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Manipulating Male Fertility in Barley (*Hordeum vulgare* L.)

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Abstract

Formation of secondary wall thickening in the endothecium layer of anther is important for anther dehiscence and pollen release. In *Arabidopsis* this process has been shown to be regulated by bHLH transcription factor *MYB26* (Yang et al., 2007) and two NAC domain transcription factors, *NAC Secondary Wall Promoting Factor 1* (*NST1*) and *NST2* (Mitsuda et al., 2005). Characterisation of the regulation of endothecium development and anther dehiscence in agronomically important cereals such as barley will enable better understanding of pollen dispersal process in barley and other important crops. Moreover, in the longer term it may also facilitate identification of a stable sterile line for hybrid seed production in barley and potentially other cereals such as wheat.

This project was aimed at identification and characterisation of the putative *NST1* and *NST2* orthologues in barley using reverse genetics approaches. The putative orthologous sequences in barley were identified by initially identifying the rice and *Brachypodium* putative sequences, prior to obtaining barley putative orthologues. The authenticity of the identified sequences was confirmed by comparison of their expression patterns and the level of synteny between *Arabidopsis*, rice, *Brachypodium* and barley sequences. These sequences were analysed by repression and overexpression in barley Golden Promise cultivar. SRDX repression of the putative sequences in barley resulted in reduction of fertility and associated reduction in plant height and spike size. The overexpression lines of *HvNST1* and *HvNST2* also showed reduction in fertility coupled with reduced spike size and plant height. Expression analysis in the barley transgenic lines suggested that the *NST2* putative orthologue was regulated by *NST1* and that *MYB26* seemed to be upstream of these two

genes. Further analysis is needed to confirm the nature of this interaction in barley.

Moreover, this project explored the possibility of degradation of *Arabidopsis* Male Sterile 5 protein via the N-end rule proteolysis pathway. The preliminary results of this experiment suggested that the N-terminus of AtMS5 could be important in its degradation under *in vitro* conditions; however there needs to be further analyses to confirm this behaviour *in vivo*.

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Abbreviations

ml	Millilitre
µg/ ml	Microgram per millilitre
Mg/ ml	Milligram per millilitre
g/l	Gram per litre
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
cm	Centimetres
min	Minute
dUTP	Deoxynucleoside triphosphate
mM	Milimolar
v/v	Volume to volume ratio
w/v	Weight to volume ratio
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription- PCR
bp	Base pair
rpm	Revolutions per minute
EDTA	Ethylenediamine tetra acetic acid
TBE	Tris-borate EDTA
LB	Lysogeny broth

Chapter One:

Introduction

1.1 Introduction

Food production has always been a major challenge for humans even with today's modernised agriculture. In early ages hunter gatherers would spend hours and days searching for a hunt to feed from. Over time the hunter gatherers were turned into farmers who bred animals and crops for their needs. This effort resulted in generation of the ancestral parents of the current domesticated cultivars and also the birth of agriculture and societies. Perhaps the hidden power and energy that plants provided enabled humans to further develop new skills as they were less worried about spending days looking for food. The small grains of barley and wheat and other species provided security to the first developed societies. These plants were also a secure source for preparing textiles and clothing. These also provided better protection during colder seasons and again further assisted the communities to develop securities against the adverse climatic conditions. Over the last 50 years or so the human diet has changed with animal sources becoming the major part of our diet.

Food production has become a major concern during recent decades. This is highlighted by predictions that the world population will reach 9 billion by 2050 (FAO, 2012). Nonetheless, the world population is not the only concern, as there are a number of additional hurdles such as climate change on the way of food production in the coming years. The intergovernmental panel for climate change (IPCC) report in 2012 states that extreme weather events are rare, and therefore, there is not enough data available to assess the frequency and intensity of that specific extreme (IPCC, 2012). However, available data based on the global trends, show that it is very likely that there has been a decrease in the number of cold days and nights and general increase in the number of hot days and nights (Alexander et al., 2006). Based on these data, it is clear that the climate

change may directly affect food security by altering the crop yield due to the fact that the current cultivated crops are not compatible to the predicted adverse climates. Thus there is a need for immediate action towards better understanding of developmental processes and the gene regulatory networks in cereals, and in particular during anther and pollen development. This knowledge can be used to meet the growing demand for food in the coming decades through production of crops with particular focus towards more resilient flowers to adverse conditions such as heat or cold stresses. This knowledge can also be used for generating male sterile lines that can be ultimately used for hybrid seed production.

Cereals are considered to be the major source of energy for humans. It has been reported that the world cereal production at the end of October 2012 reached 2.29 billion tones, which showed 0.4% reduction to previous projections (FAO, 2012). This reduction was mainly related to the severe drought conditions in the U.S, Europe and central Asia that affected production. Furthermore, the same survey shows cereal utilisation in 2012-13 marketing season was 2.31 billion tones that is slightly higher than the production (FAO, 2012). These data show that current world production, which is nearly the same as its global utilisation, will not meet the demand that is predicted by FAO in 2015. The available data on crop yield shows that the yield of the four major cereals has been doubled over the period between 1961 and 2013 (Figure 1.1). Moreover, food consumption has been increasing in the same pace and it is estimated that by 2030 the consumption will be around 3000 Kcal/ person/ day (FAO, 2002) (Figure 1.1). This shows the importance and urgency of higher yield production to supply enough food for the growing population in the near future. The increase in yield is linked to improvements in agronomy and transition from traditional agriculture to use of technology in all aspects of farming from plantation to harvest. Furthermore, development of superior cultivars such

as dwarf and semi dwarf phenotypes released during the green revolution and utilisation of new methods for production of more pest and disease resistant lines have contributed towards the yield increase seen over the past 50 years. However, as the climate change alters the conditions for plant growth and the availability of the land and water becomes more scarce (IPCC, 2012) ; there will be less land available for cultivation. Thus, there is urgent need for the development of resistant crops to adverse climatic conditions such as high and low temperature or low available water. More importantly, the need for more food production requires crops with higher yield.

Flower development is an important process during plant development that secures the formation of seeds. Better understanding of mechanisms that regulate flower, anther and pollen development in plants is key to addressing the challenge of sustaining the current level of seed production and improving on the factors that can benefit higher yield production such as hybrid vigour via identification of reliable male sterile lines. Therefore there needs to be more focus on unravelling the mechanisms and gene regulatory networks affecting anther and pollen development in plants.

The advancements in plant biology in the last decades have been mostly made through the study of model plants such as *Arabidopsis thaliana*. However, this knowledge may not be of any practical use if it is not linked to the economically important crops. Therefore, the translation of knowledge from model plants into crops is the next important stage towards better understanding of plant developmental biology, especially given that cereals are globally used.

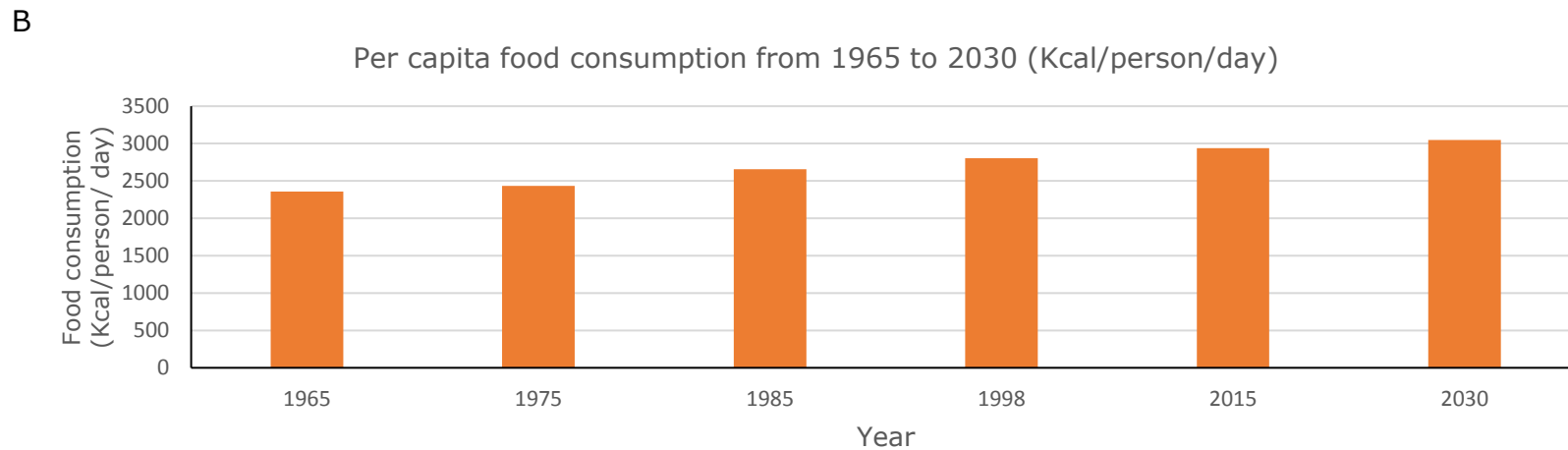
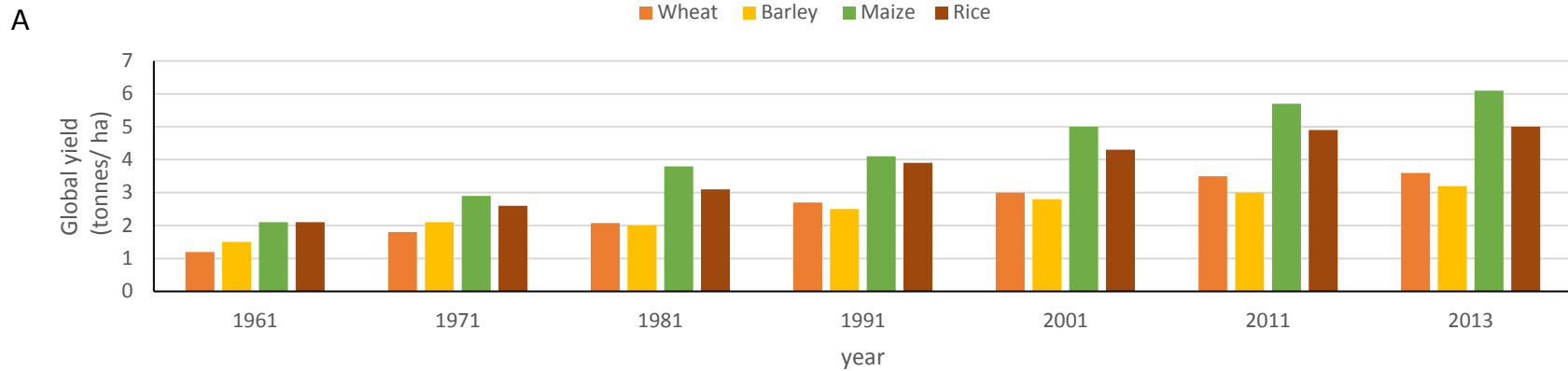


Figure 1. 1 The comparison between the yield and harvested area for four major cereals (FAO stats <http://www.fao.org/faostat/>). A: these data show that the yield of the four major cereals has been at least doubled. B: while there has been a positive increase in crop yield, the projections for food consumption in the coming decades also predicts an increase that is linked to increased population. This further shows the necessity of higher yield production to match the demand in the near future.

A good example of this translation is the role *Male Sterile 1 (MS1)* gene. Wilson *et al.* (2001) characterised the role of *Arabidopsis MS1* gene in pollen development and showed that in *ms1* mutants, the abnormal degradation of tapetal cells does not allow normal development of pollen grains, hence causing male sterility. Furthermore, it was shown that the function of this gene is conserved in barley and the male sterile plants are not able to produce viable pollen due to malfunction of the *MS1* gene (Fernandez Gomez and Wilson, 2014; Li et al., 2011; Wilson et al., 2001). Therefore, barley *MS1* gene could be a potential candidate for providing male sterility in hybrid seed production system. This example shows the importance of translation of anther and pollen development knowledge to crops. The characterisation of the gene regulatory networks affecting anther and pollen development in commercial crops can offer new tools for altering fertility and providing higher yield per unit of land.

1.2 Hybrid seed production

The projected increase in world population will bring about a big change in all aspects of human life such as education, housing, communication and most importantly food production. It is currently estimated that one in nine people (795 million people) are undernourished and the increase in the population will worsen this figure if food production is not increased proportionally (FAO, 2012).

Having said all this, this does not seem to be the first challenge in agronomy and food production. The green revolution in 1960s and use of dwarf parental lines to integrate the short stature phenotype into the cultivated material in Mexico and then India was a great success in increasing food production. Current advancements in science and

technology that has led to the availability of the full genome sequences of some of model and commercial species and identification of majority of gene regulatory network in model plants will provide a better playground for plant scientists to address the global food challenge.

One of the methods of increasing food production is to increase yield by producing superior progeny lines compared to their parents. This system is known as hybrid seed that utilises heterosis effect in the progenies. Heterosis or hybrid vigour is the superiority of the F_1 progeny compared to its parental lines (Fu et al., 2011). The hybrid vigour does not last long and disappears through the generations as the plants inbreed (Breseghello and Coelho, 2013). However, the superiority of the first generation is attractive in terms of higher yield that has persuaded plant breeders to employ the hybrid system despite its challenges to their breeding programmes. However this system has its own limitations. Firstly it requires generation and improvement of two genetically distant populations that can produce strong heterosis in the progenies (Breseghello and Coelho, 2013). Secondly, it has to be ensured that the cost of seed production is economical and profitable. This issue has been addressed by identification of male sterility genes or hand emasculation and also by further increasing the yield so that the hybrid seed system will be more cost effective (Breseghello and Coelho, 2013).

Heterosis has been used in hybrid lines to mainly improve yield in crops such as maize, rice and oilseed rape (Zhang et al., 2015). The hybrid seed production has proved to be a bigger challenge in self-pollinating crops. This is because the emasculation process that will be easy in maize by detasseling the male organ requires more time and attention in crops such as wheat, barley or rice (Breseghello and Coelho, 2013). In barley hybrid seed has been gaining importance compared to inbred line production. For instance, it has been estimated that barley hybrid seed in Germany was

sown for around 11% of feed barley (Philipp et al., 2016). Furthermore, the hybrid barley has been shown to have more yield stability compared to inbred lines (Muhleisen et al., 2014), which is an important factor for breeding companies as they can ensure their seeds have stable performance. The performance of a hybrid seed can be analysed based on the general combining ability (GCA) of parents or mid-parental values (Philipp et al., 2016).

The effectiveness of hybrid seed production depends on the availability of stable male sterile lines. In this case the breeder ensures there are no self-pollination events in the female lines and seeds generated are the result of out-crossing. There are various male sterility induction systems identified for hybrid seed production in wheat. These systems can be, depending on their availability and feasibility of the system, used in hybrid seed production (Whitford et al., 2013). Chemical hybridising agents (CHA) is a male sterility induction method used in wheat. It selectively causes male sterility, but depending on the dosage and mode of action it can also trigger female fertility that is not favourable. Furthermore, this method can be applied in large scale in the field, is not genotype dependent and has systemic activity; however, the efficiency of this method can be affected by weather so that the application time has to be extended for a good result. The CHA product must not have mutagenic activity, should not be affecting the seed set in F₁ generation and has to be environmentally safe. Therefore, the listed limitations make this method less attractive to breeders (Whitford et al., 2013).

Cytoplasmic male sterility (CMS) is another method available for generation of male sterile lines. The CMS is caused due to maternally inherited defects in establishing the genomic harmony between the organellar (mitochondrial) and nuclear genomes (Touzet and Meyer, 2014) that ultimately impairs the communication between cytoplasm and nucleus and

results in generation of non-functional pollen without posing any negative effects on agronomic performance (Bohra et al., 2016; Hanson and Bentolila, 2004). The identification of restorer lines made the cytoplasmic male sterility an applicable approach in three line breeding methods (Zhang et al., 2015). Moreover, by utilisation of the CMS system breeder eliminates the need for anther emasculation, which makes the hybrid system more economical and less time consuming.

Nevertheless, there have been reports of cytoplasmic male sterility imposing pleiotropic effects on female fertility, flower structure and chlorophyll content and yield (Bohra et al., 2016). In wheat, the effect of cytoplasm induced male sterility can be restored by nuclear encoded fertility restorer (Rf) genes that are classified as sporophytic or gametophytic based on the affected tissue. Although the identification of restorer lines has made this system attractive to breeders the potential affiliated inflexibilities of this system such as unavailability of restorer genes, yield penalties associated with the mutation, or sensitivity of CMS mutants to environmental factors make the CMS system less attractive choice for hybrid seed production (Whitford et al., 2013). The need for fertility restorers makes it difficult to adapt this system to the majority of crops, and from time to time the mutation responsible for the CMS is linked with yield penalties or phenotypic abnormalities (Whitford et al., 2013). Therefore, the utilisation of cytoplasmic male sterility requires careful consideration of all its potential effects on the F₁ generation. An example of CMS application in crop breeding is the deployment of a CMS system by Paul Bury in New Farm Crops Ltd. (Syngenta Seeds) resulted in generation the first commercial barley variety, Colossus, in 2002. Since then, Syngenta has released more than ten hybrid six-rowed barley varieties that were grown in more than 200000 hectares around the world. However, none of the commercial hybrid varieties have been used in the brewing

industry as the segregation in the F₂ generation could influence the malting process (Longin et al., 2012).

Self-incompatibility (SI) is a further male sterility inducing system, which forces selective outcrossing. However, though available in some grass species such as rye, it is not fully studied in other more commercially important species such as wheat and barley. Thus, the application of this system as a reliable male sterility provider for hybrid seed production is less attractive (Whitford et al., 2013).

The last method for generation of male sterility is genic male sterility system (GMS), in which the nuclear encoded genes are mutated. This method broadens the choice of parental lines compared to CMS system and it also avoids problems associated with complete male fertility restoration negative alloplasmic and cytoplasmic effects on yield (Whitford et al., 2013).). The spontaneous mutations have been blamed as the cause of this type of sterility. These types of natural mutations have been observed and maintained by breeders. Furthermore, the genic mutant lines can be temperature or photoperiod sensitive that is also important in the reversible induction of sterility under different climatic conditions, even though the induction of sterility under some climatic conditions has proved to be difficulty (Whitford et al., 2013).

As mentioned previously, there are a variety of methods that can be used for hybrid seed production. The use of each method depends on its availability in a particular crop and the preference of the breeder for using each method. Anther and pollen development has been widely studied in model plants such as *Arabidopsis* and rice. These studies have shown pollen development in both species follow a similar pattern; thus the regulatory network is conserved between species (Fernandez Gomez et al., 2015). Translation of knowledge of anther and pollen development to

commercially important cereals such as barley will provide the opportunity for controlling fertility and producing higher yields.

1.3 Barley (*Hordeum vulgare* L.)

Around 7000 B.C., the domestication of crops led to formation of small societies reliant on farming (Harlan and Zohary, 1966; Pourkheirandish et al., 2015). As opposed to its lower importance in today's food industry, the wild progenitor of barley (*Hordeum vulgare* ssp. *spontaneum* L.) played a pivotal role in the life of the newly emerged farmers in Asia, Europe and Africa as one of the first domesticated crops (Pourkheirandish et al., 2015; Zohary et al., 2012). Presence of the wild ancestor of barley in the pre-pottery pre-agricultural Neolithic sites (the barley remnants discovered in these sites date back to 23000 years ago) is an indication to the importance of crops as a source of food and energy for humans, long before humans moved from hunter gathering to farming (Kislev et al., 1992; Pourkheirandish et al., 2015; Weiss et al., 2008).

The domestication of crops was key in the establishment of farming villages in the region known as the fertile crescent stretching from southern province of Khuzestan in Iran to Taurus mountains in Iraq and Turkey and westwards towards Anatolia and southwards into Palestine (Harlan and Zohary, 1966). Discovery of the wild relative of barley (*Hordeum vulgare* ssp. *spontaneum*) in the sites other than the Fertile Crescent such as Tibet, central Asia, Crete, Morocco, Egypt, Ethiopia indicates that there were more than one centre of domestication for barley (Dai et al., 2012; Molina-Cano et al., 2002; Molina-Cano et al., 2005; Ren et al., 2013). This crop along with other cereals is the major source of nutritional intake for both humans and animals (Goetz et al., 2001). Cereal consumption is estimated

to be 173 kg/ person/ year representing 56 percent of calorie intake (FAO, 2003). Despite this, due to the changes in human diet, the global demand for cereals is declining. Nevertheless, cereals remain the major contributor to human diet and form more than 50 percent of our diet (FAO, 2003). .

Taxonomically, barley (*Hordeum vulgare* L.) belongs to the section *Hordeum* of the genus *Hordeum* that are part of the tribe Triticeae of grass family Poaceae (Forster et al., 2007; Von Bothmer, 1992; Von Bothmer et al., 1995). The tribe Triticeae is comprised of more than 350 species including important grain cereals such as barley, bread and durum wheats (*Triticum spp.*), rye (*Secale cereale*) and several forage grass species (Forster et al., 2007; Von Bothmer, 1992; Von Bothmer et al., 1995). The genus *Hordeum* includes nearly 32 species and 45 taxa (von Bothmer et al., 2003). The flower unit of Triticeae comprises of lemma, palea, a pair of lodicules, three stamens and a single carpel (Komatsuda, 2014; Sakuma et al., 2011). All species in this genus have similar morphological characteristics. They all comprise of three one-flowered spikelets at each rachis node called a triplet. Two lateral flowers that are pedunculate can be sterile that produce two-rowed barley or fertile that results in six-rowed barley (von Bothmer et al., 2003) (Figure 1.2).

The spikelet is comprised of reproductive organ, floret, comprising stamens and pistil and non-reproductive organs such as lemma and palea and lodicules. During floral development, a central spikelet meristem (CSM) and two lateral spikelet meristems (LSMs) are differentiated from the spikelet meristem (SM) in the developing barley inflorescence. This is followed by differentiation of each spikelet meristem into a floral meristem (FM) and a pair of glume primordial (GPs) (Houston et al., 2012). Further development results in a mature barley spikelet consisting of the rachis (floral stem) and sessile spikelets, containing reproductive organs and two bracts known as

outer glumes, attached at the rachis nodes (Houston et al., 2012; Ramsay et al., 2011).

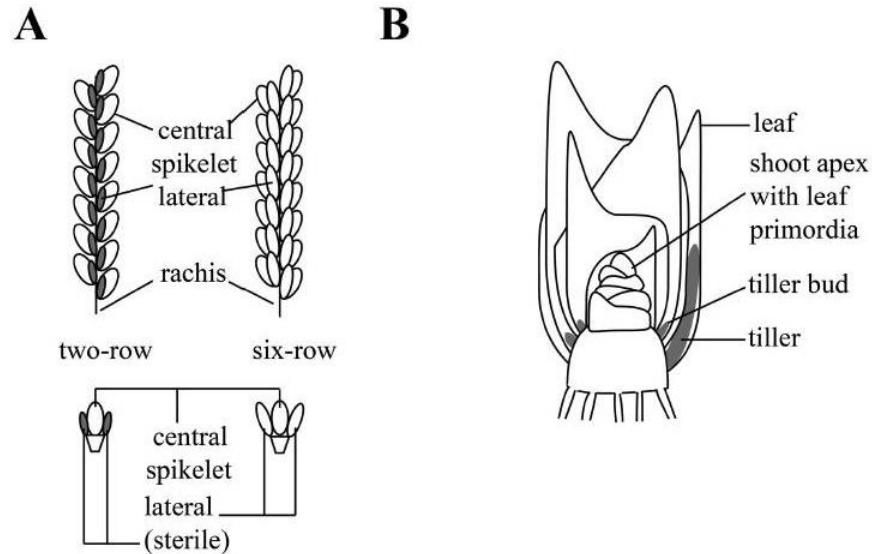


Figure 1.2 Barley Inflorescence structure (Liller et al., 2015). Barley floret comprises three one-flowered spikelets at each rachis node (A). Fertility of all three spikelets results in the formation of six-rowed spike, as opposed to a two-rowed spike that only has the middle spikelet fertile (A). Shoot apical meristem coordinates of formation various above ground tissues including axillary meristem and inflorescences. Axillary meristems, able to give rise to side shoots, are located at the most basal nodes of the plant (B).

Unlike other members of Triticeae family (e.g maize and rice) the spike of barley is not branched; hence, the spikelets are directly attached to the rachis node. The inflorescence meristem in barley produces lateral spikelet meristems that form three spikelets (Derbyshire and Byrne, 2013). In six-rowed spikes all three spikelets are fertile, whereas the sterility of the lateral spikelets and their reduced size give rise to a two-rowed spike (Komatsuda et al., 2007). *Third Outer Glume1 (TRD1)* gene in barley regulates formation of leaf-like structure arising from the lower half of the rachis nodes of the spike, these structures are called third outer glumes (Houston et al., 2012) (Figure 1.3). The malfunction of *Trd1* results in the

development of extra leaf-like structures that extends under each rachis node. Similar phenotypes were observed in the *extra glume1* (*eg1*) and *neck leaf 1* (*nl1*) mutants in rice, which develop an extra glume outside of the lemma (Li et al., 2009). Furthermore, in the orthologue of *N1* in maize, *Tassel Sheath 1* (*Tsh1*), the bracts subtend branches and spikelet pairs in both tassel and ear. This led to the conclusion that formation and differentiation of floral organs is conserved between these species (Houston et al., 2012; Whipple et al., 2010).

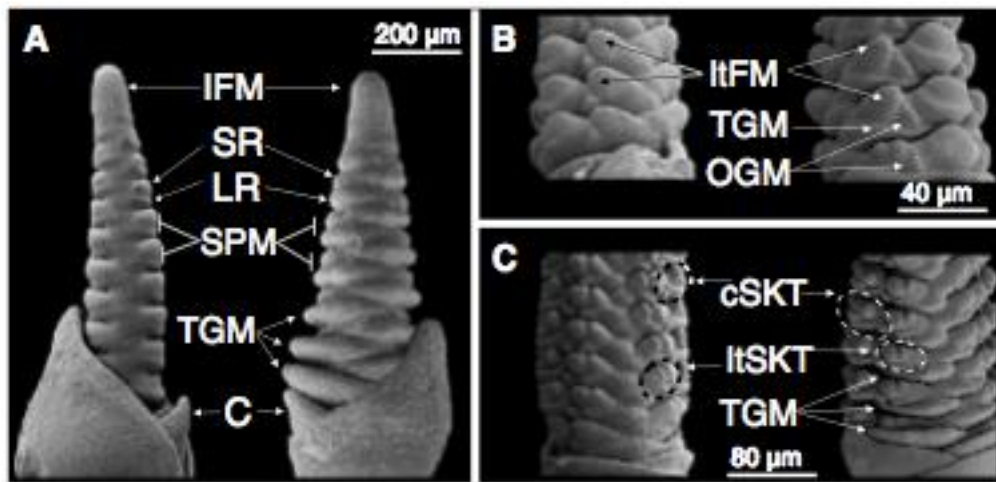


Figure 1. 3 Scanning electron micrographs of the developing barley inflorescence (Houston et al., 2012). It shows the inflorescence of cultivar Bowman (right) and *trd1* mutant (left) at 10-14 days (A), 3 weeks (B) and 4 weeks (C) after germination. After 10-14 days imbibition the shoot apical meristem has differentiated into an inflorescence reaching the double ridge stage, whereas in the mutant spikelet meristems appear more similar to leaf primordia (A). Three weeks after germination the cells of the spikelet differentiated into a central and two lateral spikelet meristems (B). Four weeks after germination pairs of sterile glumes are formed, and surrounded by reproductive structures of the barley spikelet (C). IFM: inflorescence meristem, SPM: spikelet meristem, TGM: third glume primordia, C: collar, NL: neck leaf, SR: spikelet ridge, LR: leaf ridge, OGM: outer glume primordia, ItFM: lateral floret meristem, cSKT: central spikelet primordium, ItSKT: lateral spikelet primordium

In two-rowed barley only the central spikelet is fertile and lateral spikelets are small and infertile as opposed to fully fertile and developed spikelets in six-rowed phenotype (Shang et al., 2014). It has been shown in barley that *Six-Rowed Spike 1* (*VRS1*) gene (Komatsuda et al., 2007), encoding a homeodomain-leucine zipper (HD-Zip) transcription factor, suppresses the development of the lateral spikelet in two-rowed phenotypes (Shang et al., 2014). Six-rowed barley is thought to have evolved at least four times, both temporally and spatially, through a series of independent mutations in the *VRS1* gene (Komatsuda et al., 2007; Saisho et al., 2009; Shang et al., 2014). Another gene affecting lateral spikelet development in two-rowed barley is *INTERMEDIUM* (*INT*). The phenotypic effect of *Vrs1* locus can be influenced by ten independent *INT* genes (Lundqvist and Lundqvist, 1988; Shang et al., 2014). The effects of alleles at the *int-c* locus has also caused a quantitative variation in the size and fertility of the lateral spikelets has been observed in natural populations, and in particular among the offspring derived from a cross between two-rowed and six-rowed barley (Lundqvist and Lundqvist, 1988; Shang et al., 2014).

Almost all cultivated six-rowed barley carry recessive *vrs1.a* allele and the alternative *Int-c.a* allele that enables complete fertility of the lateral spikelets (Lundqvist and Lundqvist, 1988; Shang et al., 2014). Moreover, the presence of *Int-c.b* in barley was showed to trigger the suppression of tillering and male fertility of the lateral spikelets causing formation of two-rowed spikes (Komatsuda et al., 2007; Ramsay et al., 2011). Recently, the orthologue of maize *Teosinte Branched 1* (*ZmTB1*) in barley (*HvTB1*) has been identified (Ramsay et al., 2011) that belongs to TCP gene family that are involved in the control of organ growth. The *HvTB1* is thought to act as *INT.C*, therefore, it was speculated this allele had an important role during domestication process and adaptation of six-rowed cultivars (Ramsay et al., 2011).

Another regulator of lateral spikelet development in barley is *Poly-row-and-branched spike (prbs)* (Huang and Wu, 2011; Huang et al., 2004; Shang et al., 2014). In *prbs* mutants, the rudimentary lateral spikelets in two-rowed barley change into fully developed fertile spikelets similar to the six-rowed phenotype, and additional spikelets are added in the middle of the spike (Huang and Wu, 2011; Shang et al., 2014). In the spike of *prbs* mutants the middle spikelet meristem is altered into a branched meristem developing frequently branched spikes (Shang et al., 2014). The two lateral spikelets are converted into floral meristems, developing fully fertile florets without being regulated by the alleles at *Vrs1* and *Int-c* loci (Shang et al., 2014). It is therefore shown that *Prbs* alters inflorescence development at the triple-mound stage, in which the immature triple primordial at each rachis node can be observed (Shang et al., 2014). It was also concluded that *Prbs* might be a key gene in the evolutionary process of spike inflorescence from the panicle (Shang et al., 2014).

Improving the number of fertile spikelets and number of spikelets per spike at the awn primordium (AP) stage determines the maximum yield potential per spike; thus it is a good target for achieving higher yield in crops. It was shown that the critical stage for the spikelet survival is the time between the formation of awn primordium and the tipping stage where the awns are visible from the last sheath (Alqudah and Schnurbusch, 2014).

On the tip of each spikelet there is an apical extension of the distal end of the lemma known as an awn, which is probably a modified leaf blade (Yuo et al., 2012). It is estimated that 90% of gas exchange within barley ears occurs through the awns (Hosseini et al., 2012). In barley *short awn 2 (Lks2)*, a member of *Short Internodes (SHI)* transcription factors, regulates awn elongation along with pistil morphology (Yuo et al., 2012).

Development of the barley floret occurs inside a concentric folded leaf sheath that forms a pseudo stem. The spike development starts around

twenty days before spike emergence, which is happening in parallel to stem elongation (Fernandez Gomez and Wilson, 2012; Kirby, 1988). The life cycle of cereals has been subdivided into different stages. The two main ways of this subdivision is either based on exterior morphology or internal morphology of the shoot apical meristem (Fernandez Gomez and Wilson, 2012; Landes and Porter, 1989; Large, 1954; Zadoks et al., 1974). While the internal scale is more precise, it has the disadvantages of being time consuming and destructive. Thus, in order to predict the anther pollen developmental stages in barley a non-destructive method was developed (Fernandez Gomez and Wilson, 2012). This method is an adaptation of Zadoks (Zadoks et al., 1974) staging in cereal; though the later staging from Zadoks was modified to match the external morphology of barley cultivar Optic. Although this method accurately predicts anther and pollen stages in cultivar Optic, there may be slight changes in different barley cultivars that are worth considering before using this method. Nonetheless, the stages defined in this method follow similar progression pattern in majority of barley cultivars (Fernandez Gomez and Wilson, 2012).

In this method the developmental stages from the onset of the elongation phase to Zadoks stage 34 in which the last flag sheath emerges progress the same as described in Zadoks staging. For the stages after 34, due to overlapping that occurred in many stages, an alternative approach was adopted to match the external development of the plant with spike development. Hence, last flag elongation (LFE) scale was introduced as an alternative to Zadoks stages 39-53 (Fernandez Gomez and Wilson, 2012).

The length of the last flag was used to predict the development of the inflorescence inside the sheath (Figure 1.4). In stage LFE1 the last sheath has elongated between 0.5-5 cm and the pollen inside the anthers is at mitosis I stage. At LFE2 stage the last sheath elongates further and reaches the size between 5-10 cm and the microspores are at the end of mitosis I

stage. The LFE3 stage is defined when the inflorescence grows further up inside the stem and enters the last sheath. In this stage the spike has entered up to half inside the last sheath and the last sheath is grown more than 10 cm and the pollen development corresponds to mitosis II. Finally, in the LFE4 stage the spike is fully inside the last sheath and the awns are emerging from the last sheath. At this stage anthers contain the tri-nuclear pollen (Fernandez Gomez and Wilson, 2012).

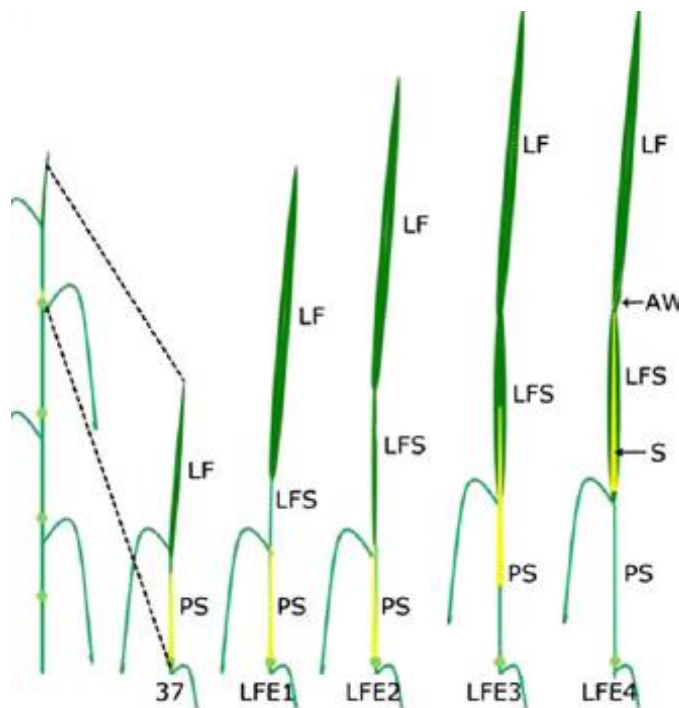


Figure 1. 4 Correlation between the external development and spike development in barley (Fernandez Gomez and Wilson, 2012). Development of last flag sheath and the position of the spike inside the last flag were used to predict anther and pollen developmental stages. PS: previous sheath, LFS: last flag sheath, LF: last flag, AW: awn, S: spike.

The sizes of spike in this study were correlated at a specific anther and pollen development stages (Fernandez Gomez and Wilson, 2012) (Figure 1.5). During barley inflorescence development, 0-1cm spikes are showing

sporogenous cells and three anther cell layers. By the time spikes reach 1-2cm in length, four cell layers are seen. At 2-3cm stage, pollen mother cells enter meiosis (Figure 1.5C), and by 3-4cm microspore release has occurred (Fernandez Gomez and Wilson, 2012) (Figure 1.5D). Microspore release and degeneration of the middle layer happen at the stage when the spike size is 4-5cm. Spikes 5-6cm show vacuolated microspores and tapetum degeneration (Figure 1.5 F). Anther and pollen development enter final stages of maturation before anthesis, during which tapetum degeneration has finished and microspores entered into two mitosis cycles (mitosis I, LFE1-2; mitosis II, LFE3) (Fernandez Gomez and Wilson, 2012) (Figure 1.3 G-I). The anther and pollen development is concluded at LFE4 stage with tri-nuclear pollen (Fernandez Gomez and Wilson, 2012).

Despite their impact on pollen development and thus crop yield there are not many examples of characterised genes related to anther and pollen development in barley. One of the few examples is the characterisation of orthologue of *AtMS1*, a PHD finger transcription factor, in barley (Fernandez Gomez and Wilson, 2014). This study showed the expression of the barley *MS1* orthologue, similar to its *Arabidopsis* orthologue, was specific to the tapetum and can functionally rescue the homozygote *AtMS1* mutant plants. It was also shown that overexpression of *HvMS1* results in generation of male sterile plants due to the failure of the tapetum layer to fully degenerate (Fernandez Gomez and Wilson, 2014). Despite the fact that pollen was viable in the *HvMS1* overexpression lines, the anthers failed to dehisce. This was due to the formation of abnormal substances on the surface of the pollens that made them stuck to the one another; hence, they were unable to be dispersed (Fernandez Gomez and Wilson, 2014).

The characterisation of orthologous genes affecting anther and pollen development in economically important crops such as wheat and barley is of paramount importance. This will provide breeders with tools that can be

incorporated into breeding programmes that can be from using male sterile lines to achieve hybrid seed or using the characterised genes to achieve higher yield targets.

Morphological diversification of grasses inflorescences is closely associated with changes in the expression pattern and copy number of MADS-box genes (Malcomber et al., 2006; Zhang and Yuan, 2014). For instance, *SEPALATA* (*SEP*) clade and its sister group *APETALA1* (*AP1*)/ *FRUITFUL* (*FUL*) originated from multiple duplications and functional diversifications before the extant angiosperms (Malcomber et al., 2006; Preston and Kellogg, 2006; Zahn et al., 2005; Zhang and Yuan, 2014). *OsMADS34*, also known as *PANICLE PHYTOMER 2* (*PAP2*), is a grass-specific *SEP* MADS-box gene that regulates the inflorescence architecture by suppression of the number of primary branches and prevention of early differentiation of early primary branch meristems into secondary branches and spikelets (Gao et al., 2010; Zahn et al., 2005; Zhang and Yuan, 2014). In barley, a number of MADS-box genes have been identified.

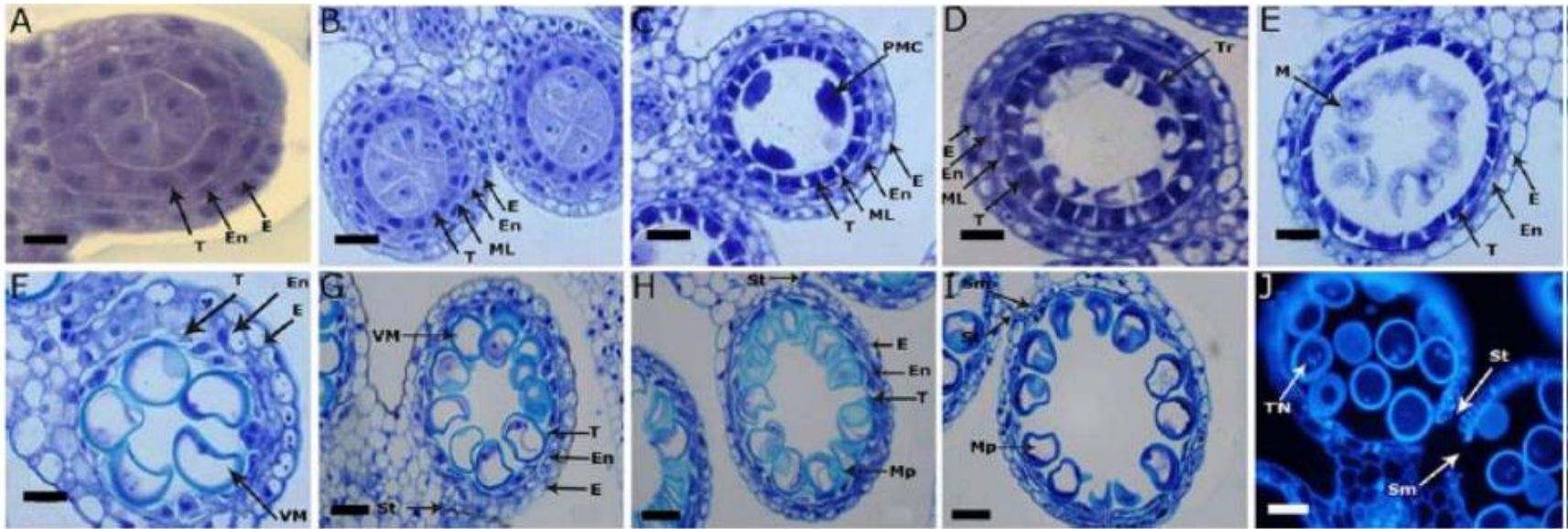


Figure 1.5 Anther and pollen development in barley correlated to spike size (Fernandez Gomez and Wilson, 2012). The adaptation of Zadoks decimal code to barley helps to relate the morphological growth of the plant to its internal anther and pollen development. A: Primary sporogenous cells; three layers are observed surrounding the anther locule. B: Formation of secondary sporogenous cells to pollen mother cells. C: Pollen mother cells enter into meiosis. D: Vacuolation of tapetum and microspore release. E: Degeneration of middle layer and free microspores, tapetum layer starts to degenerate. F: Tapetum degeneration continues while microspore becomes vacuolated. G-H: Mitosis I, tapetum layer present though still is being degenerated. I: mitosis II, binuclear pollen. J: Septum breakage, trinuclear pollen.

1.4 Flower formation

Flower development is the final stage of plant development, and is the basis of plant reproductive development. Flowers are typically comprised of four whorls organs that from outside to the centre are called, sepals, petals, stamens and carpels (Wuest et al., 2012).

The switch from vegetative to generative state triggers the establishment of a group of specialised stem cells that together form floral meristems (Fletcher, 2002; Immink et al., 2010; Zik and Irish, 2003). Formation of stamens and carpels and the synchrony between male and female organs is necessary to secure successful pollination and formation of seeds. There are a large number of gene regulatory networks, along with environmental cues such as temperature and photoperiod involved in transition between shoot apical meristem to inflorescence meristem (O'Maoileidigh et al., 2014).

These different signals are finally converged to activate expression of a small number of floral meristem identity genes in the juvenile floral meristem. These floral meristems arise from a region of the inflorescence meristem where the phytohormone auxin accumulates (Liu et al., 2009; O'Maoileidigh et al., 2014; Reinhardt et al., 2000; Reinhardt et al., 2003; Siriwardana and Lamb, 2012; Wellmer and Riechmann, 2010). Figure 1.6 shows the initiation of floral meristem in the surrounding area of the inflorescence meristem. The two main genes involved in floral meristem identity are *LEAFY* (*LFY*) and *APETALA1* (*Ap1*) that both encode transcription factors (O'Maoileidigh et al., 2014). It has been shown that loss of function of *LEAFY* gene results in transformation of flowers into the inflorescence shoots (O'Maoileidigh et al., 2014; Weigel et al., 1992). Mutation in *AP1* gene triggers formation of secondary flowers in the axils of first whorl organ in addition to showing floral organ identity defects. Observation of increased floral defects in *lfyap1* double mutant suggests

that these two genes redundantly control floral development (O'Maoileidigh et al., 2014; Weigel et al., 1992).

The role of *LEAFY* in establishment of the floral meristem was further confirmed through identification of an auxin response factor *Monopteros* (*MP*) that controls the expression of this gene in *Arabidopsis* (O'Maoileidigh et al., 2014; Yamaguchi et al., 2013). In the absence of auxin, auxin response factor Monopteros (*MP*) interacts with AUX/ IAA protein Bodenlos (*BDL*) that inhibits transcriptional activation of *LFY*. However, presence of auxin causes *BDL* to be ubiquitinated and finally degraded through interaction with Skp1 (S-phase kinase associated protein)- Cullin-F-Box (*SCF*) ubiquitin complex (Cardozo and Pagano, 2004; Mockaitis and Estelle, 2008), containing Transport Inhibitor Response 1 (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; O'Maoileidigh et al., 2014) (Figure 1.6 b and c).

These two genes have been shown to regulate expression of genes involved in hormonal pathways. For instance, *LFY* controls inhibition of auxin biosynthesis and promotes auxin signalling in the emerging floral meristems (Li et al., 2013; O'Maoileidigh et al., 2014; Yamaguchi et al., 2013), and *AP1* functions as a regulator of genes involved in gibberellin metabolism and signal transduction (Kaufmann et al., 2010; O'Maoileidigh et al., 2014).

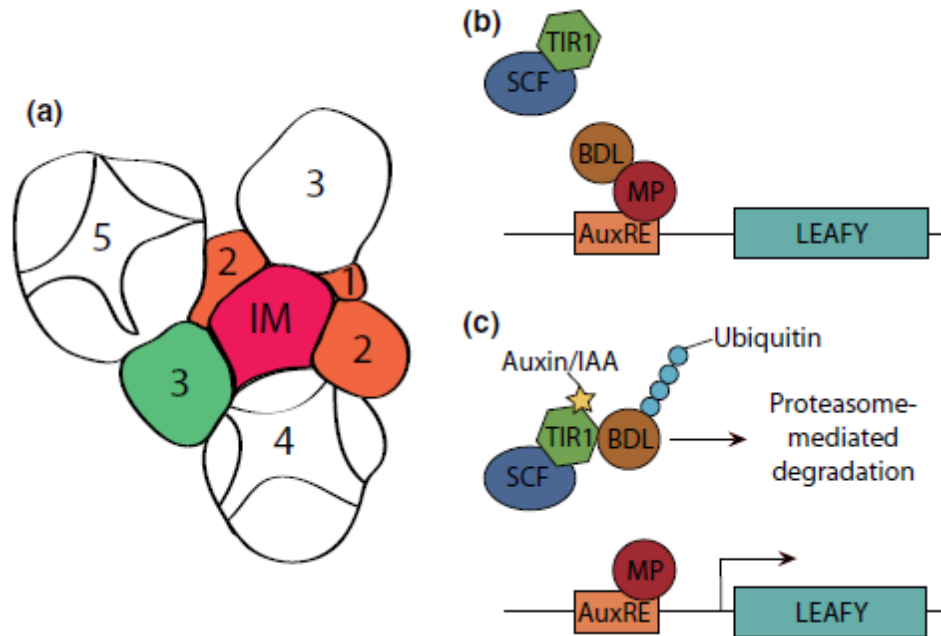


Figure 1.6 Development of inflorescence and floral meristems in *Arabidopsis thaliana*. (O'Maoileidigh et al., 2014). (a) Formation of floral meristem (orange) on the flanking regions of inflorescence meristem (red). The stages at which transcription factors *LEAFY* (*LFY*) and *APETALA 1* (*AP1*) are expressed in the floral meristem are shown as orange colour, whereas the green colour roughly represents the stage at which B and C class genes are expressed. Floral stages are shown as numbers on the floral meristems. (b and c): The effect of auxin on promotion of *LFY* expression. In the absence of auxin (b) Monopteros (MP) interacts with AUX/IAA protein BDL and blocks transcriptional activation of *LFY*. In the presence of auxin (c), the BDL interacts with Skp1 (S-phase kinase associated protein)-Cullin-F-box (SCF) and Transport Inhibitor Response 1 (TIR1) complex, and is polyubiquitylated.

Genetic analysis of *Arabidopsis* homeotic genes helped the formulation of ABC model for floral organ identity specification. It is proposed that the A function genes affect sepals and petals (Figure 1.7), B function genes affect petals and stamens, and finally the fate of the stamens and carpels depends upon C function genes. Therefore, interactions between these genes forms four floral organs (Bowman et al., 1991; Coen and Meyerowitz, 1991; O'Maoileidigh et al., 2014).

Further studies showed that except for the A function gene *Apetala1* (*AP1*) that is a member of *Ethylene Response Factor* (*ERF*) transcription factor family (O'Maoileidigh et al., 2014; Okamuro et al., 1997), the rest of the *Arabidopsis thaliana* ABC genes code for MIKC^c-type MADS-domain transcription factors (O'Maoileidigh et al., 2014). It was suggested that the floral organs were modified leaves, that in the absence of all three classes of floral organ identity genes leaf-like organs grow instead of flowers (O'Maoileidigh et al., 2014).

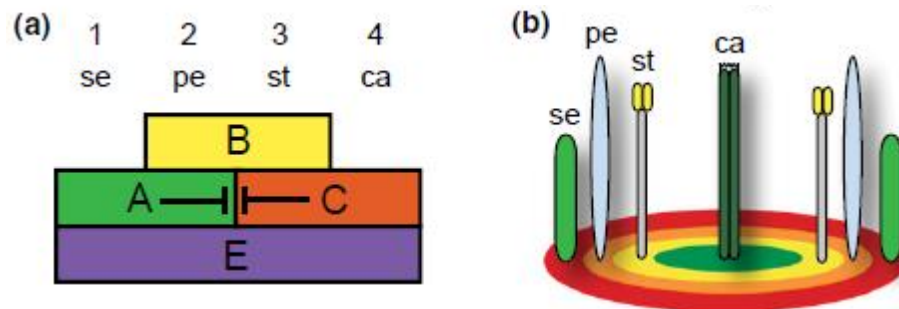


Figure 1.7 The ABCE model for floral organ identity genes in *Arabidopsis thaliana* (O'Maoileidigh et al., 2014). A: The model that has been proposed for the formation of floral organs through the interaction between different genes. B: formation of floral organs sepal, petal, stamen and carpels in *A. thaliana* flower from the outer to the inner whorls.

Furthermore, it was shown that the A and C type genes function mutually antagonistic, so that the class A genes activity expands in class C mutant flowers and *vice versa* (Krizek and Fletcher, 2005). The overexpression of floral organ identity genes did not result in formation of flowers instead of leaves (Honma and Goto, 2001; O'Maoileidigh et al., 2014).

This suggested the presence of a flower specific co-factors regulating formation of flowers. It was later shown that four closely related and

partially redundant *SEPALLATA* genes (*SEP1-SEP4*), that encode for MADS-domain transcription factors and have flower specific expression, function along with the floral identity genes. Furthermore, in the *SEP* genes quadruple mutants (*sep1sep2sep3sep4*) leaf-like structures were formed instead of floral organs (Ditta et al., 2004; Pelaz et al., 2000). Also the overexpression of these genes, in conjunction with certain floral organ identity genes, results in formation of floral organs instead of leaves (Ditta et al., 2004; Honma and Goto, 2001; O'Maoileidigh et al., 2014; Pelaz et al., 2000). These studies expanded the floral organ formation model to ABCE, that shows the E function genes (*SEP* genes) encode flower specific co-factors for organ specification in all floral whorls (Krizek and Fletcher, 2005).

Addition of homeotic class D function genes that regulate formation of ovules completes this model. The identity of the ovule in *Arabidopsis thaliana* is governed by four closely related genes, *AG*, *Seedstick* (*STK*) and *Shatterproof* (*SHP1* and *SHP2*) (Krizek and Fletcher, 2005; Pinyopich et al., 2003). The ectopic expression of either *STK* or *SHP* induces the conversion of sepals into carpeloid organs (Favaro et al., 2003).

The floral organ identity formula is a good explanation for what happens in eudicots. However, the floral formation in monocots does not follow the same pattern as what is present in the outer whorls compared to eudicots. For instance, the liliaceae family have two outer whorls of showy petal-like tepal organs. Whereas in grasses, sepals and petals are replaced with palea, lemma and lodicules (Krizek and Fletcher, 2005) (Figure 1.8).

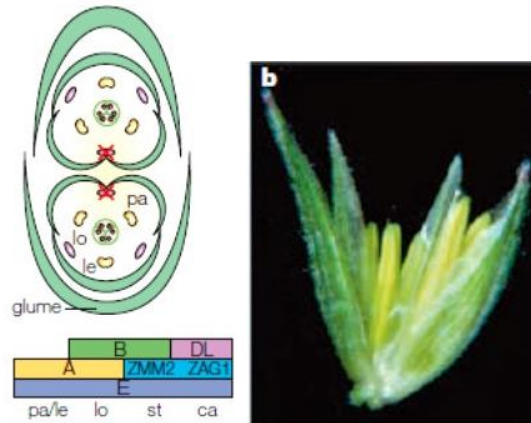


Figure 1.8 Floral organisation in grasses (Krezik and Fletcher, 2005). In majority of grasses, the formation of sepals and petals are inhibited and instead lemma and palea are formed that are surrounding the lodicule and the stamen and carpels. Lo: lodicule, pa: palea, le: lemma.

The lower expression or absence of *Apetala 3* or *Deficiens*-like genes' expression in the outermost whorl of other monocots, especially grass family, produces distinct sepals and petals that are known as lemma and palea (Krizek and Fletcher, 2005). Loss of function of class B homeotic genes in rice and maize, *ZmSilky1* and *OsSuperwoman1*) resulted in replacement of lodicules by palea or lemma, or palea-like organs respectively (Ambrose et al., 2000; Krizek and Fletcher, 2005). In maize there are two class C homeotic genes, *ZmZAG1* and *ZMM2*, which function redundantly. Mutated *ZAG1* does not affect organ identity but affects floral determinacy. Identification of other mutants in the grass family such as *Drooping Leaf (DL)* further explained the acquisition of floral identity in the grass family as the mutation in *DL* resulted in complete transformation of carpels into stamens (Nagasawa et al., 2003). (Figure 1.8). As discussed previously the co-expression and interaction between the floral organ identity genes is important for the formation and specification of flower structure. However, the environmental cues can also trigger flowering via different pathways.

The switch from vegetative to reproductive growth ensures plants set their flowers at an optimum time for pollination, seed development and dispersal (Cockram et al., 2007). The identification of flowering time mutants in *Arabidopsis* has opened the door to identification of various floral inductive pathways (Fornara et al., 2010). The floral pathways such as photoperiod and vernalisation pathways, converge to regulate a set of floral integrators genes, which integrate the output of the different floral pathways and under certain circumstances directly activate floral meristem identity genes (Jung and Muller, 2009). Floral repressors present in the plant genome also play key role in regulation of flower formation via prevention of early induction of reproductive development (Jarillo and Pineiro, 2011). The response to photoperiod requires measurement of the day length in leaves. It has been shown that in *Arabidopsis*, this then generates a mobile flowering signal (known as florigen) that is transported from leaves to shoot apical meristem where it promotes flowering (Turck et al., 2008). One of the essential components of florigen (flower-former) in *Arabidopsis* and rice is a phosphatidyl-ethanolamine binding protein known as *Flowering Locus T* (*FT*) (Corbesier et al., 2007; Jarillo and Pineiro, 2011). Moreover, The *FT* activation depends on the function of *Constans* (*CO*), a zinc finger transcription factor, which responds to light and circadian clock (Milec et al., 2014; Tiwari et al., 2010) (Figure 1.9). In *Arabidopsis*, *Constans* transcription is activated via interaction with plant specific proteins such as *Gigantea* (*GI*), *Flavin Klech F Box 1* (*FKF1*) and photoreceptors such as phytochromes and cytochromes (Milec et al., 2014).

Studies in *Arabidopsis* have shown that expression of *CO* in the vascular tissues of leaves, in response to circadian clock, activates the transcription of *FT* and its homologue *Twin Sister of FT* (*TSF*) (Jarillo and Pineiro, 2011). The *FT* then moves to shoot apical meristem and interacts with transcription factor *Flowering Locus D* (*FD*), which activate *Squamosa*

Binding Protein Like 1 (SPL) family genes that are transcription factors that involved in floral transition (Wang et al., 2009). The elevated levels of SPL in the floral meristem initiates the expression of genes encoding MADS-box transcription factors such as *Suppressor of Overexpression of CO1 (SOC1)* and *Apetala 1 (AP1)* and *Fruitful (FUL)* in order to promote floral meristem identity (Jarillo and Pineiro, 2011). The interaction between *SOC1* and another MADS-box transcription factor, *Agamous like 24 (AGL24)*, regulates the expression of *Leafy (LFY)*, which as discussed earlier regulates flower development (Lee et al., 2008).

The expression of *AtCO* is stimulated under long days where it peaks late in the day that overlaps with the light period. Under short day conditions the *CO* expression only happens in the dark, where it is rapidly degraded (Jarillo and Pineiro, 2011).

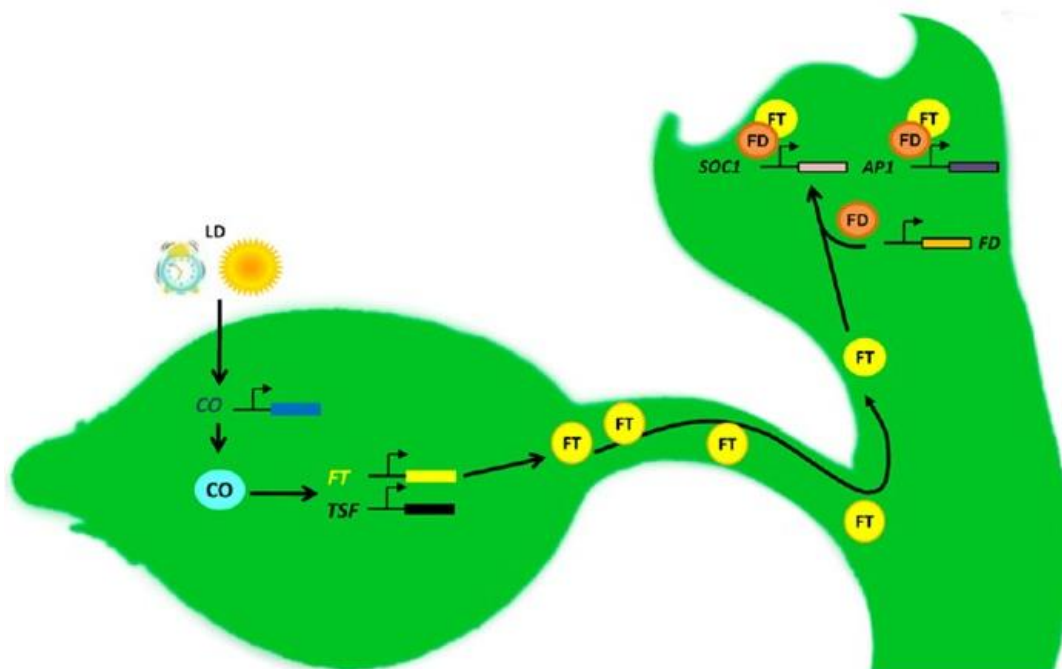


Figure 1.9 Flowering signal initiation pathway in *Arabidopsis* (Jarillo and Pineiro, 2011). The response to circadian clock generates a cue that triggers the floral meristem identity.

Studies in rice has identified *Heading date 3a* (*Hd3a*) that is the homologous gene to *Flowering Locus T* (*FT*) in *Arabidopsis* (Milec et al., 2014). (Figure 1.10). Similar to the pathway seen in *Arabidopsis* (*GI-CO-FT*), flowering induction in rice occurs through the interaction between *OsGI- Heading date 1* (*Hd1*) - *Hd3a* (Milec et al., 2014). The accumulation of *OsGI* at the end of light period activates *Hd1* that is orthologous gene for *CO* in rice and this activation up-regulates the expression of *Hd3a* (Ishikawa et al., 2009). The *Hd3a* is also regulated via *Early heading date 1* (*Ehd1*) that acts independently from *Hd1* that does not have any orthologue in *Arabidopsis* (Doi et al., 2004). Long day conditions in rice converts *Hd1* into a repressor and it suppresses the expression of *Hd3a* (Izawa et al., 2002; Milec et al., 2014) (Figure1.10).

It has been observed that rice can flower under long day conditions. This observation has resulted in identification a florigen encoded by *Rice Flowering Locus T 1* (*RFT1*) and is closest paralog of *Hd3a* (Komiya et al., 2008). Increased levels of *RLT1* in the phloem under long days is transported to the shoot apical meristem where it initiates flowering. Hence, it has been concluded that rice maintains two florigens (Milec et al., 2014). The homologous genes of the flowering time genes identified in *Arabidopsis* (*CO*, *GI* and *FT*) have also been identified in other cereals such as barley and wheat (Nemoto et al., 2003; Yan et al., 2006). The response to photoperiod in wheat is known to be regulated via *Photoperiod* (*Ppd*) genes located on chromosomes 2A, 2B and 2D as homeoloci *Ppd-A1*, *Ppd-B1* and *Ppd-D1* respectively (Mohler et al., 2004).

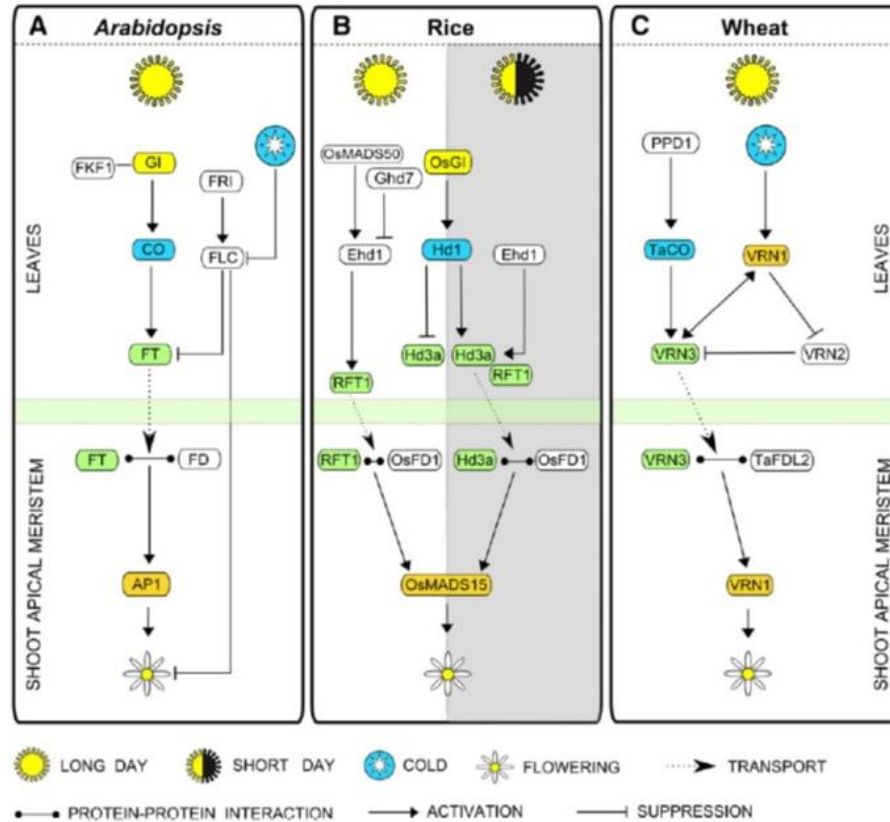


Figure 1.10 The floral initiation network in *Arabidopsis*, rice and wheat (Milec et al., 2014). The identified genes in various species have shown the conservation of the main regulators of this network in *Arabidopsis*, rice and wheat. Flowering in both *Arabidopsis* and wheat is induced in long days, which is firstly measured by *Gigantea* (*GI*). The signal produced by *GI* then activates the transcription of *Constans* (*CO*), which in turn up regulates the expression of *Flowering Locus T* (*FT*). The protein produced by *FT*, known as florigen, moves to the shoot apical meristem and activates floral initiation via interaction with *Flowering Locus D* (*FD*) and further initiation of *Apetala1* gene transcription (A). This pathway has been shown to be conserved in other species. As it is shown here the main regulators of flowering in rice and wheat have been identified (B & C). Moreover, the need for vernalisation in temperate crops has developed different floral initiation regulatory network (C).

The orthologue of this gene has also been identified in barley (*Ppd-H1*) and is located on chromosome 2HS (Laurie, 1997; Milec et al., 2014). Under long days, *Ppd-H1* was expressed predominantly during the early part of

the day. It is shown that *Ppd-H1* gene in barley is under circadian control. Under long day conditions (16 hours photoperiod) mutation in *Ppd-H1* affect other floral initiation genes via reduction in *HvCO1* expression at 8 and 12 hours during a 16-hour photoperiod (Turner et al., 2005). This mutation also reduced the expression of *HvCO2* throughout the light period. Furthermore, the expression of barley homologue of *FT* (*HvFT/VRN3*) was also markedly reduced in *ppd-H1* mutants in long days (Turner et al., 2005). This observation resulted in the conclusion that the reduced expression of *FT* in barley alters the circadian timing of *CO* expression and therefore late flowering phenotype in the mutant *ppd-H1* lines (Turner et al., 2005).

In temperate crops, the gene regulatory network of floral induction and response to photoperiod is similar to *Arabidopsis*; however, the need for cold treatment in majority of temperate crops such as barley and wheat adds vernalisation genes to the floral initiation regulators that act upstream of the *Flowering Locus T* (Milec et al., 2014). Response to cold is regulated via floral repressor genes, which repress the interaction between the *CO* and *FT* through various mechanisms and pathways such as chromatin remodelling (Jarillo and Pineiro, 2011). There has been a number of these floral repressors identified in *Arabidopsis* and include the RAV transcription factors *Tempranillo1* and 2 (*TEM1* and *TEM2*), *Apetala2* (*AP2*), *Flowering Locus C* and some other repressing factors (Wellmer and Riechmann, 2010).

In temperate crops the response to vernalisation is governed by interaction between *Vernalisation 1* (*VRN1*), *VRN2* and *VRN3* and their consequent interaction with floral induction genes. *VRN2* locus is a homologue of the *FLC* gene in *Arabidopsis* and has been shown to contain a zinc finger motif and acts as a repressor of flowering under long days (Milec et al., 2014). The expression of *VRN2* is down regulated under cold treatment by

upregulation of the expression of *VRN1* in the leaves. This ultimately activates the transcription of *VRN3* and floral initiation (Chen and Dubcovsky, 2012) (Figure 1.10). However, the changes occurred during vernalisation (such as histone acetylation) though mitotically stable, are reset in the next generation so that the inhibition of flowering in the cold season will remain intact.

Flowering time and responses to the environmental cues such as photoperiod and temperature have allowed the adaptation of plants to new environments, which enabled plants to travel with the farmers. For example, the spring wheat varieties were favoured in nomadic agricultural systems where they allowed more harvests in a year, the shorter days and colder winters in Europe and North America required targeted selection of spring varieties for better adaptation to this environment. The expansion of the cultivation area of rice from 28° northern latitude to 45° is another example of the diversity within the plants in terms of flowering time and response to photoperiod compared to their ancestors (Milec et al., 2014).

1.5 Anther and Pollen development

The stamen is the major organ for male reproductive development (Goldberg et al., 1993). There are various floral morphologies found within angiosperms; however, in most of these species the stamen is located in the interior position next to the sterile perianth organs and surrounds the central gynoecium (Ma, 2005). The number of stamens varies from species to species and differs from a few to dozens (Ma, 2005). Male reproductive system includes cells that undergo meiosis to produce microspores or haploid male spores (Goldberg et al., 1993). Further mitotic divisions and

differentiation in these cells produce multicellular cells that contain sperm cells called male gametophytes or pollen grains (Goldberg et al., 1993).

The stamen consists of two parts, the anther and the filament. The filament, is a tube of vascular tissues, which acts as a channel to provide water and nutrients for the anther. The anther includes reproductive and non-reproductive tissues that are responsible for producing pollen grains (Goldberg et al., 1993). Anther development requires synchronised steps through which the male organ differentiates and develop anthers harbouring viable pollen grains. The male organ differentiation starts through differentiation of a group of cells within the developing anther into archesporial cells (Caryl et al., 2003). These cells further develop to produce primary sporogenous cells that differentiate into pollen mother cells. Meiosis in pollen mother cells results in formation of haploid microspores that after two rounds of mitosis produce male gametophytes (Caryl et al., 2003).

Smyth *et al.* (1990) divided *Arabidopsis* flower development, from initiation until the bud opening of the buds, into twelve stages (Ma, 2005; Smyth et al., 1990). In the early stages of flower development floral meristems start to grow peaking at stage 3 where the sepal primordial forms. During stages 5 and 6 the initiation of stamen and petal primordial occurs. Cell divisions and differentiation occurs between stage 6 to 9 (Ma, 2005). At stage 7 four long and more developed stamens and two shorter ones are present that produce anthers on the top and stalk at the base. During stage 8 the anthers develop four lobes within which pollens will form. From stages 9 to 12 meiotic division in male microspores and further mitotic divisions result in formation of mature pollens. The flower development is finally concluded when the flower opens and anther dehisces (Ma, 2005).

Formation of anthers inside the developing flower is important as they generate pollen grains that are vital for fertilisation. Thus, the importance

of anther and pollen development led to detailed study of anther and pollen development within growing flower. Anther and pollen development in *Arabidopsis* is divided into 14 stages (Sanders et al., 1999). During stage 1, division in layers L1, L2 and L3 of floral meristem occurs that results in formation of stamen primordium. In stages 2 until 5, anticlinal divisions in L1 layer happen that cause the expansion of the surface area of the anther to form the epidermis (Ma, 2005; Sanders et al., 1999). The L3 layer is also subjected to division and differentiation during this period that form connective vascular tissues (Ma, 2005).

During stage 2 of anther development four clusters of archesporial cells are formed that are derived from periclinal divisions of L2 cells. The archesporial cells are also divided in stage 3, which results in formation of a parietal layer and sporogenous cells (Ma, 2005; Sanders et al., 1999). Formation of two secondary parietal layers during this stage is caused by cell division of the primary parietal layer. The outer layer of this secondary parietal layer in stage 4 is subjected to further division and differentiation to form the endothecium and middle layer. The inner cells of secondary parietal layer after division develop into the tapetum layer (Ma, 2005; Sanders et al., 1999). The four-lobed structure of the anther is formed by stage 5. Each lobe contains four non-reproductive layers that, from outer layer to the inner, are: the epidermis, the endothecium, the middle layer and the tapetum (Figure 1.11) (Ma, 2005; Sanders et al., 1999; Wilson et al., 2011).

Deposition of callose on the wall of the meiotic cells and meiosis of the pollen mother cells (PMC) are the major events in stage 6. Furthermore, the dissociation of meiotic cells from each other and formation of tapetum layer also happens during this stage. This dissociation results in formation of a space interior to the tapetum called the locule (Ma, 2005).

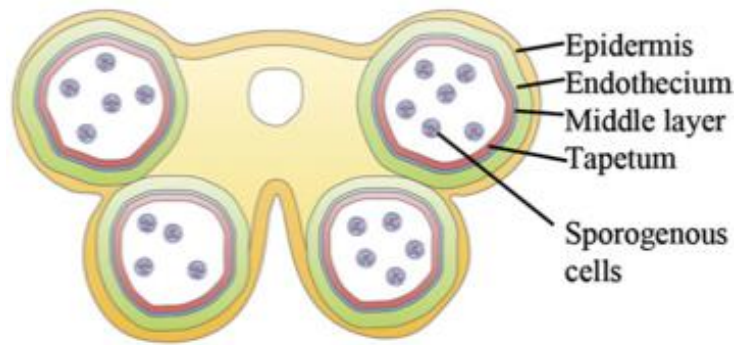


Figure 1.11 Anther structure by the end of stage 5 of anther development showing four maternal cell layers (epidermis, endothecium, middle layer and tapetum) and sporogenous cells (Wilson et al., 2011).

From stages 7-8 four haploid microspores in the shape of tetrad are formed and after the degradation of callose wall, microspores are released from the tetrad (Ma, 2005; Sanders et al., 1999). The following stages (9-12) include the development of pollen grains via mitotic divisions and degeneration of tapetum. Stages 13 and 14 of anther development coincide with dehiscence of the anther and shrinkage of anther cells (Ma, 2005; Sanders et al., 1999) (Figure 1.12). Anther dehiscence and consequent pollen release exposes pollens to the environment outside the anther lobes. The released pollen must withstand the new environment until it reaches the stigma. Thus formation of multiple layer around the mature pollen during its development ensures its longevity after anther dehiscence.

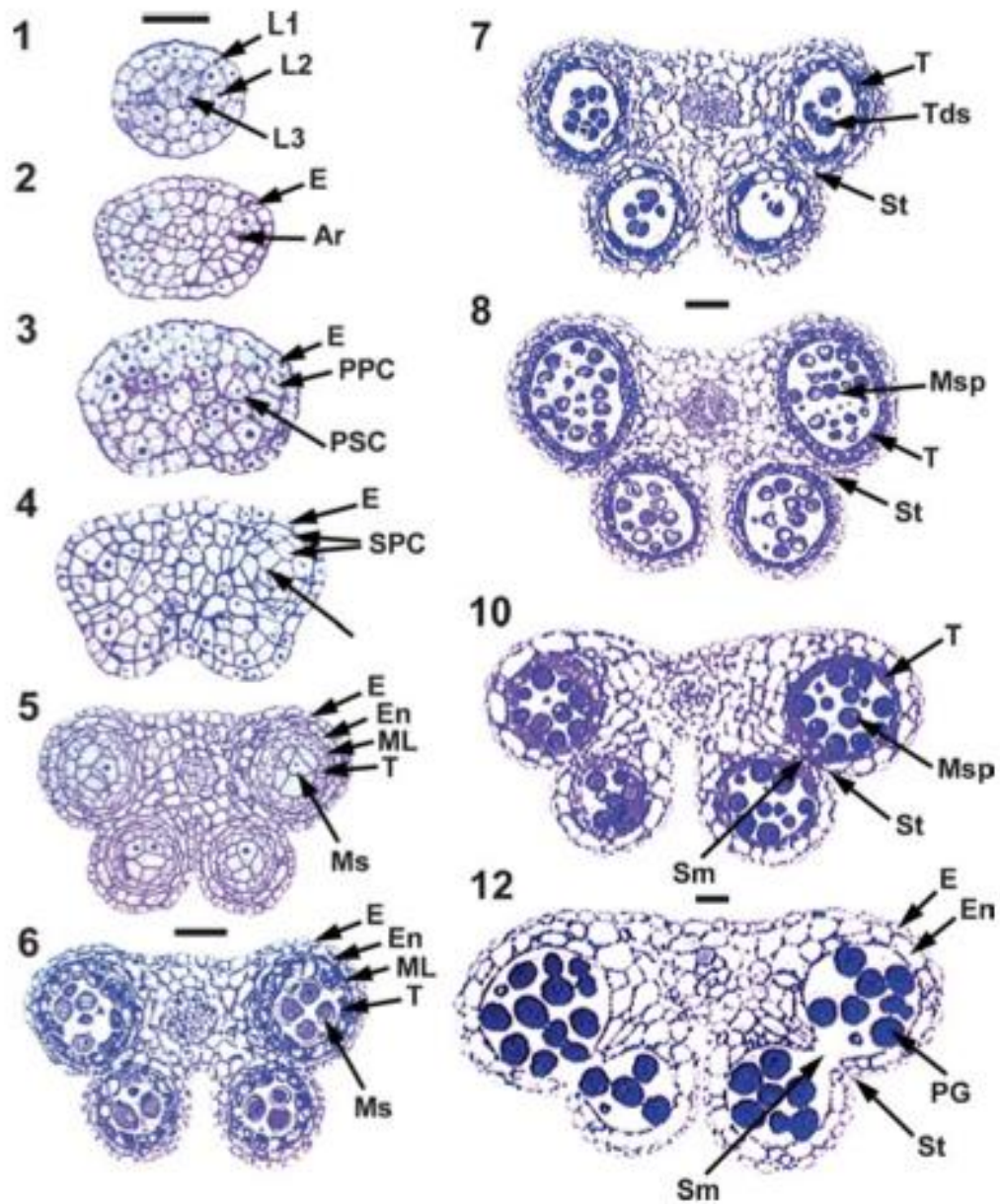


Figure 1.12 Anther development stages in *Arabidopsis thaliana*. Ar, archesporial; E, epidermis; En, endothecium; L1, L2, L3, Layer 1, 2, 3; ML, middle layer; Ms, microsporocytes; Msp, microspore; PG, pollen grain; PPC, primary parietal cell; PSC, primary sporogenous cell; Sm, septum; SPC, secondary parietal cell; St, stomium; T, tapetum; Tds, tetrads. Bar = 25 μ m, stages 1 to 5, 6 and 7, 8 and 10, and 12 alone have same bar size (Sanders et al., 1999).

Pollen grains are typically covered by exine that is the outer cell wall layer that is chemically and physically resistant (Ariizumi and Toriyama, 2011). The exine provides the structural and physical support of the cytoplasm containing the sperm and protects the microspore against the harsh conditions such as high temperature, prolonged desiccation, ultraviolet light and the mechanical damage caused by microbial attacks. It also maintains pollen grains viability and prevents water loss from the pollen (Ariizumi and Toriyama, 2011).

Formation of exine around pollen grains provides further nutrients for the developing pollen. Majority of the exine layer constituents is sporopollenin that is made up of aliphatic and aromatic components (Hemsley et al., 1994; Qin et al., 2013). A number of genes such as *AtABCG26/ WBC27*, *OsC6* have been identified that are linked to exine formation and the export and translocation of sporopollenin precursors from their site of synthesis to the tapetum (Qin et al., 2012). Sporopollenin is exported from the tapetum between tetrad to early bicellular pollen stages (Gong et al., 2015). This is one of the toughest and most durable plant material against desiccation and various kinds of chemical attack. In fact, the presence of this compound has protected the pollen grains in fossils better than any other parts of the plant (Ariizumi and Toriyama, 2011).

As mentioned previously, the ATP-binding cassette (ABC) transporter proteins are directly involved in active transport of a wide range of molecules across the membranes, hence, they play an important role during pollen development and formation of the exine layer (Qin et al., 2012). The ABC superfamily have been studied in *Arabidopsis* and rice and were shown to encode 130 and 132 proteins respectively that are categorised into different subfamilies (Qin et al 2012). The *ABCG15* belongs to the ABCG subfamily, which was shown to be highly expressed in the tapetum at the young microspore stage. The mutant *abcg15* lines showed

decreased accumulation of the monomers of cutin and wax, glossy anther epidermal surface. More importantly, the absence of orbicules and exine in *abcg15* mutant lines resulted in generation of male sterile lines (Qin et al., 2013).

The diversity of the pollen morphology is mostly associated with the differences in the exine structure. Moreover, the formation of exine layer helped the plants to colonise the land as pollen grains could manage to resist more adverse conditions compared to a naked pollen grain (Ariizumi and Toriyama, 2011).

The exine is also capable of preventing fertilisation. As the exine first comes in contact with the female stigma it decides whether the pollen grains could adhere to the female papillae cells. The exine is comprised of two layers, the outer layer sexine (ektesine) and the inner nexine layer (Figure 1.13). The sexine layer determines the species specific structure of the pollen grains and is formed of the tectum and the bacula (columellae) that are radially directed rods (Ariizumi and Toriyama, 2011). Laid on the multi-layered intine, the nexine layer is composed of nexine I (foot layer) and nexine II (endexine). The underlying layer of exine is the intine that is formed of hydrolytic enzymes, hydrophobic proteins, cellulose, hemicellulose and pectic polymers that resemble to the primary cell wall components in plants (Ariizumi and Toriyama, 2011).

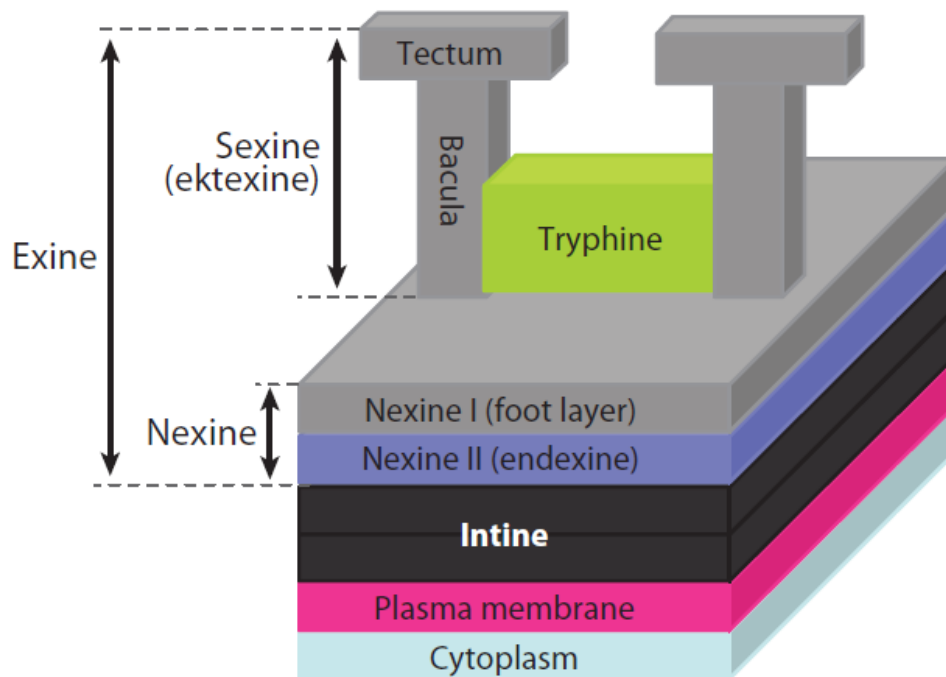


Figure 1. 13 The exine layer is formed of two major layers the sexine and nexine (Ariizumi and Toriyama, 2011). The sexine layer is subdivided into tectum and bacula and the nexine layer is divided into two layers of nexine I (foot layer) and nexine II (endexine).

1.5.1 Anther and Pollen development gene network

One of the critical stages in plant life cycle is anther and pollen development. The development of viable pollen is an important step in the plant life cycle as it ensures the production of the next generation. Moreover, pollen release has to be at the correct time when the gynoecium is ready to be fertilised. Therefore, the synchrony between the two processes determines self or cross-fertility (Wilson and Zhang, 2009; Yang et al., 2007). It is estimated that approximately 15000 genes are involved in anther and male gametophyte development (Wilson and Zhang, 2009). Identification of these genes has revealed the main pathway that controls the process of anther and pollen development in *Arabidopsis thaliana* (Figure 1.14). During the early stages of anther development two genes, *Wuschel* (*WUS*) and *AGAMOUS* (*AG*) in the centre of floral meristem initiate

Leucine-rich repeat receptor kinase *Extra Sporogenous Cells/ Excess Microsporocytes1* (*EXS/EMS1*) and its ligand *Tapetal Determinant* (*TPD1*) control archesporial cell number and tapetal cell fate (Canales et al., 2002; Jia et al., 2008; Wilson et al., 2011; Zhao et al., 2002). Tapetum development is initiated by the expression of *Dysfunctional Tapetum* (*DYT1*), and *Defective in Tapetal Development and Function1* (*TDF1*) (Zhang et al., 2006; Zhu et al., 2008). As the tapetum develops, tapetal maturation, pollen wall formation, and tapetal programmed cell death (PCD) occur. These events are linked to the expression of *Aborted Microspores* (*AMS*), *Male Sterility1* (*MS1*) and *Defective in Tapetal Development and Function 1* (*TDF1*) (Wilson et al., 2001; Wilson et al., 2011; Zhang et al., 2006; Zhu et al., 2008). Furthermore, *AMS* plays an important role in biosynthesis and secretion of pollen wall material from the tapetum (Ma et al., 2012). A bHLH transcription factor *Dysfunctional Tapetum 1* (*DYT1*) has also identified as an important regulator of tapetum function (Zhang et al., 2006). Further studies have shown that the *DYT1* functions genetically upstream of *AMS* and *MS1* and downstream of *SPL* and *TPD1/EMS1* (Feng et al., 2012; Zhang et al., 2006). In *dyt1* mutant meiocytes undergo meiosis and produce four haploid cells; however, the abnormal tapetum does not allow the development of the microspores and resulting in the absence of any developing pollen grains (Zhang et al., 2006). A closer look at the *dyt1* mutants showed that 450 genes were down regulated out of which 22 were transcription factors including *AMS*, *MYB103/ MYB80* and *MYB35* (Feng et al., 2012). Moreover, the expression of 33 genes involved in cell wall modifications that encode enzymes involved in polysaccharide's catalysis was also decreased (Feng et al., 2012).

Hormonal signalling has also been shown to be involved during microspore and tapetum developments. In rice anthers, Gibberelin (GA) signalling has

been shown to act via *OsGAMYB* transcription factors, which regulates tapetum secretory function and programmed cell death (PCD) (Aya et al., 2009; Plackett et al., 2014). GA signalling acts via degradation of DELLA proteins that inhibit GA-dependent changes of the downstream genes (Hou et al., 2008). In *Repressor of ga1-3 (RGA)* and *GA Insensitive (GAI)* double mutants, that are both encoding DELLA proteins, the flowers showed a pollenless phenotype. In the double mutants the formation of tetrads was affected and abnormal meiotic products resulted in male sterility (Plackett et al., 2014). The GA signal transduction protein in rice *Slender Rice 1 (SLN1)* and its homologue in barley, *Slender 1*, causes sterility and absence of pollen in *sln1* mutant (Ikeda et al., 2001; Plackett et al., 2014).

A comparison between *Arabidopsis* and rice anther gene regulatory networks shows that the function of genes are conserved between these two species (Fernandez Gomez et al., 2015) (Figure 1.15). In the early stages of pollen development rice *Multiple Sporocyte1 (MSP1)* has high degree of similarity to its orthologue *EMS1/EXS* in *Arabidopsis*. *MSP1* encodes for a Leucine-rich repeat (LRR) receptor like kinase, and the mutants show a similar phenotype to the *ems/exs1* mutants. These mutants show increased numbers of male and female sporocytes and disrupted anther wall layers that lack the tapetum cell layer (Nonomura et al., 2003; Wilson and Zhang, 2009). However, the network in rice may show slightly different patterns due to differences between the two species such as gene duplication. For instance, *Agamous* function is conserved in rice but it is partitioned into two MADS box genes, *OsMADS3* and *OsMADS58* (Wilson and Zhang, 2009; Yamaguchi et al., 2006).

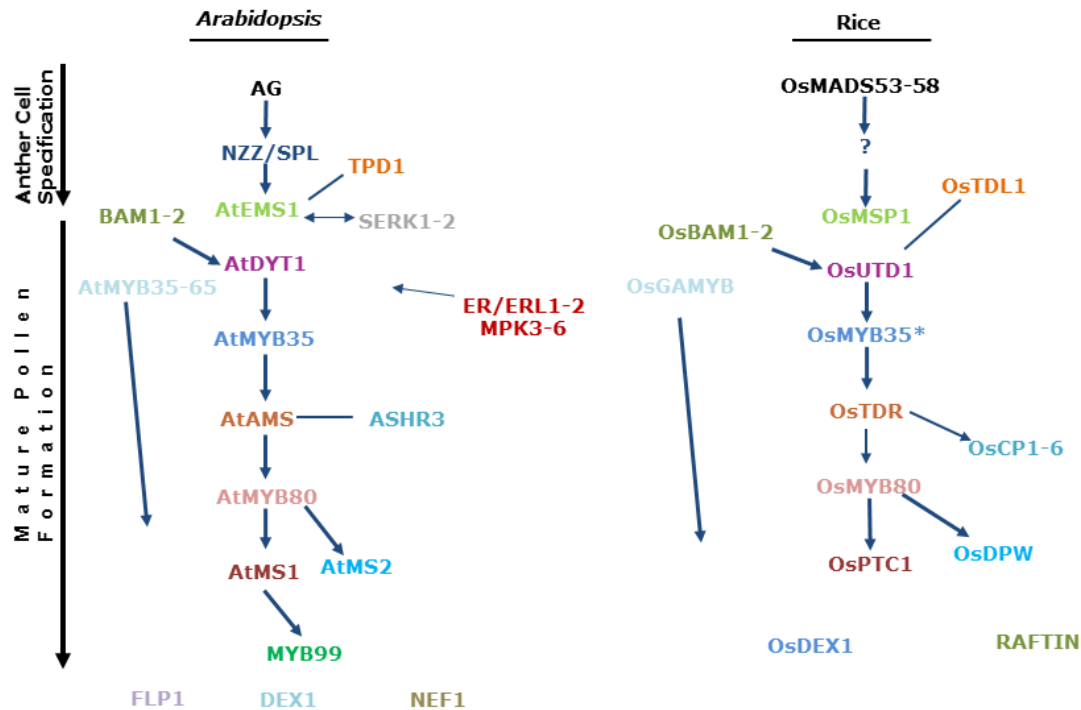


Figure 1.15 Comparison between anther and pollen development gene regulatory network in *Arabidopsis* and rice (Fernandez Gomez et al., 2015). The matching colours indicate similar function and show that the regulatory network is highly conserved between these two species.

1.5.2 Anther dehiscence

The anther opening is the last stage following the maturation of pollen that facilitates the dispersal of pollen grains. There are two types of specialised cells involved in this process. Firstly, is the septum cells that separate two lobes of the anthers, which their break down causes the formation of a single locule in the anther. The second type of cells is the stomium that are modified epidermal cells and their cleavage facilitates anther opening and pollen release (Figure 1.16) (Wilson et al., 2011). The dehydration of epidermis layer also contributes to anther opening. In tomato, selective

conversion of starch to sugar in the connective tissues in the anther generates increased osmotic potentials and further removal of water from the anthers (Nelson et al., 2012). Moreover, aquaporins have also been mentioned as major contributor towards water transport from the anthers tissues that are linked to anther and pollen dehydration (Nelson et al., 2012; Tyerman et al., 2002).

The biomechanical model for *Arabidopsis* and lily anther opening showed the formation of secondary thickening in the endothecium layer of the anther is crucial for generating forces required for the anther opening via differential resistance to bending and stretching (Nelson et al., 2012). Therefore, secondary thickening in the anther allows the dehydration-driven epidermal contraction forces to change the anther's shape (Nelson et al., 2012). This model explained that the uniform curvature of the epidermis in all parts of the anther causes uniform anther opening due to epidermal dehydration. However, localised deviations of the epidermal dehydration in the biological configuration did not allow the uniform opening to happen. It was also speculated that because the anthers are wider at the centre than the tip, this variation could contribute towards opening of the tip of the anthers first (Nelson et al., 2012).

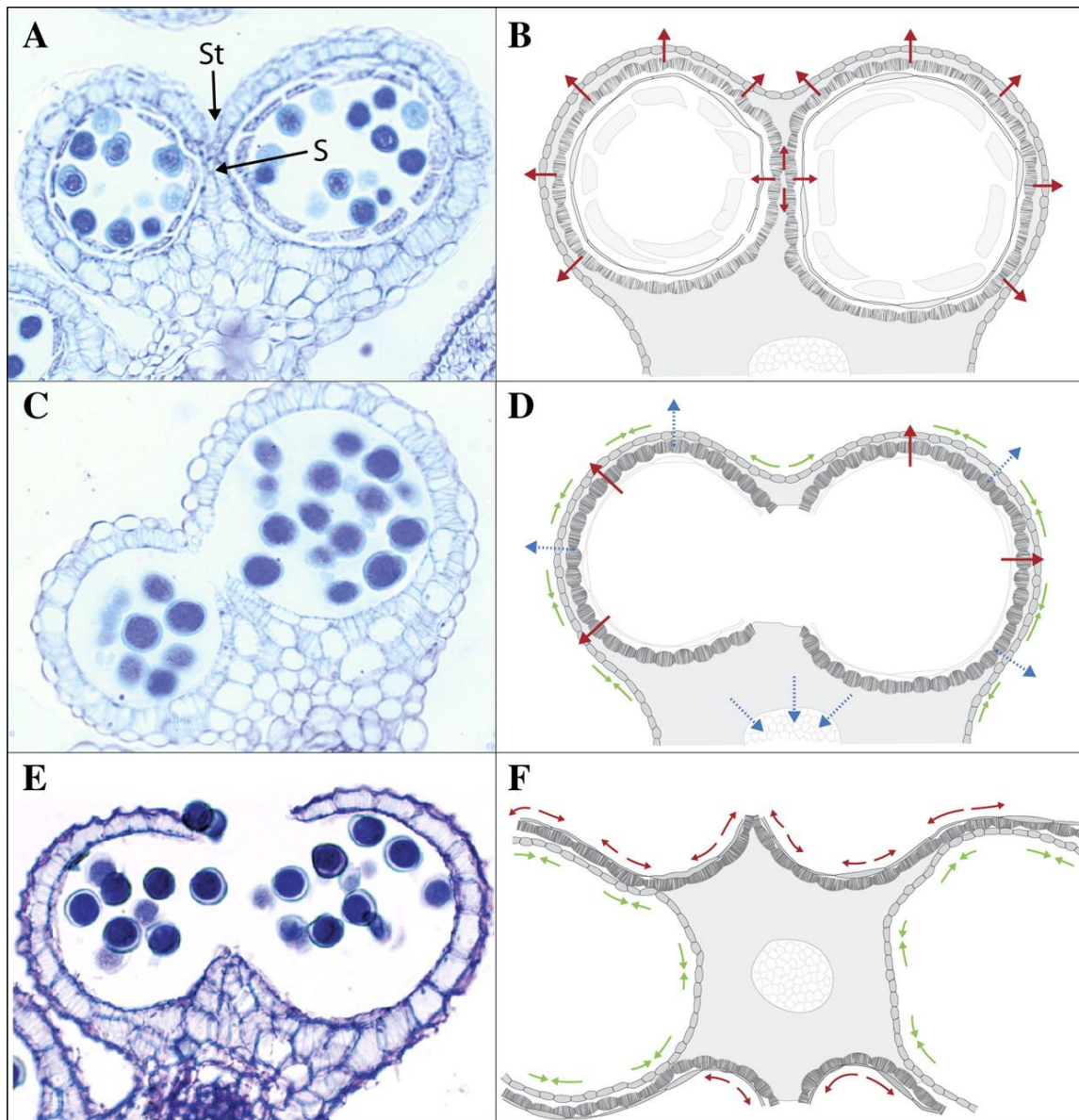


Figure 1.16 Pollen release relies on the inner and outer forces that are helping the anther to open. (Wilson et al., 2011). These forces are generated by the growing pollen inside the anther lobes, water evaporation from the anther layers and secondary wall thickening formed in the endothecium that inhibits the anthers to expand further (B, D and F). Furthermore, the degeneration of the septum cells create a single lobe that upon stomium degeneration facilitate pollen release. En, endothecium; St, stomium; S, septum. Bar=50 μm .

Secondary thickening in the endothecium layer of anther is an important parameter in anther opening. Lignified thickening and cellulose are deposited in endothecium during microspore maturation (Dawson et al., 1999; Yang et al., 2007). During anther dehiscence swelling of the endothecium creates an inwardly directed force resulting in generation of rupture in already weakened stomium (Figure 1.16 D). Differential shrinkage in the cell wall of the endothecium caused by desiccation of the endothecium then provides an outwardly bending force causing the full opening of the stomium (Figure 1.16 F) (Bonner and Dickinson, 1989; Keijzer, 1987; Wilson et al., 2011; Yang et al., 2007). A number of genes are involved in formation of secondary thickening in the endothecium during anther development including three transcription factors *MYB26*, *NST1* and *NST2* (Mitsuda et al., 2005; Yang et al., 2007).

Anther dehiscence as described above requires deposition of lignin and cellulosic components in the endothecium layer of the anther. It is the presence of these components that provide the forces required for anther opening (Nelson et al., 2012). During their anther and pollen development studies on mutant lines created through X-ray mutagenesis of *Arabidopsis* seeds, Dawson et al. (1999) characterised a mutant, *ms35*, in which anthers fail to open and release the pollen grains, however, there were no defects in terms of pollen development and the pollen was viable. Lack of anther opening in *Arabidopsis*, a self-pollinated plant, resulted in sterile plants. Pollination of *ms35* plants with wild-type pollens resulted in normal silique development that confirms the cause of male sterility was due to failure of anthers to open not the malfunction of female organ (Dawson et al., 1999).

The pattern of lignification of *ms35* mutants in vascular tissues is the same as wild-type, suggesting that this mutation only affects the secondary thickening in anthers (Yang et al., 2007). It was shown that defects in

anther dehiscence in *ms35* are due to the *MYB26* gene, which has homology to R2R3-type *MYB* transcription factors (Steiner-Lange et al., 2003; Yang et al., 2007). This type of transcription factor is thought to be involved in transcriptional activation or repression through controlling development and the determination of cell fate along with regulation of biosynthetic pathways (Stracke et al., 2001; Yang et al., 2007). Analysis of formation of secondary thickening in anthers of *ms35* mutant showed there was no lignification or secondary thickening in the endothecium (Figure 1.17) (Yang et al., 2007).

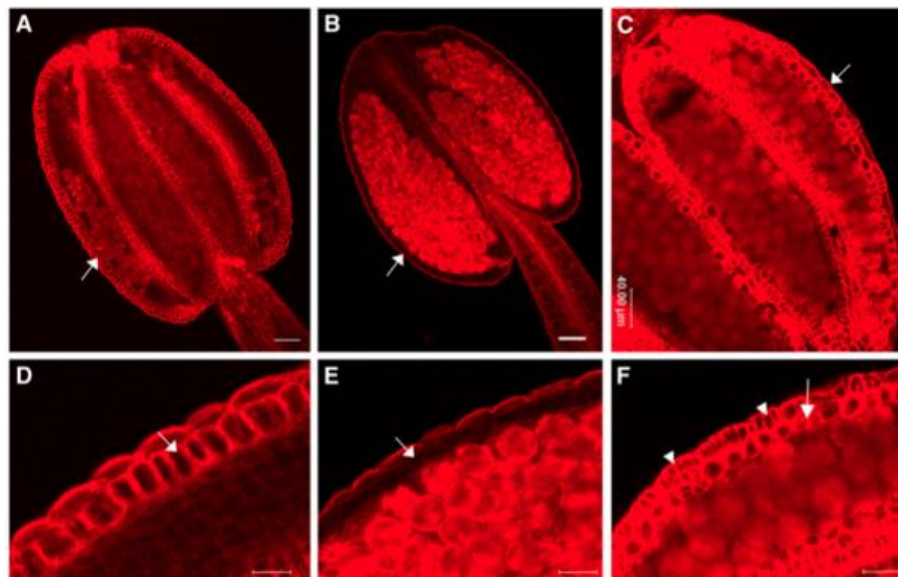


Figure 1.17 Secondary thickening in *Arabidopsis* anthers. A) Semi-dehiscent wild-type anther with formation of secondary thickening (arrow). B) Lack of secondary thickening in *ms35* anthers (arrow). C) Overexpression of *MYB26* gene and ectopic secondary thickening in the endothecium (arrow). D) Close up of A. E) Close up of B. F) Close up of C. Bar= 40 μ m (Yang et al., 2007).

Moreover, Yang *et al.* (2007) showed that *MYB26* was able to partially complement *ms35* mutants by restoration of lignin deposition in the endothecium that allows anther to open. Expression of this gene was detected in various floral organs such as style, nectaries of the flowers;

however, no expression was detected in vegetative tissues (Yang et al., 2007).

The formation of secondary wall thickening in the anthers are also regulated by two NAC domain transcription factors *NAC Secondary Wall Thickening Promoting Factor 1 (NST1)* and *NST2*. These two transcription factors have shown to be functionally redundant and share 63% amino acid identity (Mitsuda et al., 2005). The homozygote T-DNA tagged knock out lines of either *NST1* or *NST2* genes in *Arabidopsis* did not show any abnormal effects; however, the double mutant lines were indehiscent due to lack of secondary wall thickening in the anthers. Repression of *Arabidopsis NST1* showed the same phenotype as wild-type apart from the indehiscent anthers of the repressed lines (Mitsuda et al., 2005). Overexpression of *NST1* and *NST2* showed ectopic secondary thickening in multiple tissues including anthers, stamens, ovules, stems and leaves and it was less frequent in root tissues. The level of thickening in *NST1* overexpression plants was higher than *NST2* that suggests the higher activity for *NST1* (Mitsuda et al., 2005).

1.5.3 Pollination

As plants evolved traits that make the flower more attractive to the pollinators such as the inflorescence height, colour, fragrance or mechanical traits such as corolla tube length were gradually selected (Newman et al., 2014).

The release of mature pollen grains from the anthers is not the end of their life cycle. The pollen grain has yet to make its way towards the ovule and fertilise the egg cell. This journey is normally facilitated by wind, animals or insects such as bees. During its development, pollen is soaked inside the

locular fluid that in part provides forces for pollen release. The pollen then loses its water content at the maturity stage. However, the germination of pollen grain on the stigma requires hydration. For this, plants produce an ovular secretion, known as pollen drop, which forms a liquid carpet for pollen to land on and provides a humid surface for pollen growth (Pirone-Davies et al., 2016). The apertures and openings of the pollen grain in some species allows the water absorption and they provide a path for the pollen tube to grow. While in the species without porous pollen rupture in the pollen grain is required for pollen tube germination (Edlund et al., 2016).

Pollen growth rate depends on the water supply and the ability of stigma to transport water and the degree in which the pollen grains absorb water. Moreover, species specificity of the stigma determines whether the pollen received or rejected (Di Giorgio et al., 2016). In *Arabidopsis thaliana*, the absorption of water on the side of contact between the pollen and the stigma provides fluid influx along with directional cues and the turgor pressure required for pollen tube formation (Edlund et al., 2016).

The developing pollen tube requires directional growth and signalling between the stylar transmitting tract and pollen tube. It is known that the stylar exudate contains hydroxyproline-rich glycoproteins that in some cases can promote pollen tube growth and the tube guidance (Cheung et al., 1995). The guidance of the pollen tube to the embryo sac is a two-step process. During the first step the pollen tube is directed to the ovule through the funiculus. Second step is the orientation of the tube towards the micropyle and further to the embryo sac (Iwano et al., 2012). The orientation of the tube towards the micropyle is thought to be due to the attractants generated by the synergids. In *Torenia fournieri* and *Arabidopsis* the cysteine-rich proteins that are accumulated in the wall of the filiform of the synergid cells is linked to directing the pollen tube (Okuda

and Higashiyama, 2010). Furthermore, the malfunction of the auto-inhibited Ca^{2+} ATPase (*ACA9*) in *Arabidopsis* activity present in the plasma membrane of the pollen tube resulted in partial male sterility. This was shown to be due to the failure of the pollen tube to discharge the sperm cells (Iwano et al., 2012; Schiott et al., 2004).

Study of accumulation and oscillation of Ca^{2+} at the tip of the pollen tube and along with the synergid cells showed no changes in the calcium concentration and oscillation before pollen tube rupture (Iwano et al., 2012). Moreover, the concentration of the synergid cells reached its maximum at the time of pollen tube burst. This shows the involvement of calcium signalling in pollen tube-synergid interaction (Iwano et al., 2012).

The entrance of pollen tube to ovule and its contact with the synergid cell causes programmed cell death to the cell that receives with the tube. This ensures the accessibility of sperm cell to the egg and central cells for the double fertilisation (Punwani and Drews, 2008).

1.6 Plant Cell Wall

The cell wall in plants is a dynamic structure that plays a major role in plant development and growth that also enables cells to adapt to biotic and abiotic stresses (de Castro et al., 2014). Cell walls are mainly composed of polysaccharides cellulose, hemicellulose and pectins. The components of cell wall vary between plant species; hence, this variation generates a certain degree of variability in the cell wall structure and composition (de Castro et al. 2014). The ability of plants to be able to produce polysaccharide-rich cell walls and conduct photosynthesis was considered as a key innovation in plant evolution (Banasiak, 2014; Popper and Tuohy, 2010). It is suggested that this acquisition happened when cyanobacteria

invaded the host cell and gave rise to the common ancestor of Archaeplastida group including glaucophytes, red and green algae and land plants (Banasiak, 2014). Unlike cyanobacteria with cell walls composed of peptidoglycan, plant cell walls are formed of polysaccharide. It is hypothesised that this is due to horizontal gene transfer of the endosymbiont genes involved in carbohydrates biosynthesis, providing the genetic background for cell wall formation in land plants (Banasiak, 2014; Popper and Tuohy, 2010). The high mass of fermentable sugars embodied within the polysaccharides of the cell wall, has drawn attention towards production of biofuel from plant material (Burton and Fincher, 2014).

Cellulose is considered to be the most abundant biopolymer in vascular plants, but it is also found in lower plants such as algae, Oomycetes, tunicates as well as various bacterial species (Kumar and Turner, 2015). Cellulose provides strength in the xylem fibres that enables plant to grow taller and be able to withstand external forces and also the forces linked to transpirational pull on a column of water (Kim et al., 2013). The chemistry and organisation of cell walls differentiate cell types in plants, even the same type of cells adjacent to each other can differ based on the fine structures of some components related to their developmental fate or the result of exposure to the environmental conditions (Farrokhi et al., 2006).

The deposition of callose and polyphenols such as lignin in the cell wall is part of the plant defence against pathogens during plant development (Farrokhi et al., 2006). Based on the wall formation and the structural contents, cell walls are divided into two types of primary and secondary cell walls (Ruprecht et al., 2011). The primary wall in the majority of higher plants comprises of cellulose, hemicellulose and pectin while the secondary cell wall is mainly consists of cellulose, xylans and lignin (Ruprecht et al., 2011). The composition of the primary cell wall allows growth and expansion of the cells, whereas production of secondary cell wall thickening

is an indication of cell growth termination that happens in specialised cells (Kumar and Turner, 2015).

The production of carbohydrate-based cell wall components happens in the Golgi apparatus, these are then transported to the cell surface where they are incorporated into the growing cell wall matrix (Geisler et al., 2008; Ruprecht et al., 2011). The secretion of these components from the Golgi also depends upon the transportation of nucleotides and sugars to the Golgi. The defects in polysaccharide synthesis in mutants with impaired Golgi-associated transporters further underscores the importance of nucleotide-sugar transport into the Golgi membranes in cell wall formation (Carpita, 2012; Zhang et al., 2011).

Cellulose in plant cells is synthesised via a multi-meric cellulose synthase complexes (CSC) in the plasma membrane (Kim et al., 2013; Somerville, 2006). The cellulose synthase complexes were visualised in vascular plants as hexagonal rosettes (Lei et al., 2014). Despite a lack of full knowledge regarding the composition of CSCs, a popular model predicts that each of the six rosette subunits contains six individual CESA proteins meaning that each CSC could synthesise an elementary cellulose microfibril containing 36 glucan chains (Lei et al., 2014).

Ten *CESA* genes have so far been identified in *Arabidopsis* (Holland et al., 2000). Studies of these genes showed *CESA1*, 3 and 6 proteins are involved in primary wall synthesis and *CESA4*, 7 and 8 are active during secondary wall formation (Desprez et al., 2007; Ruprecht et al., 2011). Immunological studies have shown that the CESAs are the components of the terminal rosette formed at the end of cellulose strands (Kimura et al., 1999; Persson et al., 2007). The mutation in *CESA1* and 3 caused pollen defects and hence sterility, while null *CESA6* mutants showed only subtle growth phenotypes that implies redundancy between these genes (Persson et al., 2007). Persson et al. (2007) further showed that cellulose synthase complex

requires three *CESA* components; however, they added *CESA2* and *CESA9* to these components that are acting redundantly with *CESA6*.

The co-expression analysis of the *CESA* genes related to primary and secondary wall synthesis showed higher expression of secondary wall *CESAs* in stems and roots, and more uniform expression of primary wall *CESAs* across other tissues (Ruprecht et al., 2011).

Genome wide association studies (GWAS) in barley showed association between nine regions in barley genome and cellulose content in three subpopulation of two-rowed barleys (Houston et al., 2015). The identified co-expressed genes in primary cell wall synthesis in barley were *HvCobra1*, *HvCsIf6* that are orthologous to similar functioning genes in different species such as maize and *Arabidopsis*. For instance, the maize *BK2L3* is thought to be the likely orthologue of *HvCobra1* and is shown that it co-expressed in with primary cell wall *CesA* genes (Held et al., 2008; Houston et al., 2015). Moreover, *HvCsIf6* has been shown to be down regulated by a small interfering RNA antisense transcript that is an indication to the connection between these co-expressed genes (Houston et al., 2015; Held et al., 2008).

In plant cell walls, cellulose microfibrils, composed of β -1, 4-linked glucan chains, are the main load-bearing component supporting cell wall tension and also enforcing asymmetric cell expansion (Lei Lei et al., 2014). Cellulose microfibrils in wood cells are composed of arrays of individual polysaccharides that cohere laterally to each other as sheets via hydrogen bonds. These sheets then cohere to each other via Van de Waals' forces (Strabala and Macmillan, 2013). Laid down outside of the plasma membrane of the plant cells, the cellulose microfibrils are arranged in transverse orientation to the elongation axis (Lei Lei et al., 2014). This pattern of microfibrils orientation enables longitudinal cell expansion while restricts radial cell expansion. Furthermore, during cell growth the cortical

microtubules undergo constant re-organisation that allows cell expansion (Lei et al., 2011).

In dicotyledons a matrix of complex polysaccharides, mainly consisting of xyloglucans and pectic polysaccharides, surrounds the cellulosic network that is the main body of primary cell wall (Farrokhi et al., 2006). The walls of Poales and other related commelinoid monocots including wheat and barley are organised in the same manner, even though there are higher levels of glucuronoarabinoxylan and (1,3; 1,4)- β -D-glucans (Farrokhi et al., 2006; Smith and Harris, 1999). Further deposition of cellulose and other matrix phase polysaccharides such as heteroxylans and heteromannans and in some cells lignin during the secondary wall formation, provides a rigid complex capable of withstanding compressive forces (Farrokhi et al., 2006). The primary wall complexes are thought to be guided via microtubules at the cell cortex (Ruprecht et al., 2011).

1.7 Secondary wall thickening

Deposition of the secondary cell wall during the evolution of vascular plants has enabled them to dominate the planet. Secondary wall thickening occurs inside the primary walls after the cessation of cellular elongation. The thick-walled cells enable plants to transport water from roots to the shoot, and also provide mechanical support for the plant (Zhong et al., 2007). The primary cell wall in flowering plants is categorised into two types. Found in dicots, non-commelinoid monocots and gymnosperms, type I cell walls comprise of cellulose fibres enclosed by a network of xyloglucan, pectin and structural proteins (Vogel, 2008). Type II cell walls on the other hand are specific to commelinoid monocots such as grasses and are comprised of cellulose fibres surrounded by glucuronoarabinoxylans, high levels of

hydroxycinnamates and very low levels of pectins and structural proteins (Vogel, 2008). Secondary cell wall is mostly consists of cellulose, lignin and hemicellulose and are deposited between the plasma membrane and primary wall following termination of cell enlargement (Goue et al., 2013; Mohnen, 2008; Popper and Fry, 2004; Turner et al., 2007). Secondary wall containing cells, treachery elements and/or fibres in tree species are the main constituents of wood that is used for pulping, papermaking and other commodities, and also due to its high contribution to the plant biomass it is of high value for bioenergy industries (Goue et al., 2013; Zhong et al., 2007). Furthermore, in *Arabidopsis thaliana* formation of secondary wall thickening in anthers provides forces required for anther opening and dehiscence (Mitsuda et al., 2007; Wilson et al., 2011; Yang et al., 2007).

The diversity of involvement of secondary wall formation, from development of lateral roots, water transport from root to shoot and reproductive organs shows the importance of secondary cell wall formation in plant life cycle. This diversity requires a coordinated biosynthesis of these complex polymers. Therefore study of the gene network involved in the biosynthesis of the major secondary cell wall components, such as cellulose, hemicellulose and lignin is of important value (Goue et al., 2013; Zhong et al., 2007). It has been reported that several transcription factors in the MYB and NAC (*MYB26*, *NST1*, *NST2*, *SND1/ NST3*, *VND6* and *VND7*) transcription factor families contributing to various steps in cell wall thickening in plants (Olsen et al., 2005; Stracke et al., 2001; Zhong et al., 2007). Therefore, better understanding of this trait in relation to flowering and reproductive organs in agronomically important crops is beneficial in tackling plant's productivity.

These differences in the composition of cell walls and also the morphological differences between monocots and dicots indicates potential variation in cell wall gene regulatory network. Detailed analysis of

transcriptional regulation of cell wall biosynthesis in *Arabidopsis* has showed the involvement of various activator and repressors in regulation of cell wall formation. Transcription factors *WRKY12* and *MYB32* have been indicated as repressor of secondary wall biosynthesis in *Arabidopsis* (Handakumbura and Hazen, 2012; Preston et al., 2004; Wang et al., 2010). Furthermore, secondary wall NACs (SWNs) are major positive regulators of secondary wall biosynthesis in plants. The main contributors of SWNs are *NAC Secondary Wall Promoting Factor 1 (NST1)*, *NST2*, *Secondary Wall-Associated NAC Domain Protein 1 (SND1/ NST3)* and *Vascular Related NAC Domain 6 (VND6)* and *VND7* that will be discussed further in chapter 3.

1.8 NAC domain transcription factors

The NAC domain gene family is one of the largest groups of plant transcription factors with around 110 genes in *Arabidopsis* and 151 identified members in rice (Negi et al., 2016; Nuruzzaman et al., 2010; Olsen et al., 2005; Pereira-Santana et al., 2015). The domain name is short for the first three discovered mutants (*No Apical Meristem (NAM)*, *Arabidopsis Transcription Activator Factor 1/ 2 (ATAF1/ 2)* and *Cup-shaped Cotyledon (CUC2)*) that contained a particular domain (NAC domain) (Aida et al., 1997; Pereira-Santana et al., 2015; Souer et al., 1996a). The first cDNA encoding for a NAC protein was reported as the *Responsive to Dehydration 26 (RD26)* in *Arabidopsis* (Nakashima et al., 2012; Yamaguchi-Shinozaki et al., 1992). Located at the N-terminal of the protein, NAC domain consists of 150-160 amino acids that are further divided into five sub-domains (A-E) (Olsen et al., 2005).

It has been suggested that the subdomain A is involved in formation of homo and heterodimers, the B and E subdomains are responsible for functional diversity of NAC genes, and the highly conserved C and D domains are responsible for DNA binding (Chen et al., 2011; Ooka et al., 2003; Zhao et al., 2016). However, subdomains are not always the vital parts of NAC genes that means they could be functional without the subdomains. There are examples of genes without these subdomains and some with two tandemly repeated NAC domains (Christiansen et al., 2011; Jensen et al., 2010). The presence of a different conserved region before the NAC domain at the N-terminal is another example of structural variations within the NAC domain gene family (Jensen et al., 2010; Mitsuda et al., 2004; Zhao et al., 2016).

The C-terminal of the NAC domain contains a transcriptional activation region (TAR) that unlike the NAC domain is not conserved between species and acts as either activator or repressor (Ooka et al., 2003; Pereira-Santana et al., 2015). It has been shown that the presence of an α -helical transmembrane motif (TMM) at the C-terminal region of NAC transcription factors plays a major role in regulation of these transcription factors. These transmembrane motifs cause the inactivation of the NAC transcription factors via anchoring the NAC protein to the intracellular membranes that is an efficient regulatory method and provides a prompt response to environmental changes (Christiansen et al., 2011; Pereira-Santana et al., 2015). The NAC domain is known to be involved in various aspects of plant development such as formation of lateral root, meristem formation and floral organ identity (Olsen et al., 2005). The regulatory network of NAC domain transcription factors will be discussed in detail in chapter 3 (section 3.1).

1.9 Aims of the study

The aims of this study are to extend the knowledge of anther and pollen development to commercially important crops especially in barley. This was done through identification and characterisation of two main NAC domain transcription factors *NST1* and *NST2* involved in formation of secondary wall thickening in the anther. It has previously been shown that the formation of secondary wall thickening in anthers contributes to generating forces required for anther dehiscence and pollen release. In *Arabidopsis thaliana*, the deposition of lignin in the endothecium layer of the anther has also been shown to be mainly regulated by three major transcription factors *MYB26*, *NAC Secondary Wall Thickening Promoting Factor 1 (NST1)* and *NST2*.

The following chapters (as described below) will further expand on the work that was done towards identification and characterisation of orthologous *NST1*, *NST2* and *MS5* genes in barley. Furthermore, the degradation of *Arabidopsis* MS5 protein through N-end rule pathway was investigated that is discussed in chapter six. In summary:

- 1- The putative orthologous sequences of *AtNST1* and *AtNST2* in barley were identified through bioinformatics analysis using the available database (Chapter Three).
- 2- The identified putative orthologues were then characterised through reverse genetics approach. Therefore, the identified putative orthologues were repressed and overexpressed in barley and the effects of these alterations were studied (Chapter Four).

- 3- Complementation of the *Arabidopsis nst1* and *nst2* mutants using barley putative orthologues was explored in order to confirm the function of identified putative orthologous genes in barley (Chapter Four).
- 4- Identification and functional characterisation of orthologous *Male Sterile 5 (MS5)* (Chapter Five).
- 5- The involvement of N-end rule pathway in degradation of the *Arabidopsis MS5* protein was investigated. This study will determine the potential role of the N-end rule pathway in anther and pollen development, and also whether plants sense ambient oxygen to stabilise proteins required for developing reproductive organs. This will include generating both stable (MA) and unstable (MC) types of the protein and studying the stability of the protein both *in vitro* and *in vivo* (Chapter Six).

Chapter Two:

Material and Methods

2.1 Plant material and growth conditions

Barley cultivars (Golden Promise and Optic) were kindly provided by Dr. Jose Fernandez Gomez from Plant and Crop Sciences Division of the University of Nottingham. *Arabidopsis* mutant and wild-type lines (Columbia and Landsberg) were provided by Nottingham *Arabidopsis* Stock Centre (NASC) and Prof. Zoe A. Wilson laboratory.

Barley seeds (Golden Promise) were sown in twelve-cell trays (25 × 23.5 × 6.6 cm) containing John Innes N°3 compost. Seedlings were grown under 16 hours day and 8 hours night conditions with 15°C day and 12°C night temperature regimes. The growth room was set at light intensity of 500 $\mu\text{mol/ m}^2/\text{s}$ provided by metal halide lamps (HQI) with relative humidity of 80% (v/v). The seedlings at the two to three leaves stage were transferred into 5-litre pots containing Levington C2 compost (200 mg/l nitrogen, 150 mg/l phosphorus and 120 mg/l potassium, pH= 5.3-5.7), with 4 seedlings in each pot.

Arabidopsis seeds were sown in 9cm pots containing Levington M3 compost (280 mg/l nitrogen, 160 mg/l Phosphorous, 350 mg/l potassium, pH= 5.3-5.7). The pots were kept in glasshouse at 22°C under 22 hours photoperiod, 111 $\mu\text{mol/ m}^2/\text{s}$, and 75% (v/v) humidity conditions.

2.2 Bioinformatics analysis

Putative orthologous genes were identified using the information available on various databases. The databases used for identification of target genes were Gramene, The *Arabidopsis* Information Resource (TAIR), EnsemblPlants, National Centre for Biotechnology Information (NCBI), Rice

Expression Profile Database (RiceXPro), Rice genome Browser, Barley RNA-seq Database and The International Barley Sequencing Consortium (IBSC) sequences available at IPK BLAST server (Table 2.1).

Table 2.1 Genomic database used for identification of putative sequences

<u>database</u>	<u>URL Address</u>
The Arabidopsis Information Resource (TAIR)	http://www.arabidopsis.org/
EnsemblPlants	http://plants.ensembl.org/index.html
The National Centre for Biotechnology Information	https://www.ncbi.nlm.nih.gov/
Gramene	http://www.gramene.org/
The Rice Expression Profile Database (RiceXPro)	http://ricexpro.dna.affrc.go.jp/
Rice Genome Browser	http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/
IPK BLAST server	http://webblast.ipk-gatersleben.de/barley/
Barley RNA-seq Database	https://ics.hutton.ac.uk/morexGenes/

For this purpose, the orthologous genes in close relatives of barley, such as rice and *Brachypodium*, were identified in order to obtain the putative orthologue in barley. The *Arabidopsis* target coding sequence was first analysed using the BLAST algorithm against the rice database in NCBI. The rice sequence with the highest identity to the *Arabidopsis* gene was considered as potential putative orthologues of the target gene in rice. The identified rice putative sequence's expression, found in RiceXpro database, was compared to its counterpart in *Arabidopsis*, from the TAIR database. The rice sequence with closest nucleotide identity and also expression

pattern to the *Arabidopsis* gene was chosen as the rice putative orthologue. The identified rice putative orthologue was then used to identify the putative sequence of the target gene in *Brachypodium* using the Gramene orthologue service.

The *Brachypodium* sequence with highest nucleotide identity to the rice putative sequence of the target genes was selected as the *Brachypodium* putative orthologue. As for *Brachypodium* sequence, the lack of available expression data made it difficult to compare the expression pattern of the identified putative sequences with their rice and *Arabidopsis* counterparts. The identified *Brachypodium* sequence was aligned against its rice and *Arabidopsis* orthologues in order to assess the level of identity between the sequences. The *Brachypodium* sequence with highest identity to its rice and *Arabidopsis* sequences was chosen as putative sequence.

The identified orthologous sequence in *Brachypodium* was finally used to identify the barley putative orthologue using International Barley Sequencing Consortium database (IPK BLAST server; Table 2.1). The identified barley putative sequences were then aligned with their rice and *Brachypodium* sequences to further confirm the barley putative orthologue sequence with high identity to its rice and *Brachypodium* sequences at the nucleotide level.

2.3 Primer design

Primer 3 programme version 0.4.0 ([http:// Frodo.wi.mit.edu](http://Frodo.wi.mit.edu)) was used to design primers. The primers were designed using the identified putative orthologous sequence. These oligos were designed considering melting temperature (T_m) range of 55-65°C with the length of 21 bp (the length varied depending on the purpose of the primer).

2.4 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify the DNA sequences of interest. The conditions for PCR reactions were based upon the polymerase that was used (Table 2.2)

In order to obtain high fidelity in the amplification Phusion polymerase with the proofreading ability was used (Table 2.3).

Table 2.2 Thermocycle conditions for Phusion polymerase and Red Taq polymerase

Phusion Polymerase enzyme	Temperature	Time
Initial denaturation	98°C	30 seconds
Denaturation	98°C	30 seconds
Annealing	X*	30 seconds
Extension	72°C	30 seconds**
Final extension	72°C	6 minutes
Hold	8°C	
Red Taq Polymerase enzyme	Temperature	Time
Initial denaturation	94°C	6 minutes
Denaturation	94°C	30 seconds
Annealing	X*	30 seconds
Extension	72°C	30 seconds**
Final extension	72°C	6 minutes
Hold	10°C	

* Annealing temperature depended on primers melting temperature (T_m) and was normally 3-5°C below the primers melting temperature.

** Extension temperature varied based on the length of the amplified sequence and was based upon 30 seconds per 1 Kbp.

*** The number of cycles was normally 35 cycles, but varied depending on the purpose of the PCR.

Table 2.3 The component of a typical PCR reaction set up using Phusion polymerase (Thermo Scientific).

<u>Component</u>	<u>Volume</u>
5X Phusion HF buffer (Thermo Scientific)	2 μ l
DMSO (Thermo Scientific)	0.3 μ l
dNTPs (2.5 mM) (Fisher)	0.2 μ l
Forward and Reverse primers (10 mM)	0.23 μ l
Template	X*
Phusion Polymerase (0.04 U/ μ l)	0.15 μ l
Molecular grade water (Sigma)	Adjusted to 10 μ l

(*): the amount of template for plasmid DNA was 1 pg-10 ng and for highly complex genomic regions 50- 250 ng.

The Red Taq polymerase enzyme, a mixture of Taq DNA polymerase mixed with inert red dye and buffer components, was used for amplifications that did not require high stringency in the amplification. Table 2.4 shows the components of a typical PCR reaction using Red Taq polymerase.

Table 2.4 A typical PCR reaction using Red Taq (Sigma Aldrich) Polymerase.

Red Taq polymerase	5 μ l
Forward and Reverse primers (10 mM)	0.3 μ l
Template	Up to 200 ng
Molecular grade water	Adjusted to 10 μ l

2.4.1 Agarose gel preparation

The separation of the DNA samples by agarose gel electrophoresis was carried out using 0.8% (w/v) agarose gels prepared in 0.5x Tris-borate EDTA (TBE) buffer (89 mM Tris (pH 7.6), 89 mM boric acid, 2 mM EDTA). The amplified PCR products were run at 5-8 V/cm of the gel tank size containing 0.5x TBE buffer.

2.5 Genomic DNA extraction

Genomic DNA was extracted from various tissues using Bioline Isolate Plant DNA mini kit (Bioline Ltd.) according to the manufacturer's protocol.

2.6 RNA extraction

Total RNA from plant tissues was extracted using RNeasy Plant mini kit (Qiagen) according the manufacturer's protocol. The optional DNase treatment was carried out to remove the residual genomic DNA in the samples. This was done by adding the DNase I solution (150 Kunitz) to the columns and incubating them at 37 °C for an hour before the final wash step. In the final step the RNA was re-suspended in 30 µl of molecular grade water.

2.7 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesised from RNA was performed according to the following protocol. The synthesis was carried out by mixing

1-5 µg of RNA, 1 µl of oligo dT (10 µM) primer and 10mM dNTPs and adjusting this mixture to 13 µl with molecular grade water. The tubes were then incubated at 65°C for 5 minutes to allow the RNA secondary structure to denature and immediately chilled in ice for at least one minute to allow the primer to bind to the RNA. After a brief centrifuge the reverse transcription mix (5x First Strand buffer (Invitrogen), 0.1M DTT (Invitrogen), RNaseOUT recombinant RNase inhibitor (Fisher Scientific), superscript III Reverse Transcriptase (Invitrogen)) were added to the tube. The tube was incubated at 50°C for 60 minutes to allow the reverse transcriptase to synthesise the complementary strand. The reaction was then inactivated at 70°C for 15 minutes.

2.8 Transformation of *E.coli* competent cells

The transformation was performed by adding 1-5 µl (10 pg- 100 ng) of the DNA product into 20- 50 µl of the pre-thawed chemically competent cells, DH5α (Hanahan, 1983), without pipetting and agitating the cells. The tube was then kept on ice for 30 minutes followed by 90 seconds incubation at 42°C and 1 minute incubation on ice. To this reaction, 250 µl of S.O.C medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) was added and the tube was kept shaking at 37°C for one and a half hours. Aliquots of the reaction (50 and 150 µl) were plated on LB agar medium containing appropriate antibiotic and the plates were incubated at 37°C overnight.

2.9 DNA purification from agarose gel

The purification of DNA from agarose gels was done using GenElute™ Gel Extraction Kit (Sigma) in accordance with the manufacturer's instructions.

2.10 PCR purification

The purification of PCR product from impurities was done using QIAquick PCR Purification Kit from Sigma according to the manufacturer's instructions.

2.11 Cloning

The putative target genes were expressed in barley and *Arabidopsis* using various constructs. These constructs were prepared via different cloning techniques from conventional digestion and ligation of the fragment of interest inside a vector to insertion of the fragment other methods such as gateway or Gibson assembly.

2.11.1 Restriction Enzyme Digestion

The plasmid or amplified DNA was digested using restriction enzymes (New England Biolabs, NEB) according to the manufacturer's conditions. The ability of the restriction enzyme to cut in more than one site on the plasmid or the DNA was also assessed using the NEB online tool

(<http://tools.neb.com/NEBcutter2/>). In case of double digestion the optimum buffer that is efficient with both enzymes was used.

The digestion fragment was set up by adding 100 ng of DNA or plasmid, 1.5 µl of buffer, 1 µl of BSA (1:100 dilution), 0.2 µl restriction enzyme and the rest of the reaction until 15 µl was reached by adjustment with molecular biology water. The digestion reaction was incubated at 37°C overnight. The reaction was then deactivated at 65°C for 20 minutes.

2.11.2 Ligation

A typical ligation reaction was set up containing digested plasmid (50 ng), digested DNA fragment (100 ng), 1.7µl of T4 DNA ligase and 2 µl of 10x T4 ligase buffer were added into the 75 µl ligation reaction. The amount of insert per each ligation reaction was calculated according the equation below:

$$ng\ of\ insert = \frac{\text{desirable DNA concentration (e.g.100ng)} \times \text{insert size}}{\text{plasmid size}} \times \frac{3}{1} \text{ (the ratio of insert to plasmid)}$$

The reaction tube was kept at 16°C for 18 hours. From this reaction 5 µl was used to transform *E.coli* competent cells (section 2.8).

2.11.3 Gateway Cloning

The gateway cloning technology relies upon site specific recombination between the properties of bacteriophage lambda (Landy, 1989). In this system the heterologous DNA fragment flanked by the lambda

recombination sites with modified *att* sites is transferred between the Gateway compatible vectors (Hartley et al., 2000). This cloning method was used in order to transfer the DNA sequences to the entry and destination vectors of interest without digestion and ligation steps as described above.

2.11.3.1 TOPO cloning

The TOPO cloning vectors are based on two major parameters that ease the cloning of the gene of interest inside the vector. Firstly, TOPO vectors are created based on non-template-dependent terminal activity of Taq-polymerase that adds a single deoxyadenosine (A) to the 3' end of the PCR products. Therefore, the vector has a 3' deoxythymidine (T) tail residue that facilitates efficient ligation of the PCR product inside the vector. This type of cloning is also known as TA cloning method. Furthermore, the TOPO vectors are also based on Topoisomerase I activity from *vaccinia* virus. Topoisomerase I binds to the duplex DNA at specific region (CCCTT) that cleaves the phosphodiester backbone in one strand. This cleavage releases energy that is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and Tyrosyl (Tyr-274) residue of Topoisomerase I. The phospho-Tyrosyl bond can further be attacked by 5' hydroxyl of DNA strand that leads to reversion of the reaction and release of Topoisomerase I. This feature in TOPO cloning ensures efficient cloning of the PCR product inside the vector.

The PCR products that are amplified by Taq polymerases with proofreading ability, such as Phusion polymerase, are blunt ended; hence they need to have 3' A tailing to be able to be compatible with this cloning method.

The TA cloning reaction was set up by using the following components, 1 µl of entry vector (pCR/8GW/TOPO), 4 µl of A tailed DNA fragment and 1 µl of salt solution. The reaction was then incubated at 22 °C overnight. From this reaction 5 µl was used to transform *E.coli* competent cells (section 2.8), and the reaction is then streaked on LB medium containing spectinomycin (100 µg/ml).

2.11.3.2 LR reaction

The LR reaction facilitates the recombination between two recombination sites existing in both entry and destination vectors on the both sides of the insertion site. This enables the transfer of the DNA fragment of interest from the entry vector into the destination vector (Figure 2.1)

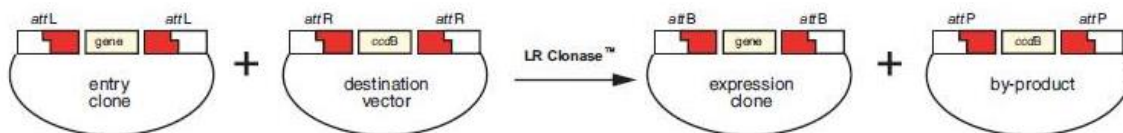


Figure 2.1 The process of LR reaction, which is the recombination between the recombination sites of entry vector and expression vector

The LR reaction was set up by mixing 50 ng entry vector containing the fragment of interest, 100ng of destination vector and 1.5 µl of LR clonase II enzyme (the enzyme that facilitates the recombination between two recombination sites of the vector) (ThermoFisher Scientific) to a total volume of 6.5 µl that was adjusted with molecular grade water. The reaction was then incubated at 25 °C for 18 hours.

The reaction was then terminated by adding 1.5 µl of proteinase K (20 mg/ml) followed by 10 minutes incubation at 37°C. This reaction was used to transform *E.coli* competent cells.

2.12.3.1 Adenine tailing

In order to add an adenine (A) tail to the PCR product the following components were added into an Eppendorf tube: 100 ng of the PCR product, 1.5 µl of 10x standard Taq reaction buffer (NEB), 0.5 µl of dATP (10 mM) (Fisher) and 0.3 µl of qTaq polymerase (NEB), and made up to 15 µl with molecular grade water. The reaction was kept at 72°C for 15 minutes.

2.12 *Agrobacterium tumefaciens* transformation

The strain of *Agrobacterium* that is used for transformation varies depending on the plant species (Gelvin, 2003). In this study *Agrobacterium* strain AGL1 and Gv3101 were used for barley and *Arabidopsis* transformation respectively. In order to transfer the destination vector into the desired *Agrobacterium* strain, 100 ng of the destination vector containing the desired DNA product and 100 ng of the helper plasmid (the helper plasmid was only used for binary vectors, otherwise there was no need for co-electroporation of helper plasmid) were added into 50 µl of electro competent *Agrobacterium* strain. The reaction was transferred into pre-chilled cuvettes (50×0.2 mm). The cells were electroporated using the Biorad GenePulser set for 2.5 KV, 25 µFD and 400 Ohms. Immediately after application of current, 250 µl of LB medium was added into the cuvette and

the reaction was transferred into Eppendorf tube. The tube was then incubated with shaking at 28 °C (150 rpm) for 3 hours. Two aliquots of the reaction (50 and 150 µl) were plated on LB agar medium containing the appropriate antibiotics and the plates were incubated at 28 °C for three days.

2.13 Plant transformation

2.13.1 *Arabidopsis* transformation

Transformation of *Arabidopsis* was done using the floral dip method (Clough and Bent, 1998). The transformation was done by preparing an overnight *Agrobacterium* culture containing the DNA fragment of interest in the destination vector and selection antibiotics. For this purpose, appropriate selective antibiotics were added to 100 ml of LB medium and the mix was poured into a 200 ml Erlenmeyer flask to allow better aeration and growth of *Agrobacterium*. Into this a positive single colony from *Agrobacterium* electroporation (Section 2.13) was propagated and the flask was incubated at 28 °C overnight.

The overnight culture was transferred into two 50 ml sterile falcon tubes and the tubes were spun down at 5000 xg for 10 minutes to remove the excess of antibiotics in the culture. The pellet was dissolved in 5% (w/v) sucrose and 0.05% (v/v) Silwet solution by pipetting up and down. At this stage the culture was ready to be used for floral dip. The newly opened flower buds of *Arabidopsis* plant were selected and dipped into the prepared culture for 30 seconds. The inoculated plants were kept under the bench in low light inside closed plastic covers overnight. The covers were opened on the following day and the end of the plants life cycle, the seeds were

collected. These seeds were allowed a month to pass their dormancy period and were selected on half strength MS medium plus appropriate antibiotic selection.

2.13.2 Barley transformation

Transformation of barley was carried out using the Harwood *et al.* (2009) protocol (Harwood et al., 2009). The protocol was developed based on *Agrobacterium*-mediated transformation of barley that is believed to have more advantages over the other methods such as biolistics.

The transformation was done using immature barley embryos isolated from developing seeds. Immature embryos ranging between 1.5-2 mm in size were isolated under sterile conditions and their axis were removed before placing the embryos scutellum side up on Murashige and Skoog (MS) medium (Appendix 9.1) without antibiotics. These embryos were pre-cultured at 22 °C for 24 hours before inoculating with an overnight culture of *Agrobacterium* harbouring appropriate destination vector. Inoculation of embryos were carried out adding a drop of the inoculum on each embryo using a pipette. The inoculated embryos were kept in contact with the inoculum for one minute. Thereafter, the plate containing the embryos was tilted for about two minutes to remove the excess of the *Agrobacterium*.

The inoculated embryos were then transferred onto a clean plate containing MS medium without antibiotics, but this time scutellum side down (from this step forward the embryos were kept scutellum side down). The plates were incubated at 22 °C in the dark for 72 hours. This step allows the *Agrobacterium* to develop and infect the embryos. After 72 hours of co-culture, the embryos were transferred onto selection medium containing antibiotics for 14 days (22 °C dark). The selection step contains three

intervals of two weeks each in which the growing calli were transferred onto freshly prepared plates after two weeks. The antibiotics used during selection until regeneration of transformed plantlets were Hygromycin (50 mg/l) for selecting transformed calli and Timentin (160 mg/l) for removal of bacterial infection. After three rounds of selection the calli were transferred onto transition medium (Appendix 9.3) containing Hygromycin (50 mg/l) and Timentin (50 mg/l) for two weeks. The transition plates were kept under low light condition at 22 °C provided by placing a thin sheet paper on the plates while the plates were kept under light conditions in tissue culture room. During this step the transformed calli generated green spots that in the subsequent stages developed into green shoots. The calli that generated green spots were transferred onto regeneration medium (Appendix 9.4) with the same level of antibiotics for two weeks under light conditions and kept at 22 °C. The green spots on the calli subsequently developed into green shoots containing a developed root system.

The independent small plantlets were then transferred into test tubes containing MS medium without growth regulators (i.e Dicamba and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) but with antibiotics. The plantlets in this step were kept on MS medium until the plants to reach the top of the tubes and developed a good root system.

The plantlets that reached to the top of the test tube were transferred to compost (John Innes No.3), covered with closed plastic sleeves to maintain the humidity and allow the plantlet to acclimatise to the new environment (the sleeves were opened after a week). These plantlets were kept at the normal temperature that was used for growing barley (16 hours photoperiod, 15 °C day and 12 °C night).

2.14 Half strength Murashige and Skoog (MS) medium

This medium was prepared with a lower concentration of MS medium.

This medium was prepared by adding the components listed in Table 2.5 in 500 ml of sterile deionised distilled water. The pH of the medium was adjusted to 5.8 and the medium was autoclaved before preparing the plates.

Table 2.5 Half strength MS medium components.

<u>Component</u>	<u>Amount</u>
Murashige and Skoog medium	1.23 g
Sucrose	2.5 g
Agar	3.5 g

2.14 Expression analysis

The quantification of the expression of the target genes was done using quantitative reverse transcription Polymerase Chain Reaction (qRT-PCR). For this purpose a set of primers that amplified a relatively small region (~200 bp) of the coding sequence of interest was designed (Table 2.6) using Primer 3 programme version 0.4.0 ([http:// Frodo.wi.mit.edu](http://Frodo.wi.mit.edu)). The primers were designed considering an intron region between the primers in order to detect genomic DNA contamination. These primers were tested in an RT-PCR in order to be sure about the specificity of the amplified target.

Moreover, the efficiency of the primers to amplify the target sequence was tested by quantifying the target amplification using diluted template (1/5, 1/25 and 1/125 dilutions) along with the non-diluted template and with three replicates per each set of dilution. This analysis should produce four separate amplification events in which all three replicates of each template

are amplified at the same cycle and the amplification rate of each sample (diluted and non-diluted) is clearly spaced. This is to see whether the primers are able to detect that the amplicon amount is doubled at each cycle; thus the efficiency of 2 per pair of primers.

The primers were then used to quantify the expression of the target genes using a 480 LightCycler (Roche).

Table 2.6 Sequence of the primers used for quantification of the expression of putative *HvNST1* and *HvNST2* using barley anther staging samples.

<u>Primer name</u>	<u>Sequence (5'...3')</u>
HvNST1-qPCR-F1	GGTTCCGTTTCCATCCAACG
HvNST1-qPCR-R1	GTCCCCGTTGGGTACTTCTT
HvNST2-qPCR-F1	GGTTCCGGTTCCATCCGAC
HvNST2-qPCR-R1	GCTGCTCACCTACTACCTCG
HvTubuline-F	AGTGTCTGTCCACCCACTC
HvTubuline-R	AGCATGAAGTGGATCCTTGG

Expression analysis was carried out by preparing a master mix using 4.5 µl of SYBR Green (Maxima SYBR Green qPCR Master Mix, Fermentas), 0.4 µl cDNA (1.5 µg) and 3.6 µl of molecular grade water, 0.2 µl of forward primer (10 mM) and 0.2 µl (10 mM) of reverse primer per reaction. The samples were analysed in three technical replicates containing positive and negative controls. The expression data from this were analysed by one to one pairing of the expression of each sample compared to the one in the housekeeping gene. The conditions used for this analysis are shown in Table 2.7.

Table 2.7 Quantitative reverse transcription polymerase chain reaction conditions used for expression analysis of the target genes.

Initial denaturation	95 °C	10 minutes
Denaturation	95 °C	30 seconds
Annealing	X °C*	30 seconds
Extension	72 °C	1 minutes

The amplification was done in 45 cycles.

* The annealing temperature varies on each pair of primers.

2.15 *In vitro* assay

In order to perform *in vitro* assay for analysis of the involvement of N-end rule pathway in *AtMS5* degradation TNT® Coupled Reticulocyte Lysate System (Promega) was used. In order to perform the *in vitro* assay 10 µl of each (*AtMS5*-MC and *AtMS5*-MA) construct with final concentration of 50 ng/µl was prepared. A mixture of 12.5 µl rabbit reticulocyte extract, 1µl TNT buffer, 0.5 µl T7 polymerase (1000U), 0.25 µl amino acids- Leucine (1mM), 0.25 µl amino acids- Methionine (1mM) and 0.5 µl of RNaseOUT (total volume of 15 µl), was added into each tube and the tubes were incubated at 30°C. Before taking the first sample after 30 minutes of incubation, 1 µl of cyclohexamide (2.6 mM) was added into the reaction in order to stop further protein synthesis. The first sample was then taken (7 µl) and added into tubes containing a mixture of 7 µl loading buffer and 21 µl of molecular grade water. Thereafter, in time courses of 45 and 105 minutes, after the first sampling time, samples were taken. After taking the last samples, the tubes were incubated at 95°C for 10 minutes followed by 5 minutes incubation in ice.

2.16 Western blot analysis

A 12% (w/v) SDS-Polyacrylamide gel was prepared for separation of proteins (Table 2.8). The samples were run in the gel for the first 20 minutes at 80V and 120V for 90 minutes. The proteins were then transferred onto nitrocellulose membrane (BioRad Immune Blot pVDF membrane).

Table 2.8 Components of SDS-Polyacrylamide gel

Component	Running gel- 10ml (12%)	Stacking gel- 5ml
Distilled water	3.3ml	3.4ml
Acryl	4ml	0.83ml
Tris (pH: 8.8)	2.5ml	(pH:6.8) 0.63ml
SDS (10%)	0.1ml	0.05ml
APS	0.1ml	0.05ml
Tetramethylethylenediamine (Temed)	4µl	4µl

The semi dry apparatus (Bio Rad) was used to transfer proteins onto to the membrane at 80 V for two hours.

For the transfer step the gel was placed on a layer of sponge and blotting paper that were soaked in running buffer (700 ml deionised water, 200 ml methanol, 100 ml Tris-Glycine (TG) buffer). The nitrocellulose membrane, rinsed briefly with methanol and soaked in deionized water, was then placed on the gel while removing the excess of bubbles formed in the space between the gel and membrane. Finally a blotting paper and sponge were placed on the top of membrane before closing the sandwich. The sandwich was then placed into an electrophoresis tank for 2 hours at 80 V.

The membrane was then blocked in 10 ml of blocking buffer (1x PBS containing 0.1% (v/v) Tween was used to prepare 5% (w/v) non-fat milk) containing 1:2000 dilution of the primary antibody (mouse Tubuline- α HA and α -HA). The membrane was incubated in the buffer shaking at 45 rpm for 2 hours. This was followed by three wash steps of the membrane with blocking buffer. The membrane was then incubated for an hour at 45 rpm in 10 ml of the blocking buffer containing 1:10,000 dilution of the secondary antibody (Goat- α -mouse antibody). The membrane was finally visualised via exposure to chemiluminescence film (GE Healthcare) for 15 seconds to 5 minutes or until a clear signal was detected.

2.17 Selection of *Arabidopsis* seeds

Arabidopsis seeds from transformation were selected on half strength MS medium with appropriate antibiotic. Seeds were transferred to Eppendorf tubes and 1 ml of 70% (v/v) ethanol added to the tube. The tube was vortexed for three minutes followed by three minutes centrifuge at full speed. The supernatant was removed and this repeated one more time. After removing the supernatant 1 ml of absolute ethanol was added to the tube and mixed well with the seeds by shaking the tube. The seeds were then poured on sterile filter papers in the flow hood until they dry. Finally, the seeds were evenly spread on half MS medium containing antibiotic. The plates were kept under full light until the seeds germinate and grow until two true leaves stage. At this stage the transformed seedlings appear strong and healthy with dark green leaves, while the non-transformant plants had pale green towards yellow leaves and gradually die. The transformant seedlings were then transferred to compost and the pot was placed inside transparent plastic sleeve. The sleeve was kept closed for a

week until the seedling established itself in compost and adopted to the new environment.

The seeds collected from transformation with vectors harbouring resistance gene to glufosinate herbicide were sown directly onto compost for selection. The seeds were allowed to grow until the two leaf stage. At the stage the plantlets were sprayed with glufosinate (stock concentration: 150 g/L (w/v), the stock was diluted 1:1000 (v/v) in distilled water to prepare the working concentration). Plants that survived the selection were sprayed for the second time a week after to make sure that they were truly resistant to the herbicide; thus carrying the transgene.

Chapter Three:

Identification of putative orthologues of the *NAC* *SECONDARY WALL THICKENING PROMOTING FACTOR 1* (*NST1*) and *NST2*

3.1 Introduction

Recent advances in plant sciences and technology have enabled researchers to identify the mechanisms and regulatory genes involved in variety of developmental processes. This includes identification of gene regulatory network controlling key phases in plant development. Despite their vital importance in research, model species are not necessarily commercial crops; hence there is a need to transfer the knowledge to agronomically important species such as cereals.

Cereals are major staple crops around the world, which also contribute to the brewing industry and animal feed and are the main source of energy in developing countries. It has also been estimated (FAO, 2003) that the world population in the coming decades will surpass current cereal production, this poses a significant problem for food security in the near future. Therefore, the developed knowledge obtained from *Arabidopsis* and other model plants need to be transferred to more economically important species in order to be able to address food security concerns.

The availability of genomic databases that contain the annotated sequences of multiple plant species has made the task of identification of the genes in cereal less challenging than before. Nevertheless, it is important to be mindful when choosing putative orthologous sequences as inadequate attention can result in identification of the wrong sequence. In particular genes with high similarity, for example genes with conserved domains, e.g. the NAC domain, and those with high numbers of family members and redundancy, are easy to be confused when identifying orthologues.

The divergence of major cereal crops (wheat, maize, rice and barley and sorghum) from their common ancestor happened 50-70 million years ago (Gaut, 2002) (Figure 3.1). It was shown that there is a genetic synteny

between *Arabidopsis* and rice at the level of individual genes and occasionally across short uninterrupted clusters of genes up to 26 Kb in total length (Liu et al., 2001). Furthermore, it was noted that rice genome contains substantially larger introns compared to *Arabidopsis* that could contribute to the hypothesis that rice genes are confined to clusters of genes accounting 12-24% of the genome that are separated by large gene-poor regions (Liu et al., 2001). Analyses of rice, barley, wheat, sorghum and maize genome and ESTs showed that nearly 70% of the genes retained conserved structural motifs, 20% remained orthologous and 40% remained conserved as single copies (Bolot et al., 2009).

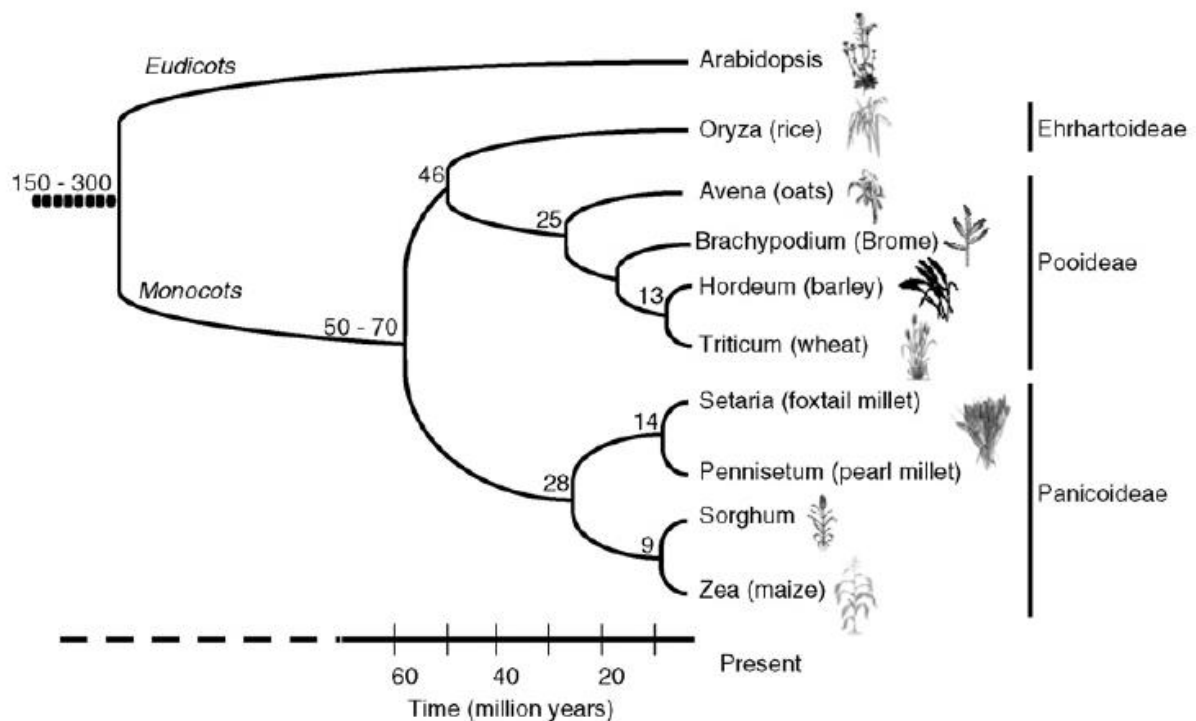


Figure 3. 1 Phylogenetic tree representing the divergence of cereals from their common ancestor (Bolot et al., 2009).

Analysis of over 1000 genetically mapped barley loci and their comparison with rice genome showed collinear organisation between chromosomes 3H

of barley and rice Os01 and 6H and Os02 over all their length (Stein et al., 2007). However, only 46% of the mapped barley genes had homologous sequences at a syntenic position in the rice genome (Stein et al., 2007). Although there is existing synteny between rice and barley, *Brachypodium* provides better insight in identifying the putative orthologous genes in barley in a transition from *Arabidopsis* to barley. The small genome size (300Mb) of *Brachypodium distachyon*, and its closer genetics links to wheat and barley makes this model plant an invaluable tool for cloning agronomically important in wheat and barley (Opanowicz et al., 2008). Furthermore, the strong synteny between *Brachypodium* and rice genomes is useful resource in translation of genetic information from rice to temperate cereals such as barley (Opanowicz et al., 2008).

3.1 The NAC domain Transcription Factor Family

The NAC domain genes are responsible for various stages and aspects of plant development such as apical meristem development, organ formation and development, leaf senescence, fruit ripening, hormonal signalling, anther dehiscence and responses to biotic and abiotic stresses (Guo and Gan, 2006; Hao et al., 2011; Shan et al., 2012; Souer et al., 1996b; Wu et al., 2015; Yamaguchi et al., 2010b). Therefore, the potential large impact of the NAC transcription factors in various plant's developmental processes makes them useful targets for characterisation in economically important species such as barley.

Formation of secondary wall thickening in plants is regulated by large number of transcription factors that are categorised into three tiers (Figure 3.2). These include NAC transcription factors in tier three or as master regulators, and two MYB transcription factors in tier two and many other in

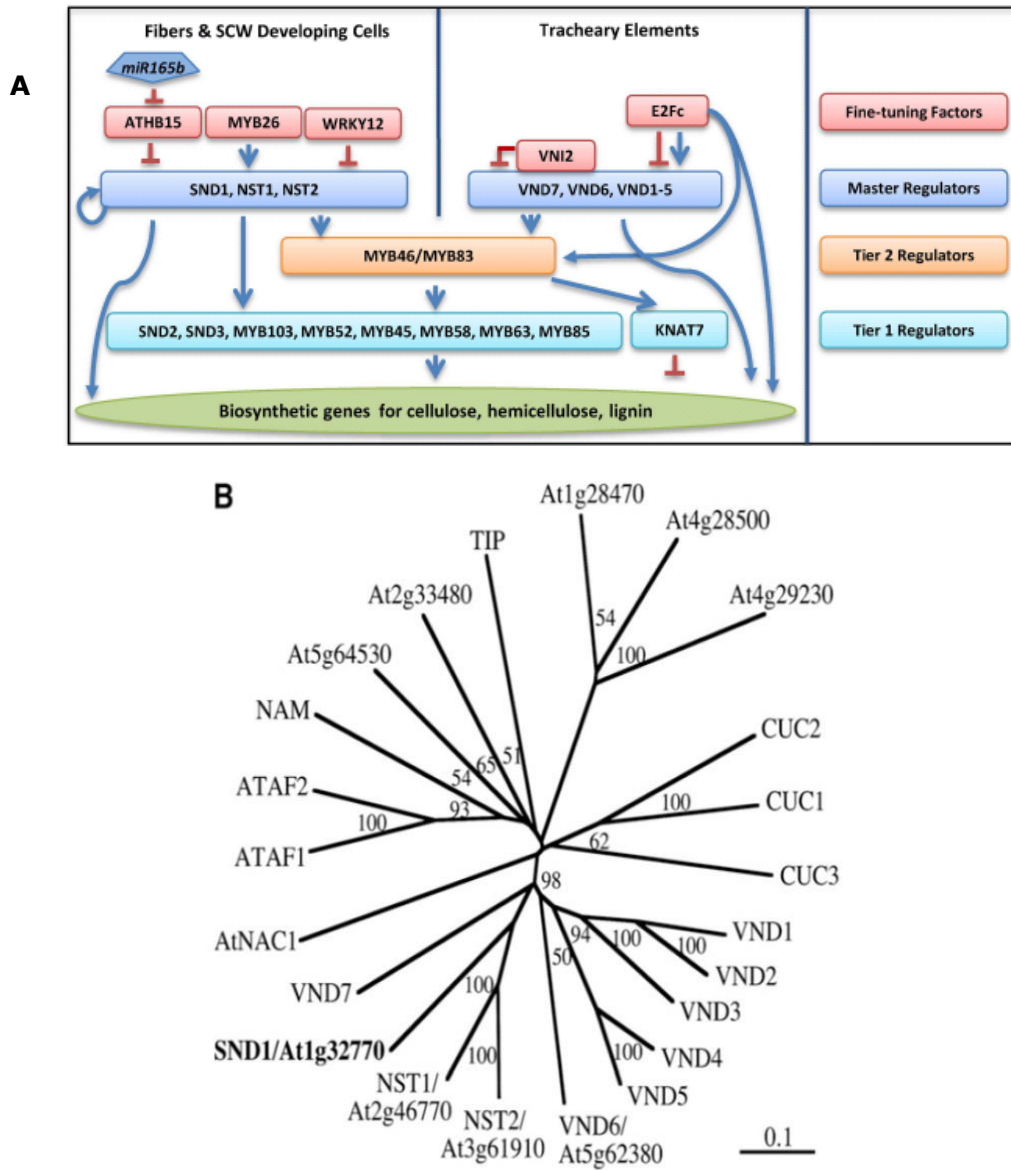


Figure 3. 2 A: The gene regulatory network of secondary wall formation in *Arabidopsis thaliana* (Yang and Wang, 2016). There have been three tiers of genes identified as regulators of secondary wall thickening in which NAC domain genes are considered as master regulators. B: Phylogenetic analysis of *VND* genes and the stem related NAC genes showed that *SND1* belongs to a subgroup of NAC genes including *VND6*, *VND7*, *NST1* and *NST2* (Zhong et al., 2006).

tier one (Yang and Wang, 2016) (Figure 3.2 A). NAC domain genes also play part in plant responses to biotic and abiotic stresses. Nakashima et al. (2012) have divided stress responsive NACs (SNAC) in *Arabidopsis*, rice, *Selaginella* and *Physcomitrella* into three subgroups, SNAC-A, SNAC-B and SNAC-C and proposed that six major groups including SNACs were established in ancient moss lineages (Nakashima et al., 2012).

Early Responsive to dehydration 1 (*ERD 1*) is a NAC domain protein identified in *Arabidopsis* that is apart from being induced during dehydration has also been shown to be upregulated during senescence and dark induced etiolation via encoding a Clp protease regulatory subunit (Nakashima et al., 1997; Nakashima et al., 2012). A number of stress responsive NAC (SNAC) genes (ANAC019, ANAC055 and ANAC072) were identified that bind to the promoter region of *ERD1* and hence involved in responding to dehydration stress. The expression of these genes was induced during drought and high salinity stresses and also via phytohormones ABA and methyl jasmonic acid (MeJA) (Mitsuda et al., 2006; Tran et al., 2004). Jasmonate (JA and MeJA) are important plant hormones involved in plant defence responses against biotic stresses (Bu et al., 2008); therefore, NAC genes could be involved in the plant responses to biotic or abiotic stress conditions.

The *Vascular-Related NAC Domain 6* (*VND6*) and *VND7* are NAC domain transcription factors regulating differentiation of metaxylem and protoxylem respectively in primary roots of *Arabidopsis*. Moreover, *VND7* regulates xylem vessel element formation including secondary cell wall deposition and cell death (Kubo et al., 2005; Yamaguchi et al., 2010a; Yamaguchi et al., 2008; Zhong et al., 2007). *VND* gene family is phylogenetically close to *NST1* and *NST3* in *Arabidopsis*, which are known to be the key regulators of differentiation of fibre cells (Yamaguchi et al., 2011; Zhong et al., 2007) (Figure 3.2 B). Yamaguchi et al. (2011) showed

that *VND7* restores secondary cell wall formation in fibres cells of inflorescence of *nst1 nst3* double mutant. Moreover, a number of genes identified that are directly regulated by *VND7* in *Arabidopsis*. Among these are genes responsible for xylem vessel differentiation such as *MYB46* and *LBD30/ASL19/JLO* (Nakano et al., 2010; Soyano et al., 2008; Yamaguchi et al., 2011), cellulose synthase subunits including *CesA4/ IRX5* and *CesA8/ IRX1* (Taylor et al., 2003; Yamaguchi et al., 2011). Changes in the expression level of *MYB46* and *MYB103* due to alteration in *VND7* expression are consistent with the previous studies that showed *NST3* is the direct regulator of *MYB46* and *MYB103* and possibly by *VND7* (McCarthy et al., 2009; Yamaguchi et al., 2011; Zhong et al., 2007).

It was showed that regulation of xylem vessels by *VND7* is done indirectly through regulation of *MYB* transcription factors *MYB46*, *MYB83* and *MYB103* (Yamaguchi et al., 2011). *MYB46* and *MYB83* have already been shown to function in secondary wall deposition through activating biosynthetic pathways of cellulose, hemicellulose and lignin (Ko et al., 2009; McCarthy et al., 2009; Yamaguchi et al., 2011; Zhong et al., 2007). Direct regulation of *IRX1/CesA8* and *IRX5/CesA4* genes that are downstream of *MYB46*, *MYB83* and *MYB103* by *VND7* protein suggests an indirect regulation of these genes.

Other *VND* genes (*VND1* to *VND5*) have been shown to be upregulated during xylem differentiation in *Arabidopsis* (Kubo et al., 2005). Zhou et al. (2014) showed that *VND1* to *VND5* are specifically expressed in vessels. Overexpression of these genes activates the expression of secondary wall biosynthesis genes for cellulose, xylan and lignin and consequently induces ectopic deposition of secondary walls (Zhou et al., 2014). Expression of *VND1* to *VND5* under control of *SND1* promoter rescued *snd1 nst1* double mutant and restored the formation of lignified secondary walls in fibres (Zhou et al., 2014). This is an indication that these genes are functional

homologues of *SND1* and are involved in transcriptional regulation of secondary wall biosynthesis (Zhou et al., 2014). *VND1* to *VND5* have also been shown to be capable of activating the entire secondary wall biosynthesis pathway (Zhou et al., 2014). On the other hand, *VND7* expression is negatively regulated via *VND interacting 2* (*VNI2*), which directly binds to *VND7* protein and inhibits its expression (Yamaguchi et al., 2010a; Yang and Wang, 2016).

Highly expressed in xylem during tracheary element (TE) differentiation, *Xylem NAC Domain1* (*XND1*) is another gene from NAC family that is important for the regulation secondary wall synthesis and programmed cell death (PCD) in xylem (Zhao et al., 2008). Loss of function of *XND1* results in reduction of plant height and length of tracheary elements in stems and hypocotyls. Overexpression of *XND1* suppressed the synthesis of secondary wall and formation of PCD in xylem resulting in replacement of functional xylem conducting cells with living parenchyma-like cells. Therefore, it was hypothesised that *XND1* expression blocks the formation of secondary wall and PCD in xylem. Moreover, slight ability of *XND1* to suppress the terminal events related to tracheary elements (TE) differentiation was regarded as its positive effect on TE length (Zhao et al., 2008). There are other genes shown to negatively regulate NAC genes.

The *Secondary Wall-Associated NAC Domain Protein1* (*SND1*) is a transcription factor responsible for secondary wall synthesis in fibres (Zhong et al., 2006). Expression of *SND1* is limited to interfascicular and xylary fibres in stems (Zhong et al., 2007). *NST1* has also been implicated in the control of secondary wall synthesis in stems as it is also expressed in interfascicular fibres (Mitsuda et al., 2005). Therefore, this suggests a potential interaction between *SND1/ NST3* and *NST1*. Moreover, single mutants of *snd1* or *nst1* do not show any alteration of secondary wall thickening in fibres nor did any changes in plant morphology (Zhong et al.,

2007). Nevertheless, simultaneous repression of *SND1* and *NST1* resulted in drastic reduction in stem strength. It also blocked the biosynthesis of all three major components of secondary cell wall including cellulose, xylan and lignin, in fibre walls (Zhong et al., 2007). Drastic reduction in the expression level of the cellulose synthase genes, *CesA7* and *CesA8* (Taylor et al., 2004), xylan biosynthesis-related gene *Fragile Fibers8* (*FRA8*) (Zhong et al., 2005a; Zhong et al., 2005b), lignin biosynthetic genes *4CL1* and *CCoAOMT* (Boerjan et al., 2003) were also seen in the fibre cells of the stem of *SND1/ NST1* RNAi lines (Zhong et al., 2007).

Formation of secondary wall thickening in the anther is also regulated by NAC transcription factors. Two NAC genes, *NAC Secondary Wall Promoting Factor 1* (*NST1*) and *NST2*, are redundantly regulate formation of secondary wall thickening in the endothecium layer of the anther. The inability of anthers to deposit secondary wall thickening results in indehiscent anthers; thus male sterile plants despite developing viable pollens (Mitsuda et al., 2005; Yang et al., 2007). Although these two NAC domain genes have higher expression in the reproductive tissues, in particular the anthers, they also interact with other NAC domain transcription factors regulating secondary thickening in other tissues. The expression of *AtNST1* was mainly observed in reproductive tissues including anthers and filaments and in vegetative tissues such as leaf midrib and inflorescence stem. *AtNST2* expression was also detected mainly in the anther walls and pollen grains (Mitsuda et al., 2005) and recently it was shown that *AtNST2* together with *NST1* has fibre-specific expression in stems (Zhong and Ye, 2015). Repression of *Arabidopsis NST1* interaction with its target genes (via SRDX motif), resulted in failure of anthers to dehisce and thus sterility while there was no other phenotype observed compared to wild-type. Moreover, repression of *NST2* gene did not result in unusual phenotype, nor did it produce sterile plants (Mitsuda

et al., 2005). This was linked to the fact that NST2 is considered to be less effective in inducing secondary thickening compared to NST1. Also, the redundant function of these two transcription factors in the anther means that repression of NST2 interaction with its targets is recovered by normal function of NST1. Therefore, lack of sterile phenotype in 35S::NST2SRDX plants was an indication to this redundant regulation. Moreover, this shows that gene knock-down methods such as RNAi silencing may not be the best way of characterising these two redundant transcription factors. However, independent repression of *NST1* and *NST2* under their own promoters (Pro_{NST1}::*NST1SRDX* or Pro_{NST2}::*NST2SRDX*) resulted in indehiscent anthers in both genes, suggesting anther specific activity of these two genes in *Arabidopsis* (Mitsuda et al., 2005).

Overexpression of these two transcription factors resulted in formation secondary wall thickening in various tissues other than the floral organs including anther and stamens. The overexpression lines in both *NST1* and *NST2* were smaller than their wild-type counterparts, but the frequency of ectopic secondary wall formation was higher in *NST1* overexpression lines (Mitsuda et al., 2005).

The importance of secondary wall formation in the anthers indicates a regulatory mechanism that controls expression of genes involved in this pathway including *MYB26*, *NST1* and *NST2*. Secondary wall formation in the anthers is negatively regulated by *TCP24* transcription factor (Wang et al., 2015). This transcription factor belongs to *TCP* family (*Teosinte Branched 1*, *Cycloidea* and *PCF*) that contain bHLH motif and is highly expressed during stages 4 and 5 in *Arabidopsis* anther development (Martin-Trillo and Cubas, 2010; Wang et al., 2015). *TCP24* expression decreases as secondary wall is formed in the endothecium layer of the anther, although it is still present in vascular regions. Overexpression of *TCP24* in *Arabidopsis* resulted in lack of secondary wall thickening whereas

its repression resulted in higher deposition of secondary wall thickening. Overexpression of *TCP24* downregulated *NST1* and *NST2* expression indicating direct negative regulation while it had no effects on *MYB26* (Wang et al., 2015).

A number of NAC genes have been identified in barley via bioinformatics analyses, which have been shown to be involved in variety of developmental processes such as leaf senescence, ABA induction and secondary wall biosynthesis (Christiansen et al., 2011). Although it was not possible to identify clear *Arabidopsis* orthologue, a few of these *HvNAC* genes showed high conservation both in NAC domain and C terminal motifs in *Arabidopsis*, rice and *Brachypodium* (Christiansen et al., 2011).

HvNAC033, *HvNAC034* and *HvNAC039* in barley were indicated to be involved in secondary wall biosynthesis in elongating stem tissue (Christiansen et al., 2011). *HvNAC033* was found to have high protein identity to ANAC043 (*Arabidopsis* NST1) and NST1 orthologue in alfalfa (MtNST1); therefore it was suggested as the putative orthologue of *NST1* in barley. Furthermore, *HvNAC034* was found to be closely related to *HvNAC033* and was also upregulated in the elongating stem tissues that could indicate this maybe an orthologue of NST2 in barley (Christiansen et al., 2011). However, there was no thorough expression analysis across various developmental stages or analysis of the effects of these putative orthologues in barley for neither *HvNAC033* nor *HvNAC034*.

This project aimed at identification and characterisation of putative orthologous *NST1* and *NST2* in barley. For this purpose, genomic database were used in step by step identification of the putative orthologous sequences in rice, *Brachypodium* and barley. The high conservation across the grass species makes it possible to identify the putative orthologous genes based on their high protein identity. It was shown that the identified barley NAC proteins had more than 70% identity to their *Brachypodium*

counterparts or 60% to rice. However, this high level of identity cannot be achieved between barley and *Arabidopsis* due to small level of protein similarity (Christiansen et al., 2011). Nevertheless, the availability of an intermediate species such as rice and *Brachypodium* makes the identification of putative orthologous sequences in barley feasible. The identified sequences were analysed for their authenticity through synteny and expression analysis of the identified sequences.

3.2 Identification of the putative orthologous sequences in barley

The identification of the putative targets was done using the information available online. The step-by-step identification of putative orthologues was done by aligning the *Arabidopsis* nucleotide sequence against NCBI (the National Centre for Biotechnology Information; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) rice database. This allowed selection of highly similar sequences in rice as putative orthologues for *NST1* and *NST2*.

Moreover, the expression pattern of the identified rice sequence was acquired from RiceXPro database (<http://ricexpro.dna.affrc.go.jp>) in order to ensure that the identified sequence shared common expression pattern to the *Arabidopsis* counterpart. The rice putative orthologous sequence was then used to identify the *Brachypodium* putative sequence using the Gramene database (<http://www.gramene.org/>). The Gramene database offers orthologue identification service for each sequence based on the availability of the sequences and their identity. The *Brachypodium* sequence with the highest identity in nucleotide level with the rice sequence was chosen as the putative orthologue of the target genes. The *Brachypodium* putative sequence (nucleotide sequence) was then aligned against the barley BLAST server (<http://webblast.ipk->

gatersleben.de/barley/) available from International Barley Sequencing Consortium website. The barley sequence with the highest sequence identity to the *Brachypodium* putative orthologue was selected as the putative orthologue in barley. The identified barley putative sequence was also aligned against the rice putative sequence in order to more accurately determine the putative orthologue in barley. The expression analysis of the identified putative orthologues was done by quantification of the expression in reproductive and non-reproductive tissues using qRT-PCR (see section 2.14).

3.3 Results

3.3.1 Bioinformatics analysis

The putative barley sequences of *AtNST1* (At2g46770) and *AtNST2* (AT3G61910) were identified via step-by-step identification of the putative orthologues from *Arabidopsis* to barley. The sequences of the genes in *Arabidopsis* were available on the *Arabidopsis* Information Resources (TAIR) (<http://www.arabidopsis.org/>) and Gramene (<http://www.gramene.org/>) databases. The sequence of the target genes in *Arabidopsis* along with other complementary information such as the size, number of exon and introns, their distribution, the location of the gene on the chromosome and the neighbouring genes were extracted using the two aforementioned databases.

This information was eventually used to help confirm the putative orthologues in comparison to their counterparts in *Arabidopsis*. The next step was to identify the rice putative orthologue of the target genes. For this purpose the *Arabidopsis* sequence was BLASTed against the rice

database of National Centre for Biotechnology Information (NCBI) in order to localise the sequences with highest identity to the *Arabidopsis* genes. These rice sequences were chosen as potential putative *NST1* and *NST2* orthologues and were then analysed for the similarity of their expression profile compared to the *Arabidopsis* genes.

The gene structure of the identified rice putative orthologue was examined using the MSU rice genome annotation project database (Table 2.1). Furthermore, the expression profile of the target genes was also studied using this database that later on was compared against that gathered from RiceXPro database (Table 2.1).

The identified rice putative orthologue was used to find the *Brachypodium* putative orthologue in the Gramene database. The identified *Brachypodium* putative orthologue was aligned against the rice and *Arabidopsis* sequences in order to ascertain the most similar sequences in nucleotide level. As there was not enough expression data available for *Brachypodium* it was not possible to check the expression similarity of the putative orthologue in *Brachypodium* to ones in rice and *Arabidopsis*.

The *Brachypodium* putative sequence of *NST1* and *NST2* was BLASTed against the coding sequence database available from the International Barley Sequencing Consortium in order to localise the barley putative orthologues. Moreover, the identified putative sequences of rice and *Brachypodium* were aligned against the barley putative orthologue in order to finalise the selection of the correct putative sequence in barley. The barley sequences with high identity to the *Brachypodium* sequences were selected as barley putative orthologue of *AtNST1* and *AtNST2*.

Further confirmation of the identified sequences was achieved via synteny analysis. This analysis provided a basic comparison between the flanking genes found on the adjacent sites to the putative targets in barley

compared to their counterparts in *Brachypodium*, rice and *Arabidopsis*. The identified putative targets and their neighbouring genes' location on the chromosome were localised. These data were used to make a synteny map and assess the level of conservation between the species in terms the other genes on the chromosome. This was done based on the sequence identity between the genes that was provided by the Gramene database. Although the sequence identity between the genes from different species may not be reliable to confirm orthologue identity and all the gene's functions might not have been characterised, these data provide a good basis of information for further confirmation of the accuracy of selection of the putative orthologues in barley.

Furthermore, the phylogenetic tree analysis of the identified putative sequences and all possible NST1 and NST2-like sequences with some identity to *Arabidopsis* NST1 and NST2 showed the relationship between the identified sequences and the distance between the orthologous genes in different species.

The comparison between the expression profiles of *Arabidopsis* and rice putative orthologues was also important to identifying the correct putative sequences with similar expression patterns. This information for *Arabidopsis* was obtained from TAIR expression database, and Rice Expression Profile Database (RiceXPro) expression profiles and MSU rice genome annotation database were used to compare with their *Arabidopsis* counterparts. This helped to take the correct rice putative orthologue with higher expression similarity to the *Arabidopsis* genes forward.

3.3.1.1 *HvNST1* identification

The BLAST analysis of nucleotide sequence of *AtNST1* (At2g46770) coding sequence in NCBI rice database led to identification of putative rice orthologue (LOC_Os08g02300) (Appendix 9.5.1) with the highest nucleotide identity to the *Arabidopsis* sequence. This putative sequence was also confirmed in Gramene database as the putative orthologue of *AtNST1* in rice. The alignment of the two sequences in amino acid level showed 47% identity between the *Arabidopsis* and rice putative orthologue (Table 3.1) (Figure 3.3). The alignment between the two sequences showed high level of conservation especially across NAC domain (amino acids 14- 55) (Figure 3.4). The expression pattern of the identified rice putative orthologue was analysed using rice expression database available from RiceXPro. The identified rice sequence was more similar expression pattern to its *Arabidopsis* orthologue was selected as rice orthologue (Figure 3.3).

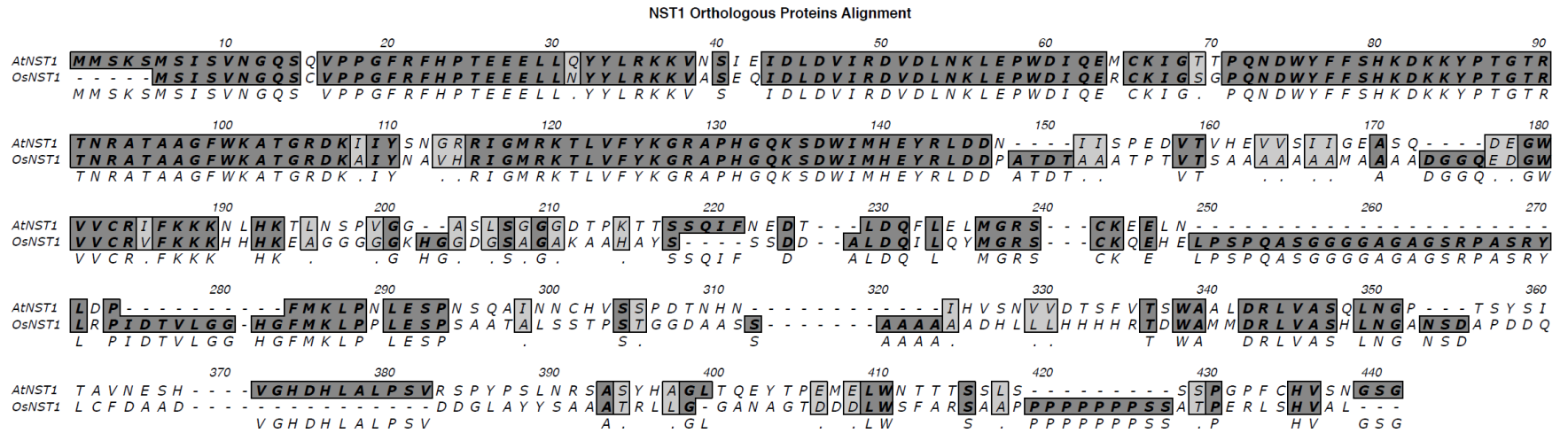


Figure 3. 4 Protein alignment between *Arabidopsis* and identified rice NST1 putative orthologue. The alignment showed high level of conservation in the two proteins especially in NAC domain region (14- 55 amino acid).

The putative rice sequence was subsequently used to identify the putative *Brachypodium* sequence. The putative orthologue in *Brachypodium* was found using the orthologue service available on Gramene database. This option offers various putative orthologues based on the identity of sequences and depending on the species and accessibility of genome sequence there may be more than one putative orthologue suggested. The alignment of rice putative *NST1* orthologous sequence to the database resulted in only one putative orthologue in *Brachypodium* (Bradi3g13727.1) (Appendix 9.5.2) with 65% amino acid identity to the rice putative orthologue. Table 3.1 shows the protein identity between the identified putative sequences and the *Arabidopsis NST1* sequence.

Table 3.1 Protein identity comparisons between the identified putative sequences and *Arabidopsis NST1*.

<i>Protein identity</i>				
	AtNST1	OsNST1	BradiNST1	HvNST1
AtNST1	100%	47%	49%	50%
OsNST1		100%	65%	70%
BradiNST1			100%	66%
HvNST1				100%

The identified *Brachypodium* putative orthologue was used to identify a barley sequence with the highest identity as barley putative orthologue of *NST1* (AK248449) (Appendix 9.5.3). This was done using the International Barley Sequencing Consortium database. The BLAST analysis of putative *BradiNST1* coding sequence (nucleotide sequence) against barley CDS database resulted in identification of a barley coding sequence with 66% amino acid identity to its *Brachypodium* counterpart. This sequence was

considered as the putative orthologue of *AtNST1* in barley. The Protein alignment of barley putative *NST1* sequence against the other identified sequences (*Arabidopsis*, rice and *Brachypodium*) showed a high level of conservation in the NAC domain (Figure 3.5). This high conservation rate between the identified putative orthologous sequences and the *Arabidopsis NST1* sequence was supported correct identification of the putative orthologue sequence in barley.

Arabidopsis NST1 is comprised of three exons and two introns, and is located on chromosome two and contains 1382 base pairs that encodes for a 365 amino acid protein. The rice orthologue coding sequence contains three exons and encodes for a 400 amino acid protein. The identified putative *Brachypodium NST1* orthologous sequence is located on chromosome three and also contains three exons. This coding sequence contains 1170 nucleotides that encodes for a 390 amino acid protein. In barley, the identified *NST1* putative orthologue is located on chromosome 7H comprising of three exons and 1197 nucleotide base pairs, which encode for a 399 amino acid protein and also contains three exons (Figure 3.6). As it can be seen from Figure 3.6, all the identified putative sequences (rice, *Brachypodium* and barley) had the same number of exons as the *NST1* in *Arabidopsis*.

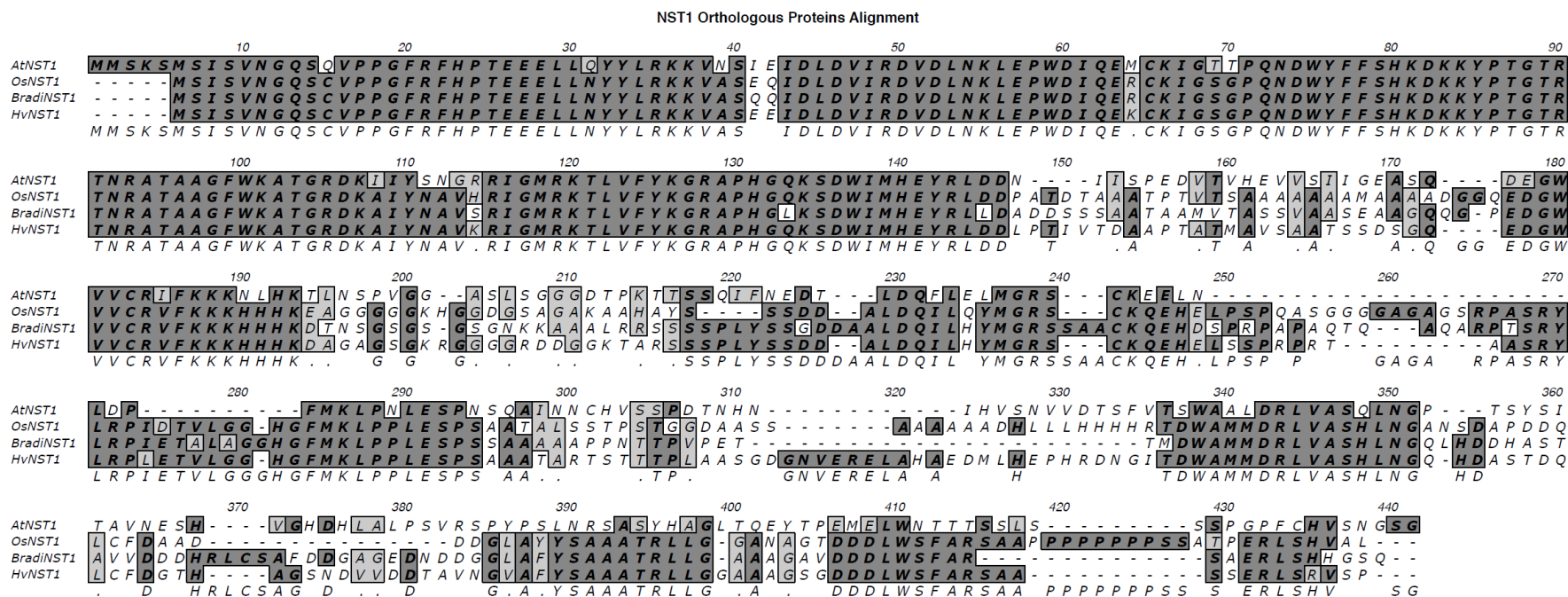


Figure 3. 5 Protein alignment of HvNST1 putative orthologue against *Arabidopsis*, rice and *Brachypodium* sequences. This alignment shows a high level of conservation in NAC domain region between all sequences. The conserved domain in Arabidopsis sequence is between 19-60 amino acids, in rice, *Brachypodium* and barley the conserved domain is between 14-55 amino acid.

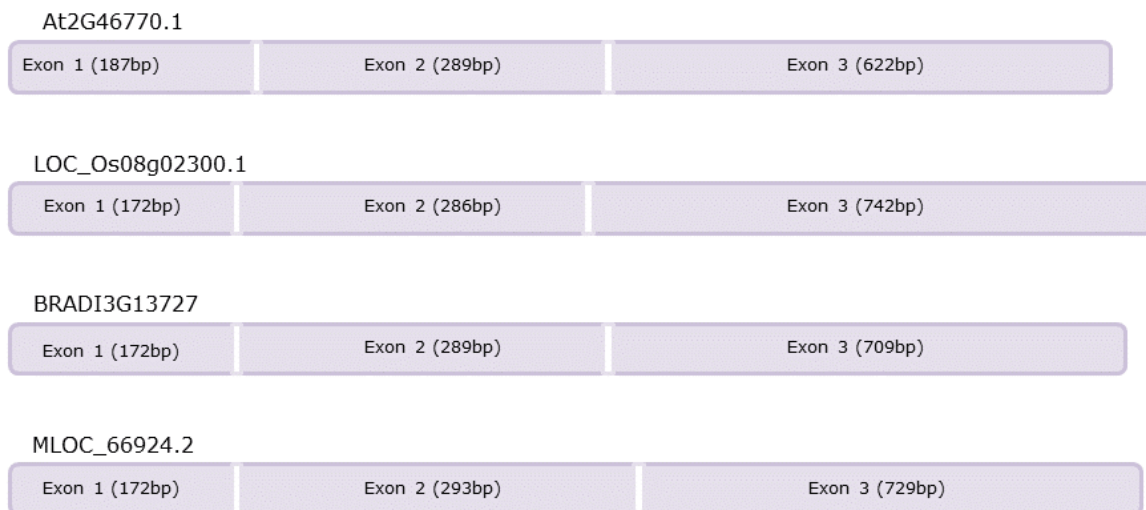


Figure 3. 6 Schematic presentation of identified putative orthologous *NST1* and *Arabidopsis* gene structures. All the identified putative sequences (rice: LOC_Os08g023001.1, *Brachypodium*: BRADI3G13727, barley: MLOC_66924.2) and the *Arabidopsis* *NST1* (At2G46770.1) gene possessed similar number of exons.

3.3.1.2 Phylogenetic analysis

The protein sequence of the identified barley putative orthologous coding sequence was blasted against NCBI protein database to further identify similar proteins. The proteins highly similar to the identified putative barley *NST1* protein were mainly NAC domain related genes. The phylogenetic tree was made using online platform (<http://www.phylogeny.fr/>) (Dereeper et al., 2008). The phylogenetic analysis of the identified putative Hv*NST1*-like proteins indicated that the identified barley sequence (AK248449.1) clustered with the *NST1* related proteins in monocots. Furthermore, the identified putative *NST1* sequence in barley showed high identity to HvNAC033, which was suggested as putative Hv*NST1* (Christiansen et al., 2011) (Figure 3.7). More importantly, the identified

Brachypodium and rice putative orthologues were also clustered together with barley putative NST1 protein.

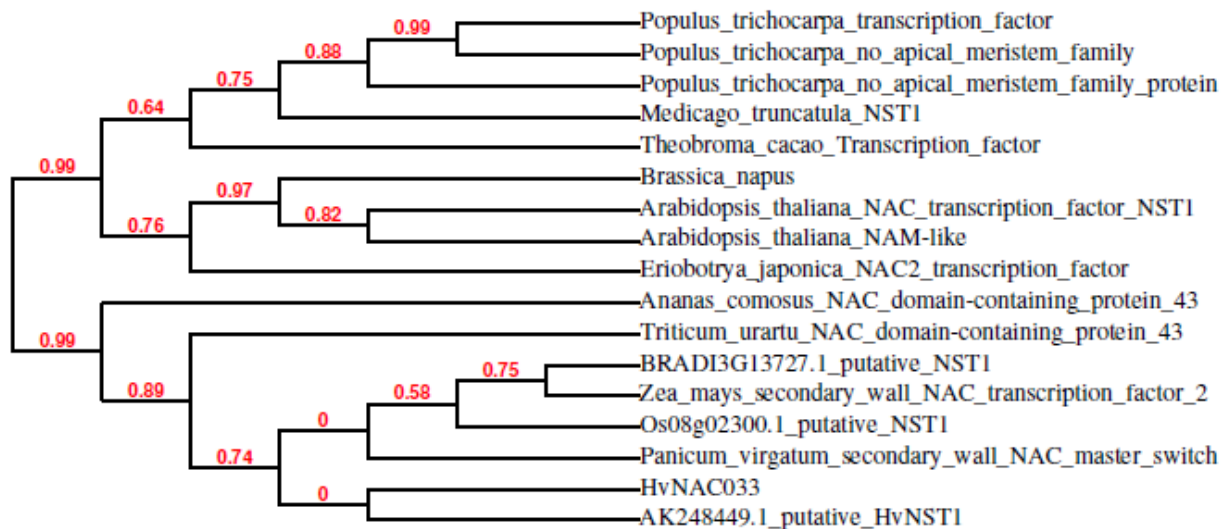


Figure 3. 7 Phylogenetic tree analysis for the identified putative NST1. The sequences of this tree were obtained by BLAST analysis of HvNST1 putative orthologous protein against NCBI protein database. The analysis showed close relationship between barley NST1 and the identified *Brachypodium* and rice putative sequences. The analysis was done using online platform (<http://www.phylogeny.fr/>) (Dereeper et al., 2008).

Using NCBI and IPK databases, reciprocal BLASTs were made between the identified barley putative orthologue (protein sequence) and *Brachypodium*, rice, *Arabidopsis* and other crops. This analysis identified a number of sequences with high identity to the barley sequence. These sequences were used to make a phylogenetic tree to further study the relationship between the identified putative orthologues and other similar sequences to NST1 (Figure 3.8). The phylogenetic tree analysis of these sequences showed a close relationship between *NST1* putative orthologue in barley (MLOC_66924/ AK248449.1) with *Brachypodium* and rice putative orthologues (Figure 3.8). There was also a close relationship between *NST1* and *NST2* (AK249120) putative orthologues in this analysis.

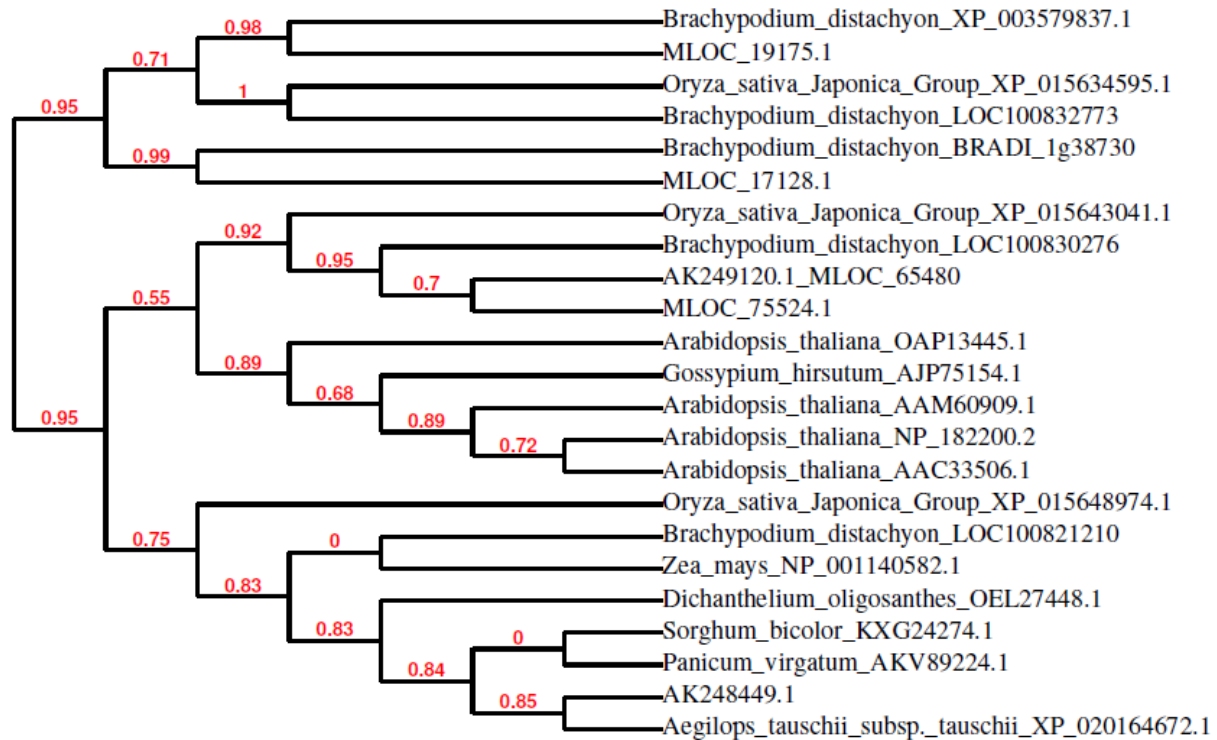


Figure 3. 8 Phylogenetic analysis of the sequences generated in reciprocal BLASTs between putative barley, *Brachypodium*, rice protein sequences and *Arabidopsis* NST1. This analysis showed that putative barley NST1 (AK248449.1) was closely related to its rice and *Brachypodium* orthologues. *Arabidopsis thaliana* NP_182200.2: NAC (No Apical Meristem) domain transcriptional regulator superfamily protein (At2g46770: AtNST1); *Arabidopsis thaliana* AAC33506.1: NAM (no apical meristem)-like protein; *Arabidopsis thaliana* OAP13445.1: SND1; *Arabidopsis thaliana* AAM60909.1: NAM-like protein NST2; *Oryza sativa* Japonica Group XP_015648974.1: PREDICTED NAC domain-containing protein 43 (putative rice NST1 LOC_Os08g02300.1); *Oryza sativa* Japonica Group XP_015643041.1: PREDICTED NAC domain-containing protein 43. *Oryza sativa* Japonica Group XP_015634595.1: PREDICTED NAC domain-containing protein 7. *Brachypodium distachyon* LOC100821210: uncharacterized protein (putative *Brachypodium* NST1 BRADI3G13727.1); *Brachypodium distachyon* LOC100830276: uncharacterized protein; *Brachypodium distachyon* BRADI_1g38730: hypothetical protein; *Brachypodium distachyon* XP_003579837.1: PREDICTED protein BEARSKIN1-like (BRADI_5g11247); *Brachypodium distachyon* LOC100832773 uncharacterized protein (BRADI_3g50067); *Hordeum vulgare* AK248449.1 (MLOC_66924): putative NST1; *Hordeum vulgare* AK249120.1 (MLOC_65480): putative NST2; *Aegilops tauschii* subsp. *tauschii* XP_020164672.1: NAC domain-containing protein 43-like; *Panicum virgatum* AKV89224.1: secondary wall NAC master switch; *Sorghum bicolor* KXG24274.1

hypothetical protein SORBI_007G018100; *Dichanthelium oligosanthes* OEL27448.1: NAC domain-containing protein 43; *Zea mays* NP_001140582.1: secondary wall NAC transcription factor 2; *Gossypium hirsutum* AJP75154.1: NAC transcription factor L1.

3.3.1.3 Synteny analysis

The preserved order of genes derived from the same ancestor is known as synteny. The utilisation of synteny between the species is a good tool for verification of the authenticity of identified putative orthologues. Synteny between two species does not indicate the function of an identified putative orthologous sequence, however it confirms that the identified sequence shares the same flanking region in other species. Thus, this is a good tool in reverse genetics when the function of the identified sequence in the target species is unknown, but there are information available in other taxonomically related species.

Syntenic analysis of the NST1 regions showed the chromosomal arrangement of the flanking loci for the target genes (in this case *Arabidopsis* NST1 and NST2) compared with that of the identified putative orthologues. The location of genes on the chromosome in various species may change due to evolution and other alterations within the genome such as duplication and deletion. These changes depend on the taxonomical distance between the species. Nonetheless, the majority of the genes in the flanking regions of the target locus of a species are likely to be present in a different species, however they could also be reshuffled within the same chromosome. This information can be used to help verify the identified putative sequences in various species based on the identity of their flanking regions.

The synteny analysis for *HvNST1* putative orthologue showed a high level of conservation between barley, *Brachypodium* and rice in the flanking regions of the identified putative *NST1* (Figure 3.9). This analysis was performed by identification of the target gene's location (*NST1*) on the chromosome in Gramene database for each species separately (i.e *Arabidopsis*, rice, *Brachypodium* and barley). The chromosome map shows the location of the gene of interest on the chromosome along with the gene codes of the genes in the flanking regions. The gene codes of the genes in the flanking regions were used to identify the putative orthologous sequences in the different species using the orthologue service in Gramene database. For this purpose, the synteny between *Arabidopsis* and rice was first studied, then rice and *Brachypodium* and finally *Brachypodium* and barley. Figure 3.9 is a simplified presentation of the synteny observed between *Arabidopsis*, rice, *Brachypodium* and barley in relation to *NST1* gene. The gene codes indicate the codes gathered from Gramene database that may not necessarily represent a characterised gene.

This study showed a very low level of synteny between *Arabidopsis* and rice genomes. This could most likely be linked to evolutionary processes such as divergence of monocots from dicots genomes and/or whole genome duplication. The high level of conservation and synteny between the genomes of barley, *Brachypodium* and rice observed in the flanking regions of the putative *NST1* was an indication to aid identification of the putative orthologous sequences.

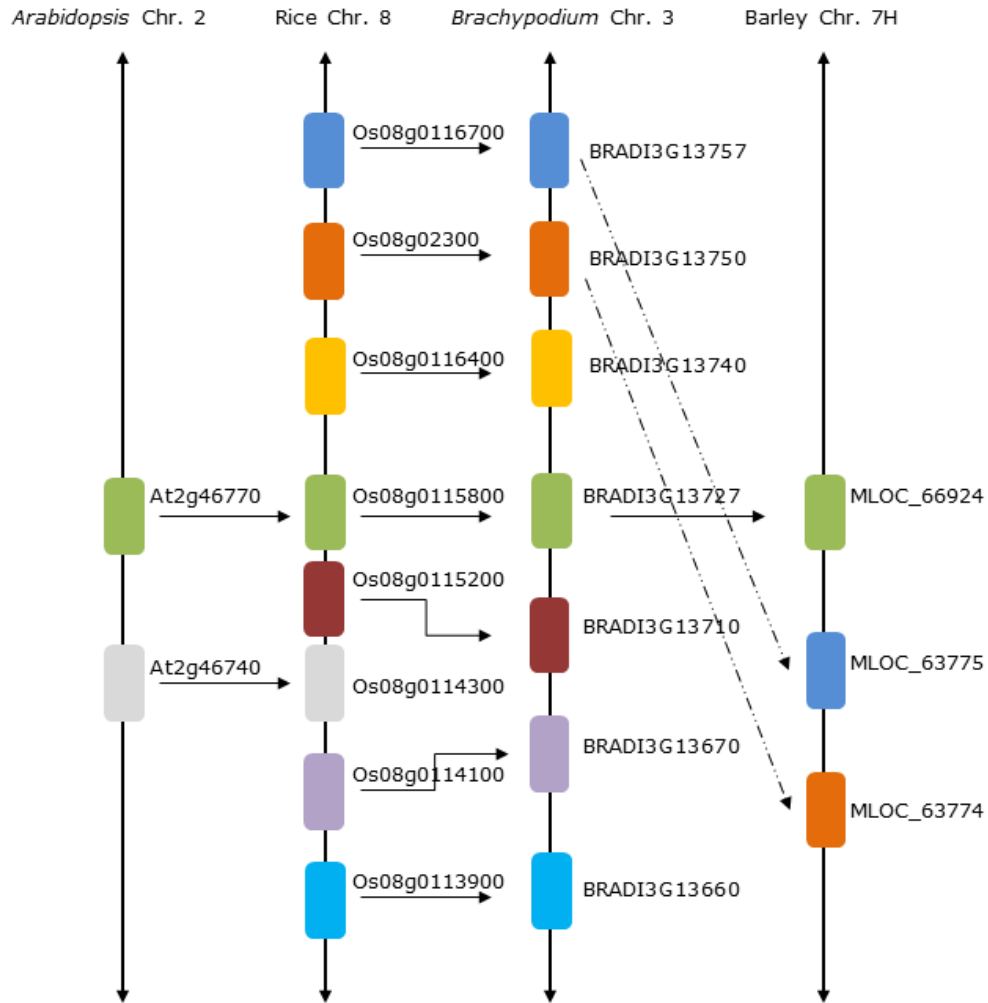


Figure 3. 9 A schematic representation of the synteny analysis for the putative *HvNST1* orthologue. The level of gene order conservation in the flanking regions of the identified *Arabidopsis* to barley putative orthologues suggest that the identified sequence in barley is likely to be orthologous to *AtNST1*. Similar colours represent conserved genes. The gene codes represent the gene names found in Gramene database.

3.3.2 Expression analysis

3.3.2.1 Expression analysis *HvNST1*

The expression of the putative *HvNST1* in barley flower stages were quantified via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Flower samples from barley cultivar Optic were staged and collected according to Fernandez and Wilson (2012) non-destructive staging protocol.

This expression of *HvNST1* showed similar pattern compared to *Arabidopsis NST1* (<https://www.cpib.ac.uk/tools-resources/resources/flowernet/>) (Figure 3.10 B). In *Arabidopsis*, *NST1* expression increases at pollen mitosis I and peaks at bicellular pollen stage. Following the entrance to pollen mitosis II *NST1* expression sharply decreases. This analysis showed that expression of the putative orthologue *NST1* starts from free microspore stage and gradually increases until mitosis I stage (Figure 3.10 A). The expression was then slightly reduced towards the end of mitosis I with a slight increase at tri-nuclear pollen stage. The putative *NST1* orthologue in barley showed some expression in vegetative tissues mainly in stem and nodes, although this was lower than floral tissues' expression.

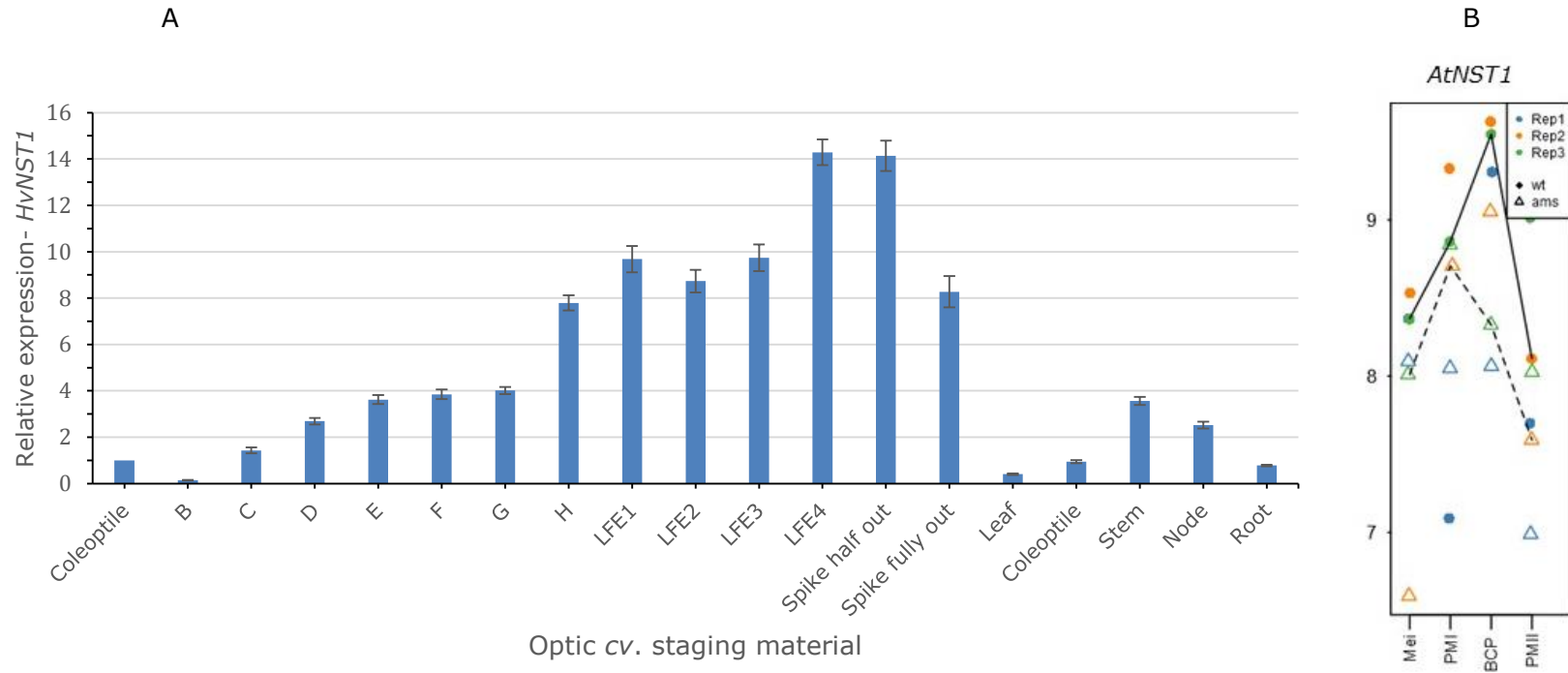


Figure 3. 10 Expression pattern of the putative *HvNST1* and *AtNST1* genes. A: The expression of the putative orthologous gene in barley peaked at LFE1 stage, as the anthers started entering mitosis I. The *HvNST1* expression was then slightly decreased until trinuclear pollen stage when the expression increased to even higher levels compared to mitosis I stage. The expression stayed high until pollen release and dropped as the fertilisation happened and grains started filling that is the end of the anther life span. B: In *Arabidopsis* *NST1* expression increases at pollen mitosis I and peaks at bicellular pollen stage. It then drops at pollen mitosis II. Anther stages that have been used in this analysis were as follows: B: secondary sporogenous cells to pollen mother cells. C: Pollen mother cells undergo meiosis. D: microspores are released from tetrad followed by tapetum vacuolation. E: free microspores, prominent tapetum layer starts to degenerate. F-G: microspores become vacuolated and tapetum degeneration. H-LFE2: mitosis I, tapetum is degenerating. LFE3: mitosis II occurs, binuclear pollen. LFE4: trinuclear pollen, septum breakage. Spike half out: pollen release. Spike fully out: fertilisation has happened and grains start filling. All the expressions were calculated relative to the one in coleoptile.

3.3.2.2 *HvNST2* identification

The identification of barley putative *NST2* sequence was done in the same manner as *NST1*. The *Arabidopsis NST2* coding sequence and expression profile were obtained from TAIR website. This coding sequence was aligned against rice sequence database available in NCBI in order to obtain the most identical sequence to the original gene in *Arabidopsis*. This BLAST analysis showed a rice sequence (Os06g04090.1) (Appendix 9.5.4) with higher similarity (42%) than the other identified sequences to the *Arabidopsis NST2* gene. To further confirm this putative orthologue the expression profile of these two sequences were compared (Figure 3.11). The comparison of the expression profiles of the two sequences showed similar tissue specificity. Both genes are highly expressed in floral tissues with some expressions in vegetative tissues, mainly in stem. This is an indication of identical putative functions for both sequences, hence, the rice sequence could be regarded as a putative orthologue of the *Arabidopsis NST2* gene. Moreover, protein alignment between *Arabidopsis* and rice *NST2* orthologues showed conservation between the two genes especially in NAC domain (Figure 3.12).

The coding sequence (nucleotide sequence) of the identified rice putative orthologue was used to identify the orthologous gene in *Brachypodium* via Gramene database. This led to the identification of *Brachypodium* orthologue (Bradi1g50057) (Appendix 9.5.5) with 57% amino acid identity to the rice gene. This sequence was then used to identify barley putative *NST2* sequence using the international barley sequencing consortium database (<http://webblast.ipk-gatersleben.de/barley/index.php>).

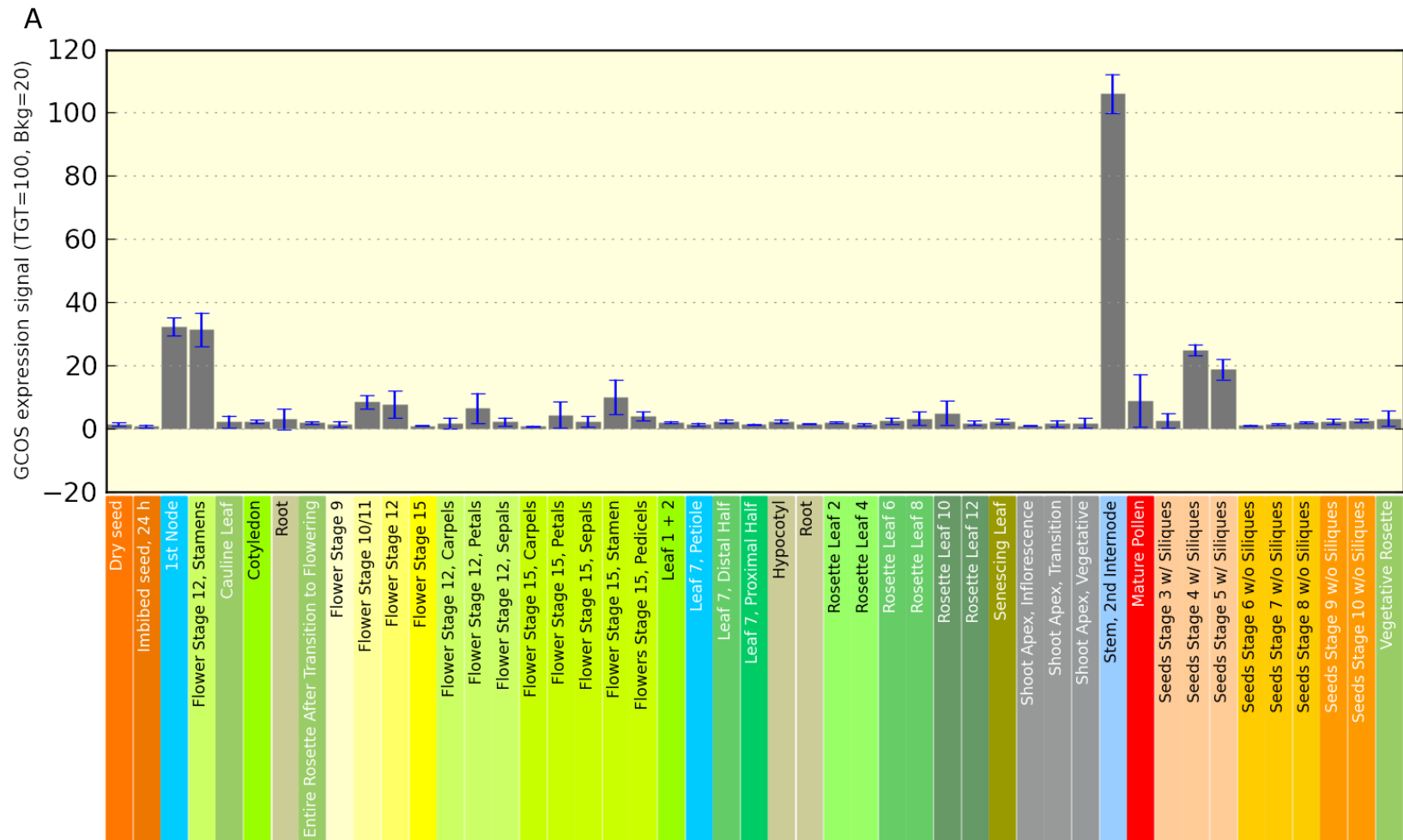


Figure 3. 11 A: Expression profile of *Arabidopsis* *NST2*. The expression profile of *NST2* in *Arabidopsis* was predominantly present in floral organs as it was reported by Mitsuda et al., (2005) (A). The expression data were obtained from TAIR database.

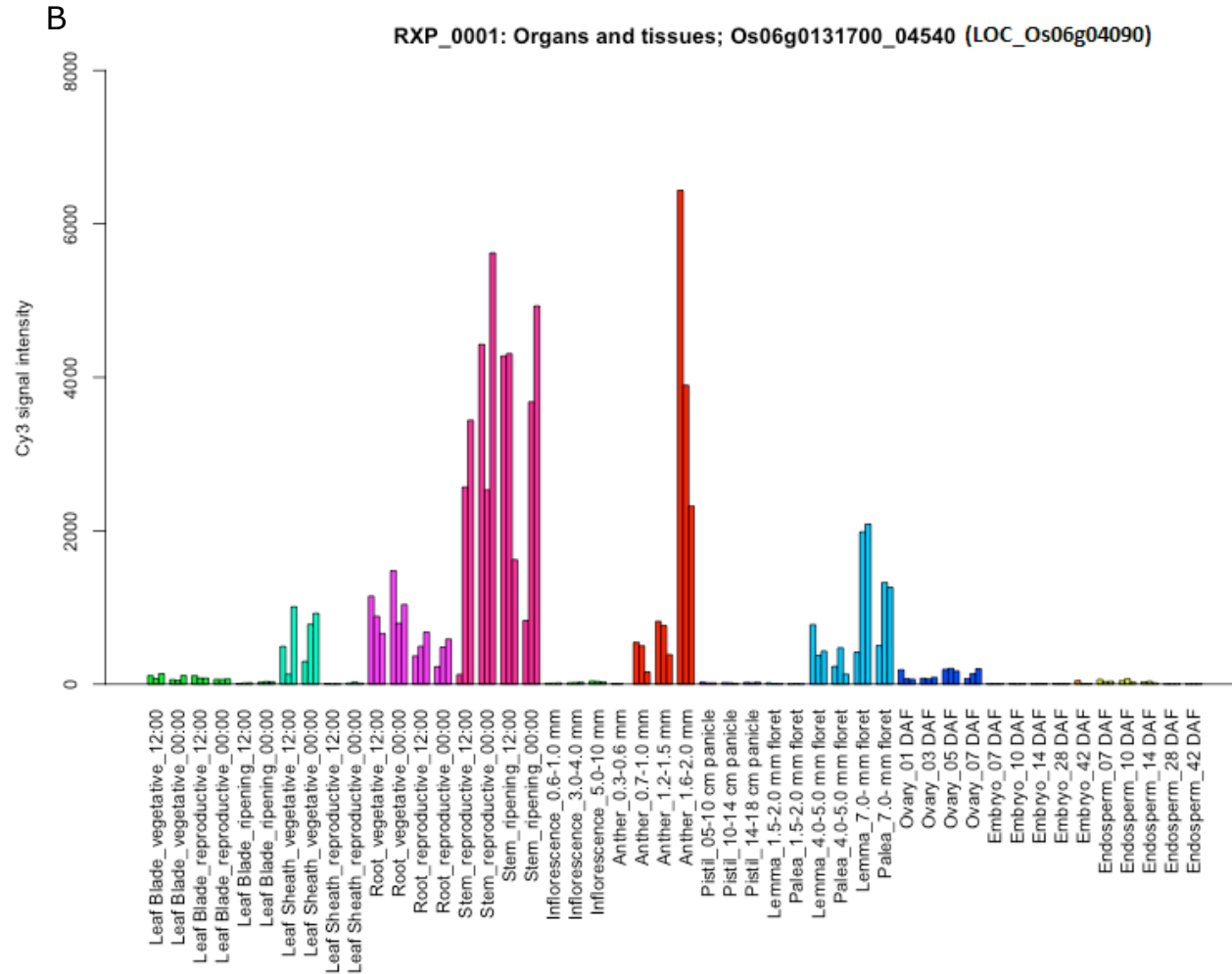


Figure 3. 11 B: Expression profile of putative NST2 in rice. The expression pattern in the rice putative orthologue (LOC_Os06g04090) showed a similar expression pattern to its *Arabidopsis* orthologue (Figure 3.11 A), with highest expression seen in the anthers and stem tissues. The expression data was obtained from RiceXPro database.

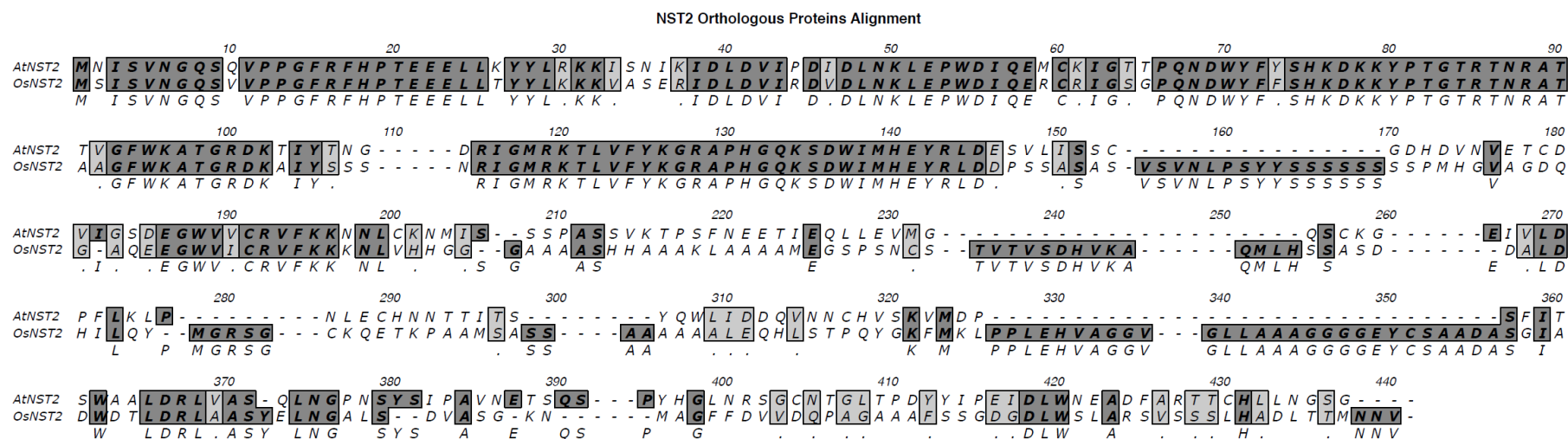


Figure 3. 12 Protein alignment between *Arabidopsis* NST2 and rice putative orthologue. The alignment showed high level of conservation in NAC domain (14-55 amino acid).

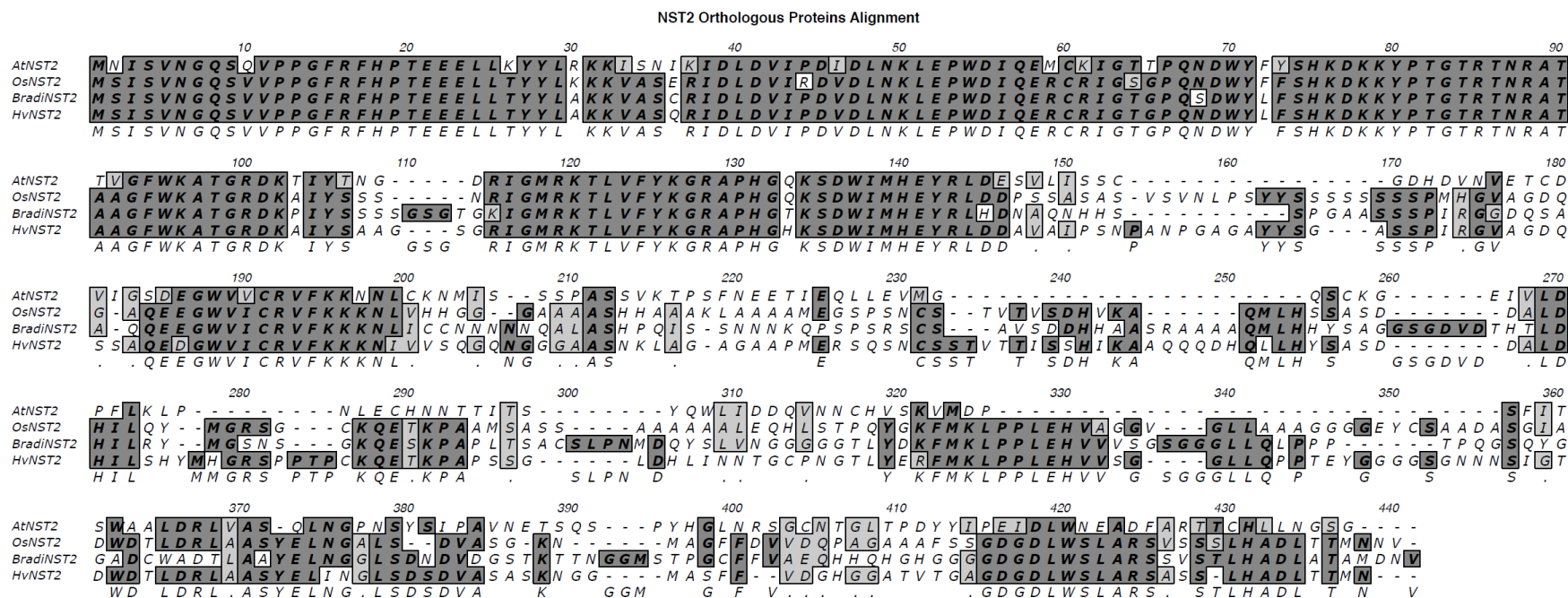


Figure 3. 13 Protein alignments of the putative *HvNST2* sequence against the *Arabidopsis*, rice and *Brachypodium* protein sequences. The alignment showed a high level of conservation between the putative orthologue and the rest of the sequences at the NAC domain. The conserved domain for all putative orthologues and *Arabidopsis* NST1 gene is between 14- 55 amino acids.

The putative orthologue in barley (AK249120.1) (Appendix 9.5.6) showed 58% (amino acid) identity to the *Brachypodium* sequence was identified. Protein alignment between the identified putative orthologous sequence and *Arabidopsis*, rice and *Brachypodium* sequences showed a high level of conservation in the NAC domain (Figure 3.13). Similar to the *NST1* putative orthologue (Table 3.1, section 3.3.1.1), this high level of conservation in the NAC domain regions was a good implication for correct identification of the putative targets.

Table 3.2 shows protein identity between the identified putative NST2 orthologues and their *Arabidopsis* NST2 counterpart. Although the identity does not seem to be high between *Arabidopsis* and the identified protein in monocots, the relatively good identity in the NAC domain region indicated that these proteins could have similar function and belonged to similar NAC family.

Table 3.2 Protein identity between the identified NST2 putative orthologues and *Arabidopsis* NST2. There is a low identity between the putative orthologues in rice, *Brachypodium* and barley and the *Arabidopsis* native protein. However, this identity increases between the identified putative orthologues in monocots.

Protein Identity				
	AtNST2	OsNST2	BradiNST2	HvNST2
AtNST2	100%	42%	39%	40%
OsNST2		100%	57%	65%
BradiNST2			100%	59%
HvNST2				100%

The *Arabidopsis* *NST2* gene is located on chromosome 3 and comprises 1227 base pairs and encodes for a 334 amino acid protein. This sequence is predicted to contain two exons and one intron. The identified putative

orthologue in rice encodes for a 396 amino acid protein (1188 bp) and comprised of three exons. In the close relative of barley, *Brachypodium*, the identified putative orthologous sequence is located on chromosome 1 and contains 1245 nucleotides in the form of three exons and two introns, and encodes for a 415 amino acid protein (Figure 3. 14).

The barley putative orthologue of *NST2* is located on chromosome 7H and comprises 1221 base pairs nucleotide (407 amino acids) containing three exons. The similarity between the gene structure of all identified putative sequences and the *Arabidopsis* native gene also suggested that these could have putative identical functions in various species.

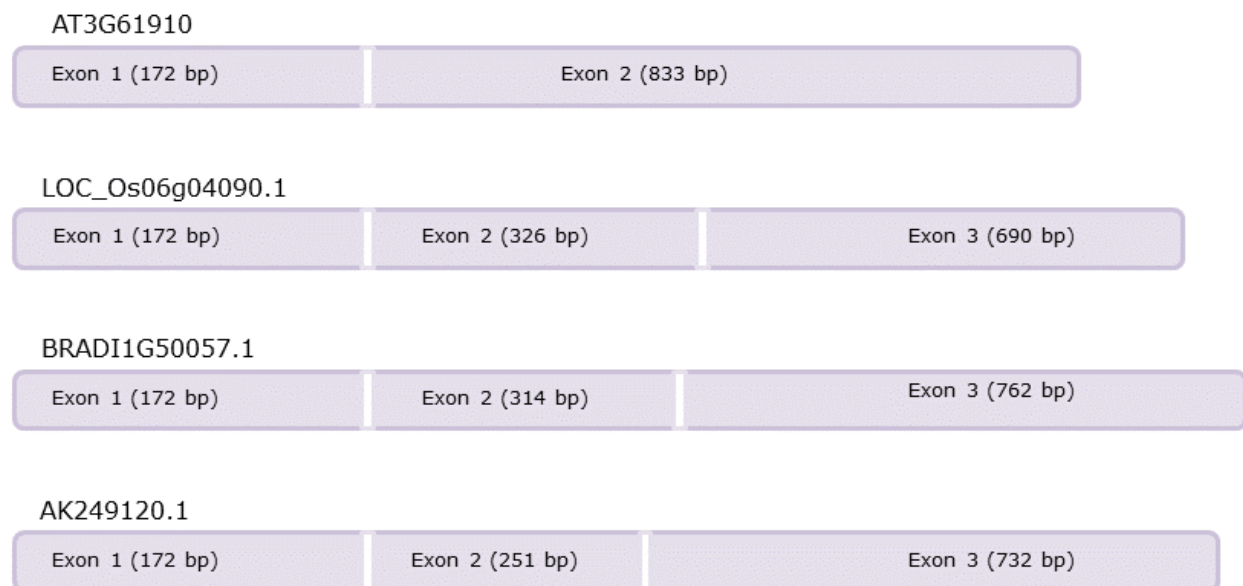


Figure 3. 14 Schematic presentation of gene structure *Arabidopsis NST2* and its putative orthologs. All putative sequences in rice, *Brachypodium* and barley appear to have retained the same number of exons.

The identification of the putative orthologous sequence in barley was further confirmed by going from barley towards *Arabidopsis*. This was done by using barley RNA-seq database (<https://ics.hutton.ac.uk/morexGenes/>). This approach was also resulted in identification of the same putative genes for rice, but did offer *Arabidopsis* NST1 gene as the ortholog instead. This could be due to the high level of identity between *NST1* and *NST2* in *Arabidopsis*.

3.3.2.3 HvNST2 Synteny

In order to further confirm the identified putative orthologs, a synteny analysis was performed. For the loci flanking *NST2* in *Arabidopsis*, rice, *Brachypodium* and barley. This showed that even though there were limited link between *Arabidopsis* and rice due to their evolutionary and taxonomic distance, there was still homologous sequences in the *NST2* flanking region between both species. This further confirmed identification of the putative ortholog in rice. Synteny between rice and *Brachypodium* and *Brachypodium* and barley also revealed a good syntenic relationship that further supported ortholog identification (Figure 3.15; appendix 9.7).

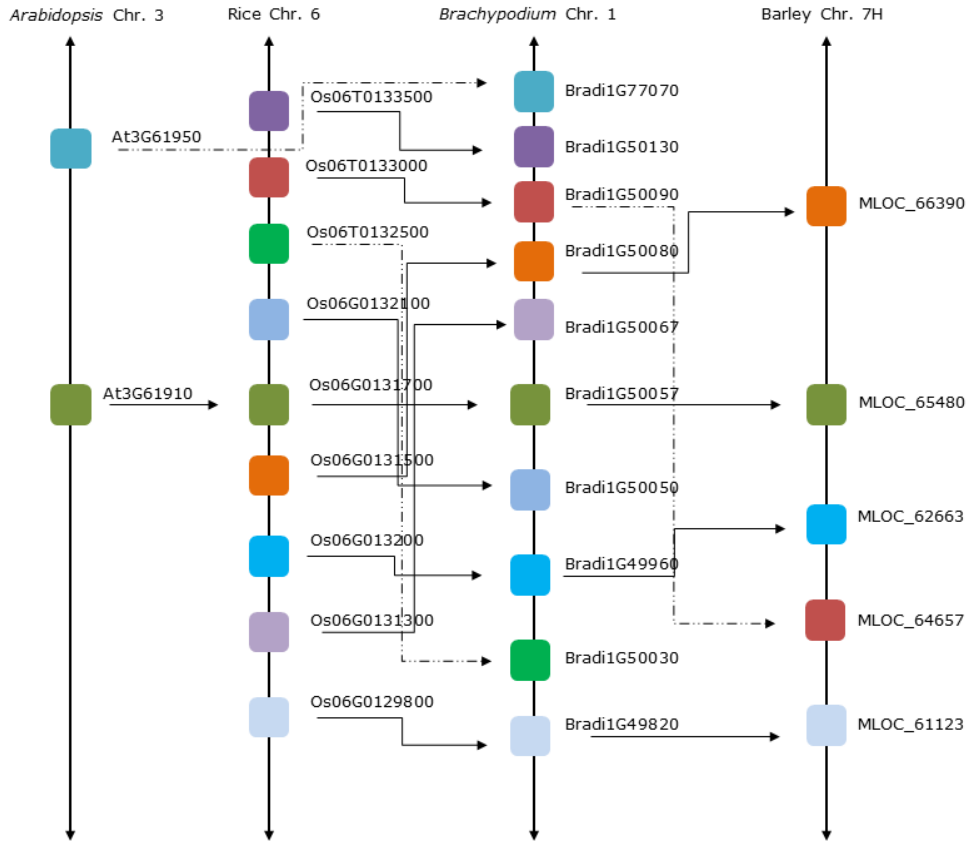


Figure 3. 15 A schematic representation of the synteny analysis between *Arabidopsis*, rice, *Brachypodium* and barley putative *NST2* flanking loci. High level of synteny between rice, *Brachypodium* and barley was observed in relation to the flanking regions of the identified putative *NST2*. The similar colours represent homologous genes.

3.3.2.4 Phylogenetic analysis

The protein sequence of the identified barley putative orthologue was blasted against the NCBI *Arabidopsis*, rice and *Brachypodium* protein databases along with IPK barley database to further identify similar proteins. These reciprocal BLASTs resulted in identification of a number of proteins showed similarity to the identified *NST2* gene. Further analysis of the relationship between these proteins and the identified putative orthologues in rice, *Brachypodium* and barley and also their relationship to

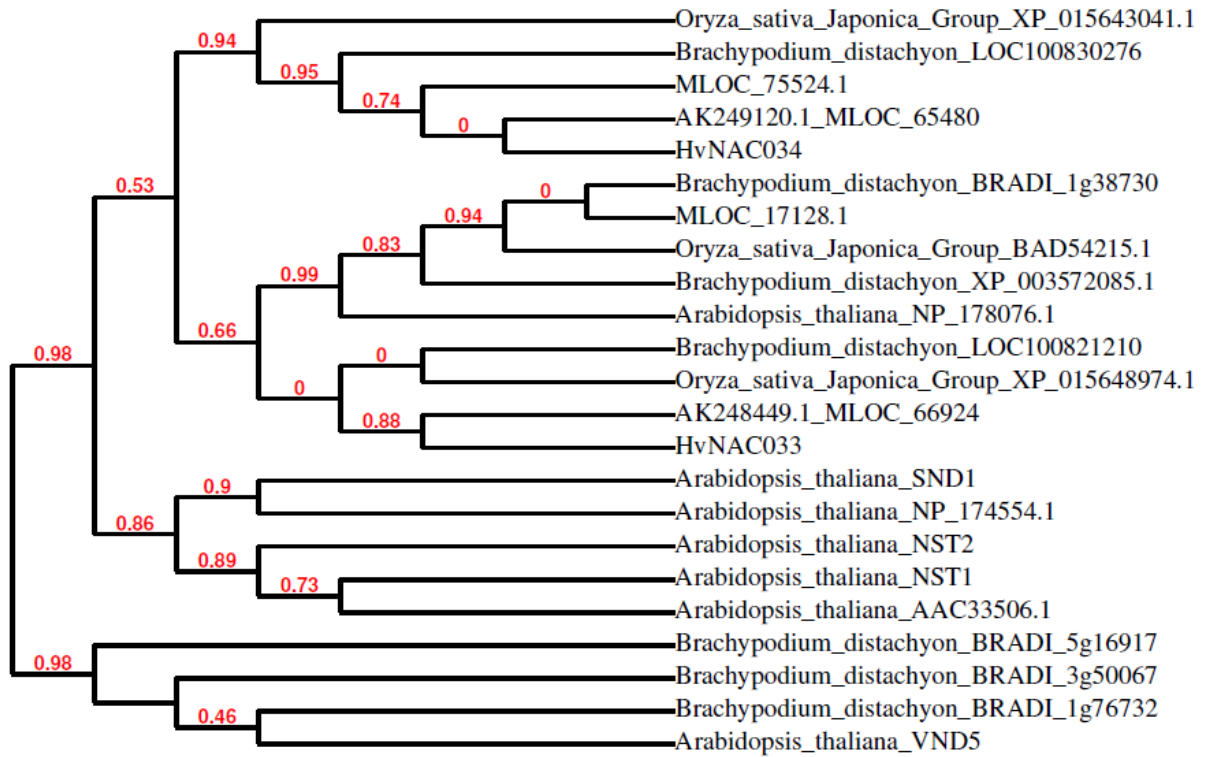


Figure 3. 16 Phylogenetic tree analysis for the identified putative NST2 and its similar protein sequences in *Arabidopsis*, rice and *Brachypodium*. This analysis showed close relationship between the identified putative orthologue in barley and its rice and *Brachypodium* counterparts. The analysis was done using online platform (<http://www.phylogeny.fr/>) (Dereeper et al., 2008). *Hordeum vulgare* AK249120.1/ MLOC_65480: NST2 putative orthologue; *Hordeum vulgare* AK148449.1/ MLOC_66924: NST1 putative orthologue; *Brachypodium distachyon* LOC100830276: putative NST2; *Brachypodium distachyon* LOC100821210: putative NST1; *Brachypodium distachyon* XP_003572085.1: predicted NAC domain-containing protein 76-like; *Brachypodium distachyon* BRADI_1g38730: hypothetical protein; *Brachypodium distachyon* BRADI_5g16917: hypothetical protein; *Brachypodium distachyon* BRADI_3g50067: hypothetical protein; *Brachypodium distachyon* BRADI_1g76732: hypothetical protein; *Oryza sativa* Japonica Group XP_015643041.1: putative NST2; *Oryza sativa* Japonica Group XP_015648974.1: predicted NAC domain-containing protein 43; *Oryza sativa* Japonica Group BAD54215.1: putative OsNAC7 protein; *Arabidopsis thaliana* AAC33506.1: NAM (no apical meristem)-like protein; *Arabidopsis thaliana* NP_174554.1: NAC domain containing protein 12.

Arabidopsis NST2 gene was explored by constructing their phylogenetic tree. The phylogenetic tree was made using online platform (<http://www.phylogeny.fr/>) (Dereeper et al., 2008). This analysis showed that the proposed HvNAC034 was identical to the identified NST2 putative orthologue in barley (Figure 3.16). Moreover, the identified putative orthologues in rice and *Brachypodium* were clustered with their barley counterpart

Furthermore, the identified putative protein sequence in barley was BLASTed against NCBI protein database to identify the similar proteins to the barley sequence. Phylogenetic analysis of the identified sequences showed that the identified rice and *Brachypodium* putative *NST2* and two sequences in barley of which one was the identified putative orthologues in *NST2* (AK249120) were clustered together along with other NAC related genes in wheat and maize (Figure 3.17). However, the identified putative orthologous *NST2* protein sequence in barley (AK249120) and MLOC_75524.1 were 65% identical. Therefore, based on a lower level of similarity to the putative *Brachypodium NST2* and MLOC_75524 cDNA coding sequences (64%) and the expected value of $2e-173$ for MLOC_75524 as opposed to zero for AK249120, it was decided to consider AK249120 as putative *NST2* sequence in barley.

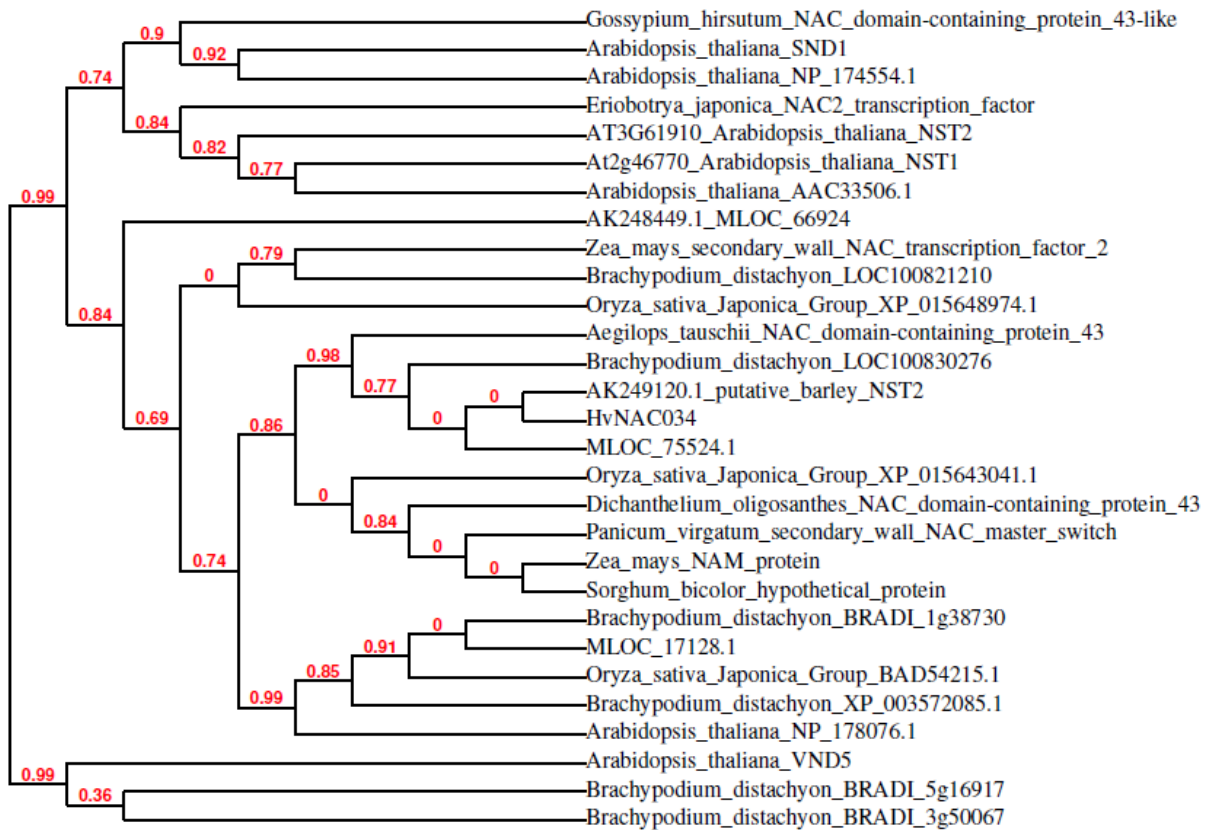


Figure 3. 17 Phylogenetic analysis of sequences similar to putative *Brachypodium* cDNA sequence in barley. This analysis showed a closely linked *Brachypodium* and rice putative NST2 genes with the identified barley putative *NST2* orthologue. *Hordeum vulgare* AK249120.1/ MLOC_65480: NST2 putative orthologue; *Hordeum vulgare* AK148449.1/ MLOC_66924: NST1 putative orthologue; *Brachypodium distachyon* LOC100830276: putative NST2; *Brachypodium distachyon* LOC100821210: putative NST1; *Brachypodium distachyon* XP_003572085.1: predicted NAC domain-containing protein 76-like; *Brachypodium distachyon* BRADI_1g38730: hypothetical protein; *Brachypodium distachyon* BRADI_5g16917: hypothetical protein; *Brachypodium distachyon* BRADI_3g50067: hypothetical protein; *Oryza sativa* Japonica Group XP_015643041.1: putative NST2; *Oryza sativa* Japonica Group XP_015648974.1: predicted NAC domain-containing protein 43; *Oryza sativa* Japonica Group BAD54215.1: putative OsNAC7 protein; *Arabidopsis thaliana* AAC33506.1: NAM (no apical meristem)-like protein; *Arabidopsis thaliana* NP_174554.1: NAC domain containing protein 12.

3.3.2.5 Expression analysis *HvNST2*

The expression analysis of *HvNST2* putative target was done using barley cDNA prepared from RNA extracted from staged flowers. The spikelets from developmental staging material were collected from cultivar Optic, according to non-destructive staging protocol (Fernandez Gomez and Wilson, 2012). The analysis of expression of the *HvNST2* putative orthologue in barley flower stages showed high expression at the tapetum degeneration stage before dropping at mitosis I (Figure 3.18). The expression peaked again at mitosis II till tri-nuclear stage. It was also observed that the *HvNST2* putative gene also has a high level of expression in vegetative tissues such as leaf, more expression observed in unopened leaf samples, stem and root (Figure 3.18).

The identified putative orthologous amino acid sequences in rice, *Brachypodium*, barley and the *Arabidopsis NST1* and *NST2* were compared (Table 3.3). This comparison showed medium level (maximum 52%) of identity between the identified putative orthologues. Although the sequence identity was not as high as the *Arabidopsis* orthologues, the potential differences in gene function in different species and the evolution pathways could cause these differences.

Table 3.3 the comparison between the proteins of the identified NST1 and NST2 putative orthologues and the *Arabidopsis* genes.

	AtNST1	OsNST1	BradiNST1	HvNST1
AtNST2	63%	44%	44%	45%
OsNST2	45%	52%	52%	52%
BradiNST2	42%	44%	47%	45%
HvNST2	44%	48%	49%	49%

Moreover the expression patterns of both *NST1* and *NST2* were aligned together to see any differences between the two putative genes (Figure 3.20). The expression profile of *HvNST2* was lower than the expression of *HvNST1* throughout anther developmental stages in barley. However, the expression of both putative orthologous genes were almost similar in stem and higher in root tissue (Figure 3.20).

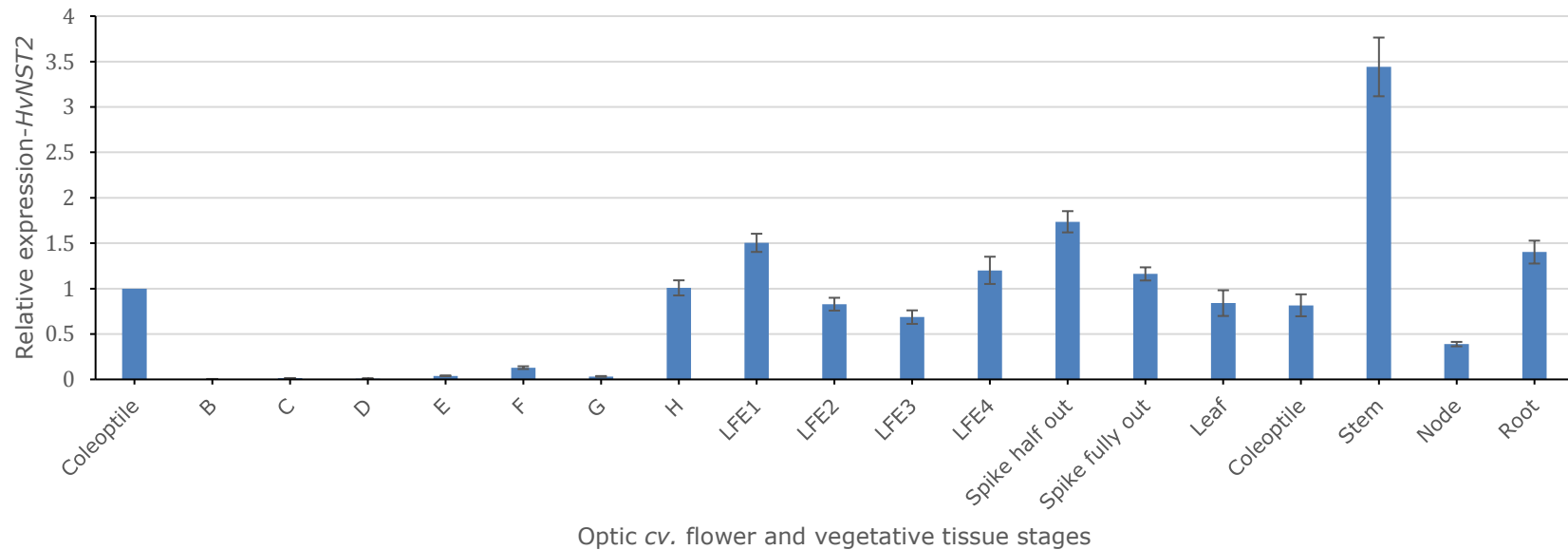


Figure 3. 18 Expression analysis of *HvNST2* putative orthologue in staged barley flowers. The expression showed a peak at mitosis I stage that was reduced gradually until the tri-nuclear stage that starts to increase right to the pollen release stage. This gene showed high level of expression in vegetative tissues especially stem and root compared to the floral organs' expression. The stages shown in the graph are as follows: B: secondary sporogenous cells to pollen mother cells. C: Pollen mother cells undergo meiosis. D: microspores are released from tetrad followed by tapetum vacuolation. E: free microspores, prominent tapetum layer starts to degenerate. F-G: microspores become vacuolated and tapetum degeneration. H-LFE2: mitosis I, tapetum is degenerating. LFE3: mitosis II occurs, binuclear pollen. LFE4: trinuclear pollen, septum breakage. Spike half out: pollen release. Spike fully out: fertilisation has happened and grains start filling. All the expressions were calculated relative to coleoptile expression levels.

The expression seen in barley flowers of the putative orthologues of *NST1* and *NST2* genes was similar to the expression profile compared to *Arabidopsis* *NST1* and *NST2* (Figure 3.19). However, in *Arabidopsis* there seems to be higher expression of *NST1* and *NST2* genes during meiosis. Moreover, there is higher expression of *NST2* at meiosis than *NST1* in *Arabidopsis* that was not observed in the barley putative orthologues.

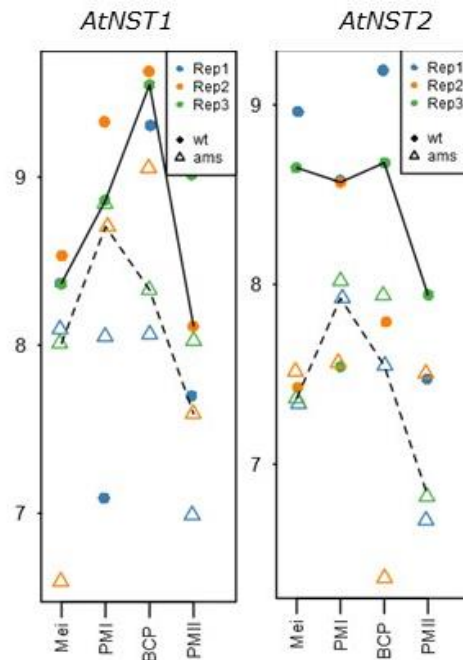


Figure 3. 19 The expression pattern of *AtNST1* and *AtNST2* (solid black line) and their expression in the *ams* mutant (dashed lines) (<https://www.cpib.ac.uk/tools-resources/resources/flowernet/>). In *AtNST1* the expression starts to increase from meiosis stage until bicellular pollen stage that is sharply reduced in pollen mitosis II. *AtNST2*, however, is expressed at meiosis stage but the expression reduces at pollen mitosis I stage. This is followed by a slight increase in expression at binuclear pollen stage and then the expression is reduced sharply at pollen mitosis II.

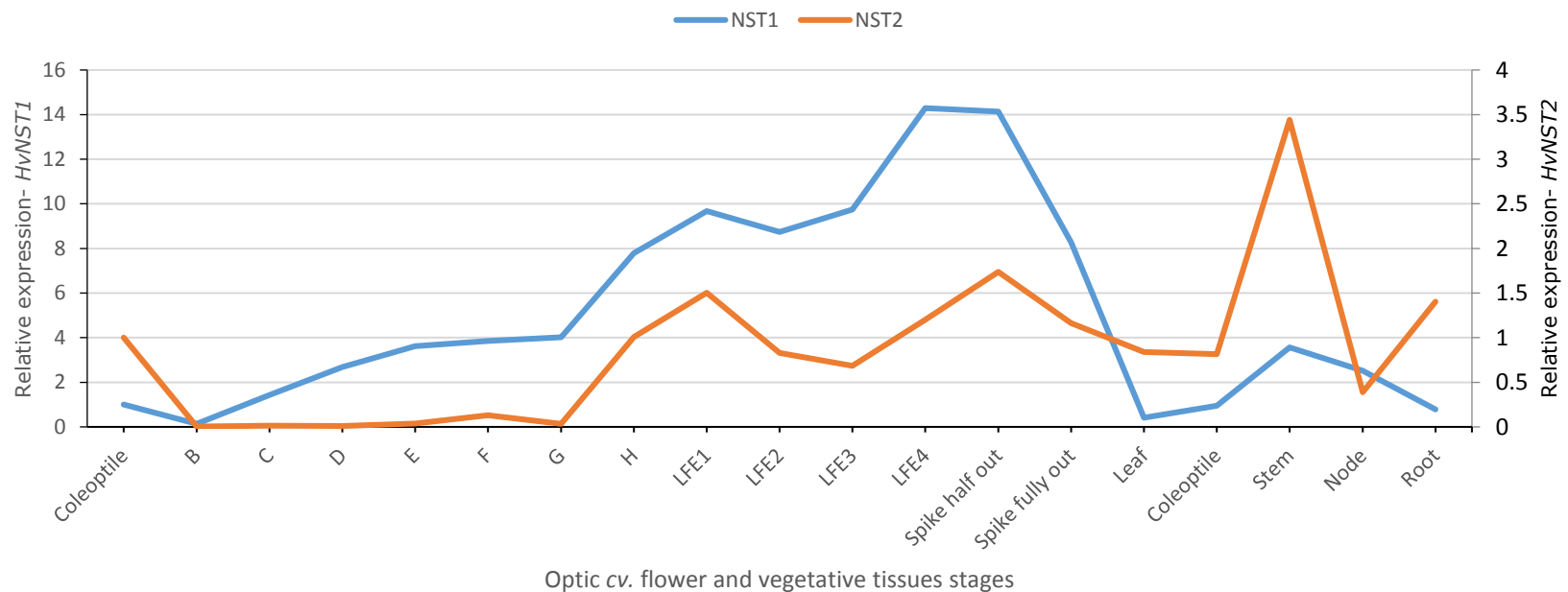


Figure 3. 20 The alignment of the expression profiles of both *HvNST1* and *HvNST2* putative orthologues in barley cultivar optic floral and vegetative tissues. It can be inferred from this that the *NST1* plays a major role during the anther development with higher expression compared to *NST2*. The expression of both candidate genes was similar in vegetative tissues, with slightly higher expression of *NST1* in stem and higher *NST2* expression in coleoptile. The stages shown in the graph are as follows: B: secondary sporogenous cells to pollen mother cells. C: Pollen mother cells undergo meiosis. D: microspores released from tetrad followed by tapetum vacuolation. E: free microspores, prominent tapetum layer starts to degenerate. F-G: microspores become vacuolated and tapetum degeneration. H-LFE2: mitosis I, tapetum is degenerating. LFE3: mitosis II occurs, binuclear pollen. LFE4: trinuclear pollen, septum breakage. Spike half out: pollen release. Spike fully out: fertilisation has happened and grains start filling. All the expressions were calculated relative to the one in coleoptile.

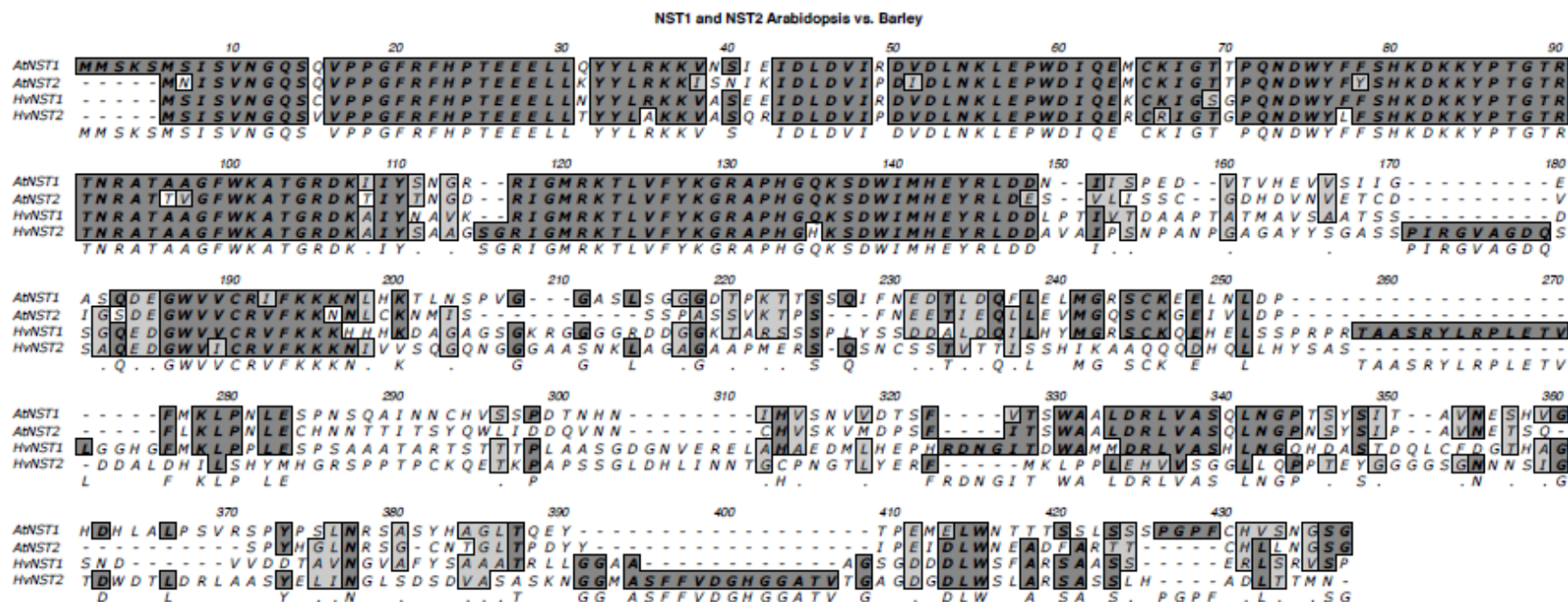


Figure 3. 21 Protein alignment between *Arabidopsis* and barley NST1 and NST2. This alignment showed high level of conservation in the NAC domain (between 14 until 55 amino acid) region.

NST1 expression in *Arabidopsis* is first seen at the meiosis stage, expression increases at pollen mitosis I stage and reaches its highest level at binuclear pollen stage, followed by a drop in expression at pollen mitosis II (Figure 3.19). Similar to *Arabidopsis*, barley *NST1* putative orthologue showed expression at meiosis stage and started to increase expression until pollen mitosis I (Figure 3.20). However there was no expression of barley *NST2* putative orthologue seen at this stage, which could potentially suggest a role of *Arabidopsis NST2* for the *HvNST1* putative orthologue. The expression of *HvNST1* showed a peak at mitosis stage that was also similar to that seen in *Arabidopsis* (Figure 3.20).

Contrary to *Arabidopsis*, the *NST1* expression in barley was then slightly dropped at pollen mitosis II stage but then increased and stayed high until anther senescence. This may be similar to *Arabidopsis* data is not available for stages after pollen mitosis II to compare these results.

Furthermore, the alignment of *Arabidopsis NST1* and *NST2* proteins against their barley counterparts showed high level of conservation in conserved domain (Figure 3.21).

3.4 Discussion

3.4.1 Identification of *HvNST1* and *HvNST2* putative sequences

Homology screening using the cDNA sequence comparison via BLAST analysis identified two putative orthologue sequences of *NST1* and *NST2* in barley (Figures 3.4, 3.6, 3.9, 3.10). These sequences were confirmed via synteny analysis between *Arabidopsis*, rice, *Brachypodium* and barley putative sequences. The synteny study showed high levels of conservation

between rice, *Brachypodium* and barley putative orthologues and their flanking loci for both *NST1* and 2 putative sequences. However, there was a lower correlation and similarity between *Arabidopsis* and rice targets. This is likely to be due to the genetic distance between the dicots and monocots that occurred around 200 million years ago (Salse et al., 2002). The erosion caused by evolution between *Arabidopsis* and rice genomes resulted in less homology between the two species that was estimated to be between 17% and in some reports up to 39% (Liu et al., 2001; Salse et al., 2002). Therefore, this indicates the small likelihood of observing a simple and one to one relationship between the target genes in *Arabidopsis* and their identified putative orthologous sequences in rice, *Brachypodium*, barley and other grass species. This was shown in the synteny analysis that there was not a clear and strong conservation in the flanking regions of the identified putative orthologous genes in rice compared to *Arabidopsis* (Figures 3.9 and 3.15). Furthermore, the lack of available linear ordering of barley genome makes the construction of synteny map for barley more challenging.

Syntenic analysis showed conservation between rice, *Brachypodium* and barley chromosomes in the flanking regions of the putative *NST1* and *NST2* genes. Although synteny analysis on its own does not provide a strong validation to correct identification of the putative orthologous sequences, it can provide useful information regarding conservation between the regions of the genome where the putative orthologous genes are located. The synteny analysis performed using the available genetic map from *Arabidopsis*, rice, *Brachypodium* and barley in Gramene database. However, in the case of barley genetic map it is possible that incorporation of more chromosomal bins into current barley genetic map could affect the arrangements of the genes and thus alteration in the synteny map between barley and rice and *Brachypodium* genomes. Nevertheless, the current

synteny analysis showed a high level of conservation between the crop species used, and incorporation of more information to the barley genetic map may as well improve and show stronger conservation in the current (Figures 3.9 and 3.15) synteny maps.

Moreover, the separation of *Arabidopsis* and grasses protein clades in the phylogenetic studies for both putative HvNST1 and HvNST2 (Figures 3.8, 3.16 and 3.17) indicates that there may not be a one to one correspondence between the genes in grass species and *Arabidopsis*. The high linkage between *Arabidopsis* NST1 and NST2 proteins in the phylogenetic tree was expected due to the paralogous nature of these genes. However, the lower protein identity between the two identified putative orthologues in barley and their separation in the phylogenetic tree resulted in the assumption that their phenotype may be distinct from that of *Arabidopsis*. Therefore, this shows the importance of generation of RNAi, SRDX repression, and overexpression lines for the identified putative orthologues in order to confirm their function in barley.

Furthermore, the observation that MLOC_75524 showed close phylogenetic relationship to the identified putative HvNST2 orthologue (Figure 3.17) indicates that there may have been potential duplication of the *NST* genes in barley compared to their *Arabidopsis* counterparts. Thus, this means that the putative phenotype that was expected from the RNAi and repression lines may not be similar to the observed phenotype in *Arabidopsis*, due to the presence of additional transcripts and potential interaction between the putative duplicate of NST2 and NST1 and NST2 in barley. Confirmation of the identified putative orthologous sequences could be achieved by the ability to complement the *Arabidopsis* mutants. Therefore, this further analysis is needed to complement both *nst1* and *nst2* mutants in *Arabidopsis* with their putative orthologous genes in barley.

The comparison of the expression profiles of *Arabidopsis NST1* and *NST2* against the identified putative sequences in rice was also performed to confirm the selection of the correct putative sequences. The *Arabidopsis NST1* is highly expressed in reproductive organs such as flowers and seeds and in non-reproductive tissues such as the stem (Figure 3.3 A). The expression of the rice putative orthologue was observed in the reproductive organs, but it was also seen in non-reproductive tissues such as root, leaf blade and stem (Figure 3.3 B). The expression of *Arabidopsis NST2* was more specific to the floral tissues and seen mostly in the anthers and some expression in stem (Figure 3.11 A).

The expression of *NST2* rice putative was also more prominent in the anthers and it was expressed in the stem (Figure 3.11 B). Using the identified rice putative orthologue, the *Brachypodium* putative sequence of *NST1* and *NST2* were identified. These sequences were used with the IPK barley BLAST server (<http://webblast.ipk-gatersleben.de/barley/>) to identify the sequences in barley coding sequence database that showed high level of identity to the *Brachypodium* sequence.

Moreover, these sequences were further confirmed by using the identified barley putative orthologue moving backwards from barley towards *Arabidopsis* using barley RNA-seq database (<https://ics.hutton.ac.uk/morexGenes/>). This analysis was done as quality control for identification of the putative targets in barley. As a result of this analysis, the same putative orthologues in rice and *Arabidopsis* for both *NST1* and *NST2* were identified. Although in the case of *NST2* the server offered *Arabidopsis NST1* as the orthologue that could be due to high level of identity between *Arabidopsis NST1* and *NST2* genes.

Moreover two NAC domain transcription factors (*HvNAC033* and *HvNAC034*) have been proposed as putative *NST1* and *NST2* respectively in barley (Christiansen et al., 2011). This conclusion was drawn based on

protein identity with *Arabidopsis NST1* and *NST2* and *Medicago NST1* without any further expression analysis in vegetative and reproductive stages in barley. The identification of putative orthologous sequences of barley *NST1* and *NST2* in the current study resulted in identification of the same sequences as Christiansen et al. (2011). These were identified and confirmed by performing expression analysis in barley and comparing the results to the available data on *Arabidopsis* and rice. Furthermore, synteny analysis of the identified putative orthologues also confirmed to correct identification of these sequences.

3.4.2 HvNST1 and HvNST2 expressions

The expression of the identified *NST1* and *NST2* in barley was used to help determine the potential role of these two transcription factors in barley anthers. The putative barley *NST1* gene showed to be expressed mainly in floral tissues, and slightly less in non-reproductive tissues such as stem and nodes (Figure 3.10). The *NST2* putative orthologue in barley was also expressed in floral tissues, but in a lower level than its paralog (Figure 3.18). However, it was also showed that the *NST2* putative orthologue expressed in stem and root tissues (Figure 3.18).

The comparison between the expression pattern of putative *NST1* and *NST2* in barley reproductive and vegetative tissues suggested a major role for *NST1* in the anthers as compared to *NST2* (Figure 3.20). The barley putative orthologue of *NST1* showed higher expression in early mitosis I (almost seven fold) and in trinuclear pollen (nearly 14 fold) stages. Both *HvNST1* and *HvNST2* showed nearly four-fold increased expression in the stem tissue (Figure 3.20). This was in accordance to the observation that *NST1* in *Arabidopsis* in interaction with *SND1* regulates secondary wall

thickening in the fibres, interfascicular fibres and the xylary fibres of the stem (Mitsuda et al., 2007; Zhong and Ye, 2015). Furthermore, the observation that the putative barley *NST2* was expressed in stem tissues nearly in the same level as *NST1* (Figures 3.20), was an indication to the correct identification of this putative orthologue and also potential similar role in barley as the *Arabidopsis NST2* is also expressed, in lower level, in the stem (Zhong and Ye, 2015).

The expression of *NST2* putative orthologue in barley followed a similar pattern as its *Arabidopsis* counterpart. *NST2* expression in *Arabidopsis* can be seen at the meiosis stage. This expression drops slightly at pollen mitosis I, however, it shows a minor increase at binuclear pollen stage followed by a sharp reduction in expression at pollen mitosis II (Figure 3.12).

Contrary to *Arabidopsis*, the barley putative orthologue of *NST2* did not show any expression at the pollen meiosis stage, but the putative barley *NST1* showed some expression at meiosis stage in barley (Figure 3.20). This could potentially mean that barley *NST1* could act similar to *Arabidopsis NST2*. However, this requires detailed study of expression of these two putative genes in flower stages and analysis of their interaction with their putative targets in barley. The expression of *HvNST2* putative orthologue was observed at mitosis I stage. This was then reduced at the end of mitosis I and showed even lower expression at mitosis II stage (Figure 3.18). This was again increase at tri-nuclear pollen stage and stayed high until anther senescence.

The two identified putative orthologues showed similar expression patterns, although *NST2* showed a lower expression compared to *NST1*. Higher expression of *HvNST1* in floral tissues suggests a major role for this transcription factor in the anther and especially during anther dehiscence. Despite its lower expression in floral tissues, *HvNST2* as the paralogue of

NST1 followed similar expression profile that was an indication that the function of *HvNST2* in the anthers is to co-regulate anther dehiscence via *NST1*.

Chapter Four:

Characterisation of *HvNST1* and *HvNST2* putative orthologues

4.1 Introduction

Functional characterisation of genes affecting flower development in cereals is of utmost importance as there is escalating need to improve cereal crop yield, and to increase food supply and help address the global food security challenge. This is more important in relation to genes affecting fertility of plants as they directly contribute to crop yield. Typically, geneticists have relied on identification of a mutation in order to define components in a particular pathway without prior knowledge of the genes involved (Lawson and Wolfe, 2011).

This characterisation of genes of interest can be performed via either forward or reverse genetic approaches. The forward genetics approach relies upon observation of phenotype without any prior knowledge about the genes affecting this. One of the ways of localising the underlying genes causing the phenotype is screening mutant populations containing random mutation in their genome (Lawson and Wolfe, 2011). Generation of heritable mutations is the first step for forward genetics approaches. In plants these mutations can be generated by a variety of methods such as physical, chemical and biological agents (Alonso and Ecker, 2006).

Due to their high efficiency, chemical agents such as ethyl methane sulphonate (EMS) and nitrosomethylurea (NMU) are frequently the preferred method of choice. Under optimal conditions, EMS treated seeds generate approximately 400 mutations per genome (Alonso et al., 2003). The chemical mutagens generate a broad range of alterations, these are mostly single base-pair substitutions, but also include small deletions and insertions (Alonso and Ecker, 2006; Till et al., 2003).

However, despite advances in science and technology such as the availability of whole genome sequence, large number of mapped

polymorphisms and cheaper and quicker genotyping methods, forward genetics approaches can still take a long time to identify the particular gene corresponding to a specific mutation (Alonso and Ecker, 2006).

On the other hand, reverse genetics is based upon selecting a gene and generating targeted mutations for this in the organism under study. In this approach the goal is to characterise the phenotype for the target gene in order to assign gene function and phenotype. The development of novel molecular methods such as gene silencing, ectopic expression, Targeting Induced Local Lesions in Genome (TILLING), Chimeric Repressor Gene Silencing Technology (CRES-T) and many other techniques has made reverse genetics approaches significantly more efficient than before. Furthermore, the development of successful *Agrobacterium* mediated transformation for model plants such as *Arabidopsis* and more commercially important species such as barley and wheat has provided a more efficient way study targeted mutations (Comai and Henikoff, 2006; Mitsuda et al., 2011).

Proteins that contain sequence specific DNA-binding that are able to activate or repress transcription are called transcription factors (Riechmann et al., 2000). Transcription factors bind via their DNA binding domain to a specific region on the promoter or enhancers of their target genes, known as *cis*-regulatory elements, to activate their target genes (Melzer and Theissen, 2011). It is estimated that around 10% of higher plant genome encodes for transcription factors. These transcription factors are important in regulation of intercellular signalling, plant development, reproduction, cell cycle and metabolism and response to environment (Yuan and Perry, 2011).

In *Arabidopsis*, transcription factors (TFs) comprise a large gene families compared to other species such as *D. melanogaster* or *C. elegans* (Mitsuda and Ohme-Takagi, 2009). For instance, it is estimated that zinc finger

transcription factors form half of the all TFs in *D. melanogaster*, whereas this only represents nearly 20% of the TFs in *Arabidopsis* (Hiratsu et al., 2003; Riechmann et al., 2000). The regulatory role of transcription factors involves activating or repressing the downstream targets.

Therefore, large number of transcription factors and their important effect on variety of developmental processes in plants, necessitates the identification of their functions especially in commercially important crops. Fusion of activating or repressor domains is one of the approaches used for studying transcription factors.

The identification of class II ERF and a number of TFIIIA-type zinc-finger transcription factors that act as repressors showed that they contain a repression domain at the C-terminal of the protein that includes a conserved ERF-associated amphiphilic repression (EAR) motif (L/FDLNL/FXP) (Ohta et al., 2001). This suppression domain is essential for normal development of plant and as it was shown in *Superman* (*SUP*) protein the suppressive activity of SUP is important for normal development of flowers (Hiratsu et al., 2002). This observation led to utilisation of the repressor domain as an effective tool in transcription factors studies.

Chimeric REpressor gene Silencing Technology (CRES-T) is a tool that enables functional analysis of transcription factors in plant systems. This gene silencing method relies on fusion of EAR-motif (EAR-associated amphiphilic repression) domain to the transcription factor under study (Mitsuda et al., 2011). This plant specific domain is present in various transcription factors and has been shown to bring about changes in chromatin structure, and thus reduce gene expression (Viola and Gonzalez, 2016).

It is reported that EAR repression domain functions via its interaction with other co-repressors such as TOPLESS and SAP18 (Kagale and

Rozwadowski, 2011; Viola and Gonzalez, 2016). This dominantly suppresses the target genes of that particular transcription factor over that endogenous and functionally redundant transcription factors (Mitsuda et al., 2011) (Figure 4.1). As a result of this suppression, the transgenic plants exhibit loss of function phenotype that is desired in functional analysis of functionally redundant transcription factors (Mitsuda et al., 2011). This gene repression method is important as due to gene duplication in plants' genome there are a large number of redundant genes and transcription factors that regulate various developmental processes (Sasaki and Ohtsubo, 2016).

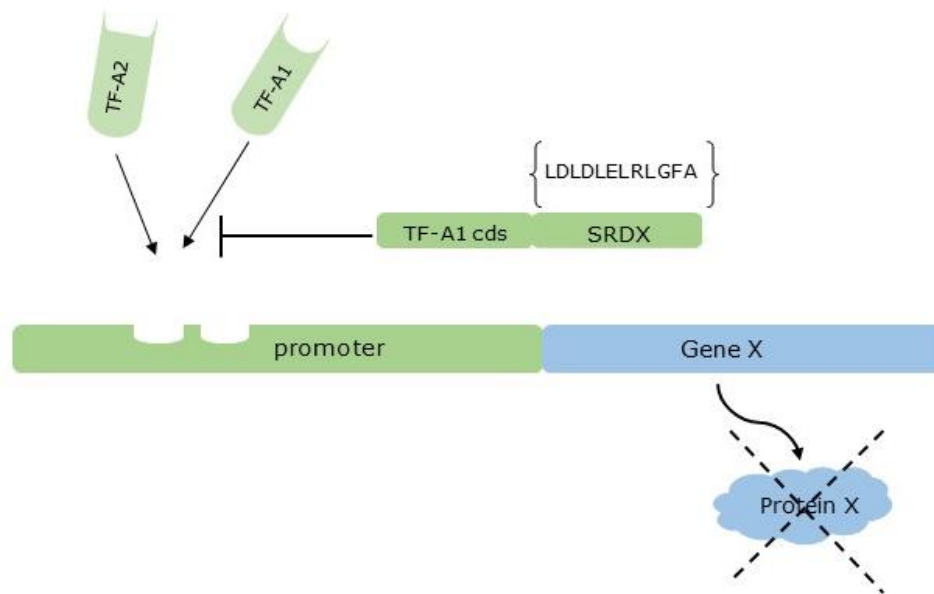


Figure 4.1 Schematic presentation of SRDX function in plants (Sasaki and Ohtsubo, 2016). In the absence of the SRDX domain the transcription factors (in this case two redundant imaginary transcription factors A1 and A2) recognise their binding sites in the target gene's promoter. However, the presence of SRDX blocks this interaction; hence, repression of the gene's expression.

A modified version of this repressor domain that is used in repressing transcription factors in plants is called Superman Repression Domain X (SRDX). The requirement for only this short amino acid sequence (LDLDLELR LGFA) in a fusion to various transcription factors is the advantage of the SRDX motif (Viola and Gonzalez, 2016).

RNA interference (RNAi) is another technique that can be used in reverse genetics studies. This technique is based on the insertion of a complimentary short nucleotide sequence that form a hairpin. The transcribed double stranded RNA is then recognised and cleaved by a nuclease called DICER, that generates small interfering RNAs (siRNA) (Senthil-Kumar et al., 2010). These small fragments then integrate into the effector complex RNA-induced silencing complex (RISC), during which the siRNA is unwound and is able to bind to the target mRNA. This results in sequence specific degradation of corresponding endogenous homologous mRNAs in the cytoplasm (Senthil-Kumar et al., 2010; Travella et al., 2006). The RNAi silencing technology has been used to study the effects of changes in a gene expression in order to identify their underlying importance in plant's development and further investigation of their involvement in responses to biotic and abiotic stresses (Senthil-Kumar et al., 2010).

Functional characterisation of the identified putative *NST1* and *NST2* orthologues (Chapter 3) was carried out by RNAi silencing and/ or SRDX repression, and ectopic expression of the putative orthologous genes in the target species. The ability of the characterised putative orthologous genes to complement their corresponding *Arabidopsis* mutants was also explored.

4.2 Materials and Methods

The characterisation of the identified targets was done via reverse genetics approaches. The identified sequences (see sections 3.3.1.1 and 3.3.1.2) were used to design silencing or repressing constructs for both targets (*HvNST1* and *HvNST2*).

4.2.1 Silencing constructs

The analysis of the function of the identified putative targets was carried out via silencing or SRDX repression of the putative target genes (*HvNST1* and *HvNST2*) in barley. For this purpose two separate constructs, RNAi silencing and SRDX repressor, were prepared for each target gene. The RNAi silencing method relies upon the formation of hairpin between two complementary non-coding RNA fragments introduced to the cell (Senthil-Kumar et al., 2010). This hairpin is then recognised and digested by the dicer in the cell that results in the generation of small fragments that bind to their complementary fragments of the endogenous gene. The small double stranded fragments is then digested by the dicer enzyme that causes changes in general down regulation of the target gene's expression.

The silencing fragments were amplified targeting a unique region of the gene of interest with sizes between 200 to 500bp. The primers used for these amplifications and for further analysis of the insertion of the gene inside the plasmid are shown in Table 4.1.

Table 4.1 Primers used for preparation of silencing, repression, overexpression and complementation constructs.

<u>Name</u>	<u>Sequence (5'... 3')</u>
HvNST1RNAi Forward	GCTTGACCAGATCCTGCACT
HvNST1RNAi Reverse	TAGAAGGCAACCCCATTGAC
HvNST2RNAi Forward	TCATCTGCAGGGTGTTCAAG
HvNST2RNAi Reverse	GTCGGTGCCGATGCTATTAT
HvNST1CDS Forward	ATGAGCATCTCGGTGAATGG
HvNST1CDS Reverse	TGGTGACACACGGCTCAGCC
HvNST2CDS Forward	ATGAGCATCTCGGTGAACGG
HvNST2CDS Reverse	ACCACCGCCGTACCCGTACGT
<i>HvNST1</i> SRDX Reverse	TTAAGCGAAACCCAAACGGAGTT CTAGATCCAGATCCAGTGGTGACA CACGGCTCAGCC
<i>HvNST2</i> SRDX Reverse	TTAAGCGAAACCCAAACGGAGTTCTAGATCCAGAT CCAGACCACCGCCGTACCCGTACGT
M13 Forward	TGTAACACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
Ubi proF1	ATGCTCACCTGTTGTTTGG
Iv2F1	CCAAAATTTGTTGATGTGCAG
HvNST1CDS-BP-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATG AGCATCTCGGTGAATGG
HvNST1CDS-BP-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATG GTGACACACGGCTCA
HvNST2CDS-BP-F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATG AGCATCTCGGTGAACGG
HvNST2CDS-BP-R1	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAC CACCGCCGTACCCGT
pBract214 Forward	TTTAGCCCTGCCTTCATACG
pBract214 Reverse	CGCGCAATTAACCCTCACTA

Figures 4.2 shows the location of the primers used for the amplification of the desired fragments (i.e CDS, RNAi, SRDX) and also qRT-PCR analysis in *HvNST1* and *HvNST2* putative orthologues.

4.2.2 Barley transformation

Barley transformation was done according to Harwood *et al.* (2009) protocol. The immature barley embryos from Golden Promise cultivar were isolated and inoculated in order to generate transgenic lines (Section 2.11.2).

4.2.3 Fertility analysis

The fertility of the plants were calculated based on the ratio between the number of florets in spike and the number of seeds set per spike. This was done by selecting spikes from five central tillers of the plant.

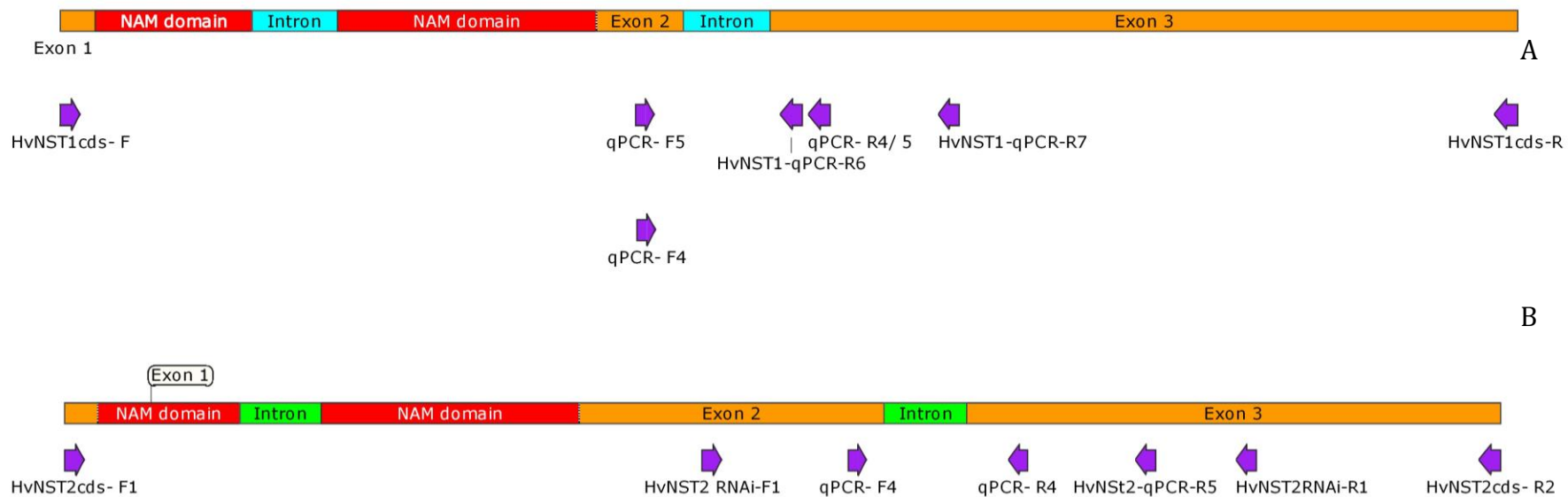


Figure 4.2 Primer locations used for coding sequence, RNAi and SRDX silencing/ repression fragments amplification and qRT-PCR analysis in *HvNST1* (A) and *HvNST2* (B) putative orthologues.

4.3 Results

4.3.1 RNAi construct

4.3.1.1 *HvNST2*^{RNAi} construct

In order to prepare this construct a unique region of *NST2* gene was amplified (464bp) using *HvNST2*^{RNAi} forward and reverse primers (Table 3.1) (Figure 4.3 A). The amplified fragment was then cloned into entry vector (pCR/8GW/TOPO). Positive plasmid containing the fragments in the correct orientation (Figure 4.3 B) was purified and used to transfer the fragment into the destination vector (pBract207) via the LR reaction. The presence of both arms of the RNAi transgene into the destination vector in the correct orientation was confirmed via colony PCR (Figure 4.3 C) and sequencing of the destination vector.

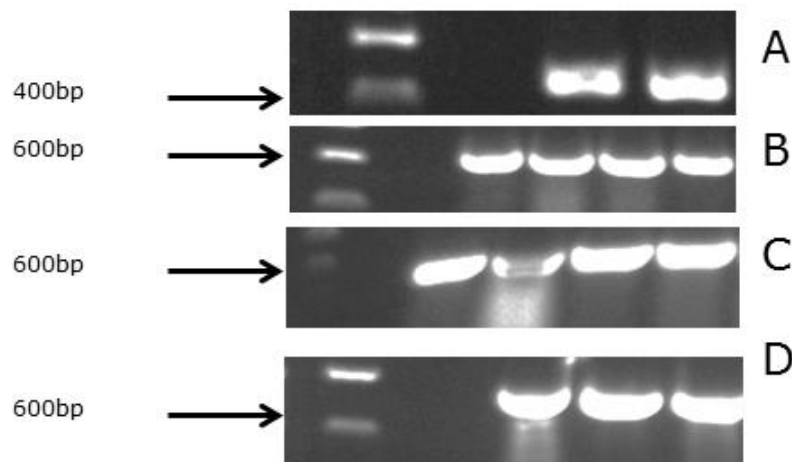


Figure 4.3 Preparation of RNAi silencing construct for *HvNST2*^{RNAi}. The first lane on each image indicates the DNA marker, and following lanes are DNA amplification of the same template. A: *HvNST2*^{RNAi} fragment amplification using *HvNST2*^{RNAi} forward and reverse primers. B: Analysis of the presence of *HvNST2* RNAi fragment inside entry vector using M13 forward and *HvNST2*^{RNAi} reverse primers. C: Analysis of the colonies grown after LR reaction. The analysis was performed using Ubi promF1 and *HvNST2*^{RNAi} reverse primers. D: Analysis of colonies after electroporation of *HvNST2*^{RNAi}:pBract207 into *Agrobacterium* using Ubi promF1 and *HvNST2*^{RNAi} primers.

The destination vectors containing the RNAi fragment were electroporated into *Agrobacterium* AGL1 strains along with the helper plasmid (pSoup) (Hellens et al., 2000). The colonies grown after the electroporation were analysed for the presence of the transgene (Figure 4.3 D). The colonies containing the plasmid with the insert inside were used for preparing a glycerol stock and plant transformation.

4.3.2 Repressor constructs

4.3.2.1 *HvNST1*^{SRDX} construct

The SRDX repressor construct of barley *NST1* putative orthologue was prepared by amplifying the fragment using *HvNST1*CDS forward and *HvNST1*^{SRDX} reverse primers (Table 3.1). The coding sequence of the target gene was amplified by including the SRDX motif in the reverse primer before the stop codon (1243bp) (Figure 4.4 A). The amplified fragment was cloned into the entry vector (pCR/8GW/TOPO) (Figure 4.4 B).

The positive plasmid was confirmed for the presence of the fragment by sequencing. The plasmid containing the fragment in the correct orientation was used in LR reactions to transfer the DNA fragment into the destination vector (pBract214). The presence of the fragment inside the plasmid was confirmed via colony PCR (Figure 4.4 C) and further sequencing of the positive colonies. The destination vectors containing the fragment were electroporated into *Agrobacterium* strain AGL1. The colonies grown after the electroporation were further analysed for the fragment (Figure 4.4 D) and the positive colonies were used to prepare glycerol stock and infect plant tissues.

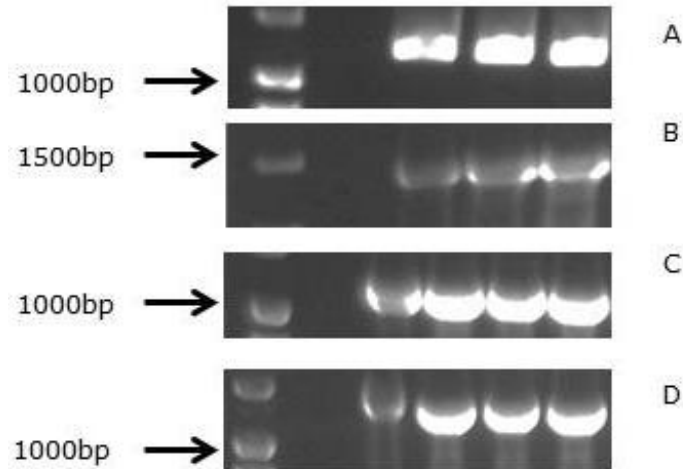


Figure 4.4 Preparation of the *HvNST1*^{SRDX} construct. The first lane of each image shows the DNA marker and the following lanes are DNA amplification of the same product. A: Amplification of full length of *HvNST1* including SRDX motif at the 5' end of the sequence. B: Analysis of the colonies grown after cloning of *HvNST1*^{SRDX} fragment into pCR/8GW/TOPO vector. This analysis was performed using M13 forward and *HvNST1* cDNA reverse primers. C: Analysis of the colonies after cloning *HvNST1*^{SRDX} fragment into destination vector pBract214. The analysis was performed using pBract214 forward and *HvNST1* cDNA reverse primers. D: Analysis of the colonies grown after transformation of *Agrobacterium* competent cells with pBract214 forward and *HvNST1*^{RNAi} reverse primer.

4.3.2.2 *HvNST2* repression construct (*HvNST2*^{SRDX})

This construct was prepared by PCR amplification of the full length of the putative coding sequence using *HvNST2*cds forward and *HvNST2*^{SRDX} reverse primers (Table 4.1). The amplified fragment that contained the SRDX motif at the 3' region of the coding sequence before the stop codon was TOPO cloned into the entry vector (pCR/8GW/TOPO). The entry vector containing the fragment in the correct orientation was used for LR reaction and transference of the fragment into expression vector (pBract214) (Figure 4.5). The expression vector containing the fragment of interest was

then confirmed via sequencing and was used for transformation of *Agrobacterium* competent cells.

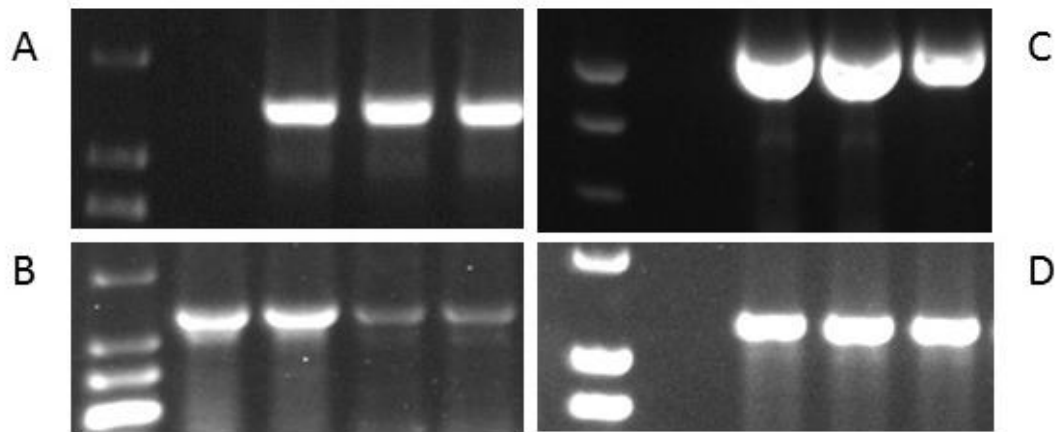


Figure 4.5 preparation steps for *HvNST2* repression construct. The first lane of each image indicated the DNA marker and the following lanes after the marker are the amplification of the same DNA product. The putative *HvNST2* coding sequence with the SRDX domain attached to its 3' end was amplified (A) and TOPO cloned into entry vector pCR/8GW/TOPO (B). The fragment was then transferred to the destination vector via the LR reaction (C). The destination vector containing the putative *HvNST2*^{SRDX} fragment was then used for transforming AGL1 *Agrobacterium* competent cells and the presence of the fragment in the AGL1 competent cells was confirmed afterwards (D).

4.3.3 Overexpression constructs

4.3.3.1 *HvNST1* overexpression construct

The overexpression construct was prepared via amplification of the full length of the gene including the stop codon with *HvNST1*cds forward and reverse primers (Table 4.1). This fragment was purified and TOPO cloned into the entry vector (pCR8GW/ TOPO). After confirmation of the presence

of the fragment inside the vector via colony PCR and sequencing, the positive plasmid was used to transfer the fragment into the destination vector (pBract214) via LR reaction (2.11.2) (Figure 4.6). The plasmid containing the fragment inside the vector (confirmed by sequencing) was electroporated into *Agrobacterium* strain AGL1. The colonies containing the fragment were used for preparing glycerol stock and infecting plant tissues.

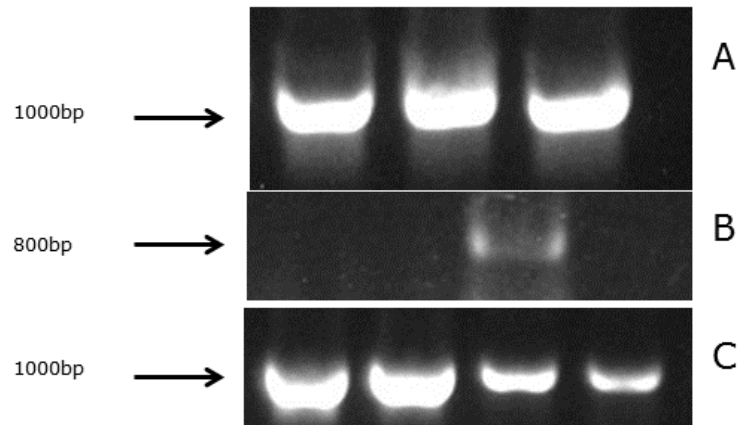


Figure 4.6 Preparation of the *HvNST1* overexpression construct. The first lane on each gel image indicates DNA marker and the lanes following the marker are the amplification of the same DNA product. A: amplification of the coding sequence of the putative orthologue (1197bp). B: TOPO cloning of the coding sequence into entry vector, pCR8GW/TOPO, and colony PCR for the confirmation of the presence of the gene. C: LR reaction to clone the fragment into expression vector, pBract214, and colony PCR to confirm the presence of the gene into the expression vector.

4.3.3.2 *HvNST2* overexpression

The overexpression construct for *HvNST2* was prepared by amplification of the full length of the coding sequence using *HvNST2*cds forward and reverse primers (Table 4.1). The amplified fragment was TOPO cloned into entry vector (pCR/8GW/TOPO). The entry vector containing the coding

sequence in the correct orientation was used for LR reaction to transfer the fragment into destination vector (pBract214). The presence and correct orientation of the fragment in the destination vector was confirmed by sequencing and the positive vector was used to transform *Agrobacterium* competent cells (Figure 4.7).

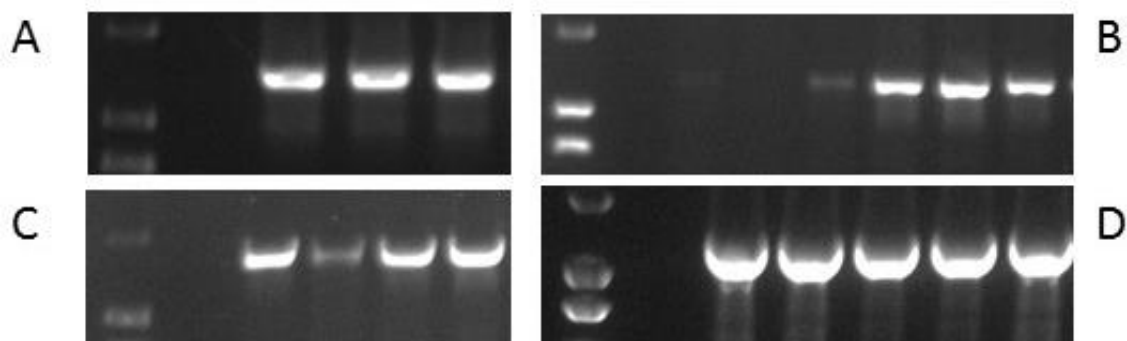


Figure 4.7 Preparation of *HvNST2* overexpression construct. The first lane on each gel image indicates DNA marker and the lanes following the marker are the amplification of the same DNA product. The putative *HvNST2* coding sequence containing stop codon was amplified (A) and TOPO cloned into entry vector pCR/8GW/TOPO (B). The fragment was then transferred to destination vector via the LR reaction and the presence of the fragment in the correct orientation was confirmed (C). Finally, the *Agrobacterium* competent cells AGL1 were transformed with the destination vector containing the putative *HvNST2* coding sequence and the presence of the fragment was then confirmed (D) before inoculating immature barley embryos.

4.3.4 Complementation constructs

Functional analysis of the putative orthologous sequences was done via study of the complementation of the *Arabidopsis* mutant by the barley putative orthologue. For this purpose the full length of the putative coding sequence was ectopically expressed in *Arabidopsis nst1NST2* heterozygote lines. Furthermore, the putative coding sequences of both *HvNST1* and *HvNST2* were used for ectopic expression in *Arabidopsis* wild-type plants.

4.3.4.1 *HvNST1* complementation construct

In order to prepare the complementation construct for *HvNST1*, the full length of the putative coding sequence was amplified using the primers listed in Table 4.1 and cloned to entry vector pDonr221 via the BP reaction. The presence of the fragment in the entry vector was confirmed by colony PCR and sequencing (Figure 4.8). The putative fragment was then transferred to the destination vector (pUB::YFP) vector (Grefen et al., 2010) containing *Arabidopsis* ubiquitin promoter via the LR reaction. The vector containing the fragment of interest (Figure 4.9) was then used to transform *Agrobacterium* Gv3101 competent cells.

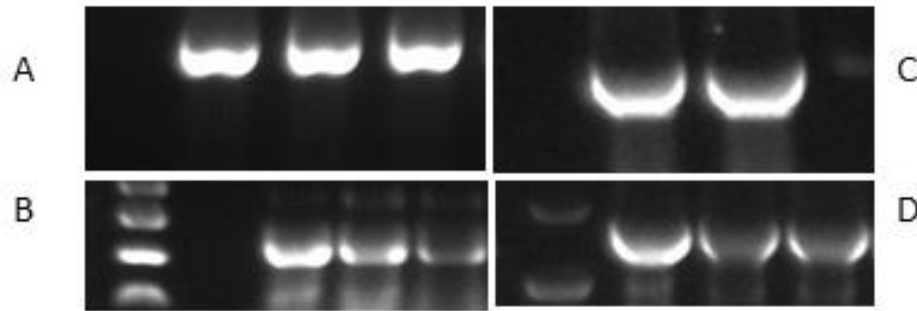


Figure 4.8 Preparation of overexpression construct for complementation of *Arabidopsis nst1NST2* genotype using barley *NST1* putative orthologue. The first lane on each gel image indicates DNA marker and the lanes following the marker are the amplification of the same DNA product. This construct was prepared by amplifying the full length of *HvNST1* coding sequence was amplified (A) and cloned into entry vector pDonr221. The plasmids containing the fragment in the correct orientation (B) was then used for LR reaction to transfer the fragment into destination vector pUbi::YFP containing *Arabidopsis* ubiquitin promoter. The presence of *HvNST1* coding sequence in the destination vector was confirmed (C) and the positive plasmids were used for transformation of *Agrobacterium* Gv3101 competent cells. Finally the presence of the fragment in the *Agrobacterium* competent cells was conformed (D).

4.3.4.2 *HvNST2* complementation construct

The complementation constructs for the putative *HvNST2* was prepared in the same way as the one for *HvNST1*. Firstly, the full length of the putative *HvNST2* coding sequence was amplified using the primers in Table 4.1 and the fragment was cloned to the entry vector (pDonr221) via the BP reaction (Figure 4.9). The entry vector containing the fragment in the correct orientation were used for the LR reaction to transfer the putative *HvNST2* fragment to the destination vector pUB::YFP (Grefen et al., 2010). The destination vector containing the fragment in the correct orientation (Figure 4.9) was used for transformation of *Agrobacterium* Gv3101 competent cells (Figure 4.9).

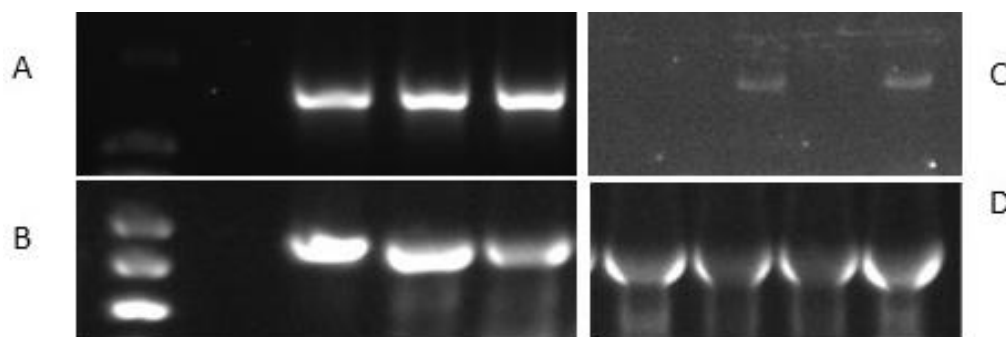


Figure 4.9 Preparation of overexpression construct for complementation of *Arabidopsis nst1NST2* genotypes using the putative *NST2* sequence. The first lane on each gel image indicates DNA marker and the lanes following the marker are the amplification of the same DNA product. The full length if barley *NST2* putative orthologue was amplified (A) and cloned into the entry vector pDonr221. The presence of the fragment in the correct orientation was then confirmed (B). The plasmid containing the *HvNST2* coding sequence in the entry vector was used in LR reaction and the coding sequence was transferred to destination vector pUbi::YFP. The presence of the fragment in the destination vector was confirmed (C) and the plasmid was used for transforming the Gv3101 *Agrobacterium* competent cells. The presence of the transgene in the *Agrobacterium* competent cells was also confirmed (D).

4.3.5 Barley transformation

Immature embryos of barley cultivar Golden Promise were isolated and used for inoculation with *HvNST1*^{SRDX}, *HvNST1*^{Ovex}, *HvNST2*^{SRDX}, *HvNST2*^{Ovex} and *HvNST2*^{RNAi} constructs. In total, 972 embryos were isolated and inoculated with the aforementioned constructs. The embryos (303 for *HvNST1*^{SRDX}, 108 for *HvNST1*^{Ovex}, 141 for *HvNST2*^{SRDX}, 270 for *HvNST2*^{Ovex} and 125 for *HvNST2*^{RNAi}). Five embryos per inoculation event were kept as control. The control embryos were not inoculated but went through the same steps as the inoculated embryos so that the regeneration ability of the protocol could be observed. This was helpful to track any abnormal growth in the transgenic lines compared to the non-inoculated plants generated considering they both went through the same process, except inoculation. The generated control (non-inoculated) plants were also used to compare their growth and fertility to that of transformant plants in T₀ generation.

The embryos inoculated with the inoculum and co-cultured for three days in dark. They were then transferred onto callus induction medium (Appendix 9.1) containing Hygromycin (50 mg/ml) and Timentin (160 mg/ml) for selecting for the transformed embryos. Along with the inoculated embryos; one control plate containing five embryos that were not inoculated were kept on callus induction medium without any antibiotics. Furthermore, to assess the effect of antibiotics on embryos a plate of non-inoculated embryos were kept on callus induction medium containing the antibiotics (Hygromycin and Timentin). The calli growing from the inoculated embryos were transferred onto transition and consequently regeneration media (Appendices 9.3 and 9.4). Plantlets generated from each callus were then transferred onto callus induction tubes without growth regulators and the plantlets were allowed to grow

until they reach the top of the tubes. The plantlets were then transferred to compost and further analysed for the presence of the transgene.

4.3.6 *HvNST1* Characterisation

4.3.6.1 *HvNST1* repression

The transformation using the repression construct resulted in generation of 11 T₀ transgenic *HvNST1*^{SRDX} plants. These plants were further analysed for the presence of transgene. Out of these, six plants survived (lines 2e, 3, 6c, 10b, 7 and 2) that were analysed for alterations in fertility and general phenotype compared to wild-type. Moreover, these lines were grown to further analyse the expression of the target genes (*NST1* and *NST2*) in T₁ generation.

4.3.6.1.1 *HvNST1*^{SRDX}: Analysis of presence of transgene and transgene copy number in T₁ generation

Four seeds per generated T₀ lines (above) were sown. These plants were named 1 to 4 (e.g 10b-1, 10b-2, 10b-3 and 10b-4). Prior to expression analysis of the target genes in the T₁ generation of the transgenic lines, the plants were examined for the presence of the transgene in their genome. The analysed lines showed positive amplification of the target putative sequence and part of the vector from genomic DNA (Figure 4.10). Out of the analysed lines, two lines (2-1 and 6c-1) that did not show any amplification for the putative target along with wild-type were used as negative controls. The sample collection was done from both positive and negative lines along with wild-type so that the negative lines could also be

used as control. This could show any potential effects of transgene in the expression analysis.

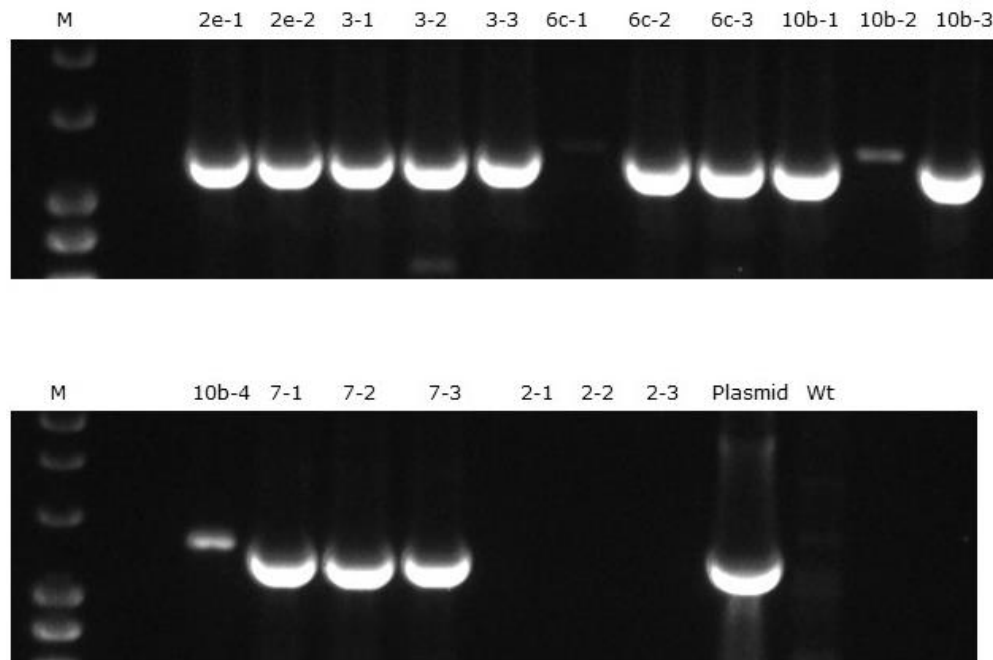


Figure 4.10 PCR analysis of the T₁ generation of *HvNST1* repression lines for the transgene. Out of all examined plants, three (lines 6c-1, 10b-2 and 10b-4) showed lack of amplification of the transgene. M: DNA marker, Plasmid: destination vector pBract214 containing *HvNST1*^{SRDX} fragment and Wt: genomic DNA from Golden Promise used as negative control.

Moreover the fertility of these transgenic lines was calculated via counting the seed number per spike (Section 4.2.3) (Figure 4.11).

As the expression of the transgene depends, at least in part, on the number of copies presence in the genome, transgene copy number in T₁ generation of *HvNST1*^{SRDX} lines was analysed. This analysis was done based on the presence of antibiotic hygromycin resistance gene in the genome compared to wild-type and positive control lines.

This analysis showed that lines positive for the presence of transgene (lines 6c-1, 6c-2, 3-3, 7-1, 7-3, 2e-2) contained at least a copy of the transgene

(Figure 4.11). There was no amplification of the hygromycin gene in the wild-type, the lines that were negative for the transgene (lines 2-1 and 6c-1) also failed to show amplification. This could be due to the fact that the copy number analysis was based on the presence of hygromycin gene not the transgene. Therefore, it could be possible that line 10b-1 lost the antibiotic resistance gene but not the transgene.

This analysis provided an indication of the number of transgenes in comparison to observations of fertility in the plants, although absolute transgene copy number cannot be entirely confirmed since it was calculated based on the presence of antibiotic resistance gene rather than the putative orthologous sequence.

4.3.6.1.2 *HvNST1*^{SRDX} Expression analysis

The expression of the putative barley *NST1* and its potential target genes (*NST2* and *MYB26*) was analysed in the transgenic lines (SRDX and overexpression). The aim of this analysis was firstly quantify the expression changes of the putative targets, and secondly to establish the relationship between the three main transcription factors involved in formation of secondary wall thickening in the anthers.

The expression of the putative barley *NST1* in the lines containing the SRDX repression transgene was conducted (Figure 4.11). Flower tissues from stage LFE1 (Fernandez Gomez and Wilson, 2012) from each transformed line and wild-type were collected and used for expression analysis. The transformed line that did not carry the repression construct (line 2-1) (Figure 4.10) was used as a negative control alongside wild-type. This analysis showed a correlation between the expression of transgene and reduction in fertility in plants. As shown in Figure 4.11, line 2e-2 exhibited

high expression of *HvNST1*^{SRDX} and had a higher sterility than line 3-3 with lower transgene expression.

Further analysis was done by quantification of the expression putative *HvNST2* and *HvMYB26* in the *NST1* repression lines in order to assess the potential expression changes in these two genes (Figure 4.12). It was observed that the *HvNST2* putative orthologue had lower expression compared to control (wild-type) in both positive and negative *HvNST1*^{SRDX} lines. As the expression of *HvNST2* in wild-type was showed to be higher at this stage (Figure 3.18), this could be an indication for regulation of *NST2* by *NST1* in barley. Also, there was very low expression of all three target genes in line 2-1 that was used as negative control (line 2-1) that was a confirmation for the correct analysis of the target genes expression. However, this reduction was not observed in line 10b-1 that was positive for the transgene.

This could potentially be due to the fact that the transgene was inserted in a location in the genome that could not have substantial effects on its targets, although the expression of the putative orthologue in this line was two times of that of wild-type. Furthermore, the repression of *HvNST1* seemed to also affect the expression of putative *HvMYB26* (Figure 4.12). The putative orthologue of *MYB26* in barley was reduced compared to wild-type control. However, the expression of *HvMYB26* in all transgenic lines remained constant, although lower than the control. The lower expression of *HvMYB26* could mean that there was a potential interaction between *NST1* and *MYB26* in barley meaning that repression of *NST1* interaction with its target genes, could reduce the expression of *MYB26*.

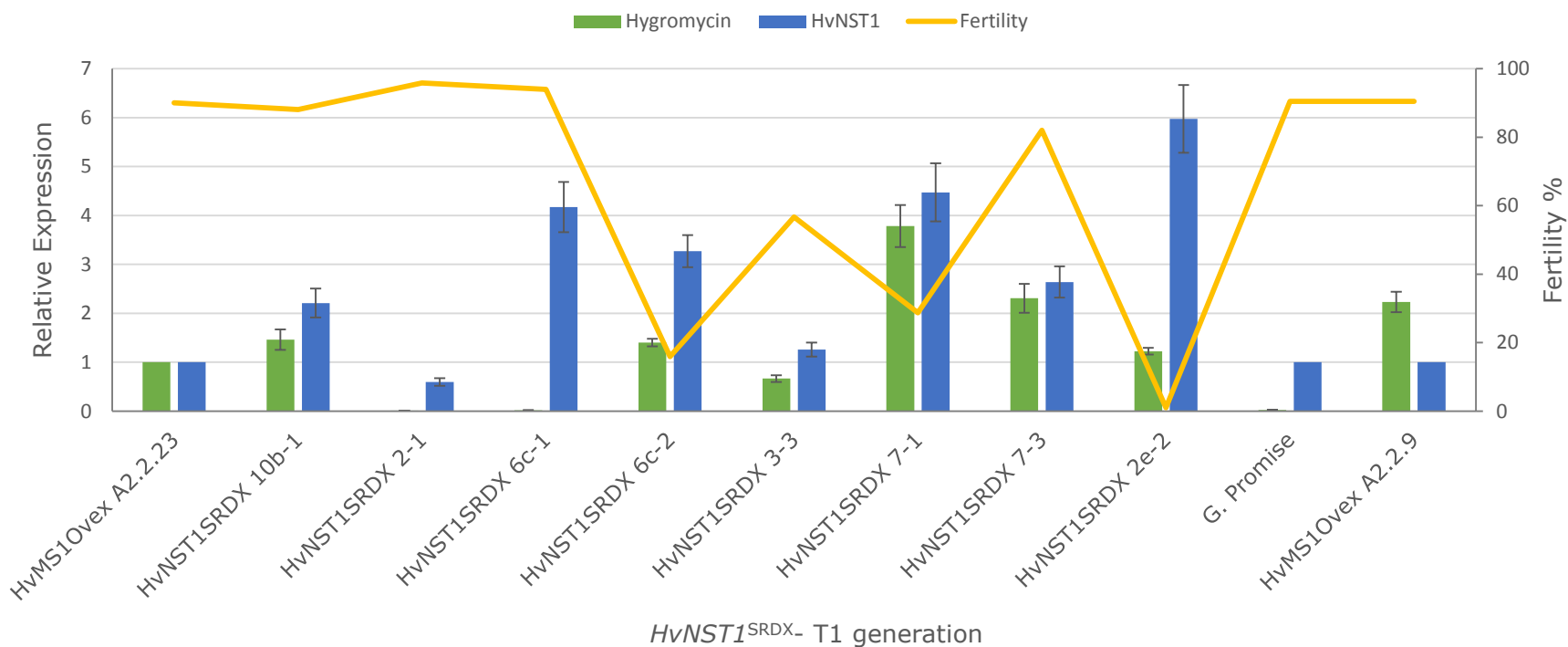


Figure 4.11 Transgene copy number, expression and fertility analysis in *HvNST1^{SRDX}* plants. The copy number of antibiotic resistance gene (hygromycin) was detected as in *HvNST1* repression lines. This analysis showed that the lines homozygote for the transgene possess lower fertility than the heterozygote lines. Though this may not be true for the lines with only one copy present (line 2e2). The detection of number of copies was done using *HvMS1^{Ovex}* lines with confirmed number of transgene copies as control. *HvMS1^{Ovex}* A2.2.23 has one copy and line A2.2.9 contains two copies of transgene. Wild-type (Golden Promise) genomic DNA was used as negative control. Increased expression of the target was observed in the transgenic lines containing the repression construct. This was in accordance with the fact that the transgenic lines that did not contain the transgene showed lower expression similar to wild-type compared to the positive transgenic lines. The expression of the samples was calculated relative to wild-type flower sample at LFE1 stage.

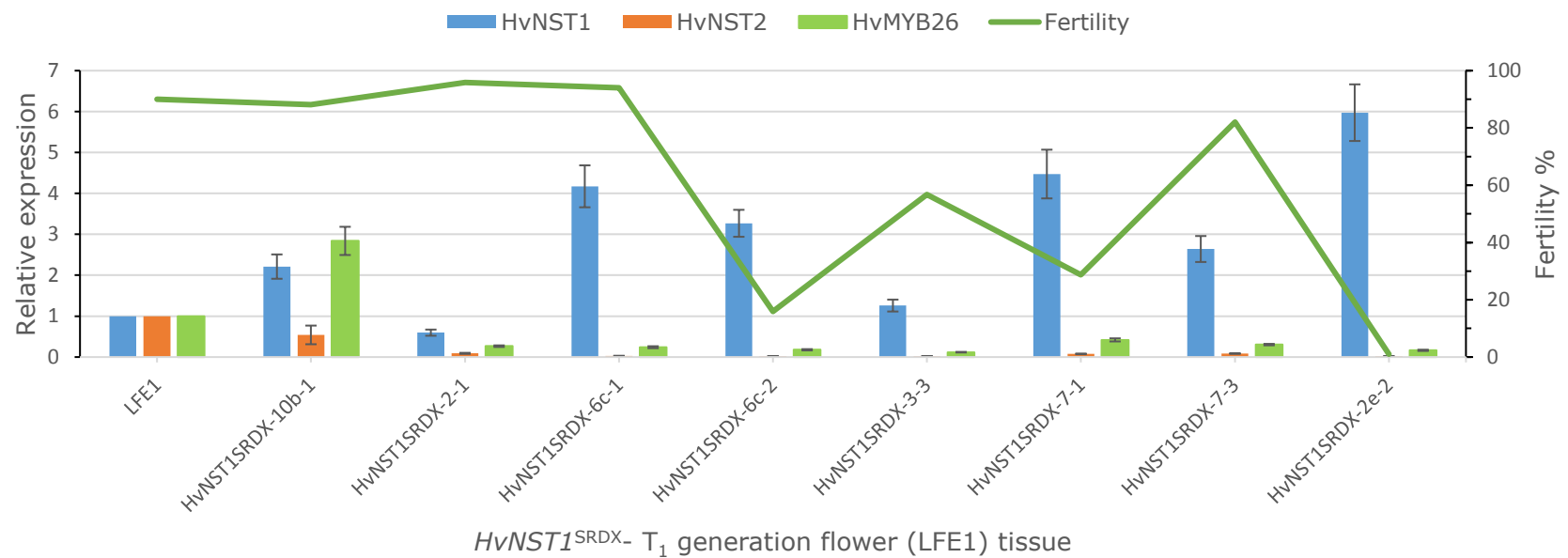


Figure 4.12 Analysis of the expression of putative *HvNST1*, *HvNST2* and *HvMYB26* in transgenic plants containing *HvNST1* repression construct. The expression analysis showed higher expression of *HvNST1* in the transgenic plants and reduced expression of *HvNST2* in the transgenic lines. Also the expression of *HvMYB26* showed reduction in the repression lines. The flower sample collected at the early mitosis I stage (LFE1) from T₁ generation.

4.3.6.1.3 Phenotypic analysis of *HvNST1^{SRDX}* lines

All the generated transgenic plants were analysed for confirmation of the presence of transgene (Figure 4.13). The majority of the positive transformant plants showed reduction in plant height and spike size compared to wild-type plants (Figures 4.13, 4.14). This was despite the fact that some transgenic plants carrying the transgene were the same height as the wild-type with the same fertility level.



Figure 4.13 Analysis of the first generation of *HvNST1^{SRDX}* plants. The first generation of *HvNST1^{SRDX}* plants showed a high level of sterility compared to wild-type. A: The transformed plants were analysed for the presence of the transgene (*HvNST1^{SRDX}*), using genomic DNA and the combination of one primer specific to the target gene and the other specific to the vector. All except one line (2) the rest contained the transgene. B: The comparison between *HvNST1^{SRDX}* lines and wild-type (Golden Promise cv.). The spike size of the transgenic plants was reduced in size and hence there were lower seed numbers in each spike. M: DNA marker, 1-7: generated *HvNST1^{SRDX}* transgenic lines, -C: Golden Promise genomic DNA used as negative control, +C: pBract214 destination vector containing *HvNST1^{SRDX}* fragment used as positive control.

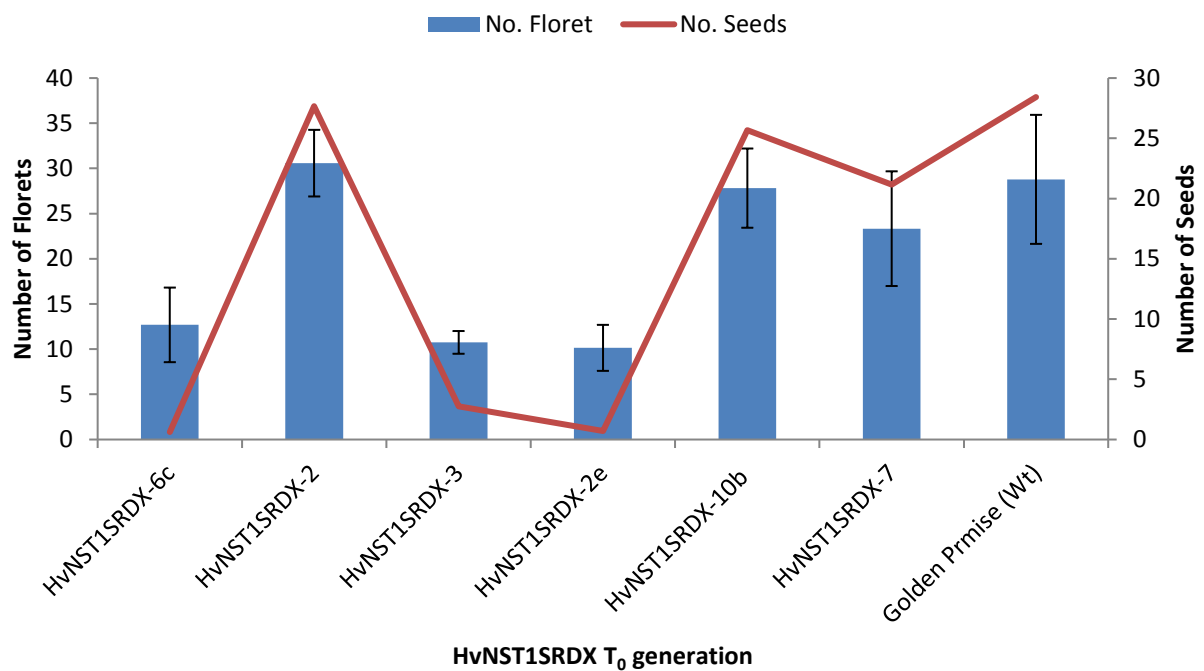


Figure 4.14 Number of florets and seeds in *HvNST1^{SRDX}* transgenic lines. Fertility reductions were seen in *HvNST1^{SRDX}* transgenic lines. This varied between lines with some of the transgenic lines showed high level of sterility coupled with reduction in spike size.

As shown in Figure 4.14, three plants from three independent transformation events (lines 6c, 3 and 2e) showed reductions in fertility and seed number compared to the wild-type grown in the same growth conditions. These lines had smaller spike size and were reduced in height compared to wild-type (Golden Promise cv.) (Figure 4.15). Moreover, these lines also possessed weaker and weedier stems compared to wild-type. Nonetheless, despite having weaker stems the plants were standing upright.

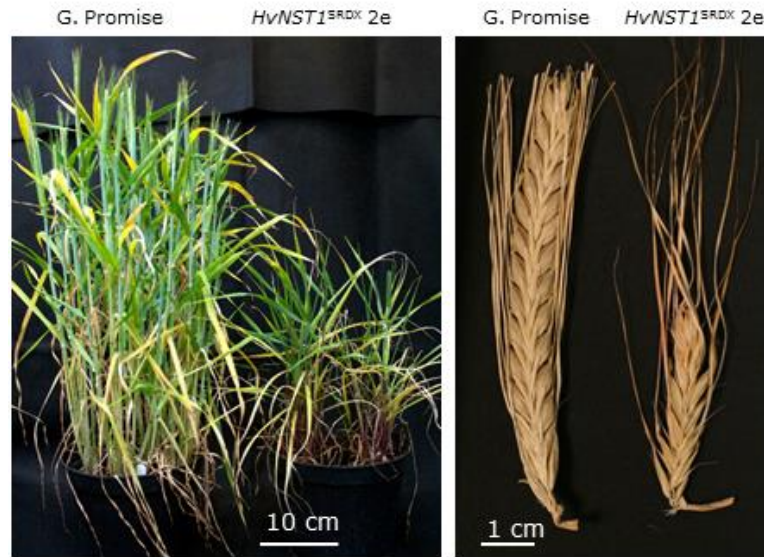


Figure 4.15 Phenotypic comparison between wild-type (Golden Promise cv.) and repression line ($HvNST1^{SRDX} 2e$) for plant height and spike size. Some of the transgenic lines repressed for the putative $HvNST1$ showed a high reduction in plant height and spike size.

4.3.7 $HvNST1$ overexpression

4.3.7.1 Phenotypic analysis of $HvNST1^{Ovex.}$

The overexpression of $HvNST1$ putative orthologue in barley resulted in generation of three lines, two of which were independent transformant plants. The overexpression lines in T_0 generation showed normal plant height compared wild-type control. Unlike repression lines the overexpression did not result in generation of plants with weaker stems. However, some lines showed a mixture of normal spikes and smaller spike sizes that eventually affected the fertility of the plant (Figure 4.16 and 4.17).



Figure 4.16 Phenotypic comparison of the plant and spike phenotype of wild-type and *HvNST1* overexpression line 1a. The ectopic expression of *HvNST1* putative orthologue in barley did not affect the plant height compared to wild-type. However it affected the spike size and fertility of the transgenic plants. The overexpression lines generated a mixture of normal sized and shorter spikes.

The fertility of the overexpression lines was reduced by 47.3% compared to wild-type (Figure 4.17). The reduction in fertility was not as high as what was seen in the repression lines (Figure 4.11). Furthermore, plants produced the same tiller number as wild-type and this could potentially compensate for the lack of seed number seen in the other spikes.

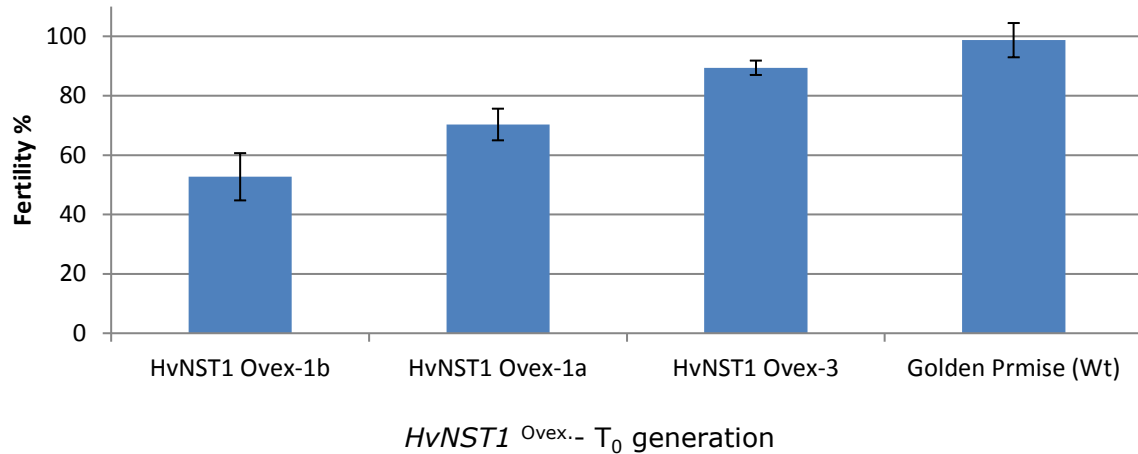


Figure 4.17 Fertility changes in the *NST1* overexpression lines compared to wild-type. The fertility of the lines were reduced by almost 50%.

The T₁ generation of the overexpression lines showed reduction in plant height compared to wild-type (Figure 4.18). The plants overexpressing *HvNST1* grew slower than wild-type under the same growth conditions. Further analysis of these lines will be carried out in the future.

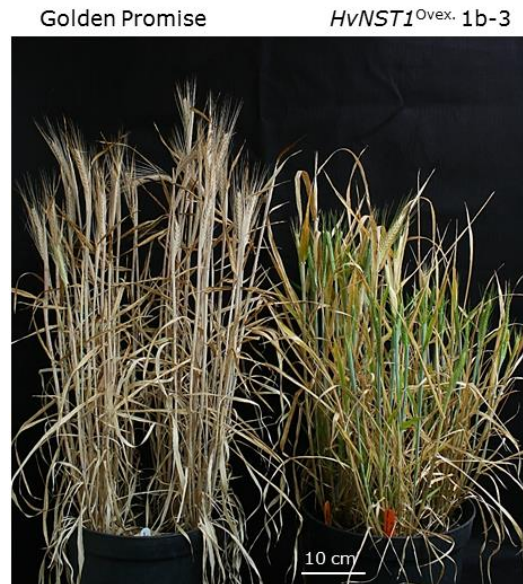


Figure 4.18 T₁ generation of *HvNST1* overexpression lines. The overexpression lines showed reduction in height compared wild-type. These plants also had slower growth compared to control plants.

4.3.8 *HvNST2* characterisation

4.3.8.1 *HvNST2*^{RNAi} phenotypic analysis

The analysis of the three generated transformant plants showed that they were positive for the transgene (Figure 4.19). The *NST2*^{RNAi} T₀ generation plants did not result in major changes in plant growth and spike size as compared to wild-type (Figure 4.20). However, these lines showed a reduction in fertility compared to wild-type, in specific lines this was as low as 39% fertility (Line 2b; Figure 4.21).

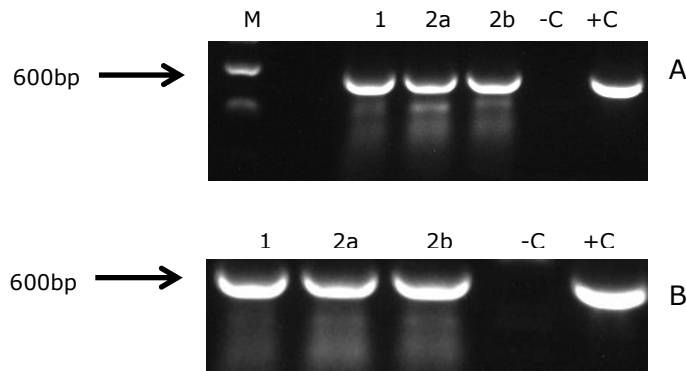


Figure 4.19 Analysis of *HvNST2*^{RNAi} mutants. The transgenic plants (plants 1, 2a and 2b) were analysed for the presence of the transgene, along with negative control (water) and plasmid (*HvNST2*^{RNAi}:pBract207) as positive control, in the T₀ generation u vector specific primers (Ubi promF1 and iv2F1) and gene specific primers (*HvNST2*^{RNAi} forward and reverse (Table 4.1)). A: PCR analysis of the transgenic plants with Ubi promF1 and *HvNST2*^{RNAi} reverse primers (expected size of the product 547bp). B: Analysis of the transgenic plants with *HvNST2*^{RNAi} forward and iv2F1, with the expected size of 547bp. M: DNA marker, -C: Golden Promise cv. genomic DNA used as negative control, +C: destination vector pBract207 containing the RNAi fragment used as positive control.

The RNAi silencing lines did not show any alterations in plant or spike size. Also, the stem of the plants had the same apparent strength as the wild-

type lines. This could be due to the silencing the target gene using RNAi silencing that could not completely knock out the function of *NST2* in barley. Moreover, considering *NST1* along with *NST2* act redundantly to regulate secondary thickening, *NST2* knock down was not sufficient to produce a phenotype similar to SRDX repression lines.



Figure 4.19 The T_0 generation of *HvNST2*^{RNAi} plants. The first generation did not show any phenotypic changes compared to wild-type. However, some spikes had lower number of seeds and reduced fertility compared to wild-type which could be due to the silencing of the gene.

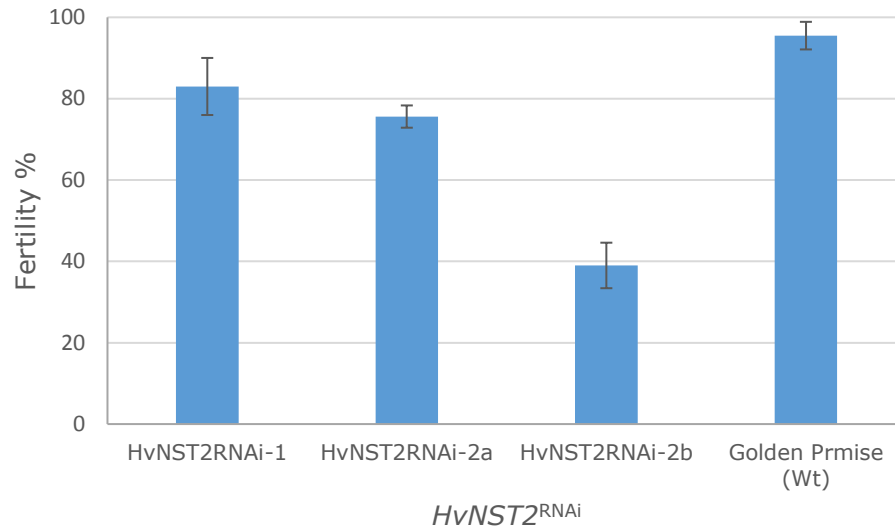


Figure 4.20 Fertility percentage in RNAi silencing lines. The reduction in fertility was as high as 61 percent compared to wild-type.

4.3.8.2 *HvNST2*^{RNAi} expression analysis

The second generation of the RNAi lines was analysed in order to further confirm the expression changes of the putative *HvNST2* gene in the silencing lines. These plants were first genotyped for the presence of the sense and antisense RNAi fragments (Figure 4.22).

The expression of the putative targets in the RNAi knock down lines was analysed. Prior to this, the silencing lines they were tested in order to estimate the number of transgenes in each line and thus to be able to better interpret the expression data. The transgene copy number analysis was done using the amplification of antibiotic resistance gene, hygromycin, and quantifying its level through quantitative real time PCR (Figure 4.23). This analysis showed that lines 1-1, 1-2 and 2b-7 have more than two copies of the transgene, while it was predicted that there was two copies of transgene in lines 2a-1, 2a-2 and 2b-5 (Figure 4.23).

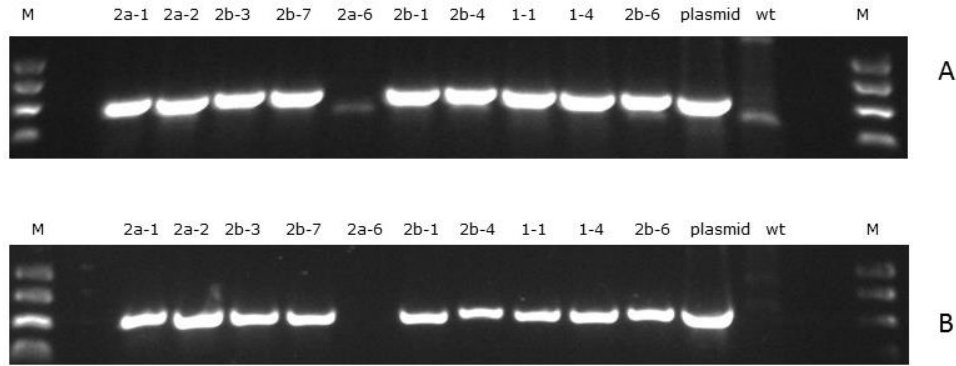


Figure 4.21 PCR analysis for confirmation of the presence of both sense and antisense RNAi fragments in the T₁ generation of *HvNST2*^{RNAi} lines. Out of all analysed lines, only line 2a-6 was negative for both sense and antisense fragments. M: marker. Plasmid: *HvNST2*^{RNAi}::pBract207 plasmid used as positive control. Wt: Golden Promise genomic DNA was used as negative control.

The flower samples from the early mitosis I stage (LFE1 and 2) stages were collected in order to compare the expression changes between wild-type and silencing lines. All the analysed samples showed expression increase in *HvNST2* compared to wild-type in both LFE1 and LFE2 stages. This increase was up to 20-fold more than that observed in of wild-type (Figure 4.23), despite the target that RNAi silencing could reduce the expression of the native gene. This could also be due to the functional *NST1* gene that could potentially increase the expression of *NST2* to compensate for expression reduction. Nonetheless, the increase level of *NST2* expression was correlated with increased fertility (Figure 4.23). On the other hand, lower expression was also showed to be linked to lower fertility (Line 2a-1; Figure 4.23), however, this was not always the case (Line 2a-2; Figure 4.23).

Further analysis of the silencing lines was done by analysis of the potential expression changes of the putative *HvNST1* gene in the RNAi knock down

lines. This analysis did not show any substantial expression changes compared to wild-type (Figure 4.24). Majority of the lines showed similar expression profile compared to wild-type. The only exception was *HvNST2*^{RNAi} 2a-1 line that showed lower expression than the wild-type in both stages that was correlated in lower fertility compared to wild-type. However, line 2b-7 showed similar expression compared to wild-type and line 2b-5, but the fertility was lower than that observed in line 2a-1 (Figure 4.24). This could be due to higher transgene copy number in line 2b-7 (Figure 4.23), and higher expression of *NST2* that could be disruptive to fertility.

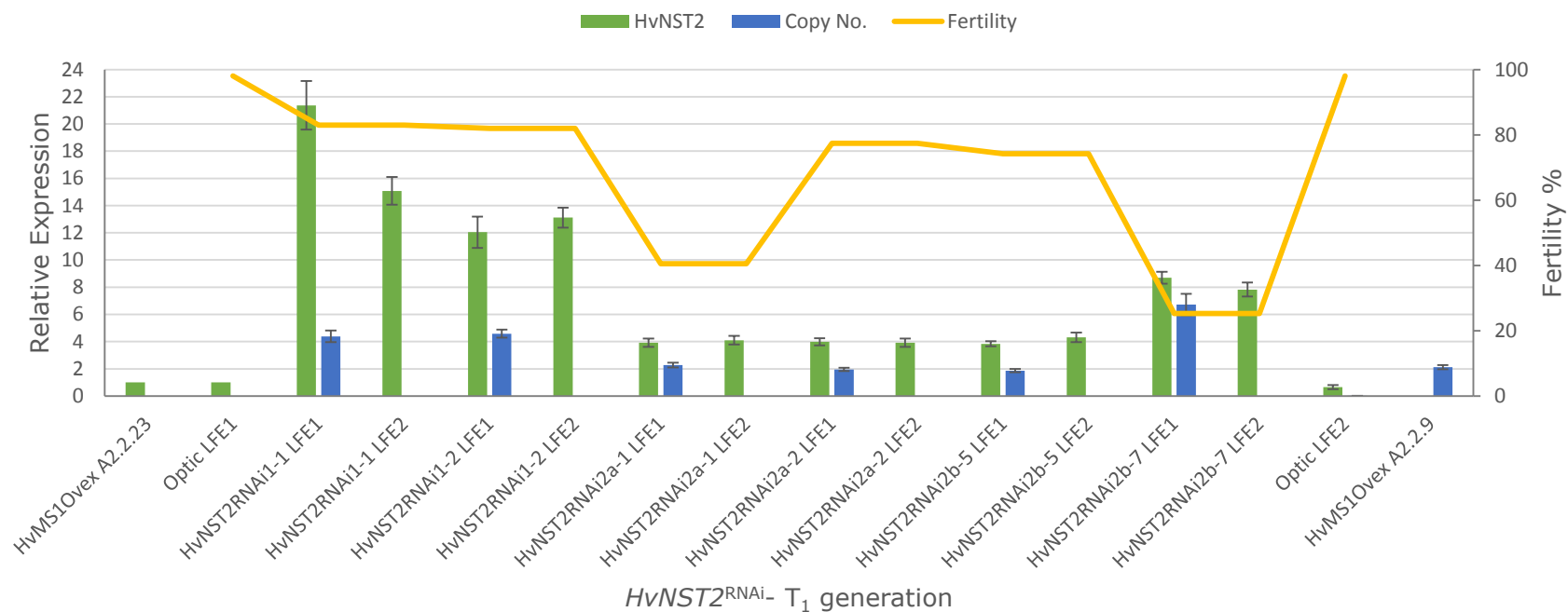


Figure 4.223 Transgene copy number, expression and fertility analyses in *HvNST2*^{RNAi} T₁ generation. This analysis was carried out by amplification and quantification of antibiotics resistance, hygromycin, gene in the transformant and control lines. The Golden Promise genomic DNA was used as negative control alongside *HvMS1*^{Ovex} lines that were used as positive control. The expression of the putative target was increased compared to similar stages in wild-type (LFE1 and LFE2).

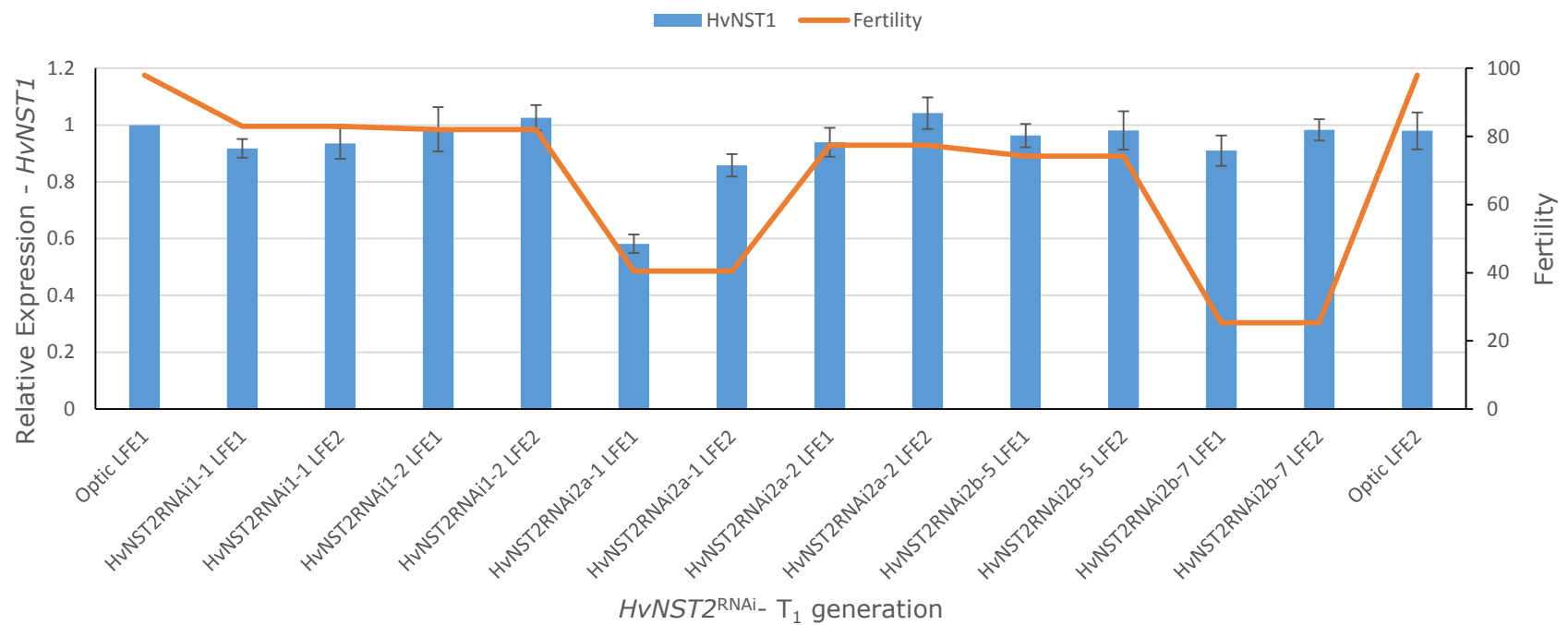


Figure 4.23 Expression analysis of the putative *HvNST1* sequence in the *HvNST2*^{RNAi} knock down lines. The majority of the analysed lines did not show any alteration of the putative *HvNST1* gene compared to wild-type in neither of the LFE1 nor LFE2 stages. The only line with lower expression of putative NST1 in both stages compared to wild-type was line 2a-1. The expression changes in the analysed lines was also compared to their fertility score.

4.3.9 *HvNST2* repression

4.3.9.1 Phenotypic analysis

The three generated *HvNST2*^{SRDX} lines were analysed for the presence of transgene and changes in their phenotype and fertility compared to wild-type. The analysis of *HvNST2*^{SRDX} lines showed that they were all positive for the transgene (Figure 4.25).

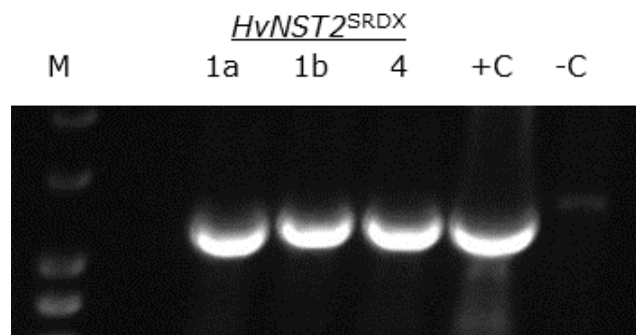


Figure 4.24 PCR analysis of T₀ generation of *HvNST2*^{SRDX} plants. The analysis was done using pBract214 forward and *HvNST1*^{RNAi} reverse primers on the genomic DNA extracted from the transformant plants along with wild-type (Golden Promise) as negative control. M: DNA marker, +C: the destination vector pBract214 containing the repression fragment used as positive control, -C: Golden Promise genomic DNA used as negative control.

These lines were similar to the *HvNST1*^{SRDX} lines with reduced plant height and spike size (Figure 4.26). The reduction in plant height was at a moderate level, so the plants were semi-dwarf compared to wild-type (Golden Promise). Moreover, the plants developed stems that were appeared weaker than wild-type, but at the same time were not as weak as the phenotype observed in *HvNST1*^{SRDX} lines, and the plants were able to stand upright (Figure 4.15).

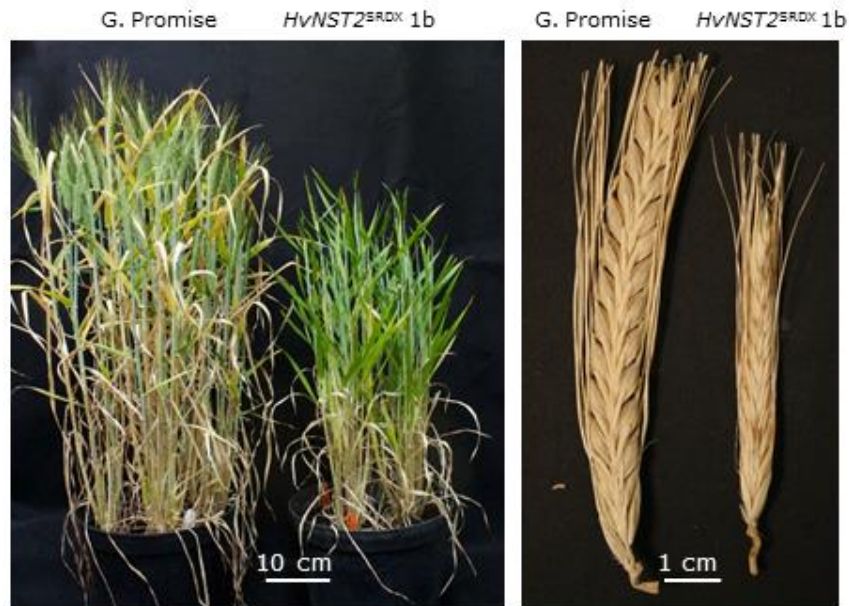


Figure 4.25 The repression of putative *HvNST2* gene in barley resulted in reduction of fertility along with reduction in plant height. The repression of the putative target resulted in reduction of spike size as compared to wild-type that is also associated with fertility reduction.

Repression of putative *NST2* orthologue in barley caused a high level of sterility, which reduced fertility to a maximum of 16% (Figure 4.27).

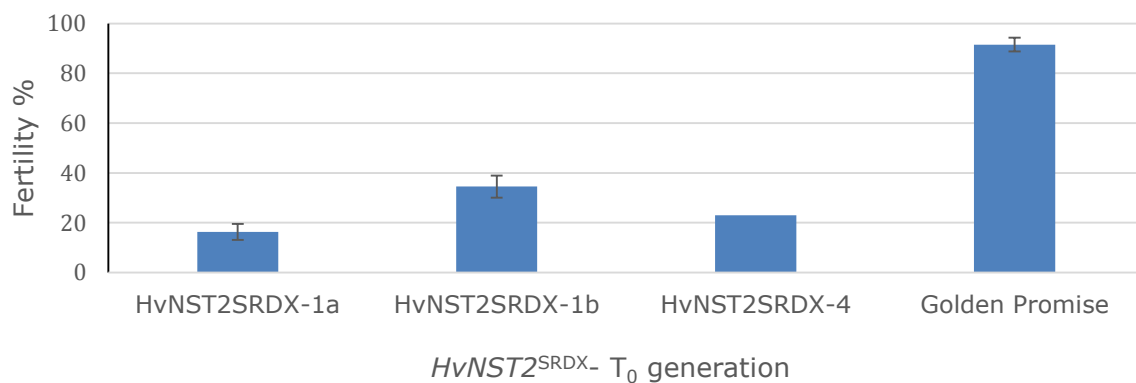


Figure 4.26 Fertility of the T₀ generation of *HvNST2*^{SRDX} lines. The repression of *NST2* putative orthologue caused fertility loss by up to 84% (line 1a) compared to wild-type (Golden Promise).

4.3.9.2 Expression analysis

The transgenic lines were tested for transgenes number presence (Figure 4.29). This analysis revealed that line 4 had more than two copies of the transgene and lines 1a and 1b contained at least one copy.

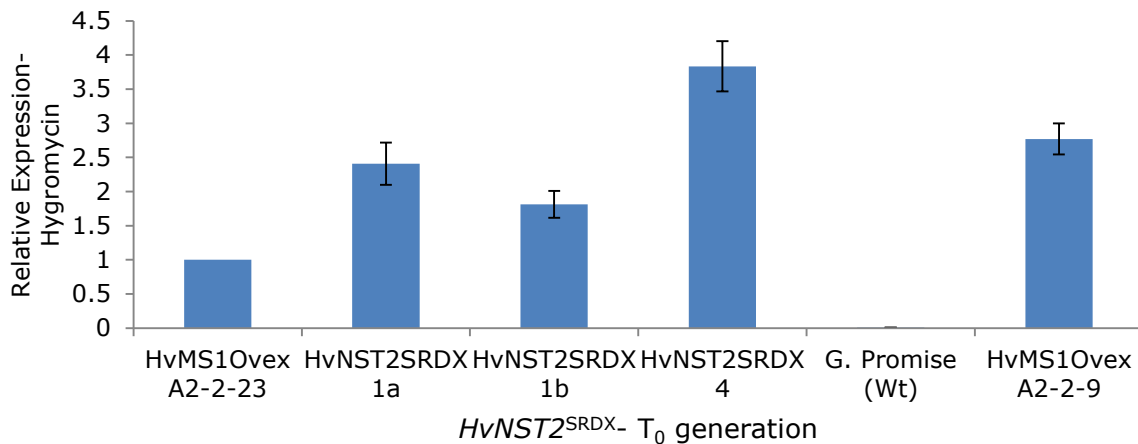


Figure 4.28 Transgene copy number analysis of *HvNST2^{SRDX}* lines. This analysis showed that lines 1a and 1b have at least one copy and line 4 more than two copies of the transgene. In this analysis the copy numbers were calculated in relation to *HvMS1^{Ovex}* lines with known number of transgene. Golden Promise genomic DNA was also used as negative control.

The expression of putative *NST1* and *NST2* genes was analysed in *HvNST2^{SRDX}* lines. This analysis showed a seven-fold increase in the expression of *HvNST2* putative target compared to the similar stage (LFE1) in wild-type (Figure 4.29). The increase in the level of expression was expected and was due to the constitutive promoter that was driving *NST2* and repression fragment. Furthermore, the expression of the *NST1* gene was not affected in the repression lines and remained constant in both analysed lines (Figure 4.30). This may be an indication that the putative

NST2 gene in barley is downstream of *NST1* in the secondary wall thickening gene regulatory network.

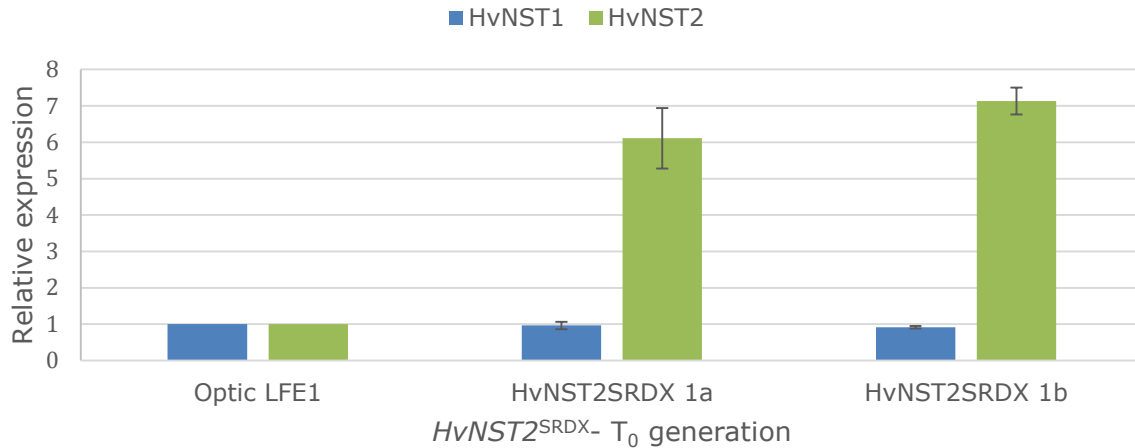


Figure 4.29 Expression analysis of *HvNST2*^{SRDX} lines. This analysis showed that the expression of putative *NST2* orthologue was increased compared to wild-type (Stage LFE1). The repression of *HvNST2* did not affect the expression of *HvNST1* putative orthologue and it remained constant in all samples.

4.3.10 *HvNST2* overexpression

The generated overexpression lines of *HvNST2* were tested for the presence of transgene. All of the generated lines contained the overexpression construct in their genome (Figure 4.30). The fertility analysis of the first generation showed reduced fertility by up to 50% (Figure 4.31 and 4.32). This reduction in fertility accompanied with reduction in plant and spike size. This was despite the fact that the spike sizes in the overexpression lines were a mixture of normal size and smaller and sizes (Figure 4.31). Furthermore, the overexpression lines had slower growth compared to wild-type plants that were grown under the same growth conditions.

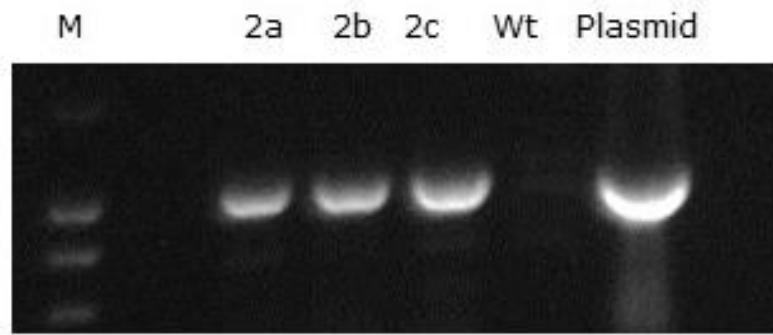


Figure 4.27 Analysis of the T₀ generation of *HvNST2*^{Ovex} lines. All the generated plants were positive for the putative *HvNST2* fragment. M: marker. 2a-2c: the generated sister lines of *HvNST2* overexpression. Wt: wild-type Golden Promise genomic DNA was used as negative control. Plasmid: the overexpression plasmid (pBract214) containing the full length of the putative target was used as positive control.



Figure 4.28 Phenotypic characterisation of lines overexpressing *HvNST2*. The transgenic plants also showed reduction in plant and spike size compared to wild-type.

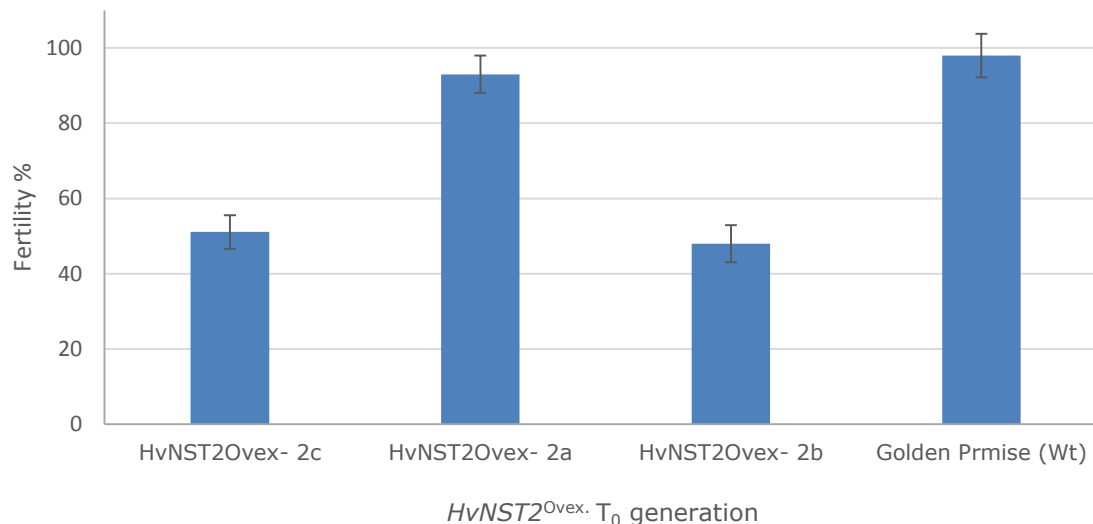


Figure 4.29 Fertility analysis of the putative *HvNST2* overexpression lines. The ectopic expression of putative *HvNST2* in barley resulted in 50% reduction in seed set in the T₀ generation.

Table 4.2 summarises the phenotypes observed from transgenic *HvNST1* and *HvNST2* plants.

Table 4.2 Phenotypic summary of *HvNST1* and *HvNST2* transformant plants.

Line	Phenotype	Number of transgenic lines
<i>HvNST1</i> ^{SRDX}	Reduction in plant and spike sizes High sterility (~95%)	T₀ : 11 generated (6 survived) T₁ : 6 lines analysed
<i>HvNST1</i> ^{Ovex.}	Reduction in plant height Medium sterility (~50%)	T₀ : 3 lines generated
<i>HvNST2</i> ^{RNAi}	Normal plant and spike sizes Medium sterility (~60%)	T₀ : 3 lines generated T₁ : 2 plants per line analysed
<i>HvNST2</i> ^{SRDX}	Reduction in plant and spike sizes High sterility (~85%)	T₀ : 3 lines generated
<i>HvNST2</i> ^{Ovex.}	Reduction in plant and spike sizes Medium sterility (~50%)	T₀ : 3 lines generated

4.3.11 Complementation of *Arabidopsis nst1* and *nst2* mutants

As part of functional characterisation of the putative orthologous *NST1* and *NST2* genes in barley it is important to show whether these genes are able to complement the corresponding mutant phenotypes in *Arabidopsis*. For this purpose, the heterozygote lines of *Arabidopsis nst1* and *NST2nst2* were used (kindly provided by Dr. Nobutaka Mitsuda (The National Institute of Advanced Industrial Science and Technology, Japan)). The heterozygote lines were sown and inoculated with either *HvNST1* or *HvNST2* overexpression constructs that were driven by the *Arabidopsis thaliana* ubiquitin promoter (*HvNST1*cds::pUbi::YFP, *HvNST2*cds::pUbi:YFP). The seeds collected from this transformation were sown on compost and selected with glufosinate herbicide (Section 4.19). However, unfortunately the selection of these seeds did not produce any transformed plants, indicating that the transformation was not successful.

It was also attempted to express these two targets in *Arabidopsis* wild-type (Ler.) in order to test whether this would induce increased production of secondary wall thickening and the associated smaller plants and phenotypes as seen with *Arabidopsis NST1/2* and further help confirm the function of the putative *NST1* and *NST2* in barley. However, similar to the complementation experiment there was no success in generating transformed plant overexpressing the putative orthologous targets.

4.4 Discussion

The vascular tissues in plants are composed of xylem, phloem and intervening cambial and procambial cells. The development of secondary cell wall in the xylary fibres and tracheary elements during their differentiation the xylary fibres and tracheary elements develop secondary

cell wall (Yang and Wang, 2016). The formation of secondary wall thickening is important process during plant development. It enables the plant to withstand the external pressures and also provide a reliable channel for transportation of water and nutrients throughout the plant through developing xylem and phloem (Yamaguchi et al., 2010a). The NAC domain transcription factors are major regulator of the components of secondary wall biosynthesis in plants. Ten major NAC transcription factors (*NST1-3* and *VND1-7*) have been identified as key activators of secondary wall biosynthesis in the xylary fibres (Yang and Wang, 2016).

It has been demonstrated that in *Arabidopsis* fibre cells, *NST1* and *NST3/SND1* regulate secondary cell wall biosynthesis (Mitsuda et al., 2007; Zhong et al., 2006). *NST1* and *NST3* have been shown to regulate secondary wall formation in *Arabidopsis* siliques and are important for seed dispersal (Mitsuda and Ohme-Takagi, 2008). The formation of secondary wall thickening in the interfascicular and xylary fibres of the stem is controlled by *NST1*, *NST2* and *SND1*. The absence of these three transcription factors in *Arabidopsis thaliana* results in complete loss of secondary wall thickening in the fibres of stem and sterility due to indehiscent anthers (Zhong and Ye, 2015).

In the reproductive tissues, *NAC Secondary Wall Thickening Promoting Factor 1* (*NST1*) and *NST2* redundantly regulate the formation of secondary wall thickening in the endothecium layer of the anthers. The lack of secondary thickening does not allow the formation of the forces that are required for the anther to open; thus the plants are sterile (Mitsuda et al., 2005; Yang et al., 2007). The repression of either *NST1* or *NST2* is shown to cause sterility in *Arabidopsis*, and ectopic expression of these genes causes ectopic deposition of secondary wall thickening in the plant (Mitsuda et al., 2005). In *Arabidopsis*, *MYB26* transcription factor regulates the deposition of secondary wall components in the endothecium layer of the

anther, and it has been speculated that this is done via activation of *NST1* and *NST2* (Yang et al., 2007). The aim of this study was to functionally characterise the two transcription factors that may be orthologous to the *Arabidopsis* genes affecting formation of secondary wall thickening known as *NST1* and *NST2* in barley anthers.

The *HvNST1*^{SRDX} repression lines of the putative *NST1* in barley showed high reduction in fertility by up to 78.6%. This was accompanied by further reduction in plant height and spike size that ultimately affected the fertility of the plant. The ectopic expression of *HvNST1* putative orthologue in barley also resulted in reduction in fertility by up to 47.3%. This reduction in fertility was not as high as that observed in the repression lines of *HvNST1*. This perhaps shows the importance of *HvNST1* interaction with its target genes, in that its repression resulted in drastic decrease in plant height and seed number. The overexpression of this gene did not cause the severe reduction in plant height and spike size as it was observed in the repression lines nor was it similar to generation of short plants seen in *Arabidopsis NST1* overexpression. Nevertheless, the ectopic expression of *HvNST1* reduced the fertility by almost half. This perhaps can indicate the existence of other regulatory genes in the pathway that can regulate the overproduction of *HvNST1*.

As for *HvNST2* characterisation, the generated *HvNST2*^{RNAi} lines did not show noticeable reduction in fertility compared to wild-type. This may be due to the fact that *NST1* and *NST2* act redundantly to control secondary wall thickening and the loss of function of one can be complemented by the other (Mitsuda et al., 2005). Hence, silencing of *HvNST2* may not show any significant changes in the anther secondary wall formation and the plant fertility due to the normal function of *HvNST1*. Furthermore, the silencing method used in this case, RNAi silencing, might not have been the best

choice for the functionally redundant transcription factors; therefore, the reduction in the *HvNST2* protein could not have been noticeable.

Generation of *HvNST2*^{SRDX} lines can also be useful in terms of investigating the role of *NST2* in formation of secondary wall formation in barley. Chimeric Repressor Gene-Silencing Technology (CRES-T) is a gene silencing method in which a chimeric repressor is produced through fusion of a plant-specific ERF associated Amphiphilic repression (EAR)-like motif repression domain to a transcription factor (Hiratsu et al., 2003; Mitsuda et al., 2006). It has been shown that the expression of the target genes of the transcription factors fused to the chimeric repressor domain was dominantly suppressed, and hence the resultant transgenic plants showed loss-of-function phenotypes specific for the target genes at high frequency (Hiratsu et al., 2003; Mitsuda et al., 2006). This system has an advantage over the other silencing methods such as RNAi since it can suppress target gene function dominantly even in the presence of functionally redundant transcription factors. Whereas RNAi can reduce the expression of transcription factor, but the presence of small amounts of expression could still be enough for normal activation of downstream genes (Mitsuda et al., 2006).

Overexpression of any of secondary wall NAC genes in *Arabidopsis* causes the activation of secondary wall biosynthesis components (Zhou et al., 2014). The ectopic expression of *NST1* and *NST2* in *Arabidopsis* caused reduction in plant size and excessive deposition of secondary wall components in various tissues with higher proportion of cells with secondary wall thickening in *NST1* overexpression lines than *NST2* (Mitsuda et al., 2005). The ectopic expression of *NST1* and *NST2* putative orthologues in barley resulted in reduction in fertility that was accompanied by reduction in spike size. However, the plant height remained the same as wild-type. Further analysis of the overexpression lines is required to

assess the amount of lignification in the plant tissues compared to wild-type.

Complementation of the *AtNST1* and *AtNST2* heterozygote mutants (*nst1NST2*) in *Arabidopsis* with barley putative orthologous gene was also attempted to help confirm the function of the genes, further analysis of this would be interesting. However, considering the fact that the putative *HvNST1* has 50% protein similarity to the *Arabidopsis* gene and also there is 40% protein similarity between *HvNST2* putative orthologue and *AtNST2* it seems likely that these are the equivalent barley genes. Furthermore, both *Arabidopsis* and barley genes share the same conserved domain that is important in the conclusion that they have similar functions.

The generated suppression and overexpression of both *HvNST1* and *HvNST2* will be further analysed for alteration in secondary wall formation especially in the anthers. The anthers from mitosis I stage until stages after anthesis will be observed for changes in formation of secondary wall thickening. This will better elucidate the role of these two putative genes in formation of secondary wall thickening and fertility in barley. Furthermore, analysis of expression pattern of putative *MYB26*, *NST1* and *NST2* in repression and overexpression lines will elucidate potential interaction between these three transcription factors in barley anther.

Chapter Five:

Identification and characterisation *HvMS5* putative orthologue

5.1 Introduction

Meiosis process is an important process that ensures the formation of the next generation with the same genetic content via production of cells with the half chromosome numbers as their mother cells, and generation of genetic variation via formation of crossovers. However, meiotic division has to be stopped after the second cell otherwise the formation of next generation will be compromised.

Cyclin dependent kinases (CDKs) promote the progression through meiosis and mitosis. The increased activity of cyclin dependent kinase- cyclin (CDK-cyclin) complexes during prophase I promotes entry to the first meiotic division, followed by their degradation that allow the dissociation of homologous chromosomes at anaphase I. However, this degradation is not complete and the presence of small amount transcript allows the second meiotic division to happen (Cifuentes et al., 2016; Marston and Amon, 2004).

The *Arabidopsis MS5* mutant has been described as the mutant with more than two rounds of division in meiosis (Glover et al., 1998). This mutant has also been reported as in earlier studies as *tdm1* (*Three Division Mutant 1*), which undergoes an apparent third cell division in meiosis II without DNA replication (Ross et al., 1997). The production of octads of microspores is the hallmark of an attempt for additional second division in *ms5* mutant. This results in generation of approximately eight microspores with variable DNA content (Ross et al., 1997). MS5 protein encodes for a 434 amino acid protein with no clear homology to other known proteins. However, it shows limited similarities to the synaptonemal complex protein (SCP1) from rat (Meuwissen et al., 1992). Synaptonemal complexes as mentioned above are involved in chromosome pairing, recombination and segregation and hence are important part of the meiotic machinery. *Arabidopsis ms5* mutants have a similar phenotype to *Drosophila* mutant

Roughex (*RUX*) in which the cells undergo normal meiosis but undergo an additional third division (Glover et al., 1998; Thomas et al., 1994). MS5 protein shows small similarity to Suc1/ Cks1 class of proteins in *Xenopus laevis*, which is a component of cyclin dependent kinase complexes that regulate progression of cell cycle (Patra and Dunphy, 1996). Although, there is no identity between MS5 protein is and *Arabidopsis* cyclin-dependent kinase related proteins such as Cks1, it is thought that MS5 may have similar functions to regulate cell cycle progression (Glover et al., 1994). The two identified mutant alleles of *MS5* exhibit a sterile phenotype when homozygous. However the semi-dominant ability of *ms5-2* allele, generates partial sterility in the heterozygote genotypes (Ross et al., 1997).

The identification and characterisation of *TDM1/ MS5* in barley provides opportunities to investigate the regulators of meiosis and their impact on fertility in a temperate cereal. Further understanding of meiosis in cereals may also help to generate more resistant crops to adverse climatic conditions.

5.2 Materials and Methods

The identification of the putative orthologous barley *MS5* gene was performed in by Dr. Jose Fernandez Gomez (Prof. Wilson group, University of Nottingham). The identification process used was as described in Chapter three for *HvNST1* and *HvNST2* (Sections 3.3.1.1 and 3.3.1.2).

The identified barley putative *MS5* sequence was used to prepare RNAi silencing, SRDX repression and overexpression constructs.

The generated transgenic lines were genotyped for the presence of transgene (RNAi, SRDX or overexpression constructs). The fertility of the transgenic plants was also measured. This was done based on the ratio

between the number of florets and seeds formed in the spikes chosen from the five main tillers per each plant compared to that of wild-type.

5.2.1 *HvMS5*^{RNAi} plants

The identified putative sequence of *HvMS5* gene was used to create RNAi silencing constructs (pBract207:: *HvMS5*RNAi) by Dr. Jose Fernandez that were used to generate the first generation of silencing lines.

5.2.2 *HvMS5* repression constructs (*HvMS5*^{SRDX})

The repression construct for the putative *HvMS5* was prepared by fusing the EAR repression domain to the C-terminus of the putative barley *MS5* coding sequence (see Table 5.1 for primer sequences). The amplified coding sequence containing the SRDX domain, was cloned into an entry vector (pCR/8GW/ TOPO) and the plasmid containing the fragment in the correct orientation was then cloned into the destination vector (pBract214). The pBract214 vector containing *HvMS5*^{SRDX} fragment was then used to transform *Agrobacterium* (AGL1) (2.13). Figure 5.1 shows the position of exons and primers on barley putative *MS5* sequence.

Table 5.1 Primers used for amplification of *HvMS5* coding sequence with the SRDX domain attached to the C-terminus of the coding sequence.

<u>Primer name</u>	<u>Sequence</u>
HvMS5cds-F1	ATGCCGAGCGGGGGCAGGCG
HvMS5SRDX-R1	CTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCC AGTGCAACATTTTGCTTCATCT

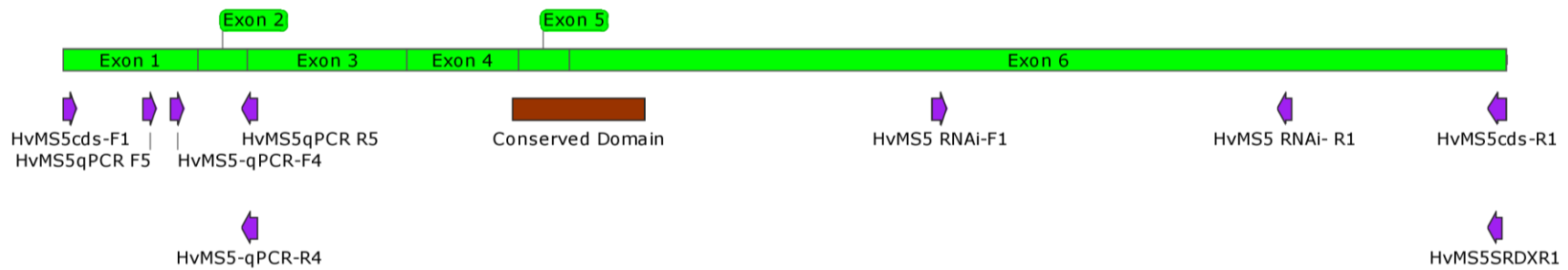


Figure 5. 1 Schematic presentation of putative MS5 cDNA coding sequence and position of primers. The RNAi fragment was designed outside the conserved domain, whereas for SRDX repression full length of the coding sequence was amplified with reverse primer containing the repression motif.

5.2.3 *HvMS5* overexpression

The ectopic expression of the putative target in barley was performed by inserting the identified coding sequence into the overexpression construct (pBract214). For this purpose, the full length of the putative *HvMS5* coding sequence was amplified (see Table 5.2 for primers) and cloned into pCR/8GW/ TOPO entry vector. The fragment was then cloned into the destination vector (pBract214) and the integrity of the sequence was confirmed by sequencing. The confirmed plasmid containing the fragment of interest was then used to transform *Agrobacterium* (AGL1) (Section 2.13).

Table 5.2 Primers used to amplify the full length of putative MS5 coding sequence.

Primer name	Sequence
HvMS5cds- F1	ATGCCGAGCGGGGGCAGGCG
HvMS5cds-R1	AGATGAAGCAAAATGTTGCATAG

5.2.4 *AtMS5* complementation

The complementation construct was prepared by amplification of the full length of barley *MS5* putative sequence and cloning the fragment into the vector pUbi::YFP (Grefen et al., 2010), for regulation by the *Arabidopsis* ubiquitin promoter.

5.3 Results

5.3.1 *HvMS5* identification

The putative orthologous sequence of barley MS5 was identified by Dr Fernandez Gomez (Prof. Zoe Wilson Group). This was carried out by step by step identification of the putative orthologous sequences of rice and *Brachypodium* prior to identification of barley putative sequence. *Arabidopsis MS5* (At4g20900) cDNA coding sequence was aligned against NCBI rice database. This BLAST analysis resulted in identification of *MS5* rice putative orthologue (Os08g0130300/ LOC_Os08g03620) with 45% nucleotide identity to *Arabidopsis MS5*.

The rice putative orthologue was then used against Gramene orthologue database to identify *Brachypodium* putative orthologue of *MS5* gene. This resulted in identification of *Brachypodium* putative orthologue (Bradi3g14297) that showed 71% nucleotide identity to rice putative orthologue of *MS5*. Finally, BLAST search of *Brachypodium* putative orthologue against barley database (IPK) resulted in identification of barley sequence (AK376532) with 75% nucleotide identity to *Brachypodium* putative *MS5* (Appendices 9.5.7- 9.5.9).

The BLAST search in IPK database resulted in identification of two highly identical sequences to *Brachypodium* (AK359744/ MLOC_37148 and AK376532/ MLOC_13664); however, phylogenetic analysis (Figure 5.2) showed close identity of AK376532/ MLOC_13664 to *Brachypodium* sequence. The phylogenetic analysis was done using the identified putative orthologue in barley (AK376532/ MLOC_13664) to BLAST against NCBI and IPK *Arabidopsis*, rice, *Brachypodium*, barley and other cereals. From these BLASTs the sequences with high identity to barley putative orthologue was

selected for constructing phylogenetic tree. Also, the tree construction helped in confirming whether the identified putative orthologues in rice, *Brachypodium* and barley cluster together considering the existence of other similar proteins. The close clustering of the three identified putative orthologues (rice, *Brachypodium* and barley) within the same clade was a good indication to correct identification of barley *MS5* orthologue. Moreover, the genetic distance between other potential barley putative orthologue AK359744 and the identified orthologues in rice and *Brachypodium*, showed that this may not be *MS5* orthologue in barley (Figure 5.2).

Therefore, this sequence was used as putative *MS5* sequence in barley.

Further confirmation of the identified putative orthologous sequence in barley was done by reverse bioinformatics analysis. For this purpose the identified barley putative orthologous gene code (MLOC_13664) was blasted against barley RNA-seq database (<https://ics.hutton.ac.uk/morexGenes/>). This database offered the same putative orthologue in rice (LOC_Os08g03620.1) that was previously identified as putative *MS5* in rice. However, it suggested an *Arabidopsis* gene (At3g51280.1) encoding for tetratricopeptide repeat (TPR)-like superfamily protein as the orthologue in *Arabidopsis*. The alignment between *Arabidopsis* *MS5* (At4g20900.1) and At3g51280.1 proteins showed low level of identity (33%). The identity between the two proteins was mainly in the conserved domain and the fact that they both encode for tetratricopeptide repeat (TPR) protein could be the reason that the server suggested At3g51280.1 instead of At4g20900.1 (*AtMS5*) as the orthologue. Moreover, the two putative barley orthologues (MLOC_13664 and MLOC_37148) were blasted against EnsemblPlants orthologue database. This resulted in identification of At4g20900 (*AtMS5*) for MLOC_13664 while there was no orthologues offered for MLOC_37148 in

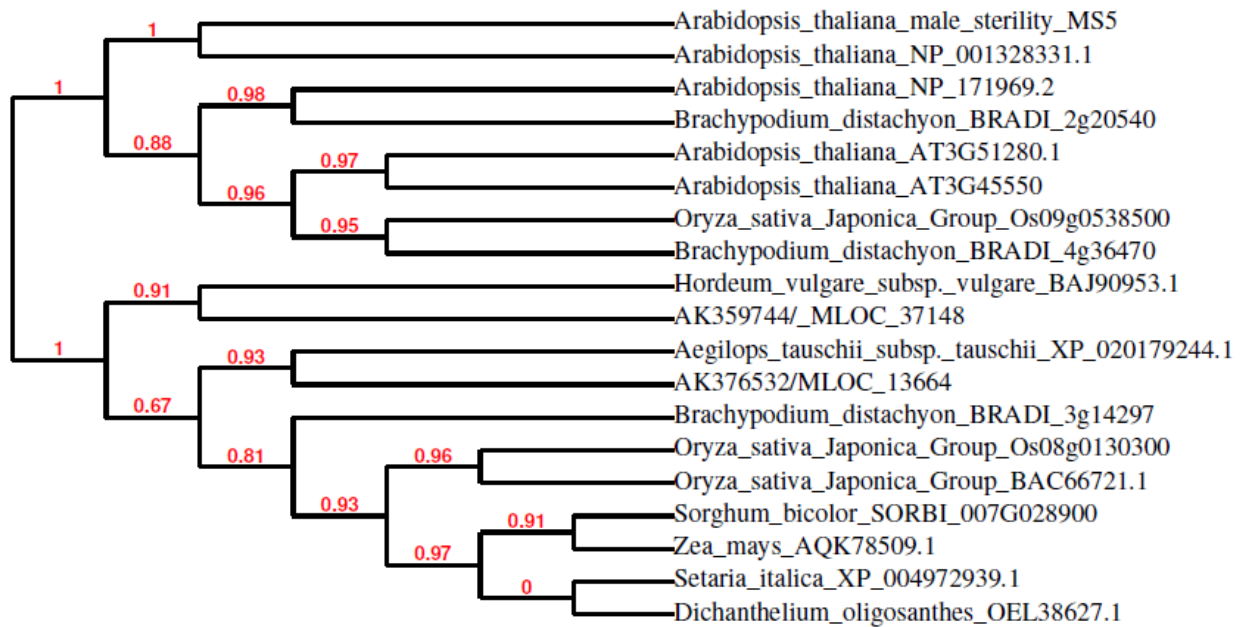


Figure 5. 2 Phylogenetic analysis of putative *MS5* sequences in barley (Dereeper et al., 2008). The BLAST search in barley database resulted in identification of a number of sequences. However, the phylogenetic analysis showed that sequence AK376532 that was considered as putative barley *MS5* had close association with rice and *Brachypodium* putative *MS5* sequences. *Arabidopsis thaliana* At3g51280.1: Tetratricopeptide repeat (TPR)-like superfamily protein; *Arabidopsis thaliana* NP_001328331.1: Tetratricopeptide repeat (TPR)-like superfamily protein; *Arabidopsis thaliana* NP_171969.2: Tetratricopeptide repeat (TPR)-like superfamily protein; *Arabidopsis thaliana* MS5: At4g20900; *Arabidopsis thaliana* At3g45550: hypothetical protein; *Oryza sativa* Japonica Group BAC66721.1: putative pollenless3 (*MS5*); *Oryza sativa* Japonica Group Os09g0538500: putative pollenless3; *Oryza sativa* Japonica Group Os08g0130300: predicted: uncharacterized protein; *Brachypodium distachyon* BRADI_3g14297: putative *MS5*; *Brachypodium distachyon* BRADI_4g36470: predicted protein POLLENLESS 3-LIKE 2; *Brachypodium distachyon* BRADI_2g20540: hypothetical protein; *Hordeum vulgare* subsp. *vulgare* BAJ90953.1 predicted protein; *Hordeum vulgare* AK376532/ MLOC_13664: putative *MS5*; *Hordeum vulgare* AK35974/ MLOC_37148: putative *MS5*-like protein; *Setaria italica* XP_004972939.1: predicted uncharacterized protein; *Aegilops tauschii* subsp. *tauschii* XP_020179244.1: uncharacterized protein; *Dichanthelium oligosanthes* OEL38627.1: Protein POLLENLESS 3-LIKE 2; *Sorghum bicolor* SORBI_007G028900 hypothetical protein; *Zea mays* AQK78509.1: Tetratricopeptide repeat (TPR)-like superfamily protein.

Arabidopsis thaliana. Therefore, the identified putative orthologous MS5 in barley was confirmed to be AK376532/ MLOC_13664. Furthermore, protein alignment between the identified putative MS5 sequences and their *Arabidopsis* counterpart showed that there was some level of conservation in the region that considered to contain the conserved domain that was thought to contain TPR domain (Figure 5.3).

The conserved domain in *Arabidopsis* contains Tetratricopeptide repeats (TPR) domain in the regions between 189-234 and 240-264 amino acids. The identified putative orthologues showed to contain different variations of this domain that could be related to their bigger protein size and their evolution (*Arabidopsis* MS5: 450 aa; rice putative MS5: 665 aa; *Brachypodium* putative MS5: 717 aa; barley putative MS5: 641 aa).

NCPCVERRAPGVYVYTPPAPRTSDHVAAMPTEERRPPYSCSSSSERRDPFHTVHKVPSGCPYVVRKAHAQLIDKFNRAISLFWTAINAGDRVDSALKD
 NPSSGGRLLPPWTSPRGAAAPRWSPCTPAGADGSGRAAHATPPASGGCSSHVTPASGGGGCYGYRVTPPTSGGCSRPPRAVSSVDSYVRAKKAQVIEKD
 NRSGGRRLLPPWTSPRGAAAEVVAPTGWSPTPAAGGGGSGSYVTPPLTAGCCSSSSYRVTPSSSGGCTSLTTPVSSGGCSRPPAPVAVDSSPYVR
 NPSGGRRLLPPWTSPRGAAAEVVAQWRWSPSPAGAAGSNHVPMSAGCCSYHVTVPVSSGGCRRPPAPKAVDSPYLRAKKAQLIEKDPNKAVPLFWAAIN
 M SGGRRLLPPWTSPRG A A VAVPQRWSPSPAGAAGSNHVPMSAGCCSYHVTVPVSSGGCRRPPAPKAVDSPYLRAKKAQLIEKDPNKAVPLFWAAIN

MAVVMKQLGRSDEIEAKISFRYLCSFESQDSINLLILEYKKSGRIEVALVLEHKLQTLQEGMGFGGRVSRARVQGHVIMTIEQEKARILGNLGVW
 PNKAVPLFWAAINSGDRIESALKDMATYLLKQANRAEEAIEAIRSFRDRCNEAQESLDNILLDLYKKCGRTHEQIEMLTILKLRIIDEELASGRWKTKLSK
 AKQQLIEKDPNKAVPLFWAAINSGDRIESALKDMATYLLKQANRAEEAIEAIRSFRDRCNEAQESLDNILLDLYKKCGRTKECIEMLTILKLRIIDEELAS
 SGERIESALKDMATYLLKQANRAEEAIEAIRSFRDRCNEAQESLDNILLDLYKKCGRTKECIEMLTILKLRIIDEELASGRWKTKLSK

HLLQLHNYGIAEQHYRFGFVTKIPNIDYCLVMRALGLERDKNKLCNLAICLMRMSRTPAERKSLDDVDRDSPAESCEGDEPFAKSYDRAVEMLAEEESKKE
 SHGRVYVLSLRDEKARLLGNLAWAHMQSENIDEAEMLYRQALAEADYNKECNLAICLMRMSRTPAERKSLDDVDRDSPAESCEGDEPFAKSYDRAVEMLAEEESKKE
 SGRWKTKLSKSHGRVYVLSLRDEKARLLGNLAWAHMQSENIDEAEMLYRQALAEADYNKECNLAICLMRMSRTPAERKSLDDVDRDSPAESCEGDEPFAKSYDRAVEMLAEEESKKE
 HARLLGNLAWAYMQSENIDEAEMLYRQALAEADYNKECNLAICLMRMSRTPAERKSLDDVDRDSPAESCEGDEPFAKSYDRAVEMLAEEESKKE

ADLSEKFYAGCSFVNRMKENIAPGTANKNYSDVSSSPASVRPNSAGLYTOPRRCLFEEETGAARALFGKQPQPFGEQHNIERGEEPEMKRKKLDQN
 TLHSPITQMKSKESLIWLAIDAENLGLHQPQSSSALTQLKSEEPHISVADAEEKQEDCNSQVFPSPITQMKREEPESLIATSENNCKLNEYDLRL
 TEMIKELESQALPSKITQMKSKDSRLLATDAENLDYIQPEILSTSSITQMKREEPESLIATSENNCKLNEYDLRL
 SQILLAADVESLEYPHQPISSSSAQLNYESLQASVLYTEKSEDCNSALPSPITQLMRKGPQIMVATDGEKNQNLQEYHDLRLFNDAAATPSQLEK

MIQYHEFVKDTADGPKSESKKWADIAEEEEEEEEERLQGEIKTAEM
 FNDAAATPSQLLEKLRKRLKEDILNISIQHQVQPSFVLCLPNSGGSTDAEENTRPEGKALVNGVRKTWADMVEEDERQLGDVSEITIGMDITKRVNSCKH
 ENQDASRLFNDAATPSQLLEKLRKRLKEDILNISIQHQVQPSFVLCLPNSGGSTDAEENTRPEGKALVNGVRKTWADMVEEDERQLGDVSEITIGMDITKRVNSCKH
 LRKRLKEDILNISIQHQVQPSFVLCLPNSGGSTDAEENTRPEGKALVNGVRKTWADMVEEDERQLGDVSEITIGMDITKRVNSCKH

ANEEMYRTPSFSQSSALKHSSVDHDPQSSSASWRHSDNISTGENVNRKFFVRTAPWRQQKVQDSNRVQRLTSHLSRAEGTEQHPWRSSTAQRS
 SKQASEQRYITPPSSQANSTLQTPAAGVRLQSSSAGSWRRNDSTGENVNRKFFVRTAPWRQQKVQDSNRVQRLTSHLSRAEGTEQHPWRSSTAQRS
 SLKTPVASVRLQSSSAGSWRRNDSTGENVNRKFFVRTAPWRQQKVQDSNRVQRLTSHLSRAEGTEQHPWRSSTAQRS

LFPDWKSKCERYGFIYVVPNDHFGQSSSHFATNRWPKKARPWRPQNRLLWFQEIITNEINQKTRALWLITYTTLLFGTTALDDTAWFLVECFHLLAGH
 RSLFHGHGLPSGESGRCHVSGHTAVNRPWPKNAATITRWRPQNRLLWFQEIITNEINQKTRALWLITYTTLLFGTTALDDTAWFLVECFHLLAGH
 ANHTEAASRWPKNAGSTRPWRPQNRLLWFQEIITNEINQKTRALWLITYTTLLFGTTALDDTAWFLVECFHLLAGH

LLLHDDQLDSVAPPSQFAEMAMOMHPATGLSTHWSQVDSPPCLKLREGANMVVLLQDQLLPMAFSIANVCEDCIMAYMSASASSVLFYTSCLDMQQLPY
 YCCCCWLAGWLAGQFFPA
 L QFAEMAMOMHPATGLSTHWSQVDSPPCLKLREGANMVVLLQDQLLPMAFSIANVCEDCIMAYMSASASSVLFYTSCLDMQQLPY

TGHVITINKSEAATIT
 TGHVITINKSEAATIT

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Arabidopsis TPR domain stretches between 189-234 and 240 until 240 amino acid. The identified putative conserved domain using NCBI conserved domain server showed that rice putative orthologue contains TPR region between 85-269 and TPR_19 region between 226-287 amino acids. *Brachypodium* putative orthologue contains TPR region (225-258 amino acids) and TPR_11 (224-284 amino acids). The identified barley putative orthologue contained TPR_7 (204-234 amino acid) and TPR_11 (201-259 amino acid). The variation within the TPR regions between *Arabidopsis* MS5 and the putative orthologues could mean that there were functional variations between these proteins.

Furthermore, it was shown that the number of exons are doubled in rice putative orthologue (LOC_Os0803620) compared to the *Arabidopsis* gene (At4g20900). However, the number of exons were the same in *Brachypodium* and barley putative orthologues (six exons) (Figure 5.4).

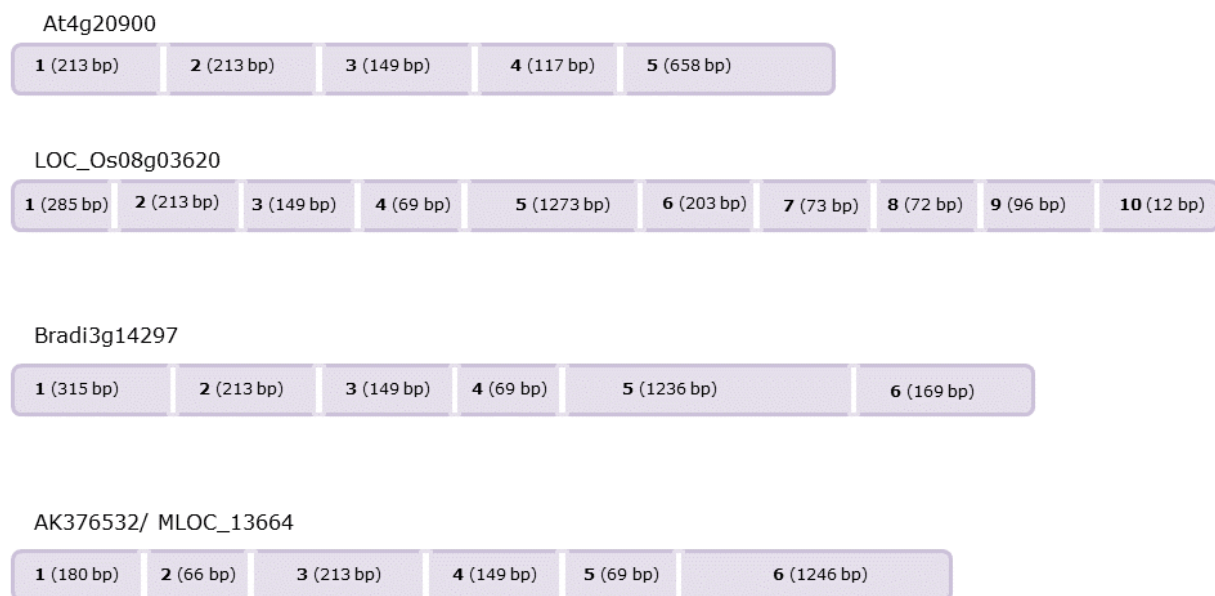


Figure 5.4 Schematic representation of exon and intron boundaries between the identified putative orthologous *MS5* genes and *Arabidopsis* *MS5*. Numbers in each box represent exon number with the size of each exon in parenthesis.

5.3.2 *HvMS5* repression (*HvMS5*^{SRDX})

The generated SRDX repression lines were genotyped for the presence of transgene. Two of the analysed plants (lines 7a and 7b) contained the transgene (Figure 5.5), however, did not show major difference compared to the wild-type in terms of fertility (Figures 5.6. and 5.7). The plants looked similar to their wild-type counterparts and there were no phenotypic differences observed between the repression lines and wild-type.

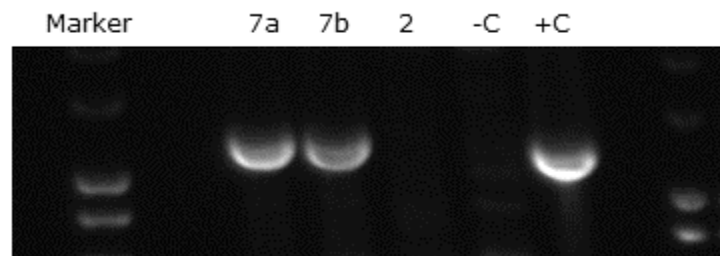


Figure 5. 4 Genotyping of the generated *HvMS5SRDX* plants. Two out of three generated transformant plants were positive for transgene; line 2 was negative for the presence of transgene. -C: Golden Promise (Wt) genomic DNA used as negative control, +C: pBract214 expression vector containing *HvMS5*^{SRDX} fragment used as positive control.



Figure 5. 5 Phenotypic comparison of *HvMS5^{SRDX}* lines to wild-type (Golden Promise). The generated transgenic lines showed no vegetative phenotypic or fertility reduction compared to wild-type.

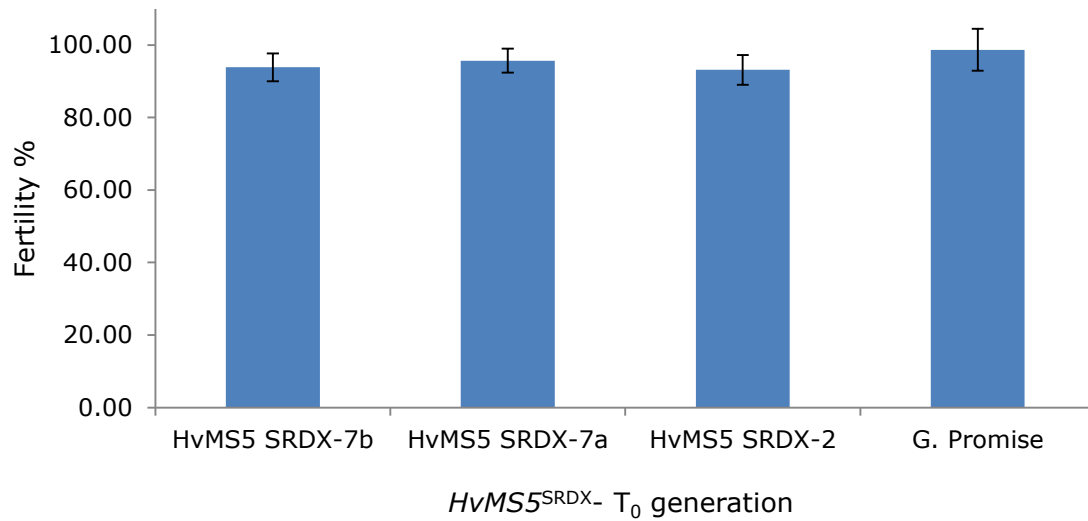


Figure 5. 6 The percentage fertility of the repression lines (*HvMS5^{SRDX}*) compared to wild-type. The presence of the repression construct in the lines did not cause any fertility alterations as compared to Golden Promise wild-type lines.

5.3.3 *HvMS5* overexpression

The overexpression lines of putative *HvMS5* were genotyped for the presence of transgene. This analysis showed that only one of the generated overexpression lines contained the transgene (Figure 5.8). The phenotypic comparison of the generated lines compared to wild-type showed that line 6 contained more sterile florets than line 4 and wild-type control (Figure 5.9). This observation that the plant without the transgene had lower fertility caused the repetition of genotyping analysis, however these results were the same and line 4 was the only positive plant for the transgene.

The fertility of the overexpression lines was measured by selecting five main tillers from each plant and counting the seeds set per spike along with the number of florets per spike in both transgenic lines and Golden Promise control plants. This analysis showed that fertility of the repression lines was reduced by up to 40 percent compared to wild-type (Figure 5.9).

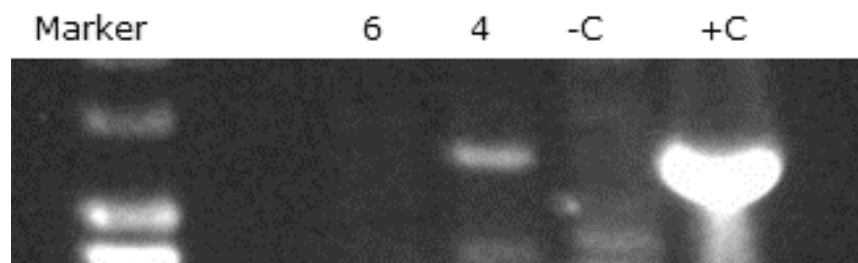


Figure 5. 7 Genotyping of *HvMS5*^{Over}. transformant plants. The analysis showed that line 4 was positive for the transgene. -C: Golden promise (Wt) genomic DNA used as negative control, +C: pBract214 expression vector containing *HvMS5*^{Over}. fragment used as positive control.



Figure 5. 8 Phenotypic comparison of *HvMS5* overexpression lines. The fertility of line 4 resembled that of wild-type; whereas the fertility in line 6 was reduced due to generation of sterile florets.

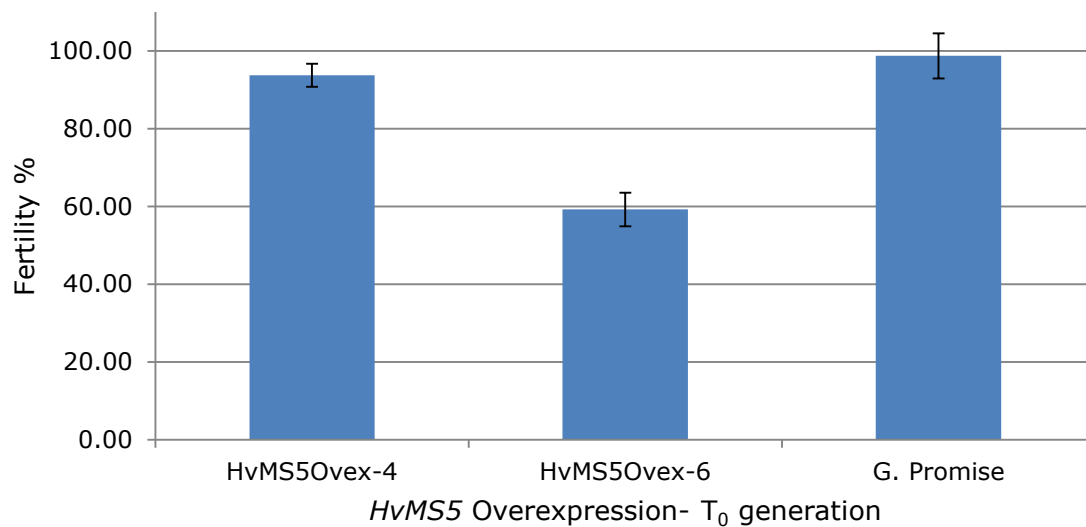


Figure 5. 9 Percentage of fertility of *HvMS5* overexpression lines. Transgenic *HvMS5* lines showed reduction in fertility, by up to 40 percent.

Unfortunately, there was no sample collected from neither overexpression nor SRDX repression lines in the T0 generation to perform expression analysis of the putative *MS5* gene.

5.4 Discussion

Cell division is a pivotal part of an organism's development. It allows the increase in the size of the cells and therefore organs, development of new cell niches within other cell structures and differentiation of new organelles that are done via mitotic cell divisions. However, evolution has enabled the reproductive organs to produce cells with half genetic content compared to their mother cells, and hence as fertilisation between the male gamete and the egg cell happens the progenies with the same genetic content as the parents are produced. This process is ensured via a reproductive tissue specific cell division known as meiosis. Meiosis also enables the recombination between homologous chromosomes that results in creation of genetic variation within the population; therefore, it is the source of genetic diversity within and between the species.

A delicate process such as meiosis requires a very refined and timed regulation between the two meiotic divisions. Any miscommunication between the regulatory genes or mutation in the genes involved in this pathway can result in formation of cells with uneven number of chromosomes or premature termination of meiosis that ultimately affects the fertility of the plant (Caryl et al., 2003; Ross et al., 1997).

The importance of meiosis for the survival of all sexually reproducing organisms has resulted in large investment in better understanding this pathway and the genes regulating it. Furthermore, the recent increase in demand for higher crop yield and availability of narrow gene pool has driven

the research towards understanding of meiotic recombination processes and possibility of alteration of crossover in order to provide more diverse gene pool (Baker et al., 2014; Higgins et al., 2012).

The final stages of meiotic division includes the separation of sister chromosomes and generation of four cells with half the genetic material as their mother cell. The failure to terminate the meiotic division results in further cell divisions without chromosome duplication and yields in formation of cells with uneven number of chromosomes. The *MS5* gene has been shown to be involved in termination of meiosis at the end of the second meiotic division in *Arabidopsis* (Cifuentes et al., 2015; Glover et al., 1994). It is also known that multiple genes, including *MS5*, that are involved in regulation and coordination between the two divisions in meiosis either by interaction with *MS5* or independently (Cifuentes et al., 2015).

In *Arabidopsis*, *MS5* is thought to be involved in the normal progression of meiosis and termination of this process at the end of the second meiotic division. The failure in termination of meiosis process results in generation of microspores with uneven number of chromosomes and sterility (Caryl et al., 2003; Glover et al., 1994). The repression of *MS5* putative orthologue in barley did not seem to have any substantial effects on fertility that suggests its lower impact on fertility in barley and perhaps other monocotyledons compared to dicotyledons.

Another explanation for not observing the expected phenotype could be that the identified putative orthologue in barley was not the correct orthologue. Therefore, a second attempt on identification of the correct putative orthologue may be advisable to rule out the possibility of working with the incorrect sequence.

The other potential reason for not observing severe effects on fertility could be that the *MS5* gene in barley follows a different regulatory pathway as compared to *Arabidopsis*. This could be due to the divergence of dicots and monocots and the fact that evolution and gene duplication have played roles in perhaps providing gene redundancy in the network controlling meiosis in barley and other monocots. Therefore, potential gene redundancy in this particular pathway could be the reason for not observing the expected phenotype. The redundancy in the meiosis network could be important in protecting cereal such as barley against possible loss of function the network that can cost the plant its next generation.

The conservation of the major contributors of the genes affecting fertility between *Arabidopsis* and rice suggests a similar pattern for the putative orthologue in barley (Fernandez Gomez et al., 2015). However, it could be possible that evolutionary processes such as whole genome duplication has made *MS5* a functionally redundant gene compared to the other regulators of meiotic progression in barley. Therefore, reverse genetic approaches, though effective, may not provide efficient information regarding a putative target and confirmation of the roles of a targets may require follow up experiments.

As the plants showed no severe effects in neither overexpression nor SRDX repression lines, perhaps the authenticity of the identified putative sequence could be revisited. This will ensure that the correct sequence and targets are being repressed or overexpressed in barley. As mentioned before, BLAST search of the barley database showed two sequences with high identity to the *Brachypodium* putative orthologue. This high identity can refer to the sequences acting as paralogs in barley, considering the fact that there is not a report for a paralogous gene to *MS5* in *Arabidopsis*. Moreover, the phylogenetic study (Figure 5.2) of the identical sequences to the identified barley putative orthologue showed a potential duplication

for the putative *MS5* gene in barley (AK359744/ MLOC_37148 and AK376532/ MLOC_13664). Thus, this potential duplication and presence of transcript could potentially complement the function of the putative *HvMS5* gene resulting in the failure of an observed phenotype in silenced lines. The use of SRDX fusions may potentially overcome this issue since they can act as dominant repressors and phenotypes can be detected despite redundancy in the gene targets (Mitsuda et al., 2005). Nevertheless, it may be worth silencing the putative paralogous *MS5* gene in barley (AK359744/ MLOC_37148) along with the identified *HvMS5* (AK376532/ MLOC_13664) to help confirm their functions.

Chapter Six:

Involvement of N-end rule Pathway in AtMS5 protein degradation

6.1 Introduction

The life span of a protein in a cell varies from less than a minute to many days. Intracellular proteolysis is responsible for a number of activities including the elimination of mis-folded or abnormal proteins, amino acid pool maintenance in cells affected by stress and production of protein fragments that act as hormones or other effectors (Graciet and Wellmer, 2010; Varshavsky, 2011). Proteolytic degradation is necessary for the regulation of the majority of the cellular signals. Nonetheless, between the 1960s and 1980s protein degradation was a neglected area and even was considered as a nonspecific or dead-end process (Glickman and Ciechanover, 2002). One of the major roles of proteolytic pathways is selective elimination of proteins during specific developmental stages (Varshavsky, 2011). It is clear now that the modulation of the activity of transcription factors and their activity is regulated through cellular signals. These signals are diverse and include changes in cell redox status, calcium spiking and protein phosphorylation or ubiquitination (Marino et al., 2013; Moore et al., 2011). Phosphorylation can regulate enzyme activity (including transcription factor activity), protein localisation, protein stability and protein-protein interactions (Kusakina and Dodd, 2012).

Ubiquitination is also a frequent feature of cell signalling and common post-translational modification (PTM), which is similar to phosphorylation except that in ubiquitination there is a covalent binding of ubiquitin (Ub) to lysine residues of the target protein (Kusakina and Dodd, 2012; Marino et al., 2013). Ubiquitin is also a signalling module for a variety of cellular processes including DNA repair, gene transcription, protein activation and selective protein degradation through 26S proteasome that is the most famous function of ubiquitin (Kusakina and Dodd, 2012; Vierstra, 2009). Ubiquitination requires a cascade of enzymes that includes Ub-activating (E1), Ub-conjugating (E2) and Ub-ligase (E3) enzymes (Marino et al.,

2013) (Figure 6.1). HECT (Homologous to E6-associated protein C-Terminus), RING (Really Interesting New Gene), U-Box and Cullin-RING ligases (CRLs) are four different types of E3s that are known in plants (Vierstra, 2009). These E3 proteins are classified by their mechanism of action and composition (Marino et al., 2013; Vierstra, 2009). Marino et al. (2013) showed that MIEL1 (MYB30-Interacting E3 Ligase1) is an active E3 ligase, which interacts with MYB30. This interaction leads to degradation of MYB30 by the 26S proteasome through its ubiquitination via MIEL1 (Marino et al., 2013).

The Ubiquitin-proteasome (UP) system mostly regulates degradation of intracellular proteins (Varshavsky, 2011). Conjugation of 8-kDa protein ubiquitin (Ub) to Lys residues of substrate proteins serves as a signal for proteolysis by the 26S proteasome, which is governed by ubiquitin-proteasome system (UPS) in eukaryotes (Graciet and Wellmer, 2010). It is also now known that there is also reversible ubiquitylation that directs the non-proteolytic events, which affect the regulation of transcription, chromatin structure and vesicular trafficking (Mukhopadhyay and Riezman, 2007; Vierstra, 2009). Protein degradation through ubiquitin/proteasome pathway involves two consecutive steps (Glickman and Ciechanover, 2002).

In the first step, multiple ubiquitin moieties are conjugated to the substrate. This is followed by the second step in which the tagged protein is degraded by the downstream 26S proteasome complex (Glickman and Ciechanover, 2002). It has been shown that there are several established principles through which post-translational modifications mediate crosstalk. In positive crosstalk, one PTM serves as a signal for recognition by binding protein that carries out a second modification. For instance phosphorylation events such as GSK3-mediated phosphorylations requiring

positioning of a priming phosphate at +4 position is an example of positive crosstalk (Hunter, 2007).

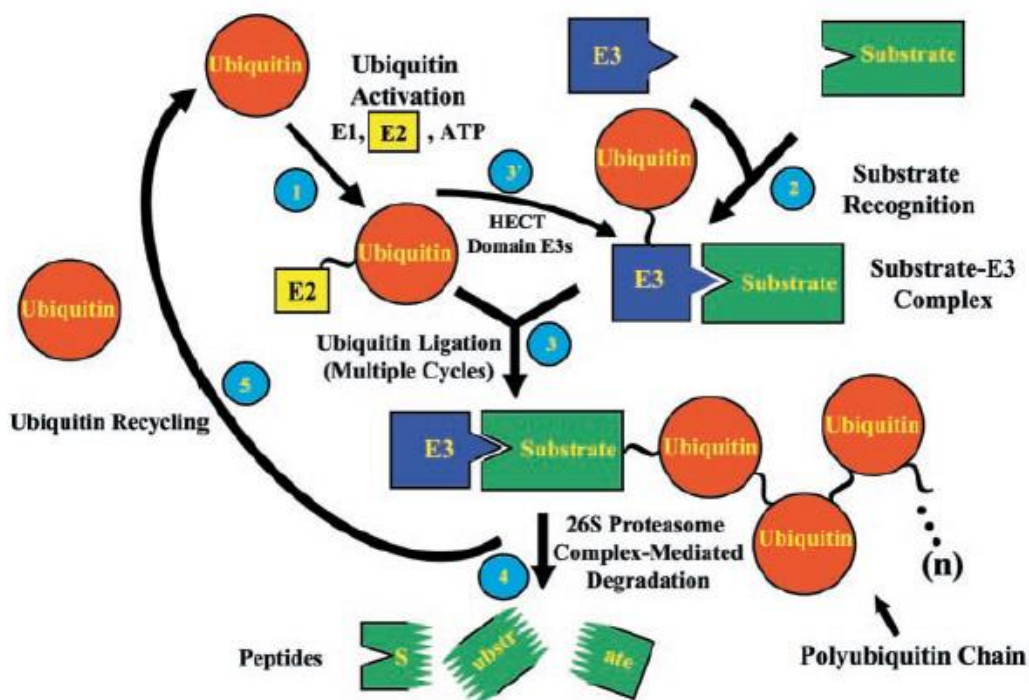


Figure 6. 1 Protein degradation via the ubiquitin proteolytic pathway (Glickman and Ciechanover, 2002). Ubiquitin is activated via the ubiquitin activating enzyme E1, a ubiquitin-carrier protein, ubiquitin-conjugating enzyme E2 and ATP. This ubiquitin activation results in formation of high-energy E2~ubiquitin thiol ester intermediate (1). The protein substrate binds to specific ubiquitin-protein ligase E3 through a defined recognition motif (2). In the next step, ubiquitin is conjugated to the target substrate in multiple cycles that results in the synthesis of polyubiquitin chain (3). E2 transfers the first activated ubiquitin moiety directly to the E3-bound substrate and in the following cycles to previously conjugated ubiquitin moiety. HECT domain E3s catalyse the direct transfer of the activated ubiquitin moiety from E2 to high-energy thiol intermediate on E3, before its conjugation to the E3-bound substrate or to the previously conjugated ubiquitin moiety (3'). The ubiquitinated substrate is degraded via 26S proteasome with release of short peptide (4) and the ubiquitin is recycled through deubiquitinating enzymes (DUBs) (5).

The negative crosstalk is the other form of PTM crosstalk, in which there is a direct competition for the modification of a single residue in a protein, or indirect event, wherein one modification covers the recognition site of the second PTM (Hunter, 2007). This is the principle when modification of a single Lys by ubiquitylation or SUMOylation, acetylation, ubiquitination and methylation (Hunter, 2007). Direct competition in Yop-J (*Yersinia* species effector protein)-mediated acetylation of Ser in the activation loops of MAP2Ks, that are normally phosphorylated by MAP3Ks is an example of this negative crosstalk (Hunter, 2007).

The N-end rule pathway was first discovered by the observation that a fusion protein was degraded differently when the N-terminal was altered. This observation was made when ubiquitin molecule (Ub) was fused at the N-terminal of a junctional amino acid residue that varied in different fusion proteins, and contained a reporter protein (β -Galactosidase) at the C terminus (Graciet and Wellmer, 2010). The expression of this fusion protein in *S. cerevisiae* resulted in cleavage of Ub by deubiquitinating enzymes (DUB) and formation of junctional protein with the reporter protein at its C-terminus (Bachmair et al., 1986). Furthermore, it was showed that the reporter gene in some N-terminal residues accumulated more than the others. They observed that some N-terminals residues (stabilising residues) resulted in accumulation of the reporter gene; whereas other so-called 'destabilising residues' caused lower level of the reporter gene (Bachmair et al., 1986; Graciet and Wellmer, 2010).

It was found that the differences in the β -Gal accumulation were correlated with half-life of the reporter proteins (Bachmair et al., 1986; Graciet and Wellmer, 2010). They showed that there is a protein degradation pathway that relates the *in vivo* stability of a protein to the nature of its N-terminal residue (Bachmair et al., 1986; Graciet and Wellmer, 2010). This pathway was therefore named N-end rule pathway (Graciet and Wellmer, 2010).

The N-end rule pathway was initially characterised in fungi and mammals (Bachmair et al., 1986; Bachmair and Varshavsky, 1989; Gonda et al., 1989; Graciet and Wellmer, 2010; Varshavsky, 1996). It has been shown that recognition of the degrons at the N-terminal of a destabilizing protein (termed N-degrons) is not the only requirement of this pathway. Furthermore, the spatial proximity of the Lys residue for Ub conjugation to the N-terminus and also a flexible region in the vicinity of these two determinants are among the requirements of this pathway (Bachmair and Varshavsky, 1989; Graciet and Wellmer, 2010; Prakash et al., 2004; Suzuki and Varshavsky, 1999).

The organisation of plants N-end rule pathway has reported to be similar to mammals through its enzymatic and destabilising residues (Garzon et al., 2007; Graciet et al., 2010; Graciet and Wellmer, 2010; Schlogelhofer and Bachmair, 2002; Yoshida et al., 2002). In this pathway the N-terminal of the proteins with the destabilising residues is recognized by E3 ligases (also known as N-recognins), which will direct the proteins for degradation by the 26S proteasome.

The destabilising residues are divided into three categories of primary, secondary and tertiary residues (Graciet and Wellmer, 2010; Holman et al., 2009). Two classes of N-recognins have been characterised in yeast and mammals that target basic and hydrophobic amino-terminal amino acids. The first characterised E3 ligase was UBR1, and there have been at least 7 proteins characterised in mammals that contain the characteristic UBR box (Gibbs et al., 2011; Holman et al., 2009). To date, two E3 ligases, *Proteolysis 1* and *Proteolysis 6* (*PRT1* and *PRT6*), have been characterised in *Arabidopsis*. *PRT6* only recognises basic amino acids, whereas *PRT1* that does not contain a UBR box recognises aromatic amino acids. It is also thought that there are other N-recognins in plants that are not currently known, due to the observation that in *prt6prt1* double mutants the N-

degrons are unstable (Garzon et al., 2007; Holman et al., 2009). The primary destabilising residue can be directly targeted by E3 ligases. The primary residues can be further subdivided into basic residues or type 1 (Arg, Lys and His), and type 2 or bulky hydrophobic residues (Phe, Trp, Tyr, Leu and Ile) (Graciet and Wellmer, 2010; Tasaki et al., 2012) (Figure 6.2).

Unlike primary residues, Secondary destabilising residues must be enzymatically changed before targeting for degradation. This change can be done through modification by arginyl-tRNA-protein transferase (ATE) that conjugates Arg to the N-terminal of substrates with Asp, Glu or oxidized Cys at their N-terminal (Graciet and Wellmer, 2010; Tasaki et al., 2012) (Figure 6.2). Proteins with tertiary destabilising residues have to be modified before arginylation by R-transferases. For instance, Asn is modified by deamidation into secondary destabilizing residue, Asp, by N-terminal amidase enzyme (Graciet and Wellmer, 2010) (Figure 6.2).

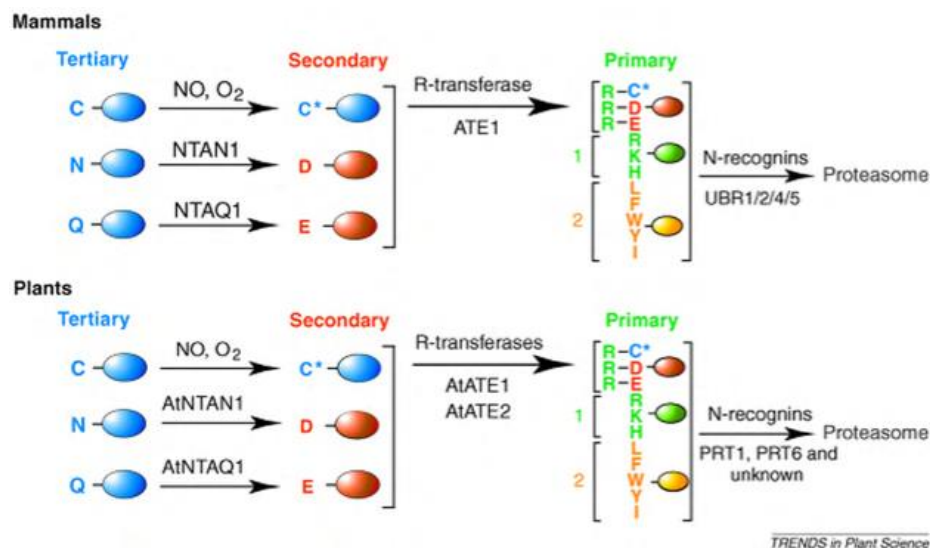


Figure 6. 2 N-end rule pathway in mammals and plants (Graciet and Wellmer, 2010). Targets of this pathway are divided into three groups of substrates with primary, secondary and tertiary destabilising residues. These substrates after modification will be degraded by proteasome.

The role of N-end rule pathway in plant development has been investigated. Seed germination is an important part of the plant life cycle, which involves the breakage of the testa layer that follows endosperm ruptures opposite the emerging embryo radicle. Penetration of radicle to the endosperm is a sign for completion of germination (Holman et al., 2009). The role of N-end rule pathway in germination is through regulation of Absciscic acid (ABA) sensitivity that acts as the repressor of germination and stabilises the dormant state (Holman et al., 2009). Study of the *prt6* mutant showed extreme ABA-hypersensitivity and inhibition of germination in comparison with the other published ABA-hypersensitive mutants (Holman et al., 2009). The mutant seeds show increased after-ripening time, indicating *PRT6* plays an important role to potentiate after-ripening (Holman et al., 2009).

Yoshida et al. (2002) isolated the *delayed leaf senescence 1* (*dls1*) mutant in *Arabidopsis* caused by T-DNA insertion. The *dls1* mutant was shown to affect age-dependent progress of senescence (Yoshida et al., 2002). In wild-type plants grown under 16 hours light and 8 hours dark conditions for 49 days, the third or fourth leaves died whereas the ones of *dls1* turned only partially yellow (Yoshida et al., 2002). Furthermore, the fifth and sixth rosette leaves of the wild-type plants partially senesced in the same conditions, while those of *dls1* remain green (Yoshida et al., 2002). Further analysis of *dls1* mutant showed that the mutation was caused by T-DNA insertion in the fourth intron of *AtATE1* gene, a component of N-end rule pathway (Yoshida et al., 2002). The full coding sequence of *AtATE1* plus its promoter region (2.1 kb upstream of the coding sequence) was able to rescue *dls1* phenotype (Yoshida et al., 2002). It was also shown that the chlorophyll content of the resultant T2 lines grown in continuous light for 10 days followed by five days incubation in dark decreased as the same level as the wild-type (Yoshida et al., 2002). It was therefore shown that

the *ATE1* rescued *dls1* mutant was responsible for delayed-senescence phenotype of *dls1* mutant (Yoshida et al., 2002).

Gibbs et al. (2011) investigated the possibility of degradation of *Arabidopsis* group VII ERFs such as SUB1A-1 through N-end rule pathway. Mutation in the second amino acid of ERFs protein enhances the stability of ERFs *in vitro*, indicating that all group VII ERFs are potentially substrate of this pathway (Gibbs et al., 2011). Transcriptome analysis of the N-end rule pathway mutant *ate1 ate2* and *prt6* showed constitutive expression of the genes responsible for anaerobic metabolism and survival hypoxia such as *ADH1*, *SUS4* and *PDC1* in high levels in both mutants (Gibbs et al., 2011). Moreover, hypoxia-induced genes were highly expressed in N-end rule pathway mutants suggesting resistance of these mutants to hypoxic (low oxygen content) conditions (Gibbs et al., 2011).

Recent studies showed *Arabidopsis* ethylene response factor (ERF) group VII transcription factors including *Hypoxia Responsive1 (HRE1)*, *HRE2* and *Related to AP2 2 (RAP2.2)* enhance responses to hypoxia and anoxia (Gibbs et al., 2011; Licausi et al., 2010). Analysis of regulation of two ERF proteins HRE1 and HRE2 through N-end rule pathway confirmed activity of N-end rule pathway towards specific MC-containing substrates (Gibbs et al., 2011). Ectopic expression of wild-type and mutant (HRE1 (C2A)), (HRE2 (C2A)), (C2A: the second amino acid was changed from cysteine to alanine) proteins showing only the mutant C2A proteins could be detected at high levels (Gibbs et al., 2011). Study of HRE1 (C2A) and HRE2 (C2A) proteins under normal and low oxygen conditions showed that after transfer of seedlings to hypoxia conditions, HRE2 protein was elevated within two hours, while HRE1 could not be detected (Gibbs et al., 2011). Upon return to normoxic (normal oxygen content) conditions HRE2 became destabilised (Gibbs et al., 2011). . These results show that stabilisation of these proteins

under hypoxia conditions leads to survival under low oxygen conditions (Gibbs et al., 2011).

The *Arabidopsis male sterile 5* (*MS5*) mutant has been shown to be linked formation of normal tetrads in developing microspores; hence an essential gene for male fertility in *Arabidopsis* (Glover et al., 1998) (section 1.2) The *Arabidopsis MS5* gene encodes for a 434 amino acid protein with predicted molecular weight of 49.2 kDa (Glover et al., 1998). This protein has a limited similarity with synaptonemal complex protein (SCP1) cloned from rat (Glover et al., 1998; Meuwissen et al., 1992). Synaptonemal complexes (SCs) are formed during the prophase stage of the first meiotic division, and thought to function in chromosomal pairing, recombination and segregation of chromosomes (Glover et al., 1998; Moens, 1994).

In the *ms5* mutant microsporocytes appear initially normal but soon degenerate (Caryl et al., 2003; Taylor et al., 1998). Further examination of these mutants showed that the pollen mother cells seem to undergo two rounds of normal meiotic division. However, at the end of meiosis II cells undergo a further division without any further DNA replication (Caryl et al., 2003; Glover et al., 1998). The result of this further division is approximately eight microspores with variable DNA content (Caryl et al., 2003; Glover et al., 1998).

The *Arabidopsis MS5* protein contains the amino acid cysteine at the second position of the protein that makes it a possible target of the N-end rule pathway. As explained previously, the cysteine in the N terminus of the protein is a tertiary residue of the N-end rule pathway, which after oxidisation and arginylation is targeted by N-recognins for degradation by the 26S proteasome. *Arabidopsis MS5* protein contains amino acid cysteine after the first amino acid, methionine. This makes *MS5* protein a potential tertiary target of N-end rule pathway. Therefore, the aim of this work was to determine whether the alteration of the second amino acid from cysteine

to alanine could stabilise the protein under *in vitro* conditions and establish whether MS5 protein is regulated via N-end rule proteolysis.

6.2 Materials and Methods

The analysis of the involvement of N-end rule pathway in degradation of MS5 protein was done by amplification of two versions of the gene. The MC or unstable version of the gene (the same sequence as the native protein in *Arabidopsis* that starts with methionine followed by cysteine) and MA or stable version that begins with methionine and the second amino acid was changed to alanine to protect it against degradation via N-end rule pathway. These two versions were amplified using the primers in Table 6.1 and cloned into destination vector (pTNT3xHA) containing HA tag. The primers (Table 6.1) included restriction sites (*KpnI* was used for forward primers and *XbaI* for the reverse) at both ends of the amplified gene, for cloning into the destination vector.

Table 6.1 Primers used for cloning *AtMS5* into pTNT3xHA expression vector

Name	Sequence
AtMS5MC-KpnI-Forward	AAAAGgtaccATGTGTCCCTGCGTAGAGCGT
AtMS5MA-KpnI-Forward	AAAAGgtaccATGgcTCCCTGCGTAGAGCGT
AtMS5-XbaI-Reverse	AAAAtctagaCATCTCTGCGGTTTTAAGCTCT

The destination vectors containing the two versions of the gene (MC, MA) were used in TNT® Coupled Reticulocyte Lysate System for *in vitro* analysis of the degradation of two protein variants (Section 2.15). The protein samples were collected after 30, 45 and 105 minute from the start of the

assay. The collected samples were visualised in polyacrylamide gel following western blot (Section 2.16).

6.3 Results

6.3.1 Analysis of involvement of N-end rule pathway (NERP) in *AtMS5* degradation

The N-end rule pathway (NERP) is a protein degradation pathway, in which the N-terminal amino acids of proteins with destabilising residues are recognised and the protein is directed for ubiquitination by the proteasome. The destabilising residue can be directly recognised by the N-recognins or they can be recognised after modifications such as oxidation, deamidation or arginylation. Proteins bearing methionine followed by cysteine at their N-terminal are the tertiary targets of this pathway. The *Arabidopsis MS5* gene has the potential of being the target of NERP due to its protein combination that starts with methionine and followed by cysteine (MC) (Figure 6.3).

In order to analyse the positive involvement of NERP in the degradation of *MS5* protein two different versions of this protein (unstable version denoted as MC and stable version denoted as MA) were generated. The stable version of the protein starts with methionine followed by alanine, which is not a substrate of this pathway. The unstable version is the normal *MS5* protein that starts with methionine followed by cysteine, which is a substrate of NERP and therefore may potentially be degraded via this pathway.

A:

MCPCVERRAPPGVYYTPPPARTSDHVAAMPMTERRRPPYSCSSSSERRDPFHIVHKVPSGDSPLYVR
AKHAQLIDKDPNRAISLFWTAINAGDRVDSALKDMAVVMKQLGRSDEGIEAIKSFYRLCSFESQDS
IDNLLLELYKKSGRIEEEEAVLLEHKLQTLQGMFGGGRVSRAKRVQGKHVIMTIEQEKARILGNLGW
VHLQLHNYGIAEQHYRFGFVTKIPNIDYCLVMRALGLERDKNKLCNLAICLMRMSRIPEAKSLDDV
RDSPAESECGDEPFAKSYDRAVEMLAEIESKKPEADLSEKFYAGCSFVNRMKENIAPGTANKNYSD
VSSSPASVRPNSAGLYTQPRRCRLFEEETRGAARKLLFGKPQPFQSEQMKILERGEEEPMKRKKLDQ
NMIQYLHEFVK DTADGPKSES KKSADIAEE EEAEEEEER LQGELKTAEM

B:

MCPCVERRAPPGVYYTPPPARTSD EEEAEEEEERLQGELKTAEM

A

AtMS5-MC

MAPCVERRAPPGVYYTPPPARTSD EEEAEEEEERLQGELKTAEM

B

AtMS5-MA

Figure 6. 3 Schematic presentation of *Arabidopsis* MS5 protein. A: full length of Arabidopsis MS5 protein. This protein contains cysteine at the second amino acid, which is a target of N-end rule pathway after oxidation and arginylation. B: Alteration of the second amino acid to alanine can stabilise this protein as alanine is not a target of the NERP (B).

The two versions of the gene were amplified using the primers in Table 6.1 (Figure 6.4). Both versions of the gene were cloned into expression vector pTNT3xHA to generate HA tagged fusion proteins, which were used for detecting the degradation of the proteins under *in vitro* conditions (section 2.15).

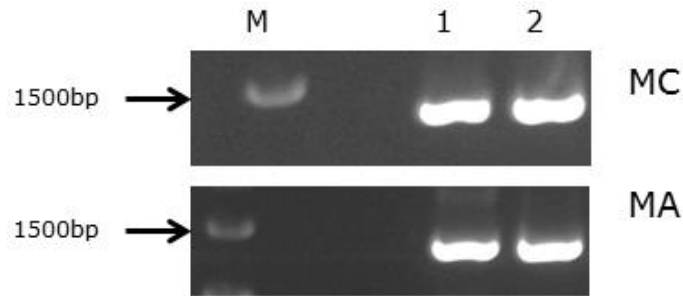


Figure 6. 4 Amplification of two versions of the gene (Mc, MA) for cloning into destination vector. The expected size for both fragments was 1353bp. M: DNA marker, 1-2: the amplified DNA products.

In order to assess the involvement of NERP in AtMS5 degradation the unstable (MC) and stable (MA) versions of the gene were translated to protein *in vitro* using TNT® Coupled Reticulocyte Lysate System (section 2.15). The samples were collected at 30, 45 and 105 minutes after the start of the *in vitro* assay. The protein stability of the samples (both MC and MA versions) were compared by western blot (section 2.16) probed with goat anti HA tag. The preliminary results showed that the MC or unstable version of the gene was degraded after 105 minutes, whereas the stable (MA) version of the gene was stable throughout the whole sampling period (Figure 6.5).

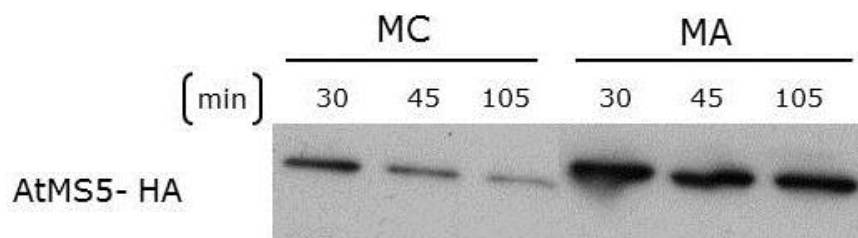


Figure 6. 5 Western blot analysis of AtMS5-MC and MA versions. Both versions of the protein were cloned into pTNT3xHA expression vector that was tagged with mouse HA protein (Hemagglutinin) at the C-terminus. The expression of AtMS5 MC and MA versions were detected using goat α -HA antibody. This result showed that the MC version is unstable and is degraded after 105 minutes, in comparison to the stable (MA) version that has constant presence from 30 to 105 minutes.

This showed that AtMS5 protein could be a potential target for degradation via NERP. To further confirm this, the activity of proteasome was inhibited by adding MG132 (5 mM) to both AtMS5-MC and AtMS5-MA reactions before *in vitro* assay sampling. This was to determine whether this inhibition of proteasome activity could stabilise the degradation of MC version that was previously observed (Figure 6.5).

The addition of MG132 did not affect the protein degradation of MC or MA version (Figure 6.6). Furthermore, contrary to what was seen before the reduction of MC version was not observed. The assay was repeated using the same amount (50 ng/μl) of MC and MA constructs per reaction. The *in vitro* assay was also repeated with freshly prepared plasmid aliquots, freshly prepared MG132, and freshly prepared primary and secondary antibodies to rule out the possible problems that could inhibit observation of the first degradation. However, all these efforts did not result in observation that the AtMS5 protein is degraded during the sampling period, so that the alteration of second amino acid to alanine could rescue this degradation.

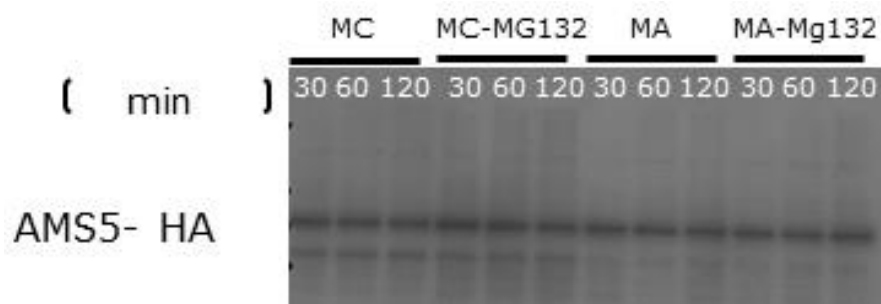


Figure 6. 6 Western blot analysis of AtMS5- MC and MA versions using MG132 as control. The repetition of *in vitro* assay and addition one replicate per each construct for proteasome inhibitor (MG132) did not result in stabilisation of AtMS5-MC protein. This assay also showed that the protein degradation observed previously was not replicable. The protein samples were collected at 30, 45 and 120 minutes intervals during *in vitro* assay prior to western blot.

6.4 Discussion

Initial analysis suggested that MS5 may be a target if the N-end rule pathway. However, the current study is not at the stage to conclude whether MS5 is degraded via N-end rule pathway or not. Further and detailed study of this protein *in vivo* is required to fully understand its degradation. The initial results from an *in vitro* assay showed that the MS5 protein is a potential target of this pathway.

The observation that the native MS5 protein was degraded during the sampling period (Figure 6.5) compared to the stable version of the protein (MA) was an indication to potential involvement of N-end rule pathway in its degradation. This was due to the fact that the stabilised version of the protein (MA), showed similar expression throughout the sampling time points (Figure 6.5). However, further attempts to stabilise the MC version by inhibiting proteasome failed to reproduce the initial results. Although all possible reason for this failure were addressed in subsequent repetition of the *in vitro* assay and western blots, there still could be a reason for this un-repeatable observation. Therefore, it is important to observe the behaviour of this protein *in vivo* in order to determine whether there is any possibility of involvement of NERP in MS5 and plant fertility regulation.

The N-end rule pathway has been shown to be involved in plant developmental processes including responses to abiotic stresses (Gibbs et al., 2011; Holman et al., 2009). The stabilisation of the target protein can enhance the response of plants to adverse conditions (Gibbs et al., 2011; Holman et al., 2009; Yoshida et al., 2002).

The living organisms rely on oxygen for energy production; however, this has proved to be a double edged sword as oxygen (O₂) is toxic by nature and all aerobes including plants experience molecular damage in exposure to high concentrations of O₂ (Schippers et al., 2012). The presence of

aerobic conditions provides a chance for organisms to use oxygen as an electron acceptor and use its reactive properties for metabolism and signalling (Schippers et al., 2016; Schippers et al., 2012). The oxygen content, however, has been shown to be important during reproductive organs development in maize. It is reported that there is 1.2 to 1.4% oxygen hypoxic condition between the tassel innermost leaf during archesporial cell specification (Kelliher and Walbot, 2012). This condition is transient and it changes to higher oxygen content (more than 5%) after a few days past the hypoxic condition (Kelliher and Walbot, 2012).

The maize *Multiple Archesporial Cells 1 (MAC1)*, the orthologue of rice *TDL1A*, is expressed in the initial archesporial cells (Wang et al., 2012). It is a secreted protein that is thought to have dual role of binding to a receptor kinase in archesporial cells and regulating their proliferation and regulation of periclinal division in the adjacent L2-d cells via binding to different receptor kinases during archesporial cell specification (Wang et al., 2012). This work has, therefore, demonstrated that oxygen, one of the regulators of N-end rule pathway, is pivotal for organ specification during anther development. Administration of oxygen and nitrogen into the major leaf whorl alters cell proliferation and results in more cell divisions that mimics *mac1* mutant phenotype (Kelliher and Walbot, 2012).

This is an indication for the potential role of oxygen sensing during MS5 protein expression in *Arabidopsis* and how this may impact on normal progression towards meiosis in the developing pollen. The additional cell division in *ms5* mutant (Glover et al., 1998; Ross et al., 1997) that results in uneven distribution of chromosomes could be related to the oxygen level in the developing buds. So that the minimum level of oxygen allows the protein to be functional and upon exposure to the higher levels of oxygen the protein is removed from the cells through degradation by N-end rule pathway. However, if MS5 is degraded in *Arabidopsis* it is not the case if

other plant species such as barley. The identified putative orthologue in barley protein sequence did not show to be the potential target of this pathway, nor is it the case for the other identified putative orthologues in rice and *Brachypodium*. Therefore, this raises the question whether evolution of flower and anthers in crops has removed the need for such involvement, or other protein degradation pathways act to regulate these proteins instead of the NERP. However, there has been reports in barley regarding the involvement of the N-end rule pathway in response to abiotic stress. For instance, down regulation of *HvPRT6* in barley has been linked to increased tolerance to waterlogging. Furthermore, *prt6* mutant lines showed higher expression of hypoxia related genes and contained more chlorophyll compared to wild-type plants under waterlogging condition.(Mendiondo et al., 2016). This shows conservation of the components of this pathway in crops such as barley. However, genome evolution may remove the necessity of protein turnover of certain genes through N-end rule pathway.

Stabilisation of MS5 protein in *Arabidopsis* under normoxia conditions can shed the light on developing plants that are more resistant under low oxygen conditions. The role of oxygen in plant fertility can be further studied by exposing the plants to lower levels of oxygen and quantify the level of MS5 protein in those plants. Furthermore, study of MS5 protein degradation *in vivo* and in wild-type and the plants that lack the functional N-recognins (*PRT1* and *PRT6*) (Garzon et al., 2007; Stary et al., 2003) will be important to determine the function of N-end rule pathway in MS5 protein degradation in *Arabidopsis*.

Chapter Seven:

General Conclusions and Future Work

7.1 *HvNST1* and *HvNST2* identification and characterisation

The NAC domain transcription factors have been shown to be involved in various developmental processes during plant development. In conjunction with their target genes they regulate the formation of vascular tissues, play a role in defense mechanism against biotic and abiotic stresses and regulate deposition of secondary wall thickening in the reproductive organs that help the pollen release and protect seeds from early shattering (Welner et al., 2016). They have also become important in recent years due to their potential use of lignin in biofuel production as cell wall components such as lignin and cellulose and pectin account for majority of the plant dry weight (Ohtani et al., 2011). The identification and characterisation of the NAC domain genes in cereals such as barley is therefore important for understanding the function of these genes in cereals. This will provide tools with potentials in breeding programmes via producing stable male sterile lines.

A reverse genetic approach was taken to identify and functionally characterise the orthologous *NST1* and *NST2* in barley. Identification of the putative orthologous sequences was done by step by step process across a range of species that showed close evolutionary relationships. Identification of the putative orthologous *NST1* and *NST2* in rice, and *Brachypodium* was therefore conducted prior to identification of the barley putative orthologues. The identified sequences were cross checked by moving from the identified putative sequence in barley back to *Arabidopsis* increase the stringency of this analysis. This process resulted in identification of two putative targets in barley for *NST1* and *NST2*. However, identification of the putative *HvNST2* orthologue showed a potential duplication. The identified putative *HvNST2* orthologue (AK249120/ MLOC_65480) showed higher identity to the identified

Brachypodium sequence compared to the AK367801/ MLOC_75524 sequence. Phylogenetic analysis of the identified sequences showed that MLOC_75524 clustered together with the identified putative NST2 and other NST2-like sequences in the grass family. Therefore, despite its low identity to the *Brachypodium* putative orthologue of NST2, MLOC_75524 could potentially be a duplication event for the *NST2* gene. The authenticity of this could be checked by further analysis of the expression pattern of the identified *NST2* putative orthologue and MLOC_75524 as potential *NST2* or *NST2*-like candidate.

These approaches included silencing the putative genes in barley, study of the phenotypic and expression changes in the transgenic plants and complementation of *Arabidopsis* mutants with barley putative orthologous sequences. The putative orthologous sequences of *NST1* and *NST2* in barley were identified using the available sequence database. These sequences were further confirmed to be the correct orthologue by performing synteny and expression analyses.

The SRDX repression lines of both *HvNST1* and *HvNST2* showed substantial reduction in fertility that was expected from repressing these genes. The *NST1*^{SRDX} lines exhibited reduction in fertility, spike size and plant height compared to wild-type. The reduction in fertility was expected as these two transcription factors are involved in formation of secondary wall thickening in *Arabidopsis* anthers and in the absence of these transcription factors the fertility impaired. The *Arabidopsis* data has shown that these two transcription factors are redundantly regulate formation of secondary wall formation and the suppression of one does not result in sterility.

The same reduction of fertility was observed in *HvNST2*^{SRDX} plants. The transgenic plants showed a reduction in plant height and spike size compared to wild-type. Analysis of the expression of *NST1* and *NST2* in the transgenic lines showed that, *NST2* in barley seemed to be regulated by

NST1 as its expression was changed in the SRDX repression lines. Moreover, in barley vegetative and reproductive stages the expression of *NST2* was lower than that of *NST1*, while both genes showed similar expression in the stem. The barley expression results showed similarity to that observed in *Arabidopsis NST1* and *NST2* (Mitsuda *et al.*, 2005). Although further studies is required to compare the alteration in secondary wall thickening in the anthers in the SRDX repression lines compared to wild-type.

Generation of transgenic *HvNST1* and *HvNST2* overexpression lines also showed a reduction in fertility. These plants were also showed reduced height and spike size. However, these lines will require to be further analysed for the alteration of secondary wall thickening in the anthers. A more detailed analysis of the expression of *NST1* and *NST2* in overexpression lines will need to be carried out to be able to establish the secondary wall thickening network in barley.

Functional complementation of *Arabidopsis* mutant using the *NST1* and *NST2* putative orthologues in barley, are also needed to further confirm the function of these genes. Unfortunately, overexpression of *HvNST1* and *HvNST2* under *Arabidopsis* ubiquitin promoter in *nst1NST2* mutant and wild-type (Ler) plants did not result in generation of transformant plants. Therefore, complementation of *Arabidopsis* mutants with putative *NST1* and 2 is required to confirm the function of these two genes. Moreover, due to disruptive and excessive formation of secondary wall thickening across the plant in overexpression lines, it is also important to use *Arabidopsis NST1* and *NST2* promoters for functional complementation so that the will be targeted to floral and very limited vegetative tissues.

Based on the results observed from *NST1* and *NST2* repression and overexpression lines, a putative model was suggested (Figure 7.1). This model suggests that *MYB26* in barley regulates expression of *HvNST1*.

Furthermore, it is predicted that there may be a feedback loop towards *HvMYB26* from *HvNST1* so that NST1 self-regulates its expression. There also seemed that *HvNST1* regulates *HvNST2* expression.

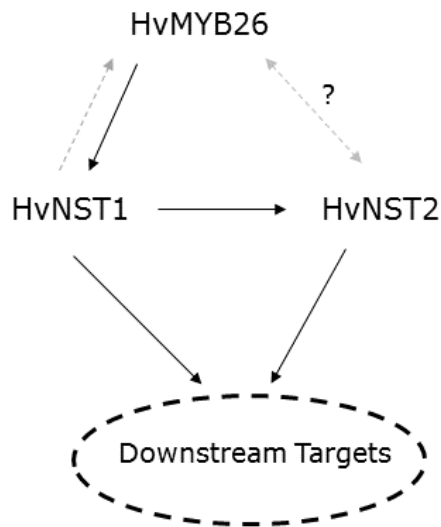


Figure 7. 1 Model proposed for interaction between *HvMYB26* and *HvNST1* and *HvNST2*.

Functional characterisation of genes affecting fertility in cereals is not only important to better understand the mechanism of fertility and seed set, but to use this knowledge to devise new systems for hybrid seed production. Hybrid seed production has been a success story in a few allogamous species such as sorghum, sunflower and maize (Longin et al., 2012). Hybrid seed production has been a bigger challenge in autogamous cereals such as wheat and barley as due to issues such as lower amount of heterosis, higher seed densities and difficulties in establishing a cost effective hybrid seed production system (Longin et al., 2012). In barley, the development of cytoplasmic male sterile (CMS) lines (Ahokas, 1982) have made hybrid seed production feasible. However, incomplete restoration of fertility in CMS lines and yield penalties associated with this (Muhleisen et al., 2015) makes this system less attractive for hybrid seed production.

Study of gene regulating anther dehiscent can potentially contribute towards a new hybrid system. Hence, *NST1* and *NST2* in barley can potentially be targets for producing reliable male sterile lines for hybrid seed production. Furthermore, smaller plants in the mutant lines could also be a good marker for visual selection of sterile lines in the field.

7.2 Identification and characterisation of *MS5* putative orthologue in barley

The identification of *MS5* putative orthologue in barley was done in Prof. Zoe Wilson laboratory at University of Nottingham. RNAi silencing, SRDX repression and overexpression constructs for the identified sequence were prepared and used to transform barley embryos. The transformant RNAi, SRDX plants did not show major reduction in their fertility compared to wild-type. The overexpression of these lines resulted in nearly 40% reduction in fertility compared to wild-type. Nonetheless, the level of *MS5* expression in the SRDX repression or overexpression lines was not quantified. Therefore, it is important to analyse the next generation of these transgenic lines and quantify the expression of the putative *HvMS5* orthologue in the vegetative and reproductive tissues.

It is also worth to have a detailed expression analysis of putative *MS5* gene in barley floral and vegetative stages. This will help to determine if the identified putative orthologue was the correct candidate for *Arabidopsis MS5*. Moreover, analysis of the next generation of SRDX repression and overexpression lines is important to quantify the level of *MS5* expression in both SRDX repression and overexpression lines.

It is also suggested to carry out the putative orthologous identification in barley one more time to make sure that the identified sequence is in fact

the correct gene. As the BLAST search in IPK barley server resulted in identification of two barley sequences highly identical to putative *Brachypodium* sequence, it could be possible that there may be other genes that redundantly regulate *MS5* in barley. Hence, a more detailed bioinformatics analysis and expression analysis for both putative barley *MS5* sequences could clarify this.

7.3 Involvement of N-end rule pathway in AtMS5 degradation

The initial examination of *Arabidopsis* *MS5* protein showed that it could be the target for degradation through N-end rule pathway (NERP). The initial observations showed that the unstable version of the protein (AtMS5-MC) was degraded over the course of sampling period in *in vitro* assay, whereas the expression level of the stable version (AtMS5-MA) was constant throughout the sampling period.

The supplementary experiments to confirm the involvement of NERP in AtMS5 protein degradation through inhibition of the proteasome and stabilisation of the MC version were not successful and therefore could not clarify if AtMS5 was being degraded by N-end rule proteolysis. Moreover, the observation that AtMS5 was degraded during the course of sampling was not reproducible and this protein degradation was not observed in the repetition of *in vitro* assay.

However, because protein degradation was observed earlier during this experiment, it was decided to follow the rest of this experiment *in vivo*.

Thus, it is important to continue with this experiment at the *in vivo* level in order to confidently determine whether there is a link between NERP and *MS5* degradation in *Arabidopsis*. This will involve overexpression of both AtMS5-MC and AtMS5-MA in wild-type and AtMS5-MC in *prt6* mutant lines

(*prt6* mutant lines lack the N-recognins element of N-end rule pathway that directs the tagged/ ubiquitinated proteins for degradation via 26S proteasome). This can prove the role of the N-terminus of AtMS5 protein in its degradation *in vivo*.

Further elucidation of the involvement of AtMS5 N-terminus in its degradation is important to address important questions such as the development of floral organs within a closed whorls of tissues. However, the identified MS5 protein in barley, *Brachypodium* and rice did not seem to be the potential target of this pathway (their protein started with methionine but did not follow by cysteine). Nonetheless, as it was shown before in maize alteration of oxygen levels can alter the cell fate and their differentiation (Kelliher and Walbot, 2012). It is interesting to see whether oxygen level triggers progression of cells through meiosis and its unnatural fluctuation causes abnormal meiosis, generation of cells with uneven DNA content and therefore sterility. The elucidation of this pathway could contribute to our understanding of how plants have evolved mechanisms to sense and measure ambient gasses and response to that by changing their developmental progression.

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9. Appendices

9.1 Callus Induction medium preparation

Murashige and Skoog medium preparation for barley transformation according to Harwood et al. (2009).

Table 9.1 Components of MS medium for barley transformation.

Component	Quantity
1-Murashige & Skoog Plant salt base (Duchefa M0221)	4.3 g/l
2-Maltose	30 g/l
3-Casein Hydrolysate	1 g/l

9.1.1 MG/ L medium preparation

MG/L medium was prepared according to the following table and the pH was adjusted to 7.2. The medium was autoclaved and biotin was added to the medium.

Table 9.2 MG/L medium components.

Component	Amount
Tryptone	5 g/l
Mannitol	5 g/l
Yeast extract	2.5 g/l
L-Glutamic acid	1 g/l
KH ₂ PO ₄	250 ml/l
NaCl	100 mg/l
MgSO ₄ .7H ₂ O	100 mg/l
Biotin	10 µl (0.1 mg/l Stock)

Phytigel was prepared at two times the required concentration in advance. To prepare 2x phytigel, 7g phytigel was added to 1000ml of distilled water and autoclaved.

9.2 Callus induction medium components

Table 9.3 Callus induction medium components.

Total volume	Component	Stock Conc.
100 ml (50 ml CI + 50 ml Phytigel)	1-Myo-inositol	35 g/l
	2-Proline	65 g/l
	3-Thiamine HCl	10 g/l
	4-Dicamba (Sigma-Aldrich D5417)	2.5 g/l
	5-CuSO ₄ .5H ₂ O	1.25 g/l
	6-Hygromycin (Roche)	50 mg/ml
	7-Timentin (Duchefa)	160 mg/ml

9.3 Transition Medium

This medium was prepared in advance at two times the required concentration. To prepare transition medium, following components were added to final volume of 500 ml:

Table 9.4 MS medium preparation for transition medium.

Component	Quantity
1-Murashige and Skoog modified Plant salt base (without NH ₄ NO ₃) (Duchefa M0238)	2.7 g/l
2- Maltose	20 g/l
3- NH ₄ NO ₃	165 mg/l
4- Glutamine	750 mg/l

Table 9.5 Transition medium components.

Total volume	Component	Stock Conc.
100 ml (50 ml Transition + 50 ml Phytigel)	1- Myo-inositol	35 g/l
	2-Thiamine HCl	10 g/l
	3- 2,4-dichlorophenoxy acetic acid (2,4-D) (Duchefa)	2.5 mg/l
	4- 6-benzylaminopurine (BAP) (Duchefa)	1 mg/l
	5-CuSO ₄ .5H ₂ O	1.25 g/l
	6-Hygromycin (Roche)	50 mg/ml
	7-Timentin (Duchefa)	160 mg/ml

9.4 Regeneration Medium

To prepare this medium (2x) following components were added to final volume of 500ml:

Table 9.6 MS medium components for regeneration medium preparation.

Component	Quantity
1-Murashige and Skoog (without NH ₄ NO ₃) (M0238 Duchefa)	2.7 g/l
2-Maltose	20 g/l
3- NH ₄ NO ₃	165 mg/l
4- Glutamine	750 mg/l

Table 9.7 Regeneration medium preparation.

Total volume	Component	Stock Conc.
100 ml (50 ml Regeneration + 50 ml Phytigel)	1- Myo-inositol	35 g/l
	2- Thiamine HCl	10 g/l
	3- Hygromycin	50 mg/ml
	4- Timentin	160 mg/ml

CACAAGGACAAGAAGTACCCAACGGGGACGCGGACGAACCGAGCGACGGCGGCCGGGTTCTGGAAGGCCACCGG
 CCGCGACAAGGCCATTTACAACGCTGTCAAACGTATCGGCATGCGCAAGACGCTCGTCTTCTACAAGGGCCGCG
 CTCCCCATGGCCAGAAGTCGGACTGGATCATGCACGAGTACCGCCTCGACGACCTTCTACCATCGTCACGGAC
 GCCGCCCCACCGCCACCATGGCTGTTAGTGCCGCCACATCGTCGGACTCCGGCCAGGAGGACGGCTGGGTGGT
 TTGCAGAGTGTTCAAGAAGAAGCACCACCATAAGGATGCGGGGGCCGGAAGTGGCAAGCGCGGCGGGGGGAC
 GAGATGATGGGGGCAAGACCGCACGCTCCTCCTCGCCGCTCTACTCCAGCGACGATGCGCTTGACCAGATCCTG
 CACTACATGGGCCGCTCGTGCAAGCAGGAGCACGAGCTTTCCTCGCCGCGCCCGAGAACGGCCGCGTCGCGGTA
 TCTGCGGCCCCCTGGAGACGGTTCTCGGCGGCCACGGTTTCATGAAGCTGCCACCGCTGGAAAGCCCCCTCGGCGG
 CGGCCACGGCGCGGACGTCGACTACCACGCCGCTCGCAGCGAGCGGAGATGGAATGTGAGCGCGAGCTGGCG
 CACGCCGAGGACATGCTGCACGAACCCACCGCGATAACGGGATCACGGACTGGGCCATGATGGACCGGCTCGT
 CGCGTCCCACCTCAATGGCCAGCACGACGCGTCGACCGACAGCTCTGCTTCGACGGCACCCATGCAGGCAGCA
 ACGACGTCGTCGACGACACGGCCGTCAATGGGGTTGCCTTCTACTCCGCCGCGGCTACAAGGCTTCTCGGAGGT
 GCCGCCGCTGGTAGCGGCGACGACGACCTGTGGAGCTTCGCCCCGATCCGCGGCGTCGTGAGCGGGCTGAGCCG
 TGTGTCACCATAA

9.5.4 Rice putative *NST2* sequence (OS06T0131700)

ATGAGCATATCGGTGAACGGGCAGTCGGTGGTGCCACCGGGGTTCCGGTTCCACCCAACGGAGGAGGAGCTCCT
 GACATACTACCTGAAGAAGAAGGTGGCCTCGGAGCGCATCGACCTCGACGTCATCCGCGACGTCGACCTCAACA
 AGCTCGAGCCATGGGACATCCAAGAGAGGTGCAGGATTGGGTGAGGGCCGAAAACGACTGGTACTTCTTCAGC
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 CCCCCACGGCCAGAAATCAGACTGGATCATGCACGATACCGCCTCGACGACCCCTCCTCCGCTCCGCCTCC
 GTCTCCGTCAACCTCCCTCCTACTACTCTTCCTCCTCATCGTCATCTTCCCAATGCACGGTGTGCGCGGAGA
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 CATCCTCCAGTACATGGGCCGCTCCGGCTGCAAGCAGGAGACCAAGCCGGCGGCGATGTGCGCGTCGTGCGCGG
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 CACGTGCGCGGCGGCGTGGGCCCTCCTGGCGGCGGCGGCGGCGGCGGCGAGTACTGCTCCGCCGCCGACGCGTC
 GGGGATCGCCGACTGGGACACGCTGGACCGGCTCGCGGCGTCGTACGAGCTCAACGGCGCCCTCTCCGACGTCG
 CCTCCGGCAAGAACATGGCCGGCTTCTTCGACGTCGTGATCAGCCCGCCGGCGCCGCCGCTTCTCCTCCGGC
 GACGGCGACCTGTGGAGCCTTGCGCGGTGCGGTGTATCGTCGCTACACGCAGACTTGACGACCATGAATAACGT
 ATAA

9.5.5 *Brachypodium* putative *NST2* sequence (BRADI1G50057)

ATGAGCATCTCTGTGAACGGGCAGTCGGTGGTGCCGCCGGGGTTCCGGTTCCACCCGACGGAGGAGGAGCTGCT
 GACCTACTACCTGGCCAAGAAGGTGGCCTCATGCCGCATCGACCTCGACGTCATCCCCGACGTCGACCTCAACA
 AGCTCGAGCCATGGGACATCCAAGAGCGTTGTAGGATCGGGACGGGCCCCGAGAGCGACTGGTACCTGTTTACG
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 CGGCGCCGCTGACGTCAGCTTGAGTTTGCCAAATATGGACAGTATAGCCTCGTCAACGGCGGCGGCGGCGG
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 GCAGCTCCCTCCGCCTACGCCGAAGGCTCGCAGTACGGAGGCGCCGACTGCTGGGCGGACACGCTCGCGGCGT
 ACGAGCTCAACGGCGGCTATCCGACAACGACGTCGACGGGTCGACCAAGACCACCAACGGCGGCGATGTGACG

CCCGGCTGCTTCTTCGTGGCCGAACAGCATCATCAGCACGGCCATGGCGGGCGGGCGACGGAGACCTGTGGAG
CCTGGCGGGTTCGTGGTGTGACGCTGCACGCGGACTTGGCGACGGCCATGGATAACGTCTAG

9.5.6 Barley putative *NST2* sequence (AK249120)

ATGAGCATCTCGGTGAACGGGCAGTCGGTGGTGCCGCCGGGGTTCCGGTTCCATCCGACGGAGGAGGAGCTGCT
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GCTCGAGCCATGGGACATCCAAGAGCGGTGCAGGATCGGCACCGGCCCCGAGAACGACTGGTACCTGTTAGCC
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CGGGACAAGGCCATCTACTCCGCCGCCGGGTCCGGCCGCATCGGCATGCGCAAGACGCTCGTCTTCTACAAGGG
CCGCGCCCCCAGCGCCACAAGTCCGACTGGATCATGCACGAGTACCGCCTCGACGACGCCGTGCAATACGAG
CAACCCCGCCAACCCCGGTGCCGGTGCCTACTACTCCGGCGCCTCGTCCCCGATTTCGTGGCGTCGCCGGGGACC
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ACTCCGCCAGCGACGACGCGCTCGACCACATCCTCAGCCACTACATGCATGGCCGCTCGCCCCGACGCCGTGC
AAGCAGGAGACCAAGCCTGCGCCCAGCTCGGGGCTTGACCACCTGATCAACAACACCGGGTGGCCGAACGGCAC
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TCGTACGAGCTGATCAACGGCCTCTCCGACTCCGACGTGCGCTCGGCCAGCAAGAACGGCGGCATGGCGTCCTT
CTTCGTGGACGGGCACGGCGGCCACCGTCACCGCGCCGGGGACGGCGACCTGTGGAGCCTGGCGCGGTCCG
CGTCGTGCTCCACGCGGACTTGACGACGATGAAGTGA

9.5.7 Rice *MS5* putative orthologue (Os08g0130300)

ATGCCGAGCGGAGGGAGGCGGGCTGCCGCCGTGGACGTGCGCGAGGGGGGCGGCGCCGAGGTGGAGCCCTGCAC
GCCTGCGGGCGCGGACGGTTTCGGGCCGCGCCGCCACGCCACGCCGCCGGCGAGCGGGCGGGTGTCTCTCGCACG
TGACGCCCCCGCGAGCGGTGGTGGCGGGTGTACGGTTACCGTGTACGCCGCCGACGAGCGGGCGGGTGTTC
CGGCCTCCTAGGGCGCCGCTGTCTGTCGGTGGATTGCGCCTACGTGCGGGCGAAGCAGGCTCAGGTGATTGAAAA
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GCAGATTGAAATGCTGACATTGAAGTTGAGAATTGTTGATGAGGAGCTAGCTTCTGGCAGGTGGAACCAAGT
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AGAAGCAGCACAGCTCAGCGTTTCGCTTTTTCTGACTGGAATCCAAGTGTGAAAGATATGGACATGGTTATGT
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GACCATGGAGGCCTCAGAACCGCCTCTGGGTCTTCCAAGAAATCACAATGAGATCAACCAAAAAGTAGTATAA

9.5.8 *Brachypodium MS5* putative orthologue (Bradi3g14297)

ATGCGGAGCGGGGGGAGGCGGCTGCCGCCGTGGACGTCGCCGAGGGGGCACGCGGCGGCGGAGGTGGTGGCGCC
GACGGGGTGGAGCCCCCGCACCCCCGCCGCGGGAGGGGGCGGCTCCGGGAGCTACGTCACGCCGCCACTGACCG
CCGGCTGCTGCTGCTCCTCCTCCTCCTACCGCGTGACACCGCCCTCGAGCGGGGGCGGGTGACCTCCCTCCTG
ACGCCGCCGGTGAGCGGCGGCGGGTGTTCCAGGCCGCCGAGGGCGCCGCCGCGCGTGGTGGATTGCGCGTACGT
GAGGGCGAAGCAGGCGCAGTTAATTGAAAAGGACCCAAACAAGGCAGTTCCATTGTTCTGGGCGGCTATAAACA
GTGGTGAACGAATTGAGAGTGCAGTGAAGGATATGGCTACCGTACTGAAACAAGCAAATAGGGCCGAAGAGGCC
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TGTTTCCGGCCACACTGAGGCGGTGAACCGCTGGCCGAAGAACGCCGCCGCAATCACGAGGCCATGGAGGGCAC
AGAACCGGCTGCGGGTCTTCCAGGAAATCACAATGAGATGAACCGAAATGTTGCATATGAACACTGCTGCTTA
CCATGCATGCAAACGCTGTGTGCTTGTACCTTTTGGCGCTACTGTTTAGATGTTACTGCATGGTTTCATTTGCTG
CAACGTACTACATGTGGCTTTTCATCTGCTATTGCTGCTGCTGCTGGCTGGCTGGCTGGCTGGCTGGACAATTTCTG
CATGA

9.5.9 Barley putative *MS5* (AK376532)

ATGCCGAGCGGGGGCAGGCGGCTGCCTCCATGGACGTCGCCGAGGGGGCGTGGCAGAGGCGGTACCTCAGA
GGTGGAGCCCCTCCCCGGCTGGCGCTGCTGGTTCCGGGAACCATGTGAGTCCGCCCATGAGCGCCGGCTG
CTGCTCCTACCACGTACCCCTGCCGGTTAGCAGCGGCGGGTGCTCCCGGCCGCCGAGGGCGCCCAAGGCG
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