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Identification of parent-to-offspring transfer of epigenetic memory in Norway spruce (*Picea abies* (L.) Karst.)

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Abstract

Conifers that live in temperate and boreal areas such as Norway spruce (*Picea abies*) must enter dormancy to survive the coldness and the time of darkness while they are maximizing the growth. Dormancy is a temporary condition in which growth and development have virtually ceased. It usually begins at the end of the growing season and budding. Day length, also called photoperiod, is used as a signal to introduce this phenomenon. For several tree species, a long photoperiod is necessary to resume growth and start bud formation, for other species, a shorter period may be sufficient. To continue the growth after dormancy, other environmental factors than light are involved, including temperature. Temperature is an important environmental factor that helps to regulate dormancy and regrowth. By stressing the plants with temperature during embryogenesis, changes in the phenology have been observed in several studies. These observations are due to changes in the expression of genes that help regulate the phenotype. The expression of genes, also called epigenetics, can occur differently in epi-temperature plants, this makes it interesting to study whether epi-temperature plants express different epigenetic traits. If the same phenological features occur over time, an epigenetic memory has taken place. This means that the stress factor led to a "permanent" change in the expression of genes. This study, it is investigated whether the F1-generation has such phenomena and whether the phenomenon is still present in the original plants. In order to look at this, a DNA methylation method, bisulfite sequencing, was used to sequence and to detect methylation patterns. Registration of bud development, called phenotyping, was also performed as a method to see if phenological features are different in F1-generation plants. The plants referred to as the F1-generation was planted for this project. The mother plants were pollinated under controlled conditions where different mothers and fathers were represented and crossed with each other. This resulted in the F1-generation consisting of several different crosses. To ensure that the pollination took place as expected, a genotyping was performed in which the genome of the F1-generation was sequenced using SSR markers and the results were used for PCA.

Results from several analyses show that there are relevant differences in the bud phenology between the seedlings. The phenotyping could establish a significant difference between several of the different crossings during the critical photoperiod and the photoperiod, which consisted of 24 hours of light. This indicates that the seedling may have inherited an epigenetic memory from the parents, as in the case of epigenetically different but genetically identical mothers with the same father resulting in phenological differences in the offspring.

Other crosses point to classical inheritance, as exemplified by the same mother but different fathers (late and early flushing) who give phenotypically different offspring as expected. The bisulfite sequencing was performed with cross 2 and 13; this indicates that the epigenetic methylations are reflected in the F1 generation, but more solid sequencing data is needed to establish this. The genotyping analyses resulted in weak percentages of variance, making it challenging to observe significant differences between individuals. On the other hand, it is confirmed that the F1 generations are closely related to the parents.

Sammendrag

Bartrær som lever i tempererte og boreale området slik som gran (*Picea abies*) må gå i vinterdvale for å kunne overleve minusgradene og mørketiden, samtidig skal de maksimere veksten. Vinterdvale er en midlertidig tilstand hvor vekst og utvikling er tilnærmet opphørt. Den blir vanligvis innledet i slutten av vekstperioden og knoppsetting. Daglengde, også kalt fotoperiode, blir brukt som et signal for å introdusere dette fenomenet. For flere trearter er en lang fotoperiode nødvendig for å kunne gjenoppta veksten og starte knuppdannelse, mens for andre arter kan en kortere periode være tilstrekkelig. For å kunne videreføre veksten etter dvale er det flere miljøfaktorer enn lys som spiller inn, blant annet temperatur. Temperatur er en viktig miljøfaktor som er med på å regulere vinterdvale og gjenvekst. Ved å stresse plantene med temperatur under embryogenese er det i flere studier observert endringer i fenologien. Disse observasjonene skylles endring i uttrykkelsen av gener som er med på å regulere fenotypen. Uttrykkelsen av gener, også kalt epigenetikk, kan forekomme forskjellig i planter som blir stresset, dermed er det interessant å undersøke om temperaturstressede planter utrykker forskjellige epigenetiske trekk. Ved å undersøke stressede planter over tid og samme fenologiske trekk forkommer, kan man si at planten ha oppnådd en epigenetisk hukommelse. Dette vil si at stressfaktoren førte til en «permanent» endring i uttrykkelsen av gener. I denne studien blir det undersøkt om F1-generasjon har slike fenomen og om fenomenet fremdeles er til stede i de originale plantene. For å kunne undersøke dette ble det brukt en DNA metylerings metode, bisulfite sekvensering, hvor ønsket sekvens blir sekvensert for å detektere metylerings mønstre. Registrering av knuppdannelse, kalt fenotyping, ble også gjennomført som en metode for å se om fenologiske trekk er forskjellig hos F1-generasjon planter. Plantene som omtales som F1-generasjon ble plantet med formål om dette prosjektet. Moderplantene ble pollinert under kontrollerte omgivelser hvor ulike mødre og fedre ble representert og krysset med hverandre. Dette resulterte i at F1-generasjonen består av flere forskjellige krysninger. For å sikre at pollineringen har skjedd som forventet ble det gjennomført en genotyping hvor genomet til F1-generasjonen ble sekvensert ved hjelp av SSR markører og resultatene ble brukt til PCA.

Resultater fra flere av undersøkelsene viser at det er relevante forskjeller i knoppfenologien mellom frøplantene. Fenotypingen kunne konstatere at det var en signifikant forskjell mellom flere av de ulike krysningene både under kritisk fotoperiode, og fotoperiode som besto av 24 timers lys. Dette indikerer at frøplanten kan ha arvet et epigenetisk minne fra foreldrene sine, som i tilfellet med epigenetisk forskjellige, men genetisk identiske mødre med samme far, noe som resulterte i fenologiske forskjeller hos avkommet. Andre kryss peker på

klassisk arv, som eksemplifisert av samme mor, men forskjellige fedre (sen og tidlig spyling) som gir fenotypisk forskjellige avkom som forventet. Bisulfite sekvenseringen ble gjennomført med krysning 2 og 13, her kan man få en indikasjon på at de epigenetiske metyleringene reflekteres i F1-generasjonen, men det er nødvendig med mer solide sekvenseringsdata for å etablere dette. Forsøket med genotypingen resulterte i svake varians prosenter som gjør det vanskelig å observere store forskjeller mellom individene. Det blir derimot bekreftet at F1-generasjonene er nært beslektet med foreldrene.

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Abbreviations

CE	Cold epitype
LD	Long day
PCA	Principal coordinates analysis
RAM	Root apical meristem
SAM	Shoot apical meristem
SD	Short day
WE	Warm epitype

Keywords

Epigenetic memory, Norway spruce, DNA methylations, Bud phenology, Photoperiodism

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1.0 INTRODUCTION

1.1 Norway spruce

Norway spruce (*Picea abies* (L.) Karst.) is one of the major tree species in the boreal temperate zones in Europe. In Europe, coniferous species play an essential role both from an economic and ecological perspective. Norway spruce covers a significant geographical area in Europe and have also been planted outside its natural range, like Denmark and Scotland (**Figure 1**). This plant can be observed from sea level up to 2300 meters above the sea level and can be found in nearly all ecosystems. Altogether, the species covers 30 million hectares in Treebreedex counties, which is 38 % of the coniferous area (Jansson, 2013).

Figure 1*: Geographical representation of Norway spruce in Europe from 2017 (Farjon, 2017). The coniferous species extends from northern Finland to southern Bulgaria. As well as from Kazan (Russia) in the east to Stavanger (Norway) in the west.*

Plants that grow in temperate and boreal zones must cope with changing temperatures. With temperatures between frost and 20 degrees, the plants need to develop processes for acclimatization (Kohmann and Johnsen, 1994, Dogramaci, 2015, Welling and Palva, 2006). Cold tolerance is a trait that has been evolved in response to various environmental signals. Due to the plants' ability to acclimatize, they can survive colder periods during the growing season, and with a subsequent temperature increase after a cold period, they can rapidly de-acclimate. Such processes are essential for plants to survive in zones with large, changing temperatures (Welling and Palva, 2006, Dogramaci, 2015). For perennials, it is also necessary to survive the winter season. Therefore, this dynamic process that controls dormancy and frost tolerance is

essential. This process ensures that the start and end of overwintering are precisely controlled (Welling and Palva, 2006). Several studies have shown that different genes take part in frost tolerance and control of the development of this phenomenon (Busov et al., 2016, Carneros et al., 2017). In this project, we want to look at genes expressed in the shoot apical meristem (SAM) in Norway spruce, see *1.5 Genes for DNA methylation pattern differences*.

A meristem is a localized region of tissues that adds new cells to the plant or a plant part by cell division. The activity of such a region increases the length or diameter of the plants. After cell growth and differentiation, such tissue areas will form organs like sporophylls, stipules, flower parts, leaves, Etc. Some meristems are self-perpetuating and can therefore be "permanent" meristems. Apical meristems and vascular cambium are examples of "permanent" meristems. Meristems that contribute to the formation of, for example, leaves and flower parts cease to function when these organs reach a specific size or shape (Beck, 2010).

Apical meristems are created at the top of the shoot apex, the most distal area of a stem or side branch (**Figure 2**). They are also found near the tip of the roots, just behind the root cap (**Figure 2**). The apical meristem that exists on the shoots is called shoot apical meristem (SAM). This area is the source of all organs above ground, such as leaves, flowers, and buds. The cells in SAM also function as stem cells to the surrounding peripheral regions. The second type of tissue created at the tip of the roots is called root apical meristem (RAM). The primary function for this tissues is the growth of roots, as well as being a reservoir for stem cells to replenish what is damaged or lost (Beck, 2010, Uchida and Torii, 2019).

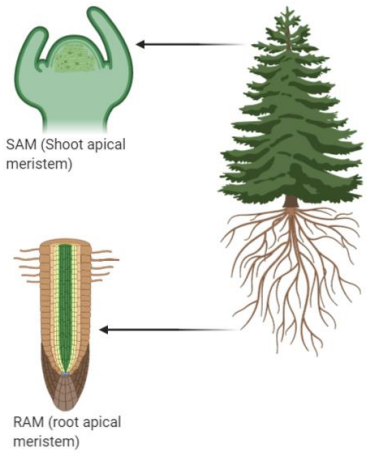


***Figure 2**: Where the shoot apical meristem and root apical meristem are located on the plant. The figure is based on a description made by Beck and Uchida and Torii (Beck, 2010, Uchida and Torii, 2019).*

1.2 Dormancy

Trees begin to prepare early for winter. After a growth period, the apical bud meristems begin to cease in response to the shortening of the day in late summer/early autumn (Busov et al., 2016, Dogramaci, 2015, Rohde and Bhalerao, 2007). Rohde and Bhalerao defined dormancy as; "the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favorable conditions" (Rohde and Bhalerao, 2007). Dormancy can be divided into three categories ecodormancy, endodormancy, and paradormancy. Ecodormancy is a temporary halt of growth caused by environmental pressures. This arrest is triggered when the stressful situation is over, such as a high temperature-induced halt in summer growth. Endodomancy is the deep sleeping state that is induced by periodic seasonal stress. Unlike ecodormancy, endodormancy cannot be reversed immediately, but it does require specific conditions such as chilling requirement. Paradormancy in most perennials occurs only in lateral buds during the growing season and can be reversed by decapitation. The different types of dormancy are often related and their interactions are essential during endodormancy (Dogramaci, 2015).

After only a few weeks, the apical and axillary buds will have come into endodormancy. At the same time as the growth slows down, the freezing tolerance in the trees begins to increase. As the trees are exposed to long-term short days (SD), the frost tolerance will increase significantly. With exposure to low and freezing temperatures, freezing tolerance will be improved, which is important for developing maximum frost tolerance. At the same time as frost hardness increases, low and zero temperatures will stimulate the release of buds from endodormancy, this results in buds that are hardened and ecodormant. Such buds will maintain a cured state of rest until the growth conditions are favorable. At an increased average temperature, the freezing tolerance will gradually decrease (Dogramaci, 2015, Welling and Palva, 2006). For the buds to start growing again, they depend on a certain heat sum. The timing of such processes is synchronized with the environment around the planet. If this synchronization is assumed to be poor, it can lead to damage that affects the buds at later stages of development (Busov et al., 2016).

The onset and release of buds is controlled by many independent genes, which indicates that dormancy is polygenic. So far, there is too deficient understanding about the mechanisms and genes that control this phenomenon (Busov et al., 2016).

1.3 Epigenetics

Plants are eukaryotic organisms that can take advantage of epigenetic regulation. There are several known mechanisms, and the most studied is DNA methylation. DNA methylation occurs in three sequence contexts, CHH, CHG, and CG (H represents A, T, or C), and reflects a balance between enzyme activities that introduce, maintain or remove methyl groups from the cytosines. Histone modification also affects the epigenetics of plants to the extent that these enzymes are encoded by relatively large gene families, enabling diversified functions. Other mechanisms such as acetylation, phosphorylation, ubiquitylation, and sumolaytion are also epigenetic mechanisms that have been identified (Weinhold, 2006, Pikaard and Mittelsten Scheid, 2014).

The appearance of epigenetic regulation in plants reflects their lifestyle, mode of development, and evolution. Unlike other eukaryotic organisms, plants regularly produce and grow new organs from self-supporting stem cells contained in the meristems. Such post-embryonic development takes place continuously and is constantly shaped by environmental cues. Plants are sessile organisms; they cannot escape the environment and are forced to deal with changes in the environment and growth conditions. Because of such circumstances, epigenetic regulatory mechanisms help to facilitate the changes in gene activity and fine-tune gene expression patterns. This allows plants to reproduce and survive in unfamiliar environments (Pikaard and Mittelsten Scheid, 2014, Iwasaki and Paszkowski, 2014).

1.3.1 Epigenetic memory

"*An epigenetic memory is defined as a heritable change in gene expression or behavior that is induced by a previous developmental or environmental stimulus*" (Yakovlev et al., 2016). Epigenetic memory that regulates bud phenology and cold acclimatization in Norway spruce, has been documented in studies where zygotic embryogenesis embryos from the same crosses were growing in mother plants in greenhouses compared with outdoors. Photoperiod and temperature during zygotic development led to changes on the epigenetic patterns in the embryos, resulting in different epitypes. Such changes can, cause bud bursts or bud sets shift in timing. If the environment around the embryos is warmer, this will delay the outbreak compared to a colder environment (Yakovlev et al., 2016, Carneros et al., 2017). In the article written by Carneros et al. it was shown that 329 of 735 genes encode putative epigenetic regulators.

Several of these regulators were related to DNA and histone methylation and may thus be important for the epigenetic memory (Carneros et al., 2017).

1.4 DNA methylation

DNA Methylation are an addition of a methyl group on the C5 position of the cytosine to form 5-methylcytosine (**Figure 3**). This important epigenetic mechanism is involved in genome stability, genomic imprinting and transposable element (TE) silencing. In promoters, DNA methylation plays a critical role in growth and development, such as regulating gene expression (Bartels et al., 2018, Moore et al., 2013, Valledor et al., 2007). The DNA methyltransferases (DNMTs) family is the reason for a methylations group to be transferred to a cytosine. DNMTs catalyzes DNA methylation to transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5-methyl-cytosine (Moore et al., 2013, Zhang et al., 2018).

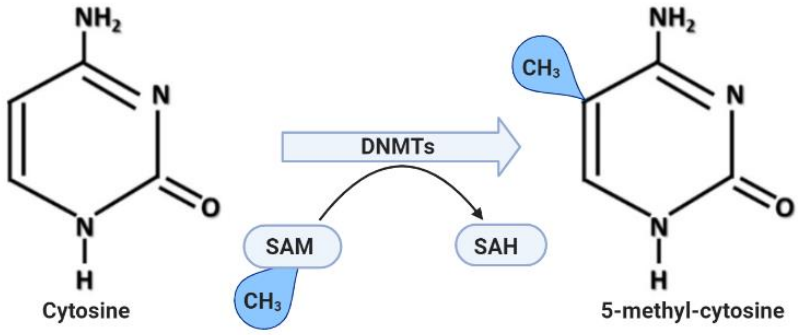


Figure 3: *Illustration of how a cytosine can be methylated. DNMTs ensure that a methylation group is transferred to a cytosine. The figure is based on descriptions from Moore et al. (Moore et al., 2013).*

A specific DNA methylation state reflects the maintenance, dynamic regulation of the establishment, and active removal. These activities are catalyzed by different enzymes that target specific genomic regions by different pathways. Plant DNA methylation occurs in all contexts of cytosine sequences: CG, CHG, and CHH (Zhang et al., 2018, Henderson et al., 2010). The first comprehensive DNA methylation mapping of an entire genome was performed on the plant *Arabidopsis thaliana*. This is also the plant with the best documentation for DNA methylation (Vanyushin, 2008). In *A. thaliana*, genomic DNA methylation is characterized by heavy methylation in heterochromatin, which is enriched with transposable elements (transposons) and other repetitive DNA sequences (Zhang et al., 2018).

1.4.1 Techniques for detecting DNA methylation

Bisulfite conversion is a technique for detecting DNA methylations. This method was developed by Frommer et al. (1992) and is considered the gold standard of DNA methylation analysis precisely because it provides a quantitative, qualitative and effective approach to identifying 5-methylcytosine at a single base-pair solution (Leontiou et al., 2015, Henderson et al., 2010, Li and Tollefsbol, 2011, Frommer et al., 1992). This treatment with sodium bisulfite will convert unmethylated cytosines to uracil's, which will become thymines in the subsequent PCR. Those cytosines that are methylated will remain unchanged (**Figure 4**) (Henderson et al., 2010). This convention will turn the usually undetectable epigenetic information into detectable sequence information. The methylation status can be determined by using either direct sequencing after PCR amplification or cloning sequencing. Compared with other DNA-methylations methods, Bisulfite-based DNA-methylations have more quantitative accuracy, high efficiency, and detection sensitivity. One of the reasons for this claim is that by comparing other DNA methylation-based methods on the sensitivity of restriction enzymes that can specifically recognize methylated cytosines within the cleavage recognition site, bisulfite-based DNA methylation has been better (Leontiou et al., 2015, Li and Tollefsbol, 2011).

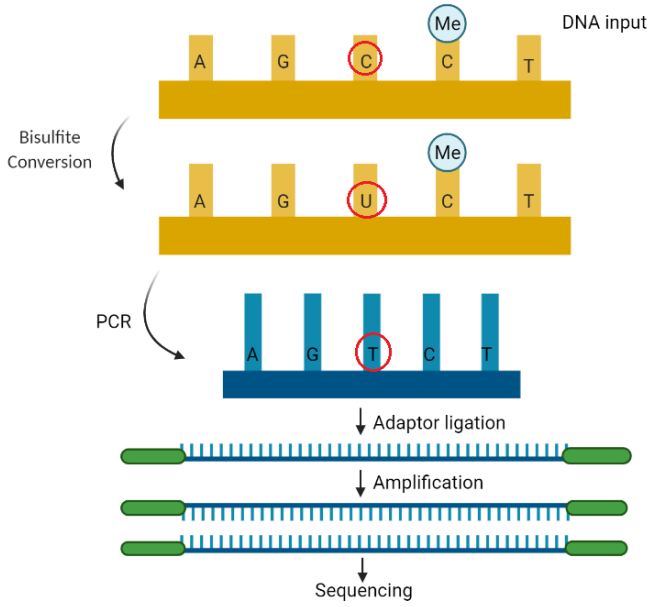


Figure 4: *From DNA to bisulfite sequencing. DNA with a methylation group will first be converted to DNA bisulfite DNA then the PCR will turn the uracil's to thymine's. Then an adaptor ligation will happen before an amplification, when this is done it is time to sequencing the sample. The red circles highlight what has happened to unmethylated cytosine.*

Bisulfited-modifying DNA is primarily the requirement for almost all DNA methylation analysis methods. Some of them require reinforcements to be able to study methylation at specific sites in the genome. Often, amplification of bisulfite-modified DNA is the most challenging part. PCR methods such as touch-down or nested PCR are often used in such samples. Regardless of the pre-optimized method, it is required that you determine your own annealing temperature based on what is best for your sample. In addition, some methods require reinforcement using nested primers. The technique of touch-up (TU) gradient PCR, which is described by Rowether et al., should be a method that requires minimal optimization and can produce specific products (Rowther et al., 2012).

1.5 Genes for DNA methylation pattern differences

As previously known, Norway spruce contains a large number of interesting genes. Through a series of analyzes from several project groups, a platform called, ConGenIE, has been formed. ConGenIE is a webpage for the Norway spruce genome project, genome assembly and expression data. This page easily finds genes and gene functions that already have been analyzed and well documented. Later in the thesis, it will be explained why the genes MA_10344604g001 and MA_8008099g001 were used during analysis.

At ConGenIE the gene known as MA_10344604g001 has the function; Homebox domain. Homebox is a globular DNA sequence found in genes involved in the regulation of patterns of morphogenesis in, among other organisms, plants. These genes encode transcription factors that help DNA bind to regulate the expression of target genes (Bürglin and Affolter, 2016). Homebox domain proteins also help regulate gene expression and cell differentiation during early embryonic development, which means that mutations in these genes can lead to developmental disorders.

MA_8008099g001 is known as a Ribonuclease III domain (RNase III). RNase III is a type of ribonuclease that contributes to the maturation of ribosomal and other structural RNAs. The involvement of RNase III in processing with ribosomal RNA makes it easier for the cell to produce more ribosomal components that are essential. The ability to cleave transcripts allows RNase III to act as both an inducer and repressor of cellular functions, and the cleavage of transcripts within coding regions enables RNase III to eliminate functional transcripts directly.

Based on these functions, these genes are considered attractive to analyze in the context of this experiment.

1.6 Microsatellites

Whole-genome sequencing can reveal the genetic relatedness between individuals. One of the goals for sequencing is to identify genetic variation among individuals, which can be utilized to develop genetic markers. Although technology is evolving so other genetic markers can be used, microsatellites are still the most used markers in this field. Microsatellites, also known as simple sequence repeats (SSRs), are highly informative genetic markers. These markers have been widely used to analyze populations' genetic diversity, gene flow, parental analyses, or the preparation of gene maps. Advantages of microsatellites are that they have high levels of detection polymorphism, reproducible, and are easily transmitted across related species (Faltinová et al., 2020, Bínová et al., 2020, Feng et al., 2016).

For the past 20 years, SSR markers have been at the forefront of genotyping because they are highly informative, codominant, and easy to reproduce. These are especially useful for wild species as, as mentioned earlier, they provide gene flow and crossing-over rates (Vieira et al., 2016). In the article by Vieira et al. it is suggested that SSRs are markers often found in the non-coded part of the genome as these sequences are "unique" to each individual. Therefore, the markers can identify parents during breeding of plants (Vieira et al., 2016).

In this experiment, controlled crosses between epitype mother trees and known fathers with known phenotypes were used. The goal is to use microsatellites to assess the similarity between parents and seedlings to ensure that they are related. Excluding any contamination during pollination is also a factor for using SSRs in this experiment. Because microsatellites collect data for many variables, this data is often used to perform a principal coordinate analysis (PCA). This analysis is a mathematical algorithm that reduces the dimensionality of the data but retains most of the variations. Here, directions are identified, principal coordinates that contain the maximum variation. Using a few coordinates, each sample can be represented by a few numbers instead of thousands of variables. These values can then be plotted and make it possible to visualize similarities and differences between samples (Ringnér, 2008, Destefanis et al., 2000).

1.7 Photoperiodism

Sunlight satisfies two very important needs for biological organisms, energy and information. Photosynthesis transforms the energy in sunlight into chemical energy. Sunlight is captured and used to convert water, carbon dioxide and minerals into oxygen and energy-rich organic compounds. Radiation, in form of lights, also provides critical information about the environment. This information is used by plants to regulate movement, mark the passage of time and activate developmental events (Hopkins, 2008). *Photoperiod is defined as day length or 'the period of daily illumination received by an organism and remains constant between years at any given geographic location* (Bloor et al., 2013).

The response to photoperiods depends on the critical daylength. Plants is divided into short-day (SD), long-day (LD) and day-neutral plants, with respect of the bud burst. This will depend on where the induction of bud burst needs a light period shorter (short-day plant) or longer (long-day plant) than the critical day length (Jackson, 2009). A short photoperiod is a key factor in introducing dormancy in most trees from cold and temperate areas (Partanen et al., 1999).

1.8 Phenology

The cycle of events associated with the passage of the seasons is one of the most familiar of all natural phenomena. This is typical in polar and temperate regions where annual changes in temperature are tremendous and are accompanied by corresponding cycles in the growth and reproduction of the plants. In tropical regions will the seasons be affected by the differences in rainfall and life-history events occurring in response to water availability. Phenology is the study of the timing of these life-history events. Under one season will bud-burst, leaf-expansion, abscission, flowering, fertilization, seedset, fruiting, seed dispersal, and germination happen in plants. These events are too familiar to attract any attention, except for changes in patterns, such as out-of-season flowering or loss of fruit (Fenner, 1998).

1.8.1 Bud burst

Environmental factors, mainly temperature, determine the timing for bud burst. This process is under strong genetic control where several genes play a role (Søgaard et al., 2008, Partanen et al., 2005). During periods of colder temperatures, such as autumn, the buds of the trees will enter a dormant state that lasts until spring comes. This dormant state means that they have no or limited ability to undergo ontogenetic development toward bud burst (Partanen et al., 2005). Trees that live in temperate and boreal zones have developed mechanisms that synchronize growth and dormancy with the seasonal changes within temperature and photoperiod. Bud burst is the first visible sign that indicates the end of the dormancy and the start of growth. Bud burst together with bud set, decides the length of growth season (Yakovlev et al., 2008, Søgaard et al., 2008).

In the paper written by Yakovlev et al. it is described that Norway spruce that has grown in areas with cold environments shows tendencies to develop bud bursts earlier than trees that have grown in areas with warmer environments, provided that they have been placed in the same climatic conditions during the analyzes (Yakovlev et al., 2008). Since Norway is an elongated country with large environmental variations within the country, research shows that there are geographical patterns for variation in bud development (Søgaard et al., 2008). Furthermore, it is described that the time of bud burst is one of the most important characteristics that affect the spruce's mortality, growth and quality (Yakovlev et al., 2008).

It has been shown that water stress-related genes can simulate the time of bud burst. This is because metabolisms in buds and twigs begin when the ambient temperature exceeds the freezing point. Shooting elongation and flushing are most likely water-demanding, including rehydration of meristems and cell expansion, which is necessary for active growth (Yakovlev et al., 2008).

Dehydrins (DHN) is a dehydration-protecting protein and is considered a late expressed gene in the stress signaling pathway. They may play a central role in the development of freezing tolerance and cold acclimatization based on the discovery that they accumulate in seeds during late embryogenesis, in plant tissues under stress, and processes that lead to water deficiency (Yakovlev et al., 2008).

1.9 Epigenetic memory in Norway spruce

Epigenetic memory can be understood as a type of adaptive phenotypic formability that might last for the next generation and is realized through specific epigenetic patterns during the development of the embryo and influencing DNA replication, gene expression, repair and recombination (Yakovlev et al., 2010). Given that epigenetic memory drives changes in gene expression without changing the primary DNA sequence, it can be estimated that mechanisms such as methylation and chromatin conversion (heterochromatin and euchromatin) lead to epigenetic memory in spruce. Epigenetic effects occur when chromosomal proteins and methylated DNA result in important phenotypic consequences. Mechanisms such as DNA methylation, non-coding RNA. and chromatin that control changes in DNA methylation and chromatin status are the basis for epigenetic effects (Henderson and Jacobsen, 2007). The basis for the epigenetic memory of spruce is most likely genetic; since there are variations in the memory response within and among the family, it is perceived as probable that the specific genetic mechanism plays a role in this phenomenon (Yakovlev et al., 2011).

As previously mentioned, spruce has the ability to adopt to the local environment within a few generations. In several plants, including *Arabidopsis thaliana*, it has been shown that the parents' environment during reproduction plays a role in the offspring's performance. Spruce plants can also «remember» temperatures and light periods experienced during their zygotic embryogenesis and seed maturation (JOHNSEN et al., 2005, Henderson and Jacobsen, 2007, Yakovlev et al., 2011, Skrøppa et al., 2010). This memory effect and the epigenetic phenomenon that act on phenological properties can affect the plant for more than 20 years after germination, and this effect is probably life-lasting (Yakovlev et al., 2011, Skrøppa et al., 2007). Thus, Norway spruce has an epigenetic mechanism that works in addition to classical genetic inheritance and allows adjustment of bud phenology in response to local environmental conditions during embryo and seed development.

In an experiment conducted by Kvaalen and Johnsen (2008), it was determined that the perceived amount of temperature (18 and 28 °C) has the same effect during in vitro somatic embryogenesis as it has during zygotic development in spruce flowers. The temperature experienced during somatic embryogenesis will thus adjust bud phenology consistently and reproducible manner that could be observed in the plants (Kvaalen and Johnsen, 2008). Embryos that are developed at different epitypes inducing temperature conditions (epi-temperature), develop into seedlings and later trees that will be pheno-typically different but genetically and are therefore referred to as «epitypes» (Yakovlev et al., 2012). Through several

years of observations and analyzes, it has been observed that there are differences in temperature-specific gene expression in different epitypes (Yakovlev and Fossdal, 2017). Epitypes produced from somatic embryogenesis have also been shown to have this long-lasting epigenetic memory. Carneros et al. (2017) can tentatively point out that even after 17 years of growth at identical conditions in the field, the bud burst timing in the various epitypes is shifted in a predictable and reproducible manner; from such claims it can be concluded that the genetic memory is maintained stably through cell division year after year in these plants (Carneros et al., 2017).

1.10 Aims of the study

The main aim for this study is to identify if the Norway spruce has any epigenetic memory that will be inherited from clonal parents (mothers) that were exposed to Epi-temperatures during their somatic embryogenesis (epitype individuals). In other words, to test whether there is any transgenerational transfer of the epigenetic memory from one generation to the next.

The specific aims for this study were:

- Develop a method to detect DNA methylation patterns on two target genes involved in epigenetic regulation in the genome of Norway spruce. The method will help to develop an understanding of potentially hereditary epigenetic traits, as if an epigenetic trait can be stable over generations.

- Identify phenological differences between offspring's from two epitypes when grown under the same bud set and bud burst inducing conditions. Significant differences between such offspring may indicate that the epigenetic memory can been inherited.

- Assess the relatedness of the parents and offspring by genotyping as so to ascertain that the effect is epigenetic and not just a classical genetic. This analysis will provide an answer to whether the parents of the given individuals are as predicted, and that the pollination (controlled crosses) was performed properly and no pollen contamination had occurred.

2.0 MATERIALS AND METHODS

2.1 Study samples

The samples used in this study were seedling from Norway spruce (*Picea abies*). The seedlings come from controlled crosses from trees at the Norwegian Institute for Bioeconomy Research field, Hogsmark (59.67°N, 10.72°E, 85 m a.s.l.). The mother trees were generated in vitro by somatic embryogenesis at 18 and 28 degrees 20 years ago and flowered in 2019 for the first time (D2V clone) (Kvaalen and Johnsen, 2008). These trees are epitypes, display early and late bud burst as previously described by Carneros et al. and were pollinated with known fathers (Carneros et al., 2017). Some flowers were also allowed to be naturally pollinated.

For the first time in 2019 the epitypes flowered and a controlled pollination could be carried out. To reduce the possibility of contamination, careful observations were done of the development of the flowers (female and male). A white pollination bag with a supervision window was placed over the female flowers to prevent fertilization of other males (**Figure 5**). The pollen we used were from unrelated and well-characterized father to avoid inbreeding effects, since our epitypes are clonal. Two of the pollen donors (induvial number 7297 and 12632) were from our national collaborators at Skogfrøverket (The Norwegian Forest Seed Center). The same mothers were also pollinated by the surrounding trees (open pollination). Seeds from an unrelated tree in the same stand that happen to produce cones that year were taken as control samples. **Table 1** gives an overview over the different crosses used in this case.

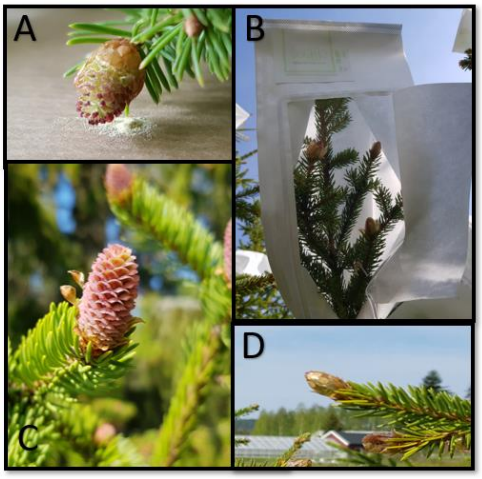


Figure 5: *How the pollination was preformed and how the female and male flowers on Norway spruce look like. A: This is a picture of a male flower that contains pollen. The main reason to harvest the flower was to collect the pollen. B: How the pollination bag we used during the pollination looked like. C: A fertilized female flower that has started to develop to a cone. D: How a female flower is before the "filter bags" are placed.*

Table 1: *Information about the different crosses from last harvest. The numbers highlighted is the crosses who will be most interesting to compared to each other. The names 97 and 32, for the father, are exactly the last part of an individual number, 7297 and 12632. The research group got this pollen from Skogfrøverkt at Hamar in 2019. The mother's, D2V and B10V, were obtained from somatic embryogenesis at different epi-temperatures (Kvaalen and Johnsen, 2008) . The number behind the epitype mother's names refers to the ambient temperature of the somatic embryos during their development.*

Mother		**Father**				
Clone	**Individual**	**Open**	**97 (Late)**	**32 (Early)**	**D2V28**	**D2V18**
D2V28	3737	1	2	3		
D2V18	3695	17				
	3694	16	13			
B10V28	3679	4			5	7
Control	Edge	12				
Unknown 1 (D2V28)	3778	10				
Unknown 2 (D2V28)	3781	11				

The seeds from the crosses shown in **Table 1** were sown in March 2020 and the resulting seedlings have been under controlled conditions in a growth room. The different crosses were randomly divided and located in arbitrary places in the room. We obtained 387 pots with two to four plants in each. The project group decided to use some of the crosses for analyses (**Table 1**), and therefore the total amount of plant was reduced from 387 to 295. For each cross there were between 30 and 40 pots with one to four plants in each. This number of plants were depending on how many seeds the project group had from the harvest and how many seeds actually began to germinate. The remaining plants that were not used for analyses during the experiment were treated in the same way as the rest of the plants.

In the initial phase of the growth period, the plants were under constant lighting (24 hours) for two months (Philips light tubes model TL-D 36W/33-640) at room temperature. The plants were also watered twice a week during this period, and fertilizer was added once a week (Pioner NPK Makro Gul and Pioner Mikro Plus with Iron from Azelis was used as the fertilizers during this period (**Table 2**)).

Table 2: *The content of the fertilizer used for the plants, as well as the percentage of the reagents.*

Fertilizer	Content	Volume (%)
Pioner NPK Makro Gul	Nitrate nitrogen	8.5
	Ammonium nitrogen	1.3
	Water soluble phosphorus	3.5
	Citrate- and water-soluble phosphorus	3.5
	Water soluble potassium	25
	Water soluble magnesium	4.4
	Water soluble sulfur	5.8
	Chloride	MAX 0.05
	Fluoride	MAX 0.05
Pioner Mikro Plus with Iron	Water soluble Boron	0.32
	Water soluble Cobber	0.13
	Water soluble Iron	1.62
	Water soluble Manganese	0.63
	Water soluble M	0.06
	Water soluble Zinc	0.32

2.2 Photoperiods and material collection

After the initial growth of the seedlings for two months, bud set was induced using short days (SD) (6 hours of darkness and 18 of light) (Kohmann and Johnsen, 1994). This critical photoperiod was used to see differences in the bud set timing among the different crosses based on previous work (Kohmann and Johnsen, 1994). When the bud set was completed, the plants were placed into long day (LD) (24 hours light) to induce bud burst. During the experiment, the photoperiod was divided into two different intervals, one interval with eighteen hours of light (SD) and an interval with twenty-four hours of light (LD) (**Table 3**).

Table 3: *Dates for the start and the end of the collection period for the phenotyping data and how many hours the different periods consisted of.*

	Start date	Stop date	Hour's light
Short day (SD)	21. September 2020	29. October 2020	18
Long day (LD)	12. November 2020	18. January 2021	24

Samples for DNA methylation were collected before inducing the SD period (five replicates), start collection; 21. September 2020 (Day 0) and collection at the end; 02. November 2020 (Day 12). Most of the samples were collected in the autumn of 2020 and some at the beginning of 2021. For the DNA methylation it was collected one bud from each cross. At 5th of May 2021, it was also collected needles for DNA methylation, this is because the group recently found that large amounts of DNA were needed to be able to perform the analysis. All

these samples were stored at -80 °C. The collection times after the induction of bud set, type of material collected, and techniques are represented in **Table 4**.

Table 4: *Weeks the group collected the samples for several analyzes and witch material we used for each analysis. The information present in parenthesis are samples that were collected at 5th of May 2021.*

	Time for collection	**Material**
Phenotyping	Day 0 – 12 and day 13 – 28	Numbers (stages)
DNA methylation	Day 0 and 12 (Week 18)	Buds (Needles)
Genotyping	Week 11	Needles

2.3 Phenotyping

Phenotyping experiment is a way to collect data from the development of bud set and bud burst. 20 plants from each cross were followed. To collect data from the plants, a table with different stages was used (**Table 5**). These stages are based on the developmental scale used in the pilot experiment that was established by our research group.

Table 5: *Description for the different stages in bud development. The description for each stage is based on earlier experiments by our research group. Stages 0-5 is known as bud set, and stages 6-10 is known as bud burst.*

Stages	**Description**	**Development stages**
0	Active growing, no signs of thicken in the upper part of the shoot.	Bud set
1	The shoot gets thicker just below the meristem.	Bud set
2	Bud scales start to appear in between the most apical needles of the shoot.	Bud set
3	There is a white bud forming that gets thicker. The apical needles are separated from each other. The bud is white, and needles are scarce or not even present.	Bud set
4	Same bud shape as stage 3 but the scales start to turn brown.	Bud set
5	Same bud shape as stages 3 and 4 but scales are completely brown.	Bud set
6	There is a visible hole in the top of the bud.	Bud burst
7	Green buds are possible to see through the whole.	Bud burst
8	Needles protrude the hole.	Bud burst
9	Needles keep elongating.	Bud burst
10	Elongation of the shoot.	Bud burst

The data collection was done on Mondays and Thursdays to obtain detailed data on the development of the buds. Pictures of the different stages for development of buds are represented in **Figure 6**.

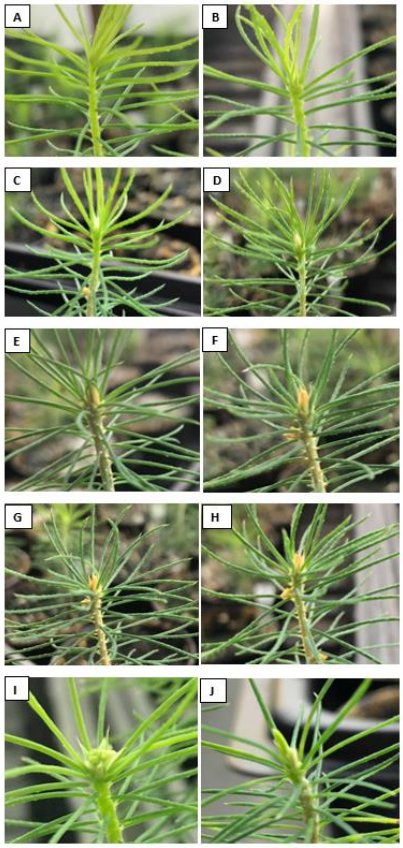


Figure 6: *Picture of the different stages correspond to the description in* ***Table 5*** *(A=1, B=2, C=3, D=4, E=5, F=6, G=7, H=8, I=9 and J=10). Because of the size for the plants, it was difficult to make a good presentation of the different stages, special stage five (E) to eight (H).*

2.4 Bisulfite sequencing

2.4.1 Target gene selection

In the pilot experiments, the methylation pattern in 2700 genes target genes of Norway spruce were analyzed. That set of data was used as a starting point for further gene selection. Based on the 2700 genes, around 500 seemed to have detectable differences in methylation and was therefore interesting for further work. The pilot experiment analyzed the patterns of methylation in two epitype embryos divided into an upper part containing the shoot apical meristem (SAM) and a bottom part containing the root apical meristem (RAM). They analyzed 2 kb of the promoter and 1 kb of the coding region (gene body) for each of the genes, as **Figure 7a** represents. The level of methylation of each of the epitype embryos were displayed in plots (**Figure 7b**). The genes that we used were selected based on the results (graphs) from the pilot experiment. The main point of these analyzes is to use these data to select target genes that can potentially show differences in their epigenetic pattern among seedlings from different crosses.

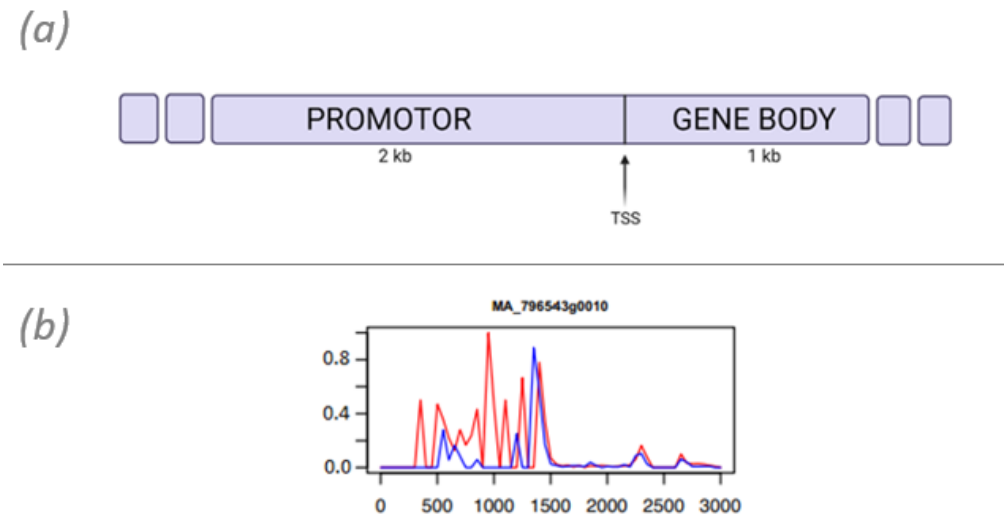


***Figure 7**: (a) Illustration of the promotor and the gene body used during the polit experiments for detection of methylations pattern in different genes. The transcriptional start site (TSS) is also represented in this illustration. (b) Example of a graph prepared by the group working on the pilot experiments. The graph represents gene MA_796543g0010 taken from SAM and it shows methylation for CHH.*

We selected target genes that were related to the epigenetics machinerys, hormones and growth and development. From 500 potential target genes, 37 target genes were left based on the methylation's levels illustrated in the plots as well as their function (**Figure 7b**).

To detect the differences in the methylation patterns a DNA method described by Valledor and his collaborators were used (Valledor et al., 2007). This method is based on bisulfite modification of the DNA. Where deoxycytosines transforms into uracyl bases, while 5-methyldeoxycytosines remain unchanged. The genome sequence can be analyses by PCR amplification using specific primers designed to amplify only if deoxycytosines corresponding with primer sequences have changed (Valledor et al., 2007). Articles by, among others, Leitao et al. and Rowther et al. were used as inspiration for optimalization of the method (Leitão et al., 2018, Rowther et al., 2012). We explored what genes displayed differences between embryo epitypes in specific regions that were flanked by non-methylated areas. That way we could design primers on the flanking regions which were likely not to be affected by the bisulfite conversion. To do this selection, we gathered information, for each target gene, from the three different types of methylation, CHH, CHG and CpG, and considered the part of the embryo (upper and lower). All the raw data was collected into the same plot (**Figure 8**). This was done using previous information from each type of methylation and entering it into the same sheet on excel. Furthermore, formulas were used to align six different information lines into one and the same line. This work was done for all the 37 selected genes. The concatenated information

took the average of methylation for each 50 base pairs, which represents the X-axis in **Figure 8**. The methylation frequency represented in the Y-axis in **Figure 8** is results from previous work done by our research group. It must also be mentioned that this was done on single strands of DNA, which means that two analyzes were performed for each of the individual genes.

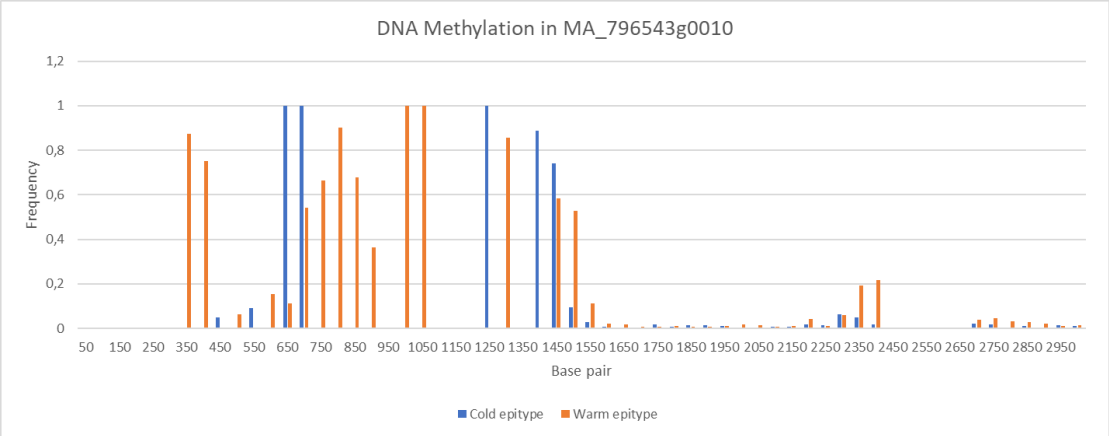


***Figure 8**: How a graph after aligning the six different information-lines looks like. The graph contains three types of methylation, as well as to different tissues. These are measurements from the same 50 base pairs.*

These new graphs made it easier to see which areas can be used to design primers and who much methylation each gene contains. In addition to designing primers, the new graphs made it easier to locate methylation patterns in the individual genes without depending on six different graphs at the same time.

2.4.2 Primer design

The primers were designed by using MethPrimer, inspired by a paper by Leitão et al. (Leitão et al., 2018). This is a program that find possible primers pairs at different places in the target sequence. The primers were chosen in areas where there was less methylation, as well as in the vicinity of large methylation differences. **Table 6** contains the six primers (out of eleven, appendix **A.1**) that amplified after DNA bisulfite conversion and PCR optimization (Touch-up PCR). Before processing the samples from the crosses, other DNA were used to make sure that the primers and the different kit works. At first, we collected dormant buds from random trees growing in NMBU campus to test and optimize the method to use the same kind of tissues that we obtained after the short day treatment in our seedlings.

Table 6: *Six of eleven (it was only possible to design primers for 11 genes) primers that was designed and tested on the DNA. These six primers were the once that amplified after DNA bisulfite conversion. Tm reference to melting temperature.*

Identification	Sequence 5' to 3'	Product length	Tm °C
MA_10344604_F	TTAAGATATGTAGGATAATAGATTAAGTA	465	55
MA_10344604_R	CTAATTCTATAAAAAAAAATAAAAATATTTCCC		56
MA_10427514_F	TGGAATTAAGAGAGTTTTAGTTGAGTA	488	57
MA_10427514_R	TTTCTTAAAAAATACATCTTTCC		50
MA_120256_F	TTTAATTAGGATATTATATGGGGA	402	52
MA_120256_R	CCTAATAAAAACAAAAAACAATATTTAACTT		55
MA_132879_F	TTTTTTAAAGATAAGGTAGTTGAAA	997	52
MA_132879_R	AAACCTAACACATAAAAAAATACTCTCAAA		57
MA_796543_F	TGAATTTTGTTTAATGGTGAAAAA	632	51
MA_796543_R	AATTAAAAAATACAATCCACTTTTA		50
MA_8008099_F	AAGATTTTGTTAAAATTATGGAAGATGAA	372	55
MA_8008099_R	TTTTAACTTACCAAAAACCTACTTA		53

As mention before, designing of primers was based on "*Locus-Specific DNA Methylation Analysis by Targeted Deep Bisulfite Sequencing*" by Leitão et al (Leitão et al., 2018). In this article, the primers were designed with adapters. In our case the primers were first design without adapters to test their potential use. The primers that amplified were then redesigned with adapters (**Table 7**).

Table 7: *Re-designed primers including the adapters (adapters marked in yellow). Tm reference to melting temperature.*

Gene	Identification	Sequence 5' to 3'	Length of the primers	Tm °C
MA_10344604	PaHMD-T7	TAATACGACTCACTATAGGGTTAAGATATGTAG GATAATAGATTAAGTA	49	67,9
	PaHMD-SP6	CATTTAGGTGACACTATAGCTAATTCTATAAAAA AAAATAAAAATATTTCCC	52	70,8
MA_10427514	PaChi1-T7	TAATACGACTCACTATAGGGTGGAATTAAGAGA GTTTTAGTTGAGTA	47	72,0
	PaChi1-SP6	CATTTAGGTGACACTATAGTTTCTTAAAAAATAC ATCTTTCC	42	69,0
MA_120256	PaBAH1-T7	TAATACGACTCACTATAGGGTTTAATTAGGATAT TATATGGGGA	44	70,4
	PaBAH1-SP6	CATTTAGGTGACACTATAGCCTAATAAAAACAA AAAACAATATTTAACTT	50	71,0
MA_132879	PaGNAT1-T7	TAATACGACTCACTATAGGGTTTTTTAAAGATAA GGTAGTTGAAA	45	70,2
	PaGNAT1-SP6	CATTTAGGTGACACTATAGAAACCTAACACATA AAAAAATACTCTCAAA	49	72,1
MA_796543	PaXSd-T7	TAATACGACTCACTATAGGGTGAATTTTGTTTAA TGGTGAAAAA	44	73,4
	PaXSd-SP6	CATTTAGGTGACACTATAGAATTAAAAAATACA ATCCACTTTTA	44	69,3
MA_8008099	PaRNB1-T7	TAATACGACTCACTATAGGGAAGATTTTGTTAAA ATTATGGAAGATGAA	49	73,6
	PaRNB1-SP6	CATTTAGGTGACACTATAGTTTTAACTTACCAAA AACCTACTTA	44	69,0

2.4.3. Melting curve

When the samples gave satisfactory results, the buds collected from Hogsmark, that had the same epitypes as the seedlings, were used for further optimization. The samples from Hogsmark were mainly used to see if there were any differences between the epitypes. For this purpose, a melting curve method were used after the bisulfite conversion. The trees we collected samples from in Hogsmark, are represented in **Table 8**.

Table 8: *These threes were used to optimize the method. It was used two different clones to make it easier to see the differences. The ''individual number'' shows exactly witch tree the group used and where it is in the field (not included data). The number that are marked with bold font is mothers to the seedlings of interest.*

Clone	Epitype	Individual
B10V	28	**3679**
D2V	28	3749
B10V	28	3677
B10V	28	3663
D2V	28	**3737**
D2V	28	3743
B10V	18	3653
B10V	18	3669
D2V	18	3695
B10V	18	3667
D2V	18	3705
D2V	18	3694

Melting curve analysis is an assessment of the dissociation characteristics of double-stranded DNA during heating. Which means that the information can be used to deduce the presence of methylation. This is because GC base pairing has more hydrogen bonding than AT base pairs. DNA with a higher GC content will have a higher melting temperature than DNA with a higher AT content. The samples from **Table 8** were used for this analysis to detect any differences in the dissociation characteristics between the two epitypes. The Applied Biosystems ViiA 7 Real-Time PCR system together with reagents shown in **Table 9** were used. The setup for the PCR were the same as in **Table 10** with 40 cycles and an annealing temperature at 65 °C.

Table 9: *Reagents, concentrations and volume used during the melting curve analysis. These were mixed as a master mix to ensure that the replicates (used three replicates) were as similar as possible.*

Reagents	Concentration	Volume for one reaction (µL)
2x SYBRGreen mix	1x	12.5
F + R primer	200 nM	2
DDWater	-	8.5
Total	25	25
DNA		2

Results from this test were not informative enough to indicate any significant difference between the two epitypes. Therefore, the group decided to not extend this analyze (results and further information in the Appendix, **A.2**).

2.4.4 DNA extraction

For DNA extraction DNeasy Plant Mini Kit (QIAGEN) or MagMAX DNA Multi-Sample Kit were used depending on the initial amount of material. From previous projects it has been shown that DNease Plant Mini Kit is better for samples with a higher amount of biomass, like needles. On the other hand, the MagMAX DNA Multi-Sample Kit works better and gives more DNA from samples with smaller amount of material, like tiny buds.

For DNeasy Plant Mini Kit, a myrtle was used to homogenize the sample. A tissuelyse was used for MagMAX DNA Multi-Sample Kit since it is more critical to have the small amount of material closed in a tube during the homogenize.

The concentration and quality of the DNA was tested with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). This identify the concentration of the sample material by measuring the type of electromagnetic radiation that is absorbed by the substance being analyzed. Using this instrument, the contents of the samples can be indicated; DNA (value ~ 1.8), RNA (value ~ 2.0) or proteins, phenol or other contaminants that absorb at or near 280 nm (value lower than either case). The DNA used in this project had acceptable concentrations of and the 260/280 ratios were respectable.

2.4.5 PCR optimization

HotStarTaq Plus DNA Polymerase (QIAGEN) kit was used for the PCR process. The protocol for this kit was followed as directed and the associated PCR setup (**Table 10**) were used as a guidance. This setup was used as a starting point for the optimization.

Table 10: *PCR cycles that were represented in the protocol from HotStaTaq Plus DNA Polymerase (QIAGEN) and it was used until the group got more satisfied results from the touch-up PCR (**Table 12**).*

Initial denaturing	Cycles	Denaturing	Annealing	Elongation	Final elongation	Rest period
95 °C, 5 min	25 - 35	94 °C, 30-60 s	50-68 °C, 30-60 s	72 °C, 1 min	72 °C, 10 min	4 °C, ∞

Results from the PCR setup in **Table 10** run in an E-gel Agarose with SYBR safe, 2% (Thermo Fisher Scientific), did not gave any band, therefore it was decided to try a new PCR method de therefore it was decided to try a new PCR method which is described by Rowther et al. (Rowther et al., 2012). The new method consisted of a touch-up PCR that increases the annealing temperature over several cycles (**Table 11**). The article describes an annealing temperature at 48 °C for the first cycles. In our case, the primers had high melting temperature which means to avoid unspecific bindings, the annealing temperature were increased to 55 °C. This change was performed based on observations of good results without increasing the time. An attempt was also made on touch-down PCR, which is described in the same article. Touch-up and touch-down PCR gave the same results, we decided to use touch-up as this reduced the analysis time.

Table 11: *The setup for the touch-up PCR that is described in the article by Rowther et al. (Rowther et al., 2012). This PCR was used for the bisulfite sequencing.*

Initial denaturing	Cycles	Denaturing	Annealing	Elongation	Final elongation	Rest period
94 °C, 5 min	5	94 °C, 45 s	55 °C, 45 s	72 °C, 60 s		
	5	94 °C, 45 s	60 °C, 45 s	72 °C, 60 s		
	30	94 °C, 45 s	65 °C, 45 s	72 °C, 60 s	72 °C, 7 min	10 °C, ∞

2.4.6 Bisulfite conversion

EZ DNA Methylation-Lightning™ Kit (Zymo Research) was used for Bisulfite conversion. The protocol was followed at the point, except the DNA were eluted in 20 µL instead of 10 µL. The idea behind this change was to increase the amount of DNA obtained from the column since the concentration was regularly high enough to dilute the samples with 10 µl extra. For this protocol, the DNA concentration should be between 10 ng/µl and 25 ng/µl for each sample. By using too much DNA it may lead to incomplete deamination, and re-annealing of complementary sequence complexity. After the bisulfite conversion, Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) was used to quantify the DNA concentration. It is normal to have some DNA loss during the bisulfite-conversion (10-20 %), therefore, we took it into account.

2.4.6.1 Bisulfite sequencing

Samples to be analyzed in this experiment were from crosses number 2 and 13, **Table 1**. These seedlings had the same father, but different mothers and were therefore relevant to compare. After DNA extraction and bisulfite conversion, a PCR was performed with two primers (PaHMD-T7/PaHMD-SP6 and PaRNB1-T7/PaRNB1-SP6) from **Table 7** and the setup for the PCR machine was touch- up from **Table 11**. The PCR product was placed on a 2% Agarose E-Gel with SYBR safe (Thermoe Fisher Sicentific) gel together with a 1 Kb Plus DNA ladder (Invitrogen by Thermo Fisher Scientific). The bands were cut out of the gel by using a clean, sharp scalpel and further used as material for the QIAquick Gel Extraction Kit (QIAGEN). The samples were labeled with barcodes and send to sequencing by Eurofins Scientific.

2.5 Genotyping

For the genotyping we collected needles from five individuals for each of the crosses 1, 2, 3, 4, 5, 7, 12, 13 and 16 (**Table 1**), also pollen from the two different known fathers were analyzed (97 and 32). The needles were collected on the 16th of March 2021 and the DNA was extracted the same day. This part of the experiment was performed to ensure that the controlled pollination was properly orchestrated, and that no pollen contamination had occurred during fertilization.

The DNA extraction was conducted with DNeasy Plant Mini Kit (QIAGEN) and checked with Nanodrop 2000 Spectrophotometer. Due to the slightly poor quality of the DNA, it was decided to test it on housekeeping genes to make sure that it would give visible results. Actin-family was the housekeeping gene that was used during the test. Microsatellite analysis (SSR markers) was performed to assess the similarities among seedling from the crosses. Procedure for this part were based on articles by Binova et al., Tollefsrud et al., Tsuda et al., Sønstebø et al. and Tollefsrud et al. (Tollefsrud et al., 2008, Sønstebø et al., 2018, Bínová et al., 2020, Tsuda et al., 2016, Tollefsrud et al., 2009). These articles have earlier been extensively used in Norway spruce by our research group. Eleven SSR markers were used divided in three mixes as shown in **Table 12**.

Table 12: *The contents of the various SSR marker mixtures used during the bisulfite sequencing.*

	Marker mix 1	**Marker mix 2**	**Marker mix 3**
Markers	B15 EATC1E03 EATC2G05 F13 O09 Pa28	EAC2C08	A19 EATC1B02 EATC2B02 H08

A Type-it Microsatellite PCR kit (QIAGEN) was used to amplify each sample. The reactions consisted of 5 µl Type-it multiplex kit, 1 µl marker mix, 2 µl Q-solution, 2 µl water and 1 µl DNA. The PCR setup from **Table 13** was used.

Table 13: *PCR customized setups for each of the three different marker mixes. This PCR setup was only used during the genotyping analyzes. It is also inspired from article by Sønstebø et al., together with Tollesfrud et al. and Binova et al. (Sønstebø et al., 2018, Bínová et al., 2020, Tollefsrud et al., 2009).*

	Mix 1 & Mix 2	**Mix 2**	**Mix 3**
Initial denaturing	95 for 5 min	95 for 5 min	95 for 5 min
	7 cycles	*30 cycles*	*30 cycles*
Denaturing **Annealing** **Elongation**	95 for 30 s 57 for 90 s 72 for 30 s	95 for 30 s 57 for 90 s 72 for 30 s	95 for 30 s 60 for 90 s 72 for 30 s
	23 cycles		
Denaturing **Annealing** **Elongation**	95 for 30 s 50 for 90 s 72 for 30 s		
Final elongation	60 for 30 min	60 for 30 min	60 for 30 min
Rest period	4 for ever	4 for ever	4 for ever

After the PCR, the products were diluted 20 times and 1 µl diluted PCR product was mixed with 9 µl HiDi Formamide and 0.18 µl of GeneScan-500 (LIZ) size standard (Life Technologies) (Sønstebø et al., 2018). The kit (Type-it Microsatellite PCR kit) is a recurring theme in several of the articles, while the PCR setup is based on the article by Sønstebø et al. together with some temperatures inspires by Tollefsreud et al. and Binova et al. (Tollefsrud et al., 2009, Bínová et al., 2020).

The fluorescently labelled PCR products were analyzed on SeqStudio Genetic Analyzer System (Thermo Fisher Scientific). Genotyping and allele identification were performed using GeneMapper Software Version 4.1.

2.6 Statistical analysis

During the whole experiment, excel (Microsoft 365) was used in all the analyzes. All the graphs that are represented in the thesis were made in this program.

The data was analyzed by a repeated measures model through the 'nlme' package in R (Pinheiro J, 2013). The initial saturated model included day, cross and photoperiod and their interaction as predictors. Individual was included as a random effect in these models. However, preliminary analyses showed not enough degrees of freedom, so new analyses were conducted separately for each photoperiod. The final model includes growth as dependent variable, and day, cross and their interaction as fixed factors, being individual the random factor. Analyses were conducted using R v1.3.1093 (R Core Team 2020). Statistical significance was set at $\alpha = 0.05$. ANOVA were then used to specify any differences and present the results.

For genotyping, Excel was mainly used where the raw data was processed to form PCA plots. COORD. 1 and COORD. 2 were used to produce the score plot.

3.0 RESULTS

3.1 Development of buds

The phenotype observation were divided into two periods, SD (bud set) and LD (bud burst). The purpose of this sub-experiment was to confirm whether it was possible to find any significant difference in the phenotype between the different crosses. The experiment should also answer whether the phenological features found in the parents are passed on to the offspring.

3.1.1 Bud set development

The plant was under SD conditions in six weeks during the first period of the phenotyping. It was collected data from all the crosses, represented in **Table 1** (graph for all crosses during bud set are represented in the appendix, **A.3**). Some of the crosses were more interesting to compare than others. These have the same parent, which makes it possible to see whether the choice of parents influences the expression of phenological features. It was decided to compare crosses 1, 2, 3, 13, and 16. As shown in **Table 1**, crosses 1, 2, and 3 have the same mother (D2V28) but different fathers. Cross 13 and 16 also have the same mother (D2V18) and different fathers. Both groups 1 and 16 are fertilized with open pollination, while 2 and 13 are fertilized with a pollen donor that shows "late" phenological features. Based on this, a figure has been created that contains four different graphs (**Figure 9**). **Figure 9a** shows the comparison between cross 2 (Late D2V28) and 13 (Late D2V18) where cross 13 has become further in the development of bud. Comparison between cross 1 (Open D2V28) and 16 (Open D2V18) (**Figure 9b**) illustrate that development of buds in cross 1 are slower than in cross 16. In **Figure 9c** the comparison between cross 1 (Open), 2 (Late) and 3 (Early) indicates that cross 2 are develop buds slower than the other two crosses, as expected. Cross 13 (Late) and 16 (Open) were also compared (**Figure 9d**) which shows that cross 16 are further in the development of buds.

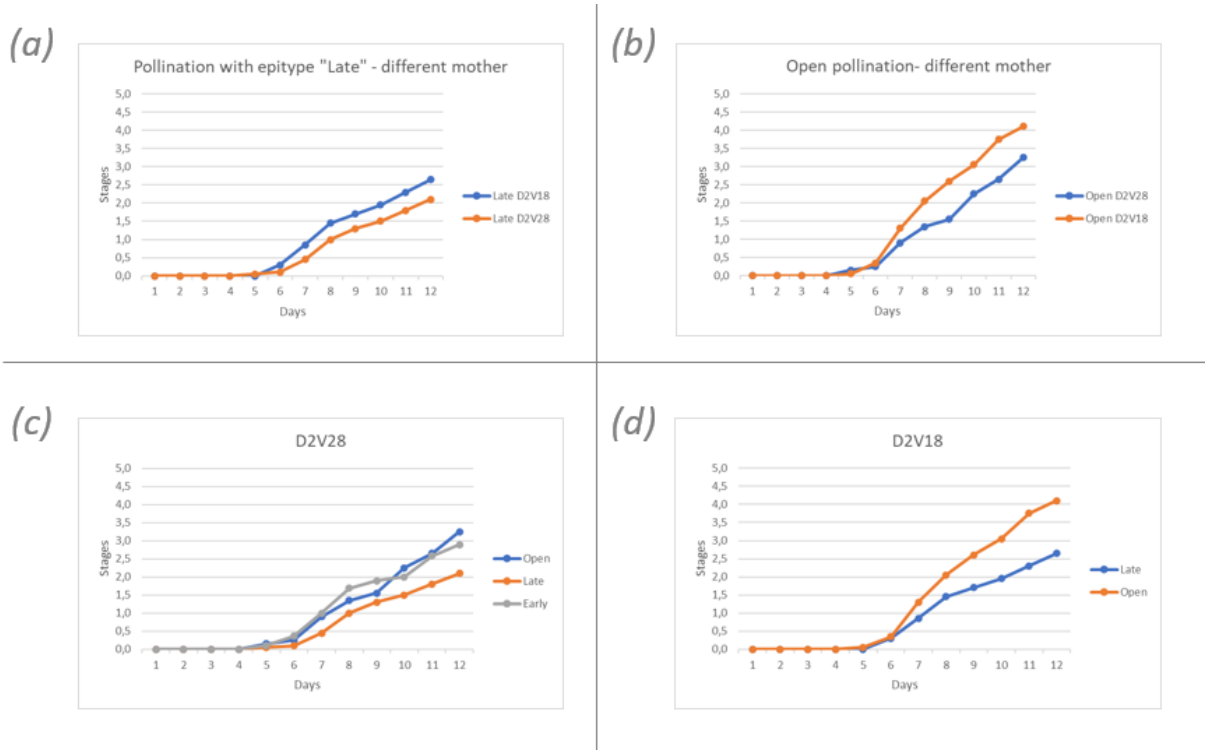


***Figure 9**: Graphs based on results from phenotyping data during SD period. (a) The comparison between cross 2 (Late D2V28) and 13 (D2V18). Observed that the development of "Late D2V28" is slower than the development of "Late D2V18". (b) Comparison between cross 1 (Open D2V28) and 16 (Open D2V18). Observed a difference where "Open D2V18" has come further in development than "Open D2V28". (c) The comparison between crosses 1 (Open), 2 (Late) and 3 (Early). As expected, "Late" is later in development than the other two crosses. (d) The comparison between cross 13 (Late) and 16 (Open). Here, the development of "Open" has come a lot longer than "Late".*

For the statistics part of the experiment, a nonlinear mixed-effects model (NLME) was performed in R. This method gives a P-value that indicates whether there is a difference between the crosses. **Table 14** represents results from the bud set where all the interesting crosses were compared to each other. The statical significance was set to $\alpha = 0.05$. Based on the table, it was shown that most crosses were significantly difference from each other. Exceptions are the comparison between 1 and 3, where no significant difference was presented.

Table 14: *Statistical results where the different groups have been compared to each other. Further data for these results are shown in the appendix (**A.3**, **A.4**, **A.5** and **A.6**).*

Crosses versus	Value	Standard error	DF	T-value	P-value
1 VS 2	-0.3375000	0.09479762	1165	-3.560216	0.0004 ***
1 VS 3	-0.0375000	0.09479762	1165	-0.395580	0.6925 ns
1 VS 16	0.4083333	0.09479762	1165	4.307422	0.0000 ***
2 VS 3	0.3000000	0.09479762	1165	3.164637	0.0016 **
2 VS 13	0.2416667	0.09479762	1165	2.549291	0.0109 *
13 VS 16	0.5041667	0.09479762	1165	5.318348	0.0000 ***

*Significance codes: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns = not significant*

The effect of the interaction between time and the various crosses was also analyzed. This analysis was performed to see when two given crosses are different in the development stage. Results from this analysis are shown in **Table 15**. Only an excerpt of the results values is represented in this text; the rest is in the appendix (**A.7**, **A.8**, **A.9**, and **A.10**). Based on the figures, the value highlighted in yellow shows significant differences and green highlighting represent a marginal difference. Cross 1 compared to 2 indicate a significant difference at day 12, and a marginal difference at day 11. In comparison between 1 and 16, a significant difference appears in day 9 and day 11. In day 12 a marginal difference is represented. When we compared cross 13 and 16, significant differences from day 9 to day 12 occurs. The other comparisons did not show any significant difference during the bud set and SD period.

***Table 15**: Results from comparison of the different crosses during SD, taking time into account. The table is divided into six different tables where the most interesting comparisons are represented. Values highlighted in yellow represented significant differences when values highlighted in green show marginal differences. For more information and values, see appendix (**A.8**, **A.9**, **A.10**, and **A.11**).*

Cross 1 vs 2	T- value	P-value
day2: cross 1 vs 2	0.000000	1.0000
day3: cross 1 vs 2	0.000000	1.0000
day4: cross 1 vs 2	0.000000	1.0000
day5: cross 1 vs 2	-0.218600	0.8270
day6: cross 1 vs 2	-0.327901	0.7430
day7: cross 1 vs 2	-0.983702	0.3255
day8: cross 1 vs 2	-0.765102	0.4444
day9: cross 1 vs 2	-0.546501	0.5848
day10: cross 1 vs 2	-1.639504	0.1014
day11: cross 1 vs 2	-1.858104	0.0634
day12: cross 1 vs 2	-2.513906	0.0121

Cross 1 vs 3	T-value	P-value
day2: cross 1 vs 3	0.000000	1.0000
day3: cross 1 vs 3	0.000000	1.0000
day4: cross 1 vs 3	0.000000	1.0000
day5: cross 1 vs 3	-0.109300	0.9130
day6: cross 1 vs 3	0.218600	0.8270
day7: cross 1 vs 3	0.109300	0.9130
day8: cross 1 vs 3	0.546501	0.5848
day9: cross 1 vs 3	0.546501	0.5848
day10: cross 1 vs 3	-0.765102	0.4444
day11: cross 1 vs 3	-0.437201	0.6620
day12: cross 1vs 3	-1.093002	0.2746

Cross 2 vs 3	T-value	P-value
day2: cross 2 vs 3	0.000000	1.0000
day3: cross 2 vs 3	0.000000	1.0000
day4: cross 2 vs 3	0.000000	1.0000
day5: cross 2 vs 3	0.109300	0.9130
day6: cross 2 vs 3	0.546501	0.5848
day7: cross 2 vs 3	1.093002	0.2746
day8: cross 2 vs 3	1.311603	0.1899
day9: cross 2 vs 3	1.093002	0.2746
day10: cross 2 vs 3	0.874402	0.3821
day11: cross 2 vs 3	1.420903	0.1556
day12: cross 2 vs 3	1.420903	0.1556

Cross 2 vs 13	T-value	P-value
day2: cross 2 vs 13	0.000000	1.0000
day3: cross 2 vs 13	0.000000	1.0000
day4: cross 2 vs 13	0.000000	1.0000
day5: cross 2 vs 13	-0.109300	0.9130
day6: cross 2 vs 13	0.437201	0.6620
day7: cross 2 vs 13	0.874402	0.3821
day8: cross 2 vs 13	0.983702	0.3255
day9: cross 2 vs 13	0.874402	0.3821
day10: cross 2 vs 13	0.983702	0.3255
day11: cross 2 vs 13	1.093002	0.2746
day12: cross 2 vs 13	1.202303	0.2295

Cross 1 vs 16	T-value	P-value
day2: cross 1 vs 16	0.000000	1.0000
day3: cross 1 vs 16	0.000000	1.0000
day4: cross 1 vs 16	0.000000	1.0000
day5: cross 1 vs 16	-0.218600	0.8270
day6: cross 1 vs 16	0.218600	0.8270
day7: cross 1 vs 16	0.874402	0.3821
day8: cross 1 vs 16	1.530203	0.1262
day9: cross 1 vs 16	2.295305	0.0219
day10: cross 1 vs 16	1.748804	0.0806
day11: cross 1 vs 16	2.404605	0.0164
day12: cross 1 vs 16	1.858104	0.0634

Cross 13 vs 16	T-value	P-value
day2: cross 13 vs 16	0.000000	1.0000
day3: cross 13 vs 16	0.000000	1.0000
day4: cross 13 vs 16	0.000000	1.0000
day5: cross 13 vs 16	0.109300	0.9130
day6: cross 13 vs 16	0.109300	0.9130
day7: cross 13 vs 16	0.983702	0.3255
day8: cross 13 vs 16	1.311603	0.1899
day9: cross 13 vs 16	1.967404	0.0494
day10: cross 13 vs 16	2.404605	0.0164
day11: cross 13 vs 16	3.169707	0.0016
day12: cross 13 vs 16	3.169707	0.0016

3.1.2 Bud burst development

Previously it has been explained that some crossings are more interesting to look at than others (graph for all crosses during bud burst are represented in the appendix, **A.12**). It is therefore relevant to follow the same crosses that were followed during the SD period. In **Figure 10**, four of the most interesting comparisons that have been under LD conditions for 8 weeks are represented. After the end of the LD growth period, it looks like the differences occur to be smaller than previously appeared through the graphs (**Figure 10**). **Figure 10a** shows the comparison between cross 2 (Late D2V28) and 13 (Late D2V18) where cross 2 has become further in the development of bud. Comparison between cross 1 (Open D2V28) and 16 (Open D2V18) (**Figure 10b**) illustrate that development of buds in cross 1 are slower than in cross 16, det same results as from bud set. In **Figure 10c** the comparison between cross 1 (Open), 2 (Late) and 3 (Early) indicates that cross 1 are develop buds slower than the other two crosses. Cross 13 (Late) and 16 (Open) were also compared (**Figure 10d**) which shows that cross 16 are further in the development of buds, but they are quite similar to each other.

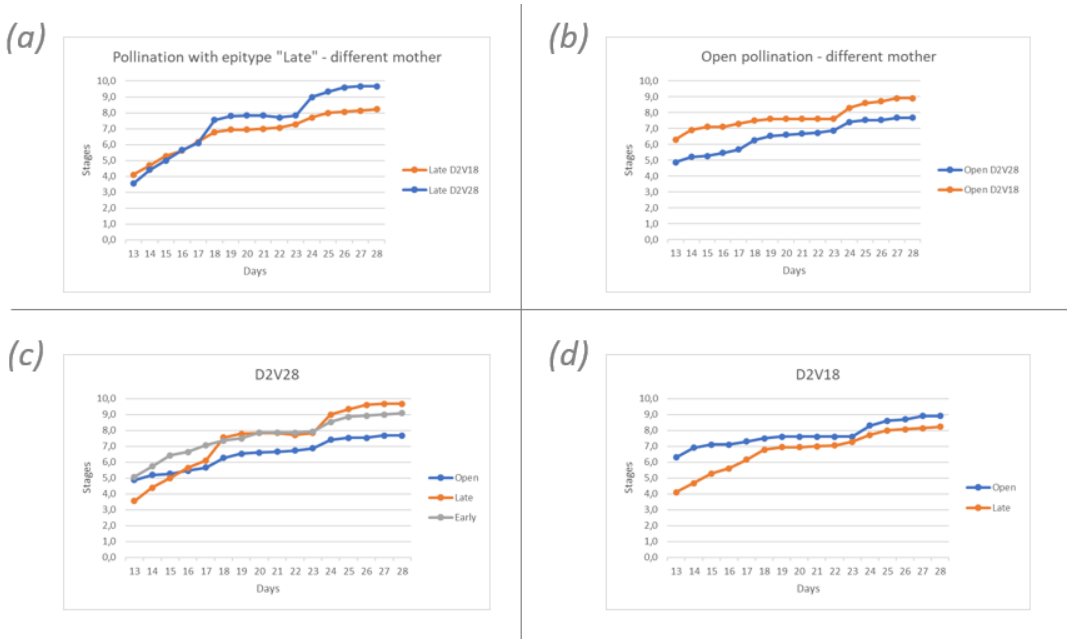


Figure 10: *Graphs based on results from phenotyping data during LD period. (a) The comparison between cross 2 (Late D2V28) and 13 (D2V18). Observed that the development of "Late D2V18" is slower than the development of "Late D2V28". (b) Comparison between cross 1 (Open D2V28) and 16 (Open D2V18). Observed a difference where "Open D2V28" has come further in development than "Open D2V18". (c) The comparison between crosses 1 (Open), 2 (Late) and 3 (Early). In* ***Figure 9****, "Late" had a later development than the other two crosses. In this case "Late" has evolved to be in the same stage at "Early". (d) The comparison between cross 13 (Late) and 16 (Open). In Figure, we expected that the "Open" should evolve faster than "Late", which was the case. Here, the development of "Late" and "Open" is close to similar.*

A nonlinear mixed-effects model (NLME) was used for the statistical analyse during the LD period. The statistical significance in this period was set as $\alpha = 0.05$. In **Table 16**, the results for the different crosses are represented. In the table, P-values that express a significant difference are marked, which show that all the crosses demonstrate a significant difference to each other.

Table 16: *Statistical results where the different groups have been compared to each other. Further data for these results are shown in the appendix (**A.11**, **A.12**, **A.13** and **A.14**).*

Crosses versus	Value	Standard error	DF	T-value	P-value
1 VS 2	2.807086	0.2562249	1097	10.955556	0.0000 ***
1 VS 3	1.239827	0.2461384	1097	5.037112	0.0000 ***
1 VS 16	2.742942	0.2987977	1097	9.179930	0.0000 ***
2 VS 3	-1.567260	0.2436548	1097	-6.432296	0.0000 ***
2 VS 13	-1.431178	0.2605552	1097	-5.492803	0.0000 ***
13 VS 16	1.367034	0.2947805	1097	4.637464	0.0000 ***
*Significance codes: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ns = not significant*					

As for the SD period, an analysis for looking at the effect of crosses between time and groups was performed. The results are represented in **Table 17,** and description for each cross are shown in **Table 1.** An excerpt of the results is represented, and further information is therefore presented in the appendix (**A.17**, **A.18**, **A.19**, and **A.20**). Cross 1 compared to cross 2 shows a significant difference from day 17 to day 28. This table also indicate a marginal difference at day 16. The comparison between cross 2 and 3, and 2 and 13 represent a significant difference from day 18 to day 28. The other comparisons do not demonstrate any significant differences.

Table 17: *Results from comparison of the different crosses during LD, taking time into account. The table is divided into six different tables where the most interesting comparisons are represented. Values highlighted in yellow represented significant differences when values highlighted in green show marginal differences. For more information and values, see appendix (**A.17**, **A.18**, **A.19**, and **A.20**).*

Cross 1 vs 2	T-value	P-value
day14: cross 1 vs 2	0.822645	0.4109
day15: cross 1 vs 2	1.354945	0.1757
day16: cross 1 vs 2	1.935636	0.0532
day17: cross 1 vs 2	2.177591	0.0297
day18: cross 1 vs 2	3.145409	0.0017
day19: cross 1 vs 2	3.338972	0.0009
day20: cross 1 vs 2	3.338972	0.0009
day21: cross 1 vs 2	3.290581	0.0010
day22: cross 1 vs 2	3.242190	0.0012
day23: cross 1 vs 2	3.145409	0.0017
day24: cross 1 vs 2	3.484145	0.0005
day25: cross 1 vs 2	3.629318	0.0003
day26: cross 1 vs 2	3.822881	0.0001
day27: cross 1 vs 2	3.774490	0.0002
day28: cross 1 vs 2	3.774490	0.0002

Cross 1 vs 3	T-value	P-value
day14: cross 1 vs 3	0.152575	0.8788
day15: cross 1 vs 3	0.460658	0.6451
day16: cross 1 vs 3	0.592694	0.5535
day17: cross 1 vs 3	0.619101	0.5360
day18: cross 1 vs 3	0.545748	0.5854
day19: cross 1 vs 3	0.809820	0.4182
day20: cross 1 vs 3	0.809820	0.4182
day21: cross 1 vs 3	0.759939	0.4475
day22: cross 1 vs 3	0.710059	0.4778
day23: cross 1 vs 3	0.654311	0.5131
day24: cross 1 vs 3	0.607365	0.5437
day25: cross 1 vs 3	0.683652	0.4943
day26: cross 1 vs 3	0.727664	0.4670
day27: cross 1 vs 3	0.671916	0.5018
day28: cross 1 vs 3	0.715928	0.4742

Cross 2 vs 3	T-value	P-value
day14: cross 2 vs 3	-0.695389	0.4870
day15: cross 2 vs 3	-0.935987	0.3495
day16: cross 2 vs 3	-1.402514	0.1611
day17: cross 2 vs 3	-1.625508	0.1044
day18: cross 2 vs 3	-2.696465	0.0071
day19: cross 2 vs 3	-2.631914	0.0086
day20: cross 2 vs 3	-2.631914	0.0086
day21: cross 2 vs 3	-2.631914	0.0086
day22: cross 2 vs 3	-2.631914	0.0086
day23: cross 2 vs 3	-2.587902	0.0098
day24: cross 2 vs 3	-2.984010	0.0029
day25: cross 2 vs 3	-3.057363	0.0023
day26: cross 2 vs 3	-3.212872	0.0014
day27: cross 2 vs 3	-3.218740	0.0013
day28: cross 2 vs 3	-3.174728	0.0015

Cross 2 vs 13	T-value	P-value
day14: cross 2 vs 13	-0.590969	0.5547
day15: cross 2 vs 13	-0.910229	0.3629
day16: cross 2 vs 13	-1.226092	0.2204
day17: cross 2 vs 13	-1.249866	0.2116
day18: cross 2 vs 13	-1.980087	0.0480
day19: cross 2 vs 13	-2.119338	0.0343
day20: cross 2 vs 13	-2.119338	0.0343
day21: cross 2 vs 13	-2.068393	0.0389
day22: cross 2 vs 13	-2.068393	0.0389
day23: cross 2 vs 13	-1.966501	0.0495
day24: cross 2 vs 13	-2.170284	0.0302
day25: cross 2 vs 13	-2.204248	0.0277
day26: cross 2 vs 13	-2.343499	0.0193
day27: cross 2 vs 13	-2.340103	0.0195
day28: cross 2 vs 13	-2.289157	0.0223

Cross 1 vs 16	T-value	P-value
day14: cross 1 vs 16	0.216411	0.8287
day15: cross 1 vs 16	0.346257	0.7292
day16: cross 1 vs 16	0.346257	0.7292
day17: cross 1 vs 16	0.346257	0.7292
day18: cross 1 vs 16	0.259693	0.7952
day19: cross 1 vs 16	0.324616	0.7455
day20: cross 1 vs 16	0.324616	0.7455
day21: cross 1 vs 16	0.281334	0.7785
day22: cross 1 vs 16	0.238052	0.8119
day23: cross 1 vs 16	0.151487	0.8796
day24: cross 1 vs 16	0.259693	0.7952
day25: cross 1 vs 16	0.367898	0.7130
day26: cross 1 vs 16	0.432821	0.6652
day27: cross 1 vs 16	0.476104	0.6341
day28: cross 1 vs 16	0.476104	0.6341

Cross 13 vs 16	T-value	P-value
day14: cross 13 vs 16	0.018290	0.9854
day15: cross 13 vs 16	-0.036580	0.9708
day16: cross 13 vs 16	-0.265206	0.7909
day17: cross 13 vs 16	-0.457251	0.6476
day18: cross 13 vs 16	-0.740747	0.4590
day19: cross 13 vs 16	-0.722457	0.4702
day20: cross 13 vs 16	-0.722457	0.4702
day21: cross 13 vs 16	-0.768182	0.4426
day22: cross 13 vs 16	-0.768182	0.4426
day23: cross 13 vs 16	-0.859632	0.3902
day24: cross 13 vs 16	-0.868777	0.3852
day25: cross 13 vs 16	-0.859632	0.3902
day26: cross 13 vs 16	-0.841342	0.4004
day27: cross 13 vs 16	-0.759037	0.4480
day28: cross 13 vs 16	-0.804762	0.4211

3.2 Detection of methylation pattern in bisulfited DNA

A sequencing was performed on a PCR product containing the desired amplicon to detect methylation patterns in bisulfite-converted DNA. For this analysis, two different crosses were used, 2 and 13, (**Table 1)** together with primers for the genes MA_10344604g0010 and MA_8008099g0010.

The samples sent for sequencing were purified from the gel. **Figure 11** shows a picture of the ladder (**Figure 11a**) and a gel from 10 of the 18 samples (**Figure 11b**). Most of samples had similar strength on the bands, as well as the correct size. The bands presented on the gel show individual bands that were purified and sent for sequencing.

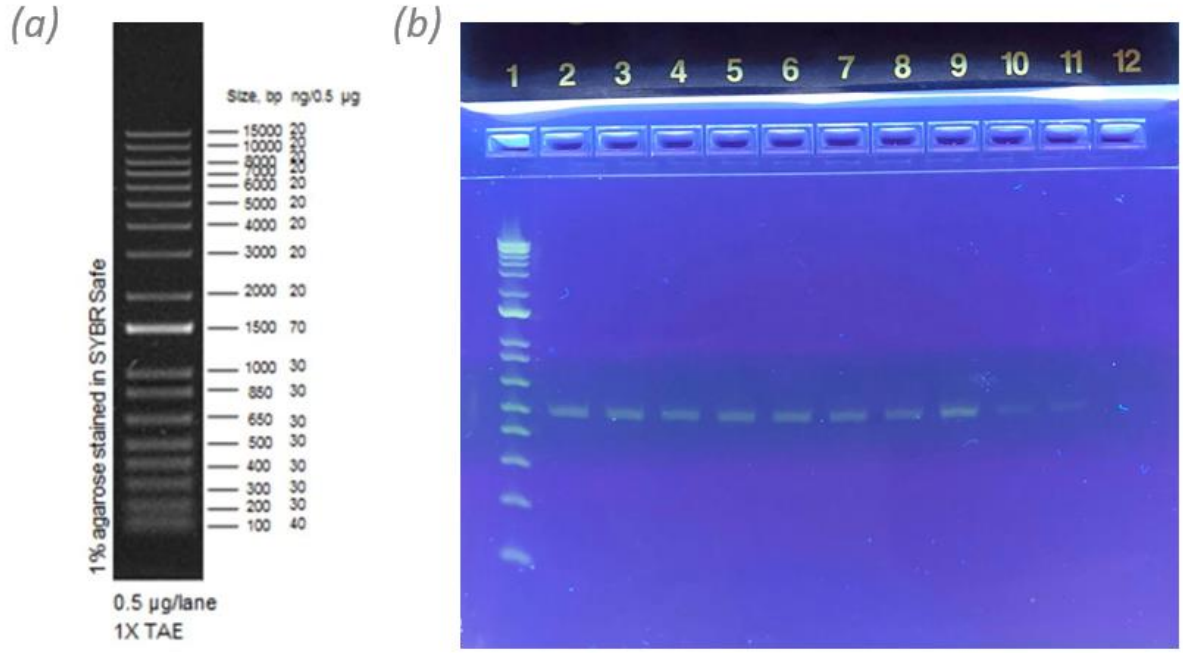


Figure 11: *Overview of the gel result before the samples were sent for sequencing. (a) Represent the ladder used for this gel (1 Kb Plus DNA Ladder, Thermo Fisher Scientific). An overview of which bands correspond to which base pairs are illustrated. (b) Pictures of the gel with 10 of the 18 samples. All samples of the gel are amplified with primer from the gene MA_10344604g0010 and each cross (crosses 2 and 13) has five samples (Well 1 = ladder, well 2 = cross 2, well 3 = cross 2, well 4 = cross 2, well 5 = cross 2, well 6 = cross 2, well 7 = cross 13, well 8 = cross 13, well 9 = cross 13, well 10 = cross 13 and well 11 = cross 13).*

The results from the sequencing showed varying quality; however, by aligning the sequence, the results were good enough to detect the DNA methylations differences within the various crosses. **Figure 12**, which represents MA_10344604, methylation occurred on the sequence from cross 13. However, no methylation change was detected at cross 2. Both crosses lacked parts of the sequences since only two satisfactory replicas were obtained. Two sequences lead to difficulties in determining the nucleotide if the sequences are different at some part. Thus, the sequence for cross 2 reverse was not used during this comparison, based on uncertainty of the nucleotides.

```
sequence        CTAAGACATGTAGGACAACAGATCAAGTAATGCATCATTTAATAATTTTAATGAACTAAA
cross13         ----------------------------------TCATTTAATAATTTTAACGAACTAAA
cross2          --------------------------NAATGCATCATTTAATAATTTTAACGAACTAAA
                                          ****************** ********

sequence        TTACACCAATTCATCAATAACTCATTGTCACAGTTAAAATATCAATGAGAAATCAAAGAG
cross13         TTACACCAATTCATCAATAACTCATTGTCACATTTAAAATATCATTAGGAAATCAAAGAG
cross2          TTACACCAATTCATCAATAACTCATTGTCACATTTAAAATATCATTAGGAAATCAAAGAG
                ********************************** *********** *  ************

sequence        GGTTTTAAACATGGCAGGA---ACCAAG---------TGAATAAACAATAGTGGCATCTC
cross13         GGTTTTAAACATGGCAGGATTGACCAAGMTTMAACATCAAAAAAMAAAAAGTMTTA----
cross2          GGTTTTAAACATGGCAGGA---ACCAAG---------TGAGTAAACAATAGT--------
                ******************   ******          *  **  ** ***

sequence        TAATCCCCAATCAGTATTCTCAACTAACATCGAAGCATCATTCCTTCCTAACTTACTTCC
cross13         ------------------------------------------------------------
cross2          ------------------------------------------------------------

sequence        ATCTTCCATCCTATCCTTTCCTCTGTTGGACTAAGCTGGAGCGTCAACTATATTAACCTA
cross13         ------------------------------------------------------------
cross2          ------------------------------------------------------------

sequence        ACTCCAGATAGAACCCATAAGCGGAAGTGTCATACATCTTGACCAAGCTTCACCAACAGG
cross13         ------------------------------------------------------------
cross2          ------------------------------------------------------------

sequence        CAACTAGCAGTCGTCATCACACATCTATGGGAGTGTGACTGCACACAACCCTCCCACGAC
cross13         --------------ATCAAAAATMTATGGGAGTGTGAMTGCACACAAMCMTCCMAC---
cross2          ------------------------------------------------------------

sequence        TCTAGGTTTTGGAAGATAAACGGTGGGAAATACCTCCACCCCTCCTCATAGAACTAG
cross13         ------------------------------------------------------------
cross2          ------------------------------------------------------------
```

***Figure 12**: Comparison between the seedlings and the original sequence for gene MA_10344604g0010. Marked nucleotides show where methylation occurred and represented as M in the sequence.*

The sequences were also compared with the mothers' sequences for the different crosses to prove that the individuals have inherited methylation patterns from the parents. In **Figure 13**, the sequence from D2V18 ("coldmother" in figure 13 and 15) corresponds to cross 13. The sequence from D2V28 ("warmmother" in figure 13 and 15) corresponds to cross 2. As mentioned earlier, there is significant lack of data in this comparison. No direct connection can be found between methylation and the mothers. However, this does not mean that the methylation patterns are not inherited since the father is not analyzed.

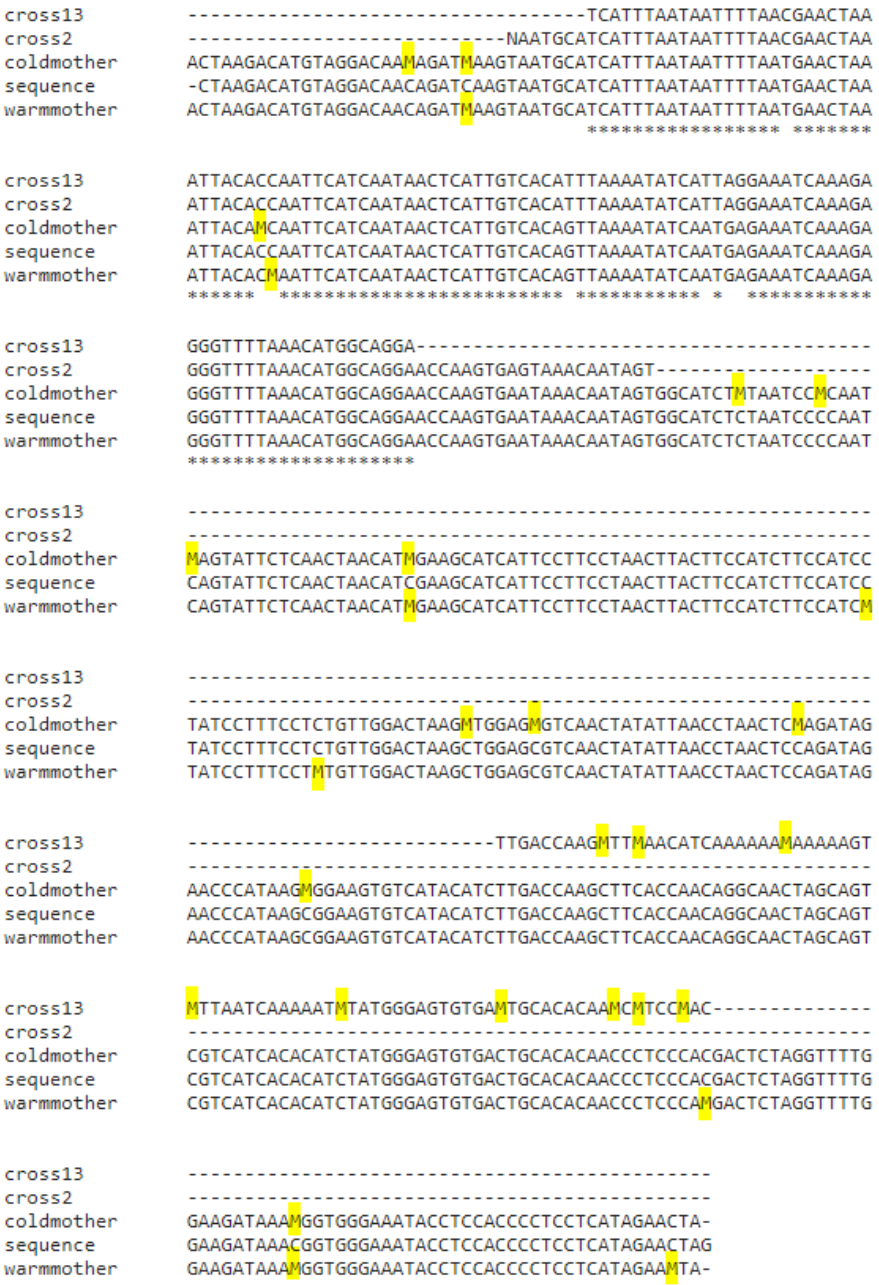


Figure 13: *Comparison between the individuals, the original sequence, and the mother's sequence for gene MA_10344604g0010. Marked nucleotides show where methylation occurred and represented as M in the sequence. Cross 13 corresponds to the F1 from "coldmother", while cross 2 corresponds to the F1 "warmmother".*

In contrast to **Figure 12**, **Figure 14** shows more coverage of the sequence. The gene MA_8008099g0010 (**Figure 14**) had satisfactory sequences that made it easier to align. The figure shows that methylation occurred at both crossings, 13 and 2. Even though in the analysis there was only methylation change in one base for cross 2, it still indicates that epigenetic changes occur. The methylation differences between the two crosses also happen at different places in the amplicon.

```
cross13         AAGCMTTTCMAACMATAAT---------ATMAAAGTCMTTCATTTGTGGGGATTTMAAGC
sequence        AAGACTTTGCTAAAACCATGGAAGATGAATCAAAGTCCTTCATTTGTGGGGATTTCAAGC
cross2          -------------------------GATTAAAAGTCCTTCATTAGTGGG----------
                                          * ****** ****** *****

cross13         CTCCAAAAGTTTTGGCAGACATAGTGGAATCCATAGCC----------------------
sequence        CTCCAAAAGTTTTGGCAGACATAGTGGAATCCATAGCCGCTGCAGTGTTCGTTGACAGTG
cross2          ------------------------------------------------------------


cross13         ---------------------GGCTAGTAATTCTATC------GCAACAATCACAATTCC
sequence        GGCGCTCTGCAGATCGAGTGTGGGAGGTATTTCTTTTAACTTGGTTTCCCTCAAACTTTC
cross2          --CGCTCTGCAGATCGAGTGTGGGAGGTATTTCTTTTAACTTGGTTTCCCTCAAACTTTC
                        **   *** **** *       *   *  *** * ** *

cross13         AAACCATTGACAAGTAAGATTCATGTCATGTGTTGTATGCAAGATGCAGGTGTTCCGGCC
sequence        AAACCATTGACAAGTAAGATTCATGTCATGTGTTGTATGCAAGATGCAGGTGTTCCGGCC
cross2          AAACCATTGACAAGTAAGATTCATGTCATGTGTTGTATGCAAGATGCAGGTGTTCCGGCC
                ************************************************************

cross13         TTTGTTAGAACCATTGATAAGTCTAGAGAAGCTTGAGTTGCACCCAGTGACAGAGCTAAC
sequence        TTTGTTAGAACCATTGATAAGTCTAGAGAAGCTTGAGTTGCACCCAGTGACAGAGCTAAC
cross2          TTTGTTAGAACCATTGATAAGTCTAGAGAAGCTTGAGTTGCACCCAGTGACAGAGCTAAC
                ************************************************************

cross13         ACAGCTATGCCAAAAGCAAGCCAAACACATCGAGTACAAAGCCTCCATGGAGGGTGATAC
sequence        ACAGCTATGCCAAAAGCAAGCCAAACACATCGAGTACAAAGCCTCCATGGAGGGTGATAC
cross2          ACAGCTATGCCAAAAGAAAGCCAAACACATCGAGTACAAAGCCTCCATGAAGGGTGATAC
                **************** ********************************** **********

cross13         GATTAGAGTTATGGCGGTGATAGATGGTAATGTCGTGGGGATTGCTGAACGCCAAAAGAA
sequence        GATTAGAGTTATGGCGGTGATAGATGGTAATGTCGTGGGGATTGCTGAACGCCAAAAGAA
cross2          GATTAGAGTTATGGCGCTGATAGATGGTAATGTMGTGGGGATTGCTGAACGCCAAAAGAA
                **************** **************** ************************

cross13         AGTACTGG----------------------------------------
sequence        AGTACTGGCAAAGAGAATGGCCGCTAAGCAGGCTCTTGGCAAGTTAAAA
cross2          A---------------------------------------------
                *
```

***Figure 14**: Comparison between the seedlings and the original sequence for gene MA_8008099g0010. Marked nucleotides show where methylation occurred and represented as M in the sequence.*

As for MA_10344604g0010, the sequences here were also compared with sequences from the mothers. **Figure 15** shows a complete alignment for both crossings. In this comparison, the methylations in the seedlings are similar to the methylation patterns in the mothers. Cross 13, several methylations are identical to both warm and cold mother, while cross 2 has only one methylation that corresponds well with D2V28 ("warmmother").

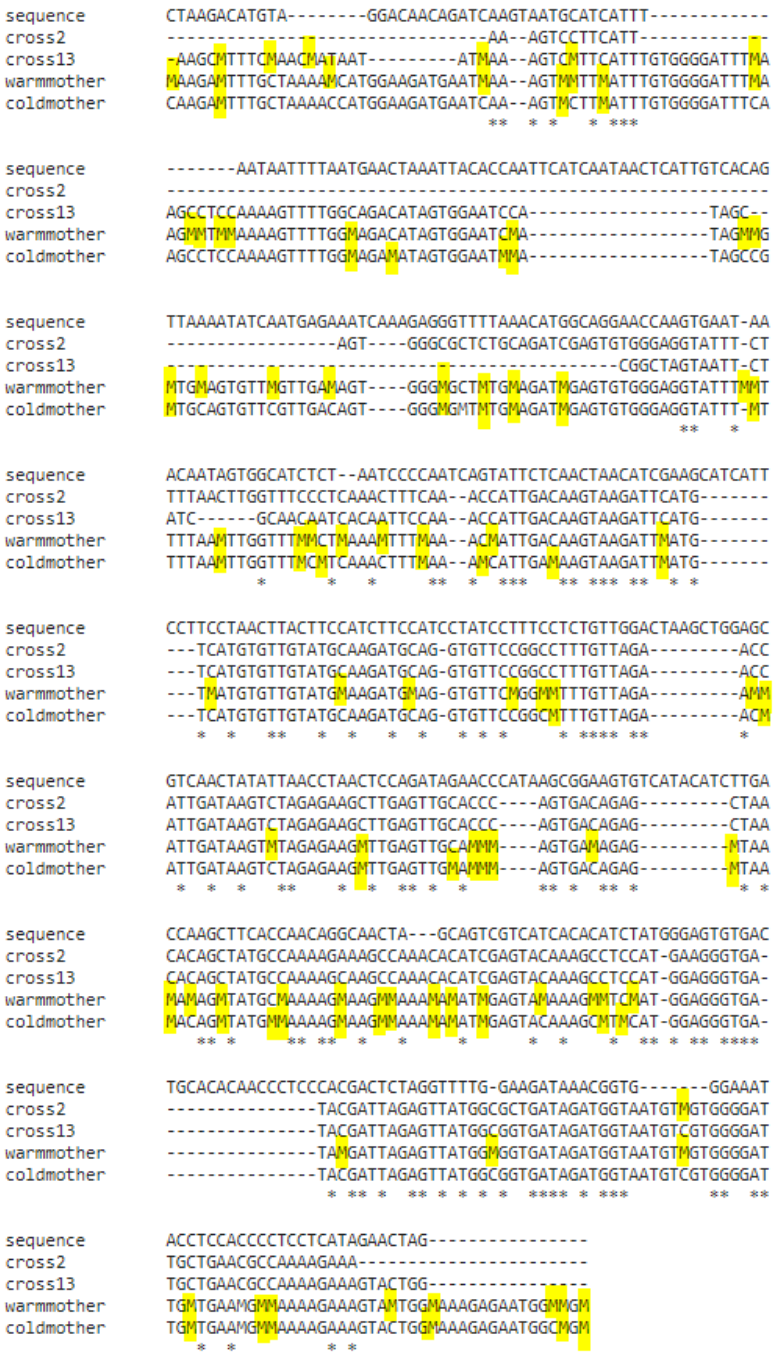


Figure 15: *Comparison between the individuals, the original sequence, and the mother's sequence for gene MA_8008099g0010. Marked nucleotides show where methylation occurred and represented as M in the sequence. Cross 13 corresponds to the F1 from "coldmother", while cross 2 corresponds to the F1 "warmmother".*

3.3 Relatedness between seedling and parents

To check whether the pollination had taken place as predicted and whether the individuals are genetic as expected, a genotyping analysis was performed. Here, individuals from the seedlings, pollen fathers from Skogfrøverket, and epitype trees growing at Hogsmark were analyzed. The analysis will give an indication of how closely related the various samples are.

The raw data from the analysis were used to perform a Principal Coordinate Analysis (PCA) (**Figure 16**). At the first sight, it seems like the samples separated into two different clusters. The cluster on the right are individuals who belongs to a previous SSR characterization of clonal tree epitypes taken from Hogsmark. This cluster includes the D2V clonal mothers that were used in this study. The cluster on the left (crosses 1, 2, 3, 4, 5, 7, 12, 13, and 16, **Table 1**) are samples taken from the seedling and the mothers (D2V18 and D2V28). This may indicate that the seedling is more related to the parents than the other trees from Hogsmark. Seedlings who are pollinated with open pollination conditions demonstrate more genetic variability in the resulting seedlings than the controlled crosses. This results in the openly pollinated individuals spreading more than the rest of the individuals.

COORD. 1 has a variance percentage of 13.26, and COORD. 2 has a variance percentage of 4.24. This gives a total variance percentage of 17.51, which is relatively low. Significant gaps in the detection of the markers may be the reason for this low percentage, which means that there are a lot of "zero" values in the raw data. With a variance percent at 17.51, relatedness between the seedling and the parents can be proven.

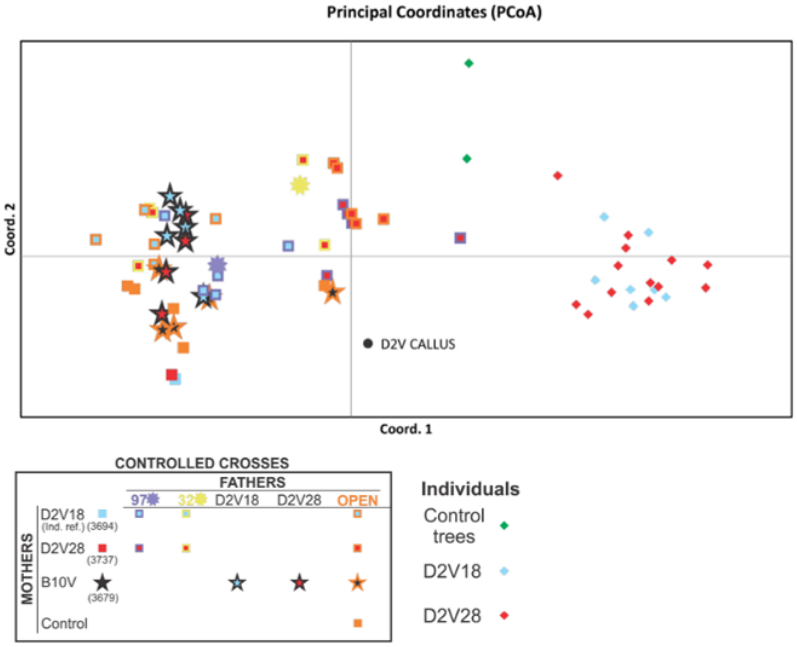


Figure 16: *Score plot from PCA analysis performed on data from genotyping. The plot illustrates the formation of two clusters, indicating that the individuals in each cluster are different from each other. The individuals within the clusters are more similar to each other and are thus more closely related. The cluster on the left is samples from the seedling, as well as the parents. The cluster to the right is samples from trees that have grown near the parents on Hogsmark. The figure also contains an overview of which individuals correspond to which crossing.*

4.0 DISCUSION

Different temperatures during embryogenesis have previously been shown to influence the resulting plants in terms of phenological features suggesting and epigenetic memory effect resulting in epitypes (Henderson and Jacobsen, 2007, JOHNSEN et al., 2005, Yakovlev et al., 2011). In this study, plants bred from well characterized epitype mothers, have been used to observe any epigenetic memory pattern that may be transgenerational in nature. In Norway spruce epigenetic marks such as DNA methylation have previously been poorly explored due to the technical limitations and the lack of an annotated genome that could help to link epigenetics with known genes with specific functions. Thanks to the accumulated knowledge, our aim in this thesis was to show the first time that it is possible to transmit certain phenological traits such as bud set and bud burst timing through concrete DNA methylation patterns in specific genes to the next generation.

4.1 Epigenetic memory effects the phenological pattern in seedlings

In the past, phenological features for the two epitypes used as mothers for the seedling in this study, have been documented (Kvaalen and Johnsen, 2008). These two epitypes, also known as cold-epitye (CE) and warm-epitype (WE), should after observations give the buds late and early features. This means that in CE, buds should occur earlier than in WE. These epitypes are used as mothers for the seedlings and considering to how they were bred, it is expected to observe a similar effect in the seedling. According to Skogfrøverket, the fathers of the seedling (97 (Late) and 32 (Early), **Table 1**) have shown similar effect as observed in CE and WE.

As represented in the results, two different analyses of the phenological features have been performed. First to compare the crossing independently of time, while the other analysis takes the time into account. In the first analysis, represented in **Table 15** and **Table 17**, there is a significant difference between almost all the crossings which indicate that differences are expressed phenological. During the bud burst, a significant difference is more visible than at bud set. This is almost expected as the plants used during the experiment were of small size and thus difficult to distinguish which stages the buds corresponded to. Phenological differences related to the memory of temperature during somatic and zygotic embryonic development have previously been reported (Kvaalen and Johnsen, 2008), but never in a transgenerational fashion.

The comparison between crosses 1 and 2 shows a significant difference. For the bud set period, this difference has been demonstrated at day 12; during the bud burst a significant difference appears from day 17 to day 28. These two crossings have the same epitype mother but different fathers. The difference between these two crosses may simply indicate that the traits inherited from the father are classical genetic traits since the fathers are genetically different. For the comparison between crosses 2 and 3, and 2 and 13, the significant difference emerges at day 18 during the bud burst period. Crosses 2 and 3 have the same mother but different fathers, therefore the phenological features inherited from the father have an impact. In crosses 2 and 13, these have the same father but different epitype mothers (phenologically different but genetically the same). Here, on the other hand, it will indicate that epigenetic traits (possibly DNA methylation differences) inherited from the mother are reflected in the phenology and affect the epigenetic memory of the seedling, suggesting an transgenerational epigenetic effect. Comparing crosses 1 and 16 indicates a significant difference during the bud set period at day 9 and day 11. At the same time, a marginal difference on day 12 occurs, also supporting a transgenerational epigenetic effect. However, it appears that this difference it is not detectable during bud burst. Cross 1 and 16 are both open pollinated and have epigenetically different mothers. Due to open pollination, it is not possible to say with certainty how well the fathers are related. This change could indicate that the difference occurs randomly because the plants were small and difficult to distinguish from the various stages of development. This cessation of significant difference also occurs in the comparison between crosses 13 and 16. These crosses have the same mother but different fathers. The comparison between 1 and 3 indicates no significant difference. Crosses 1 and 3 have the same mother but a different father. Cross 1, as mentioned earlier, is pollinated under open pollination conditions, while cross 3 is pollinated with pollen donor 32 (early bud break tree). Since there is no significant difference, neither during bud set nor bud burst, it is conceivable that cross 1 may be pollinated by nearby trees with similar phenological features as pollen donor 32.

The article by Kohman and Johnsen inspired the SD period performed on the seedlings from the controlled crosses. This is a photoperiod referenced as the critical photoperiod for Norway spruce (Kohmann and Johnsen, 1994). It is defined as a lower limit for daylight required for bud set. The conditions under SD should trigger the plants to perform bud formation indicating differences that epigenetic differences (between otherwise genetically similar plants) may cause. The SD period may elicit minor differences that are of interest in this study. Furthermore, LD was performed where the light conditions return to continuous light.

The cessation of the differences in the comparison between 1 and 16, and 13 and 16 may indicate that the plant has achieved an intense stimulation during bud set, which causes great growth during bud burst. If this is the case, the plants will be perceived as more similar, thus not finding the significant difference. As mentioned, cross 13 and 16 have the same mother. At the same time, cross 16 are pollinated under open pollination conditions, which indicates a larger gene pool than when using the same father under controlled conditions, and this diversity can mask the mothers' effect. This may also explain why the significant difference ceases.

Through the phenotyping analysis, several interesting observations have been made. The fact that significant differences are observed between crosses that have different mothers and fathers indicates that genes inherited from the parents influence the bud phenology. Several studies indicate that Norway spruce can remember temperature and light periods during embryogenesis, and this memory can last for at least 20 years (Skrøppa et al., 2007, Skrøppa et al., 2010, Yakovlev et al., 2011). In this study, the F1 generation was used, which means seedlings from previously documented plants. This has made it interesting to see how the different parents may have affected the plants.

4.2 Method development and bisulfite sequencing

Bisulfite sequencing was preformed to detect DNA methylation differences in from Norway spruce materials. At this time, availability for a method that present repetitive and informative results for DNA methylations in spruce is limited. Therefore, several previous experiences were used to optimize a reliable method, including methylation differences in several genes from pilot experiments done by the group. Articles from several projects were included to compose a method for our purpose.

4.2.1 Designing primers

As described in the material and methods, determining genes to be examined and reviewing previous data from the pilot experiment were time consuming. The selection was based on previous results showing differences between methylation patterns in somatic embryos with containing heat sum temperatures (grown at 18 and 28 °C). Genes were selected based on significant differences and the function of the genes. In order to design primers for the selected genes, the program Methprimer was used, which has previously been proved to be

reliable for designing primers (Li and Dahiya, 2002, Leitão et al., 2018). After the selection, only two primers remained that work optimally. Factors such as GC content, melting temperature and incorrectly creation could be reasons why the remaining primers did not work. The primers designed in this project were made to fit bisulfite converted DNA which is a more complex process than making primers on regular DNA. Methprimer is designed to find suitable primers for the desired sequence (Li and Tollefsbol, 2011). Not possible to design primers, together with desired function were some reasons to why this study had limited number of primers compare to the earlier project within this group.

4.2.2 Confirmation for methylations

Before the samples were sequenced, a gel was used to confirm (**Figure 11**) that they contained the correct amplicon size and that the primers worked adequately. The bands were purified from the gel and passed on for sequencing. The results from the sequencing showed varying quality, which appears in the sequences. Since we know that the gel showed good and clear bands, it will be difficult to blame bad DNA. However, DNA may have been lost during the purification process, making the sequence challenging to detect. The samples sent for sequencing had five replicas where all the samples were used to make a final sequence representing the cross. Some of the replicas were deficient or not detected. Only two replicas from one of the samples (cross 2 reverse MA_10344604g0010), were satisfying. Here, was impossible to make a final sequence as there will be a 50 % probability of which nucleotide will be in place. Due to this shortcoming, **Figure 12** lacks part of the sequence, which means that it was not as easy to compare the different crosses against the original sequence. **Figure 14**, which contains the two crosses, including the original sequence, also illustrates some shortcomings. However, here the sequences cover more of the amplicon, which gives a better picture of the methylation pattern in the gene. Although there are some deficiencies, we can still state that methylation was present at both crosses at both genes. The methylation is marked in the figures (**Figure 12, Figure 13, Figure 14** and **Figure 15**), so it is easier to see where these changes occur and see which cross contains more methylation. The sequences were also compared with the mother's sequences to see if the seedling inherited the methylation patterns. In **Figure 13**, it is difficult to conclude whether the methylation comes from the mother since no direct connection occurs. This means that methylations from seedling are not at the exact same spot as the methylations for the mothers. Nevertheless, this does not mean that the methylation is not inherited since the father is not considered. For MA_8008099g0010 (**Figure**

15), there were several cases where methylation from the seedlings corresponded to the mothers. It is easy conclude that parts of the sequence for the seedlings are inherited from the mother. As we can observe, the sequence is different for some nucleotides. This differences in the sequencing are due not only to natural variation but also to the occurrence of recombination during sexual reproduction. The sequence we also used as the original ("sequence" in the figures 12 to 15) are from mother plants, and it will therefore have some distinctions compared to the seedlings.

Because this is the first time anyone looks at the inheritance of methylation patterns in the F1 generation in Norway spruce, there is no literature we can compare the results to. The observations in this project will then be an indication that inheritance occurs. To ensure that the results and conclusions are real, more analyzes must be performed on a more significant number of samples than this experiment contains. In any case, our results show that our seedlings have tendencies towards inherited material that contains epigenetic marks from the parents.

4.3 Demonstration of relatedness

Controlled pollination of the plants used in this study was performed. To confirm the expectations for the parents (**Table 1**), a genotyping analysis was performed. This analysis can potentially tell if the individuals are related and indicates who the father is (relevant for the open pollinated crosses).

The score plot represented in **Figure 14** illustrate results from a PCA based on the data from individuals used during this analysis, as well as raw data from previous analysis on the generation from the parents. The score plot indicate two clusters. Cluster to the right contains individuals from trees at Hogsmark and the cluster to the left involve the seedling, mothers (D2V18 and D2V28) and the pollen donors (97 and 32, **Table 1**). These clusters demonstrate which individuals are most likely related. As illustrated in the plot, individuals from Hogsmark are different from the individuals we have used during this study. Based on the expectations we assumed that the mother and father should be in the same cluster as the seedling, which they are. Crosses 1 and 2 are slightly further from the rest of the individuals this could indicate that they are somewhat different from the others. The conditions for COORD. 1 and COORD. 2 should also be considered. The COORD.1 and COORD.2 have a relatively low percentage (17.51 %), although the first coordinates have more variance than later coordinates, they only consist of 17.51 % of the original variance of 90% (Ringnér, 2008).

This low percentage are most likely a result of not-detected markers in the data. During the analysis, detection of all the SSR markers were not confirmed which can lead to unconvinced values.

As mentioned in the materials and methods section, the markers were already mixed, which should not have any impact in the results, except there have been observed slightly poor result by using this finished mix in previous experiments. These not-detected markers could occur for several reasons; the mixed markers that have been stored at -20 degrees over time could have incurred damage, or it could be poor DNA that contained other components which may interfere the analysis. From the DNA extractions, it appears that the DNA did not have the optimal quality, therefore, by using housekeeping genes we could perform a control test to prove the presence of DNA. To perform this test, the housekeeping gene actin were used. Actin is a ~100 bp housekeeping gene which are expressed in the Norway spruce sequence (Schwarzerová et al., 2010). The control test presented weak bands at the right size, even if the bands were feeble the machine (SeqStudio Genetic Analyzer) have a high detection and would be able to recognize the amplicons.

To summarize the results from this analysis, the fathers and mothers are more related to the individuals than the rest of the trees at Hogsmark. Therefore, the expected fathers and mothers are considered to be the right parents and the controlled crosses were successful. Even if the variance percentage was low (17% of variance explained by coordinates 1 and 2), the results indicated relatedness.

5.0 CONCLUSIONS

In conclusion, we have identified relevant differences in bud phenology between seedling breed from epigetically different mothers (epitypes) and different fathers. Some of these differences indicate that the seedling may have inherited an epigenetic memory from their parents, as in the case with epigenetically different but genetically identical mothers with the same father resulting in phenological differences in the offspring. Other crosses point to classically inheritance, as exemplified by same mother but different fathers (late and early flushing) giving phenotypically different offspring as expected. For the bisulfite sequencing, we found methylations patterns for some bases that can indicate that the epigenetic methylations are reflected in F1-generation, but much more solid sequencing data is needed to establish this. The genotyping results also confirm that the F1-generations are closely related to the parents, which means we can use this and similarly generated plant material to later confirm or reject that the epigenetic memory mechanism in Norway spruce can be inherited.

6.0 REFERENCES

BARTELS, A., HAN, Q., NAIR, P., STACEY, L., GAYNIER, H., MOSLEY, M., HUANG, Q. Q., PEARSON, J. K., HSIEH, T. F., AN, Y. C. & XIAO, W. 2018. Dynamic DNA Methylation in Plant Growth and Development. *Int J Mol Sci,* 19.

BECK, C. B. 2010. *An Introduction to Plant Structure and Development: Plant Anatomy for the Twenty-First Century*, Cambridge University Press.

BÍNOVÁ, Z., KORECKÝ, J., DVOŘÁK, J., BÍLÝ, J., ZÁDRAPOVÁ, D., JANSA, V. & LSTIBŮREK, M. 2020. Genetic Structure of Norway Spruce Ecotypes Studied by SSR Markers. *Forests,* 11**,** 110.

BLOOR, I. S. M., ATTRILL, M. J. & JACKSON, E. L. 2013. Chapter One - A Review of the Factors Influencing Spawning, Early Life Stage Survival and Recruitment Variability in the Common Cuttlefish (Sepia officinalis). *In:* LESSER, M. (ed.) *Advances in Marine Biology.* Academic Press.

BUSOV, V., CARNEROS, E. & YAKOVLEV, I. 2016. EARLY BUD-BREAK1 (EBB1) defines a conserved mechanism for control of bud-break in woody perennials. *Plant Signal Behav,* 11**,** e1073873.

BÜRGLIN, T. R. & AFFOLTER, M. 2016. Homeodomain proteins: an update. *Chromosoma,* 125**,** 497-521.

CARNEROS, E., YAKOVLEV, I., VIEJO, M., OLSEN, J. E. & FOSSDAL, C. G. 2017. The epigenetic memory of temperature during embryogenesis modifies the expression of bud burst-related genes in Norway spruce epitypes. *Planta,* 246**,** 553-566.

DESTEFANIS, G., BARGE, M. T., BRUGIAPAGLIA, A. & TASSONE, S. 2000. The use of principal component analysis (PCA) to characterize beef. *Meat Science,* 56**,** 255-259.

DOGRAMACI, M., FOLEY, M.E., CHAO, W.S., CHRISTOFFERS, M.J., ANDERSON, J.V., HORVATH, D.P., 2015. *Advances in Plant Dormancy* Springer International Plublishing.

FALTINOVÁ, Z., KORECKY, J., DVOŘÁK, J., BÍLÝ, J., ZÁDRAPOVÁ, D., JANSA, V. & LSTIBŮREK, M. 2020. Genetic Structure of Norway Spruce Ecotypes Studied by SSR Markers. *Forests,* 11**,** 110.

FARJON, A. 2017. *Picea abies. The IUCN Red List of Theatened Species 2017.* [Online]. Available: https://dx.doi.org/10.2305/IUCN.UK.2017-2.RLTS.T42318A71233492.en. [Accessed 26. November 2020].

FENG, S., HE, R., LU, J., JIANG, M., SHEN, X., JIANG, Y., WANG, Z. A. & WANG, H. 2016. Development of SSR Markers and Assessment of Genetic Diversity in Medicinal Chrysanthemum morifolium Cultivars. *Frontiers in Genetics,* 7.

FENNER, M. 1998. The phenology of growth and reproduction in plants. *Perspectives in Plant Ecology, Evolution and Systematics,* 1**,** 78-91.

FROMMER, M., MCDONALD, L. E., MILLAR, D. S., COLLIS, C. M., WATT, F., GRIGG, G. W., MOLLOY, P. L. & PAUL, C. L. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 1827-1831.

HENDERSON, I. R., CHAN, S. R., CAO, X., JOHNSON, L. & JACOBSEN, S. E. 2010. Accurate sodium bisulfite sequencing in plants. *Epigenetics,* 5**,** 47-49.

HENDERSON, I. R. & JACOBSEN, S. E. 2007. Epigenetic inheritance in plants. *Nature,* 447**,** 418-424.

HOPKINS, W. G., HUNER, N. P. A 2008. *Introductin of plant Physiology* WILEY.

IWASAKI, M. & PASZKOWSKI, J. 2014. Epigenetic memory in plants. *The EMBO journal,* 33**,** 1987-1998.

JACKSON, S. D. 2009. Plant responses to photoperiod. *New Phytologist,* 181**,** 517-531.

JANSSON, G., DANUSEVICUS, D., GROTEHUSMAN, H., KOWALCZYK, J., KRAJMEROVA, D., SKRØPPA, T., WOLF, H. 2013. *Forest Tree Breeding in Europe* Springer.

JOHNSEN, Ø., FOSSDAL, C. G., NAGY, N., MØLMANN, J., DÆHLEN, O. G. & SKRØPPA, T. 2005. Climatic adaptation in Picea abies progenies is affected by the temperature during zygotic embryogenesis and seed maturation. *Plant, Cell & Environment,* 28**,** 1090-1102.

KOHMANN, K. & JOHNSEN, Ø. 1994. The timing of bud set in seedlings of Picea abies from seed crops of a cool versus a warm spring and summer. *Silvae Genetica,* 43**,** 329-333.

KVAALEN, H. & JOHNSEN, Ø. 2008. Timing of bud set in Picea abies is regulated by a memory of temperature during zygotic and somatic embryogenesis. *New Phytol,* 177**,** 49-59.

LEITÃO, E., BEYGO, J., ZESCHNIGK, M., KLEIN-HITPASS, L., BARGULL, M., RAHMANN, S. & HORSTHEMKE, B. 2018. Locus-Specific DNA Methylation Analysis by Targeted Deep

Bisulfite Sequencing. *In:* JELTSCH, A. & ROTS, M. G. (eds.) *Epigenome Editing: Methods and Protocols.* New York, NY: Springer New York.

LEONTIOU, C. A., HADJIDANIEL, M. D., MINA, P., ANTONIOU, P., IOANNIDES, M. & PATSALIS, P. C. 2015. Bisulfite Conversion of DNA: Performance Comparison of Different Kits and Methylation Quantitation of Epigenetic Biomarkers that Have the Potential to Be Used in Non-Invasive Prenatal Testing. *PloS one,* 10**,** e0135058-e0135058.

LI, L. C. & DAHIYA, R. 2002. MethPrimer: designing primers for methylation PCRs. *Bioinformatics,* 18**,** 1427-31.

LI, Y. & TOLLEFSBOL, T. O. 2011. DNA methylation detection: bisulfite genomic sequencing analysis. *Methods in molecular biology (Clifton, N.J.),* 791**,** 11-21.

MOORE, L. D., LE, T. & FAN, G. 2013. DNA Methylation and Its Basic Function. *Neuropsychopharmacology,* 38**,** 23-38.

PARTANEN, J., HÄNNINEN, H. & HÄKKINEN, R. 2005. Bud burst in Norway spruce (Picea abies): preliminary evidence for age-specific rest patterns. *Trees,* 19**,** 66-72.

PARTANEN, J., KOSKI, V. & HÄNNINEN, H. 1999. Effect of photoperiod and temperature on the timing of bud burst in Norway spruce (Picea abies). *Tree physiology,* 18**,** 811-816.

PIKAARD, C. S. & MITTELSTEN SCHEID, O. 2014. Epigenetic regulation in plants. *Cold Spring Harb Perspect Biol,* 6**,** a019315.

PINHEIRO J, B. D., DEBROY S, SARKAR D, 2013. nlme: Linear and Nonlinear Mixed Effects Models. .

RINGNÉR, M. 2008. What is principal component analysis? *Nature Biotechnology,* 26**,** 303-304.

ROHDE, A. & BHALERAO, R. 2007. Plant dormancy in the perennial context. *Trends in plant science,* 12**,** 217-23.

ROWTHER, F. B., KARDOONI, H. & WARR, T. 2012. TOUCH-UP gradient amplification method. *Journal of biomolecular techniques : JBT,* 23**,** 1-3.

SCHWARZEROVÁ, K., VONDRÁKOVÁ, Z., FISCHER, L., BORÍKOVÁ, P., BELLINVIA, E., ELIÁSOVÁ, K., HAVELKOVÁ, L., FISEROVÁ, J., VÁGNER, M. & OPATRNÝ, Z. 2010. The role of actin isoforms in somatic embryogenesis in Norway spruce. *BMC plant biology,* 10**,** 89-89.

SKRØPPA, T., KOHMANN, K., JOHNSEN, Ø., STEFFENREM, A. & EDVARDSEN, Ø. 2007. Field performance and early test results of offspring from two Norway spruce seed

orchards containing clones transferred to warmer climates. *Canadian Journal of Forest Research,* 37**,** 515-522.

SKRØPPA, T., TOLLEFSRUD, M. M., SPERISEN, C. & JOHNSEN, Ø. 2010. Rapid change in adaptive performance from one generation to the next in Picea abies—Central European trees in a Nordic environment. *Tree Genetics & Genomes,* 6**,** 93-99.

SØGAARD, G., JOHNSEN, O., NILSEN, J. & JUNTTILA, O. 2008. Climatic control of bud burst in young seedlings of nine provenances of Norway spruce. *Tree Physiol,* 28**,** 311-20.

SØNSTEBØ, J. H., TOLLEFSRUD, M. M., MYKING, T., STEFFENREM, A., NILSEN, A. E., EDVARDSEN, Ø. M., JOHNSKÅS, O. R. & EL-KASSABY, Y. A. 2018. Genetic diversity of Norway spruce (Picea abies (L.) Karst.) seed orchard crops: Effects of number of parents, seed year, and pollen contamination. *Forest Ecology and Management,* 411**,** 132-141.

TOLLEFSRUD, M. M., KISSLING, R., GUGERLI, F., JOHNSEN, Ø., SKRØPPA, T., CHEDDADI, R., VAN DER KNAAP, W. O., LATAŁOWA, M., TERHÜRNE-BERSON, R., LITT, T., GEBUREK, T., BROCHMANN, C. & SPERISEN, C. 2008. Genetic consequences of glacial survival and postglacial colonization in Norway spruce: combined analysis of mitochondrial DNA and fossil pollen. *Mol Ecol,* 17**,** 4134-50.

TOLLEFSRUD, M. M., SØNSTEBØ, J. H., BROCHMANN, C., JOHNSEN, Ø., SKRØPPA, T. & VENDRAMIN, G. G. 2009. Combined analysis of nuclear and mitochondrial markers provide new insight into the genetic structure of North European Picea abies. *Heredity,* 102**,** 549-562.

TSUDA, Y., CHEN, J., STOCKS, M., KÄLLMAN, T., SØNSTEBØ, J. H., PARDUCCI, L., SEMERIKOV, V., SPERISEN, C., POLITOV, D., RONKAINEN, T., VÄLIRANTA, M., VENDRAMIN, G. G., TOLLEFSRUD, M. M. & LASCOUX, M. 2016. The extent and meaning of hybridization and introgression between Siberian spruce (Picea obovata) and Norway spruce (Picea abies): cryptic refugia as stepping stones to the west? *Molecular Ecology,* 25**,** 2773-2789.

UCHIDA, N. & TORII, K. U. 2019. Stem cells within the shoot apical meristem: identity, arrangement and communication. *Cellular and Molecular Life Sciences,* 76**,** 1067-1080.

VALLEDOR, L., HASBÚN, R., MEIJÓN, M., RODRÍGUEZ, J. L., SANTAMARÍA, E., VIEJO, M., BERDASCO, M., FEITO, I., FRAGA, M. F., CAÑAL, M. J. & RODRÍGUEZ, R. 2007.

Involvement of DNA methylation in tree development and micropropagation. *Plant Cell, Tissue and Organ Culture,* 91**,** 75-86.

VANYUSHIN, B. F., ASHAPKIN, VASILI V. 2008. *DNA Methylation in Plants* New York, United Stated Nova Science Publishers Inc.

VIEIRA, M. L. C., SANTINI, L., DINIZ, A. L. & MUNHOZ, C. D. F. 2016. Microsatellite markers: what they mean and why they are so useful. *Genetics and molecular biology,* 39**,** 312-328.

WEINHOLD, B. 2006. Epigenetics: the science of change. *Environmental health perspectives,* 114**,** A160-A167.

WELLING, A. & PALVA, E. T. 2006. Molecular control of cold acclimation in trees. *Physiologia Plantarum,* 127**,** 167-181.

YAKOVLEV, I., FOSSDAL, C. G., SKRØPPA, T., OLSEN, J. E., JAHREN, A. H. & JOHNSEN, Ø. 2012. An adaptive epigenetic memory in conifers with important implications for seed production. *Seed Science Research,* 22**,** 63-76.

YAKOVLEV, I. A., ASANTE, D. K. A., FOSSDAL, C. G., JUNTTILA, O. & JOHNSEN, Ø. 2011. Differential gene expression related to an epigenetic memory affecting climatic adaptation in Norway spruce. *Plant Science,* 180**,** 132-139.

YAKOVLEV, I. A., ASANTE, D. K. A., FOSSDAL, C. G., PARTANEN, J., JUNTTILA, O. & JOHNSEN, Ø. 2008. Dehydrins expression related to timing of bud burst in Norway spruce. *Planta,* 228**,** 459-472.

YAKOVLEV, I. A., CARNEROS, E., LEE, Y., OLSEN, J. E. & FOSSDAL, C. G. 2016. Transcriptional profiling of epigenetic regulators in somatic embryos during temperature induced formation of an epigenetic memory in Norway spruce. *Planta,* 243**,** 1237-1249.

YAKOVLEV, I. A. & FOSSDAL, C. G. 2017. In Silico Analysis of Small RNAs Suggest Roles for Novel and Conserved miRNAs in the Formation of Epigenetic Memory in Somatic Embryos of Norway Spruce. *Frontiers in Physiology,* 8.

YAKOVLEV, I. A., FOSSDAL, C. G. & JOHNSEN, Ø. 2010. MicroRNAs, the epigenetic memory and climatic adaptation in Norway spruce. *New Phytologist,* 187**,** 1154-1169.

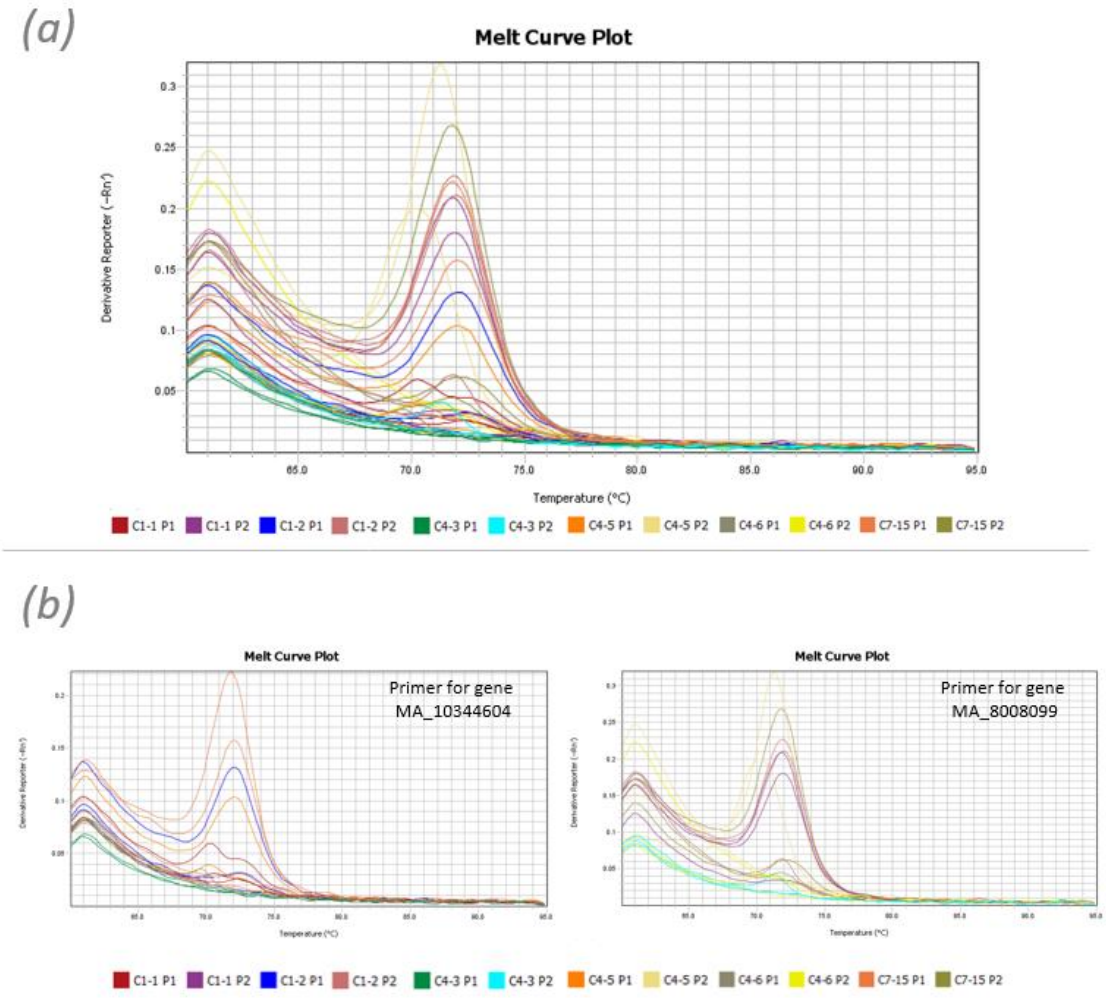
ZHANG, H., LANG, Z. & ZHU, J.-K. 2018. Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology,* 19**,** 489-506.

7.0 APPENDIX

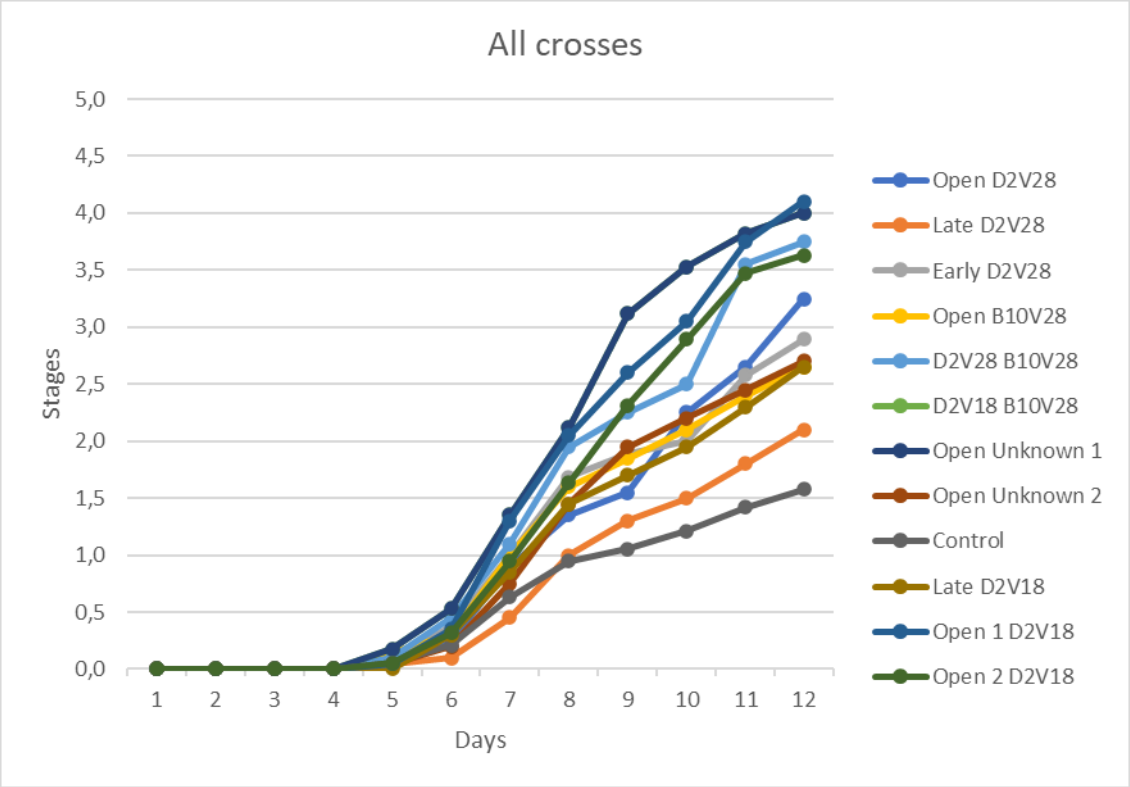
A.1: All primers ordered without adapters. Ten primers were designed to be used on bisulfited DNA. None of the mentioned primers gave satisfactory results, which led to the design of new primers with adapters. Several primers were designed for the same gene, as shown in the table, the reason for this is that primer design was performed on single stranded DNA.

Name	Sequence (5' – 3')	Tm °C	Length
MA_10048467_LF	TTAATAATGGGATTTTAAAGTATTGAA	53	27
MA_10048467_LR	ACATCAAAAATTAAAATACATAACTTA	51	27
MA_10048467_UF	GTATAAAATTTATAATTTAAATTTTAATA	48	29
MA_10048467_UR	TCAAAAAATCTATATTAATTATATCATAT	51	29
MA_10344604_F	TTAAGATATGTAGGATAATAGATTAAGTA	55	29
MA_10344604_R	CTAATTCTATAAAAAAAAATAAAAATATTTCCC	56	33
MA_10425853_LF	TAAGTGAATAAGTTTTTTTATATAGGG	54	27
MA_10425853_LR	AAAACAACTATCATCAACAATCTCTAT	56	27
MA_10425853_UF	ATATTGGAGAGTATGATATGGAATATG	57	27
MA_10425853_UR	TTATATATATAAACAATTAAAATTACATTA	50	30
MA_10427514_F	TGGAATTAAGAGAGTTTTAGTTGAGTA	57	27
MA_10427514_R	TTTCTTAAAAAATACATCTTTCC	50	23
MA_120256_F	TTTAATTAGGATATTATATGGGGA	52	24
MA_120256_R	CCTAATAAAAACAAAAAACAATATTTAACTT	55	31
MA_120256_LF	TAATTAGGATATTATATGGGGATAGGT	57	27
MA_120256_LR	CTTCTCTTAACACTTTTAAAACACAAA	56	27
MA_120256_UF	GGAATTAATATAGTAATAAATTTTTAA	50	27
MA_120256_UR	TTAAATTATAAAACCTATATCCATCTTA	53	28
MA_130726_LF	TGGGTATGAGGTTAAATTGTTATTAAGT	58	28
MA_130726_LR	AATTAAATTCATAATTTACATAAAAAA	48	27
MA_130726_UF	TTGAGTTTAATGGATAATTAAATTTTT	51	27
MA_130726_UR	CATTTAAATTTTATACATAATTAAAAATTA	50	30
MA_132879_F	TTTTTTAAAGATAAGGTAGTTGAAA	52	25
MA_132879_R	AAACCTAACACATAAAAAAATACTCTCAAA	57	30
MA_132879_LF	GTTGTAATAATTAAGATTATAAGAAGAAAT	54	30
MA_132879_LR	CTCTTACTATATTATCTAAAAATCAAATT	54	29
MA_132879_UF	GATAATATTATTAATAATTATAAATATTTGA	51	31
MA_132879_UR	ACATCACAATTAAAATTACAAATAAAT	51	27
MA_138058_LF	GATATTTAGTGTTAAAAAATGTAAGTGT	55	28
MA_138058_LR	ATTATTAAAATTCACTCACTAAATTCT	53	27
MA_138058_UF	TGTTTTTTTTAGATATTTAGTGTTAAA	51	27
MA_138058_UR	TAAAAAAAAAAACCAACATTACAATT	51	26
MA_796543_F	TGAATTTTGTTTAATGGTGAAAAA	51	24
MA_796543_R	AATTAAAAAATACAATCCACTTTTA	50	25
MA_796543_LF	TATGTGATTTTTTTTTAAAGTTTGTTT	51	27
MA_796543_LR	CAAATTTAAAAATAAATTACACCTCC	54	26
MA_796543_UF	TAAATAAATTTAAAAGTGGATTGTATT	51	27
MA_796543_UR	ACTTTTATATAACCTTCTCTTAAAACT	54	27
MA_8008099_F	AAGATTTTGTTAAAATTATGGAAGATGAA	55	29
MA_8008099_R	TTTTAACTTACCAAAAACCTACTTA	53	25
MA_96853_UF	TTTTTTATTATAAAATTAGTTTTTTTTA	48	28
MA_96853_UR	ATATATCACTCTCAAAAATAAATAAAA	51	27

A.2: Melting curve results. Two different genes were used for six DNA samples and all DNA samples had three replicas, a total of 36 samples. The challenge with this analysis was to get the replicates to be similarly identical. (a) The melting curve for all 36 samples. (b) The separated melting curve of the two genes to make it easier to see the differences.



A.3: During the analysis with phenotyping, bud development was registered for all the crosses produced after the pollination. Comparing all the crossings can be perceived as difficult to read, and it is challenging to compare relevant crossings. The figure represents results from the SD period where the individuals were under 18 hours of light for 6 weeks.



A.4: Results from ANOVA analysis where cross 1 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 1 and the other groups are represented.

BUD SET – CROSS 1 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	0.0125000	0.13753915	1165	0.090883	0.9276
day2	0.0000000	0.14685984	1165	0.000000	1.0000
day3	0.0000000	0.14685984	1165	0.000000	1.0000
day4	0.0000000	0.14685984	1165	0.000000	1.0000
day5	0.0700000	0.14685984	1165	0.476645	0.6337
day6	0.2700000	0.14685984	1165	1.838488	0.0662
day7	0.8900000	0.14685984	1165	6.060200	0.0000
day8	1.4900000	0.14685984	1165	10.145728	0.0000
day9	1.7900000	0.14685984	1165	12.188493	0.0000
day10	2.1300000	0.14685984	1165	14.503625	0.0000
day11	2.5900000	0.14685984	1165	17.635863	0.0000
day12	2.9700000	0.14685984	1165	20.223365	0.0000
Cross 1 vs 2	-0.3375000	0.09479762	1165	-3.560216	0.0004
Cross 1 vs 3	-0.0375000	0.09479762	1165	-0.395580	0.6925
Cross 1 vs 13	-0.0958333	0.09479762	1165	-1.010926	0.3123
Cross 1 vs 16	0.4083333	0.09479762	1165	4.307422	0.0000

A.4: Results from ANOVA analysis where cross 2 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 2 and the other groups are represented.

BUD SET – CROSS 2 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	-0.3250000	0.13753915	1165	-2.362964	0.0183
day2	0.0000000	0.14685984	1165	0.000000	1.0000
day3	0.0000000	0.14685984	1165	0.000000	1.0000
day4	0.0000000	0.14685984	1165	0.000000	1.0000
day5	0.0700000	0.14685984	1165	0.476645	0.6337
day6	0.2700000	0.14685984	1165	1.838488	0.0662
day7	0.8900000	0.14685984	1165	6.060200	0.0000
day8	1.4900000	0.14685984	1165	10.145728	0.0000
day9	1.7900000	0.14685984	1165	12.188493	0.0000
day10	2.1300000	0.14685984	1165	14.503625	0.0000
day11	2.5900000	0.14685984	1165	17.635863	0.0000
day12	2.9700000	0.14685984	1165	20.223365	0.0000
Cross 2 vs 1	0.3375000	0.09479762	1165	3.560216	0.0004
Cross 2 vs 3	0.3000000	0.09479762	1165	3.164637	0.0016
Cross 2 vs 13	0.2416667	0.09479762	1165	2.549291	0.0109
Cross 2 vs 16	0.7458333	0.09479762	1165	7.867638	0.0000

A.6: Results from ANOVA analysis where cross 3 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 3 and the other groups are represented.

BUD SET – CROSS 3 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	-0.0250000	0.13753915	1165	-0.181766	0.8558
day2	0.0000000	0.14685984	1165	0.000000	1.0000
day3	0.0000000	0.14685984	1165	0.000000	1.0000
day4	0.0000000	0.14685984	1165	0.000000	1.0000
day5	0.0700000	0.14685984	1165	0.476645	0.6337
day6	0.2700000	0.14685984	1165	1.838488	0.0662
day7	0.8900000	0.14685984	1165	6.060200	0.0000
day8	1.4900000	0.14685984	1165	10.145728	0.0000
day9	1.7900000	0.14685984	1165	12.188493	0.0000
day10	2.1300000	0.14685984	1165	14.503625	0.0000
day11	2.5900000	0.14685984	1165	17.635863	0.0000
day12	2.9700000	0.14685984	1165	20.223365	0.0000
Cross 3 vs 1	0.0375000	0.09479762	1165	0.395580	0.6925
Cross 3 vs 2	-0.3000000	0.09479762	1165	-3.164637	0.0016
Cross 3 vs 13	-0.0583333	0.09479762	1165	-0.615346	0.5384
Cross 3 vs 16	0.4458333	0.09479762	1165	4.703002	0.0000

A.7: Results from ANOVA analysis where cross 13 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 13 and the other groups are represented.

BUD SET – CROSS 13 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	-0.0833333	0.13753915	1165	-0.605888	0.5447
day2	0.0000000	0.14685984	1165	0.000000	1.0000
day3	0.0000000	0.14685984	1165	0.000000	1.0000
day4	0.0000000	0.14685984	1165	0.000000	1.0000
day5	0.0700000	0.14685984	1165	0.476645	0.6337
day6	0.2700000	0.14685984	1165	1.838488	0.0662
day7	0.8900000	0.14685984	1165	6.060200	0.0000
day8	1.4900000	0.14685984	1165	10.145728	0.0000
day9	1.7900000	0.14685984	1165	12.188493	0.0000
day10	2.1300000	0.14685984	1165	14.503625	0.0000
day11	2.5900000	0.14685984	1165	17.635863	0.0000
day12	2.9700000	0.14685984	1165	20.223365	0.0000
Cross 13 vs 1	0.0958333	0.09479762	1165	1.010926	0.3123
Cross 13 vs 2	-0.2416667	0.09479762	1165	-2.549291	0.0109
Cross 13 vs 3	0.0583333	0.09479762	1165	0.615346	0.5384
Cross 13 vs 16	0.5041667	0.09479762	1165	5.318348	0.0000

A.8: Results from ANOVA analysis where cross 1 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 1 and the other groups are represented.

BUD SET – CROSS 1 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	0.00	0.2384986	1121	0.000000	1.0000
day2	0.00	0.3234699	1121	0.000000	1.0000
day3	0.00	0.3234699	1121	0.000000	1.0000
day4	0.00	0.3234699	1121	0.000000	1.0000
day5	0.15	0.3234699	1121	0.463722	0.6429
day6	0.25	0.3234699	1121	0.772869	0.4398
day7	0.90	0.3234699	1121	2.782330	0.0055
day8	1.35	0.3234699	1121	4.173495	0.0000
day9	1.55	0.3234699	1121	4.791791	0.0000
day10	2.25	0.3234699	1121	6.955825	0.0000
day11	2.65	0.3234699	1121	8.192416	0.0000
day12	3.25	0.3234699	1121	10.047303	0.0000
Cross 1 vs 2	0.00	0.3234699	1121	0.000000	1.0000
Cross 1 vs 3	0.00	0.3234699	1121	0.000000	1.0000
Cross 1 vs 13	0.00	0.3234699	1121	0.000000	1.0000
Cross 1 vs 16	0.00	0.3234699	1121	0.000000	1.0000
day2: cross 1 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 1 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 1 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 1 vs 2	-0.10	0.4574555	1121	-0.218600	0.8270
day6: cross 1 vs 2	-0.15	0.4574555	1121	-0.327901	0.7430
day7: cross 1 vs 2	-0.45	0.4574555	1121	-0.983702	0.3255
day8: cross 1 vs 2	-0.35	0.4574555	1121	-0.765102	0.4444
day9: cross 1 vs 2	-0.25	0.4574555	1121	-0.546501	0.5848
day10: cross 1 vs 2	-0.75	0.4574555	1121	-1.639504	0.1014
day11: cross 1 vs 2	-0.85	0.4574555	1121	-1.858104	0.0634
day12: cross 1 vs 2	-1.15	0.4574555	1121	-2.513906	0.0121
day2: cross 1 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 1 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 1 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 1 vs 3	-0.05	0.4574555	1121	-0.109300	0.9130
day6: cross 1 vs 3	0.10	0.4574555	1121	0.218600	0.8270
day7: cross 1 vs 3	0.05	0.4574555	1121	0.109300	0.9130
day8: cross 1 vs 3	0.25	0.4574555	1121	0.546501	0.5848
day9: cross 1 vs 3	0.25	0.4574555	1121	0.546501	0.5848
day10: cross 1 vs 3	-0.35	0.4574555	1121	-0.765102	0.4444

day11: cross 1 vs 3	-0.20	0.4574555	1121	-0.437201	0.6620
day12: cross 1 vs 3	-0.50	0.4574555	1121	-1.093002	0.2746
day2: cross 1 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 1 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 1 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 1 vs 13	-0.15	0.4574555	1121	-0.327901	0.7430
day6: cross 1 vs 13	0.05	0.4574555	1121	0.109300	0.9130
day7: cross 1 vs 13	-0.05	0.4574555	1121	-0.109300	0.9130
day8: cross 1 vs 13	0.10	0.4574555	1121	0.218600	0.8270
day9: cross 1 vs 13	0.15	0.4574555	1121	0.327901	0.7430
day10: cross 1 vs 13	-0.30	0.4574555	1121	-0.655801	0.5121
day11: cross 1 vs 13	-0.35	0.4574555	1121	-0.765102	0.4444
day12: cross 1 vs 13	-0.60	0.4574555	1121	-1.311603	0.1899
day2: cross 1 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 1 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 1 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 1 vs 16	-0.10	0.4574555	1121	-0.218600	0.8270
day6: cross 1 vs 16	0.10	0.4574555	1121	0.218600	0.8270
day7: cross 1 vs 16	0.40	0.4574555	1121	0.874402	0.3821
day8: cross 1 vs 16	0.70	0.4574555	1121	1.530203	0.1262
day9: cross 1 vs 16	1.05	0.4574555	1121	2.295305	0.0219
day10: cross 1 vs 16	0.80	0.4574555	1121	1.748804	0.0806
day11: cross 1 vs 16	1.10	0.4574555	1121	2.404605	0.0164
day12: cross 1 vs 16	0.85	0.4574555	1121	1.858104	0.0634

A.9: Results from ANOVA analysis where cross 2 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 2 and the other groups are represented.

BUD SET – CROSS 2 VS THE REST					
	Value	**Standard Error**	**DF**	**T-value**	**P-value**
(Intercept)	0.00	0.2384986	1121	0.000000	1.0000
day2	0.00	0.3234699	1121	0.000000	1.0000
day3	0.00	0.3234699	1121	0.000000	1.0000
day4	0.00	0.3234699	1121	0.000000	1.0000
day5	0.05	0.3234699	1121	0.154574	0.8772
day6	0.10	0.3234699	1121	0.309148	0.7573
day7	0.45	0.3234699	1121	1.391165	0.1645
day8	1.00	0.3234699	1121	3.091478	0.0020
day9	1.30	0.3234699	1121	4.018921	0.0001
day10	1.50	0.3234699	1121	4.637217	0.0000
day11	1.80	0.3234699	1121	5.564660	0.0000
day12	2.10	0.3234699	1121	6.492104	0.0000
Cross 2 vs 1	0.00	0.3234699	1121	0.000000	1.0000
Cross 2 vs 3	0.00	0.3234699	1121	0.000000	1.0000
Cross 2 vs 13	0.00	0.3234699	1121	0.000000	1.0000
Cross 2 vs 16	0.00	0.3234699	1121	0.000000	1.0000
day2: cross 2 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 2 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 2 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 2 vs 1	0.10	0.4574555	1121	0.218600	0.8270
day6: cross 2 vs 1	0.15	0.4574555	1121	0.327901	0.7430
day7: cross 2 vs 1	0.45	0.4574555	1121	0.983702	0.3255
day8: cross 2 vs 1	0.35	0.4574555	1121	0.765102	0.4444
day9: cross 2 vs 1	0.25	0.4574555	1121	0.546501	0.5848
day10: cross 2 vs 1	0.75	0.4574555	1121	1.639504	0.1014
day11: cross 2 vs 1	0.85	0.4574555	1121	1.858104	0.0634
day12: cross 2 vs 1	1.15	0.4574555	1121	2.513906	0.0121
day2: cross 2 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 2 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 2 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 2 vs 3	0.05	0.4574555	1121	0.109300	0.9130
day6: cross 2 vs 3	0.25	0.4574555	1121	0.546501	0.5848
day7: cross 2 vs 3	0.50	0.4574555	1121	1.093002	0.2746
day8: cross 2 vs 3	0.60	0.4574555	1121	1.311603	0.1899
day9: cross 2 vs 3	0.50	0.4574555	1121	1.093002	0.2746
day10: cross 2 vs 3	0.40	0.4574555	1121	0.874402	0.3821

day11: cross 2 vs 3	0.65	0.4574555	1121	1.420903	0.1556
day12: cross 2 vs 3	0.65	0.4574555	1121	1.420903	0.1556
day2: cross 2 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 2 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 2 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 2 vs 13	-0.05	0.4574555	1121	-0.109300	0.9130
day6: cross 2 vs 13	0.20	0.4574555	1121	0.437201	0.6620
day7: cross 2 vs 13	0.40	0.4574555	1121	0.874402	0.3821
day8: cross 2 vs 13	0.45	0.4574555	1121	0.983702	0.3255
day9: cross 2 vs 13	0.40	0.4574555	1121	0.874402	0.3821
day10: cross 2 vs 13	0.45	0.4574555	1121	0.983702	0.3255
day11: cross 2 vs 13	0.50	0.4574555	1121	1.093002	0.2746
day12: cross 2 vs 13	0.55	0.4574555	1121	1.202303	0.2295
day2: cross 2 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 2 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 2 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 2 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day6: cross 2 vs 16	0.25	0.4574555	1121	0.546501	0.5848
day7: cross 2 vs 16	0.85	0.4574555	1121	1.858104	0.0634
day8: cross 2 vs 16	1.05	0.4574555	1121	2.295305	0.0219
day9: cross 2 vs 16	1.30	0.4574555	1121	2.841806	0.0046
day10: cross 2 vs 16	1.55	0.4574555	1121	3.388308	0.0007
day11: cross 2 vs 16	1.95	0.4574555	1121	4.262710	0.0000
day12: cross 2 vs 16	2.00	0.4574555	1121	4.372010	0.0000

A.10: Results from ANOVA analysis where cross 3 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 3 and the other groups are represented.

BUD SET – CROSS 3 VS THE REST					
	Value	**Standard Error**	**DF**	**T-value**	**P-value**
(Intercept)	0.00	0.2384986	1121	0.000000	1.0000
day2	0.00	0.3234699	1121	0.000000	1.0000
day3	0.00	0.3234699	1121	0.000000	1.0000
day4	0.00	0.3234699	1121	0.000000	1.0000
day5	0.10	0.3234699	1121	0.309148	0.7573
day6	0.35	0.3234699	1121	1.082017	0.2795
day7	0.95	0.3234699	1121	2.936904	0.0034
day8	1.60	0.3234699	1121	4.946365	0.0000
day9	1.80	0.3234699	1121	5.564660	0.0000
day10	1.90	0.3234699	1121	5.873808	0.0000
day11	2.45	0.3234699	1121	7.574121	0.0000
day12	2.75	0.3234699	1121	8.501564	0.0000
Cross 3 vs 1	0.00	0.3234699	1121	0.000000	1.0000
Cross 3 vs 2	0.00	0.3234699	1121	0.000000	1.0000
Cross 3 vs 13	0.00	0.3234699	1121	0.000000	1.0000
Cross 3 vs 16	0.00	0.3234699	1121	0.000000	1.0000
day2: cross 3 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 3 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 3 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 3 vs 1	0.05	0.4574555	1121	0.109300	0.9130
day6: cross 3 vs 1	-0.10	0.4574555	1121	-0.218600	0.8270
day7: cross 3 vs 1	-0.05	0.4574555	1121	-0.109300	0.9130
day8: cross 3 vs 1	-0.25	0.4574555	1121	-0.546501	0.5848
day9: cross 3 vs 1	-0.25	0.4574555	1121	-0.546501	0.5848
day10: cross 3 vs 1	0.35	0.4574555	1121	0.765102	0.4444
day11: cross 3 vs 1	0.20	0.4574555	1121	0.437201	0.6620
day12: cross 3 vs 1	0.50	0.4574555	1121	1.093002	0.2746
day2: cross 3 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 3 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 3 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 3 vs 2	-0.05	0.4574555	1121	-0.109300	0.9130
day6: cross 3 vs 2	-0.25	0.4574555	1121	-0.546501	0.5848
day7: cross 3 vs 2	-0.50	0.4574555	1121	-1.093002	0.2746
day8: cross 3 vs 2	-0.60	0.4574555	1121	-1.311603	0.1899
day9: cross 3 vs 2	-0.50	0.4574555	1121	-1.093002	0.2746
day10: cross 3 vs 2	-0.40	0.4574555	1121	-0.874402	0.3821

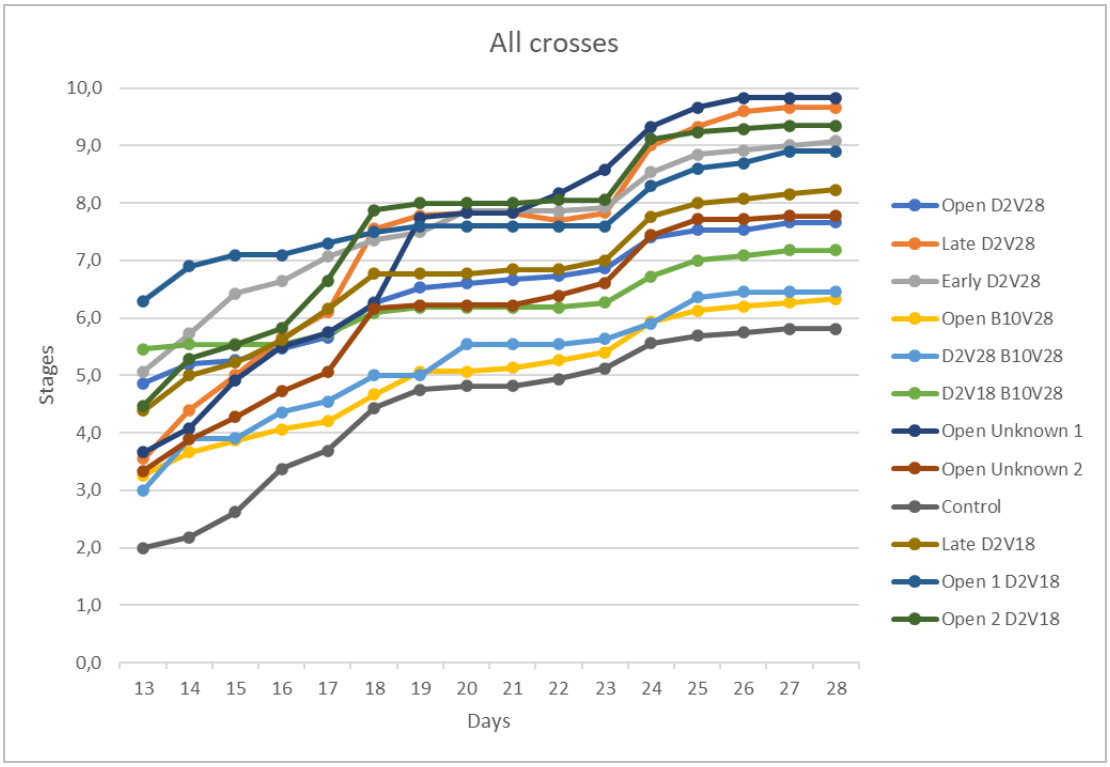
day11: cross 3 vs 2	-0.65	0.4574555	1121	-1.420903	0.1556
day12: cross 3 vs 2	-0.65	0.4574555	1121	-1.420903	0.1556
day2: cross 3 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 3 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 3 vs 13	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 3 vs 13	-0.10	0.4574555	1121	-0.218600	0.8270
day6: cross 3 vs 13	-0.05	0.4574555	1121	-0.109300	0.9130
day7: cross 3 vs 13	-0.10	0.4574555	1121	-0.218600	0.8270
day8: cross 3 vs 13	-0.15	0.4574555	1121	-0.327901	0.7430
day9: cross 3 vs 13	-0.10	0.4574555	1121	-0.218600	0.8270
day10: cross 3 vs 13	0.05	0.4574555	1121	0.109300	0.9130
day11: cross 3 vs 13	-0.15	0.4574555	1121	-0.327901	0.7430
day12: cross 3 vs 13	-0.10	0.4574555	1121	-0.218600	0.8270
day2: cross 3 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 3 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 3 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 3 vs 16	-0.05	0.4574555	1121	-0.109300	0.9130
day6: cross 3 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day7: cross 3 vs 16	0.35	0.4574555	1121	0.765102	0.4444
day8: cross 3 vs 16	0.45	0.4574555	1121	0.983702	0.3255
day9: cross 3 vs 16	0.80	0.4574555	1121	1.748804	0.0806
day10: cross 3 vs 16	1.15	0.4574555	1121	2.513906	0.0121
day11: cross 3 vs 16	1.30	0.4574555	1121	2.841806	0.0046
day12: cross 3 vs 16	1.35	0.4574555	1121	2.951107	0.0032

A.11: Results from ANOVA analysis where cross 13 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud set to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 13 and the other groups are represented.

BUD SET – CROSS 13 VS THE REST					
	Value	**Standard Error**	**DF**	**T-value**	**P-value**
(Intercept)	0.00	0.2384986	1121	0.000000	1.0000
day2	0.00	0.3234699	1121	0.000000	1.0000
day3	0.00	0.3234699	1121	0.000000	1.0000
day4	0.00	0.3234699	1121	0.000000	1.0000
day5	0.00	0.3234699	1121	0.000000	1.0000
day6	0.30	0.3234699	1121	0.927443	0.3539
day7	0.85	0.3234699	1121	2.627756	0.0087
day8	1.45	0.3234699	1121	4.482643	0.0000
day9	1.70	0.3234699	1121	5.255512	0.0000
day10	1.95	0.3234699	1121	6.028382	0.0000
day11	2.30	0.3234699	1121	7.110399	0.0000
day12	2.65	0.3234699	1121	8.192416	0.0000
Cross 13 vs 1	0.00	0.3234699	1121	0.000000	1.0000
Cross 13 vs 2	0.00	0.3234699	1121	0.000000	1.0000
Cross 13 vs 3	0.00	0.3234699	1121	0.000000	1.0000
Cross 13 vs 16	0.00	0.3234699	1121	0.000000	1.0000
day2: cross 13 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 13 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 13 vs 1	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 13 vs 1	0.15	0.4574555	1121	0.327901	0.7430
day6: cross 13 vs 1	-0.05	0.4574555	1121	-0.109300	0.9130
day7: cross 13 vs 1	0.05	0.4574555	1121	0.109300	0.9130
day8: cross 13 vs 1	-0.10	0.4574555	1121	-0.218600	0.8270
day9: cross 13 vs 1	-0.15	0.4574555	1121	-0.327901	0.7430
day10: cross 13 vs 1	0.30	0.4574555	1121	0.655801	0.5121
day11: cross 13 vs 1	0.35	0.4574555	1121	0.765102	0.4444
day12: cross 13 vs 1	0.60	0.4574555	1121	1.311603	0.1899
day2: cross 13 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 13 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 13 vs 2	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 13 vs 2	0.05	0.4574555	1121	0.109300	0.9130
day6: cross 13 vs 2	-0.20	0.4574555	1121	-0.437201	0.6620
day7: cross 13 vs 2	-0.40	0.4574555	1121	-0.874402	0.3821
day8: cross 13 vs 2	-0.45	0.4574555	1121	-0.983702	0.3255
day9: cross 13 vs 2	-0.40	0.4574555	1121	-0.874402	0.3821
day10: cross 13 vs 2	-0.45	0.4574555	1121	-0.983702	0.3255

day11: cross 13 vs 2	-0.50	0.4574555	1121	-1.093002	0.2746
day12: cross 13 vs 2	-0.55	0.4574555	1121	-1.202303	0.2295
day2: cross 13 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 13 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 13 vs 3	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 13 vs 3	0.10	0.4574555	1121	0.218600	0.8270
day6: cross 13 vs 3	0.05	0.4574555	1121	0.109300	0.9130
day7: cross 13 vs 3	0.10	0.4574555	1121	0.218600	0.8270
day8: cross 13 vs 3	0.15	0.4574555	1121	0.327901	0.7430
day9: cross 13 vs 3	0.10	0.4574555	1121	0.218600	0.8270
day10: cross 13 vs 3	-0.05	0.4574555	1121	-0.109300	0.9130
day11: cross 13 vs 3	0.15	0.4574555	1121	0.327901	0.7430
day12: cross 13 vs 3	0.10	0.4574555	1121	0.218600	0.8270
day2: cross 13 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day3: cross 13 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day4: cross 13 vs 16	0.00	0.4574555	1121	0.000000	1.0000
day5: cross 13 vs 16	0.05	0.4574555	1121	0.109300	0.9130
day6: cross 13 vs 16	0.05	0.4574555	1121	0.109300	0.9130
day7: cross 13 vs 16	0.45	0.4574555	1121	0.983702	0.3255
day8: cross 13 vs 16	0.60	0.4574555	1121	1.311603	0.1899
day9: cross 13 vs 16	0.90	0.4574555	1121	1.967404	0.0494
day10: cross 13 vs 16	1.10	0.4574555	1121	2.404605	0.0164
day11: cross 13 vs 16	1.45	0.4574555	1121	3.169707	0.0016
day12: cross 13 vs 16	1.45	0.4574555	1121	3.169707	0.0016

A.12: During the analysis with phenotyping, bud development was registered for all the crosses produced after the pollination. Comparing all the crossings can be perceived as difficult to read, and it is challenging to compare relevant crossings. The figure represents results from the LD period where the individuals were under 24 hours of light for 8 weeks.



A.13: Results from ANOVA analysis where cross 1 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 1 and the other groups are represented.

BUD BURST – CROSS 1 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	2.512729	0.4873584	1097	5.155815	0.0000
day14	0.661972	0.4462216	1097	1.483505	0.1382
day15	1.000000	0.4462216	1097	2.241039	0.0252
day16	1.281690	0.4462216	1097	2.872318	0.0042
day17	1.619718	0.4462216	1097	3.629852	0.0003
day18	2.253521	0.4462216	1097	5.050229	0.0000
day19	2.422535	0.4462216	1097	5.428996	0.0000
day20	2.422535	0.4462216	1097	5.428996	0.0000
day21	2.450704	0.4462216	1097	5.492124	0.0000
day22	2.464789	0.4462216	1097	5.523688	0.0000
day23	2.535211	0.4462216	1097	5.681508	0.0000
day24	3.211268	0.4462216	1097	7.196576	0.0000
day25	3.464789	0.4462216	1097	7.764727	0.0000
day26	3.563380	0.4462216	1097	7.985675	0.0000
day27	3.661972	0.4462216	1097	8.206622	0.0000
day28	3.690141	0.4462216	1097	8.269750	0.0000
Cross 1 vs 2	2.807086	0.2562249	1097	10.955556	0.0000
Cross 1 vs 3	1.239827	0.2461384	1097	5.037112	0.0000
Cross 1 vs 13	1.375908	0.2571239	1097	5.351149	0.0000
Cross 1 vs 16	2.742942	0.2987977	1097	9.179930	0.0000

A.14: Results from ANOVA analysis where cross 2 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 2 and the other groups are represented.

BUD BURST – CROSS 2 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	5.319816	0.4874540	1097	10.913472	0.0000
day14	0.661972	0.4462216	1097	1.483505	0.1382
day15	1.000000	0.4462216	1097	2.241039	0.0252
day16	1.281690	0.4462216	1097	2.872318	0.0042
day17	1.619718	0.4462216	1097	3.629852	0.0003
day18	2.253521	0.4462216	1097	5.050229	0.0000
day19	2.422535	0.4462216	1097	5.428996	0.0000
day20	2.422535	0.4462216	1097	5.428996	0.0000
day21	2.450704	0.4462216	1097	5.492124	0.0000
day22	2.464789	0.4462216	1097	5.523688	0.0000
day23	2.535211	0.4462216	1097	5.681508	0.0000
day24	3.211268	0.4462216	1097	7.196576	0.0000
day25	3.464789	0.4462216	1097	7.764727	0.0000
day26	3.563380	0.4462216	1097	7.985675	0.0000
day27	3.661972	0.4462216	1097	8.206622	0.0000
day28	3.690141	0.4462216	1097	8.269750	0.0000
Cross 2 vs 1	-2.807086	0.2562249	1097	-10.955556	0.0000
Cross 2 vs 3	-1.567260	0.2436548	1097	-6.432296	0.0000
Cross 2 vs 13	-1.431178	0.2605552	1097	-5.492803	0.0000
Cross 2 vs 16	-0.064144	0.2896779	1097	-0.221433	0.8248

A.15: Results from ANOVA analysis where cross 3 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 3 and the other groups are represented.

BUD BURST – CROSS 3 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	3.752556	0.4832213	1097	7.765710	0.0000
day14	0.661972	0.4462216	1097	1.483505	0.1382
day15	1.000000	0.4462216	1097	2.241039	0.0252
day16	1.281690	0.4462216	1097	2.872318	0.0042
day17	1.619718	0.4462216	1097	3.629852	0.0003
day18	2.253521	0.4462216	1097	5.050229	0.0000
day19	2.422535	0.4462216	1097	5.428996	0.0000
day20	2.422535	0.4462216	1097	5.428996	0.0000
day21	2.450704	0.4462216	1097	5.492124	0.0000
day22	2.464789	0.4462216	1097	5.523688	0.0000
day23	2.535211	0.4462216	1097	5.681508	0.0000
day24	3.211268	0.4462216	1097	7.196576	0.0000
day25	3.464789	0.4462216	1097	7.764727	0.0000
day26	3.563380	0.4462216	1097	7.985675	0.0000
day27	3.661972	0.4462216	1097	8.206622	0.0000
day28	3.690141	0.4462216	1097	8.269750	0.0000
Cross 3 vs 1	-1.239827	0.2461384	1097	-5.037112	0.0000
Cross 3 vs 2	1.567260	0.2436548	1097	6.432296	0.0000
Cross 3 vs 13	0.136082	0.2435814	1097	0.558670	0.5765
Cross 3 vs 16	1.503116	0.2797449	1097	5.373166	0.0000

A.16: Results from ANOVA analysis where cross 13 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. All values from the result of the comparison between cross 13 and the other groups are represented.

BUS BURST – CROSS 13 THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	3.888638	0.4907379	1097	7.924063	0.0000
day14	0.661972	0.4462216	1097	1.483505	0.1382
day15	1.000000	0.4462216	1097	2.241039	0.0252
day16	1.281690	0.4462216	1097	2.872318	0.0042
day17	1.619718	0.4462216	1097	3.629852	0.0003
day18	2.253521	0.4462216	1097	5.050229	0.0000
day19	2.422535	0.4462216	1097	5.428996	0.0000
day20	2.422535	0.4462216	1097	5.428996	0.0000
day21	2.450704	0.4462216	1097	5.492124	0.0000
day22	2.464789	0.4462216	1097	5.523688	0.0000
day23	2.535211	0.4462216	1097	5.681508	0.0000
day24	3.211268	0.4462216	1097	7.196576	0.0000
day25	3.464789	0.4462216	1097	7.764727	0.0000
day26	3.563380	0.4462216	1097	7.985675	0.0000
day27	3.661972	0.4462216	1097	8.206622	0.0000
day28	3.690141	0.4462216	1097	8.269750	0.0000
Cross 13 vs 1	-1.375908	0.2571239	1097	-5.351149	0.0000
Cross 13 vs 2	1.431178	0.2605552	1097	5.492803	0.0000
Cross 13 vs 3	-0.136082	0.2435814	1097	-0.558670	0.5765
Cross 13 vs 16	1.367034	0.2947805	1097	4.637464	0.0000

A.17: Results from ANOVA analysis where cross 1 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 1 and the other groups are represented.

BUD BURST – CROSS 1 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	3.848438	0.7675823	1037	5.013714	0.0000
day14	0.266667	0.9741594	1037	0.273740	0.7843
day15	0.266667	0.9741594	1037	0.273740	0.7843
day16	0.266667	0.9741594	1037	0.273740	0.7843
day17	0.466667	0.9741594	1037	0.479045	0.6320
day18	0.800000	0.9741594	1037	0.821221	0.4117
day19	0.800000	0.9741594	1037	0.821221	0.4117
day20	0.800000	0.9741594	1037	0.821221	0.4117
day21	0.866667	0.9741594	1037	0.889656	0.3739
day22	0.933333	0.9741594	1037	0.958091	0.3382
day23	1.066667	0.9741594	1037	1.094961	0.2738
day24	1.600000	0.9741594	1037	1.642442	0.1008
day25	1.733333	0.9741594	1037	1.779312	0.0755
day26	1.733333	0.9741594	1037	1.779312	0.0755
day27	1.866667	0.9741594	1037	1.916182	0.0556
day28	1.866667	0.9741594	1037	1.916182	0.0556
Cross 1 vs 2	-1.005636	0.9776373	1037	-1.028639	0.3039
Cross 1 vs 3	0.444274	0.9478077	1037	0.468738	0.6394
Cross 1 vs 13	-0.014186	0.9939891	1037	-0.014272	0.9886
Cross 1 vs 16	2.276218	1.0963485	1037	2.076181	0.0381
day14: cross 1 vs 2	1.133333	1.3776695	1037	0.822645	0.4109
day15: cross 1 vs 2	1.866667	1.3776695	1037	1.354945	0.1757
day16: cross 1 vs 2	2.666667	1.3776695	1037	1.935636	0.0532
day17: cross 1 vs 2	3.000000	1.3776695	1037	2.177591	0.0297
day18: cross 1 vs 2	4.333333	1.3776695	1037	3.145409	0.0017
day19: cross 1 vs 2	4.600000	1.3776695	1037	3.338972	0.0009
day20: cross 1 vs 2	4.600000	1.3776695	1037	3.338972	0.0009
day21: cross 1 vs 2	4.533333	1.3776695	1037	3.290581	0.0010
day22: cross 1 vs 2	4.466667	1.3776695	1037	3.242190	0.0012
day23: cross 1 vs 2	4.333333	1.3776695	1037	3.145409	0.0017
day24: cross 1 vs 2	4.800000	1.3776695	1037	3.484145	0.0005
day25: cross 1 vs 2	5.000000	1.3776695	1037	3.629318	0.0003
day26: cross 1 vs 2	5.266667	1.3776695	1037	3.822881	0.0001
day27: cross 1 vs 2	5.200000	1.3776695	1037	3.774490	0.0002
day28: cross 1 vs 2	5.200000	1.3776695	1037	3.774490	0.0002
day14: cross 1 vs 3	0.203922	1.3365357	1037	0.152575	0.8788

day15: cross 1 vs 3	0.615686	1.3365357	1037	0.460658	0.6451
day16: cross 1 vs 3	0.792157	1.3365357	1037	0.592694	0.5535
day17: cross 1 vs 3	0.827451	1.3365357	1037	0.619101	0.5360
day18: cross 1 vs 3	0.729412	1.3365357	1037	0.545748	0.5854
day19: cross 1 vs 3	1.082353	1.3365357	1037	0.809820	0.4182
day20: cross 1 vs 3	1.082353	1.3365357	1037	0.809820	0.4182
day21: cross 1 vs 3	1.015686	1.3365357	1037	0.759939	0.4475
day22: cross 1 vs 3	0.949020	1.3365357	1037	0.710059	0.4778
day23: cross 1 vs 3	0.874510	1.3365357	1037	0.654311	0.5131
day24: cross 1 vs 3	0.811765	1.3365357	1037	0.607365	0.5437
day25: cross 1 vs 3	0.913725	1.3365357	1037	0.683652	0.4943
day26: cross 1 vs 3	0.972549	1.3365357	1037	0.727664	0.4670
day27: cross 1 vs 3	0.898039	1.3365357	1037	0.671916	0.5018
day28: cross 1 vs 3	0.956863	1.3365357	1037	0.715928	0.4742
day14: cross 1 vs 13	0.304762	1.4020549	1037	0.217368	0.8280
day15: cross 1 vs 13	0.590476	1.4020549	1037	0.421151	0.6737
day16: cross 1 vs 13	0.947619	1.4020549	1037	0.675879	0.4993
day17: cross 1 vs 13	1.247619	1.4020549	1037	0.889850	0.3738
day18: cross 1 vs 13	1.557143	1.4020549	1037	1.110615	0.2670
day19: cross 1 vs 13	1.628571	1.4020549	1037	1.161560	0.2457
day20: cross 1 vs 13	1.628571	1.4020549	1037	1.161560	0.2457
day21: cross 1 vs 13	1.633333	1.4020549	1037	1.164957	0.2443
day22: cross 1 vs 13	1.566667	1.4020549	1037	1.117408	0.2641
day23: cross 1 vs 13	1.576190	1.4020549	1037	1.124200	0.2612
day24: cross 1 vs 13	1.757143	1.4020549	1037	1.253263	0.2104
day25: cross 1 vs 13	1.909524	1.4020549	1037	1.361947	0.1735
day26: cross 1 vs 13	1.980952	1.4020549	1037	1.412892	0.1580
day27: cross 1 vs 13	1.919048	1.4020549	1037	1.368739	0.1714
day28: cross 1 vs 13	1.990476	1.4020549	1037	1.419685	0.1560
day14: cross 1 vs 16	0.333333	1.5402813	1037	0.216411	0.8287
day15: cross 1 vs 16	0.533333	1.5402813	1037	0.346257	0.7292
day16: cross 1 vs 16	0.533333	1.5402813	1037	0.346257	0.7292
day17: cross 1 vs 16	0.533333	1.5402813	1037	0.346257	0.7292
day18: cross 1 vs 16	0.400000	1.5402813	1037	0.259693	0.7952
day19: cross 1 vs 16	0.500000	1.5402813	1037	0.324616	0.7455
day20: cross 1 vs 16	0.500000	1.5402813	1037	0.324616	0.7455
day21: cross 1 vs 16	0.433333	1.5402813	1037	0.281334	0.7785
day22: cross 1 vs 16	0.366667	1.5402813	1037	0.238052	0.8119
day23: cross 1 vs 16	0.233333	1.5402813	1037	0.151487	0.8796
day24: cross 1 vs 16	0.400000	1.5402813	1037	0.259693	0.7952
day25: cross 1 vs 16	0.566667	1.5402813	1037	0.367898	0.7130
day26: cross 1 vs 16	0.666667	1.5402813	1037	0.432821	0.6652
day27: cross 1 vs 16	0.733333	1.5402813	1037	0.476104	0.6341
day28: cross 1 vs 16	0.733333	1.5402813	1037	0.476104	0.6341

A.18: Results from ANOVA analysis where cross 2 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 2 and the other groups are represented.

BUD BURST – CROSS 2 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	2.842802	0.7676432	1037	3.703286	0.0002
day14	1.400000	0.9741594	1037	1.437136	0.1510
day15	2.133333	0.9741594	1037	2.189922	0.0288
day16	2.933333	0.9741594	1037	3.011143	0.0027
day17	3.466667	0.9741594	1037	3.558624	0.0004
day18	5.133333	0.9741594	1037	5.269500	0.0000
day19	5.400000	0.9741594	1037	5.543240	0.0000
day20	5.400000	0.9741594	1037	5.543240	0.0000
day21	5.400000	0.9741594	1037	5.543240	0.0000
day22	5.400000	0.9741594	1037	5.543240	0.0000
day23	5.400000	0.9741594	1037	5.543240	0.0000
day24	6.400000	0.9741594	1037	6.569767	0.0000
day25	6.733333	0.9741594	1037	6.911942	0.0000
day26	7.000000	0.9741594	1037	7.185682	0.0000
day27	7.066667	0.9741594	1037	7.254117	0.0000
day28	7.066667	0.9741594	1037	7.254117	0.0000
Cross 2 vs 1	1.005636	0.9776373	1037	1.028639	0.3039
Cross 2 vs 3	1.449910	0.9471625	1037	1.530793	0.1261
Cross 2 vs 13	0.991450	0.9948887	1037	0.996544	0.3192
Cross 2 vs 16	3.281853	1.0938839	1037	3.000185	0.0028
day14: cross 2 vs 1	-1.133333	1.3776695	1037	-0.822645	0.4109
day15: cross 2 vs 1	-1.866667	1.3776695	1037	-1.354945	0.1757
day16: cross 2 vs 1	-2.666667	1.3776695	1037	-1.935636	0.0532
day17: cross 2 vs 1	-3.000000	1.3776695	1037	-2.177591	0.0297
day18: cross 2 vs 1	-4.333333	1.3776695	1037	-3.145409	0.0017
day19: cross 2 vs 1	-4.600000	1.3776695	1037	-3.338972	0.0009
day20: cross 2 vs 1	-4.600000	1.3776695	1037	-3.338972	0.0009
day21: cross 2 vs 1	-4.533333	1.3776695	1037	-3.290581	0.0010
day22: cross 2 vs 1	-4.466667	1.3776695	1037	-3.242190	0.0012
day23: cross 2 vs 1	-4.333333	1.3776695	1037	-3.145409	0.0017
day24: cross 2 vs 1	-4.800000	1.3776695	1037	-3.484145	0.0005
day25: cross 2 vs 1	-5.000000	1.3776695	1037	-3.629318	0.0003
day26: cross 2 vs 1	-5.266667	1.3776695	1037	-3.822881	0.0001
day27: cross 2 vs 1	-5.200000	1.3776695	1037	-3.774490	0.0002
day28: cross 2 vs 1	-5.200000	1.3776695	1037	-3.774490	0.0002
day14: cross 2 vs 3	-0.929412	1.3365357	1037	-0.695389	0.4870

day15: cross 2 vs 3	-1.250980	1.3365357	1037	-0.935987	0.3495
day16: cross 2 vs 3	-1.874510	1.3365357	1037	-1.402514	0.1611
day17: cross 2 vs 3	-2.172549	1.3365357	1037	-1.625508	0.1044
day18: cross 2 vs 3	-3.603922	1.3365357	1037	-2.696465	0.0071
day19: cross 2 vs 3	-3.517647	1.3365357	1037	-2.631914	0.0086
day20: cross 2 vs 3	-3.517647	1.3365357	1037	-2.631914	0.0086
day21: cross 2 vs 3	-3.517647	1.3365357	1037	-2.631914	0.0086
day22: cross 2 vs 3	-3.517647	1.3365357	1037	-2.631914	0.0086
day23: cross 2 vs 3	-3.458824	1.3365357	1037	-2.587902	0.0098
day24: cross 2 vs 3	-3.988235	1.3365357	1037	-2.984010	0.0029
day25: cross 2 vs 3	-4.086275	1.3365357	1037	-3.057363	0.0023
day26: cross 2 vs 3	-4.294118	1.3365357	1037	-3.212872	0.0014
day27: cross 2 vs 3	-4.301961	1.3365357	1037	-3.218740	0.0013
day28: cross 2 vs 3	-4.243137	1.3365357	1037	-3.174728	0.0015
day14: cross 2 vs 13	-0.828571	1.4020549	1037	-0.590969	0.5547
day15: cross 2 vs 13	-1.276190	1.4020549	1037	-0.910229	0.3629
day16: cross 2 vs 13	-1.719048	1.4020549	1037	-1.226092	0.2204
day17: cross 2 vs 13	-1.752381	1.4020549	1037	-1.249866	0.2116
day18: cross 2 vs 13	-2.776190	1.4020549	1037	-1.980087	0.0480
day19: cross 2 vs 13	-2.971429	1.4020549	1037	-2.119338	0.0343
day20: cross 2 vs 13	-2.971429	1.4020549	1037	-2.119338	0.0343
day21: cross 2 vs 13	-2.900000	1.4020549	1037	-2.068393	0.0389
day22: cross 2 vs 13	-2.900000	1.4020549	1037	-2.068393	0.0389
day23: cross 2 vs 13	-2.757143	1.4020549	1037	-1.966501	0.0495
day24: cross 2 vs 13	-3.042857	1.4020549	1037	-2.170284	0.0302
day25: cross 2 vs 13	-3.090476	1.4020549	1037	-2.204248	0.0277
day26: cross 2 vs 13	-3.285714	1.4020549	1037	-2.343499	0.0193
day27: cross 2 vs 13	-3.280952	1.4020549	1037	-2.340103	0.0195
day28: cross 2 vs 13	-3.209524	1.4020549	1037	-2.289157	0.0223
day14: cross 2 vs 16	-0.800000	1.5402813	1037	-0.519386	0.6036
day15: cross 2 vs 16	-1.333333	1.5402813	1037	-0.865643	0.3869
day16: cross 2 vs 16	-2.133333	1.5402813	1037	-1.385028	0.1663
day17: cross 2 vs 16	-2.466667	1.5402813	1037	-1.601439	0.1096
day18: cross 2 vs 16	-3.933333	1.5402813	1037	-2.553646	0.0108
day19: cross 2 vs 16	-4.100000	1.5402813	1037	-2.661851	0.0079
day20: cross 2 vs 16	-4.100000	1.5402813	1037	-2.661851	0.0079
day21: cross 2 vs 16	-4.100000	1.5402813	1037	-2.661851	0.0079
day22: cross 2 vs 16	-4.100000	1.5402813	1037	-2.661851	0.0079
day23: cross 2 vs 16	-4.100000	1.5402813	1037	-2.661851	0.0079
day24: cross 2 vs 16	-4.400000	1.5402813	1037	-2.856621	0.0044
day25: cross 2 vs 16	-4.433333	1.5402813	1037	-2.878262	0.0041
day26: cross 2 vs 16	-4.600000	1.5402813	1037	-2.986467	0.0029
day27: cross 2 vs 16	-4.466667	1.5402813	1037	-2.899903	0.0038
day28: cross 2 vs 16	-4.466667	1.5402813	1037	-2.899903	0.0038

A.19: Results from ANOVA analysis where cross 3 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 3 and the other groups are represented.

BUD BURST – CROSS 3 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	4.292712	0.7299351	1037	5.880950	0.0000
day14	0.470588	0.9150634	1037	0.514268	0.6072
day15	0.882353	0.9150634	1037	0.964253	0.3351
day16	1.058824	0.9150634	1037	1.157104	0.2475
day17	1.294118	0.9150634	1037	1.414238	0.1576
day18	1.529412	0.9150634	1037	1.671372	0.0949
day19	1.882353	0.9150634	1037	2.057074	0.0399
day20	1.882353	0.9150634	1037	2.057074	0.0399
day21	1.882353	0.9150634	1037	2.057074	0.0399
day22	1.882353	0.9150634	1037	2.057074	0.0399
day23	1.941176	0.9150634	1037	2.121357	0.0341
day24	2.411765	0.9150634	1037	2.635626	0.0085
day25	2.647059	0.9150634	1037	2.892760	0.0039
day26	2.705882	0.9150634	1037	2.957043	0.0032
day27	2.764706	0.9150634	1037	3.021327	0.0026
day28	2.823529	0.9150634	1037	3.085611	0.0021
Cross 3 vs 1	-0.444274	0.9478077	1037	-0.468738	0.6394
Cross 3 vs 2	-1.449910	0.9471625	1037	-1.530793	0.1261
Cross 3 vs 13	-0.458460	0.9637720	1037	-0.475693	0.6344
Cross 3 vs 16	1.831944	1.0670302	1037	1.716862	0.0863
day14: cross 3 vs 1	-0.203922	1.3365357	1037	-0.152575	0.8788
day15: cross 3 vs 1	-0.615686	1.3365357	1037	-0.460658	0.6451
day16: cross 3 vs 1	-0.792157	1.3365357	1037	-0.592694	0.5535
day17: cross 3 vs 1	-0.827451	1.3365357	1037	-0.619101	0.5360
day18: cross 3 vs 1	-0.729412	1.3365357	1037	-0.545748	0.5854
day19: cross 3 vs 1	-1.082353	1.3365357	1037	-0.809820	0.4182
day20: cross 3 vs 1	-1.082353	1.3365357	1037	-0.809820	0.4182
day21: cross 3 vs 1	-1.015686	1.3365357	1037	-0.759939	0.4475
day22: cross 3 vs 1	-0.949020	1.3365357	1037	-0.710059	0.4778
day23: cross 3 vs 1	-0.874510	1.3365357	1037	-0.654311	0.5131
day24: cross 3 vs 1	-0.811765	1.3365357	1037	-0.607365	0.5437
day25: cross 3 vs 1	-0.913725	1.3365357	1037	-0.683652	0.4943
day26: cross 3 vs 1	-0.972549	1.3365357	1037	-0.727664	0.4670
day27: cross 3 vs 1	-0.898039	1.3365357	1037	-0.671916	0.5018
day28: cross 3 vs 1	-0.956863	1.3365357	1037	-0.715928	0.4742
day14: cross 3 vs 2	0.929412	1.3365357	1037	0.695389	0.4870

day15: cross 3 vs 2	1.250980	1.3365357	1037	0.935987	0.3495
day16: cross 3 vs 2	1.874510	1.3365357	1037	1.402514	0.1611
day17: cross 3 vs 2	2.172549	1.3365357	1037	1.625508	0.1044
day18: cross 3 vs 2	3.603922	1.3365357	1037	2.696465	0.0071
day19: cross 3 vs 2	3.517647	1.3365357	1037	2.631914	0.0086
day20: cross 3 vs 2	3.517647	1.3365357	1037	2.631914	0.0086
day21: cross 3 vs 2	3.517647	1.3365357	1037	2.631914	0.0086
day22: cross 3 vs 2	3.517647	1.3365357	1037	2.631914	0.0086
day23: cross 3 vs 2	3.458824	1.3365357	1037	2.587902	0.0098
day24: cross 3 vs 2	3.988235	1.3365357	1037	2.984010	0.0029
day25: cross 3 vs 2	4.086275	1.3365357	1037	3.057363	0.0023
day26: cross 3 vs 2	4.294118	1.3365357	1037	3.212872	0.0014
day27: cross 3 vs 2	4.301961	1.3365357	1037	3.218740	0.0013
day28: cross 3 vs 2	4.243137	1.3365357	1037	3.174728	0.0015
day14: cross 3 vs 13	0.100840	1.3616580	1037	0.074057	0.9410
day15: cross 3 vs 13	-0.025210	1.3616580	1037	-0.018514	0.9852
day16: cross 3 vs 13	0.155462	1.3616580	1037	0.114171	0.9091
day17: cross 3 vs 13	0.420168	1.3616580	1037	0.308571	0.7577
day18: cross 3 vs 13	0.827731	1.3616580	1037	0.607885	0.5434
day19: cross 3 vs 13	0.546218	1.3616580	1037	0.401142	0.6884
day20: cross 3 vs 13	0.546218	1.3616580	1037	0.401142	0.6884
day21: cross 3 vs 13	0.617647	1.3616580	1037	0.453599	0.6502
day22: cross 3 vs 13	0.617647	1.3616580	1037	0.453599	0.6502
day23: cross 3 vs 13	0.701681	1.3616580	1037	0.515313	0.6064
day24: cross 3 vs 13	0.945378	1.3616580	1037	0.694285	0.4877
day25: cross 3 vs 13	0.995798	1.3616580	1037	0.731313	0.4648
day26: cross 3 vs 13	1.008403	1.3616580	1037	0.740570	0.4591
day27: cross 3 vs 13	1.021008	1.3616580	1037	0.749827	0.4535
day28: cross 3 vs 13	1.033613	1.3616580	1037	0.759084	0.4480
day14: cross 3 vs 16	0.129412	1.5036027	1037	0.086068	0.9314
day15: cross 3 vs 16	-0.082353	1.5036027	1037	-0.054770	0.9563
day16: cross 3 vs 16	-0.258824	1.5036027	1037	-0.172136	0.8634
day17: cross 3 vs 16	-0.294118	1.5036027	1037	-0.195609	0.8450
day18: cross 3 vs 16	-0.329412	1.5036027	1037	-0.219082	0.8266
day19: cross 3 vs 16	-0.582353	1.5036027	1037	-0.387305	0.6986
day20: cross 3 vs 16	-0.582353	1.5036027	1037	-0.387305	0.6986
day21: cross 3 vs 16	-0.582353	1.5036027	1037	-0.387305	0.6986
day22: cross 3 vs 16	-0.582353	1.5036027	1037	-0.387305	0.6986
day23: cross 3 vs 16	-0.641176	1.5036027	1037	-0.426427	0.6699
day24: cross 3 vs 16	-0.411765	1.5036027	1037	-0.273852	0.7843
day25: cross 3 vs 16	-0.347059	1.5036027	1037	-0.230818	0.8175
day26: cross 3 vs 16	-0.305882	1.5036027	1037	-0.203433	0.8388
day27: cross 3 vs 16	-0.164706	1.5036027	1037	-0.109541	0.9128
day28: cross 3 vs 16	-0.223529	1.5036027	1037	-0.148663	0.8818

A.20: Results from ANOVA analysis where cross 13 is compared with the remaining groups are demonstrated in the table. The ANOVA analysis was performed on data from the phenotyping on the bud burst to see if a significant difference between the individuals would appear. This analysis is also taken time into account, which indicates that differences between the groups on the various days will occur. All values from the result of the comparison between cross 13 and the other groups are represented.

BUD BURST – CROSS 13 VS THE REST					
	Value	**Standard error**	**DF**	**T-value**	**P-value**
(Intercept)	3.834252	0.7901164	1037	4.852769	0.0000
day14	0.571429	1.0083508	1037	0.566696	0.5710
day15	0.857143	1.0083508	1037	0.850044	0.3955
day16	1.214286	1.0083508	1037	1.204229	0.2288
day17	1.714286	1.0083508	1037	1.700089	0.0894
day18	2.357143	1.0083508	1037	2.337622	0.0196
day19	2.428571	1.0083508	1037	2.408459	0.0162
day20	2.428571	1.0083508	1037	2.408459	0.0162
day21	2.500000	1.0083508	1037	2.479296	0.0133
day22	2.500000	1.0083508	1037	2.479296	0.0133
day23	2.642857	1.0083508	1037	2.620970	0.0089
day24	3.357143	1.0083508	1037	3.329340	0.0009
day25	3.642857	1.0083508	1037	3.612688	0.0003
day26	3.714286	1.0083508	1037	3.683525	0.0002
day27	3.785714	1.0083508	1037	3.754362	0.0002
day28	3.857143	1.0083508	1037	3.825199	0.0001
Cross 13 vs 1	0.014186	0.9939891	1037	0.014272	0.9886
Cross 13 vs 2	-0.991450	0.9948887	1037	-0.996544	0.3192
Cross 13 vs 3	0.458460	0.9637720	1037	0.475693	0.6344
Cross 13 vs 16	2.290404	1.1096656	1037	2.064048	0.0393
day14: cross 13 vs 1	-0.304762	1.4020549	1037	-0.217368	0.8280
day15: cross 13 vs 1	-0.590476	1.4020549	1037	-0.421151	0.6737
day16: cross 13 vs 1	-0.947619	1.4020549	1037	-0.675879	0.4993
day17: cross 13 vs 1	-1.247619	1.4020549	1037	-0.889850	0.3738
day18: cross 13 vs 1	-1.557143	1.4020549	1037	-1.110615	0.2670
day19: cross 13 vs 1	-1.628571	1.4020549	1037	-1.161560	0.2457
day20: cross 13 vs 1	-1.628571	1.4020549	1037	-1.161560	0.2457
day21: cross 13 vs 1	-1.633333	1.4020549	1037	-1.164957	0.2443
day22: cross 13 vs 1	-1.566667	1.4020549	1037	-1.117408	0.2641
day23: cross 13 vs 1	-1.576190	1.4020549	1037	-1.124200	0.2612
day24: cross 13 vs 1	-1.757143	1.4020549	1037	-1.253263	0.2104
day25: cross 13 vs 1	-1.909524	1.4020549	1037	-1.361947	0.1735
day26: cross 13 vs 1	-1.980952	1.4020549	1037	-1.412892	0.1580
day27: cross 13 vs 1	-1.919048	1.4020549	1037	-1.368739	0.1714
day28: cross 13 vs 1	-1.990476	1.4020549	1037	-1.419685	0.1560
day14: cross 13 vs 2	0.828571	1.4020549	1037	0.590969	0.5547

day15: cross 13 vs 2	1.276190	1.4020549	1037	0.910229	0.3629
day16: cross 13 vs 2	1.719048	1.4020549	1037	1.226092	0.2204
day17: cross 13 vs 2	1.752381	1.4020549	1037	1.249866	0.2116
day18: cross 13 vs 2	2.776190	1.4020549	1037	1.980087	0.0480
day19: cross 13 vs 2	2.971429	1.4020549	1037	2.119338	0.0343
day20: cross 13 vs 2	2.971429	1.4020549	1037	2.119338	0.0343
day21: cross 13 vs 2	2.900000	1.4020549	1037	2.068393	0.0389
day22: cross 13 vs 2	2.900000	1.4020549	1037	2.068393	0.0389
day23: cross 13 vs 2	2.757143	1.4020549	1037	1.966501	0.0495
day24: cross 13 vs 2	3.042857	1.4020549	1037	2.170284	0.0302
day25: cross 13 vs 2	3.090476	1.4020549	1037	2.204248	0.0277
day26: cross 13 vs 2	3.285714	1.4020549	1037	2.343499	0.0193
day27: cross 13 vs 2	3.280952	1.4020549	1037	2.340103	0.0195
day28: cross 13 vs 2	3.209524	1.4020549	1037	2.289157	0.0223
day14: cross 13 vs 3	-0.100840	1.3616580	1037	-0.074057	0.9410
day15: cross 13 vs 3	0.025210	1.3616580	1037	0.018514	0.9852
day16: cross 13 vs 3	-0.155462	1.3616580	1037	-0.114171	0.9091
day17: cross 13 vs 3	-0.420168	1.3616580	1037	-0.308571	0.7577
day18: cross 13 vs 3	-0.827731	1.3616580	1037	-0.607885	0.5434
day19: cross 13 vs 3	-0.546218	1.3616580	1037	-0.401142	0.6884
day20: cross 13 vs 3	-0.546218	1.3616580	1037	-0.401142	0.6884
day21: cross 13 vs 3	-0.617647	1.3616580	1037	-0.453599	0.6502
day22: cross 13 vs 3	-0.617647	1.3616580	1037	-0.453599	0.6502
day23: cross 13 vs 3	-0.701681	1.3616580	1037	-0.515313	0.6064
day24: cross 13 vs 3	-0.945378	1.3616580	1037	-0.694285	0.4877
day25: cross 13 vs 3	-0.995798	1.3616580	1037	-0.731313	0.4648
day26: cross 13 vs 3	-1.008403	1.3616580	1037	-0.740570	0.4591
day27: cross 13 vs 3	-1.021008	1.3616580	1037	-0.749827	0.4535
day28: cross 13 vs 3	-1.033613	1.3616580	1037	-0.759084	0.4480
day14: cross 13 vs 16	0.028571	1.5621304	1037	0.018290	0.9854
day15: cross 13 vs 16	-0.057143	1.5621304	1037	-0.036580	0.9708
day16: cross 13 vs 16	-0.414286	1.5621304	1037	-0.265206	0.7909
day17: cross 13 vs 16	-0.714286	1.5621304	1037	-0.457251	0.6476
day18: cross 13 vs 16	-1.157143	1.5621304	1037	-0.740747	0.4590
day19: cross 13 vs 16	-1.128571	1.5621304	1037	-0.722457	0.4702
day20: cross 13 vs 16	-1.128571	1.5621304	1037	-0.722457	0.4702
day21: cross 13 vs 16	-1.200000	1.5621304	1037	-0.768182	0.4426
day22: cross 13 vs 16	-1.200000	1.5621304	1037	-0.768182	0.4426
day23: cross 13 vs 16	-1.342857	1.5621304	1037	-0.859632	0.3902
day24: cross 13 vs 16	-1.357143	1.5621304	1037	-0.868777	0.3852
day25: cross 13 vs 16	-1.342857	1.5621304	1037	-0.859632	0.3902
day26: cross 13 vs 16	-1.314286	1.5621304	1037	-0.841342	0.4004
day27: cross 13 vs 16	-1.185714	1.5621304	1037	-0.759037	0.4480
day28: cross 13 vs 16	-1.257143	1.5621304	1037	-0.804762	0.4211

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