit color, 300 dpi resolution, reflective document type, professional mode. Pictures were analysed with GrainScan software (Whan *et al.*, 2014) which determines area, perimeter [mm], length [mm], width [mm] and ration [L/W] for each scanned grain. Subsequently, the average of all parameters was calculated with focus on grain length using MS excel.

3.2.14.3 gDNA extraction and purification

Seeds from 132 accessions from landrace collection were sterilized with 70% ethanol for 30 sec and 4 minutes in 4% NaOCl. Afterwards, seeds were washed four times in sterile H_2O. Such prepared seeds were placed into Petri dishes with wet cotton wool and stratified in fridge (4°C) for three days. After that, the Petri dishes were placed for 5 days into phytotron with 16 hours photoperiod, 16°C/18°C night/day and 60% humidity. From plants, only green parts were collected for DNA extraction. Plant material was frozen with liquid nitrogen and stored in -80°C.

A small piece of tissue (approximately 1 mm^2) was transfer to 1,5 ml Eppendorf tube together with two tungsten beads, 300 µl of DNA extraction buffer II and 100 µl of chloroform-IAA (24:1, v:v). Samples were shaking two times for 1 minutes at 25 rpm in mixer. After that, samples were transferred to the centrifuge and spin for 3 minutes at 20 000 x g. Supernatant was transferred to the new Eppendorf tube and 700 µl of 96% ethanol was added. Samples were mixed by inverting by several times immediately after adding ethanol and after that they were incubated 5 minutes in room temperature. After incubation samples were centrifuged for 15 minutes, 14 000 x g at room temperature and supernatant was removed. 70 µl of 70 % ethanol was added and samples were centrifuged for 15 minutes, 20 000 x g at room temperature. Supernatant was removed and 50 µl of TE buffer was using for dissolving gDNA. Concentration was measured by NanoDrop One^C spectrophotometer. The PCR mix was prepared according to Tab 15 with primers screenTGW6a (Tab 14). The PCR reactions were run on thermocycler according the following parameters. Initial denaturation: 95°C for 2 min; 35 cycles: 95°C for 30 sec, 60 °C for 30 sec, 72°C for 1 min; final extension in 72°C for 2 min. Tm for each primer pair is described in table X. The PCR reaction was loaded on 1% agarose gel containing ethidium bromide and ran in 1X TAE at 90 volts for 30 min. Gels were visualized using Gel Doc EZ Gel Imaging System (Bio-Rad, USA).

PCR products were purified using polyethylene glycol (PEG) before sequencing. To the PCR products there was added he same amount of PEG solution (20 % PEG, 2,5 $mol \cdot l^{-1}$ NaCl). Followed by 1hour long precipitation at room temperature. After that, the samples were centrifuged at maximal speed at room temperature,

supernatant was discarded and to the pellet 1 ml of 70% ethanol was added. Followed by centrifugation at maximal speed for 15 minutes at room temperature. Supernatant was discarded and pellet was allowed to dry at room temperature. Finally, the pellet was dissolved in 15 µl of H_2O and DNA concentration was measured NanoDrop One^C spectrophotometer.

3.2.15 Tables

Table 14. List of primers

Primer set	Primer sequence (5´-3´)	Tm [°C]
TGW6a, forward	GGATCGTCCTAGCGAAGCAA	66,5
TGW6a, reverse	TCCCAACAGTGAAAAAGCAAGC	67,2
TGW6b, forward	AGCTCTCGAAACAGCGAAGT	63,2
TGW6b, reverse	ACGCATGCCCTAGGAAAAGC	67,3
OsU3P, forward	CAGGGACCATAGCACAAGAC	62,2
Osu3T, reverse	TCAGCGGGTCACCAGTGTTG	70,1
M13, reverse	GGAAACAGCTATGACCATGA	60,6
M13, forward	TGTAAAACGAGGGCCAGTG	64,1
pMiniT 2.0 vector, forward	ACCTGCCAACCAAAGCGAGAAC	70,6
pMiniT 2.0 vector, reverse	GCGAGTACTCTGTTATTGGGACT	62,7
pSH91, reverse	AATGTGGCGCCGTAAATAAG	63,9
Cas9, forward	TGGTTAGGGCCCGGTAGTTC	67,3
Cas9, reverse	TTAATCATGTGGGCCAGAGC	65,3
Hygromicin, forward	GAATTCAGCGAGAGCCTGAC	64,5
Hygromicin, reverse	ACATTGTTGGAGCCGAAATC	64,2
seqTGW6a (T_0), forward	GGGTCGTACCTCATTAGCCG	65,5
seqTGW6a (T_0), reverse	GCTCAAGTGGAACGGCGA	67,8
seqTGW6b (T_0), forward	CTCTCCGTGGCCGTCTCC	68,6
seqTGW6b (T_0), reverse	GACCCGTCCGCCGTTATTAT	66,7
ScreenTGW6a, forward	CAAGTCAACAATGGCGAGCG	57,3
ScreenTGW6a, reverse	ACGACGGACACATAGCGAAG	57,2
qTIP41, forward	TGGTTGGTTTCTGCTCTTGC	65,7
qTIP41, reverse	CGGCTTTGCTTCCTCCTTAC	65,2

Table 15. PCR mixtures (from left): amplification of TGWa and TGW6b in order to clone and characterize putative of HvTGW6; colony PCR after cloning TGW6a and TGW6b into vectors; colony PCR for verification during CRISPR-Cas9 construct preparation; control PCR with RNA in order to detect DNA contamination after DNase treatment; screen PCR in order to identified T_0 plants with cloned CRISPR-Cas9 construct and amplification of targeted sequencies in order to detect mutations; screen PCR of landrace collection of barley in order to identified natural variations in TGW6a sequencies

Component	Amplification: TGW6a, TGW6b	Colony PCR: TGW6 cloning	Colony PCR: CRISPR/Cas9	Control PCR: RNA	Screen PCR: Osu, Cas9, hpt, oligo.	Screen PCR: TGW6a
GoTaq Flexi DNA polymerase	-	0,01 $U \cdot \mu l^{-1}$	0,025 $U \cdot \mu l^{-1}$	1.639 $U \cdot \mu l^{-1}$	0,05 $U \cdot \mu l^{-1}$	0,025 $U \cdot \mu l^{-1}$
Phusion DNA Polymerase	0,02 $U \cdot \mu l^{-1}$	-	-	-	-	-
5X Green Go Taq Flexi Buffer	-	1X	1X	1,639 X	1X	-
5X colourless Go Taq Flexi Buffer	-	-	-	-	-	1X
5X Phusion GC/HF Buffer	1X	-	-	-	-	-
DMSO	-	-	-	-	2%	-
$MgCl_2$	-	2500 $\mu mol \cdot l^{-1}$	2500 $\mu mol \cdot l^{-1}$	1640 $\mu mol \cdot l^{-1}$	2500 $\mu mol \cdot l^{-1}$	2000 $\mu mol \cdot l^{-1}$
dNTPs	200 $\mu mol \cdot l^{-1}$	100 $\mu mol \cdot l^{-1}$	250 $\mu mol \cdot l^{-1}$	328 $\mu mol \cdot l^{-1}$	500 $\mu mol \cdot l^{-1}$	200 $\mu mol \cdot l^{-1}$
Forward Primer	0,5 $\mu mol \cdot l^{-1}$	0,5 $\mu mol \cdot l^{-1}$	0,25 $\mu mol \cdot l^{-1}$	0,328 $\mu mol \cdot l^{-1}$	0,5 $\mu mol \cdot l^{-1}$	0,5 $\mu mol \cdot l^{-1}$
Reverse Primer	0,5 $\mu mol \cdot l^{-1}$	0,5 $\mu mol \cdot l^{-1}$	0,25 $\mu mol \cdot l^{-1}$	0,328 $\mu mol \cdot l^{-1}$	0,5 $\mu mol \cdot l^{-1}$	0,5 $\mu mol \cdot l^{-1}$

3.3 RESULTS

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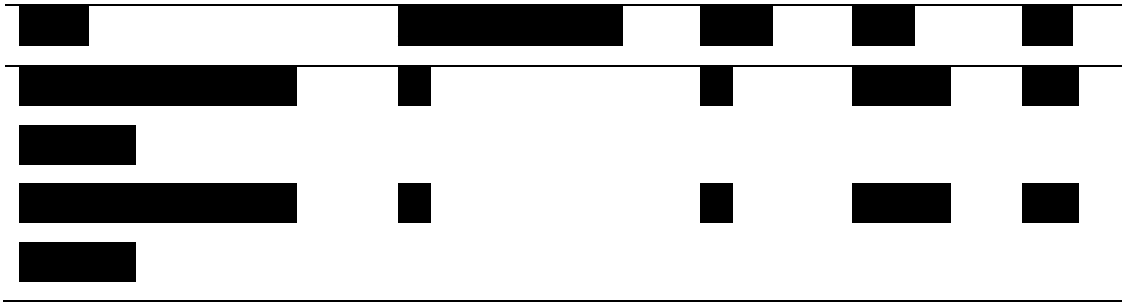

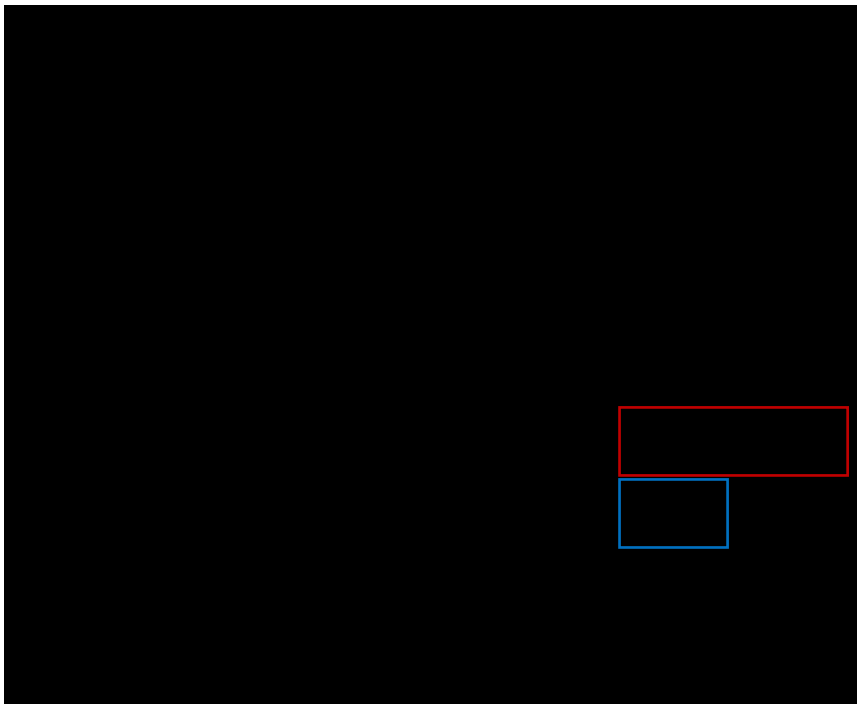

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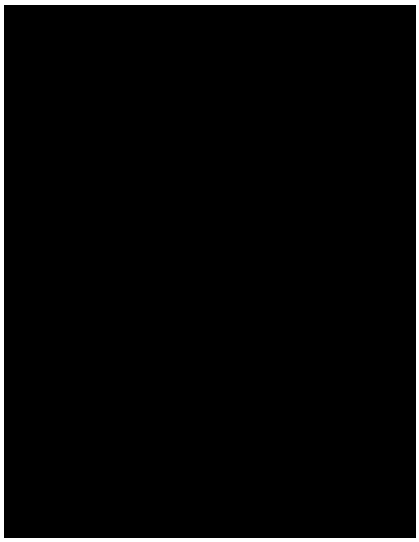

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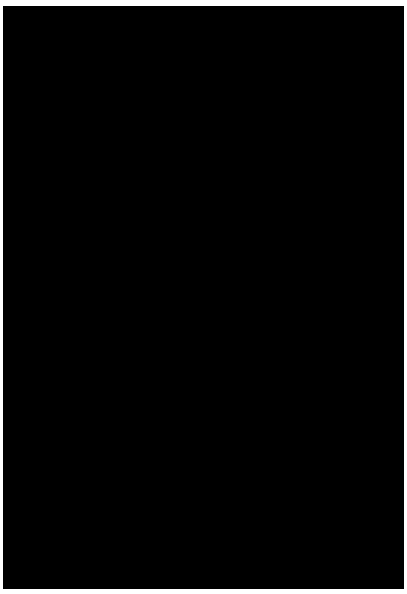

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4 DISCUSSION

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5 CONCLUSION

In the theoretical part of this thesis, there was summarized why it is important to study new genes in order to increase yield of important crops, current knowledge about TGW6 and CRISPR-Cas9 genome editing technology.

In the experimental part, we reported several approaches in order to study TGW6 in barley. We identified, that *TGW6a* is located on chromosome 7, sharing 91 % homology with OsTGW6 including strictosidine synthase-like domain and GFPase residues which are important for the activity of the protein. The qPCR results showed that *TGW6a* is highly expressed in ovule during tipping and in flag leaf before tipping, whereas its expression in the floret before tipping or during heading is significantly lower and barely detectable. The second candidate, *TGW6b*, localized on the chromosome 5 was not expressed in any of the samples analysed, suggesting that it is not involved in grain size development despite its high homology with TGW6a. Based on the experimentally obtained sequences, we predicted an endoplasmic reticulum localization of the protein. Further, using a spring barley landrace collection, 9 haplotypes of TGW6a were identified could be sorted into 3 groups based on amino acid sequence. The polymorphism could have an important effect on the functionality of the protein. Unfortunately, we were not able to find a relationship between haplotypes and grain size. Finally, we initiated the CRISPR-Cas9 mediated knock-out of *TGW6a* and *b*: we successfully prepared seven T_0 generation plants showing apparent mutations in targeted site and phenotypic characterization will be determined in a close future.

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7 LIST OF ABBREVIOTIONS

cDNA	Complementary DNA
CRISPR	Clustered repetitive interspaced palindromic repeats
crRNA	CRISPR RNAs
CKX	Cytokinin dehydrogenase
DAA	Days after anthesis
DAP	Days after pollination
DNA	Deoxyribonucleic acid
DSBs	Double-stranded breaks
HR	Homologous recombination
IAA	Indole-3-acetic acid
LDL	Low density lipoprotein
MT	Mega tonne
NHEJ	Non-homologous end joining
NIL	Near-isogenic lines
NLS	Nuclear localization signal
PAM	Protospacer-adjacent motifs
QTL	Quantitative trait locus
RNA	Ribonucleic acid
sgRNA	Single guide RNA
SNP	Single-nucleotide polymorphism
TALEN	Transcription activator-like effector nucleases
TGW	Thousand grain weight
tracrRNA	Transactivating CRISPR RNA