JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
198

Teija Tenhola-Roininen

Rye Doubled Haploids

Production and Use in Mapping Studies

JYVÄSKYLÄN YLIOPISTO

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 198

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ylistönrinteellä, salissa FYS3
maaliskuun 27. päivänä 2009 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
in Ylistönrinne, Auditorium FYS3, on March 27, 2009 at 12 o'clock noon.

UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2009

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Publishing Unit, University Library of Jyväskylä

Jyväskylä Studies in Biological and Environmental Science

Cover picture by Teija Tenhola-Roininen

URN:ISBN:978-951-39-3523-8
ISBN 978-951-39-3523-8 (PDF)

ISBN 978-951-39-3496-5 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä 2009

ABSTRACT

Tenhola-Roininen, Teija
Rye doubled haploids – production and use in mapping studies
Jyväskylä: University of Jyväskylä, 2009, 93 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 198)
ISBN 978-951-39-3523-8 (PDF), 978-951-39-3496-5 (nid.)
Yhteenveto: Rukiin kaksoishaploidit – tuotto ja käyttö kartoituksessa
Diss.

The aim of this research was to find DNA markers linked to the gene affecting dwarfism (*Ddw1*) and the gene(s) controlling pre-harvest sprouting resistance in rye using homozygous doubled haploid plants. Two different rye populations were established, one segregating for short straw and the other for pre-harvest sprouting. The former used doubled haploid parents and the latter doubled haploid progeny. The anther culture technique, incorporating cold and heat stress treatments, was optimized to improve embryo induction and green plant regeneration. In general, cold pre-treatment was beneficial for all cultivars and lines tested. A combination of cold and heat treatments increased regeneration in some breeding lines not tested previously, but did not improve anther culture response of the most recalcitrant cultivars.

The best DNA marker for *Ddw1* was a combination of the microsatellite REMS1218 and the SNP marker created from it, located 13 cM from the QTL for plant height.

The first linkage map of rye using doubled haploids was constructed. The map comprised 281 loci, with a total length of 747 cM, and all the seven rye chromosomes were recognized. *a*-Amylase activity correlates negatively with falling number, which determines the sprouting damage in rye. One major QTL affecting *a*-amylase activity was found on chromosome 5RL, and was located at the microsatellite loci SCM74, RMS1115, and SCM77. These new DNA markers can be used in marker-assisted selection for the short-straw growth habit and pre-harvest sprouting resistance in rye breeding.

Keywords: Anther culture; DNA marker; doubled haploid; dwarf; mapping; pre-harvest sprouting; *Secale cereale* L.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers and a manuscript. These are referred to in the text by their Roman numerals I-IV.

I Tenhola-Roininen, T., Tanhuanpää, P. & Immonen, S. 2005. The effect of cold and heat treatments on the anther culture response of diverse rye genotypes. Euphytica 145: 1-9.

II Tenhola-Roininen, T., Immonen, S. & Tanhuanpää, P. 2006. Rye doubled haploids as a research and breeding tool – a practical point of view. Plant Breeding 125: 584-590.

III Tenhola-Roininen, T. & Tanhuanpää, P. 2009. Tagging the dwarfing gene *Ddw1* in a rye population derived from doubled haploid parents. Euphytica. In press.

IV Tenhola-Roininen, T., Kalendar, R., Schulman, A. & Tanhuanpää, P. 2009. A doubled-haploid rye linkage map with a QTL affecting *a*-amylase activity. Manuscript.

RESPONSIBILITIES OF TEIJA TENHOLA-ROININEN IN THE PAPERS AND THE MANUSCRIPT INCLUDED IN THIS THESIS

Paper I:

I am responsible, with the help of laboratory technicians, for the practical work, including growing the rye plants and the anther culturing. Sirkka Immonen mainly planned the experiments. I did all the statistical analyses in consultation with the statistician. I wrote the paper with Sirkka Immonen and with the help of Pirjo Tanhuanpää.

Paper II:

I am responsible, with help of laboratory technicians, for all the work done in this paper. I wrote the paper with the help of Sirkka Immonen and Pirjo Tanhuanpää.

Paper III:

I am responsible with Pirjo Tanhuanpää for the scoring of markers and for the interpretation of the results. DNA extractions and PCR amplifications were mainly done by laboratory technicians. I did the SNP analyses with the help of Pirjo Tanhuanpää. MegaBACE runs and DNA sequencings were done at the laboratory of genomics in MTT. I did all the statistical, mapping, QTL, and comparative analyses for this paper. I wrote the paper with the help of Pirjo Tanhuanpää.

Paper IV:

I am responsible with Pirjo Tanhuanpää for the scoring of markers and the interpretation of the results. DNA extractions, PCR amplifications and α-amylase activity measurements were mainly done by laboratory technicians. Retrotransposon-based primers were provided by Ruslan Kalendar and Alan Schulman. I performed the AFLP analyses. MegaBACE runs were done at the laboratory of genomics in MTT. I did all the mapping and QTL analyses. I wrote the paper with the help of Pirjo Tanhuanpää.

All the studies were performed under the supervision of Dr Pirjo Tanhuanpää.

ABBREVIATIONS

ABA	abscisic acid
AFLP	amplified fragment length polymorphism
α-Amy	*α*-amylase gene
β-Amy	*β*-amylase gene
AP	albino plant
CI	callus induction
cM	centiMorgan
Ddw1	dominant dwarf 1 gene in rye
DH	doubled haploid
GA	gibberellin
GP	green plant
GRE	green plant regeneration efficiency
IRAP	inter-retrotransposon amplified polymorphism
ISSR	inter-simple sequence repeat
MAS	marker-assisted selection
PHS	pre-harvest sprouting
QTL	quantitative trait locus/loci
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
REMAP	retrotransposon-microsatellite amplified polymorphism
REMS	rye expressed microsatellite site
Rht	reduced height gene in wheat
RMS	rye microsatellite site
SCM	*Secale cereale* microsatellite
SNP	single nucleotide polymorphism
SRAP	sequence-related amplified polymorphism
SSR	simple sequence repeat

1 INTRODUCTION

Since the Second World War rye cultivation in Finland has decreased, and rye is regarded as an unprofitable and risky crop. Rye cultivars, with their tall growth habit, are especially sensitive to lodging in the Finnish climate and are susceptible to pre-harvest sprouting during rainy seasons. This decreases the quality of the grain for the baking industry. One of the goals in breeding rye has been to develop high-yielding, short-strawed cultivars, resistant to sprouting and adapted to the Finnish climate. This would make rye cultivation more productive and more attractive to farmers. Grain sprouting in the ear is a result of many factors, including rain during harvest time, duration of dormancy of the grains, levels of abscisic acid (ABA, germination inhibitor, stress hormone) and gibberellin (GA, germination hormone), and the activities of starch-degrading enzymes in the grains. It is commonly assumed that rye bread is made from Finnish grain, but nowadays over 50% of the raw material for industrial use in Finland is imported from foreign growers. Previously, Finns made rye bread from their own grains grown in their own fields and there is current concern about the sufficiency of domestic rye production. There have been attempts to increase rye cultivation and consumption by improving yield stability and by increasing public awareness about its health-improving properties.

As a part of the research project 'Increasing efficiency of rye breeding and cultivation in the North', funded by the Finnish Ministry of Agriculture and Forestry, an attempt was made to increase rye cultivation in Finland by increasing research for better quality, cultivation and yield stability. This thesis was a part of this cooperative project. The objectives were to find DNA markers for the short-straw growth habit and pre-harvest sprouting resistance by using doubled haploid techniques and molecular biology methods for quality improvement in Finnish rye. The rye doubled haploid technique, developed at MTT (Agrifood Research Finland) by Sirkka Immonen and her co-workers, is one of the bases of this thesis. Good knowledge of DNA markers and QTL analyses at MTT, including barley, oat, and rapeseed, assisted in researching various DNA marker methods for rye. The work was performed in close

collaboration with Boreal Plant Breeding Ltd., which provided all the rye cultivars and breeding lines used in the study.

2 REVIEW OF THE LITERATURE

2.1 Rye

2.1.1 Taxonomy and origin

Cultivated rye (*Secale cereale cereale* L.) is an allogamous diploid species (2n = 2x = 14) (Evans 1976) with a gametophytic self-incompatibility system that limits self-fertilization (Lundqvist 1956). Rye belongs to the grass family *Poaceae*, tribe *Triticeae* and the genus *Secale* L., which comprises numerous species. Two distinct subspecies of *S. cereale* have been important in the evolution of cultivated rye. The first *cereale* group from the Near East area contains outbred, annual weeds such as *S. ancestrale*, *S. dighoricum*, *S. afghanicum*, and *S. segetale*, which resemble cultivated rye. The second group is the *montanum* group, which includes wild perennial, outbred races from North Africa to Iran: *S. ciliatoglume*, *S. dalmaticum*, and *S. kuprijanovii*. In addition, two annual self-pollinating species, *S. vavilovii* and *S. sylvestre*, have affected rye evolution. Nowadays, only *Secale cereale cereale* L. is widely cultivated, but perennial rye varieties and wild rye (*Secale cereale*) exist (Evans 1976, Burger et al. 2006). There is also evidence of enigmatic accessory or B chromosomes in *Secale cereale,* which have parasitic effects on fertility, decreasing it in females and their progeny (Evans 1976, Gonzáles-Sánchez et al. 2004). B chromosomes are inherited in non-Mendelian fashion, they lack genes for specific phenotypic function and are not needed for normal development, but they affect their carrier.

The centre of origin of wild rye is in Anatolia (Turkey, Iran, and Armenia). There is archaeological evidence of rye domestication from the early Stone Age (Neolithic) at the beginning of agriculture, 7000 BCE, when *Secale* migrated to Central Europe as a weed among other cereals. Rye was consequently regarded as a secondary crop. During the early Iron Age, the status of rye changed from weed to crop plant. As a crop of modest requirements, rye was easily cultivated on poor soils under unfavorable weather conditions. The cultivation of rye expanded during the Middle Ages (Behre 1992). In addition to geographic

separation, germplasm exchange has also influenced genetic diversity of rye (Isik et al. 2007).

2.1.1.1 Relatedness of rye, wheat, barley, and rice genomes

After comparative studies of cereal chromosomes and their gene orders (Moore 1995), it has been suggested that cereal chromosomes have evolved from a single ancestral chromosome. Rice (*Oryza sativa* L.), rye, wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.) share orthologous regions among their genomes (Devos et al. 1993a, 1993b, Devos & Gale 2000). This has been demonstrated through comparative mapping of barley EST-SSR (expressed sequence tagged - simple sequence repeat) markers in wheat, rye, and rice (Varshney et al. 2005). During the evolution of rye and wheat chromosomes (Devos et al. 1993b), the common ancestral *Triticeae* chromosomes were reorganized so that different chromosomes contained different parts of the ancestral chromosomes. For example, the distal ends of chromosomes 5 in rye and wheat evolved from the interchromosomal translocation between *Triticeae* 4L and *Triticeae* 5L chromosomes during the divergence of cereals (Liu et al. 1992, Devos et al. 1993b, Devos et al. 1995, Börner et al. 1998a). This means that the present rye chromosome 5RL has a distal end of the former *Triticeae* 4L chromosome, and the present rye chromosome 4RL of the former *Triticeae* 5L chromosome. These chromosome ends have been shown to be important both in decreasing plant height and in preventing pre-harvest sprouting in wheat and rye (Börner et al. 1996, 1998a, Zanetti et al. 2000, Masojć & Milczarski 2009).

2.1.2 Consumption and cultivation

Rye is an important crop in the northern parts of Europe, Belarus, and the Russian Federation. About 30% of rye production is used as food for humans and the remainder as forage. There have been changes among the dominant rye producers over the years. In 2007 (FAO, Faostat data: http://faostat.fao.org), the major rye producer was the Russian Federation (2.6 million ha), the yield being about 4 million tonnes, and the secondary producers were Germany (0.67 million ha) and Poland (1.3 million ha), which reached almost the same level of production as the Russian Federation. Belarus was also among the main producers and produced about a million tonnes of rye in 2007. In Finland, rye production was 0.09 million tonnes in 2007. The traditional rye bread is dark and sour, and is commonly eaten in Finland, the Baltic countries, Poland, Belarus, and the Russian Federation. The other main rye consumers are Denmark, Sweden, Germany, and Norway (Anon. 1998, Kujala 1999).

The cultivation of rye in Finland has decreased since the Second World War. Previously cultivation was about 250 000 hectares per annum. In 2007, rye was cultivated on 31 700 hectares (21 800 ha in 2006), about 13% of the top-year's production (FAO, Faostat data: http://faostat.fao.org). Due to reduced state subsidy and waning interest in cultivation, Finnish self-sufficiency has decreased and only one third of consumed rye was domestically produced at

the beginning of the 21st century. Rye is imported into Finland particularly from Poland, the Baltics, and Denmark (Vuori 2006). Finns consume about 16 kg of rye per capita per annum, mainly as bread, but consumption is decreasing (Anon. 1998).

2.1.3 Health effects

Whole-grain products are important in the daily diet as sources of carbohydrate, protein, vitamins, and especially dietary fiber. Rye bread is the most important source of dietary fiber in Finnish food, accounting for over 40% of intake. The daily diet should include 25-35 g of dietary fibers while currently Finns get only 15-25 g/d (Kujala 1999, Lounasheimo 2008). Dietary fibers are grouped into water-insoluble and soluble fibers. Water-insoluble fibers include cellulose and lignin, whereas arabinoxylan and β–glucan are partly water-insoluble and soluble. Dietary fibers have several health promoting effects. Due to their beneficial effects on bowel physiology (Gråsten et al. 2000), they protect against intestinal cancers (Hallmans et al. 2003). Soluble fibers control body weight by lowering available dietary energy and decreasing serum cholesterol levels and thereby their consumption reduces the risk of contracting type II diabetes (Liu et al. 2000, Murtaugh et al. 2003) and coronary heart disease (Leinonen et al. 2000, Jacobs & Gallaher 2004).

Whole-grain products contain antioxidants, especially phenolic acids, polyphenols, which protect body tissues against oxidative stress and cell death and may be important in protecting against chronic diseases, including cardiovascular diseases, neurodegenerative disorders, and cancers (Ovaskainen et al. 2008). It has been recently reported that the outer layer (pericarp and testa) of rye grain, rather than the whole grain, serves as a reservoir for antioxidants (Zieliński et al. 2007, Landberg et al. 2008). Wheat and rye grains also contain the highest amounts of alk(en)ylrecorcinols (> 500 µg/g), one group of polyphenols in cereals (Ross et al. 2003, Mattila et al. 2005). In addition, whole-grain products include other health benefiting compounds, such as phytoestrogens. A diet high in phytoestrogens can suppress the development and growth of hormone dependent cancers, such as breast and prostate cancer (Adlercreutz et al. 2000, Adlercreutz 2002). In general, people who regularly consume whole-grain foods have a lower risk of developing a chronic disease (Lang et al. 2003, Willcox et al. 2004).

2.2 Methods and goals in rye breeding

Cultivated rye is mainly an out-crossing species with a gametophytic self-incompatibility (GSI) system (Lundqvist 1956). The GSI mechanism prevents self-fertilization, contributes to high level of heterozygosity, and helps avoid inbreeding depression. Three multi-allelic loci, *S, Z* and *T* (*S5*), have been found to control GSI in rye (Lundqvist 1956, Voylokov et al. 1993, Egorova &

Voylokov 1998, Voylokov et al. 1998). They have been mapped and are located on chromosomes 1R, 2R, and 5R, respectively (Wricke & Wehling 1985, Gertz & Wricke 1989, Egorova & Voylokov 1998, Voylokov et al. 1998, Hackauf & Wehling 2005). In the GSI system, pollen tube growth is inhibited and pollination is totally restricted if the pollen genotype and the pistil genotype match at all GSI loci (Franklin-Tong & Franklin 2003, Yang et al. 2008).

Improvement of rye is possible by conventional population breeding (out-crossing or synthetic varieties) and by hybrid breeding. Due to the heterozygosity of rye, varieties are always produced as populations by crossing varieties, lines, or landraces. Breeding includes individual and line selections at different stages for special, important traits of interest. A synthetic variety is developed by combining four to six parental lines with good combining ability. These lines can be inbred, half-sibs, pair-crosses, or doubled haploids and they are open-pollinated to create a synthetic generation (Anon. 1998). Hybrid breeding is common, especially in Germany, where about three quarters of the total rye cropping area is cultivated with hybrid varieties (Miedaner et al. 2000, Falke et al. 2008). In hybrid breeding, two different inbred lines with desired traits are crossed to make an F_1 hybrid variety. Hybrids are developed using a system that utilizes maternally inherited cytoplasmic male sterile (*cms*) genes and their fertility restorer genes, which are located at least on chromosomes 1RS, 3RL, 4RL, 5R, and 6R (Geiger & Schnell 1970, Miedaner et al. 2000). Hybrids exhibit strong heterosis (improved growth power), which increases yield. However, hybrids need to be developed continuously from their parental lines, making seed more expensive.

Finnish rye varieties are freezing tolerant and relatively resistant to low-temperature fungal infection. On the other hand, they have long straw and are lodging-sensitive, low yielding, susceptible to pre-harvest sprouting and susceptible to snow mould (Anon. 1998, Hovinen et al. 2004). In Finland, important goals of rye breeding have been to develop varieties with high yield, short straw, lodging and sprouting resistance, and adaptation to the Finnish climate. Furthermore, interest in synthetic varieties has been stimulated. The introduction of hybrid rye varieties in Finland during the 1990s improved annual yields more than was expected (Öfversten et al. 2004). To date, winter rye varieties have been more popular than spring varieties. However, due to insufficient supply of domestic rye, cultivation of spring rye has started to become more common in southern Finland, and its good baking quality has been noticed by small bakeries. On the other hand, global climate change could increase the use of winter rye varieties due to their adaptability and improved drought tolerance in comparison with spring varieties (Aaltonen 2008).

2.2.1 Short straw

Plant height in cereals is known to be a complex trait under the control of many contributing genes and environmental effects (e.g. rye: Börner et al 1999a, wheat: Zhang et al. 2008a). However, major genes exist (dwarfing genes) that decrease plant height. A reduction in final plant height is usually achieved by

introducing height suppressing genes (major or minor dwarfing genes) into the genotype or removing strong height promoting genes from the genotype. Since the so-called 'Green Revolution - food for all' of the 1960s and 1970s, new cereal varieties with shorter straw and greater grain yield and stability have been adopted by farmers. Average heights of wheat varieties in the United Kingdom dropped from 150 cm to 90 cm with the use of dwarfing genes (Worland et al. 2001). Short straw is an advantage as plants do not lodge, but extremely short varieties are not desired due to problems during harvest and associated yield loss. The maximum grain yield is achieved when plants of intermediate height are used. A plant breeder's decision about the final plant height is a compromise between adequate lodging resistance and an acceptable yield level. In addition, a plant breeder should pay attention to genotype x environment interactions (Flintham et al. 1997). For example, the yield of semi-dwarfs is greater in an unstressed (e.g. no drought) environment (e.g. Gale & Youssefian 1985, Mathews et al. 2006).

Dwarfing genes cause semi-dwarfism or dwarfism and they have both epistatic and additive effects (e.g. Konzak 1988, Flintham et al. 1997). In addition, they can have positive or negative pleiotropic effects on other plant traits, such as grain yield (e.g. positive: increased spikelet fertility, grain number, harvest index; negative: reduced protein level, 1000-grain weight) and quality parameters (Gale & Youssefian 1985, Milach & Federizzi 2001, Li et al. 2006). Examples of positive relationships include also involvement of dwarfing genes in the reduction of *a*-amylase synthesis and thus decreased sprouting damage in wheat (Flintham & Gale 1982, Flintham & Gale 1988, Gale 1989, Mrva & Mares 1996, Mares & Mrva 2008). Dwarfing genes may be linked to disease resistance genes, based on linkage studies, and can be used as markers in resistance breeding (Milach & Federizzi 2001).

The plant hormone GA is involved in the determination of plant height. It has been shown that elevated GA levels are associated with taller plants and reduced GA levels with dwarfism (Richards et al. 2001). Natural or artificially induced dwarf mutants are divided into two classes: GA insensitive and sensitive. A higher number of GA sensitive than insensitive mutants have been identified in cereals. However, GA insensitive mutants have been used more frequently in plant breeding (e.g. mutants with *Rht-B1b* and *Rht-D1b* genes in wheat; Gale & Youssefian 1985). GA sensitive dwarf mutants have defects in GA synthesis (GA supply is blocked). They respond to exogenously applied GA by elongating the stem and restoring a normal growth habit, whereas GA insensitive mutants show reduced reaction to added GA (e.g. Gale & Youssefian 1985, Reid 1986, Börner & Melz 1988, Milach & Federizzi 2001). GA insensitive mutants usually have reduced coleoptile length, leaf size, and decreased internode length. GA insensitive mutants lack a transcriptional repressor that limits the expression of GA biosynthetic enzymes. Therefore, they have elevated rates of endogenous GA production and do not react to added GA. GA 20-oxidase (*GA 20ox*) is the main enzyme in GA biosynthesis and its feedback regulation (Huttly & Phillips 1995, Hedden and Kamiya 1997, Richards et al. 2001, Sasaki et al. 2002). For example, in rice, reduced height has

been achieved by introducing a defective *GA 20ox* gene in rice genotypes (called as a semi-dwarf-1 mutant) (Spielmeyer et al. 2002).

Both GA-sensitive and GA-insensitive dwarfs have been described for rye, wheat, barley, and oat (the best-known dwarfing genes are presented in Table 1). In rye, dwarfing genes are distributed throughout the genome. The most common GA-sensitive, natural, dwarf mutant in rye is the 'EM-1' mutant, which has a single dominant dwarfing gene, *Ddw1*, in its background (Dominant dwarf 1 or Dwarf 1, *Dw1*: Kobyliansky 1972, Melz 1989). *Ddw1* has been located on chromosome 5RL (Korzun et al. 1996, 1997). Other details of this gene are explained in paper III. The other dominant dwarfing gene (*Ddw2* or *Dw2*) is located on chromosome 7R (Melz 1989). The remaining dwarfing genes in rye are recessive.

Comparative analyses have revealed that dwarfing gene functions and organizations are conserved across plant genomes (e.g. Peng et al. 1999). The major GA-sensitive dwarfing gene *Ddw1* in rye (Korzun et al. 1996) and *Rht12* in wheat (Korzun et al. 1997) are homoeologous and located in comparable regions on 5RL and on 5AL, respectively (Börner et al. 1996, Korzun et al. 1997, Börner et al. 1998a). However, GA-insensitive *Rht* genes in wheat may not be related to GA-insensitive dwarfing genes of rye and barley (Börner et al. 1996, Börner et al. 1998a, Ivandic et al. 1999).

2.2.2 Pre-harvest sprouting resistance

Pre-harvest sprouting (PHS) is a global problem in cereals that leads to yield loss and reduction in grain quality. PHS is premature germination of a ripened grain in the ear (post-maturity sprouting) during harvest as a result of wet weather and reduced or no dormancy (Groos et al. 2002). PHS is difficult to manage from a plant breeder's point of view as it is affected by many genes and their interactions with the environment. Two approaches to breeding a PHS resistant cultivar are the conventional way, combining different components affecting PHS into a variety (Derera et al. 1977) and the more sophisticated way, using DNA markers linked to genes affecting sprouting for haplotyping and pyramiding these components into cultivated varieties (marker-assisted selection, MAS) (Twardowska et al. 2005, Ogbonnaya et al. 2007).

The most widely used parameters for the determination of sprouting-damaged grains are Hagberg falling number and *a*-amylase activity, which are inversely correlated (Hagberg 1960, Perten 1964). The Hagberg falling number test records the time in seconds for a plunger to fall through a gelatinized aqueous flour paste after a standard mixing procedure. Sprouted grains have lower falling number because *a*-amylase promotes starch hydrolysis by increasing the amount of fermentable sugars and liquefying the suspension (Brümmer 1999). Other methods for determining PHS also exist: germination percentages of grains, visible sprouting of spikes, viscography, pregelatinized starch procedure, immunochromatography, and diffusion methods based on *a*-amylase (Masojć & Larsson-Raźnikiewicz 1991, Skerritt & Heywood 2000, Donelson et al. 2001, Zhang et al. 2005, Ogbonnaya et al. 2008, Singh et al. 2008).

TABLE 1 The best known dwarfing genes in rye, wheat, barley, and oat

Species	Dwarfing gene	Inheritance	Chromosomal localization	GA response	Key References
Rye	*Ddw1, Dw1, Hl*	dominant	5RL	*sensitive*	Kobyliansky 1972, Sturm & Engel 1980, Korzun et al. 1996
	Ddw2, Dw2	dominant	7R	*sensitive*	Melz 1989
	dw1, d1	recessive			Sybenga & Prakken 1962
	dw2, d2	recessive	2R	*sensitive*	De Vries & Sybenka 1984
	dw3	recessive	3R		De Vries & Sybenka 1984
	dw4	recessive	1R		Melz 1989
	dw5	recessive	4R		Melz 1989
	dw6	recessive	5R	*insensitive*	Melz 1989, Börner et al. 1992
	dw7	recessive	6R		Melz 1989
	ct1	recessive	7R	*insensitive*	De Vries & Sybenka 1984, Börner 1991, Börner et al. 1992, Plaschke et al. 1993, 1995
	ct2	recessive	5RL	*insensitive*	De Vries & Sybenka 1984, Börner 1991, Börner et al. 1992, Plaschke et al. 1993
	ct3		7R		Malyshev et al. 2001
	np	recessive	4RL	*sensitive*	Malyshev et al. 2001
Wheat					
	Rht-B1a, rht1, rht3	recessive	4BS		Flintham et al. 1997
	Rht-B1b, Rht1	semi-dominant	Former 4A, 4BS	*insensitive*	Gale & Youssefian 1985, Konzak 1987, Pinthus & Gale 1990, Börner et al. 1996, Cadalen et al. 1998, Peng et al. 1999, Ellis et al. 2004
	Rht-B1c, Rht3	semi-dominant	Former 4A, 4BS	*insensitive*	Gale & Youssefian 1985, Börner et al. 1997
	Rht-B1d, Rht1S	semi-dominant	4BS	*insensitive*	Worland & Petrovic 1988
	Rht-B1e, Rht KrasnodariI	semi-dominant	4BS	*insensitive*	Worland 1986
	RhtB1f, Rht T. aetiopicum	semi dominant	4BS	*insensitive*	Börner et al. 1995

Rht-D1a, rht	recessive	4DS		Börner et al. 1996
Rht-D1b, Rht2	semi-dominant	4DS	*insensitive*	Gale & Youssefian 1985, Konzak 1987, Cadalen et al. 1998, Peng et al. 1999, Ellis et al. 2004
Rht-D1c, Rht10, Ai-bian 1	semi-dominant	4DS	*insensitive*	Gale & Youssefian 1985, Börner et al. 1996, Börner et al. 1997, Ellis et al. 2004
Rht-D1d, Rht Ai-bian 1a	semi-dominant	4DS	*insensitive*	Börner et al. 1991, 1997
Rht4	recessive	2BL	*sensitive*	Gale & Youssefian 1985, Börner et al. 1996
Rht5	partially dominant	3BS	*sensitive*	Gale & Youssefian 1985, Konzak 1987, Börner et al. 1996
Rht6	recessive		*sensitive*	Gale & Youssefian 1985, Konzak 1987, Börner et al. 1996
Rht7	recessive	2A	*sensitive*	Worland et al. 1980
Rht8	semi-dominant	2DS, 2DL	*sensitive*	Gale & Youssefian 1985, Worland & Law 1986, Korzun et al. 1998a, 1998b, Worland et al. 2001, Ellis et al. 2004
Rht9	semi-dominant	7BS, 5AL	*sensitive*	Gale & Youssefian 1985, Worland et al. 1990
Rht11	recessive		*sensitive*	Konzak 1987, Ellis et al. 2004
Rht12	dominant	5AL	*sensitive*	Sutka & Kovacs 1987, Konzak 1987, Korzun et al. 1997
Rht13	partially dominant	7BS	*sensitive*	Börner et al. 1996, Ellis et al. 2005
Rht14	semi-dominant		*sensitive*	Konzak 1987, 1988
Rht15	partially recessive		*sensitive*	Konzak 1987
Rht16	semi-dominant		*sensitive*	Konzak 1987
Rht17	recessive		*sensitive*	Konzak 1987, Ellis et al. 2004
Rht18	semi-dominant		*sensitive*	Konzak 1987
Rht19	semi-dominant		*sensitive*	Konzak 1987
Rht20	partially dominant		*sensitive*	Konzak 1987

	Rht21	partially dominant	2AS	*sensitive*	Yang et al. 1995
	Rht-L (Leeds)	partially dominant		*sensitive*	Gale & Youssefian 1985
	D1		2D		Hermsen 1967, Gale & Youssefian 1985, Worland & Law 1986
	D2		2B		Hermsen 1967, Gale & Youssefian 1985
	D3		4B		Hermsen 1967, Gale & Youssefian 1985
	D4		2D		Hermsen 1967, Worland & Law 1986, Gale & Youssefian 1985
	us1	recessive			Gale & Youssefian 1985
	us2	recessive			Gale & Youssefian 1985
	Ppd1				Worland & Law 1986
Barley					
	dwf1			*sensitive*	Falk & Kasha 1982
	Dwf2	dominant	4HS	*insensitive*	Falk 1995, Ivandic et al. 1999
	sdw1, sdw	recessive	3HL	*sensitive*	Hellewell et al. 2000
	denso	recessive	3HL	*sensitive*	Rasmusson et al. 1973, Barua et al. 1993, Laurie et al. 1993, Milach & Federizzi 2001
	sdw2, sdw-b		3HL		Lundqvist et al. 1997, Franckowiak et al. 2005
	GPert	recessive	5H	*sensitive*	Thomas et al. 1984, Milach & Federizzi 2001
	sdw3, gai, GA-ins, Rht-H1	recessive	2HS	*insensitive*	Börner et al. 1999b, Gottwald et al. 2004
	gal, GA-less	recessive	2HL	*sensitive*	Börner et al. 1999b
	sln1				Franckowiak 1987, Chandler et al. 2002
	brh1	recessive	7HS	*insensitive*	Franckowiak 1987, Lundqvist et al. 1997, Li et al. 2001, Dahleen et al. 2005
	brh2, ari-1		4HS, 4HL		Tsuchiya 1980, Hang & Tsuchiya 1980, Franckowiak 1987, Dahleen et al. 2005

	brh3, ert-t,		2HS		Franckowiak 1987, Dahleen et al. 2005
	cud1		5HL		Franckowiak 1987, Lundqvist et al. 1997
	cud2		1HL		Lundqvist et al. 1997
	lzd		3HS		Franckowiak 1987, Lundqvist et al. 1997
	min1, min		4HL		Lundqvist et al. 1997
	mnd1, m				Franckowiak 1987, Lundqvist et al. 1997
	nld		5HL		Franckowiak 1987, Lundqvist et al. 1997
	sid, nls		4HL		Franckowiak 1987, Lundqvist et al. 1997
	sld1, dw-1		3HL		Franckowiak 1987, Lundqvist et al. 1997
	sld2		2HS		Franckowiak et al. 2005, Lundqvist et al. 1997
	uzu, uz	recessive	3HL		Hoskins & Poehlman 1970, Jing & Wanxia 2003
	hcm, h		2HL		Franckowiak et al. 2005, Lundqvist et al. 1997
	wnd	recessive	7HS		Lundqvist et al. 1997
Oat					
	Dw1				Marshall & Murphy 1981
	Dw2				Marshall & Murphy 1981
	Dw3				Marshall & Murphy 1981
	Dw4				Marshall & Murphy 1981
	Dw5	recessive			Marshall & Murphy 1981
	Dw6	dominant	18	*sensitive*	Brown et al. 1980, Milach et al. 1997, 1998
	Dw7	partially dominant	19	*sensitive*	Marshall & Murphy 1981, Milach et al. 1997, 1998
	Dw8	dominant		*sensitive*	Milach et al. 1997, 1998

2.2.2.1 Factors influencing pre-harvest sprouting

PHS is a complex and quantitatively inherited trait that is affected by many factors in cereals, including dormancy, water absorption by the grains, responses to hormones, enzyme activities, grain color, and spike morphology, in addition to environmental conditions (e.g. Flintham et al. 1999, Roy et al. 1999, King & von Wettstein-Knowles 2000, Flintham et al. 2002, Groos et al. 2002, Chen et al. 2008). A major factor that inhibits PHS is grain dormancy, the inability of a viable, mature seed to germinate under favorable conditions (Bewley 1997). It is mainly measured as germination percentage. Dormancy (primary dormancy) prevents germination of a grain after harvesting. A dormant grain with low water capacity can tolerate stress more than grains at other stages of maturation. After dormancy release, germination occurs, resulting in physical, chemical, and physiological changes and interactions in the embryo, endosperm, and testa (with pericarp) (Nonogaki 2006). The grain pericarp includes germination inhibitors, such as phenolic acids, which have a significant role in grain dormancy and protect grain against environmental stress (Enari & Sopanen 1986, Weidner et al. 1999, 2000). Dormancy can also be an undesirable trait during the malting process in barley when there is a demand for rapid germination (Oberthur et al. 1995).

The plant hormones ABA and GA play important roles in the regulation of dormancy and germination. ABA (also referred to as a stress hormone) levels and embryogenic sensitivity to ABA are key factors in maintaining dormancy during the middle and late stages of embryogenesis (e.g. Walker-Simmons 1987, Corbineau et al. 2000, Suzuki et al. 2000, Romagosa et al. 2001, Gubler et al. 2005). In contrast, GAs act as germination hormones and take part in degradation of starch endosperm and sugar modifications by regulating enzyme synthesis (e.g. Huttly & Phillips 1995, Perata et al. 1997, Bethke et al. 2001). The balance of forces (antagonism) between ABA and GA actions possibly triggers seed germination (Gómez-Cadenas et al. 2001, Gubler et al. 2005, Nonogaki 2006). Additionally, other hormones, such as auxins and cytokinins, affect enzyme synthesis during germination (Enari & Sopanen 1986). In particular, auxin IAA (indole acetic acid) is involved in inhibition of germination in dormant embryos and plays an inhibitory role in PHS (Ramaih et al. 2003).

A group of starch degrading enzymes, *α*-amylases (*α*-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1.), are the principal enzymes taking part in increasing PHS susceptibility in cereals. *α*-Amylases are endo-enzymes that hydrolase *α*-1,4-glucosidic linkages of starch into metabolizable sugars and significantly reduce starch viscosity. *α*-Amylase isozymes are products of a multi-gene family. They are divided into three subgroups based on amino acid homology and gene structure in rye, wheat and rice (Huang et al. 1992): the high pI (*α-Amy1* on the group 6 chromosomes) group and the low pI group (*α-Amy2* on the group 7 chromosomes) are expressed in the scutellar epithelium of the embryo and in aleurone cells (Gale & Ainsworth 1984, Salinas & Benito

1985, Masojć 1987, Masojć & Gale 1991, Huttly & Phillips 1995, Perata et al. 1997), and the third small multi-gene group (*a-Amy3* on the group 5 chromosomes) is only expressed in the outer pericarp layer during early grain development (Salinas & Benito 1985, Baulcombe et al. 1987, Masojć & Gale 1991, Huang et al. 1992). In barley, only gene families *a-Amy1* and *a-Amy2*, are found (Knox et al. 1987). The enzyme product of *a-Amy1* also known as 'late maturity' or 'malt' *a*-amylase is induced by GA during later grain maturation, but especially during germination and PHS. The enzyme product of *a-Amy2* is known as 'green' or 'pericarp' *a*-amylase and is synthesized during germination and can also be found in the pericarp during early grain development (Gale 1989). However, high *a*-amylase activities can be measured without visible sprouting of a grain (Flintham and Gale 1988, Mares & Mrva 2008).

At least two types of *a*-amylase inhibitors are present in the endosperm of cereal grains during grain maturation and germination (Gale 1989). The first type consists of exogenous inhibitors, albumin and globulin proteins, which inhibit mammalian and insect *a*-amylases but not cereal amylases, and are controlled by a multigene family in cereals. Their levels drop rapidly during germination (Carbonero & García-Olmedo 1999). The second type, located at least on group 2 chromosomes, consists of endogenous *a*-amylase inhibitors (e.g. *Isa1* on 2R in rye: Masojć & Gale 1990, Masojć et al. 2001), which are active against native *a*-amylases during germination.

In addition, other genetic factors influence PHS, including coat color genes of grain and dormancy-related transcription factors. It has been observed that red-grained wheat is more tolerant to PHS than white-grained wheat due to grain coat color genes tightly linked to dormancy/PHS genes, or pleiotropic effects of the genes (Mares 1993, Flintham et al. 1999, Roy et al. 1999, Groos et al. 2002, Himi et al. 2002). In addition, Viviparous1 (*Vp1*) homologues encoding dormancy-related transcription factors play an important role in controlling embryogenesis and seed germination, for example by repressing *a*-amylase transcription and thereby reducing sprouting damage in wheat (Groos et al. 2002, Yang et al. 2007, Xia et al. 2008, Utsugi et al. 2008).

PHS is highly influenced by environmental factors including both biotic (e.g. pathogen attack) and abiotic stresses such as light, temperature, and duration of seed storage (Koornneef et al. 2002, Nyachiro et al. 2002). The favorable weather conditions for PHS, cool or moderate temperature (10-15ºC), high humidity and rainfall, cause breakdown of grain dormancy, increasing the precocious germination of grain. Substantial genotype differences across environments are seen in PHS effects (e.g. wheat: Kato et al. 2001, Flintham et al. 2002, Mares et al. 2005, Torada et al. 2005, Tan et al. 2006, Biddulph et al. 2005, 2007; barley: Chono et al. 2006, Bonnardeaux et al. 2008; wild oat: Jones et al. 1997a). Therefore, breeders should screen potential sprouting resistant candidates in the appropriate environment and evaluate the trait in various environments.

2.2.2.2 Quantitative trait loci affecting sprouting

Several QTL (quantitative trait loci) affecting PHS have been found. These QTL are dependent on population, year, and environment (e.g. Kato et al. 2001, Kulwal et al. 2005, Mori et al. 2005). In Table 2, examples of QTL controlling PHS in rye, wheat, and barley are presented.

In general, loci affecting sprouting seem to be mainly additive (e.g. Anderson et al. 1993, Romagosa et al. 1999, Kulwal et al. 2005, Mares et al. 2005, Hori et al. 2007). The variance explained by the QTL affecting PHS ranges from small (1.5%) to large effects (77.3%) (Table 2). At least 13 QTL control visible sprouting (sprouting damage) in rye, and 16 QTL control *a*-amylase activity, some of them being the same in different populations (Table 2). There are common chromosomal regions controlling both visible sprouting and *a*-amylase activity in addition to some chromosomal regions that do not overlap in rye (Masojć & Milczarski 2005, Masojć et al. 2007, Twardowska et al. 2005, Masojć & Milczarski 2009). The distribution of the QTL for *a*-amylase activity across all seven rye chromosomes might indicate that the direct and indirect regulatory mechanisms coordinate the expression of *a*-amylase genes (Masojć & Milczarski 2009). Some QTL for *a*-amylase activity have been located near *a*-amylase inhibitor and structural genes, and some QTL for visible sprouting might be regulatory genes activated during water stress (Masojć et al. 2007, Masojć & Milczarski 2009).

At least 27 different QTL linked to PHS resistance have been reported on every chromosome of wheat and barley (Table 2, different studies can include the same QTL). Generally, the QTL with the strongest effect on PHS, including dormancy, are located on chromosome groups 4L and 5L in rye, wheat, and barley (rye: Masojć et al. 2007, Masojć & Milczarski 2009; wheat: Zanetti et al. 2000, Nakamura et al. 2007, Chen et al. 2008; barley: Han et al. 1996, Li et al. 2003, 2004, Hori et al. 2007, Ullrich et al. 2008). Furthermore, a gene encoding GA 20-oxidase was identified as a candidate gene controlling seed dormancy/preharvest sprouting QTL on 5HL in barley, which exhibits synteny with wheat chromosome 4AL and rice chromosome 3L (Li et al. 2004). In addition, the regions that carry QTL for PHS include other QTL that affect free amino acid nitrogen, malt extract, diastatic power, total protein content, and early vigor of seed (Marquez–Cedillo et al. 2000, Li et al. 2003, 2004), which also implies that the genes controlling PHS have both epistatic and pleiotropic effects.

TABLE 2 QTL controlling traits affecting PHS in rye, wheat, and barley

Species	Trait	Mapping population	Chromosomal localization of the QTL	Phenotypic variance explained by the QTL	References
Rye	*Seed dormancy*	*RXL10 x DS2*	1RS, 2RS, 5RL	*11.4-53.0%*	Masojć et al. 1999
	Sprouting damage	*Ot1-3 x 541*	1RS, 1RL, 2RL, 3RS, 4RL, 5RS, 5RL, 6RS, 6RL, 7RS, 7RL	*4.0- 63.8%*	Masojć et al. 2007, Masojć & Milczarski 2009
		RXL10 x DS2	1RS, 2RS, 2RL, 5RL, 5RL	*10.8-53.0%*	Masojć et al. 1999, Masojć & Milczarski 2009
	Alpha-amylase activity	*Ot1-3 x 541*	1RS, 1RL, 2RS, 3RS, 4RS, 4RL, 5RS, 5RL, 6RL, 7RS	*1.5 – 59.8%*	Masojć & Milczarski 2009
		RXL10 x DS2	1RS, 1RL, 2RS, 2RL, 3RL, 4RL, 5RS, 5RL, 6RS, 6RL, 7RL	*3.0-38.7%*	Masojć et al. 1999, Masojć & Milczarski 2005, 2009
Wheat	*Seed dormancy*	*Halberd x Cranbook*	2AL, 2DL, 4AL	*9-11%*	Mares et al. 2002, Mares & Mrva 2001
		Kyle x CI13102	1A		Knox et al. 2005
		AC Domain x Haruyutaka	4AL, 4BL, 4DL	*13.9-77%*	Kato et al. 2001
		Zenkoujikomugi x Chinese Spring	3AS, 3AL, 4AL, 4BL	*11.6-44.8%*	Osa et al. 2003, Mori et al. 2005
		Zenkoujikomugi x Spica	3AS, 4AL		Kottearachchi et al. 2006
		Soleil x Boxer	7D		Flintham et al. 1999
		Kitamoe x Münstertaler, Haruyutaka x Leader, OS-21-5 x Haruyokoi	4AL	*28.5%-43.3%*	Torada et al. 2005
		AUS1408 x SW95-50213, AUS1408 x Janz, AUS1408 x Cascades,	4AL	*25-38%*	Mares et al. 2005

	SW95-50213 x Cunningham, AUS1490 x Janz			
	CN19055 x Annuello, several germplasm	3DL, 4AL	*45%*	Ogbonnaya et al. 2007, 2008
	W7984 x Opata85	3AL		Lohwasser et al. 2005
	AUS 1408 x Cascades	4AL, 5BL	*5-15%*	Tan et al. 2006
	Triticum monococcum L. (KT3-5) x Triticum boeticum L. Boiss (KT1-1)	3AL, 4AL, 5AL	*10-27%*	Nakamura et al. 2007
	Totoumai A x Siyang 936	4AL	*28.3%*	Chen et al. 2008
	Halberd x Cranbrook, AUS1408 x Janz, Cunningham x SW95-50213	4AL		Zhang et al. 2008b
Sprouting damage	*Timgalen x RL4137, Boxer x Soleil*	1BS, 4BL, 7AS		Flintham et al. 2002
	Renan x Récital	3AL, 3BL, 3DL, 5AS	*4.2-11.4%*	Groos et al. 2002
	SPR8198 x HD2329	3AL, 6BS, 7DL	*24.7-35.2%*	Roy et al. 1999, Kulwal et al. 2005, Varshney et al. 2001
	CN19055 x Annuello, several germplasm	3DL, 4AL	*20%, 43%*	Ogbonnaya et al. 2007, 2008
	NY6432-18 x Clark's cream, NY6432-18 x NY6432-10	1AS, 2S, 2L, 3BL, 4AL, 5DL, 6BL, one unknown	*44- 51%*	Anderson et al. 1993, Sorrells & Anderson 1996
	Chahba88 x Vic, IACT12 x Ben	5BS, 6BL	*17.4-26.5%*	Gelin et al. 2006
	Totoumai A x Siyang 936	4AL	*30.6%*	Chen et al. 2008

		W7984 x Opata85	1AL, 2BL, 2DS, 3BL, 3DL, 4AL	*2.2-19.8%*	Kulwal et al. 2004, Lohwasser et al. 2005
		Rio Blanco x NW97S186, Rio Blanco x NW97S078	2B, 3AS	*4.5-58%*	Liu et al. 2008
		Halberd x Cranbrook, AUS1408 x Janz, Cunningham x SW95-50213	4AL		Zhang et al. 2008b
		AC Domain x White-RL4137	3A, 3B, 3D, 5D	*7-44%*	Fofana et al. 2009
	Alpha-amylase (AA), falling number(FN)	*Forno x Oberkulmer*	1BS, 2AL, 3AS, 3BL, 4DL, 5AS, 5AL, 6A, 6DL, 7BL	*AA: 7.2-38.5 %, FN: 6.6-49.7%*	Zanetti et al. 2000, Flintham et al. 2002
		Halberd x Cranbrook	3BL, 7BL	*AA: 13-31%*	Mrva & Mares 2002
		Batis x Syn022, Zentos x Syn086	1B, 2D, 3A, 4B, 6B, 7B	*FN: 4.7-9.7%*	Kunert et al. 2007
		AC Domain x White-RL4137	3B, 3D	*FN: 7-33%*	Fofana et al. 2009
Barley	*Seed dormancy*	*Steptoe x Morex*	1HL, 2HS, 3H, 4HS, 4H, 5H, 5HL, 7H, 7HL	*3%-75%*	Ullrich et al. 1993, Han et al. 1996, Larson et al. 1996, Romagosa et al. 1999, Gao et al. 2003, Ullrich et al. 2008
		Stirling x Harrington	1H, 2H, 3H, 4HS, 4HL, 5H, 5HL	*1.7-52.2%*	Bonnardeaux et al. 2008
		Wadi Qilt (Hordeum spontaneum C. Koch) x Mona	1H, 2H, 3H, 4H, 5H, 7H		Zhang et al. 2005
		Blenheim x E224/3	2H, 3H, 5H, 7H	*17-81%*	Thomas et al. 1996
		H.E.S. 4 x Russia 6, Mokusekko 3 x Ko A, Khanaqin 7 x Harbin 2-row, Turkey 6 x Harbin	1H, 3H, 4H, 5H, 6H, 7H	*3.2-77.3%*	Hori et al. 2007

	2-row, Turkey 45 x Harbin 2-row, Katana 1 x Harbin 2-row, Khanaqin 1 x Harbin 2-row, H602 x Haruna Nijo			
	Triumph x Morex, Triumph x TL43	2H, 3HL, 5H, 5HL, 6H	*9-52%*	Prada et al. 2004, 2005
	Harrington x Morex	2H, 5H		Edney & Mather 2004
	Chebec x Harrington, Stirling x Harrington	2H, 3HL, 5H, 5HL	*4-61%*	Li et al. 2003
Sprouting damage	*Steptoe x Morex*	2HS, 3H, 4HS, 5H, 5HL	*4-31%*	Ullrich et al. 2008
Alpha-amylase activity (AA), Falling number (FN)	*Harrington x Morex, Steptoe x Morex, TR306 x Harrington*	1HL, 2HS, 4HS, 5H, 5HL, 6H, 7H		Marquez-Cedillo et al. 2000
	Blenheim x E224/3	7H	*FN: 54.3%*	Thomas et al. 1996
	Chebec x Harrington, Stirling x Harrington	2H, 5HL	*AA: 8-73%*	Li et al. 2003
	Labelle x Morex	5HL	*AA*	Ayoub et al. 2003
	Steptoe x Morex	2HS, 3H, 4HS, 5H, 5HL	*AA: 3-27%*	Ullrich et al. 2008

2.3 Biotechnological tools for research and breeding

2.3.1 Doubled haploid technology

Tissue culture is a useful tool to produce plant tissues artificially on special growth media for cloning, gene bank, research, and breeding purposes. Tissue culture is based on cell totipotency, which means that every cell is able to regenerate into a new plant. The regeneration of a plant is induced by organogenesis via plant tissue, undifferentiated cells (calli) or by embryogenesis via embryos that develop into plants.

Haploid and doubled haploid (DH) plants (a doubled genome), derived from haploid gametophytic cells either from egg cells (gynogenesis) or male gametes (androgenesis), are especially important for research and breeding. Homozygous DHs are produced either spontaneously or by using the anti-microtubular chemical colchicine or other chromosome doubling chemicals. During androgenesis, microspores, premature pollen grains, are utilized for embryo formation following culture of anthers or microspores (Khush & Virmani 1996). For anther culture, anthers are plated on a special medium to induce embryogenesis, whereas in microspore culture anthers without the anther wall are cultured. The other method for producing haploid embryos is wide crossing with pollen from another cereal genus (e.g. *Hordeum bulbosum* or *Zea mays*). In this method, fertilization occurs, but foreign chromosomes are eliminated during grain development (Forster & Thomas 2005, Forster et al. 2007). In addition, irradiated pollen can be used in this method (Sestili & Ficcadenti 1996). For rye, anther or microspore methods have been more favorable than other methods due to their easiness and efficiency in plant production (Flehinghaus-Roux et al. 1995, Immonen & Anttila 1996, Deimling & Flehinghaus-Roux 1997, Immonen 1999, Guo & Pulli 2000, Ma et al. 2004a, Forster et al. 2007).

An androgenetic haploid embryo can be induced to develop through different pathways at the first division of the microspore; either via two asymmetric divisions or one symmetric division of a vacuolated microspore (Reynolds 1997, Touraev et al. 1997, Forster et al. 2007), although variations also exist (Zheng 2003). During the asymmetric division, either the vegetative or the generative cell of the microspore divides and forms a proembryoid, while in the symmetric division the microspore divides symmetrically to produce two equivalent cells or nuclei that both divide to form a proembryoid (Reynolds 1997, Touraev et al. 1997, Forster et al. 2007). During microspore embryogenesis some microspores die directly, some adopt pollen-like development and attempt to follow the gametophytic pathway before dying, others form callus, which is capable of generating haploid or doubled haploid plants through organogenesis, and embryogenic microspores develop into proembryoids (Seguí-Simarro & Nuez 2008a). The mechanism for the duplication of a haploid genome is currently thought to occur through nuclear fusion by coalescence of nuclear membranes of haploid cells and mixing of DNA contents after a

defective telophase. The other possible mechanisms include the endoreduplication of a cell (DNA duplication before mitosis), endomitosis with a defective cell cycle, and doubling with chemicals (Seguí-Simarro & Nuez 2008b).

DH populations are excellent material for genetic mapping and QTL studies due to their homozygosity. In homozygous populations, dominant DNA markers can be used with equal efficiency as co-dominant ones. In addition, DHs are useful in transgenic and mutagenic studies. In self-pollinating species, DHs are genetically consistent, and they can be utilized at multiple sites and seasons in replicated field trials. Recessive lethal factors affecting, for example, hybrid sterility and incompatibility, can be studied with DHs in out-crossing species. A DH mapping population can be produced in 1.5-2 years, more rapidly than by using recombinant inbred lines (RILs, inbred for 5-6 generations) and almost as quickly as with an F_2 or BC_1 population (Forster & Thomas 2003, Forster & Thomas 2005, Forster et al. 2007). DHs are completely homozygous whereas RILs are heterozygous to some degree (Chu et al. 2008). In mapping studies of rye, both homozygous inbreeding lines and RILs, but not DHs, have been used (see references in Table 3).

Completely homozygous DHs can enhance conventional population breeding or hybrid breeding by shortening breeding time and offering tools for genotype selection for desired traits. One of the benefits of DHs is that desired genotypes can be fixed in one generation. Time saving is especially important in disease resistant breeding when a new pathogen race has overcome existing disease resistance. In MAS, DHs increase the efficiency by providing a possibility to combine large numbers of genes with a minimum number of marker tests. In Europe, DH technology and MAS have become commonplace in barley, wheat, and rapeseed breeding (Forster & Thomas 2003, Forster & Thomas 2005, Forster et al. 2007). It has been estimated that DH technology can reduce the time needed for the development of a new cultivar by 50% in winter-grown crops (Tuvesson et al. 2007). Over 100 barley and rice cultivars have been developed using doubled haploidy. Furthermore, DH protocols have been generated for over 250 species (Forster & Thomas 2003).

Anther culturability is a quantitatively inherited trait controlled by nuclear-encoded genes and cytoplasmic factors (Bolibok & Rakoczy-Trojanowska 2006, Datta 2005, Zheng 2003), and a trait with additive gene effects, although both epistatic and dominant effects have been reported (Zheng 2003). Embryo induction, plant regeneration, and albino to green plant ratio are independently inherited (Agache et al. 1988, Zheng 2003). Rye chromosomes 3RL and 5R are associated with embryo formation (induction) whereas 1R, 4R, and 6RS are associated with regeneration rate (Martinez et al. 1994, Große et al. 1996). The special feature in anther or microspore culture is substantial production of albino plants (Deimling & Flehinghaus-Roux 1997, Jähne & Lörz 1995). Albino plants have extensive deletions in their plastid DNA and only small amounts of ribosomal RNA, which affects their protein synthesis (Logue 1996).

Segregation distortion of markers is quite common in DH populations. The distortion can be caused by tight linkages of markers to genes involved in anther culture response, with a prevalence of the alleles of the better-responding parent. However, the chromosomal regions controlling anther culture response and the distorted regions are not always the same (Manninen 2000, Zhang et al. 2003). Linkages of markers to other partially lethal factors, such as hybrid sterility, incompatibility, or nuclear cytoplasmic interactions can also increase the distortion of markers (Foisset & Delourme 1996, Yamagishi et al. 1996, Cheng et al. 1998, Zhang et al. 2003). To decrease segregation distortion it is better to use parental lines with the same anther culturability when creating a DH mapping population (Murigneux et al. 1993, Foisset & Delourme 1996).

2.3.1.1 Anther culture in rye

Rye has generally been considered recalcitrant in anther culture. In the 1970s, the production of calli, embryos, and green plants was reported for the first time (e.g. Wenzel & Thomas 1974, Thomas & Wenzel 1975, Wenzel et al. 1977). In those studies, either self-fertile inbred lines were used or DHs were generated from the cross *S. cereale cereale* L. x *S. vavilovii* L. (wild type). The wild type of rye is unwanted for population breeding due to its self-compatibility and other negative traits such as lodging, brittle spikes, and low grain yield. As recently as the 1990s, the first studies with true *S. cereale cereale* L. genotypes and the success in high green plant regeneration rate were reported (Flehinghaus-Roux et al. 1995, Immonen & Anttila 1996). Since the first studies on rye anther culture, it has been observed that callus induction, embryo formation, and plant regeneration ability are strongly affected by the genotype, and no universal medium exists for different rye genotypes (Deimling & Flehinghaus-Roux 1997, Rakoczy-Trojanowska et al. 1997).

The switch from the gametophytic to the sporophytic pathway (embryogenesis) depends on many endogenous and exogenous factors (Zheng 2003, Datta 2005, Maraschin et al. 2005, Shariatpanahi et al. 2006, Forster et al. 2007, Hosp et al. 2007, Seguí-Simarro & Nuez 2008a). In anther or microspore cultures of rye, callus induction and embryo formation, albino and green plant rates vary depending on genotype, physiological stage of donor plant, stress treatments, developmental stage of microspore, medium, and culture conditions (Jähne & Lörz 1995, Deimling & Flehinghaus-Roux 1997, Immonen & Anttila 1998, 1999, 2000). Callus induction and embryo formation can be triggered by stress (cold, heat or chemical treatments, nitrogen starvation, osmotic stress, water stress, irradiation) as pre-treatment before plating or as post-treatment after plating of anthers (e.g. Reynolds 1997, Touraev et al. 1997, Immonen & Anttila 1999, Immonen & Robinson 2000, Shariatpanahi et al. 2006). Usually, detached tillers (with spikes) are stored at 4-5 ºC before culturing anthers for 1-4 weeks (Deimling & Flehinghaus-Roux 1997, Immonen & Anttila 1999). The optimal cold pre-treatment for anther culture response is genotype dependent (Immonen & Anttila 1999). The combination of cold treatment with heat shock improved androgenic response in triticale (Immonen & Robinson

2000). However, cold-treatment with mannitol starvation has contrary effects on induction in triticale (Immonen & Robinson 2000). In addition, mannitol starvation suppresses response in rye anther culture, but not in microspore cultures (Immonen & Anttila 1999, Guo & Pulli 2000).

The developmental stage of microspores is crucial in induction. In rye, microspores at the late uninucleate stage or at first pollen mitosis, which corresponds to anthers 6-6.5 mm long, have been most effective (Flehinghaus-Roux et al. 1995, Immonen & Anttila 1998). Plating density of anthers affects green plant regeneration, 0.5-2.2 anthers per cm^2 being the most favorable (Immonen & Anttila 2000, Ma et al. 2004a).

Anthers are cultured on special induction (basal) medium, which comprises carbohydrates, iron, vitamins, amino acids, macro and microelements, growth regulators (auxins, cytokinins), and solidification agents. Auxin (e.g. 2,4-dichlorophenoxyacetic acid, 2,4-D) is used to trigger induction and the formation of embryo-like structures. Cytokinins (e.g. kinetin) promote cell division and are needed for callus growth. Various basal media have been tested for rye, the most common with some modifications being higher total nitrogen (35-60 mmol/l) containing media: N6 (Chu et al. 1975) and MS (Murashige & Skoog 1962), and lower total nitrogen (14.2-23.1 mmol/l) containing media: 190-2 (Wang & Hu 1984) and W14 (Ouyang et al. 1989). The basal media, W14, 190-2, and PG-96 (Guo et al. 1999), have been superior for callus induction and green plant regeneration (Immonen & Anttila 1999, Immonen & Anttila 2000, Ma et al. 2004a). Carbohydrate source in the induction medium affects the induction and regeneration rates. For rye, sucrose has been replaced with maltose due to slower carbohydrate hydrolysis and higher regeneration rates (Flehinghaus et al. 1991, Deimling & Flehinghaus-Roux 1997, Guo & Pulli 2000, Immonen & Anttila 2000). Adding glutamine to the induction medium increased embryo formation and green plant regeneration in cereals (Datta 2005) including rye (Immonen & Anttila 2000). Solidification of media (solid or liquid) with a gelling agent can affect anther culture response. Agar has inhibitory effects on anther culture and therefore media have been solidified with polysaccharides, gelrite (an agar substitute), or agarose (obtained from agar) (Deimling & Flehinghaus-Roux 1997). Moreover, Phytagel (an agar substitute) and Ficoll 400 (synthetic polymer of sucrose) have been used (Immonen & Anttila 1996, 2000).

The anthers in induction media are incubated at 24-28ºC in darkness (Deimling & Flehinghaus-Roux 1997, Immonen & Anttila 1999, Immonen & Anttila 2000, Ma et al. 2004a). After 6-10 weeks, calli and embryo-like structures are moved to regeneration medium with lower auxin concentrations at 21-25ºC in light (Deimling & Flehinghaus-Roux 1997, Immonen & Tenhola-Roininen 2003, Ma et al. 2004a). Later, regenerated green plantlets are moved to rooting medium (MS or 190-2) without growth regulators. The ploidy level of plantlets are determined with flow cytometry, or using chromosome counting from the root tips stained with orcein (Immonen et al. 1999, Immonen & Tenhola-Roininen 2003, Ma et al. 2004a).

Spontaneous doubling rate of chromosomes depends on the cereal, but can be high, approximately 70% in rye (Deimling & Fehinghaus-Roux 1997). In addition to diploid plants, also aneuploid, mixoploid, triploid, and tetraploid plants are produced. Colchicine treatment has not been favored in the production of DHs in rye due to it having only little effect on chromosome doubling or resulting in low survival rate of plants (Thomas et al. 1975, Deimling & Fehinghaus-Roux 1997, Immonen & Anttila 1999, Guo & Pulli 2000).

Albino rye plant generation rates varied from 0-100% (per 100 anthers) depending on the genotype and culture conditions (Immonen & Anttila 1996, Deimling & Flehinghaus-Roux 1997, Rakoczy-Trojanowska et al. 1997, Immonen 1999, Immonen & Anttila 2000). The best green plant regeneration has been 30.6 green plants per 100 anthers on the solid induction medium with 190-2 macroelements and MS microelements in the spring line Jo02 (Immonen & Anttila 1999). The solid medium increased green plant regeneration rate from anther-derived calli (10.9 green plants/100 calli in spring cultivar Jussi) also in other rye studies (Ma et al. 2004a). Variable fertility and morphological abnormalities were recorded in DH plants of rye and triticale (Deimling & Flehinghaus-Roux 1997, Immonen & Anttila 1999, Immonen & Robinson 2000), although plants solely with normal fertility were also reported (e.g. Guo & Pulli 2000). Enforcement to complete homozygosity in an out-crossing species might lead to inbreeding depression (Logue 1996, Keller & Waller 2002), which affects the fertility and viability of DH plants.

2.3.2 DNA markers and marker assisted selection

There is currently great interest in developing fast, robust, high throughput methodologies for DNA analyses using pipetting robots. However, this is not possible for many research laboratories, and therefore low cost and easy to use DNA markers are still commonly used. DNA markers can reveal genetic differences among genotypes. Markers can be described as co-dominant or dominant based on whether they discriminate between homo- and heterozygotes (Collard et al. 2005).

DNA markers can be used directly to tag specific, functional genes, or gene families (van Tienderen et al. 2002, Kuchel et al. 2005, 2007). They can act as 'flags' or 'signs' near the target genes. They are also useful as molecular tools for linkage mapping, diversity studies, fingerprinting, exploring genetic resources, and for diagnostic and breeding purposes. DNA markers can be divided into three categories based on methods of detection: hybridization-based, polymerase chain reaction (PCR)-based, and DNA sequence-based (Collard et al. 2005).

Hybridization-based RFLP (restriction fragment length polymorphism) markers are reliable, co-dominant markers that were extensively used in plants in the 1990s. In the RFLP marker procedure, genomic DNA is digested with restriction enzymes and the segments are blotted onto a filter (Southern blotting). The polymorphisms are detected with a labeled probe (Tanksley et al.

1989). The RFLP procedure is quite laborious and requires large amounts of DNA.

Another marker method that uses restriction enzyme digestion and PCR is the AFLP (amplified fragment polymorphism) method. In this marker method, the digested DNA is amplified with primers between known, ligated, double-stranded oligonucleotide adapters (Vos et al. 1995). The AFLP markers are inherited mostly in a dominant manner.

RAPD (random amplified polymorphic DNA) markers were the first PCR-based, randomly amplified markers used at the beginning of the 1990s (Williams et al. 1990). The RAPD protocol usually uses a 10 bp arbitrary primer, and the markers are inherited in a dominant manner (amplified/ not amplified). These markers are still in use despite problems with reproducibility.

Simple sequence repeats (SSRs) or microsatellites (usually mono-, di-, tri-, or tetra-nucleotide repeats in tandem arrays) were the first PCR-based non-random, co-dominant markers (Nakamura et al. 1987, Litt & Luty 1989, Akkaya et al. 1992, Cuadrado et al. 2008). The forward and reverse primers anneal to the sequences that flank the microsatellite. SSR markers are user-friendly, highly polymorphic, stable, and usually locus-specific. An additional microsatellite-based marker type is the inter-simple sequence repeat (ISSR) marker that is amplified by primers consisting of microsatellite repeats between closely spaced, inversely oriented microsatellites (Reddy et al. 2002, Zietkiewicz et al. 1994). These dominant markers are also termed SCIM (*Secale cereale* inter-microsatellite) in rye (Camacho et al. 2005). The combination of RAPD and microsatellite primers (RAMP, random amplified microsatellite polymorphism, Wu et al. 1994) has also been used in mapping studies (e.g. Shang et al. 2003, González et al. 2005). RAMP markers are inherited in a co-dominant manner. The microsatellite sequence–based co-dominant/dominant SAMPL (selective amplification of microsatellite polymorphic loci) procedure resembles the AFLP method. Genomic DNA is digested with restriction enzymes and pre-amplified as in the AFLP procedure. Selective amplification is done with a selective AFLP primer and a selective SAMPL primer. The SAMPL primer consists of a compound of SSR sequences with two adjacent dinucleotide repeats, or the primer is complementary to a microsatellite sequence and is anchored at the non-microsatellite sequence. SAMPLs are superior to AFLPs and RAPDs for finding polymorphisms in closely related genotypes (Rakoczy-Trojanowska & Bolibok 2004).

SRAP (sequence-related amplified polymorphism) markers use pairs of primers with AT- (anneals to an intron) or GC-rich (anneals to an exon) cores to amplify intragenic fragments (Li & Quiros 2001). These markers are inherited in a dominant or a co-dominant manner. A modification of the SRAP system, target region amplified polymorphism (TRAP) is a mainly dominant marker system that uses two primers, one of which is designed from the EST sequence and the second, arbitrary primer anneals to an intron or an exon (Hu & Vick 2003).

Retrotransposon-based markers have been developed for many plant species (Schulman 2007). Retrotransposons, also termed Type I transposable

elements, are transcribed as normal genes, and via reverse-transcription of mRNA (cDNA) they are copied back into the genome (Vicient et al. 2001, Schulman 2007). The LTR (long terminal repeat) retrotransposons seem to be the most abundant and ubiquitous in plants. They have repeats of several hundred nucleotides at both ends of the transposable element. In the dominant IRAP (inter–retrotransposon amplified polymorphism; Kalendar et al. 1999, Kalendar & Schulman 2006) marker method, the segment between retrotransposons that lie near to each other in head-to-head or tail-to-tail orientation is amplified with a single primer complementary to the 5´ or 3′ end of the LTR. In the dominant REMAP (retrotransposon-microsatellite amplified polymorphism) procedure, the region between a closely spaced microsatellite and a retrotransposon is amplified (Kalendar et al. 1999, Provan et al. 1999, Kalendar & Schulman 2006). RBIP (retrotransposon-based insertion polymorphism) is a co-dominant method in which markers are amplified with two primers, one of which is in the flanking DNA of the retrotransposon and the other in a retrotransposon (Flavell et al. 1998). In the dominant SSAP (sequence-specific amplified polymorphism) procedure (Waugh et al. 1997), genomic DNA is digested with a restriction enzyme and adapters are ligated to fragments as in the AFLP procedure. The sequence of the specific primer is based on the conserved motif at the retrotransposon and together with a selective AFLP primer it amplifies a product between a retrotransposon and a restriction site. For a variety of crops retrotransposon-based methods are more informative than the AFLP procedure (Schulman 2007).

Co-dominant SNPs (single nucleotide polymorphisms) and small insertions/deletions (indels) are the most abundant form of DNA variation, and they distinguish sequence differences between alleles. They can be used for association studies in high-resolution genetic mapping of traits and to improve the efficiency of breeding programs (Rafalski 2002, Ravel et al. 2007). EST-derived SNP markers have been developed for major cereals (Varshney et al. 2004), although the chances of finding SNPs is usually higher in a non-coding region of the genome (less stringent selection) (van Tienderen et al. 2002). With the SNP marker system, the marker density in maps can be increased 100-fold compared with microsatellite maps. With automation, the SNP system is more efficient for genetic studies (Varshney et al. 2007), but requires high-cost equipment (Bagge & Lübberstedt 2008). SNPs can also be converted to cleaved amplified polymorphic sequence (CAPS) format, which involves amplification of DNA followed by digestion with restriction enzymes (Thiel et al. 2004, Varshney et al. 2007). CAPS markers are mainly inherited in a co-dominant manner.

Random markers (e.g. AFLP, ISSR, RFLP, RAPD, REMAP, SRAP) can be converted into sequence specific SCAR (sequence characterized amplified region; McDermott et al. 1994) or STS (sequence-tagged site, Olson et al. 1989) markers by cloning and sequencing the original marker and planning a new pair of PCR primers, which detect only one specific locus. SCARs and STSs are inherited in a co-dominant manner and they are robust and reliable (Gupta et al. 1999, Semagn et al. 2006).

A microarray-based high throughput molecular technique has been developed to speed up marker analyses, scoring thousands or tens of thousands of DNAs for polymorphism in gene expression studies. This technique has evolved from Southern blotting. Two types of microarray exist: the DNA-fragment-based and the oligonucleotide-based microarray (Richmond & Somerville 2000). The collections of DNAs in arrays were first described in 1987 (Kulesh et al. 1987). These arrays contain a short section of a gene or other DNA fragment used as probes to hybridize to cDNA or cRNA labeled with fluorescent dyes. Probes are attached to a solid surface, which can be a glass, plastic, gold, or silicon chip, or a microscopic bead. The microarray is scanned in a microarray scanner to visualize fluorescence from two samples that are to be compared e.g. diseased tissue versus healthy tissue (Lockhart & Winzeler 2000). Several microarray-based SNP methods have also been developed (e.g. Wang et al. 1998, Lindroos et al. 2002). One of the microarray approaches detects insertional polymorphism of transposons using tagged primer oligonucleotides (the tagged microarray marker approach, TAM; Flavell et al. 2003). This method enables screening of large plant populations for e.g. disease resistance. Diversity array technology (DArT) is a hybridization-based method that does not need DNA sequence information (Jaccoud et al. 2001, Semagn et al. 2006). It generates whole-genome fingerprints by scoring the presence or absence of DNA fragments from genomic DNA samples. It has been used in genetic mapping and genetic relationship studies in e.g. rice, barley, and wheat (e.g. Jaccoud et al. 2001, Wenzl et al. 2004, Akbari et al. 2006, Hearnden et al. 2007, Peleg et al. 2008).

DNA markers can be used in breeding for parental or background selection, gene pyramiding, and purity control. Markers are especially important for finding superior genotypes when the trait is difficult to observe phenotypically. In addition, MAS (also called as marker assisted breeding or marker aided selection) speeds up breeding time, is unaffected by environment, prevents transfer of undesirable or deleterious genes, and can also allow selection of low-heritability traits (Koebner 2004, Collard et al. 2005). Regardless of the benefits, the use of MAS has not been unequivocally accepted by breeders due to insufficient numbers of reliable markers, high costs, and complexity of quantitative traits (Koebner 2004, Varshney et al. 2004). MAS is commonly used for major crops such as maize, wheat, barley, and rice, but not for minor crops such as rye and oat (Koebner 2004).

When developing markers for MAS, one should identify the genes that affect traits of interest and design markers within or flanking the genes. The selective marker must be diagnostic in the target population to be useful in breeding. In addition, linkage disequilibrium (LD) must be maintained between the target gene and the marker for introducing in MAS. Moreover, the QTL effect and candidate markers for the trait require validation in other genetic backgrounds to demonstrate their diagnostic value, which is usually low (Tuvesson et al. 2007). To increase adoption of MAS by breeders, researchers should create more reliable markers by using larger population sizes for mapping, more exact phenotypic data, and they should validate markers in

different genetic backgrounds. In addition, improvements in marker technology (e.g. multiplex PCR), exploitation of functional genomics and comparative mapping (consensus maps), and the construction of high-density maps will increase breeders' willingness to exploit MAS. It has been proposed that the development of a high-throughput SNP detection system will especially benefit future mapping studies and MAS. The efficiency of MAS will improve particularly when the DNA sequence of a target gene is known and a perfect marker can be designed. Before knowing all the genes that affect agronomically important traits, QTL maps and DNA markers will be the only way to tag genes of interest (Young 1999, Collard et al. 2005).

2.3.2.1 Linkage mapping and gene tagging

Linkage maps describe the position and relative genetic distances between markers along chromosomes. Linkage maps are used for QTL analyses to identify the chromosomal location of genes associated with a trait of interest (Collard et al. 2005). The construction of a linkage map can be divided into three stages: the production of a mapping population segregating for a desired trait, finding polymorphic markers in the parents and screening these markers in the mapping population, and the linkage analyses of markers. 'Anchor' markers (e.g. SSRs) are used to compare marker locations between different maps. The linkage analyses are made using algorithms that divide markers into linkage groups. Linkages are calculated using odds ratios (linkage versus no linkage), and termed logarithm of odds ratio (LOD) (Collard et al 2005). Different methods exist for the construction of linkage maps, the most common being JoinMap (Van Ooijen & Voorrips 2001) and MAPMAKER (Lander et al. 1987). The genetic distances between markers/genes are calculated from the recombination frequencies. However, the map distances (centiMorgans, cM) are not equal to recombination values due to double crossovers affecting the map length, but not the recombination of genes (Jones et al. 1997b). Two mapping functions are referred to: Kosambi's (Kosambi 1944) and Haldane's (Haldane & Smith 1947). In the first function, it is presumed that there is interference between adjacent recombinations, in the second no interference is expected.

Traits of interest are phenotypically scored in the mapping population before QTL analysis. In the QTL analysis, observations are divided into different groups according to the genotype, and significant differences between groups mean that the marker is linked to the QTL controlling the trait (Collard et al. 2005). Two common methods are used for QTL mapping: simple interval mapping (SIM), which analyses intervals between adjacent pairs of linked markers simultaneously and composite interval mapping (CIM), which combines interval mapping and linear regression, including additional genetic markers (Collard et al. 2005). Several computer programs for QTL analyses have been produced, e.g. MAPMAKER/QTL (Lander et al. 1987), MAPQTL (Van Ooijen 2004), QGENE (Nelson 1997), MQTL (Tinker & Mather 1995), WQTLCART (Wang et al. 2007), and MAPMANAGERQTX (Manly et al. 2001).

It is also possible to find genes without constructing a whole linkage map. The easiest way to tag genes is by using bulked segregant analysis (BSA, Michelmore et al. 1991) or selective genotyping (Collard et al. 2005). BSA is an effective method in detecting marker and trait associations when the traits are Mendelian in nature and are controlled by relatively few genes. In this method, the DNA samples of the opposite extremes (10-20 individuals) of phenotype in a mapping population are bulked and used for marker screening and tagging a gene of interest. The association of a marker with the trait of interest in the two different DNA bulks is statistically analyzed. Putative markers associated with the trait are screened in the whole mapping population to ensure the result and to perform subsequent QTL analysis. In selective genotyping, only individuals representing phenotype extremes from the mapping population are selected for marker genotyping, linkage mapping, and QTL analysis (Collard et al. 2005).

2.3.2.2 DNA markers and linkage maps in rye

Until now, the availability of rye-specific DNA markers has been low in comparison to those for wheat and barley. The constructed rye linkage maps with DNA markers are presented in Table 3. The first DNA-based rye linkage maps consisted of RAPDs and RFLPs. Saal and Wricke (1999) developed the first microsatellites for rye (SCM; *Secale cereale* microsatellite). Due to close relatedness of rye, wheat, and barley, some known wheat and barley markers (RFLPs, SNPs, SSRs, CAPSs) have been exploited in rye linkage maps (Devos et al. 1992, Devos et al. 1993a, Saal & Wricke 1999, Ma et al. 2001, Khlestkina et al. 2004, Khlestkina et al. 2005, Varshney et al. 2007, Cuadrado et al. 2008, Hackauf et al. 2009). The length of rye linkage maps varies between 340 cM (Loarce et al. 1996) and 1500 cM (Milczarski et al. 2007, Masojć & Milczarski 2009). This means that saturated linkage maps are needed for the identification of important genes of agronomic value in rye (Varshney et al. 2007). Rye markers have also been exploited e.g. in diversity (Matos et al. 2001, Persson et al. 2001, Chebotar et al. 2003, Shang et al. 2003, Ma et al. 2004b, Bolibok et al. 2005, Bolibok et al. 2006, Kuleung et al. 2006, Shang et al. 2006, Isik et al. 2007), wheat-rye translocation (Nagy et al. 2003, Kofler et al. 2008, Vaillancourt et al. 2008), fertility/sterility (Voylokov et al. 1998, Börner et al. 1998b, Bednarek et al. 2002a, 2002b, Stojalowski et al. 2005, 2006), somaclonal variation (de la Puente et al. 2008), and aluminum tolerance studies (Miftahudin et al. 2002, Matos et al. 2005).

TABLE 3 DNA markers exploited in the rye linkage maps

Cross	DNA markers									References
	AFLP	ISSR/ SCIM	SSR[a]	RAPD	RFLP	SCAR	SAMPL	STS/ EST	SNP	
Danko x Halo				x	x					Philipp et al. 1994
Inbred lines not reported				x	x					Senft & Wricke 1996
E x R				x	x					Loarce et al. 1996
P87 x P105			x		x					Korzun et al. 1998a, 2001
13 different rye crosses				x	x					Börner & Korzun 1998*
Inbred lines not reported	x		x	x	x					Saal & Wricke 1999, 2002
UC-90 x E-line King II x Imperial			x		x					Ma et al. 2001
DS2 x RXL10				x	x					Masojć et al. 2001
DS2 x RXL10	x			x	x					Bednarek et al. 2003
BC1 population 9953	x		x							Hackauf & Wehling 2003

P87 x P105 N6 x N2 N7 x N2 N7 x N6			x		x					Khlestkina et al. 2004*, 2005*
Ailés x Riodeva		x								Camacho et al. 2005
541 x Otl-3 DS2 x RXL10	x		x	x	x			x		Masojć & Milczarski, 2005, 2009
L318 x L9		x	x	x		x	x	x		Bolibok et al. 2007
541 x Otl-3		x	x	x				x		Masojć et al. 2007
541 x Otl-3		x	x	x		x		x		Milczarski et al. 2007
P87 x P105 N6 x N2 N7 x N2 N7 x N6			x		x				x	Varshney et al. 2007
L2053 x Altevogt 14160	x		x					x		Hackauf et al. 2009

[a] also contains microsatellites from wheat and barley
* consensus map

3 AIM OF THE STUDY

This study was a part of the collaborative rye research project initiated by the Ministry of Agriculture and Forestry in 1999 to improve rye breeding, to develop new, good quality adapted rye varieties, and to increase rye cultivation in Finland. The aim of the study was to produce rye DHs for the creation of populations segregating for short straw and PHS, to construct a rye linkage map, and to search for DNA markers closely linked to the gene affecting dwarfism and the gene(s) affecting PHS in winter rye.

The objectives of this study were:

- To establish a good anther culture method for doubled haploid production in rye (I, II)
- To create two populations using DHs: 1) a population (EM-1 x Voima) segregating for short straw and 2) a mapping population (Amilo x Voima) segregating for PHS resistance (I, II)
- To construct a linkage map using a DH mapping population (IV)
- To identify DNA markers closely linked to the dwarfing gene, *Ddw1*, in the population derived from EM-1 DH x Voima DH cross (III)
- To search for markers near the gene(s) affecting PHS in the mapping population derived from Amilo DH x Voima DH cross (IV)

4 SUMMARY OF MATERIALS AND METHODS

All the materials and methods are explained in detail in the Materials and Methods sections of the original papers and the manuscript I-IV.

4.1 Plant material and growth conditions (I-IV)

Boreal Plant Breeding Ltd. provided all the seeds used in anther culture (I, II), crossing (II), and mapping (II-IV) studies. The rye material used in the study is presented in Table 4. Plant material was grown in the greenhouse and fertilized as reported in papers I and II. Winter ryes were vernalized in an unheated greenhouse during winter or in a cold chamber during other seasons as explained in paper II.

In the crossing test (II, Table 4), anther-culture derived DHs were reciprocally crossed to assess their fertility and morphology and to study their usefulness for research and breeding. In addition, three different populations were created: 1) two populations segregating for the short-straw growth habit (II, III) and 2) a DH mapping population segregating for PHS resistance (the sprouting resistance population: II, IV). In the populations segregating for short-straw, the parents originated from the Finnish tall winter rye cv. Voima and the Russian short mutant EM-1 with the dominant dwarf 1 gene, *Ddw1* (Kobyliansky 1972). In these populations, only the parents were DHs due to too low a yield of F_1 seeds. The larger population was used to find markers linked to *Ddw1* and both populations for QTL analysis. In the sprouting resistance population, the parents were DHs derived from the sprouting-resistant Polish cv. Amilo (Masojć & Larsson-Raźnikiewicz 1991, Wolski & Pietrusiak 1988) and the susceptible Finnish cv. Voima. The sprouting resistance population was developed from F_1 plants through anther culture (II). The DH progeny was crossed with the sprouting-susceptible Finnish cv. Riihi to get seeds for α-amylase activity measurements (II, IV).

TABLE 4 Rye material used in the study

Name	Spring/ Winter type	Cultivars/Lines, Breeding company, Year of release	Used in the test / study	Paper no.
Akusti	Winter	Cultivar, Boreal Plant Breeding Ltd., Finland,1992	Test 1	I
Amilo	Winter	Cultivar, Rolimpex S.A., Poland, 1995	Test 1, Crossing test, Mapping	I, II, IV
Amilo/ Riihi/ Madar	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Crossing test	II
Auvinen	Spring	Landrace, Finland	Test 1	I
Bor 7068	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 2	I
Bor 9414	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 2	I
Bor 9214	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 2	I
EM-1	Winter	Natural mutant, Vavilov genebank collection, St. Petersburg; Russia (Kobyliansky 1972)	Mapping	II, III
Jo 02	Spring	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 1	I
Jo 8708/Madar	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 2	I
Jo 8708/Madar/ Amilo	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Crossing test	II
Riihi	Winter	Cultivar, Boreal Plant Breeding Ltd., Finland, 1999	Crossing test, Mapping	II, IV
Riihi/ Talovskaja	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 2	I
Sibirskaja/ Talovskaja	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Test 2	I
Sibirskaja/ Talovskaja/ Bor 7068	Winter	Breeding line, Boreal Plant Breeding Ltd., Finland	Crossing test	II
Voima	Winter	Cultivar, Boreal Plant Breeding Ltd., Finland, 1966	Mapping	II, III, IV

4.2 Anther culture (I-II)

For the anther culture experiments, the tillers of donor rye plants were collected and stored in the dark at 4°C, as described by Immonen and Anttila (1998). For embryo induction and green plant regeneration, cold pre-treatments of varying durations (1-4 weeks) were tested (I), and the best cold-treatment of 3 weeks was used in the production of DHs for mapping populations (II). Spikes with microspores at the mid to late uninucleate stage were used. Three induction media (differing in their macro- and microelements: W14, MS, or 190-2) were tested (I, II), but only two of them (W14 or 190-2) were used in the production of DHs for mapping purposes (II). The anther cultures were incubated in the dark at 25°C, or first at 32°C for 3 days (heat post-treatment: I, II). All calli were transferred to half strength MS differentiation medium and the nuclear DNA content of developed green plantlets was determined by flow cytometry (I, II). Haploid plantlets were colchicine treated as reported by Immonen and Tenhola-Roininen (2003). Only a solid 190-2 induction medium with no heat post-treatment was used for the creation of a DH mapping population segregating for sprouting resistance (II, IV). Callus induction (CI) rate, the number of green (GP) and albino plants (AP) and their ratio (GP:AP), green plant regeneration efficiency (GRE), and proportions of ploidy levels (%) were calculated (I, II). The fertility (including pollen viability) and morphology observations were performed as described in paper II.

4.3 Trait observations (II-IV)

4.3.1 Height measurement (II-III)

In the short straw populations of rye, the height of the plants was measured in the greenhouse from the soil surface to the top of the tiller (with the spike) just before ripening.

4.3.2 *a*-Amylase activity measurement (II, IV)

For the determination of PHS in the DH mapping population, *a*-amylase activity determination was chosen instead of Hagberg falling number. *a*-Amylase activity was measured from one grain, except for DH parents and Riihi where it was done from more grains after 2 or 4 days of germination. The sterilization, germination, drying, and grinding of grains were performed as described in paper IV. *a*-Amylase activities (Ceralpha U/g) of rye flour were measured with adjusted volume by an *a*-amylase assay procedure using amylase HR reagent (Ceralpha method, ICC standard No. 303, CER 07/00, Megazyme, Co. Wicklow, Ireland).

4.4 DNA analyses (III-IV)

4.4.1 DNA isolation and DNA bulks

DNA was extracted from 2g of young leaves by a modified CTAB (cetyltrimethyl ammonium bromide) II method (Poulsen et al. 1993) as described in paper III. The DNA concentration was measured with a GeneQuant II RNA/DNA calculator (Pharmacia Biotech, Cambridge, England). BSA was used to find polymorphic markers between 'short' and 'tall' bulks, which consisted of DNA from the nine shortest and the nine tallest individuals of the short straw population as explained in paper III.

4.4.2 DNA markers

AFLP analyses were performed with minor modifications according to the procedure developed by Vos et al. (1995) described in paper IV. Markers were generated using *Mse*I and *Eco*RI primer pairs with one selective nucleotide at their 3' end in a pre-amplification step and with three selective nucleotides in a selective amplification step.

Six different types of microsatellites (SCM: *Secale cereale* microsatellite; RMS: rye microsatellite site; REMS: rye expressed microsatellite site; WMS: wheat microsatellite marker; HVM: *Hordeum vulgare* microsatellite; BMS: barley microsatellite marker) were used for mapping as described in more detail in papers III-IV. The microsatellites were either amplified with a two-primer (one with a fluorescent dye) or with a three-primer system (a forward, a reverse, and an M13 primer, Oetting et al. 1995). In the three-primer system, the M13 primer was labeled with a fluorescent dye, and the forward primer had an M13 tail.

RAPD primers were either synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer or purchased from Operon Technologies (Alameda, CA, USA). The PCR reactions were carried out as described in paper III.

Retrotransposon-based REMAP and IRAP markers (Kalendar et al. 1999, Kalendar & Schulman 2006) in paper IV were produced with minor modifications as described by Schulman et al. (2004).

ISSR primers were run with the same PCR profile as REMAP primers as described in paper IV.

Exon-intron-based primers (ET, exon targeting, and IT, intron targeting, primers: sequence data provided from Andrzej Rafalski, IHAR, Poland; SRAP: Li & Quiros 2001) were used as described in paper III.

The REMS1218 sequence was amplified from EM-1 and Voima DH parent, the DNA fragments were ligated, transformed with the TOPO TA cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA), and several plasmid clones were sequenced with a MegaBACE™ 500 Sequencer (GE Healthcare, Buckinghamshire, UK) as described in paper III. In addition, the endosperm-specific β-amylase gene was sequenced from the DH parents using direct sequencing. One SNP marker for the microsatellite site REMS1218 (SNP-

REMS1218) and one for β-amylase gene (SNP-BAMY) were created in paper III. The SNP reactions were carried out as explained in paper III.

4.5 Statistical analyses

Parametric and nonparametric tests (SAS Institute, version 8.1, Cary, NC, USA 1999) and a nonparametric chi-square test were used to study rye anther culture efficiency (I) and to calculate associations of DNA markers linked to plant height in the short straw population (III). The statistical test was chosen according to data, data distributions and parameters used. The segregation of markers in the sprouting resistance population was tested against an expected 1: 1 ratio using the chi-square test in JoinMap® version 3.0 (Van Ooijen & Voorrips 2001) in paper IV.

MAPMAKER 3.0 software (Lander et al. 1987) was used to create the linkage map in the short straw population. Recombination values were converted to map distances, centiMorgans (cM), using the Haldane mapping function (Haldane & Smith 1947).

The DH linkage map was constructed using a JoinMap® program version 3.0 (Van Ooijen and Voorrips 2001) as described in paper IV. Map distances in centiMorgans (cM) were calculated using the Kosambi mapping function (Kosambi 1944).

QTL data for plant height were analyzed using a MAPMAKER/QTL 1.1 (Lander et al. 1987) program in paper III. The QTL analysis for sprouting resistance was performed with NQTL software (version 26-Nov-2001, Tinker & Mather 1995) with simple interval mapping as explained in paper IV.

BLASTX searches (Altschul et al. 1997), which search protein databases using a translated nucleotide query against GenBank databases, were performed using the National Center for Biotechnology Information server (Bethesda, Md., USA; http://www.ncbi.nlm.nih.gov).

5 REVIEW OF THE RESULTS

5.1 The effects of cold and heat treatments and media on anther culture response (I-II)

Immonen and Anttila (1996, 1998, 1999, 2000) developed the basis of the protocol for the rye anther culture technique used in this study. However, the effect of cold pre-treatment (at 4°C in dark for 1-4 weeks) of tillers in combination with heat treatment (post-treatment at +32°C for 3 days; control treatment at 25°C in dark) of plated anthers on green plant regeneration had not been previously tested and was studied for both spring and winter rye types. Also some previously untested breeding lines of winter rye were anther-cultured using various induction media (with W14, 190-2, or MS micro- and macro-elements) in paper I.

Significant interactions ($P < 0.1$) between genotypes and cold pre-treatment were observed for CI, AP, and GP (I). GP production was significantly increased ($P < 0.1$) by three weeks of cold pre-treatment in all tested rye genotypes, and four weeks of cold was inferior to a 2-3 week cold pre-treatment (I). The best GP production was 8.4/100 anthers for spring-rye line Jo 02 and the lowest 1.4/100 anthers for winter-rye Akusti after a three week cold-treatment of tillers (I). However, there was variation in the duration of the best cold pre-treatment for GRE and GP:AP ratio between the genotypes (I).

Heat post-treatment of plated anthers was examined in combination with cold pre-treatments of various durations in paper I. Heat with three weeks of cold pre-treatment enhanced the regeneration ability, which was seen as improvements in CI, GRE, and GP:AP in the majority of previously untested breeding lines of winter rye in paper I. In the previously most responsive line Jo 02 and in three untested breeding lines (Bor 7068, Bor 9214, Riihi/Talovskaja), the effect of the combinations of cold (2-4 weeks for Jo 02, 3 weeks for others) and heat treatments was clearly seen as improved GP production, which was mostly a consequence of enhanced GRE and the GP:AP ratio (I). Therefore, heat

post-treatment followed by three weeks of cold treatment was also used in the experiments described in paper II.

To investigate whether a broadly suitable induction medium for rye could be identified, previously untested rye lines were grown in alternative induction media for paper I. The 190-2 induction (total N 14.2 mmol/l) medium was superior ($P < 0.001$) to W14 (total N 23.1 mmol/l) in promoting CI (results not shown in paper I). Significant interactions between genotype, medium, and heat post-treatment were recorded for GP production (results not shown in paper I) as significant genotype-specific results for 190-2 medium in heat-stressed plates. Heat post-treatment increased GP production in nine out of twelve medium (MS, 190-2, W14) -genotype (breeding lines of winter rye) combinations in paper I. The effect of heat treatment on GP regeneration was positive for all cultures initially grown on 190-2 medium (I). In paper II, heat treatment resulted in a higher GP:AP ratio. The effect of heat on cultures (GP:AP) that were initiated on W14 medium was more variable in paper I. However, for breeding line Bor 7068, the W14 medium with heat treatment produced most green plants (5.7 plants/100 anthers, paper I).

5.2 The production of doubled haploids (I-II)

The ploidy level of green regenerants was analyzed using flow cytometry to detect the spontaneous doubling rate and to separate DHs from haploid plants. The proportion of all spontaneous DHs ranged from 13% (I) to 87.5% (II). The best result was achieved with cultivar Voima. The amount of plants with abnormal ploidy levels (triploid, tetraploid) ranged from 3.6% to 56% of all green plantlets analyzed in papers I and II.

Heat significantly increased ($P < 0.1$) spontaneous doubling rates across rye genotypes and cold pre-treatments in paper I. However, in paper II, heat following three weeks of cold treatment decreased spontaneous doubling in the parent cultivars (Amilo and Voima) of the sprouting resistance population. Therefore, no heat treatment was used for the F_1 cultures of the sprouting resistance population (II). The duration of cold pre-treatment (with heat post-treatment) affected spontaneous doubling, three weeks of cold pre-treatment being the best (I).

Morphology and fertility (percentage of seeds developed from hand-pollinated florets) of spontaneous DHs and colchicine-treated haploids were studied in the crossing test in paper II. The growth habits of the plants were categorized into three groups: normal, stunted, and grass-like, of which the last category was the most common. In reciprocal crossings of anther-derived plants, one fifth of the plants were fertile and developed one or more seeds, the percentage varying between 2.8% to 41.3%. In general, there were more fertile plants among spontaneous DHs than among colchicine-treated haploids. In the crossing test, the fertility of the plants increased in the following order: from the crosses between different DH populations to the crosses of DH populations

with cultivars. There was no statistically significant difference in the fertility of plants when the plant was used as a pollinator or a pollen recipient (results not shown in paper II).

5.3 The generation of populations (II-IV)

The objective was to create two populations using rye DHs. The populations segregating for short-straw (EM-1 x Voima) and for sprouting resistance (Amilo x Voima) were produced over the period of three years as reported in papers II and III. In general, high mortality (21-34%) of anther culture-derived plants was noted during the generation of populations (II).

Two populations segregating for short straw were produced from two different F_1 x F_1 crosses and the larger (109 individuals) was used to search for markers linked to plant height (II, III).

A mapping population segregating for sprouting resistance was produced for the first time using DHs of out-crossing rye (II, IV). Only 11% of all green regenerants (130/1214 plantlets) were usable because of low survival rate and low fertility (II). Eighty-nine DHs out of 130 were used for mapping because the rest of the plants were clones regenerated from the same embryos (II). The DHs of the mapping population were test-crossed with Riihi for *a*-amylase analyses. The fertility of DHs in the Riihi test-crosses was less than 30% (II). The morphology of seeds was mostly abnormal (shrunken and lighter seeds with a rough seed coat) and about 20% of the seeds from the test-crosses were not viable (II). Only the seeds of 68 of the 89 DHs could be used for *a*-amylase activity analyses (II, IV).

5.4 Tagging DNA markers linked to the dwarfing gene, *Ddw1* (III)

The objective was to identify DNA markers closely linked to the dwarfing gene, *Ddw1*, in the population (F_1 x F_1) derived from the EM-1 DH x Voima DH cross. Bulked segregant analysis was used to find DNA markers (amplified with RAPD, microsatellite, exon-intron-based primers) associated with plant height.

In total, 589 DNA markers were screened in the bulks and/or in the parents. The most promising ones were screened in the population. Four markers were significantly ($P < 0.01$) associated with plant height (RAPD: OPR-06780; exon-intron-based marker: ET2280; SNP: SNP-BAMY; microsatellite: REMS1218).

Microsatellite REMS1218 showed the strongest association ($P < 0.0001$) with plant height. Since it was a dominant marker, it was impossible to distinguish between the short homozygous plants for the EM-1 allele and the short heterozygous plants, which would be necessary for breeding purposes.

Therefore, the REMS1218 sequence carrying the microsatellite was cloned and sequenced from the parents. Sequencing revealed one base difference (A/C) in an intron between EM-1 and Voima. Based on this difference an SNP marker was developed. Sequencing also showed that two different REMS1218 loci existed in Voima and EM-1. Both Voima loci had a 5 x GA repeat, but the EM-1 loci had a 5 x GA repeat and a 7 x GA repeat. The longer repeat was associated with the short growth habit. Neither the SNP nor the microsatellite marker alone could differentiate between all the genotypes, and therefore the results of SNP and microsatellite analyses were combined (REMS1218com). However, for a rye breeder, the SNP alone is an adequate tool to identify plants homozygous for the EM-1 allele and could be used in selection for the desirable growth habit in rye.

Fourteen markers were mapped that were linked to *Ddw1* or were previously localized on 5RL. Nine of the markers were arranged in two linkage groups, which both contained markers known to be located on 5R. The rest remained unlinked. A major QTL (LOD score 15.4) for plant height, which corresponds to *Ddw1*, was found on linkage group 2. The two populations segregating for short straw were pooled (containing 158 individuals together), and when the QTL analysis was repeated with the pooled data, the LOD score increased to 22.4. The QTL for plant height (*Ddw1*) was located between the markers WMS6 and REMS1218com, 13 cM from the latter.

5.5 Construction of a linkage map using a doubled haploid population (IV)

Three hundred and ninety-two polymorphic DNA markers were analyzed in the DH mapping population (the sprouting resistance population) derived from the Amilo DH x Voima DH cross. The linkage map was composed of new marker types in rye: SRAPs, IRAPs, and REMAPs, in addition to AFLP, microsatellite, and RAPD markers. The linkage map (with LOD values of 7 to 14) covered 281 loci with a length of 747 cM. Overall, 18 linkage groups (≥ 4 markers, ≥ 10 cM) were formed. All the seven rye chromosomes (some of them divided into two or three linkage groups; 13 linkage groups in total) could be identified with the previously mapped anchor markers (mainly rye microsatellites). The number of markers varied from 27 to 48 per identified chromosome. Five linkage groups (LG14-LG18) were unassigned to a chromosome.

Thirty five per cent of the mapped markers showed distorted segregation ($P \leq 0.01$) from the expected 1:1 ratio and were distributed on all chromosomes except 7R. Nearly 70% of the distorted markers contained alleles inherited from Amilo in excess. The most severely distorted region (18 markers in total, $P = 0.0001$) was detected on chromosome 6R: 9.3% of markers were distorted.

5.6 Finding a chromosomal region affecting *a*-amylase activity (IV)

One major QTL controlling *a*-amylase activity after 4 days of germination was found on the long arm of 5R. The QTL explained 16.1% of phenotypic variation. The QTL peak controlling PHS was located at the microsatellite loci SCM74, RMS1115, and SCM77.

6 DISCUSSION

6.1 Optimization of rye anther culture technique (I-II)

To optimize the rye anther culture technique, the effects of cold pre-treatment and heat post-treatment on anther culture, and especially on GP regeneration, were studied. These stress treatments have been used to increase green plant regeneration of other cereals (barley: Huang & Sunderland 1982, Powell 1988, Devaux et al. 1993, Hou et al. 1993, Ritala et al. 2001; oat: Kiviharju & Pehu 1998; triticale: Immonen & Robinson 2000; timothy: Touraev et al. 1996, Guo et al. 1999; wheat: Zheng 2003). Cold pre-treatment of varying durations has previously increased rye GP regeneration (Immonen & Anttila 1996, Deimling & Flehinghaus-Roux 1997, Immonen & Anttila 1999, Ma et al. 2004a), which was also observed in this study. Two to three weeks of cold pre-treatments were best for GP production in the lines or cultivars tested. The reason for the good GP production was improved regeneration efficiency from embryo-like structures (GRE) rather than better induction rate. Cold pre-treatment appears to maintain microspore viability and delay development, which increases embryogenesis (Shim & Kasha 2003, Zheng 2003, Shariatpanahi et al. 2006). In addition, it has been observed that the switch from the gametophytic to the sporophytic pathway can also be induced without any stress treatments (Indrianto et al. 1999, Ohnoutkovà et al. 2001). This was also recognized in the present study, but better results were obtained with stress treatments.

The combination of cold and heat treatment enhanced regeneration ability. However, GP production rate was genotype dependent, and anther culture response in the most recalcitrant cultivars did not improve. In addition, variable results for spontaneous doubling were observed: both enhancing and decreasing. In the parents (Amilo and Voima) of the sprouting resistance population the treatments decreased spontaneous doubling. Heat probably affected the pollen developmental stage and cell divisions and did not favor nuclear fusion and DH production (Smykal & Pechan 2000, Seguí-Simarro & Nuez 2008a). The effects of stress treatments on doubling rates vary also for

other cereals. Cold stress without heat increased spontaneous doubling in wheat and barley (Indrianto et al. 1999, Oleszczuk et al. 2006). Although heat can increase GP regeneration, it can reduce doubling rate (Oleszczuk et al. 2006), as shown in the present study. Spontaneous doubling rate in rye has been about 70 % (Deimling & Fehinghaus-Roux 1997), as was achieved in this study. According to Oleszczuk et al. (2006), no universal stress treatment can be applied to increase androgenetic response in plants. Highly efficient DH production requires a different optimal stress treatment for each genotype. In conclusion, heat treatment with a combination of cold stress is recommended for increasing GP regeneration for previously untested genotypes or those genotypes considered recalcitrant.

Various induction media have been applied in rye anther culture (Chu et al. 1975, Murashige & Skoog 1962, Wang & Hu 1984, Ouyang et al. 1989, Guo et al. 1999), but no universal medium has been discovered. According to the results of this study, GP production was clearly genotype, media, and heat dependent. Nonetheless, no significant effects of genotype and medium on callus induction or AP production were recorded, which might indicate that the same induction medium with optimal culture conditions could be used for all untested rye genotypes. To date, anther culture on 190-2 (Wang & Hu 1984) induction medium has produced the best results for rye in terms of green plant production (Immonen & Anttila 1999).

6.2 Benefits and weaknesses of doubled haploids in rye research and breeding (I-II)

When DHs are used in research and breeding it is important that they are sufficiently fertile and viable. Low survival rate of regenerated plants was observed in this study, which means that even ten times more anthers than were eventually needed, based on the anther culture efficiency, should be cultured to get enough fertile DH plants for mapping studies. During DH production, variable fertility, viability, and sterility of DHs were detected as previously reported (Hoffman & Wenzel 1981, Friedt et al. 1983, Deimling & Flehinghaus-Roux 1997, Immonen & Anttila 1999). Ahokas (1980) reported that cultivar Voima can include male sterile genotypes, which could have affected fertility of some DHs. Fertility was much lower in crossings within DH than between DH populations, probably due to the self-incompatibility system of rye (Lundqvist 1956, Egorova & Voylokov 1998). Both cultivars Amilo and Voima have cultivar Kungs II in their pedigree, which might explain the low fertility among DHs derived from the Amilo DH x Voima DH cross.

In addition to self-incompatibility, other factors influence fertility and viability of DHs. Partial sterility due to aneuploidy (Hu & Kasha 1997) or inbreeding depression, especially in a small population (Dewan et al. 1998, Willi et al. 2005), might have an effect on reproductive capacity. Colchicine-treated

haploid plants usually have more mutations or other abnormalities than spontaneous DHs (Logue 1996). Moreover, morphological differences (variation in plant growth habit, plant height, spike length, spike number, seed appearance) were found in DHs and colchicine-treated haploids in this study. Due to its unwanted effects, colchicine treatment was omitted when the DH mapping population was created. Variation in morphology of DHs has been described in other cereals (Logue 1996). In addition to mutations caused by anther culture, these changes might be a consequence of chromosomal alterations, defects in cytoplasmic organelles, disruptions of signal transduction between nucleus and cytoplasm in microspores, and residual heterozygosity of parents (Khush & Virmani 1996, Mix et al. 1978). Muranty et al. (2002) reported that about 10% of spontaneous hexaploid wheat plants were heterozygous at one to three loci. In the present study, heterozygosity in a few DHs at some microsatellite loci (results not shown) was found, which might be a consequence of unreduced gametes (produced by second division restitution), aneuploid trisomic gametes, or transposition events. Those plants were excluded from the mapping procedure.

DHs shorten the time needed for breeding because desired genotypes can be fixed in a single generation, and DHs help to purify breeding lines of unwanted alleles (Forster & Thomas 2005, Forster et al. 2007). In hybrid breeding and synthetic variety breeding, DHs represent a tool to create lines with the best genetic combinations. DHs can be produced throughout the year so that a new generation for the following spring can be created in a greenhouse during the winter, which is especially important in the northern areas. In addition, DHs enable field trials in multiple environments for self-pollinating species. In the present study, the development of DH mapping populations took about three years, which is about one year more than needed for a self-pollinating species or for spring type cultivars that need no vernalization. However, creation of inbred or recombinant inbred lines under field conditions takes over five generations (> 5 years). In out-crossing species, DHs help to locate recessive lethal factors that can be hidden in heterozygous populations. In addition, they help in mutation and transformation studies. Furthermore, due to homozygosity of DHs, mapping studies are simplified (Forster & Thomas 2005), which means that dominant markers are as efficient as co-dominant ones. In conclusion, DHs of out-crossing rye can be used on a small-scale for research and breeding, but low efficiency and fertility must be borne in mind when new studies are designed.

6.3 The use of DNA markers and linkage mapping in rye research and breeding (III-IV)

In this study, to the best of our knowledge, the first linkage map was constructed using a DH mapping population from out-crossing rye. The map

(281 loci) covered all the seven rye chromosomes (divided into 13 linkage groups), in addition to five linkage groups that could not be identified. The large number of linkage groups in the map reflects the gaps inside some rye chromosomes. However, large gaps are also present in other rye linkage maps (Senft & Wricke 1996, Saal & Wricke 1999, Ma et al. 2001, Milczarski et al. 2007). Ma et al. (2001) reported polymorphic markers (21% of all polymorphic markers) not mapped to any linkage group, as was the case in this study (28% of all polymorphic markers were unlinked). The population size in the present mapping study was quite low, 89 individuals, due to low survival rate and fertility of DH plants.

About one third of the mapped markers showed segregation distortion, which has also been reported in other studies of rye (Philipp et al. 1994, Wanous et al. 1995, Loarce et al. 1996, Senft & Wricke 1996, Korzun et al. 1998a, 2001, Ma et al. 2001, Hackauf & Wehling 2002, 2003, Bolibok et al. 2007, Hackauf et al. 2009) and triticale (González et al. 2005). The distortion might have little effect on map length if there are missing values and/or errors in the map. In the presence of distorted segregation ratios, missing values might lead to shorter map lengths for more widely separated markers (Hackett & Broadfoot 2003). Probable reasons for the distortion in the present map are the anther culture technique itself (Foisset & Delourme 1996), self-incompatibility (Lundqvist 1956), and inbreeding depression (Keller & Waller 2002). In maize, it has been proposed that distorted regions might contain selective genes actively involved in androgenesis (Dufour et al. 2001). In rye, chromosomes 1R, 3RL, 4R, 5R, and 6RS have been reported to be associated with anther culture response (Martinez et al. 1994, Große et al. 1996). Gonzáles et al. (2005) found QTL associated with embryo induction and GP percentage on chromosomes 4R and 3R in triticale, respectively. In addition, QTL associated with GP regeneration were found on chromosomes 2AL, 2BL, and 5BL of wheat and on chromosomes 2H, 3H, 5H, 6H, and 7H of barley (Bregitzer & Campbell 2001, Torp et al. 2001, Muňoz-Amatriaín et al. 2008). A better-responding parent in anther culture usually has predominant alleles in distorted markers (Foisset & Delourme 1996). In the present study, most of the distorted markers had alleles inherited in excess from Amilo. However, cultivars Amilo and Voima and their F_1 plants had almost equal GP regeneration rates. Therefore, genes affecting anther culture response are not the only reason for segregation distortion of markers in the study.

Self-incompatibility loci (*S, Z,* and *T*) are located on chromosomes 1R, 2R, and 5R of rye (Wricke & Wehling 1985, Gertz & Wricke 1989, Voylokov et al. 1998, Egorova & Voylokov 1998, Hackauf & Wehling 2005), and all these chromosomes contained distorted markers in the present study. In addition to self-incompatibility loci, fertility restorer genes have been found on chromosomes 1RS, 3RL, 4RL, 5R, and 6R in rye (Miedaner et al. 2000), which might also affect the viability of DHs and thus cause distortion. Furthermore, both a hybrid sterility gene and a gametophyte gene can cause segregation distortion (Harushima et al. 1996). A hybrid sterility gene affects the abortion of a gamete or zygote, and a gametophyte gene expressed in a haploid gamete is involved in gametophytic competition due to genetic differences in pollen. In

wheat, one hypothesis for deviation of markers is existence of a pollen killer (*Ki*) gene in the distorted region (Groos et al. 2002).

Height reduction in rye is achieved using dwarfing genes. The most actively used dominant dwarfing gene, *Ddw1*, is located at the distal end of chromosome 5RL. One objective of this thesis was to identify PCR-based DNA markers closely linked to *Ddw1*. The closest markers previously identified are the wheat RFLP marker *Xwg199* (for *Hp*, hairy peduncle gene) and the isozyme marker *β-amy-R1*. The genetic distances are 5.6-5.9 cM between *Xwg199* and *Ddw1* and 11.5-12.7 cM between *Ddw1* and *β-amy-R1*, respectively (Börner et al. 1999a, Korzun et al. 1996). In the present study, the best marker associated with plant height was a combination of the microsatellite REMS1218 and the SNP marker created from it (REMS1218com). The combined marker was located 13 cM from *Ddw1*.

There are differences in map distances near *Ddw1* on 5RL between the present map and that of Korzun et al. (2001). According to Ma et al. (2001) and Tan & Fornage (2008), genetic distances in different maps can vary due to genetic differences between parents, environmental differences (e.g. temperature), population sizes, marker densities, and different mapping functions. The map distances from using the Haldane function are longer than those from using the Kosambi function (Tan & Fornage 2008), which partly explains why the marker distances near *Ddw1* are longer in the present map than in the map of Korzun et al. (2001).

A low frequency of polymorphism was observed on the distal end of 5RL, which might be one reason why a marker closer to *Ddw1* was not found. The ancestral origin of this chromosomal end might explain the low polymorphism, which has also been reported for chromosome 4DL of wheat (Milla & Gustafson 2001). In a basic *Triticeae* genome, the first reciprocal translocation event occurred between the distal end of the ancestral 4L and 5L chromosomes (Devos et al. 1993b, Börner et al. 1998a), which means that the present rye chromosome 5RL has parts of the former *Triticeae* 4L chromosome, and the present rye chromosome 4RL of the former *Triticeae* 5L chromosome. According to the known co-linearity between rye 5RL, wheat 5AL (under the translocation break point), and rice R3S, (Devos et al. 1993b, Moore 1995, Rice chromosome 3 sequencing consortium 2005, Hackauf et al. 2009), the QTL region for *Ddw1* in rye seems to be situated in a highly conserved region and is therefore protected by a low level of polymorphism. Another reason for not finding a closer marker might be the BSA method. The 'short' bulk likely contained heterozygous individuals for *Ddw1*, which prevented finding marker alleles for the tall growth habit. The REMS1218 sequence that is located near *Ddw1* had a significant genetic identity with oxalate oxidase–like proteins (paper III), and the highest sequence similarity was found on the short arm of chromosome R8 in rice (results not shown in paper III). Therefore, it might not be a coincidence that many QTL controlling plant heights on R8S have been recognized in rice (Zhang et al. 2006). Hackauf et al. (2009) also found marker sequences from rye chromosome 5R located on rice chromosome R8. It was also reported that two

different REMS1218 loci existed in Voima and EM-1. This duplication event might be of an ancient origin during the divergence of cereals (Xu et al. 2005).

PHS is a serious problem worldwide in cereals. The association of dwarfing genes with sprouting resistance was reported previously (wheat: Flintham & Gale 1982, 1988, Gale 1989, Mrva & Mares 1996, Mares & Mrva 2008; rye: Korzun et al. 1996, Masojć et al. 1999, 2007). Lodging is more evident in a taller cultivar and it easily increases sprouting damage due to favorable conditions in spikes near the ground. Other relationships between plant height and PHS also exist: e.g. GA 20-oxidase, which is the main enzyme in feedback regulation of GA biosynthesis (Huttly & Phillips 1995, Hedden & Kamiya 1997, Richards et al. 2001, Sasaki et al. 2002), is involved with both traits, plant height (Spielmeyer et al. 2002) and PHS (Li et al. 2004). A defective *GA 20-ox* gene reduces height in rice and the same gene is a candidate gene controlling seed dormancy or PHS in barley.

In this study, susceptibility to sprouting was measured as *a*-amylase activity of a grain. One major QTL controlling *a*-amylase activity was found at the marker loci SCM74, RMS1115, and SCM77 on 5RL. Previously, several genomic regions affecting *a*-amylase activity, visible sprouting, or seed dormancy (measured as germination percentage) were reported on chromosomes 5 in rye, wheat, and barley (Table 2). The QTL found in the present study might be the same as the QTL for *a*-amylase activity found earlier on chromosome 5RL (Masojć et al. 2007, Masojć & Milczarski 2009). However, the comparison is only based on the location of one common DNA marker, the microsatellite SCM77, in that region. The structural gene *a-Amy-3* has been mapped to the same region as the QTL for PHS and *a*-amylase activity, on the long arm of 5R in rye (Masojć & Gale 1991). Because *a-Amy-3* is expressed in immature seeds, it was suggested that the structural gene itself might not have a strong influence on PHS but other neighboring ABA or dormancy related genes might have (Zanetti et al. 2000). In rye, due to many QTL controlling *a*-amylase activity, it has been suggested that there is a complex network of genes associated with ABA and GA synthesis and their regulation (Masojć & Milczarski 2009). Ullrich et al. (2008) reported that due to several QTL clusters associated with dormancy or PHS there might be one gene that affects more than one trait and/or several genes that are closely linked. This means that the real number of genes associated with PHS or dormancy is greater than the number of QTL identified. Masojć and Milczarski (2009) pointed out that breeders should use major genes/QTL controlling both PHS and *a*-amylase activity in crop improvement to develop sprouting resistant cultivars.

Many QTL controlling sprouting have been found (Table 2). In the present study, only one QTL affecting *a*-amylase was detected. The low power of QTL detection could be due to a small population size, the kinship of the parents of the DH mapping population (both have the same cultivar, Kungs II, in their pedigree), the inadequate number of DNA markers in the map, and the biased structure of the progeny. In addition, due to the out-crossing nature of rye, the DH population was test-crossed with susceptible Riihi to get grains for *a*-amylase activity measurements. The number of seeds from the crosses was

quite low and seeds were partly unsuitable for the activity measurements, which decreased the sample volume and therefore the power of the QTL mapping.

Genetic maps enable breeders to use markers near the genes of agronomic and economic importance. Particularly by using MAS, new breeding lines with the best possible gene combinations can be found efficiently. However, from the breeder's point of view, it should be carefully considered whether investments in these new technologies are economically attractive. The economic value of a molecular marker for the trait of interest is determined by the aims of the breeding program, the closeness of the linkage between the marker and the desirable trait, the possibility for phenotypic screening, and the costs of phenotypic screening compared with MAS (Brennan & Martin 2007).

7 CONCLUSIONS

The production of domestic rye for industrial use has decreased in Finland due to farmers' unwillingness to cultivate the crop. Reasons include the unprofitableness of cultivation and low yield stability. In this study, as a part of rye research program 'Increasing efficiency of rye breeding and cultivation in the North', the aim was to use DHs for gene mapping and to create biotechnological tools for quality improvement in rye.

The first goal was to produce DHs for research and breeding purposes. Because cold pre-treatment together with heat post-treatment had not been previously used for rye anther culture, it was studied to find the best technique to improve green plant regeneration. Three weeks of cold-treatment appeared to improve green plant regeneration, but there was variation among rye cultivars and breeding lines. Also cold treatment in combination with heat treatment increased green plant regeneration. However, the spontaneous doubling rate was decreased when heat treatment was applied to the parent cultivars Amilo and Voima. This was the principal reason why heat treatment was not used for anther culture when creating the mapping population segregating for PHS. This decision was also supported by the observation that colchicine-treatment for chromosome doubling was not useful because it reduced viability and fertility of anther-culture-derived haploid plants.

The goals of Finnish rye breeding have been to decrease straw length and susceptibility to PHS. In the present study, two populations were created, one segregating for short straw (EM-1 x Voima) and the other for sprouting resistance (Amilo x Voima). The parents of the first population were DHs, but the population itself consisted of the progeny of an F_1 x F_1 cross due to too few seeds being produced from the parent crosses for anther culture. The SNP marker linked to the dominant dwarf 1 gene (*Ddw1*) was created from the microsatellite REMS1218. The SNP marker can be used in MAS for breeding lines having EM-1 in their pedigree. This marker has been used to purify breeding lines of the recessive allele for longer straw in the Finnish plant breeding company, Boreal Plant Breeding Ltd.

In the second mapping population, both the parents and the progeny were DHs. However, the efficiency of producing DHs was quite low due to unsatisfactory survival rate of anther-derived plants and low fertility of DHs. Because of the out-crossing nature of rye, the DH mapping population was test-crossed to PHS-susceptible cultivar Riihi to get grains for *a*-amylase measurements to gauge PHS. The first rye linkage map using DHs was constructed. The map consisted of 281 markers, of which some were new DNA marker types, and was 747 cM long. All the seven rye chromosomes were identified using anchor markers, although some of the chromosomes were divided into two or three linkage groups. Segregation distortion of markers was clearly seen in almost every chromosome. One QTL controlling *a*-amylase activity was found on 5RL. To date, it is not clear whether the QTL found is orthologous to the other *a*-amylase activity, sprouting, or dormancy QTL reported on chromosome 5RL or if it is a new previously unrecognized QTL.

Rye is an important crop in the northern parts of Europe, Belarus, and the Russian Federation. However, areas sown to rye have decreased in Finland. The profitability of rye cultivation needs to be improved so that farmers are willing to cultivate it and thereby increase national self-sufficiency in rye. In this thesis, biotechnological tools such as DH technology and DNA marker methods were introduced to study their usefulness for increasing rye quality in Finland. New DNA markers represent a possibility for use in other rye studies as well as in wheat and triticale studies. In addition, for a breeder this study offers new tools, the DNA markers affecting plant height and PHS that can be used in purity control and in MAS for cultivar improvement, particularly in Finland.

Acknowledgements

This study was carried out at the Department of Crops and Biotechnology, Plant Production Research (later Biotechnology and Food Research, Plant Genomics), MTT Agrifood Research Finland, and was a part of the comprehensive rye research project 'Increasing efficiency of rye breeding and cultivation in the North' funded by the Finnish Ministry of Agriculture and Forestry during 1999-2003.

I wish to express my warmest thanks to my kind and patient supervisor Dr Pirjo Tanhuanpää who never refused helping me during this study. I also want to thank Dr Sirkka Immonen for her valuable advice and encouraging support. In addition, I want to highlight her successful work in rye anther culture before this study.

Plant Breeder Dr Simo Hovinen, his assistants Eliisa Peltomäki and Mervi Suni, plant breeder Esa Teperi, and Dr Kirsi Peltoniemi from Boreal Plant Breeding Ltd. are warmly thanked for their co-operation during this study, not forgetting the shared seminars and trips abroad.

I am very grateful for Reviewers, Professor Sven Bode Andersen and Dr Helmi Kuittinen, for their critical reading and valuable comments of the thesis. I also want to thank Professor Jari Ylänne for his advice during the writing phase of this thesis. Dr Jonathan Robinson is thanked for the language revision of the thesis. Professor Alan Schulman and Roy Siddall are acknowledged for their language revisions of the manuscripts.

I am very grateful to Professor Aarne Kurppa, Director of Plant Production Research, and Dr Eeva-Liisa Ryhänen, Director of Biotechnology and Food Research, for the working facilities in MTT. Boreal Plant Breeding Ltd. is also thanked for the greenhouse facilities and all the seed material provided for this study.

I want to express my very special thanks to Leena Ramstedt, Marja-Riitta Arajärvi, Marika Niskanen, Maarit Tuovila, Sirpa Moisander, and Leena Lohermaa, for their excellent technical assistance during this study. Essi Haklin is warmly thanked for RAPD and BSA analyses. MSc Maria Erkkilä (diss.) is especially thanked for the β-amylase primers and for practical advice during this thesis. Yrjö Karppinen is thanked for his efficient and skilful assistance in the greenhouse. Anneli Virta and Johanna Rusi are acknowledged for their work at the laboratory of genomics. Christian Eriksson and Jaakko Tanskanen are thanked for the assistance in statistical analyses and genome searches. Pirkko Rinne (a mother figure of Plant Genomics) and Ritva Koskenoja are warmly thanked for their assistance in reporting the results, making seminars, and posters.

I also want to express my special thanks to all other staff at Plant Genomics (KGE). Without you all, I could not pass this thesis. Special thanks also go to Dr Marjo Serenius, my roommate, for support and encouragement. In addition, I want to emphasize, how warmly I reminisce about the time spent at the Department of Cell Biology, University of Jyväskylä, in 'the old garage' during my master thesis.

The Finnish Ministry of Agriculture and Forestry, Niemi Foundation, Heikki and Hilma Honkanen Foundation, the Finnish Academy of Sciences (project number 112053), and Finnish Cultural Foundation are gratefully acknowledged for financially supporting this study.

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I also want to express my special thanks to my former workmates Timo Turpeinen, Airi Tauriainen, and Jaana Laurila for their support during this study. I am also glad that there is life outside of work. Special thanks belong to my student friends Aurora, Hande, Salla, and Silja for their mental support during these years. I also want to thank my childhood friends Johanna and Leena, friends forever, who have helped me through thick and thin.

Last, but not least, I am grateful to my mom who has supported and encouraged me during this thesis. My husband Janne is warmly thanked for his love and for his support during these years. He is especially thanked for taking care of our son Aapeli. Finally, tiny thanks also belong to my belly kicker, 'Pikku-Ukolle', who reminded me to hurry and to do this work before the next 'project.'

Jokioinen, March 2009

Teija Tenhola-Roininen

YHTEENVETO (RÉSUMÉ IN FINNISH)

Rukiin kaksoishaploidit - tuotto ja käyttö kartoituksessa

Ruista on viljelty Suomessa monien vuosikymmenien ajan, ja ruisleipä on kuulunut suomalaisen ruokapöytään melkeinpä itsestäänselvyytenä. 'Ruista ranteeseen', kuten sanonta kuuluu, ei ole pelkästään sukupolvelta toiselle periytyvä sananparsi, vaan rukiin kuitujen on jo tieteellisesti todistettu edistävän terveyttä. Kuiduilla on suotuisia vaikutuksia suoliston toimintaan, ja ne todennäköisesti ehkäisevät esimerkiksi rinta- ja eturauhassyöpää ja alentavat sepelvaltimokuolleisuutta. Eniten kuitua suomalaiset saavat ennen kaikkea täysjyväruisleivästä.

Rukiin käyttö leipäviljana on kuitenkin vähentynyt toisen maailmansodan jälkeen, ja vehnä on korvannut osittain rukiin ruokapöydässämme. Rukiinviljely on myös vähentynyt huonojen hehtaarisatojen ja alentuneiden EU-tukien myötä. Rukiin sadon laatu on riippuvainen sääolosuhteista, mistä syystä ruis on hyvin riskialtis vilja. Syksyllä kylvettävää syysruista vaurioittaa talvella lumihome ja satokauden aikana syyskesällä rankat sateet lakoonnuttavat pitkää ruista ja edistävät tähkässä tapahtuvaa jyvien itämistä (tähkäidäntää) ennen sadonkorjuuta. Leipomoteollisuus käyttää rukiin leivonnan yhtenä laatukriteerinä sakolukua, joka kertoo kuinka paljon *a*-amylaasi-entsyymi on pilkkonut tärkkelystä sokereiksi vesi-jauhoseoksessa. Mitä korkeammat entsyymin aktiivisuudet ovat, sitä alhaisempi on sakoluku ja sitä liukoisempaa on vesi-jauhoseos. Ruisjauhosta, jossa on alhainen sakoluku (60 - 80), leipomot pystyvät valmistamaan pääasiassa mämmiä ja hapankorppuja. Hapanleivän ja happamattoman leivän valmistuksessa käytetään sakoluvultaan noin 120 ja näkkileivän valmistuksessa sakoluvultaan 120-200 olevaa ruista.

Ruislajikkeiden ominaisuuksia on pyritty parantamaan pohjoisille viljelyalueille sopiviksi. Yhtenä tärkeänä jalostustavoitteena pidetään suomalaisiin olosuhteisiin soveltuvan lyhytkortisen ja tähkäidännänkestävän ruislajikkeen kehittämistä. Tässä väitöskirjatyössä, osana Maa- ja metsätalousministeriön rahoittamaa yhteistyöprojektia 'Rukiin jalostuksen ja viljelyn tehostaminen pohjoisilla viljelyalueilla, 1999-2003', kehitettiin rukiinjalostajalle valinnassa käytettäviä apuvälineitä, DNA-merkkejä, joiden avulla pystytään tunnistamaan nopeasti ja vaivattomasti jalostuksellisesti hyödylliset lyhytkortiset ja tähkäidännän kestävät kasvit. Molekyylibiologisten menetelmien lisäksi tutkimuksessa käytettiin ponsiviljelytekniikkaa, jonka avulla tuotettiin kasveja rukiin ponsien sisältämistä siitepölyhiukkasten esiasteista, joista kehittyi joko haploideja tai spontaaneja kaksoishaploideja kasveja. Kaksoishaploidit kasvit ovat homotsygoottisia (samaperintäisiä) eli sisältävät siitepölyhiukkasen perimän kahdentuneena, kun taas haploideissa kasveissa on vain yksi kromosomisto, joka kahdennetaan esimerkiksi kemiallisen aineen kolkisiinin avulla. Kaksoishaploidien kasvien etuna on ominaisuuksien ja DNA-merkkien helpompi tulkinta, koska piilevät eli resessiiviset ominaisuudet eivät jää vallitsevien eli dominoivien

ominaisuuksien varjoon. Näiden menetelmien avulla rukiin lajikejalostusta voidaan nopeuttaa 3-4 vuodella.

Rukiin ponsiviljelytekniikkaa pyrittiin parantamaan stressikäsittelyillä. Tähkien kylmäkäsittely yleisesti ottaen lisäsi ponsiviljelyllä tuotettujen vihreiden kasvien määrää – kolmen viikon käsittelyn ollessa paras. Kylmä- ja lämpökäsittelyn yhdistelmä lisäsi vihreiden kasvien tuottoa ja spontaanien kaksoishaploidien määrää joillakin ruislinjoilla/lajikkeilla, mutta sillä ei ollut vaikutusta huonoimmin ponsiviljelyssä toimivien lajikkeiden tuottokykyyn. Joillakin linjoilla/lajikkeilla lämpökäsittely selvästi vähensi kaksoishaploidien muodostumista. Tutkimuksessa havainnoitiin myös spontaanien ja kolkisiinilla kahdennettujen kaksoishaploidien kasvien hedelmällisyyttä, elinvoimaisuutta ja morfologiaa. Spontaanit kaksoishaploidit kasvit olivat selvästi hedelmällisempiä kuin kemiallisesti kolkisiinilla kahdennetut kasvit. Ponsiviljelyllä tuotettujen kasvien kasvutapa vaihteli normaalista ja ruohomaisesta hyvin kitukasvuiseen kasvutapaan. Kaksoishaploidien kasvien risteytyksistä saadut siemenet saattoivat olla kurttuisia tai kehittymättömiä. Yleisesti ottaen ponsiviljelyllä tuotettujen kasvien kuolleisuus oli korkea, mikä täytyy ottaa huomioon suunniteltaessa rukiin kaksoishaploidien kasvien käyttöä myöhemmissä tutkimuksissa.

Sopivien lyhytkortisuuteen tai tähkäidännänkestävyyteen liittyvien DNA-merkkien etsimiseksi tuotettiin kaksi erillistä ruispopulaatiota, joissa nämä ominaisuudet vaihtelivat. Lyhytkortisuuspopulaatio (EM-1 x Voima, lyhyt x pitkä) koostui F_1 x F_1 jälkeläistöstä, joiden vanhemmat olivat kaksoishaploideja. EM-1 mutantti sisältää perimässään dominoivan kääpiögeenin (*Ddw1*), joka lyhentää kasvin pituutta. Tähkäidäntäpopulaatiossa (Amilo x Voima, kestävä x altis) sekä vanhemmat että itse populaatio olivat kaksoishaploideja.

Tähkäidännänkestävyyteen liittyvien DNA-merkkien löytämiseksi tehtiin geenikartta, mutta lyhytkortisuuteen liittyvien merkkien etsinnässä käytettiin ns. bulkkimenetelmää (BSA, bulked segregant analysis), jossa populaation lyhimpien ja pisimpien kasvien DNA:t yhdistettiin. DNA-merkki, joka on erilainen (monimuotoinen) näissä bulkeissa, saattoi liittyä korrenpituuteen. Tällä menetelmällä ei kuitenkaan löydetty lähellä kääpiögeeniä sijaitsevaa DNA-merkkiä. Tutkimuksessa testattiin myös muita tunnettuja 5R-kromosomissa sijaitsevia DNA-merkkejä. Yksi näistä (REMS1218-mikrosatelliitti) liittyi selvästi pituuteen. Tästä kehitettiin myös SNP (single nucleotide polymorphism) – merkki, jonka avulla rukiin jalostaja voi valita homotsygootit lyhytkortiset rukiit 13%:n virhemarginaalilla jalostusaineistosta.

Geenikarttaa tehtäessä etsittiin ensiksi DNA-merkkejä, jotka olivat monimuotoisia populaation vanhemmissa, ja tämän jälkeen näillä merkeillä analysoitiin koko populaatio. Geenikartta koostui uusista, aikaisemmin ruistutkimuksissa käyttämättömistä (IRAP, REMAP, SRAP) ja näiden lisäksi jo muissa ruistutkimuksissa käytetyistä DNA-merkeistä (AFLP, mikrosatelliitit, RAPD). Itse tähkäidäntä määritettiin jyvien *a*-amylaasiaktiivisuuksien perusteella, mikä korreloi käänteisesti sakoluvun kanssa. *a*-Amylaasi–entsyymi pilkkoo jyvän tärkkelystä pienemmiksi sokereiksi, joita kasvi tarvitsee itääkseen.

Tähkäidäntäpopulaation avulla koottiin yhteensä 281 DNA-merkkiä sisältävä ruisgeenikartta (pituudeltaan 747 cM). Tämä rukiin geenikartta on ensim-

mäinen maailmassa, jonka laadinnassa on hyödynnetty ristipölytteisen rukiin kaksoishaploideja kasveja. Kartassa jokainen rukiin seitsemästä kromosomista pystyttiin tunnistamaan jo aikaisemmin paikoitettujen ns. ankkurimerkkien avulla. Osa kromosomeista jakaantui kahteen tai kolmeen kytkentäryhmään. Yksi tähkäidäntään liittyvä geenialue (QTL, quantitative trait locus) löytyi kromosomin 5R pitkästä varresta. Jalostaja voi käyttää tällä alueella sijaitsevia DNA-merkkejä (SCM74, RMS1115 ja SCM77) hyväkseen merkkiavusteisessa valinnassa etsiessään tähkäidännänkestäviä ruislinjoja.

Ruis on tärkeä viljelykasvi Pohjois-Euroopassa, Venäjällä ja Valko-Venäjällä. Suomessa rukiin omavaraisuus on kuitenkin laskenut, ja tällä hetkellä yli puolet rukiista tuodaan ulkomailta. Meillä niin tunnettu aito suomalainen ruisleipä ei enää olekaan täysin suomalaista. Tässä väitöskirjatutkimuksessa käytettiin bioteknisiä menetelmiä luomaan jalostusta helpottavia työvälineitä rukiin laadun parantamiseksi ja uusien lajikkeiden kehittämiseksi. Löydettyjä DNA-merkkejä voidaan käyttää sekä jalostuslinjojen puhdistuksessa että valinnassa etsittäessä lyhytkortisia ja tähkäidännänkestäviä ruislinjoja. Lisäksi koottu rukiin geenikartta ja siinä oleva uusi DNA-merkkitieto helpottavat muita ruis-, vehnä- sekä ruisvehnätutkimuksia.

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