Stellingen

1. The leaf wax layer on *Hordeum chilense* does not contribute to nor hampers the orientation of the barley leaf rust germ tubes.

This thesis
Contra: Lewis and Day (1972), Wynn and Staples (1981)
Rubiales and Niks (1996) and Jenks and Ashworth (1999)

2. The role of cuticular wax in preventing directly or indirectly the entrance of stoma penetrating fungi has been overlooked or only hypothesised.

Martin and Juniper (1970), Jenks and Ashworth (1999)

3. The degree of AFLP polymorphism between sets of accessions cannot be used to conclude about species delimitations.

This thesis

4. The polygenic nature of a resistance does not imply a more durable effectiveness of that resistance.

5. When considering the introgression of QTLs controlling a certain trait, attention must be given not only to the percentages of explained phenotypic variability but also to the additive effects of the detected QTLs.

6. What we observe is not nature itself, but nature exposed to our method of questioning.

Werner Heisenberg

6.348. Everything is vague to a degree you do not realise till you have tried to make it precise.

Bertrand Russell

7. Few things are as healthy, when you find an intellectual obstacle, as giving a holiday to the problem or to yourself.

António Damásio

8. "...you will cross from place to place in the unknown worlds, and among the known worlds you will be an enchanted nomad."

La Tia Teresa (The gypsy magic)

Stellingen behorend bij het proefschrift van Maria Carlota Morais e Cunha Vaz Patto: The genetics and mechanism of avoidance of rust infection in *Hordeum chilense*. Wageningen, 8 mei 2001.

The genetics and mechanism of avoidance of rust infection in *Hordeum chilense*

Maria Carlota Morais e Cunha Vaz Patto

Promotoren: Prof. Dr. Ir. P. Stam
Hoogleraar in de plantenveredeling
in het bijzonder de selectiemethoden
en resistentieveredeling

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The genetics and mechanism of avoidance of rust infection in *Hordeum chilense*

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman
In het openbaar te verdedigen
op dinsdag 8 mei 2001
des namiddags te vier uur in de Aula

ISBN 90-5808-397-7

Contents

1

General introduction

Cereal rusts- a persistent threat

Cereal rusts are spread world-wide and produce frequent severe epidemics with substantial annual losses. Various species or special forms of *Puccinia* attack all cultivated and wild grasses, including all small grains, corn, and sugarcane. They are among the most serious diseases of cultivated plants, resulting in losses equivalent to about 10% of the world grain crop per year (Agrios, 1997).

Cereal rust fungi are obligate parasites or biotrophs, belonging to the order Uredinales. The epidemic phase of these fungi is represented by the urediospores. Urediospores germinate and produce germ tubes that grow, directed by the leaf surface, towards stomata where they cease growth and develop appressoria directly over the stomatal openings. Subsequently, an infection peg grows from the appressorium and penetrates the stomatal aperture. A substomatal vesicle is formed from continued growth of the infection peg within the substomatal cavity, which in turn gives rise to one or more infection hyphae. Further differentiation of the apices of these infection hyphae occurs when they contact mesophyll cells and form haustorial mother cells. The haustorial mother cell produces a penetration peg that penetrates the cell wall, and forms a haustorium. Nutrients are taken up via the contact zone between the plasmalemma and haustorium cell wall. Many more haustoria are formed and the fungus grows further intercellularly forming a network of hyphae. During colonisation, the fungus starts forming sporogenic tissue resulting in the production and release of new urediospores (Hoch & Staples, 1987).

Cereal rusts are among the most harmful plant pathogens as described before. However on the plant side there is a great diversity of mechanisms of defence against potential consumers what opens good perspectives for plant protection breeding.

Defence mechanisms- a world of diversity

The defence mechanisms utilised by the plants can take many different forms, ranging from passive mechanical or preformed chemical barriers, which provide non-specific protection against a wide range of organisms, to more active host-specific responses that provide host or varietal specific resistance (Jackson & Taylor, 1996).

According to Parlevliet (1981), the plant defence mechanisms may be grouped into three different classes: Avoidance, resistance and tolerance.

Avoidance, antixenosis for the entomologists, reduces the probability of contact between potential consumers and plants. It acts before establishment of intimate contact between host and parasite, including feeding and oviposition (Niks et al., 1993). Avoidance mechanisms are often of a morphological nature and are directed particularly to animal parasites and herbivores (Niks et al., 1993). This group of consumers combines sense organs with the capacity to move actively to search for food substrates. Avoidance to pathogens is rare. An example is the upright plant habit, which reduces the spore deposition in cereals (Gasowski, 1990).

Resistance is the ability of the plant to reduce the growth and/or development of the parasite after contact has been initiated or established (Niks et al, 1993). Resistance mechanisms operate especially against parasites and pathogens, rather than large herbivores (which do not establish an intimate contact with the plant). Resistance may be qualitative or quantitative. The resistance type most frequently deployed in plant breeding is characterised by a hypersensitive reaction (HR), controlled by major gene(s). The resistance genes interact in a gene-for-gene mode with avirulence genes in the pathogen to give an incompatible reaction (Flor, 1956). The great disadvantage of this type of resistance is that it is often ephemeral as the pathogen can develop virulence by a loss-of-function even by single base pair mutation of the avirulence gene. The incompatible reaction is not elicited any more and the compatibility/pathogenicity is restored (Agrios, 1997).

Tolerance is the mechanism by which plants reduce the extent of damage per unit parasite present (Niks et al., 1993). Tolerance does not reduce levels of infection, but there is little damage in terms of yield reduction or quality loss. It may be based on recovery from damage, which is important especially if the plant is exposed temporarily to the consumer (herbivores).

Though a great range of different potential defence mechanisms exist in nature, lack of durability has been a constant concern in breeding for resistance to rust fungi due to genetic adaptation of the pathogen.

Is it durable?- pertinent doubt in cereal breeding to rust fungi

The durability of disease resistance is affected by the evolutionary potential of the pathogen population (McDonald, 1997). The two most important parameters on the model of pathogen evolution are reproduction/mating system and gene/genotype flow. Pathogens that possess a mixed reproduction system, with one sexual cycle per growing season and asexual reproduction during the epidemic phase and a high potential for gene flow (wind dispersed spores) pose the greatest risk for breaking down the effectiveness of resistance genes (McDonald & Linde, 2000). Rust fungi are among these quickly evolving pathogens.

Plant protective mechanisms depending on many genes and/or that by their partial expression impose less selection pressure, face such a mutable pathogen with a potentially extended durability.

In the search for possibly durable types of defence mechanisms to rust fungi, it is useful to investigate the different phases of the infection process on various host genotypes to identify defence mechanisms that interfere with one or more stages of the infection process. Different cultivar-pathogen combinations with the same infection efficiency may inhibit growth of the rust fungus at different phases of the infection process. It is presumed that different genes determine inhibitions at different phases (Zadoks & Schein, 1979). The "components analysis" method (Zadoks, 1972) is aimed at the resolution of plant defence into components like spore germination, penetration (appressoria formation), colonisation (vesicles, hyphae and colonies formation), sporulation, latent period, infectious period, etc. (Zadoks & Schein, 1979). In a study on resistance components of *Hordeum chilense* to rust fungi, Rubiales and Niks (1992a) found variation among plant genotypes in the level of early inhibition of the infection process. Up to seven times fewer appressoria were formed on certain *H. chilense* accessions (H7, H17 and H47) than on the remaining accessions (H1 and H12).

Hordeum chilense– alternative source of defence mechanisms

Screening of germplasm may result in the discovery of alternative non-HR, quantitative, hopefully more durable defence mechanisms. Related species or related genera may be of great value in this search, whether they are host or non-host species.

Hordeum chilense Roem. & Schult. is a native South American diploid (2n=14), perennial, wild barley included in the section *Anisolepis* Nevski of the genus *Hordeum* in the *Triticeae* tribe. It originates from Chile and Argentina and is extremely polymorphic both at morphological and biochemical level. This species is found in a wide range of edaphic conditions mainly along lake shores or in wet areas. The species is mainly autogamous but some accessions readily produce hybrids in nature and in experimental plots (Martín et al., 1998).

Next to *H. vulgare* and *H. bulbosum*, *H. chilense* is likely to be the species of the genus *Hordeum* with the highest potential for cereal breeding purposes, because of its agronomic interesting traits and its high crossability with other members of the *Triticeae* tribe (Martín et al., 1998). Its hybrids with diploid, tetraploid or hexaploid wheat, after chromosome doubling, give rise to tetraploid, hexaploid or octaploid ampliploids, which are called tritordeums. The hexaploid tritordeum is considered to be a promising new cereal crop by itself (Cubero et al., 1986; Martín, 1988; Alvarez et al., 1992) and a bridge to transfer desirable agronomic traits to cultivated cereals (Martín & Cubero, 1981).

Disease resistance is one of the most interesting characteristics of *H. chilense* to be transferred to cultivated species (Rubiales et al., 1992). *H. chilense* is a non-host to the leaf rusts of wheat, rye and barley (*Puccinia triticina*, *P. recondita* and *P. hordei*, respectively). The species is a host but has a high level of resistance to the wheat stripe rust (*P. striiformis* f.sp. *tritici*) (Rubiales et al., 1991). The *H. chilense* lines differ in the level of appressorium formation by rust fungi on their stomata (Rubiales & Niks, 1992b), the level of early abortion of rust sporelings and the frequency of plant necrosis associated with the infection sites (Rubiales & Niks, 1992a).

The low appressorium induction, which is the base of the avoidance mechanism to rust fungi, can be found for several different leaf rust species in *H. chilense*. Rubiales and Niks (1992b, 1996) reported the existence of such a mechanism against barley leaf

rust, wheat leaf rust, brown rust collected from *H. jubatum* and rye leaf rust (*Puccinia hordei, P. triticina, P. recondita agropyrina* and *P. recondita*, respectively). Only *H. chilense* and four other wild barley species (*H. brachyantherum, H. marinum, H. parodii* and *H. secalinum*) were reported as having this defence mechanism to rust fungi (Rubiales et al., 1996).

This thesis will focus on analysing the low appressorium formation on some *H. chilense* lines, using as a model pathogen, barley leaf rust fungi (*P. hordei*). *P. hordei* is a specialist pathogen colonising barley, *H. vulgare*. It produces many small pustules of a rusty colour on leaves (Agrios, 1997). The formation of an appressorium over the stoma is the first critical stage in the infection process by this fungus, since invasion of the host can only occur via the stoma. In this plant/pathogen system the "hurdle" in the infection process occurs before intimate contact between the fungus and the plant and can, therefore, be regarded as a case of avoidance (Rubiales & Niks, 1992b). This avoidance mechanism may arrest the infection process in an early stage by certain features of stomata that prevent the induction of appressoria and hence, penetration of the stoma (Rubiales & Niks, 1992b, 1996).

Objectives and outline of present study

This thesis covers studies on the avoidance mechanism of *H. chilense* against the model-pathogen barley leaf rust, *P. hordei*. We performed studies on the role of the epidermal wax layer in avoidance. In addition to this, the genetic basis of the mechanism was studied. At the same time we searched for association between avoidance and other characters in *H. chilense*. Finally we concluded about the feasibility to exploit the avoidance character in cereal breeding.

In Chapter 2 we investigate the occurrence and distribution of avoidance in the species *H. chilense* and assess whether it is confined to a certain distinct ecotype or subspecific taxon of *H. chilense*. For that purpose a *H. chilense* collection of accessions was characterised for morphological and agronomic traits, AFLP fingerprint pattern, level of avoidance of barley leaf rust fungus, *P. hordei*, and habitat of origin.

In Chapter 3, we test if the germ tube growth was directed towards stomata and investigate the role of the leaf cuticular wax layer and the stoma dimensions on the early

stages of the infection process of *P. hordei* on *H. chilense* leaves. For that purpose several germ tube orientation parameters and the differentiation of appressorium were measured in *H. chilense* leaves with and without the cuticular wax layer.

The aim of Chapter 4 was to evaluate the AFLP (amplified fragment length polymorphism) marker technique and the degree of polymorphism in *H. chilense*. Two different enzyme combinations in a total of 20 different primer combinations were compared among *H. chilense* accessions that represent two different ecotypes.

In Chapter 5 we report on the construction of the first genetic AFLP linkage map for wild barley *H. chilense*. A segregating F_2 population, resulting from the cross of two *H. chilense* accessions with contrasting avoidance level, was analysed for 15 primer combinations. This linkage map, and histological evaluations on inoculated plants of this population allowed us to study the genetic basis of the quantitative trait avoidance of barley leaf rust in *H. chilense* and to evaluate the association of avoidance with leaf stoma density (Chapter 6).

In the final chapter (Chapter 7) the main results are discussed with regards to the feasibility of exploiting the avoidance character in cereal breeding.

2

Morphology and AFLP markers suggest three *Hordeum chilense* ecotypes that differ in avoidance of rust fungi[1]

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[1]Published in Canadian Journal of Botany (in press)

Abstract

In *Hordeum chilense* Roem. & Schult., a high variation in the level of avoidance of infection of barley leaf rust (*Puccinia hordei* Otth) occurs. Probably resulting from the properties of the stomata, the rust germ tube overgrows stomata and the infection process fails in an early stage.

In the present study we tested the hypothesis that the avoidance character occurs in certain morphologically and molecularly distinct ecotypes of *H. chilense*.

Eighty-eight *H. chilense* accessions were inoculated with *P. hordei* to assess the level of avoidance. The accessions were described for 30 morphological characters and three AFLP primer combinations. Cluster analysis using both morphological and AFLP fingerprint data suggested three distinct clusters of accessions. One of the clusters had a particularly high level of avoidance. This putative subspecific taxon was characterised by shorter and wider spikes, more erect culms, a greater number of stomata per square centimetre on the abaxial leaf side, and a shorter uppermost internode until flag leaf. All accessions clustered in this subspecific taxon were collected from humid habitats.

We conclude that *H. chilense* consists of at least three rather well defined, morphologically and genetically distinct subspecific taxa, one of which has a very high level of avoidance of barley leaf rust.

Keywords: AFLP fingerprinting, diversity, *Hordeum chilense*, avoidance, rust fungi, *Puccinia hordei*.

Introduction

Hordeum chilense Roemer et Schultes is a diploid perennial wild barley (2n=14), included in the section *Anisolepis* Nevski of the genus *Hordeum* in the *Triticeae* tribe.

H. chilense is confined to the zone of central Chile and the most western parts of the provinces of Neuquen and Rio Negro in Argentina, where it is found along road sides, in pastures, and in mesic, as well as xeric habitats, and at altitudes up to 2100 m (Tobes *et al.*, 1995; Giménez *et al.*, 1997). The species shows a very wide range of variation of morphological characters (Von Bothmer *et al.*, 1980, 1995) and is mainly autogamous (Martín *et al.*, 1998).

H. chilense is readily crossed with wheat, rye and also cultivated barley (Martín & Chapman 1977; Martín & Cubero, 1981; Martín *et al.*, 1996), allowing the possible transfer of traits to cultivated cereals.

One of the characters for which large variation has been found is the degree to which germ tubes of rust pathogens overgrow the stomata and fail to penetrate into the leaf tissue (Rubiales & Niks, 1992a, 1996). This trait is considered a case of avoidance (Rubiales & Niks, 1992a). Avoidance mechanisms of plants reduce the probability of intimate contact between host plant and potential consumer organism (Parlevliet, 1981). Such mechanisms are passive, and interfere with the infection process before there is question of physiological interaction. In *H. chilense*, high avoidance is associated with an extensive wax covering of the stomatal guard cells (Rubiales & Niks, 1996), and is effective against several rust species such as *Puccinia hordei, P. triticina, P. recondita agropyrina* and *P. recondita recondita* (Rubiales & Niks, 1992b, 1996).

Rubiales and Niks (1996) reported that 18 out of 37 *H. chilense* accessions showed a high level of avoidance, *i.e.* 80% or more of the germ tubes of *P. hordei* failed to form appressoria on a stoma. Thirteen of the accessions had a low level of avoidance, *i.e.* 40-60% germ tubes formed no appressorium. Accessions with an intermediate level were scarce.

To test the hypothesis that avoidance may be confined to a certain distinct ecotype or subspecific taxon of *H. chilense*, we characterised a collection of *H. chilense* accessions for morphological and agronomic traits, level of avoidance of barley leaf rust fungus *P. hordei*, habitat of origin and AFLP fingerprint pattern.

Materials and methods

Plant material and character measurement

A collection of 88 accessions of the species *H. chilense* was examined (Table 1). Accession number H_1 was obtained from Plant Breeding International (PBI), Cambridge, UK; H_7, H_8, H_{10}, H_{11} from United States Department of Agriculture (USDA), USA; H_{12} and H_{13} from Dr. Lange, Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO), The Netherlands, and H_{14}, H_{16} and H_{17} from Prof. Von Bothmer, Svalöv, Sweden. H_{33} to H_{93} have been collected previously by David Contreras. The accession numbers between H_{200}-H_{311} were collected during two expeditions in Chile and Argentina in the years 1990-92 and are maintained at the germplasm bank of the Consejo Superior de Investigaciones Científicas (CSIC), Córdoba in Spain (Tobes *et al.*, 1995; Giménez *et al.*, 1997). The geographic provenance of these accessions covered a wide range of habitats in Chile and Argentina as described by Tobes *et al.* (1995) and Giménez *et al.* (1997).

Measurements of level of avoidance

The accessions were sown at the Instituto de Agricultura Sostenible, Córdoba, Spain, in small wooden boxes, in rows of at least five seeds per accession. These seeds germinated and grew in a greenhouse compartment kept at 18-25 °C and ambient humidity. The adaxial surface of the 6th or 7th leaf of each plant was inoculated with *P. hordei* isolate 1-2-1, which is a monospore culture derived from 1-2 (Parlevliet, 1976), obtained from the Laboratory of Plant Breeding, Wageningen University, Wageningen, The Netherlands. The leaves were fixed in horizontal position and inoculum was applied to the adaxial leaf surface by dusting directly without using a settling tower. The sampling of the leaves and staining with Uvitex (Ciba-Geigy) for histological observation was as described by Rubiales and Niks (1996). Observations were made with an epifluorescence microscope (Nikon Fluophot, V-excitation, 380-425 nm) at 100x magnification.

Table 1. Collection accessions, location, habitat classification, avoidance level and clustering based on morphological characters.

Access.	Altitude	Latitude	Longitude	Place	Environment[a]	% germ tubes not forming appressorium	Cluster
H1	?	?	?	?	?	51.4	I
H7	0	31° 56'	71° 31'	?	?	82.4	III
H8	300	34° 04'	70° 56'	?	?	51.6	I
H10	?	?	?	?	?	83.6	II
H11	?	?	?	?	?	75.7	II
H12	?	?	?	?	?	55.8	I
H13	?	?	?	?	?	51.4	I
H14	?	?	?	?	?	82.2	III
H16	1750	32° 18'	71° 31'	?	?	68.6	II
H17	500	30° 0'	72° 22'	?	?	81.9	III
H33	?	?	?	?	?	56.4	II
H34	300	34° 04'	70° 56'	?	?	43.7	I
H35	300	34° 04'	70° 56'	?	?	65.1	I
H38	200	34° 03'	71° 38'	?	?	69.7	III
H39	200	34° 03'	71° 38'	?	?	73.3	III
H41	?	?	?	?	?	63.6	III
H46	?	?	?	?	?	66.8	III
H47	83	36° 45'	72° 18'	?	?	75.1	III
H49	?	?	?	?	?	85.4	III
H51	0	36° 45'	73° 09'	?	?	70.1	III
H52	0	36° 45'	73° 09'	?	?	80.1	III
H54	1000	34° 51'	70° 34'	?	?	50.1	I
H55	1000	34° 51'	70° 34'	?	?	89.3	III
H56	1000	34° 51'	70° 34'	?	?	82.4	III
H57	800	34° 45'	70° 34'	?	?	53.1	I
H58	800	34° 45'	70° 34'	?	?	48.8	I
H59	800	34° 45'	70° 34'	?	?	49.5	I
H60	800	34° 45'	70° 34'	?	?	45.3	I
H61	800	34° 45'	70° 34'	?	?	54.1	I
H68	1200	36° 45'	72° 18'	?	?	87.6	III
H74	230	33° 21'	71° 23'	?	?	63.1	II
H75	230	33° 21'	71° 23'	?	?	39.6	I
H83	?	?	?	?	?	48.9	I
H93	300	33° 06'	71° 28'	?	?	49.7	I
H200	800	34° 45'	70° 34'	?	A	63.2	I
H202	750	33° 01'	70° 54'	LA DORMIDA	A-EXTENDED DRY SUMMER	64.9	I
H203	0	32° 15'	71° 32'	LOS MOLLES	B-MISTY STEPPE	59.5	II
H204	1200	33° 00'	70° 57'	LA DORMIDA	A-EXTENDED DRY SUMMER	53.2	I
H205	350	32° 58'	71° 10'	OLMUE:CERRO LA CAMPANA	A-MEDIUM DRY SUMMER	89.1	III
H206	300	33° 06'	71° 28'	QUILPUE	A-CLOUDY DRY SUMMER (STREAM)	54.6	I
H207	500	31° 54'	72° 22'	CAIMANES	A-MISTY STEPPE	59.6	II
H208	351	32° 58'	71° 10'	OLMUE:CERRO LA CAMPANA	A-MEDIUM DRY SUMMER	78.8	I
H209	301	33° 06'	71° 28'	QUILPUE	A-CLOUDY DRY SUMMER (STREAM)	64.2	I
H210	1060	33° 39'	70° 21'	?	A	46.8	I

Table 1. (cont.)

Access.	Altitude	Latitude	Longitude	Place	Environment[a]	% germ tubes not forming appressorium	Cluster
H211	352	32° 58'	71° 10'	OLMUE:CERRO LA CAMPANA	A-MEDIUM DRY SUMMER	39.2	I
H212	1	32° 15'	71° 32'	LOS MOLLES	B-MISTY STEPPE	65.3	II
H213	1100	32° 25'	70° 55'	ALICAHUE	A-MODERATE WINTRY STEPPE	45.6	I
H216	1750	32° 18'	71° 31'	ALICAHUE	A-MODERATE WINTRY STEPPE	61.2	I
H217	300	33° 04'	70° 56'	ALHUE	A-CLOUDY DRY SUMMER	50.0	I
H218	900	33° 04'	70° 57'	LA DORMIDA	A-EXTENDED DRY SUMMER	54.1	I
H220	83	36° 45'	72° 18'	?	D	52.9	III
H222	0	36° 45'	73° 09'	?	D	84.6	III
H225	1750	32° 18'	71° 31'	ALICAHUE	A-MODERATE WINTRY STEPPE	57.1	I
H226	1750	34° 03'	71° 38'	?	A	40.1	I
H228	301	33° 04'	70° 56'	ALHUE	A-CLOUDY DRY SUMMER	47.6	I
H229	1700	33° 38'	70° 18'	?	A	_	I
H232	1100	32° 25'	70° 55'	ALICAHUE	A-MODERATE WINTRY STEPPE	43.8	I
H241	1800	33° 00'	70° 57'	LA DORMIDA	A-EXTENDED DRY SUMMER	59.3	I
H245	1500	34° 58'	70° 27'	?	C	77.4	III
H250	0	38° 42'	73° 02'	?	E	92.9	III
H251	1150	38° 26'	71° 22'	?	C	81.4	III
H252	0	38° 41'	73° 24'	?	E	92.4	III
H254	1800	34° 57'	70° 23'	?	C	75.0	III
H255	?	?	?	?	?	83.4	III
H261	700	30° 23'	70° 58'	PICHASCA	D-DRY STEPPE	59.6	III
H266	50	30° 32'	71° 42'	ALTOS	F-MISTY STEPPE	75.0	II
H283	600	30° 41'	70° 52'	LAS JUNTAS	D-DRY STEPPE	69.9	II
H286	150	29° 55'	71° 14'	LA SERENA	D-MISTY STEPPE	65.2	III
H290	25	31° 53'	71° 29	LOS VILOS	B-MISTY STEPPE	88.3	III
H292	250	30° 45'	71° 32'	SALALA	D-DRY STEPPE	74.9	II
H293	50	30° 32'	71° 42'	ALTOS	F-MISTY STEPPE	_	II
H294	150	30° 37'	71° 19'	TRAPICHE	D-DRY STEPPE	67.0	II
H295	150	30° 41'	71° 22'	VALLE DEL ENCANTO	D-DRY STEPPE	74.6	III
H296	600	30° 41'	70° 52'	LAS JUNTAS	D-DRY STEPPE	61.5	III
H297	150	30° 33'	71° 29'	TALINAY	F-MISTY STEPPE	83.1	III
H298	700	30° 21'	71° 29'	Qda SECA	D-DRY STEPPE	61.3	II
H299	1300	30° 15'	70° 41'	HURTADO	D-DRY STEPPE	89.6	III
H300	550	28° 55'	70° 45'	VALLENAR	D-TRANSITIONAL DESERT	77.9	III
H301	150	30° 41'	71° 22'	VALLE DEL ENCANTO	D-DRY STEPPE	59.4	III
H302	601	30° 41'	70° 52'	LAS JUNTAS	D-DRY STEPPE	65.6	III
H303	500	31° 54'	70° 22'	CAIMANES	A-MODERATE WINTRY STEPPE	54.1	II
H304	650	31° 48'	71° 21'	CAVILOLEN	A-MODERATE WINTRY STEPPE	38.3	I
H305	250	30° 37'	71° 14'	OVALLE	D-DRY STEPPE	_	III

Table 1. (cont.)

Access.	Altitude	Latitude	Longitude	Place	Environment[a]	% germ tubes not forming appressorium	Cluster
H307	150	29° 55'	71° 14'	LA SERENA	D-MISTY STEPPE	_	III
H308	2100	31° 47'	70° 35'	CUCUMEN	A-MODERATE WINTRY STEPPE	_	I
H309	251	30° 37'	71° 14'	OVALLE	D-DRY STEPPE	70.5	III
H310	0	31° 56'	71° 31'	LOS VILOS	B-MISTY STEPPE	_	III
H311	350	30° 48'	71° 40'	LOS LOROS	F-MISTY STEPPE	53.5	III

[a] Legend: A=Dry steppe environment, without hydromorphism (less water), C=hydromorphism, high altitude, D=hydromorphism, middle altitude, B=rocky shore, F=saline plains, low altitude, E=south cost, plants near seashore watered by the tide (more water)

The level of avoidance was measured as the frequency of germ tubes that did not form an appressorium on a stoma, counting a total of at least 100 germlings per leaf segment.

Stomatal densities were determined on the abaxial epidermis of the leaf segments (three microscope fields per leaf), in the same five leaves per accession that were used to determine the level of avoidance. These determinations were done only on the abaxial epidermis since Rubiales and Niks (1996) reported already that the avoidance level was only highly correlated with the stomatal density on the abaxial epidermis and not with the stomatal density on the adaxial epidermis.

Morphological and agronomic characterisation

Seedlings were planted in small (5 cm square) pots, one seedling per pot, in a greenhouse in late November at the Instituto de Agricultura Sostenible, Córdoba, Spain. In April, they were transferred to 12 cm square pots and placed outdoors with three replicates per accession. At about the sixth leaf stage, they were transferred to a bird-proof cage in a random order.

Each plant was evaluated for three earliness parameters, 22 other quantitative traits including stomatal densities and nine qualitative morphological characters (Table 2). Means of the three replicate plants represented the OTU (Operational Taxonomic Unit) for each accession examined in the study.

Table 2. Morphological and agronomic characteristics measured

Quantitative traits	Number of days to awn emergence (**AWN**) – *earliness I*	
	Number of days to anthesis (**ANT**) – *earliness II*	
	Number of days to complete cycle (**MAD**) – *earliness III*	
	Flag leaf length (**FFL**) – *flag leaf I*	5x, cm[a]
	Flag leaf width (**FFW**) – *flag leaf II*	5x, cm
	Spike thickness (**SPA**) – *spike I*	5x, cm
	Spike width (**SPB**) – *spike II*	5x, cm
	Spike length (**SPC**) – *spike III*	5x, cm
	Uppermost internode length (**INA**) – *up internode I*	5x, cm
	Length of uppermost internode from nodus until flag leaf (**INB**) – *up internode II*	5x, cm
	Length of uppermost internode from flag leaf until spike (**INC**) – *up internode II*	5x, cm
	Fertility main spikelets (**FEM**) – *fertility I*	4x, %
	Fertility lateral spikelets (**FEL**) – *fertility II*	4x, %
	Number of spikelets per spike (**SNR**)	4x
	Tillering (**TNR**)	Number of shoots
	Plant height (**HGT**)	cm
	Minimum culm length (**LMI**) – *culm I*	cm
	Maximum culm length (**LMA**) – *culm II*	cm
	Seed longitude of progeny (**SLG**)	3x, cm
	Seed width of progeny (**SWH**)	3x, cm
	Seed thickness of progeny (**SSN**)	3x, cm
	Number of roots at seed emergence stage (**NRR**)	2x[b]
	Coleoptile length at seed emergence stage (**LGC**)	2x, 1/<1 cm-10/>10 cm[b]
	Root length at seed emergence stage (**LGR**)	2x, 1/<1 cm-10/>10 cm[b]
	Stoma frequency (per cm^2) (**STOMAF**)	3x, 5 leaves[c]
Qualitative traits	Seed color of progeny (**SCO**)	3x, 1/amber-3/green
	Growth habit at the beginning of the cycle (**PSB**) – *initial growth habit*	1x, 1/erect culms-5/creeping culms
	Growth habit at the end of the cycle (**PSF**) – *final growth habit*	1x, 1/erect culms-5/creeping culms
	Plant color (**PCO**)	1x, 1/light green-5/dark green
	Pigmentation of nodus (**PIG**)	1x, 1/green-5/dark purple
	Biomass at end of cycle (**BIO**) – *total growth*	1x, 1-5
	Biomass at seedling stage (≈ *early growth*) (**EMG**)	1x, 1-5
	Position of mature anthers (**STA**) – *allogamy level*	1x, 1/inside-5/outside
	Hairiness of shoots at seedling stage (**HRS**)	1x, 1/smooth-5/very hairy

[a]. Five observations per plant recorded in cm
[b]. Observations were recorded from seed raised in petri dishes
[c]. Observations were recorded in the same leaves used to determine the level of avoidance

AFLP fingerprints

H. chilense accessions were sown in 12 cm square pots, one seedling per pot, at the Laboratory of Plant Breeding, Wageningen University, Wageningen, The Netherlands. Plants were raised in a greenhouse compartment at 18-25°C and ambient humidity. From one plant per accession, not fully expanded leaves were collected, frozen and stored at –80 °C. DNA was extracted following the CTAB-method (Van der Beek *et al.*, 1992). The amplified fragment length polymorphism technique (AFLP) was performed as described by Vos *et al.* (1995). The AFLP-analysis followed the protocol described by Van Eck *et al.* (1995) with modifications by Qi and Lindhout (1997). Three *Eco*RI/*Mse*I (+3+3) primer combinations (E35M48, E38M54 and E42M48) were used

that have previously shown a high rate of polymorphism in cultivated barley (*H. vulgare*) (Qi & Lindhout, 1997). Primer core sequences were according to Vos *et al.* (1995).

Analysis of character scores

Pairwise Pearson's correlations between the morphological and agronomic data were calculated. When characters correlated with a coefficient higher than r = 0.9 (chosen as an arbitrarily high coefficient), only one of these characters was retained randomly for further analysis. For example, the number of days to awn emergence (AWN) was correlated highly (r=0.98) with number of days to anthesis (ANT), which is not surprising. In such cases we discarded data on one or the other character. Thus the characters AWN, uppermost internode length (INA), minimum culm length (LMI) and maximum culm length (LMA) were removed (Table 2). Thirty characters out of the 34 then were used in the principal coordinates analysis (PCO).

PCO was performed with all the morphological and agronomic traits that remained after the correlation analysis, except for the avoidance level. Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) (Rohlf, 1993) version 2.02j, was used for this analysis. The similarity matrix was generated with standardised data to eliminate effects of different measurement scales. As similarity coefficient the average taxonomical distance was used.

Character means and ranges of the resulting clusters were calculated, and a one-way analysis of variance was performed to determine which characters differed significantly between the clusters. Duncan's multiple range test was applied when variances were homogeneous, otherwise the Dunnett T3 multiple comparisation test was used. SPSSpc (version 5.0.1) was used for basic statistics, correlation and one-way analysis of variance.

The presence or absence of AFLP fragments was scored on the autoradiograms and recorded as 1 (present), 0 (absent) or 2 (missing observation) for all OTUs. Principal co-ordinate analysis (PCO) was performed using NTSYSpc. Genetic similarities were calculated using the Jaccard coefficient (Jaccard, 1908).

The relationship between the results of the morphological and agronomic grouping and the AFLP grouping was analysed using Mantel's test for correlation between matrices (Mantel, 1967). This test is a randomisation procedure that compares the correlation between two matrices with the correlation between one of these and randomisation of the other. For each pairwise comparison, 250 randomisations were carried out.

Results

Level of avoidance

The accessions of the collection differed significantly in the level of avoidance. This variation between the accessions was continuous (Fig. 1). The frequency distribution did not deviate significantly from a normal distribution (Kolmorogorov-Smirnov test, p=0.38). The variation ranged from 38% (H_{304}) to 93% (H_{250}) of germ tubes failing to form an appressorium (Table 1).

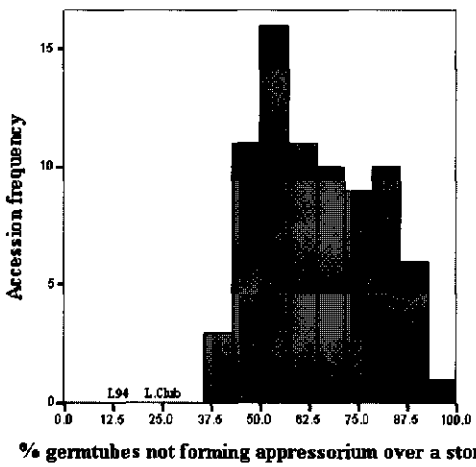

Figure 1. Frequency distribution of avoidance level, expressed as the percentage of germ tubes of *P. hordei* that did not form an appressorium over a stoma in a collection of 88 *Hordeum chilense* accessions. L94-12% (cultivated barley) and Little Club-26% (cultivated wheat) served as reference. (from Rubiales & Niks, 1996)

Morphological and agronomic characters

Significant correlations between the different morphological and agronomic characters were observed. (Table 3). Only the characters not correlated with a high coefficient ($|r|<0.9$) were kept for further analysis.

From PCO analysis based on the morphological and agronomic criteria (without the avoidance level), the accessions were plotted according to the first two principal coordinates (Fig. 2). The first two coordinates explained 37% of the total observed variation. The third coordinate increases this percentage to 47%, but does not influence the basic conclusions on the clustering. Based on this PCO, the accessions can be classified into three main clusters (I, II, and III) (Fig. 2, Table 1). Mean values for all characters of these clusters are given in Table 4.

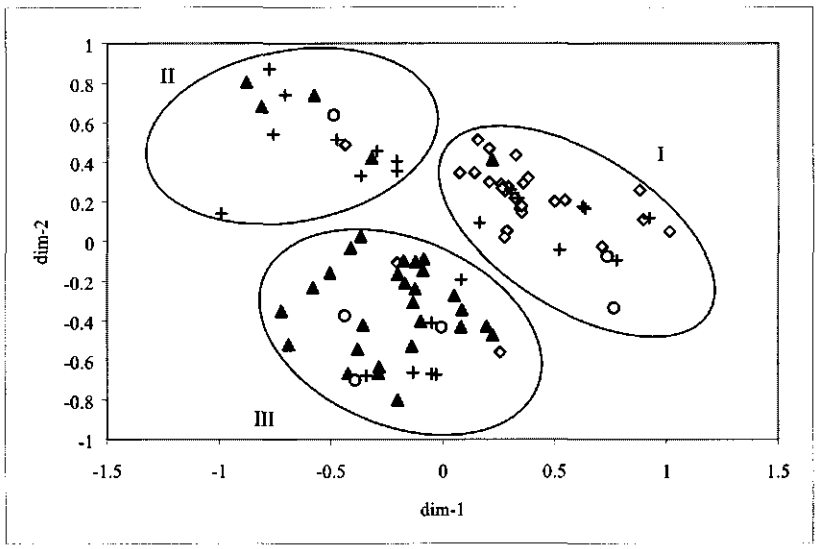

Figure 2. Principal coordinates plot of morphological data of 88 lines of *H. chilense*, with the avoidance level indication. [three arbitrary avoidance classes: ◊ 0-55% germ tubes not forming appressorium (low level of avoidance), + 55-70% germ tubes not forming appressorium, ▲ 70-100% germ tubes not forming appressorium (high level of avoidance), o represent missing data on level of avoidance]. The two principal coordinates accounted for 36.7% of the total variation.

Table 3. Pearson correlation coefficient (r) between morphological and agronomic characters[a].

ANT	MAD	FFL	FFW	SPB	SPC	INB	INC	SNR	TNR	HGT	SSN	LGC	LGR	STOMAF	PSF	BIO	EMG	STA	HRS	AVGDIAMCH	
1	0.87**																				ANT
	1																				MAD
		1	0.59**													0.57**					FFL
			1																		FFW
				1	-0.64**						0.53**			0.67**	-0.57**			-0.62**	0.50**	0.64**	SPB
					1	0.82**				0.59**				-0.56**			0.56**			-0.60**	SPC
						1				0.74**						0.58**				-0.56**	INB
							1	0.57**	0.60**	0.79**						0.66**					INC
								1		0.57**						0.56**					SNR
									1	0.51**						0.77**					TNR
										1						0.70**					HGT
											1								0.61**		SSN
												1	0.52**								LGC
													1								LGR
														1	-0.63**			-0.51**		0.62**	STOMAF
															1					-0.50**	PSF
																1					BIO
																	1				EMG
																		1			STA
																			1		HRS
																				1	AVGDIAMCH

Note: r is only presented when higher than 0.5. Characters SPA, FEM, FEL, SLG, SWH, NRR, SCO, PSB, PCO and PIG did not have a correlation coefficient higher than 0.5 with any other character, and therefore are excluded from the table.

**: Correlation is significant at the 0.01 level; *: Correlation is significant at the 0.05 level.

[a] : Abbreviations of the characters are explained in Table 2.

Table 4. Morphological and agronomic characters in the clusters*

	ANT (nº of days)	MAD (nº of days)	FFL (cm)	FFW (cm)	SPA (cm)	SPB (cm)	SPC (cm)
Cluster I(n=35)	147[b]	170[b]	4.39[b]	0.43[b]	0.22[b]	0.35[a]	9.82[b]
Cluster II(n=15)	141[a]	162[a]	3.48[a]	0.39[a]	0.21[a]	0.41[b]	7.44[a]
Cluster III(n=38)	145[ab]	168[b]	4.13[b]	0.50[c]	0.21[a]	0.49[c]	7.46[a]

	INB (cm)	INC (cm)	FEM (%)	FEL (%)	SNR (nº of spikelets)	TNR (nº of shoots)	HGT (cm)
Cluster I(n=35)	15.6[a]	25.6[b]	77.6[a]	1.71[b]	49.7[b]	75.9[b]	70.1[c]
Cluster II(n=15)	12.0[b]	12.6[a]	77.6[a]	0.03[a]	39.9[a]	49.6[a]	38.3[a]
Cluster III(n=38)	13.2[b]	25.8[b]	82.8[a]	0.32[ab]	52.0[b]	77.3[b]	60.7[b]

	SLG (cm)	SWH (cm)	SSN (cm)	NRR (nº of roots)	LGC (cm)	LGR (cm)	STOMAF (nº of stomata/cm²)
Cluster I(n=35)	8.94[a]	3.00[a]	1.37[b]	1.48[a]	5.69[a]	4.41[b]	1677[a]
Cluster II(n=15)	8.25[b]	2.97[a]	1.14[a]	1.60[a]	6.64[b]	3.92[a]	2310[a]
Cluster III(n=38)	8.36[b]	2.97[a]	1.61[c]	1.83[a]	5.04[a]	3.51[a]	4828[b]

	SCO (1-3)	PSB (1-5)	PSF (1-5)	PCO (1-5)	PIG (1-5)	BIO (1-5)	EMG (1-5)
Cluster I(n=35)	2.51[a]	1.57[a]	3.64[a]	3.40[a]	2.81[a]	3.84[b]	3.91[b]
Cluster II(n=15)	2.61[a]	1.93[a]	2.77[ab]	4.15[b]	3.86[b]	2.22[a]	2.73[a]
Cluster IIIn=38)	2.39[a]	1.92[a]	1.92[b]	3.78[ab]	2.62[a]	3.75[b]	2.52[a]

	STA (1-5)	HRS (1-5)	**Avoidance (% germ tubes not forming appressorium)**
Cluster I(n=35)	4.46[c]	1.35[a]	**51.69[a]**
Cluster II(n=15)	3.77[b]	1.35[a]	**64.29[b]**
Cluster III(n=38)	2.65[a]	2.49[b]	**77.06[c]**

*Letters in common indicate that the values are not statistically significant (P<0.05, Duncan test or Dunnett test)

The three clusters were significantly different from each other in at least five characters. These characters were position of anthers (STA), seed thickness of progeny (SSN), spike width (SPB), flag leaf width (FFW), and plant height (HGT). These are the main characters separating the clusters. Pairwise analysis between clusters showed that the clusters differed significantly for 22 (cluster I versus II), 15 (cluster II versus III) and 13 (cluster I versus III) characters.

The avoidance level showed significant differences between the three clusters (Table 4 and Fig. 2). The accessions in cluster III had the highest level of avoidance (on average 77% of germ tubes not forming an appressorium), those in cluster I the lowest level (on average 52% of germ tubes not forming an appressorium), while the accessions in cluster II had intermediate levels of avoidance. Avoidance level was significantly correlated ($r=0.64$, $p<0.001$) with spike width (SPB), one of the five characters for which the three clusters differed significantly from each other. Apart from the wider spikes, the avoidance accessions were characterised by shorter spikes (SPC), high stoma density on the abaxial leaf side (STOMAF), more erect culms (PSF) and a shorter uppermost internode until flag leaf (INB).

Clustering also was associated with the habitat of provenance of these lines. The accessions in cluster I were collected from dry places, those in the other two clusters from permanently or temporarily humid habitats. Avoidance tends therefore to be associated with such humid habitats. The subjectivity of this information (Table 1) and the lack of information on many of the accessions warrant some caution on the conclusions to be drawn on the nature of the habitat of the clusters. Avoidance level was not associated with attributes such as longitude, latitude or altitude of place of origin.

AFLP fingerprints

Analysis of the *H. chilense* accessions with 3 AFLP primer combinations identified a total of 275 scorable fragments, of which 33 were constant and 242 (88%) were not present in all accessions. The size of scored amplified fragments ranged from 62 to 551 bp.

As for the agronomic and morphological characters the accessions were plotted according to the first two principal coordinates (Fig. 3).

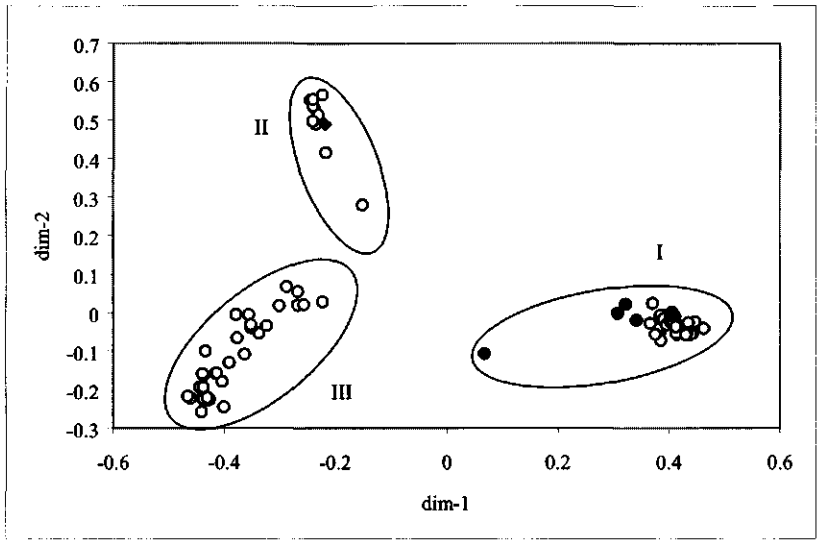

Figure 3. Principal coordinates plot of 88 lines of *H. chilense*, based on the AFLP molecular data. The two principal coordinates accounted for 46% of the total variation. (O represent all the lines that plotted in the same cluster as on the basis of the morphological data, ● represent lines that plotted in cluster II on the basis of morphological data, ♦ represent line that plotted in cluster III on the basis of morphological data, Fig. 2)

The two first principal coordinates explained 46% of the variation, and the third co-ordinate added only 3.8%. The AFLP data discriminated the OTUs again into three distinct main clusters. The PCO plots resulting from each individual primer combinations remained generally the same as the one resulting from all the data combined (data not shown). The separation between the clusters was sharper than when based on the morphological and agronomic characters (Fig. 2). All accessions clustered in the same clusters as for the morphology and agronomy characters, except for 6 accessions (Fig. 3). H_{16}, H_{33}, H_{74}, H_{203} and H_{298} were in the morphological cluster II, and were now found in cluster I and H_{290} moved from morphological cluster III to

molecular cluster II. Indeed, results from the Mantel test, showed that the genetic similarity based on AFLP is significant and positively correlated with the one based on the morphological characters (r=0.47, p=0.004). This significant correlation suggests that the three clusters represent distinct genotype groups of *H. chilense*, and that genotypic differences between the groups are reflected both by the morphological characters of the plants and by AFLP fingerprint patterns.

There were no unique bands present in all accessions of one cluster and absent in all the accessions of the other clusters. However, in each cluster some AFLP marker bands were present in all the accessions, but present only in some of the accessions in the other two clusters (Fig. 4).

Discussion

Von Bothmer *et al.* (1980) reported a wide morphological and continuous variation in *H. chilense*. As no evidence was found for distinct morphotypes, they preferred to keep *H. chilense* as a single polymorphic taxon, rather than splitting it into subspecies. Avoidance of leaf rust fungi (Rubiales & Niks, 1996) is one of the characters for which *H. chilense* shows a wide variation. Rubiales and Niks reported that the percentage of germ tubes forming an appressorium on the *H. chilense* lines is not distributed normally: accessions with intermediate levels of avoidance were scarce. From this observation Rubiales and Niks suggested that the lines with high and low levels of avoidance might represent distinct ecotypes of *H. chilense*. In the present study we tested this hypothesis.

In contrast to the result of Rubiales and Niks (1996), the frequency distribution of the avoidance character in our study did not deviate significantly from a normal distribution. Thirty-two of the accessions studied were in common with the work of Rubiales and Niks (1996), and the correlation between their and our data was high (r=0.7). Our study showed that in the work of Rubiales and Niks there was by chance an excessive representation of the two ecotypes with the largest contrast in avoidance (cluster I and III).

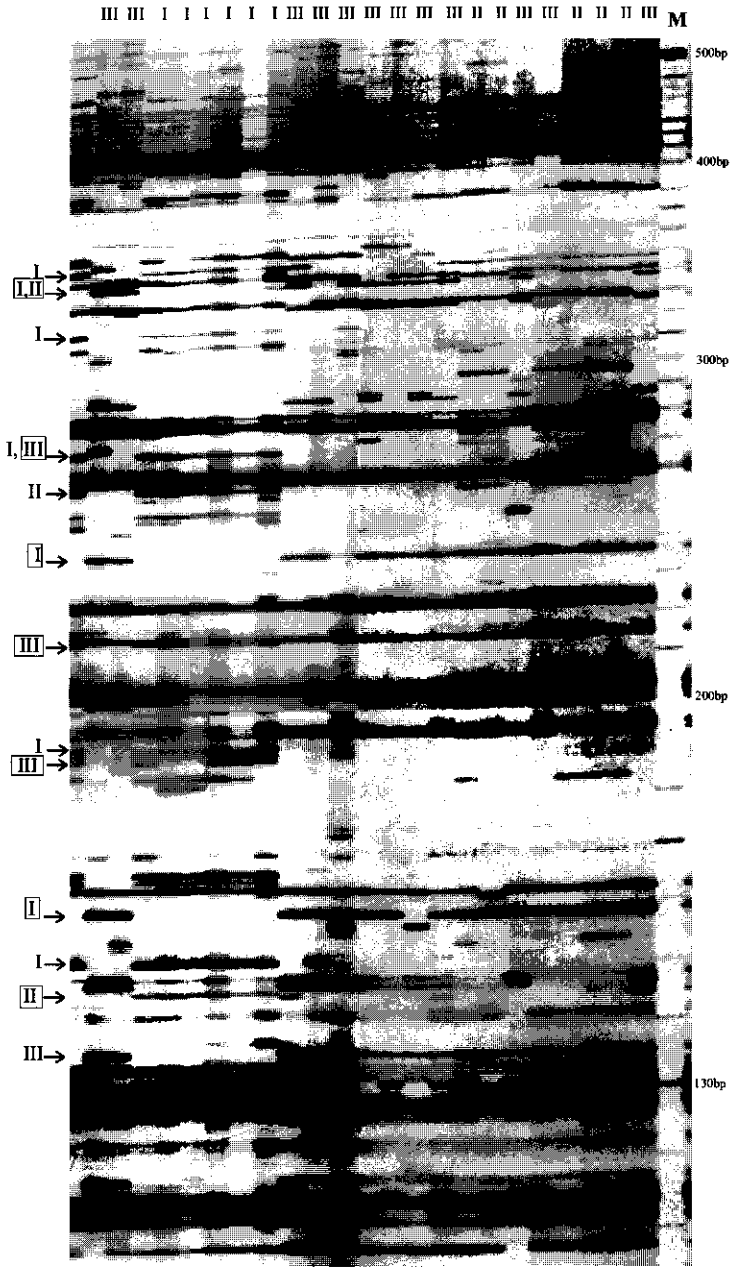

Figure 4. AFLP gel (E35M48) indicating cluster specific bands. (I, II and III - cluster number, squares - absence of band, no squares - presence of band)

The gap between these more extreme accessions was in our work, filled by accessions with an intermediate level of avoidance (Fig. 2). This resulted in a normal distribution of the avoidance trait.

Cluster analysis based on the morphological and agronomic data and on the AFLP fingerprint suggested three distinct clusters of accessions within this *H. chilense* collection. This suggests (in contrast to Von Bothmer *et al.*, 1980) that *H. chilense* does consist of at least three subspecific taxa. Representatives of the ecotypes distinguished by us are perfectly cross-fertile, implying that these are not representing different species. However, the genetic distance, based on AFLP polymorphisms, between accessions from cluster I (H_1) and cluster III (H_7 and H_{47}) was similar to the one between *H. vulgare* and *Triticum monococcum*, that are entirely different, non-crossable species belonging to different genera (Vaz Patto *et al*, 2000). This indicates that the degree of AFLP polymorphism between sets of accessions cannot be used to conclude about species delimitations.

One of the *H. chilense* morphotypes (cluster III) had a particularly high level of avoidance of *P. hordei*. The typical avoidance line could be characterised by plants with shorter and wider spikes, high stoma density on the abaxial leaf side, more erect culms and a shorter uppermost internode until flag leaf. The accessions of this cluster generally originate from humid places.

No geographical clustering was observed. Cluster III, showing the high avoidance, was not associated with a particular longitude, latitude or altitude of place of origin. The three detected main groups tend to occupy different but sometimes overlapping types of habitats. Cluster III accessions specially occurred in humid habitats.

In an F_2 population, resulting from the cross of two *H. chilense* accessions (H_1, cluster I and H_7, cluster III), no significant correlation ($r=-0.15$) between avoidance and the stoma density on the abaxial epidermis of the leaves was found (Vaz Patto *et al.*, 1998). This indicates that the association between avoidance and the stoma density was not due to pleiotropic effects of genes.

It is interesting that the avoidance seems to occur especially in *H. chilense* collected in humid habitats. This may indicate that the avoidance character of *H. chilense* could have been developed to protect the plants against rust pathogens. It is

conceivable that in such humid habitats selection pressure imposed by rust fungi is higher than in dry habitats. However, it is unknown to us what rust species, if any, occur in substantial amounts on *H. chilense* in natural habitats. Alternatively, high avoidance may merely be a consequence of morphological features that developed in the particular morphotype because of particular features of its habitat. The extensive wax covering on stomatal guard cells has been proposed to cause the avoidance (Rubiales & Niks, 1996). This morphological trait is normally associated with environments with reduced water availability or high radiation. Nevertheless, Rubiales and Niks (1996) reported that no relation existed between avoidance level and water use efficiency or stomatal conductance.

The partial association of avoidance with humid habitats, where no special requirements for water efficiency are needed, favours the hypothesis that the extensive wax covering of stomata suggested as the cause for the avoidance results from the selection due to high rust pressure.

The results showed good correlation between the large morphological and molecular variation in *H. chilense* species. In addition, we found a high variation for avoidance level, which was associated with particular morphological features and AFLP fingerprintypes. A segregating mapping population produced from accessions belonging to different clusters will allow to elucidate the genetics of the avoidance, and to identify possible molecular markers linked to those avoidance genes.

Acknowledgements

We thank Piet Stam and Pim Lindhout for critically reviewing the manuscript.
This research was financially supported by the program PRAXIS XXI, Fundação para a Ciência e a Tecnologia, Lisboa, Portugal (research fellowship number BD/9124/96).

3

Leaf wax layer prevents appressorium differentiation but does not influence orientation of the leaf rust fungus *Puccinia hordei* on *Hordeum chilense* leaves[1]

Maria Carlota Vaz Patto and Rients E. Niks

[1]Based on: 'Leaf wax layer prevents appressorium differentiation by the leaf rust fungus *Puccinia hordei* on *Hordeum chilense* leaves'(submitted) and on 'Orientation of germ tubes of *Puccinia hordei* on the *Hordeum chilense* leaf surface' Acta Phytopathologica et Entomologica Hungarica (2000) 35:213-220.

Abstract

On South American wild barley, *Hordeum chilense* Roem. & Schult., several rust fungal species hardly form appressoria on the stomata. This so called avoidance is characterised by a high percentage of germ tubes of rust fungi overgrowing the stomata which results in early failure of the infection process.

The directional growth of urediospores germ tubes along the transverse axis of a cereal's leaf and the appressorium differentiation are considered to be responses to stimuli from the plant surface.

In order to find out if the germ tube growth is directed towards stomata, and if the cuticular wax layer plays a role in this orientated growth and on appressorium differentiation, several orientation and differentiation parameters of *Puccinia hordei* Otth germ tube were measured on *Hordeum chilense* leaves with and without the wax layer.

Orientated growth of the germ tubes seems to start only upon contact with the epidermal cell junctions. The lateral growing of the germ tube over the first epidermal cell junction that it meets is longer than the subsequent lateral growings. These lateral growings, especially the first one, may help the germ tube to grow along the transverse axis of the leaf. No evidence was found of attraction of the germ tube by stomata. Removal of the cuticular wax layer did not result in poor germ tube orientation. This suggests that the leaf wax layer has no role to guide the germ tube in a certain direction.

On high avoidance accessions the removal of the wax layer allowed appressoria to develop over stomata that would otherwise be overgrown. No effect of the cell widths in stomatal complexes was found on the chance that stomata were overgrown. This suggests that the overgrowth of stomata on *Hordeum chilense* leaves by *Puccinia hordei* germ tubes is mainly due to the wax covering of the stomatal apparatus.

Key words: *Puccinia hordei*, *Hordeum chilense*, barley leaf rust, orientated growth, epicuticular waxes, appressorium, thigmotropic responses.

Introduction

Rust fungi (Basidiomycotina, Uredinales, Pucciniaceae) are important plant pathogenic obligate biotrophs. The infection process of cereal rust fungi, as barley leaf rust fungus *Puccinia hordei* Otth, starts with hydration and germination of urediniospores on the plant surface (Hoch & Staples, 1987). The germ tube grows across the longitudinally orientated epidermal cells till contact with a stoma where it ceases growth and develops an appressorium directly over the stomatal opening (Littlefield & Heath; 1979, Hoch & Staples, 1987; Hoch *et al.*, 1987). It is supposed that this orientation of the germ tube growth along the transverse axis of the leaf increases the probability of encountering stomata, which are arranged in rows on the cereal leaves (Lewis & Day, 1972; Littlefield & Heath, 1979).

Orientated growth of the germ tube and appressorium formation are considered to be responses to stimuli from the host (Staples *et al.*, 1983; Hoch & Staples, 1987; Hoch *et al.*, 1987; Allen *et al.*, 1991b; Collins & Read, 1997). These stimuli from the host may be physical as the close spacing of epidermal cell junctions (Read *et al.*, 1997) or the epicuticular wax crystals pattern (Lewis & Day, 1972), or may be chemical as pH gradient around the stomata (Edwards & Bowling, 1986).

The effect of topographical features on the orientation of rust germ tube growth and on appressorium differentiation can be studied in different ways. The rust may be applied to an inert artificial substratum with precise microtopography of defined dimensions (Staples et al., 1983; Hoch *et al.*, 1987; Allen *et al.*, 1991a and b; Collins & Read, 1997; Read *et al.*, 1997) or to leaf surface replicas made of such inert material (Collins & Read, 1997). No chemical signals from the host will be present in these cases.

Hoch et al. (1987) observed that the best signals for growth orientation of the bean rust, *Uromyces appendiculatus*, germlings were ridges or grooves in the substrate spaced 0.5 μm to 15.0 μm apart. The regularity of orientation diminished as the spacing increased, and spacing beyond 30.0 μm was not effective. This markedly perpendicular growth pattern to the orientation of the ridges and/or grooves was also observed on *Puccinia hordei* and *P. graminis tritici* germ tubes after growing over more than a few ridges or grooves on artificial membranes (Read et al., 1997). Hoch et al. (1987)

suggested that the surface depressions located at the anticlinal walls of the epidermal cells could serve as the topographical feature orienting germling growth. These depressions were spaced 15 to 30 μm apart on bean leaves what could explained that the germling orientation in vivo is not as strong as that observed with the closest spacing on the artificial membranes. These authors concluded that the depth of the signal for growth orientation is not as critical as it is for cell differentiation since orientation occurred on ridges ranging in height from 0.1 to 5.0 μm.

The appressorium differentiation pattern of response to different ridge heights varies between and even within a rust species (Allen et al., 1991a; Collins & Read, 1997). In *U. appendiculatus*, it has been suggested that topographical features of the stomatal complex provide the signals for appressorium differentiation over stomata (Hoch et al., 1987). However, the role of such topographical signals in the induction of cereal rust appressoria is much less clear (Read et al., 1997). In the bean rust, the optimal inductive artificial topographical signal closely resembles the ridge or "ledge" at the guard cell lip of the host plant *Phaseolus vulgaris* (Hoch et al., 1987). The stomatal complexes of cereal leaves do not possess such a prominent guard cell ridge (Read et al., 1997).

An important distinction between the topographical signals involved in appressorium differentiation in vivo for the bean rust and for the cereal rusts has already been stressed by Read *et al.* (1997). In the case of the bean rust, the ridge appears to be the important factor, whilst furrows (cell junctions) seem to be significant for the cereal rusts. Read *et al.* (1997), using artificial membranes, showed that not only the ridge height but also the spacing between ridges was influencing the appressorium differentiation. Germ tubes of *P. hordei* differentiated over closely spaced, multiple ridges and grooves, but not to a significant extent over single ridges or flat surfaces. Of the 1.5 μm, 2.5 μm and 50 μm ridge spacings tested, the most inductive spacing was 1.5 μm for all the heights analysed (0-2.5 μm) and the greatest level of appressorium differentiation was recorded over ridges which were 2 μm high (Read et al., 1997). The same authors suggest that cereal rust, in contrast to bean rust, respond to the close spacing of cell junctions of the dumbbell shaped cereal guard cells. Nevertheless, these authors acknowledge that they had not identified the precise topographical features that are involved in appressorium differentiation in vivo.

These previous studies with artificial membranes, suggested that physical features of the host alone are sufficient to orient germ tube growth and to induce appressorium formation but insufficient to explain the high level of appressorium formation observed *in vivo*. A reason for this discrepancy with *in vivo* observations may be that the host provides a combination of chemical and topographical signals which allows stomata to be accurately recognised by the cereal rust (Collins & Read, 1997). Also an imperfect reproduction of host physical features by the replication method, notably the cuticular wax crystals, may explain these discrepancies.

The wax crystal lattice of the cuticle has been implicated in directing germ tube growth (Lewis & Day, 1972). Nevertheless, Jenks and Ashworth (1999) hypothesised that the wax structure, covering the leaf surface, may disorient fungal hyphae growth across plant surfaces.

On some *H. chilense* accessions, it was observed that a large percentage of germ tubes overgrow one or more stomata, without differentiating an appressorium. Appressorium differentiation on these accessions may occur at 10 to 30% of the frequency of that on other *H. chilense* accessions or on cultivated cereals (Rubiales & Niks, 1996). As a consequence, no penetration of the stoma and no further infection can take place (Rubiales & Niks, 1996). This feature on *H. chilense* accessions was coined "avoidance" (Rubiales & Niks, 1992a). Overgrowth of stomata on high avoidance accessions might be due to the absence of an effective appressorium inducer structure or to the failure of the germ tube to contact the inducer structure. Scanning electron microscopy indicated the presence of an extensive wax covering over the stomata of these accessions. Extensive wax covering over the guard cells obscuring the features that normally induce appressorium differentiation was therefore suggested to be the cause of overgrowth of stomata in *H. chilense* accessions with high avoidance levels (Rubiales & Niks, 1996).

The first aim of the present study was to distinguish whether the germ tube growth of *P. hordei* on a *H. chilense* leaf surface is directed towards stomata or whether it is merely perpendicular to the long axis of the leaf. The second aim was to clarify the role of the cuticular wax layer on orientation of the germ tube to the stomata. The third aim was to determine the role of the cuticular wax layer and of the dimensions of the

stomatal cells on the appressorium differentiation by the leaf rust fungus *P. hordei* in the wild barley *H. chilense*.

Material and methods

Fungus: Urediniospores of *Puccinia hordei* Otth (isolate 1-2-1; Parlevliet, 1976) were produced in a green house on the susceptible barley (*H. vulgare* L.) line L98.

Plant: Two separate experiments were conducted. *Orientation studies* included *Hordeum chilense* Roem. et Schult. accessions H7, H46 and H47, selected on the basis of their relatively high stoma density on the abaxial leaf side (Rubiales & Niks, 1996). Susceptible barley line L94 was included as a reference plant. Countings and measurements were done on the flat abaxial leaf surface as no significant differences on wax coverage between adaxial and abaxial leaf surface were detected using SEM (Vaz Patto, unpublished). For the *appressorium differentiation studies*, eight accessions of *H. chilense* were selected on the basis of their appressorium inducing capacity. H250, H47, H46 and H7 have a low appressorium inducing capacity (high level of avoidance) and H304, H1, H211 and H75 a moderately high appressorium inducing capacity (low level of avoidance) (Vaz Patto, 2001a). Countings and measurements were done in this experiment on the upper (adaxial) leaf surface. All the *H. chilense* accessions were obtained from Prof. A. Martín (Instituto de Agricultura Sostenible, Córdoba, Spain). Plants were grown in a greenhouse compartment at 18-25°C and ambient humidity, at the Laboratory of Plant Breeding, Wageningen University, The Netherlands.

Removal of the epicuticular wax: About three cm^2 of the adaxial or abaxial (depending on the experiment) leaf surface of four sixth leaves of tillers of plants in the vegetative stage were treated with celloidin in order to remove the cuticular wax layer according to Rubiales and Niks (1996). This treated part and the adjacent untreated part were detached from the plants and placed, adaxial or abaxial surface up (depending on the experiment), on damp filter paper in petri dishes.

Inoculation procedure: Inoculations took place in an inoculation tower. Four milligrams of *P. hordei* spores (about 190 spores/cm^2), mixed with *Lycopodium* powder (1:10, v:v), were applied for the orientation studies and six milligrams of *P. hordei* spores (about 400 spores/cm^2), mixed with *Lycopodium* powder (1:10, v:v) for the appressorium differentiation studies. After inoculation the petri dishes were covered and incubated overnight in a green house compartment inside a black polyethylene bag. This procedure results in very small water droplets on the leaf surface. Large droplets of water might disturb germ tube growth. Twenty four hours later, the leaf segments were stained, taking care not to disturb the position of the germ tubes. The leaf segments were flooded in lactophenol-ethanol two to three days, gently rinsed in water, and flooded in a solution of 0.1% Uvitex in 0.1M Tris/HCl buffer for 3 min. Finally the segments were immersed in 25% glycerol solution for 5 min. Countings and measurements were made by using an epifluorescence microscope (Zeiss Axiophot, exciter filter BP 395-440, chromatic beam splitter FT 460 nm and barrier filter LP 420).

The efficiency of wax removal was checked by examining one leaf segment per plant accession and per wax treatment under cryo-scanning electron microscopy as described in Rubiales and Niks (1996).

Orientation of the germ tubes: In one trial, four leaves per accession of the accessions H7, H46 and H47 were inoculated in two consecutive replications where we measured:
-Direction of emergence of germ tube from the uredospore to determine if orientation at germination already is towards the stomatal rows, i.e. transverse direction (Fig.1).
-Length of first five lateral growings from the germ tube (Fig.1). These lateral growings develop above the lateral junctions of the epidermal cells. Where two of such lateral growings emerged opposite to each other, the longest was measured.
-Minimal distance from germ tube passing a stoma in the first row of stomata reached (Fig. 1), to determine if there is an active attraction by the stoma.

In a second trial four leaves per accession of the accessions H7, H46 and H47 were inoculated with and without the wax removal treatment where we measured:
-Length of germ tube from urediniospore to the first stoma reached.
-Length and width of the projection of the germ tube on the long axis of the leaf.

Measurements were performed on 20 germlings per leaf segment. Averages over the 20 germlings measured per leaf were used for statistical analysis.

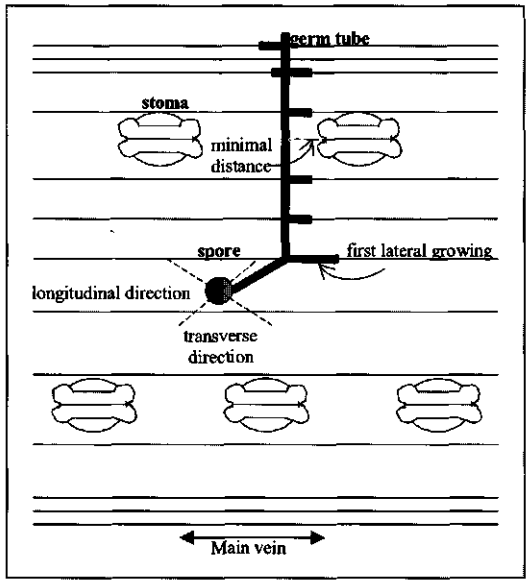

Figure 1. *Hordeum chilense* leaf surface in which are indicated possible directions of *Puccinia hordei* spore germination, germ tube lateral growings over epidermal cell junctions and the minimal distance from germ tube to stoma.

Appressorium differentiation by the germ tubes: Four leaves per accession of all the *H. chilense* accessions were inoculated with and without the wax removal treatment, in two consecutive replications. The following observations were recorded:

-Percentage of germ tubes not reaching any stoma (in 100 germlings).

-Percentage of germ tube/stoma encounters resulting in appressorium differentiation (in 100 germlings).

-Percentage of germ tubes that form an appressorium (in 100 germlings).

Statistical analysis was performed on the percentages of germ tubes per leaf.

Cell dimensions on stomatal complex: Width of guard and subsidiary cells of 20 stomata (10 overgrown, 10 with appressorium differentiation) per leaf segment was

measured on the four H75 leaf segments where no wax had been removed (Fig. 2). Measurements were done by eyepiece micrometer at 1000x magnification.

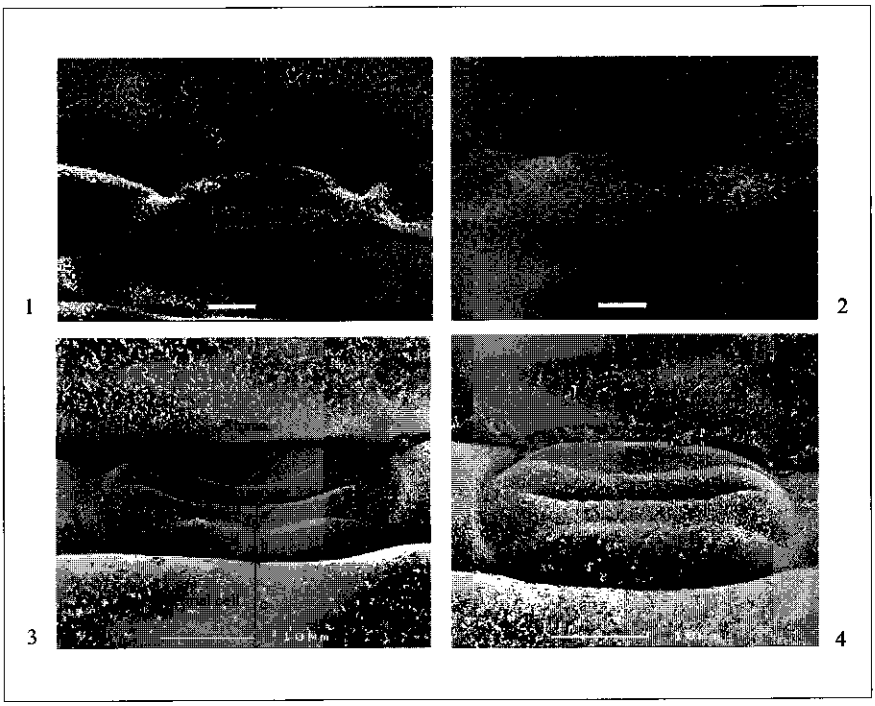

Figure 2. Ultrastructure of stomata of *Hordeum chilense* line H1 (low avoidance) [control (1) and wax removal (3)], and line H7 (high avoidance) [control (2) and wax removal (4)]. On photo 3, indication of measurements done: a= guard cell width, b= subsidiary cell width and c= adjacent epidermal cell width. Note absence of a regular lattice on the wax crystal microstructure covering stoma on *Hordeum chilense* leaf surface (Scale bars, 10 μm)

Results

Orientated-growth studies on the abaxial leaf surface

The adaxial leaf surface of *H. chilense* is much more ridged than the abaxial leaf surface. Since the ridged structure of the adaxial epidermis could compromise

orientation measurements, we decided to use the flat abaxial leaf surface to study the orientation of germ tubes.

On the abaxial surface, germ tubes showed very pronounced directional growth, perpendicular to the epidermal cell junctions (Fig. 3).

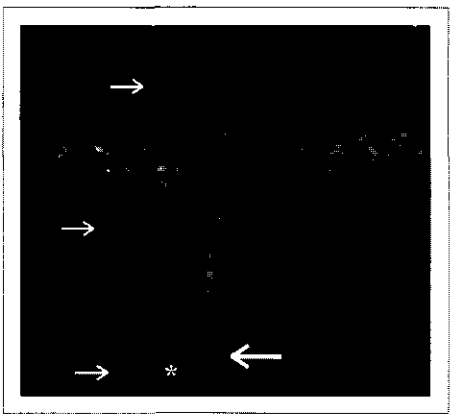

Figure 3. Marked directional growth of the germ tubes of *Puccinia hordei* perpendicular to the epidermal cell junctions on *Hordeum chilense* (H47) leaf surface. Germ tube grows from spore (*) showing a first lateral growing (←) longer than the subsequent lateral growings. Note that the first lateral growing marks the beginning of transverse orientation of the germ tube. Narrow arrow indicates stoma row.

Of all the 630 spores observed in the two repetitions, 328 started the germination in a transversal direction. This is was not significantly more than 50% of the spores. This implies that the direction of germination of the spores occurs in a random way, and is not influenced by features of the leaf.

The first encounter of the germ tube to an epidermal cell junction induced a significantly longer lateral growing than the subsequent encounters (Table 1). It appeared that the first encounter was crucial in the transition from the random germ tube growth to orientated growth perpendicular to the long axis of the leaf (Fig. 3). Germ tubes that failed to form lateral growings appeared to be poorly attached to the leaf surface. Once germ tubes grew perpendicular to the long axis of the leaf, they would either pass one or more stomatal rows, or encounter a stoma on which it might form an appressorium.

Table 1. Length (μm) of the first five germ tube lateral growings (LG) of *Puccinia hordei* on three *Hordeum chilense* and on cultivated barley (L94) leaf surface.

LG	H7	H46	H47	L94
1	23^b	21^b	23^b	24^b
2	8^a	12^a	10^a	17^a
3	6^a	10^a	10^a	17^a
4	8^a	10^a	12^a	18^a
5	7^a	10^a	12^a	16^a

Each value is the average of measurements on 20 germlings on four leaves per accession in two repetitions. Values with letter in common in each column are not statistically significant ($p<0.05$), Duncan test.

Germ tubes did not seem to change direction in order to reach a nearby stoma. Stomata that were encountered, typically were in the way of the straight growing germtube. Germ tubes could pass a stoma even at a very short distance (<1μm) without changing direction towards that stoma.

Effect of wax amount

The SEM photographs indicate that the celloidin treatment removed satisfactorily the wax layer from the leaf surface (Fig. 2).

The wax removal did not significantly change the percentage of germ tubes that failed to reach a stoma in all the accessions (Table 2).

The length of the germ tube till it reached the first stoma was not different on the leaves from which the wax had been removed (Table 3). This implies that wax removal did not result in a more erratic growth direction of the germ tubes. This conclusion can also be drawn from the lack of effect of wax removal on the length/width projection of the germ tube (Table 3). Germ tubes tend to grow at right angles to the long axis of the leaf and the projection of the germ tube on the long axis of the leaf is referred to as width. The ratio length/width of the projection of the germ tube would be expected to be high in the case of normal orientation. In the case of disoriented germ tube growth the average ratio would be close to 1.

We conclude that in general the cuticular wax does not provide essential clues for the germ tube to find the stomata.

Table 2. Germ tube growth and appressorium formation of *Puccinia hordei* on leaves of different *Hordeum chilense* accessions.

	Germ tube/stoma encounters with appressorium differentiation (%)[#]		Germlings not reaching any stoma (%)[#]	
	Control	Wax removal	Control	Wax removal
High avoidance accessions				
H250	3.3^{a}	66.7^{**a}	15.0^{bc}	11.6^{ab}
H47	9.3^{ab}	65.2^{**a}	12.7^{b}	13.0^{ab}
H46	15.1^{bc}	65.6^{**a}	7.1^{a}	10.4^{a}
H7	20.3^{c}	72.1^{**ab}	22.1^{d}	16.6^{bc}
Low avoidance accessions				
H75	55.9^{d}	74.7^{*ab}	17.8^{cd}	15.6^{abc}
H211	68.1^{e}	67.0^{a}	15.0^{bc}	16.6^{bc}
H1	75.4^{f}	72.0^{ab}	22.1^{d}	16.0^{abc}
H304	85.4^{g}	79.4^{b}	21.9^{d}	21.2^{c}

[#] Each value is the average of counts on at least 100 germ tubes or 100 stoma/germ tube encounters each on four leaves per accession in two repetitions. Half segment of each leaf was treated to remove the wax layer, while the other half was left untreated as a control. Differences between control and wax removal are statistically significant at 0.05 (*) or at 0.01 (**) level (ANOVA). Values with the same letter per column are not significantly different (Duncan test, $p<0.05$).

Table 3. Germ tube growth of *Puccinia hordei* on three *Hordeum chilense* and on cultivated barley (L94) leaf surface without (control) and with treatment to remove the wax layer.

	Germ tube length from spore to first stoma (μm)		Ratio length/width of germ tube projection	
accessions	Control	Wax removal	Control	Wax removal
H47	50	53^{ns}	11	11^{ns}
H46	50	51^{ns}	11	9^{ns}
H7	52	57^{ns}	10	11^{ns}
L94	57	57^{ns}	9	7^{ns}

[a]Each value is the average of measurements on 20 germlings on four leaves per accession. Half segment of each leaf was treated to remove the wax layer, while the other half was left untreated as a control.
*Differences between control and treatment statistically significant at 0.05 level (ANOVA). ns= no significant difference.

On the control leaf segments, *H. chilense* high avoidance accessions H250, H47, H46 and H7 showed a low percentage of germ tube/stoma encounters with differentiation of appressorium (12% average). On these accessions removal of the wax resulted in a sixfold and significant increase in appressorium differentiation (Table 2). Apparently, the removal of the wax layer allowed appressorium development over stomata that would otherwise be overgrown. Compared with the high avoidance accessions, the low avoidance accessions H211, H1 and H304 had a six to sevenfold

higher percentage of encounters leading to appressorium differentiation (68 to 85 %). Removal of the wax in these accessions did not increase the appressorium differentiation. After wax removal all accessions had a similar percentage of germ tube/stoma encounters leading to appressorium formation (Table 2).

Effect of cell dimensions on stomatal complex

On *H. chilense* accession H75 control leaf segments had an overgrowth of stomata (44%) about as common as appressorium differentiation (56%) per germ tube/stoma encounter (Table 3). Wax removal increased this appressorium differentiation to 75%. In this accession no significant differences were observed on the stomatal cell dimensions between stomata overgrown and stomata where an appressorium was differentiated (Table 4).

Table 4. Cell widths (µm) of stomata of *Hordeum chilense* accession H75, that were overgrown or on which an appressorium was formed by *Puccinia hordei*.

Dimensions	Overgrown	Appressorium formed
width of guard cells	4.0[a]	3.9[ns]
width of subsidiary cells	5.8	5.7[ns]
width of adjacent epidermal cell	11.8	10.8[ns]

[a] Each value is the average of measurements on 10 stomata on four leaves. Differences in dimensions between overgrown stomata and stomata on which an appressorium was formed are statistically not significant at 0.05 level (T-test).

Discussion

Germ tubes of several rust fungi have been shown to grow perpendicular to the epidermal cells junctions of the leaf surface (Wynn, 1976; Read *et al.*, 1997) or at right angles to the structural lines of artificial membranes (Wynn, 1976; Staples *et al.*, 1983, Hoch *et al.*, 1987; Read *et al.*, 1997). This growth is directed by physical contact of the growing tip with the surface. Close adherence is needed (Allen *et al.*, 1991a). It is supposed that this particular way of orientation permits that the germ tube can hardly miss a stoma if it grows far enough because of the arrangement of the stomata in the

leaf surface (Wynn, 1976). In addition, it is in the interest of the rust fungus to reach the stoma as efficiently as possible. Spending energy in germ tube growth will decrease the chance of success in the further infection process. Niks (1990) showed that the longer the germ tube length between spore and first stoma reached, the smaller the chance that the infection unit will establish a colony.

The present study was undertaken to understand if the germ tube growth of *P. hordei* on a *H. chilense* leaf surface is directed towards stomata or if it is merely perpendicular to the long axis of the leaf, and how the wax layer can affect the orientation and the appressorium formation.

For this propose we used leaves rather than artificial membranes to avoid imperfect reproduction of host physical structures, notably the cuticular wax crystals (Collins & Read, 1997).

From the results of this study, growth of the germ tube started in a random direction at spore germination, until it contacted an epidermal cell junction. Since the outer walls of the leaf epidermis are convex, a sharp downward longitudinal fold occurs over the junction between epidermal cells. This longitudinal fold seems to induce a lateral branching of the germ tube that follows the fold. This event appears to be associated with the germ tube taking a growth direction perpendicular to this first cell junction.

The first lateral growing was in general longer than the subsequent lateral growings. Our data suggest that the first, longer, lateral growing will initiate the orientation, while the subsequent, shorter, growings serve to maintain the direction of the growth.

Contrary to the positive attraction reported by Edwards and Bowling (1986) on the broad bean rust fungus, in our study there was no evidence of active germ tube growth to stomata. Germ tubes growing in the proximity of a stoma did not converge to it as would be expected if they were responding to stimuli from that stoma. Germ tubes did not necessarily grow towards the nearest stomata and, even when a spore germinated adjacent to a stoma, the germ tube might grow in the opposite direction.

According to Jenks and Ashworth (1999) or Lewis and Day (1972), the wax crystal microstructure might influence the orientation of germ tube growth. If this is the case, the wax removal should alter for better or for worse the orientation of the germ

tube growth. Nevertheless, no improving or worsening of germ tube orientation was observed with the removal of the wax layer. The scanning electron microscopy photos showed that the wax crystals covered the whole leaf surface except the hairs. These wax crystals were not arranged according to a regular lattice (Fig. 2), as suggested by Lewis and Day (1972) would be the case, for wheat. Because of the apparently random distribution of wax crystals, it was not surprising that the wax layer did not improve the orientation of the germ tube growth.

Rubiales and Niks (1996) studied a subset of these *H. chilense* accessions using the percentage of germ tubes forming appressoria on leaves as avoidance parameter. They observed that the wax removal treatment resulted in a substantial increase in appressorium differentiation on high avoidance accessions but not to the level of the untreated low avoidance accessions. They also reported a significant reduction of appressorium differentiation on the low avoidance accessions. They presumed that these results were due to poor germ tube orientation and suggested that the wax layer plays a role in the orientation of germ tube growth across the leaf towards the stomata (Rubiales & Niks, 1996). In none of the accessions used in our study, the wax removal significantly changed the percentage of germ tubes that failed to reach a stoma (Table 2). This is an indication, contrary to what has been suggested by Rubiales and Niks (1996), that the leaf wax layer does not contribute to nor hampers the orientation of the germ tube.

In the present study and using the same avoidance parameter as Rubiales and Niks (1996) (data not shown) wax removal resulted in a complete restoration of appressorium differentiation on high avoidance accessions and not in a significant reduction in appressorium differentiation on the low avoidance accessions. Only on the low avoidance accession H211 the wax removal reduced significantly (P=0.05) the appressorium differentiation. As no effect of the wax layer on the orientation of germ tubes was observed in our work, the differences between our results and the results of Rubiales and Niks (1996) on appressorium differentiation are possibly due to a technical artefact.

Read *et al.* (1997), using artificial membranes and the fungus *P. hordei*, found that the optimal differentiation signal were multiple, 2.0 μm high ridges, that were closely spaced (1.5 μm). This spacing does not correspond with the spacing of adjacent

cell walls in the stomatal complex. The widths of the guard cells and of the subsidiary cells reported in the present work were about 4.0 and 5.8 μm respectively. Unfortunately Read *et al.* (1997) did not use ridge spacings covering the cell widths reported here (4.0 to 5.8). Therefore it remains to be investigated whether the spacing of cell junctions of *H. chilense* stomatal complexes are the inductive signal to trigger appressorium differentiation.

From this study we can conclude that orientated growth of the germ tubes starts only upon contact with the epidermal cell junctions. The germ tube develops lateral growings over the epidermal cell junction that may help the germ tube to grow along the transverse axis of the leaf. No evidence was found of attraction of the germ tube by stomata. Removal of the cuticular wax layer did not result in poor germ tube orientation. This suggests that the leaf wax layer has no role in the orientation of the germ tube.

We also suggest that the overgrowth of stomata by the rust germ tube was mainly due to the more extensive wax covering of the stomatal apparatus on the high avoidance accessions rather than to differences in the stomatal cell dimensions (width). In the *H. chilense* high avoidance accessions the epicuticular waxes were probably covering the appressorium inducer structure of the stoma, reducing the chance for effective appressorium induction. On the low avoidance accessions, where the wax covering was not so extensive, the inducer structures of the stoma were more exposed to the germ tube tip increasing the probability of differentiation of an appressorium.

An intriguing question to be addressed in the future is what are the precise features of the stoma in *H. chilense* that induce the appressorium differentiation of rust fungi.

Acknowledgements

We thank Pim Lindhout for critically reviewing the manuscript. This research was supported by PRAXIS XXI, Fundação para a Ciência e a Tecnologia, Lisboa, Portugal (research fellowship number BD/9124/96).

4

AFLP markers in *Hordeum chilense*: A highly polymorphic species[1]

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[1]Published in Proceedings of the 8th International Barley Genetic Symposium. Adelaide, Australia, 2000. pp. 98-99.

Abstract

A molecular map of the wild barley *Hordeum chilense* Roem. & Schult. would greatly facilitate to map and efficiently transfer agronomic traits from *H. chilense* to cereal genomes. As a first step towards a map construction, we evaluated AFLP markers in *H. chilense*. We compared *Eco*RI/*Mse*I and *Pst*I/*Mse*I AFLP fingerprints among three *H. chilense* accessions (H1, H7 and H47) that represent two distinct ecotypes. One accession of *Triticum monococcum* spp. *monococcum*, one of *T. monococcum* spp. *boeoticum* and one of cultivated barley (*H. vulgare*) were included as reference.

*Eco*RI/*Mse*I AFLP fingerprints revealed more polymorphisms than *Pst*I/*Mse*I AFLP fingerprints for all species tested. *H. chilense* showed a higher percentage of polymorphisms than *T. monococcum* for both *Eco*RI/*Mse*I and *Pst*I/*Mse*I AFLP fingerprints (59 and 39% in *H. chilense* against 36 and 25% in diploid wheat respectively).

It was remarkable that, based on AFLP markers generated using 12 *Eco*RI/*Mse*I primer combinations, the cultivated barley was more similar to diploid wheat than to *H. chilense*.

Even more surprisingly, the genetic distance between the interfertile *H. chilense* accessions (H1 and H7) was almost as large as the genetic distance between the non-crossable cultivated barley and diploid wheat.

Key words: *Hordeum chilense*, AFLP markers, *Triticum monococcum*, *Hordeum vulgare*.

Introduction

In the search for durable resistance mechanisms in general and for leaf rust resistance in particular wild relatives of wheat and barley are investigated at our laboratory. *Hordeum chilense* Roem. & Schult. is a wild barley, native to South America. It shows many interesting agronomic traits, including resistance to several cereal pathogens. Its good crossability with wheat, rye and cultivated barley confers *H. chilense* a high potential for cereal breeding (Martín et al., 1998).

One of the interesting traits of *H. chilense* is the avoidance mechanism to leaf rust (Rubiales & Niks, 1992b). The avoidance mechanism is inherited in a complex way. Research is in progress to map QTLs for this trait on an AFLP-based genetic map. The map locations of these QTLs will facilitate the transfer of this trait to other cereal genomes.

AFLP (Vos et al., 1995) are widely used in almost all important crop species and with diverse objectives. AFLP were first applied in genetic mapping studies (Van Eck et al., 1995; Qi et al., 1998), but has proven also to be a competent tool in taxonomic studies (Schut et al., 1997; Kardolus et al., 1998).

The objective of the work presented here was to evaluate the AFLP polymorphism in *H. chilense* comparing *Eco*RI/*Mse*I and *Pst*I/*Mse*I AFLP fingerprints among *H. chilense* accessions that represent two distinct ecotypes.

Material and methods

H. chilense accessions were selected from two distinct ecotypes in order to maximise the chance for genetic variation in *H. chilense* (Vaz Patto et al., 2001a). *H. chilense* accessions H7 and H47 showed a high level of avoidance and *H. chilense* accession H1 a low level of avoidance. Two diploid wheat subspecies (*Triticum monococcum* spp. *monococcum* "Einkorn 1,3" (E1) and *T. monococcum* spp. *boeoticum* "1486") and cultivated barley (*H. vulgare*) "L94" were included as reference.

AFLP procedures according to Vos et al. (1995) were performed using 12 *Eco*RI/*Mse*I based primer combinations (+3+3) on all accessions and in addition 8

*Pst*I/*Mse*I based primer combinations (+2+3) on H1, H7, E1 and 1486. AFLP amplification products in the size range of 100 to 500 N were clearly separated on a poly-acrylamide gel and evaluated for polymorphisms as described by Qi and Lindhout (1997).

Genetic distances were calculated from *Eco*RI/*Mse*I data using the Jaccard coefficient (Jaccard, 1908) and the UPGMA clustering method in the software package Treecon.

Results and discussion

In general, *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations both generated informative AFLP fingerprints from *H. chilense* and *T. monococcum*. Using *Eco*RI/*Mse*I more polymorphisms were generated as compared to *Pst*I/*Mse*I for both species (Table 1). *H. chilense* showed a higher percentage of polymorphisms than *T. monococcum* for both *Eco*RI/*Mse*I and *Pst*I/*Mse*I AFLP fingerprints, 59 and 39% in *H. chilense* against 36 and 25% in diploid wheat respectively (Table 2).

Table 1. Percentage of AFLP polymorphisms per primer combination in *T. monococcum* and *H. chilense*.

T. monococcum	*H. chilense* (% polymorphisms)			
(% polymorphisms)	20-35%	36-40%	41-55%	56-71%
>20%	P11/M61	E35/M51		
20-35%	P11/M54	P14/M61	E35/M48	E32/M55
	P13/M49	P14/M54	P11/M49	E32/M61
			P13/M48	E32/M62
				E35/M55
36-40%			E32/M54	E38/M55
			P15/M49	E42/M54
41-55%				E35/M54
				E35/M61
56-71%				E35/M49

Note: Primer combination nomenclature as described in Qi and Lindhout (1997) and Haanstra et al. (1999). Data obtained from two accessions of *H. chilense* (H1 and H7) and two accessions of *T. monococcum* (E1 and 1486)

Table 2. Average number of amplification products, percentage of polymorphisms and markers per enzyme (*Pst*I/*Mse*I: P and *Eco*RI/*Mse*I: E) in *T. monococcum* (T) and *H. chilense* (H).

	# of amplification products		% polymorphisms		# markers/primer combination	
	T (n=2)	H (n=2)	T	H	T	H
P (n=8)	66 (± 12)	56 (± 14)	25 (± 8)	39 (± 7)	16 (± 8)	22 (± 7)
E (n=12)	48 (± 17)	56 (± 13)	36 (± 11)	59 (± 10)	17 (± 9)	33 (± 9)

The average number of markers found (Table 2) is similar to that reported Qi and Lindhout (1997) in cultivated barley. Using 25 *Eco*RI/*Mse*I primer combinations they found on average 22 markers per primer combination.

Primer combinations that are efficient in *H. chilense* tend also to be efficient in *T. monococcum* (Table 1).

It was remarkable that, based on 12 *Eco*RI/*Mse*I primer combinations (1366 *Eco*RI/*Mse*I markers), the cultivated barley was more similar to diploid wheat than to *H. chilense* (Figure 1).

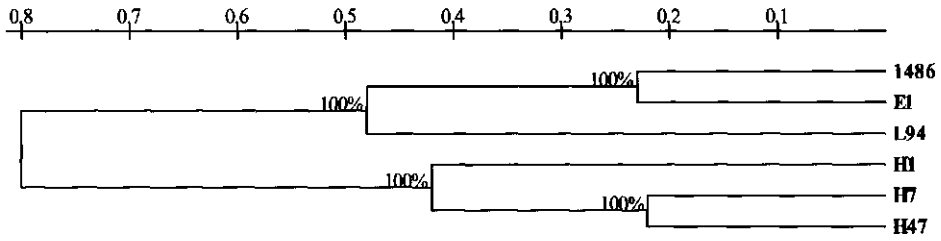

Figure 1. Genetic distances (Jaccard coefficient) between *H. chilense* H1, H7, H47, *H. vulgare* L94 and *T. monococcum* 1486, E1 based on 1366 *Eco*RI/*Mse*I markers. The bootstrap values for each branching were always 100% indicating a stable tree.

Even more surprisingly, the dissimilarity between the interfertile *H. chilense* accessions (H1 and H7) was almost as large as the dissimilarity between the non-crossable cultivated barley and diploid wheat.

Based on the number of amplification products and the polymorphism rate, 15 *Eco*RI/*Mse*I primer combinations were selected that together should provide about 450 AFLP markers, between H1 and H7. For the map construction these 15 selected *Eco*RI/*Mse*I primer combinations were used on 100 F_2 plants, resulting from the cross between genetically remote accessions H1 and H7. This molecular map will be used to locate QTLs for avoidance and other interesting traits in *H. chilense*.

Acknowledgments

We would like to thank Fien Meijer-Dekens and Petra van den Berg for the technical advice on AFLP analysis. This research was supported by PRAXIS XXI, Fundação para a Ciência e a Tecnologia, Lisboa, Portugal (research fellowship number BD/9124/96) and by NWO-STW, The Netherlands (project number WBI 44-3288).

5

A genetic linkage map of wild barley (*Hordeum chilense*) based on amplified fragment length polymorphisms

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Abstract

Hordeum chilense Roem. & Schult. (2n=2x=14) has high potential for cereal breeding, because of its agronomic interesting characteristics and its good crossability with other members of the *Triticeae* tribe. A first linkage map of this wild barley using amplified fragment length polymorphism (AFLP) was generated. The mapping population consisted of 100 F_2 plants derived from a cross between two genetically distinct *H. chilense* accessions (H1 and H7). A total of 15 primer combinations resulted in 463 polymorphic markers, most of which were dominant (presence or absence of a band), although 21 (5%) of them could be scored co-dominantly. 151 markers (33%) showed significant deviation from the expected monogenic segregation ratio. Seventy-nine markers showed complete linkage with at least one other marker. This resulted in 443 loci, of which 364 were unique, on nine long and ten shorter linkage groups, covering 714 cM. Twenty markers were not linked to any of these linkage groups. Nine of these linkage groups were assigned to *H. chilense* chromosomes using a set of *H. chilense* in *Triticum aestivum* addition and substitution lines. The average density of markers was approximately one per 2 cM. Strong clustering of AFLP markers was observed at putative centromeric regions.

Our map represents an initial step towards mapping of genes or quantitative trait loci for diverse agronomic traits in *H. chilense* and will increase transfer efficiency of these agronomic traits from *H. chilense* to cereal genomes.

Key words: AFLP, genetic linkage map, *Hordeum chilense*, wild barley

Introduction

Hordeum chilense Roem. & Schult. is a mainly autogamouos, diploid, perennial, wild barley species. Its agronomic interesting characteristics and its good crossability, producing chromosomally stable hybrids with wheat, rye and also cultivated barley (Martín & Chapman 1977; Martín & Cubero 1981; Martín et al. 1998) confer it as having high potential for cereal breeding purposes. However, the only fertile amphiploid obtained from these three hybrids is the one with wheat, tritordeum. This amphiploid represents the basic genetic material for using the *H. chilense* germplasm for wheat and triticale breeding (Martín et al. 1998). In *H. chilense,* resistance has been identified to powdery mildew, *Blumeria graminis* (Rubiales et al. 1993), to *Septoria tritici* (Rubiales et al. 1992), to common and karnal bunt (Rubiales et al. 1996b; Martín et al. 1998), to the smuts *Ustilago nuda* and *U. tritici* (Nielsen 1987), to *Pyrenophora teres* and *P. tritici-repentis* and to *Rhynchosporium secalis* (Rubiales, unpublished, 1998). *H. chilense* is also known to possess resistance to the aphids *Diuraphis noxia* (Clement & Lester 1990), *Rhopalosiphum padi* (Weibull 1987) and to the greenbug *Schizaphis graminum* (Castro et al. 1994) and to nematodes *Meloidogyne* spp (Person-Dedryver et al. 1990; Jensen & Griffin 1994). Moreover, also avoidance of rust infection was detected in *H. chilense* and has proven to be effective against several rust species such as *Puccinia hordei, P. triticina, P. recondita agropyrina* and *P. recondita recondita* (Rubiales & Niks 1992a and b, 1996). Finally, *H. chilense* has also been suggested as a source of salt and drought tolerance and high carotenoid content (Gallardo & Fereres 1989; Foster et al. 1990; Alvarez et al. 1998).

A molecular map of *H. chilense* would greatly facilitate the mapping and efficiently transfer of agronomic traits from *H. chilense* to cereal genomes. Amplified fragment length polymorphisms (AFLP, Vos et al. 1995), which combine the reliability of the RFLP technique with the power of the PCR technique, have been successfully used in the construction of genetic linkage maps for several plant species (e.g. Becker et al. 1995; Van Eck et al. 1995; Keim et al. 1997; Qi & Lindhout 1997; Wang et al. 1997; Bert et al. 1999; Haanstra et al. 1999; Remington et al. 1999).

Here, we describe the construction of the first genetic linkage map of *Hordeum chilense* based on AFLP markers. A skeletal map with a more even distribution of markers can be extracted from this map and applied to map genes and to identify QTLs.

Material and methods

Plant materials

The mapping population consisted of 100 F_2 plants obtained from a cross of H1xH7, two genetically distant *H. chilense* accessions (Vaz Patto et al. 2001a) in order to maximise the genetic variation. *H. chilense* accession H7 shows a high, and accession H1 a low, level of avoidance of rust fungi (Rubiales & Niks, 1996). *H. chilense* accessions H 1 and H7 show contrasting reactions to several cereal pathogens as *Puccinia hordei*, *P. striiformis*, *P. graminis* and *Fusarium culmorum* (Rubiales & Niks, 1992b; Rubiales et al., 1996b).

The genome symbol of *H. chilense* is H^{ch} (Wang et al. 1994). For the assignment of the linkage groups to specific *H. chilense* chromosomes we used various genetic stocks from the wheat cv Chinese Spring supplied by S. M. Reader from the John Innes Centre, Norwich, UK: 1) a set of disomic addition lines with *H. chilense* chromosomes $4H^{ch}$, $5H^{ch}$, $6H^{ch}$ and $7H^{ch}$, 2) a ditelosomic addition line for the $2H^{ch}$ α arm, 3) a substitution line of chromosome 1A for $1H^{ch}$ and the parents Chinese Spring and H1 *H. chilense* accession. Chromosome numbers of these genetic stocks were checked using Feulgen-stained seedling root tip squashes.

The AFLP protocol

DNA was extracted from leaf tissue of not fully expanded leaves, according to the CTAB-method (Van der Beek et al. 1992). The AFLP procedure was performed as described by Vos et al. (1995) with modifications by Qi and Lindhout (1997).

A total of 32 *Eco*RI/*Mse*I (+3/+3) primer combinations, chosen from Qi and Lindhout (1997), were applied to AFLP fingerprint the parental lines (H1 and H7) in order to select the most informative primer combinations. This resulted in the following

15 primer combinations E32M55, E32M61, E32M62, E33M55, E35M48, E35M49, E35M54, E35M55, E38M54, E38M55, E38M59, E38M61, E42M47, E42M48, E42M50, that were applied on the mapping population and on the set of addition and substitution lines. Primer core sequences were according to Vos et al. (1995). AFLP bands detected by radioactive labelling.

Data analysis and map construction

Clearly readable amplified fragments were visually scored predominantly as dominant genetic markers, i.e. as presence or absence of the band. To identify possible band pairs that could be used as codominant markers, we followed the same genetic criteria as in Alonso-Blanco et al. (1998). Two AFLP bands are allelic when they are derived from two different parents, generated by the same primer combination and segregate as one locus, i. e. in all progeny one or both parental bands are present, but in none both bands are absent. In addition to this, both bands in the putative heterozygous individuals should have weaker intensity than the single band in the putative homozygous individuals.

To ensure accuracy, we scored all AFLP markers at least twice independently and ambiguous marker bands were recorded as unknown in the data set.

The AFLP amplification products were designated according to Qi and Lindhout (1997).

Linkage analysis and segregation distortion tests ($P \leq 0.05$) were performed using JoinMap version 2.0 (Stam 1993; Stam & Van Ooijen 1995). The determination of linkage groups of markers was done with a LOD score of 5.5. The calculations of the linkage maps were done using all pairwise recombination estimates smaller than 0.45 and LOD score larger than 0.1 and using the Kosambi mapping function (Kosambi 1944).

When no co-dominant markers were present in a linkage group, the correspondence of H7 linkage groups to H1 linkage groups was based on the highest LOD scores of the linkage of markers in H7 linkage groups with markers in H1 linkage groups.

Linkage groups were assigned to the corresponding *H. chilense* chromosome using H1 addition and substitution lines of wheat. Bands were considered specific for *H. chilense* chromosomes if they were present in one of the addition lines and in H1, but absent in *T. aestivum* parent Chinese Spring and absent in H7. In this way, linkage groups of linked the AFLP markers were assigned to the specific *H. chilense* chromosome.

Results

DNA marker generation and data scoring

As a first step required to construct a linkage map we screened the segregating population's parental lines for polymorphism. For the 15 selected primer combinations, the number of amplification products varied from 57 to 127, while the number of clearly polymorphic bands ranged from 18 (E38M55) to 47 (E42M48) (Table 1). In total, 463 clear AFLP markers were identified, corresponding to an average of 31 markers per primer combination. Among these 463 markers, 223 were H1 specific and 219 were H7 specific and scored dominantly. The twenty-one markers left satisfied the co-dominant genetic criteria (see material and methods), and in all cases the molecular size of the two allelic bands differed only in 1 or 2 bases, except for three pairs differing in 10, 25 and 50 bases. In these markers, the putative heterozygous individuals showed both bands with consistently weaker intensity than the individuals containing only one band (Fig. 1). These pairs of AFLP marker bands were considered single locus markers, their names being composed of both allelic fragment sizes, although true allelism should be confirmed by sequence analysis.

It appeared that 79 markers showed complete linkage with at least one other marker. This was found for 28 loci that consisted of two to 14 markers.

Table 1.Total number of AFLP markers generated per primer pair using 15 *Eco*RI/*Mse*I (+3+3) primer combinations, including unlinked markers and mapped markers, with their assignment to individual linkage groups.

Primer pairs	Total markers	Unlinked markers			Mapped markers			Linkage groups[b]											
		H1[a]	H7	Codo	H1	H7	Codo	1	2a	2b	3'	4a	4b	4c	5	5"	6	7	7"	A
E32M55	33	0	0	0	14	18	1	4	2	0	5	3	2	1	1	0	4	6	2	3
E32M61	31	1	0	0	15	13	2	6	5	0	5	1	0	0	0	2	2	1	5	3
E32M62	29	1	0	0	11	17	0	7	4	1	6	2	0	0	0	1	1	3	3	0
E33M55	32	0	1	0	17	11	3	6	5	0	5	4	1	0	1	0	1	2	4	2
E35M48	34	2	1	0	19	10	2	2	5	1	5	4	0	1	1	0	7	3	2	0
E35M49	27	0	0	0	10	14	3	5	6	0	5	3	0	0	2	0	2	1	0	3
E35M54	38	5	0	0	16	16	1	3	1	1	8	9	0	0	1	1	6	2	0	1
E35M55	26	0	1	0	11	12	2	3	4	0	5	8	0	0	1	0	1	2	1	0
E38M54	23	0	1	0	10	11	1	2	3	1	5	3	2	0	1	0	1	0	2	2
E38M55	18	0	0	0	7	10	1	3	3	0	3	4	0	0	0	0	1	3	1	0
E38M59	36	1	2	0	21	12	0	5	3	2	4	5	0	0	2	1	1	2	3	5
E38M61	24	0	0	0	14	10	0	5	7	0	2	1	1	1	3	0	2	1	0	1
E42M47	31	1	0	0	12	18	0	5	4	0	5	3	0	0	2	2	5	2	1	1
E42M48	47	0	1	0	21	21	4	5	5	0	11	7	0	0	3	0	7	3	2	3
E42M50	33	0	2	0	14	17	1	5	4	0	5	5	0	0	1	0	3	7	1	1
Total	463	11	9	0	212	210	21	66	61	6	79	62	6	3	37	7	44	38	27	25

[a]: H1and H7 stand for dominant specific markers of parental accession H1and H7, respectively; Codo for co-dominant pairs of markers.

[b]: Linkage group numbers indicate the H^{ch} chromosome to which the group is assigned. Lower case letter indicates multiple linkage groups assigned to the same chromosome but not linked in this study. ' and "-indicates presumed chromosomal location. A- indicates markers in seven short linkage groups not assigned to any chromosome.

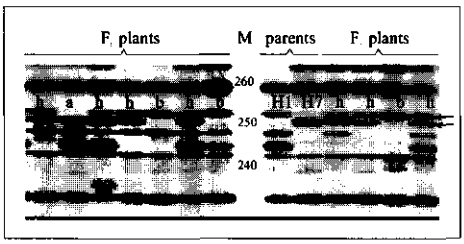

Figure 1. Part of an AFLP fingerprint showing some of the F_2 (H1xH7) plants with the co-dominant marker E35M49-252/254 indicated with arrows. a: homozygous a as on parent H7, only the 252bp-band is amplified with high intensity. b: homozygous b as on parent H1, only the 254bp-band is amplified with high intensity. h: heterozygous, both 252bp- and 254bp-bands are amplified with half of the intensity as the homozygotes. M: 10bp marker.

Map construction

Of the 463 markers, 403 were assigned to 9 long linkage groups (over 30 cM) and 40 markers to ten short groups (shorter than 30 cM) (Table 2 and Fig. 2) when a LOD threshold of 5.5 was chosen for establishing linkage groups. Only 20 markers were not linked to any other marker. The 19 linkage groups were consistent when using higher LOD threshold values.

Five of the nine long groups had co-dominant markers and dominant markers from both parental lines. Of the other four long groups, two had markers only from H1 (designated 5 and 7) and the other two only markers from H7 (designated 5" and 7").

The resulting map contained 443 markers (422 dominant and 21 co-dominant), covering a total map distance of 714.1 cM corresponding to approximately 2 cM per marker (Fig. 2, Table 2).

Inspection of the individual linkage group χ^2 values gives insight into the reliability of the obtained map. The χ^2 values of the majority of the linkage groups were < 1 (data not shown), except for linkage group 1 (χ^2=1.3), linkage group 3 (χ^2=1.6) and linkage group 6 (χ^2=1.8). Given the high densities of markers these χ^2 values indicate that the map is indeed rather reliable.

Table 2. Distribution of markers (443 AFLPs) among the linkage groups of *Hordeum chilense*.

Linkage group	No. markers	Length (cM)	Average distance (cM)	Clustering areas		Chromosome arms	
				No. markers	Distance (cM)[a]	No. markers	Distance (cM)[a]
1	66	101.0	1.5	39	7 (0.18)	27	94 (3.5)
2a	61	40.8	0.7	50	11.5 (0.23)	11	29.3 (2.7)
2b	6	14.0	2.3	-	-	6	14.0 (2.3)
3'	79	77.0	1.0	46	9 (0.20)	33	68 (2.1)
4a	62	38.4	0.6	52	7.7 (0.15)	10	30.7 (3.1)
4b	6	26.8	4.5	-	-	6	26.8 (4.5)
4c	3	10.0	3.3	-	-	3	10 (3.3)
5	19	71.3	3.8	6	2.8 (0.47)	13	68.5 (5.3)
5"	7	54.2	7.7	-	-	7	54.2 (7.7)
6	44	40.6	0.9	35	6.2 (0.18)	9	34.4 (3.8)
7	38	82.6	2.2	25	6.9 (0.28)	13	75.7 (5.8)
7"	27	78.6	2.9	18	4.7 (0.26)	9	73.9 (8.2)
A	25	78.8	3.2	3	0	22	78.8 (3.6)
Total	443	714.1	1.6	274	55.8 (0.20)	169	658.3 (3.9)

[a]. Figures between brackets indicate average markers density inside linkage group. A- indicates markers in seven short linkage groups not assigned to any chromosome.

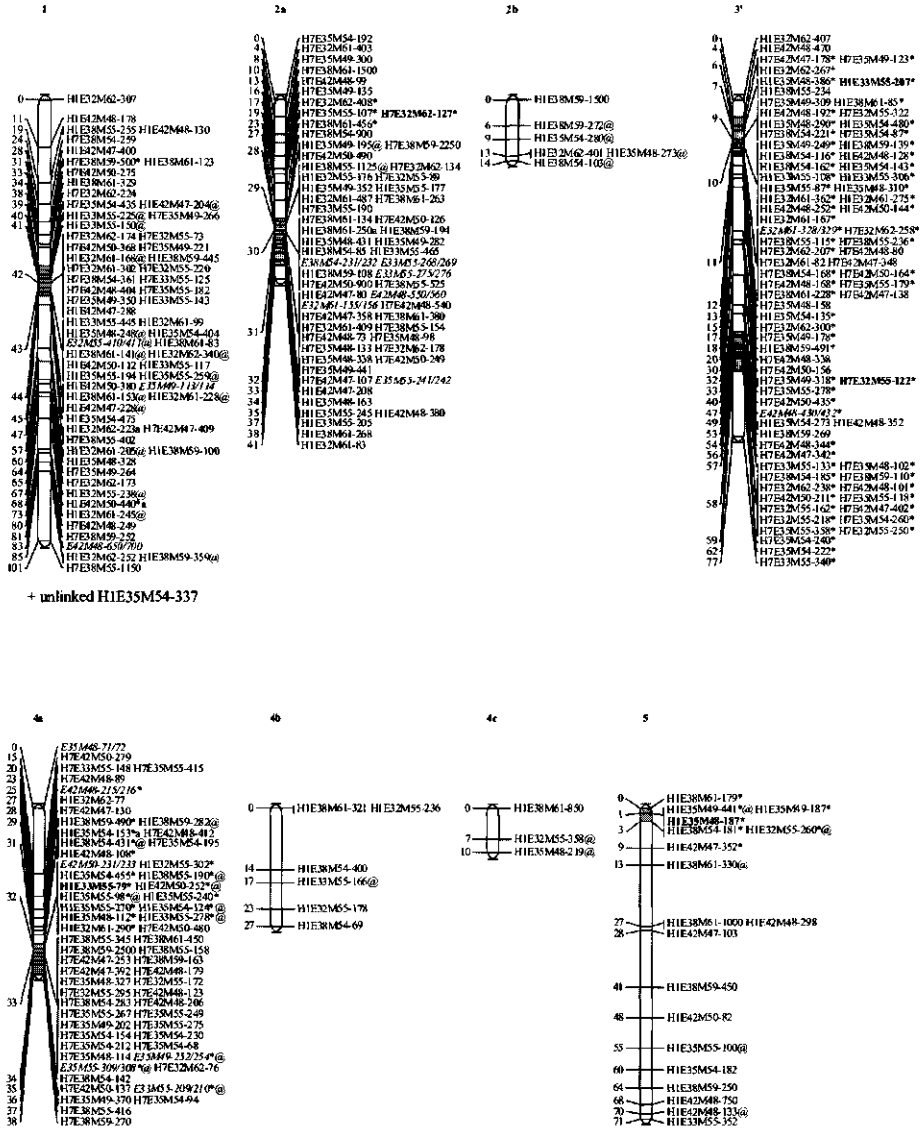

Fig. 2 See next page for legend

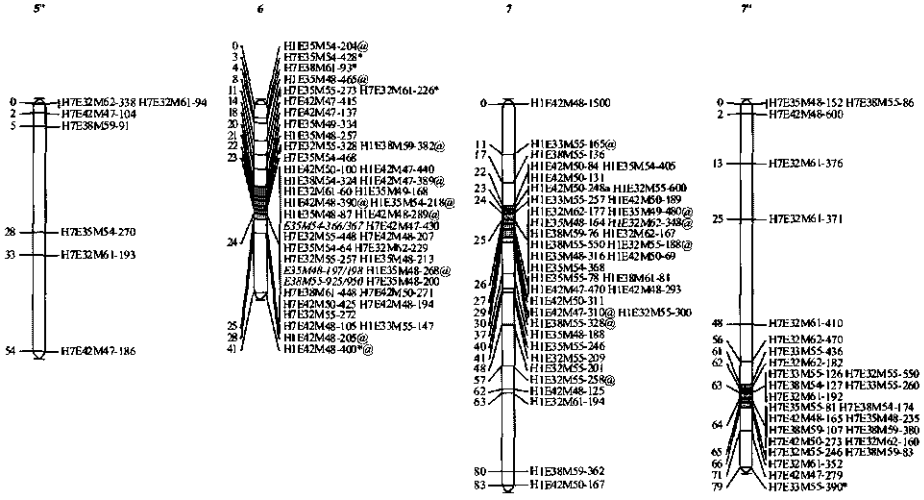

Figure 2. *Hordeum chilense* AFLP linkage map based on an H1x H7 F_2 population. Chromosome assigned linkage groups are designated 1 through 7. Lower case letter indicates multiple linkage groups assigned to the same chromosome but unlinked in this study. Presumed assigned linkage groups are indicated by ' or ". Map distances are given in cM (Kosambi mapping function). @: represents markers unambiguously assigned to a specific chromosome. a: represents doubtful amplification products. Co-dominant markers are designated in italics. *: represents skewed markers ($P \leq 0.005$). Markers with the highest distortion are printed in bold. The solid bars within the chromosomes represent the clusters of markers in the putative centromeric regions.

AFLP analysis of addition lines and assignment of groups to specific chromosomes

Of the 223 dominant specific H1 plus the 21 co-dominant markers, 63 were not present in Chinese Spring but occurred in one of the addition or substitution lines, and hence could serve to assign a linkage group to a chromosome (Fig. 2). Sixty H1 markers could not be used since they co-migrated with an amplification product in Chinese Spring. 121 H1 markers were also not used since they were not clearly visible on the fingerprints of the addition and substitution lines. Per substitution or addition line almost all informative markers belonged to the same linkage group. Only 5 exceptions were detected that may represent doubtful amplification products (Fig. 2). As only markers assigned to the same chromosome could be present in the same linkage group this was also a check for the accuracy of the composition of linkage groups.

The AFLP analysis of the set of addition and substitution lines allowed assignment of six long linkage groups and three short groups to specific *H. chilense* chromosomes. Groups with only H7 markers were tentatively combined with groups with only H1 markers using lower LOD scores. In this way, long group 7" and long group 7 both represent chromosome 7 and long group 5" and long group 5, represented chromosome 5.

Only one of the long linkage groups could not be assigned to one of the seven chromosomes of *H. chilense* since none of the H1 markers on that linkage group were found to occur in any of the addition and substitution lines. This linkage group covered at least 77 cM, and should represent either chromosome 3 or the β arm of chromosome 2, since no addition or substitution lines were available for these. However, as a long and a short group had already been assigned to the α arm of chromosome 2, covering a total of 55 cM, we believe that the length of the linkage group that was not assigned suggests that it probably corresponds to chromosome 3 H^{ch}. We refer to this group as group 3'.

Clustering of AFLP markers

The distribution of the AFLP markers within the linkage groups was not uniform. Strong clustering of markers was observed. Almost two thirds of the markers (62%) covered a small area (8%) of the actual map (Table 2, clusters of markers represented by shaded chromosome bar segments in Fig. 2). Cluster length ranged from 2.8 to 11.5 cM.

Most genetic distances between consecutive pairs of markers were smaller than 20 cM, except for one gap in group 7" and two in group 5" (with 24, 22 and 21 cM).

Distorted segregation

Thirty-three percent of the markers showed significant deviation from the expected monogenic segregation ratio. These markers clustered in several chromosome regions. A chromosomal region was regarded as skewedly segregating if four or more closely linked markers showed significant segregation distortion in the same direction, as described in Xu et al. (1997). Based on these criteria, skewed regions were observed in

linkage groups 2a, 3', 4a, and 5 (Fig. 2). Group 3' showed distorted segregation over almost its entire length with an excess of H1 alleles, while in groups 2a, 4a and 5 only minor regions showed distorted segregation. In groups 4a and 5 the skewed segregation was also in the direction of an excess of H1 alleles, while in the group 2a an excess of H7 alleles was observed. In such regions, the location of the marker(s) that show the most extreme skewness, are probably closest to genetic factors causing the distorted segregation.

Discussion

In the current paper we describe the construction of the first molecular marker map for the species *Hordeum chilense* based on AFLP. A total of 463 AFLP markers were analysed in a segregating population of 100 F_2 individuals. The present map covers a total of 714.1 cM with an average density of markers of one every 2 cM.

Map construction

The mapping effort resulted in 9 long linkage groups, longer than 30 cM, and 10 short groups. Six of the long groups and three of the short groups had markers from H1 that allowed its assignment to a specific chromosome. A similar set of addition and substitution lines had been successfully used in the past for the chromosome location of several genes and of *H. chilense* specific RAPD markers (Castro et al. 1994; Hernandéz et al. 1996; Alvarez et al. 1998). Only chromosome 3 and the β arm of chromosome 2 were not represented in the present map. The unambiguous assignment of linkage groups from this map to chromosome 3 and 2β awaits the development of the respective addition lines of *H. chilense*.

The available marker data did not allow the total merging of the H1 markers with the H7 markers into the expected 7 linkage groups, i.e. one for each chromosome. This is due mainly to the low number of markers (21 out of 463 loci, representing 4.5%) scored co-dominantly. Even so, these co-dominant markers were very useful to integrate the H1 and H7 marker linkage groups. Since no sequence confirmation of true allelism

was done on the supposed co-dominant markers, we have to be aware that an incorrect classification of one of these pair of bands as allelic may cause underestimation of the total length of the map.

In constructing a genetic linkage map, dominant markers in repulsion phase are positioned on the basis of the information obtained from the co-dominant markers. Therefore, in the present study the ordering of the dominant markers in repulsion phase is less reliable than the ordering of the co-dominant markers and dominant markers in coupling phase. If more co-dominance had been found, a complete merging of H1 and H7 AFLP markers would have been possible as well as a more reliable positioning of markers in repulsion phase.

Marker segregation

Distorted segregation ratios of genetic markers are often observed in progeny of distant crosses and may result from competition among gametes or from abortion of zygotes that carry certain genes or gene combinations (Faris et al. 1998; Cheng et al. 1998). Such genes presumably act as partially lethal factors and should be located in the neighbouring region around the marker that shows the highest skewness (Cheng et al. 1998; Bert et al. 1999). The parents of the cross, H1 and H7, represent two genetically distinct *H. chilense* ecotypes, with very distinct AFLP fingerprints (Vaz Patto et al. 2000). This low genetic similarity may be the cause of the distorted segregation observed in the F_2. Subvital plants never reaching adult stage were found repeatedly in H1xH7 progeny, indicating the existence of lethality in this cross (A. Martín, pers. com.). Large regions of the linkage groups 2a, 3', 4a and 5 consisted of markers with skewed segregation ($P \leq 0.001$).

AFLP clustering

A clustering of AFLP markers as observed in *H. chilense* has also been reported in other plant AFLP linkage maps, such as barley (Becker et al. 1995; Qi et al. 1998), *Arabidopsis* (Alonso-Blanco et al. 1998), soybean (Keim et al. 1997), tomato (Haanstra et al. 1999), ryegrass (Bert et al. 1999), sorghum (Boivin et al. 1999), maize (Castiglioni

et al. 1999) and may represent the regions around the centromeres (Bert et al. 1999; Alonso-Blanco et al. 1998; Tanksley et al. 1992). The clustering of markers may be due to the suppression of recombination around the centromere (Tanksley et al. 1992) but also to the presence of repetitive sequences in the peri-centromeric areas (Alonso-Blanco et al. 1998; Qi et al. 1998; Castiglioni et al. 1999). This is especially probable with *Eco*RI/*Mse*I AFLP markers, since the methylation-non sensitive restriction enzyme (*Eco*RI), is expected to get access to the methylated regions around centromeres (Boivin et al. 1999), allowing these regions to be amplified by using the AFLP technique.

All but one of the long linkage groups contained one AFLP marker cluster. This is consistent with the clusters marking the regions around the centromere of the chromosome. Group 3' contained two widely spaced clusters (40 cM). However, one of the clusters contained a much smaller number of markers than the other (19 versus 49 markers). The presence of an additional region of suppressed recombination on the chromosome has been observed in other plant species (Tanksley et al. 1992). Cytogenetic studies will be necessary to confirm the location around the centromere of these clusters.

Because this genetic map did not coalesce into seven linkage groups, linkage groups without major marker clusters (putative centromere location) may represent distal chromosome regions that are actually part of a centromere containing linkage group.

Applications of the map

In summary, we have developed a genetic linkage map for *H. chilense* based on AFLP. The F_2 population used for this purpose exhibits a high level of polymorphism for DNA markers, high variation in resistance and tolerance to biotic stresses (e.g. avoidance of rust fungi) and variation in morphology. The AFLP technique provided a rapid way to construct a genetic linkage map that can be used to map genes and to identify QTLs. Work is in progress to locate markers linked to the avoidance mechanism against barley leaf rust fungi (*P. hordei*) since H7 possess this mechanism and H1 does not. This avoidance mechanism is characterised by a high percentage of germ tubes of rust fungi overgrowing the stomata which results in early failure of the infection process.

The genetic map presented here could be extended using other types of markers, such as co-dominant RFLP or microsatellites. This would allow gaps between distant markers to be filled as well as the merging of all the short linkage groups consisting of dominant markers derived from only one of the parental lines.

Acknowledgements

We would like to thank Fien Meijer-Dekens, Petra van den Berg and M. Carmen Ramírez for the technical assistance. This research was supported by PRAXIS XXI, Fundação para a Ciência e a Tecnologia, Lisboa, Portugal (research fellowship number BD/9124/96).

6

QTL mapping provides evidence for lack of association of the avoidance of leaf rust in *Hordeum chilense* with stomata density

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Abstract

In cereals, rust fungi are among the most harmful pathogens. Breeders usually rely on short-lived hypersensitivity resistance. As an alternative, "avoidance" may be a more durable defence mechanism to protect plants to rust fungi. In *Hordeum chilense* avoidance is based on extensive wax covering of stomata, which interferes with induction of appressorium formation by the rust fungi. High avoidance levels seem also to be associated with a higher stoma density on the abaxial leaf epidermis as a possible compensatory gas exchange mechanism.

The avoidance level was assessed as the percentage of germ tube/stoma encounters that did not result in appressorium differentiation. One hundred F_2 individuals from the cross between two *H. chilense* accessions with contrasting levels of avoidance showed a more or less continuous distribution for avoidance of the rust fungus *Puccinia hordei* and for stoma density, indicating quantitative inheritance of the traits. No significant correlation was found between avoidance and stoma density on the F_2 segregating population. In order to map QTLs for both traits, an AFLP marker map was constructed based on the F_2 population.

"Multiple interval mapping" identified three QTLs for avoidance and three QTLs for stoma density. The QTLs for avoidance were mapped on chromosome 1, 3' and 5, those for stoma density on chromosomes 1, 3' and 7". The detected QTLs had no pleiotropic effects on both avoidance and stoma density, supporting the hypothesis of joint evolution of both traits in nature.

The wild barley *H. chilense* has a high crossability with other members of the *Triticeae* tribe. The knowledge on the location of the QTLs responsible for the avoidance trait is a prerequisite to transfer this favourable agronomic trait from *H. chilense* to cultivated cereal genomes.

Key Words: Avoidance, stoma density, barley leaf rust, QTL mapping, *Puccinia hordei*, *Hordeum chilense*.

Introduction

Fungi belonging to the group of rust fungi cause the most serious and widespread diseases in cereals (Buchenauer, 1982). The cereal rusts are spread worldwide and produce frequent severe epidemics with substantial annual losses. About 10% of the world grain crop per year is lost by the rust infections (Agrios, 1997).

Until recently, breeders concentrated on the use of single race-specific resistance genes conferring a hypersensitive reaction of plant tissue to cereal rusts. Such use of resistance often results in ephemeral disease control, because virulent genotypes are rapidly evolving from the pathogen population (Mundt & Browning, 1985).

Recently, Rubiales and Niks (1992b, 1996) identified an alternative defence mechanism in *Hordeum chilense* Roemer et Schultes (2n=14), a perennial wild barley occurring in Chile and Argentina. This alternative mechanism is designated "avoidance". Avoidance reduces the chance of contact between the prospective plant tissue and the parasite. It is different from resistance as this operates when plant tissue and the parasite come into intimate contact (Parlevliet, 1981). This avoidance is characterised by the rust germ tube overgrowing the stomata rather than forming an appressorium and penetrating the stomata to invade the leaf tissue (Rubiales & Niks, 1992b; 1996). High avoidance is associated with an extensive wax covering of the stomatal guard cells (Rubiales & Niks, 1996). The avoidance in *H. chilense* is effective against several leaf rust species such as *Puccinia hordei, P. triticina, P. recondita* and *P. recondita agropyrina* (Rubiales & Niks, 1992a, 1996).

This avoidance of rust fungi has only been found in *H. chilense* and in four other wild species of barley (*H. brachyantherum*, *H. marinum*, *H. parodii* and *H. secalinum*) (Rubiales et al., 1996b).

In previous studies on collections of *H. chilense* accessions, a high positive correlation was found between avoidance level on the adaxial epidermis and stoma density on the abaxial epidermis of the leaf (Rubiales & Niks, 1996; Vaz Patto et al., 2001a). Rubiales and Niks (1996) suggested that the close association between avoidance and high stoma density existed to compensate for the presumed less efficient gas exchange by stomata that are covered extensively by wax. This association might either be due to pleiotropy, to genetic linkage between genes for avoidance and for

stoma density or might just represent association of the characters over ecotypes. The latter idea was supported by evidence reported before (Vaz Patto et al., 2001a).

H. chilense has been employed by cereal breeders due to its agronomic interesting characteristics and its high crossability with *Triticum*, *Hordeum*, *Secale* and *Agropyron* (Martín & Chapman, 1977; Martín et al., 1996, 1998, 1999). The amphiploid *H. chilense* x *T. turgidum* conv. *durum*, called tritordeum, is being used in a breeding programme as a new crop or as bridge to transfer useful genes from *H. chilense* to wheat (Martín & Cubero, 1981; Martín et al., 1998). Genes governing avoidance would be among the useful traits to be transferred to cereal modern cultivars.

The development of molecular markers allows the identification of individual loci controlling quantitative traits (e.g. Tanksley, 1993). By statistical analysis, the quantitative trait variation can be dissected into the effect of individual genome regions, the quantitative trait loci (QTLs), that are identified by linkage to markers on a molecular marker map (e.g. Paterson et al., 1988). Determination of the number, location, and magnitude of effects of QTLs are instrumental for improving breeding and selection efficiency.

In the present study, the inheritance of the avoidance of rust fungi and of stoma density and the association between these two traits are investigated further by means of microscopic analysis and QTL analysis on an F_2 population (using amplified fragment length polymorphism, AFLP). We choose for AFLP as this DNA marker technique is most efficient in whole genome analysis.

Material and methods

Plant material

H. chilense accession H7 showing a high level of avoidance of rust fungi and high stoma density on the abaxial epidermis of the leaf and accession H1 with a low level of avoidance and a low stoma density on the abaxial leaf epidermis (Rubiales & Niks, 1996), are representatives from two distinct ecotypes of *H. chilense* (Vaz Patto et al., 2001a). An F_2 population consisting of 100 plants obtained from a cross of H1xH7 was

used for AFLP marker analysis (Chapter 5 of this Thesis) and for disease tests in the greenhouse with the two parental *H. chilense* accessions as controls.

Phenotypic evaluation

The F_2 plants were raised at the Instituto de Agricultura Sostenible, Córdoba, Spain, in small pots ($1000cm^3$), in a greenhouse compartment and kept at 18-25 °C and ambient humidity. Clones of these plants were transferred to the Wageningen University, The Netherlands, and kept under similar growing conditions.

Sixth leaves of tillers of mature plants were inoculated with *Puccinia hordei* isolate 1-2-1, which is a monospore culture derived from 1-2 (Parlevliet, 1976), obtained from the Laboratory of Plant Breeding, Wageningen University. The leaves were fixed horizontally with their adaxial surface facing up and inoculum was applied using a settling tower.

The sampling of the leaves and the staining with Uvitex for histological observations was as described by Rubiales and Niks (1996). Observations were made with an epifluorescence microscope (Nikon Fluophot, V-exitation, 380-425 nm) at 100x magnification.

The avoidance level was measured as the percentage of germ tube/stoma encounters that did not result in appressorium formation.

Stomatal densities were determined on the abaxial epidermis of leaf segments of the F_2 plants (three microscope fields per leaf, 2.6 mm^2 each, white light, 100x magnification) in the same two leaves per plant that were used to determine the level of avoidance.

Four replicate experiments were conducted in different times of the year, each with two leaves per F_2 plant.

Wax covering of the stoma

The leaves of the contrasting parental accessions (H1 and H7) and of ten F_2 plants, selected to represent the full range of avoidance levels, were observed by scanning electron microscope. Scanning electron microscopy was used to observe epicuticular

wax covering of the stomata. Samples of the leaves were mounted with the adaxial surface facing up on metal stubs and frozen in liquid nitrogen. The specimens were sputter coated with gold and examined and photographed in the scanning electron microscope (JEOL 35C at PCM Wageningen University) at an accelerating voltage of 15 kV.

QTL mapping

The F_2 population was previously used to generate an AFLP map of *H. chilense* (Chapter 5 of this Thesis). From this AFLP map, a skeletal map with markers approximately 5 cM apart was extracted and used for QTL identification.

Analysis of variance was carried out for avoidance and stoma density on the F_2 population, using a nested model, and broad sense heritability (h_b^2) was calculated. The similar LOD profiles obtained with the data from each of the four replications justified pooling the data from the four replications for QTL mapping.

A computer software package, MapQTL version 4.0 (Van Ooijen & Maliepaard, 1996), was used for interval mapping (Lander & Botstein, 1989) and multiple QTL mapping (Jansen, 1993; Jansen & Stam, 1994). In the regions of the putative QTLs, the markers with the highest LOD values were taken as co-factors. When LOD values for some markers in other regions became significant, they were gradually added as co-factors, until the LOD profile stabilised. LOD values of 3.7 (avoidance) and 3.3 (stoma density) were chosen, by permutation test (available in the MapQTL package), as significant threshold values for declaring a QTL at 95% confidence. The additive effect and the percentage of total phenotypic variation explained by each putative QTL were also estimated using the MapQTL software.

Results

Phenotypic data

The mean values of avoidance level of the two parental accessions were 23 and 94% and of stoma density 5.4 and 30.2 stoma/mm^2 respectively (Fig. 1). F_2 plants also showed a large and significant variation in avoidance and stoma density. Kolmogorov-Smirnov statistic test showed that avoidance level had no normal distribution (p=0.000). The stoma density was normally distributed (p=0.2). An arcsine square root transformation was applied to the avoidance level data in order to improve homogeneity of residual variance.

The phenotypic values of the two parental accessions were not significantly different from the values of the most extreme F_2 plants (P≥0.2), so no transgressive segregation was observed for any of the two traits.

The wide sense heritability for avoidance was 0.61 and for stoma density only 0.20. No significant correlation was found between the stoma density and the level of avoidance untransformed (r=-0.15, Fig. 2), indicating that the two traits inherit independently.

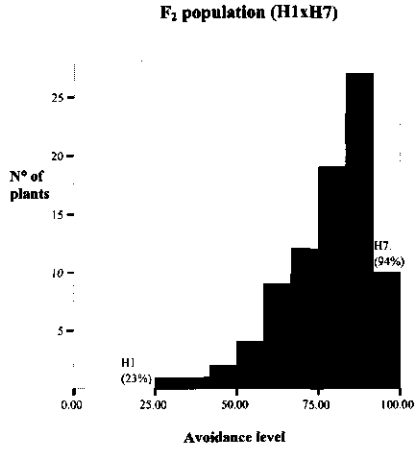

Fig. 1a See next page for legend

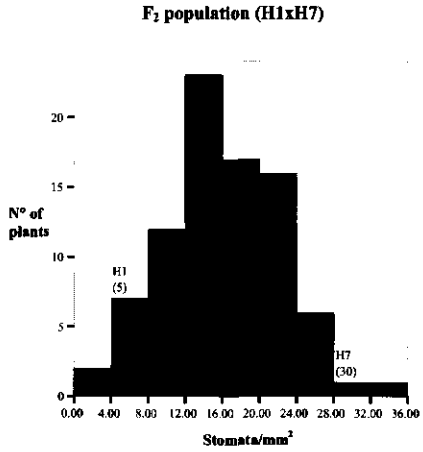

Figure 1b. Frequency distribution of the avoidance level measured on the adaxial leaf epidermis as the percentage of the germ tube/stoma encounters that did not result in an appressorium (a) and of the stoma density on the abaxial leaf epidermis (b) (averages over four experiments) in the *Hordeum chilense* F_2 population H1xH7. The average levels of avoidance and stoma density of the two parental accessions (H1 and H7) are indicated.

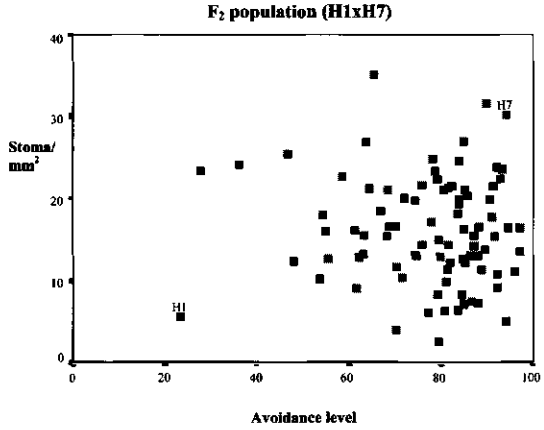

Figure 2. Avoidance level measured on the adaxial leaf epidermis as the percentage of the germ tube/stoma encounters that did not result in an appressorium and stoma density of the abaxial leaf epidermis on 100 *Hordeum chilense* F_2 plants from H1xH7 (Correlation coefficient = -0.15). The average levels of avoidance and stoma density displayed by the two parental accessions (H1 and H7) are indicated.

Scanning Electron Microscopy observations

Wax layer differences among *H. chilense* accessions are probably the cause of variation in avoidance level. The contrasting parental accessions and ten F_2 plants, representing the full range of avoidance levels, were observed by SEM (Fig. 3). Plants with high avoidance showed a more extensive wax covering of the stomata (Fig. 3, H7, A, B and C) than plants with a low level of avoidance (Fig. 3, I, J and H1). In the high avoidance plants, wax covering was so extensive that it was difficult to distinguish the stomata in the epidermis even though they were open. The extent of wax covering did not vary substantially within leaves. However, in some plants with intermediate levels of avoidance an unexpected level of wax covering of the stoma was found. Some of these plants had stomata quite severely covered by epicuticular wax (Fig. 3, G and H), while other plants had stomata almost completely uncovered (Fig. 3, E).

The wax coverage of the stomata in the abaxial epidermis was similar to the wax coverage of the stomata at the adaxial side of the leaves.

All F_2 plants showed vigorous growth indicating that the variation in wax coverage or in stomata density did not effect the fitness.

QTL mapping

QTLs controlling avoidance

Three QTLs for the avoidance trait, on three different chromosomes were detected in this F_2 population and designated *qavoi1*, *qavoi2* and *qavoi3* (Table 1, Fig. 4). The QTL with the largest effect, *qavoi3*, was located on chromosome 5, and explained about 41 % of the phenotypic variance. Together the three QTLs explained 63 % of the phenotypic variance. For all the QTLs, H7 alleles increased the level of avoidance, which is in agreement with the lack of transgressive segregation. Additive effects of the H7 alleles for *qavoi1*, *qavoi2* and *qavoi3* were +6.1, +6.2 and +15.0 % germ tube/stoma encounters that do not result in appressorium formation, respectively. Using the estimated additive effects of the H7 alleles on the three QTLs, we predicted a difference of 54.6% in avoidance level between the two parental accessions H1 and H7, based on a

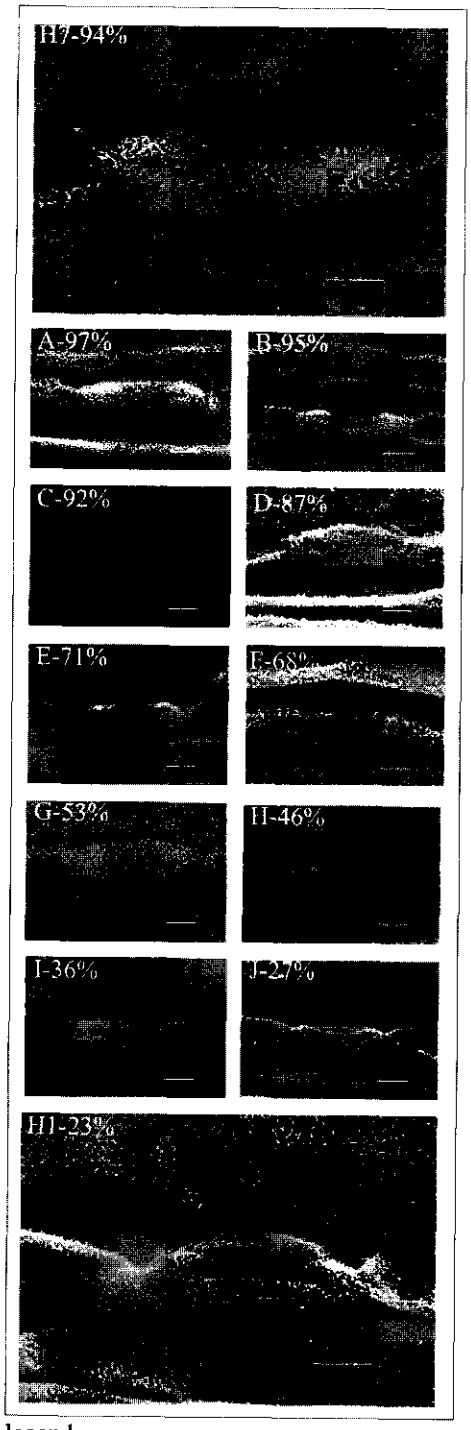

Fig. 3 See next page for legend

Figure 3. Scanning electron microscopy of *Hordeum chilense* adaxial leaf stomatal complexes. H1: low avoidance parental accession; H7: high avoidance parental accession; A to J: F_2 plants (H1 x H7) covering the all range of avoidance levels. Level of avoidance expressed has the percentage of germ tube/stoma encounters that fail to form an appressorium. Notice the extensive wax covering of the guard cells on the high avoidance parental accession H7.

simple additive model (two-times the sum of the additive effects). The observed difference between the two parental accessions H1 and H7 was of 71% of germ tube/stoma encounters with no appressorium differentiation. This implies that the detected QTLs explain 77% of the phenotypic difference between the parental accessions.

Table 1. Quantitative trait loci for avoidance level and stoma density on the abaxial leaf epidermis in an *Hordeum chilense* F_2 (H1xH7).

QTLs	co-factor marker	chromosome	Interval length[a] (cM)	QTL position (cM)	additive effect[b]	peak LOD score	% variance explained[c]
Avoidance							
qavoi1	H1E32M61-205	1	13.1	56.8	+6.1	4.2	7.4
qavoi2	H7E35M48-158	3'	2.2	12.1	+6.2	6.2	14.8
qavoi3	H1E42M47-352	5	7.8	8.5	+15.0	6.9	40.8
Stoma density							
qstod1	E35M49-113/114	1	4.2	43.8	-3.8	5.2	16.1
qstod2	H7E32M55-322	3'	4.3	8.5	+5.6	7.3	26.4
qstod3	H7E32M61-410	7"	23.0	48.1	+2.3	3.3	9.2

[a] The support interval was estimated at a LOD fall off –2.00
[b] (mean of the H7 allele genotypes – mean of the H1 allele genotypes)/2. Avoidance measured as the percentage of the germ tube/stoma encounters that does not result in an appressorium and stoma density as stomata/mm^2
[c] Percent explained phenotypic variance

QTLs controlling stoma density

Three QTLs were identified for stoma density (Table 1, Fig. 4). The three QTLs (*qstod1*, *qstod2* and *qstod3*) explained together 52 % of the phenotypic variation and were located on chromosomes 1, 3' and 7" (see Chapter 5 of this Thesis, for chromosome nomenclature). Two alleles for increasing and one for decreasing stoma density were contributed by H7 (additive effect of +5.6, +2.3 and -3.8 stomata/mm^2, respectively). However, transgressive segregation was not observed in the F_2

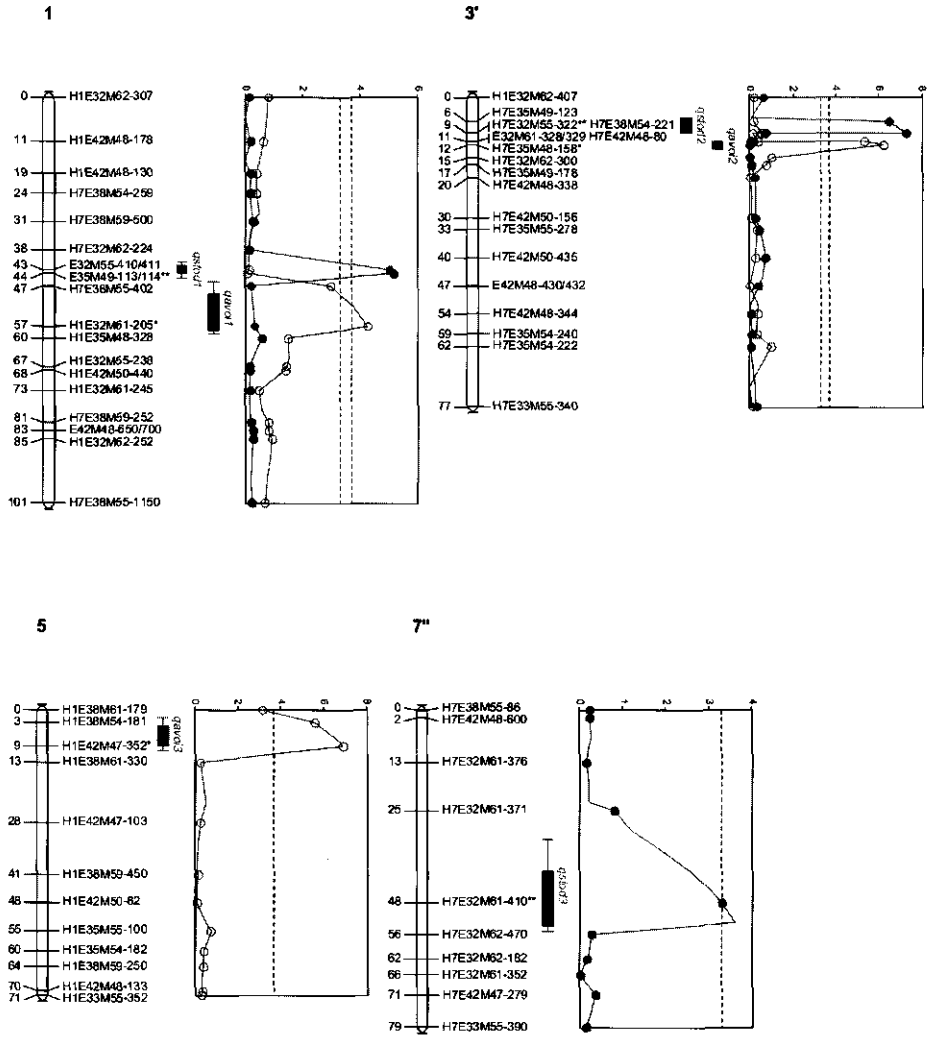

Figure 4. AFLP marker linkage map of *Hordeum chilense* chromosomes 1, 3', 5 and 7 showing the positions of QTLs for avoidance level and stoma density. The map was developed using the F_2 population H1xH7. Map positions are given in cM, using the Kosambi function. AFLP marker loci were assigned the prefix "H1" or "H7" encoding the parental line, that produced the amplified DNA fragment. Co-dominant markers in italics. Bars indicated the QTL locations with a two LOD support confidence interval on the right side of the chromosome maps (avoidance level as empty and stoma density as solid circles). QTL likelihood profiles for multiple QTL model are depicted with the LOD significance threshold values as a dotted line (3.7 for avoidance level and 3.2 for the stoma density). Markers used as co-factors are indicated by * and ** for avoidance level and stoma density respectively.

population. Some F_2 plants had a average stomata density higher than H7 or lower than H1, but these differences were not significant, maybe because of the large non-genetic variance components for this trait (heritability of only 0.20). These three QTLs explained only 33 % of the phenotypic difference between the two parental accessions.

Chromosomes 1 and 3' included QTLs for the avoidance trait and for stoma density. There was no overlap of the supporting confidence intervals, suggesting that each of these QTLs was only involved in one of the two traits.

Additive effects of QTLs

Multiple QTL mapping assumes that QTLs have only additive effects and no epistatic effects (Jansen, 1993). However, by applying a three factor (three QTLs for each trait) analysis of variance based on the genotype values of the 'peak' markers of each QTL we tested whether the QTL effects are additive or significant interactions occur between them. No significant interactions among QTLs were detected for any of the two traits (Tables 2 and 3).

Table 2. Three factor analysis of variance for avoidance level.

Source of Variation	Mean Square	F	Sig.
H7E35M48-158	0.154	7.391	0.008
H1E32M61-205	0.133	6.400	0.013
H1E42M47-352	0.211	10.145	0.002
H7E35M48-158* H1E32M61-205	0.00425	0.204	0.652
H7E35M48-158* H1E42M47-352	0.01430	0.687	0.410
H1E32M61-205* H1E42M47-352	0.00005	0.003	0.958
H7E35M48-158* H1E32M61-205* H1E42M47-352	-[a]	-	-
Error	0.00208		

[a] Not estimated because no plant with absence of H1E32M61-205 and H1E42M47-352 bands and presence of H7E35M48-158 band genotype existed in the F_2 population.

Table 3. Three factor analysis of variance for stoma density.

Source of Variation	Mean Square	F	Sig.
H7E32M61-410	264.315	8.771	0.004
H7E32M55-322	258.037	8.562	0.005
E35M49-133/114	146.435	4.859	0.011
H7E32M61-410* H7E32M55-322	0.004	0.000	0.991
H7E32M61-410* E35M49-133/114	35.993	1.194	0.309
H7E32M55-322* E35M49-133/114	9.734	0.323	0.725
H7E32M61-410* H7E32M55-322* E35M49-133/114	11.907	0.395	0.675
Error	30.136		

Discussion

From the analysis of a morphologically and molecularly very polymorphic *H. chilense* collection, Vaz Patto et al. (2001a) concluded that avoidance was associated with a particular ecotype of the species. This ecotype had, among other traits, a greater number of stomata/cm^2 on the abaxial leaf epidermis than two other, lower avoidance ecotypes.

High association between avoidance and stoma density on the abaxial leaf epidermis had already been described by Rubiales and Niks (1996) working with a subsample of the same *H. chilense* collection. These authors hypothesised that, in the high avoidance plant types, the higher total stomatal pore area caused by the high stoma density on the abaxial epidermis will compensate for a possibly reduced gas exchange per stoma, due to the extensive wax covering.

It was suggested that the association between high avoidance and high stoma density on the abaxial leaf epidermis was either due to pleiotropy, to genetic linkage between genes for avoidance and for stoma density or just to association of the characters over ecotypes.

To determine which is the nature of the association between avoidance and stoma density, the current study presents a QTL analysis of the avoidance trait to barley leaf rust and stoma density on the abaxial epidermis in the wild barley *H. chilense*. The results were obtained by repeated phenotypic analysis of avoidance level and stoma density in a segregating F_2 population and by genotyping the same population for AFLP markers.

QTL analysis revealed that the genetic control of avoidance and stoma density was indeed polygenic. Three QTLs for the avoidance of barley leaf rust and three QTLs for stoma density were detected. The number of detected QTLs may have been underestimated as QTLs may have escaped detection because of the scarcity of markers in few map regions, the small population size (100 F_2 individuals) and a relatively high LOD threshold (3.7 and 3.3) used to reduce the chance of detecting ghost QTLs (Young, 1996). Even so, the detected QTLs could explain 63% of the phenotypic variation for avoidance level and 52% of the phenotypic variation for stoma density.

Evidence was also found for lack of genetic association between avoidance and stoma density on the abaxial epidermis. In the F_2 population, a low correlation between

avoidance and stoma density was found. This observation was consistent with the fact that the QTLs for avoidance were different from the QTLs controlling stoma density. This indicates that the association between avoidance and the stoma density in the *H. chilense* collections was not due to pleiotropic effects. However, on chromosomes 1 and 3', taking into account the small population size, the non-overlapping of the two-LOD intervals of QTLs *qavoi1* and *qstod1* (chromosome 1) and *qavoi2* and *qstod2* (chromosome 3') might have been caused by the choice of particular cofactor markers for multiple QTL mapping.

Close linkage between QTLs for avoidance and stoma density, was detected on chromosomes 1 and 3' (Fig. 4). However, on chromosome 3', the H7 alleles of both QTLs, *qavoi2* and *qstod2*, contribute positively for avoidance and stoma density, whereas on chromosome 1 the H7 allele of *qavoi1* has a positive effect on avoidance level and of *qstod1* has a negative effect on stoma density. Because of these two kinds of linkage between QTLs for avoidance and stoma density in *H. chilense*, we conclude that the close linkage between QTLs controlling both traits shouldn't be the cause for the positive correlation observed in the *H. chilense* collection.

In the interaction *H. chilense-P. hordei*, extensive wax coverage over the guard cells, obscuring the features that normally induce appressorium differentiation, was suggested to be the cause of overgrowth of stomata in *H. chilense* accessions with high avoidance levels (Rubiales & Niks, 1996). The role of the epicuticular wax layer coverage on avoidance was confirmed by Vaz Patto et al. (2001b). On high avoidance accessions, the removal of the wax layer allowed appressoria to develop over stomata that would otherwise be overgrown. Removal of the wax layer on the low avoidance accessions did not significantly affect the appressorium differentiation.

In the present study, scanning electron microscopy confirms, at the F_2 level, that plants with a high avoidance level showed a more extensive wax coverage of the stomatal complexes than plants with a low level of avoidance. However, on plants with intermediate levels of avoidance this association was not as obvious. A greater variation in wax coverage among the intermediate phenotypes than among the most extreme phenotypes for avoidance was observed. This could suggest that the wax covering of stomata is not the only factor determining the level of avoidance of these plants.

As reported by Farquhar and Richards (1984) the ratio $^{13}C/^{12}C$ correlates positively with water use efficiency and negatively with stomatal conductance. Rubiales and Niks (1996) did not find significant differences for this $^{13}C/^{12}C$ ratio between avoidance and non-avoidance accessions, concluding that the extensive wax covering of *H. chilense* avoidance accessions does not decrease stomatal conductance. However, no stoma density was taken into account in their measurements. The authors compared water use efficiency per leaf, rather than per stoma. We suggest that the extensive wax covering of the stomata on the avoidance accessions may reduce stomatal conductance but the higher stoma density on the abaxial epidermis compensated for this, resulting in a similar water use efficiency as in low avoidance accessions.

No epistatic effects were found between the detected QTLs for avoidance. The QTLs identified had only additive effects. This can imply that the inheritance of this trait is not particularly complex, opening the way for relatively straightforward use of markers in avoidance breeding in general. Breeding for resistance to pathogens is an important aspect of plant breeding programmes.

The knowledge of the genetic basis and location of the QTLs responsible for the avoidance character will facilitate the transfer of this trait from *H. chilense* to the genomes of related cultivated species.

In spite of the fact that *H. chilense* crosses readily with wheat, rye, cultivated barley and crested wheatgrass, resulting in chromosomically stable hybrids, the only fertile amphiploids obtained from these four types of hybrids are the ones obtained with *Agropyron cristatum* and with wheat (tritordeum). This limits the use of *H. chilense* in cultivated barley breeding but opens good perspectives for forage crop or for wheat and triticale breeding (Martín et al., 1998, 1999). Up to now, no addition lines have been developed from the *Agropyron* amphiploid reducing the possibilities of introgression of avoidance into this forage species. However chromosome addition and substitution lines of *H. chilense* in wheat have been obtained as a means to transfer desirable agronomic traits from this wild barley to wheat (Fernández, 1989). Avoidance on the wild barley, *H. chilense*, occurs for at least four different leaf rust species (Rubiales & Niks, 1992a, 1996). Among them, barley leaf rust (*P. hordei*) and wheat leaf rust (*P. triticina*) show the same pattern of appressorium differentiation response to topographical stimuli on artificial membranes (Collins & Read, 1997). Therefore, the avoidance QTLs effective

against *P. hordei* should also be effective against *P. triticina*. Molecular markers could be used to support the introgression of the chromosomal regions harbouring the loci that confer the avoidance in *H. chilense* to wheat leaf rust into wheat. Using disomic addition wheat lines based on avoidance line H7 of *H. chilense*, future work will concentrate on investigating whether the avoidance character is expressed in a wheat genomic background. Rubiales et al. (1991) showed that, unfortunately, *H. chilense* rust avoidance is not expressed in tritordeum. Apparently, the genes for avoidance in *H. chilense* are not expressed in a wheat genomic background.

Acknowledgements

We would like to thank Fien Meijer-Dekens, Petra van den Berg and M. Carmen Ramírez for the technical assistance. This research was financially supported by the program PRAXIS XXI, Fundação para a Ciência e a Tecnologia, Lisboa, Portugal (research fellowship number BD/9124/96).

7

General discussion

In search for durable resistance mechanisms in general and for leaf rust resistance in particular, wild relatives of wheat and barley are investigated at the laboratory of Plant Breeding, Wageningen University.

Diploid wheat (*Triticum monococcum*) and the wild barley *Hordeum chilense* are among the wild species used in this search for durable protection against leaf rust. Diploid wheat has a very high level of resistance to the leaf rust of bread wheat and macaroni wheat, with prehaustorial, posthaustorial or a combination of both mechanisms (Niks & Dekens, 1991; Anker & Niks, 2001). Some *H. chilense* accessions avoid recognition of stomata by several leaf rust fungi (*Puccinia hordei*, *P. triticina* and *P. recondita*). Appressorium differentiation is substantially reduced on these accessions (Rubiales & Niks, 1996).

The research described here is an endeavour to get a deeper insight into the principles underlying the potentially durable avoidance character of the wild barley *Hordeum chilense* to the barley leaf rust fungus (*Puccinia hordei*).

State-of-the-art in avoidance research in Hordeum chilense

The high crossability of *H. chilense* with other *Triticeae* species (Martín & Chapman, 1977; Martín & Cubero, 1981; Martín et al., 1998) prompted several authors to study disease resistance in this species as a potential trait to be transferred to cereal crop species.

Rubiales and Niks (1992b) studied the histological reaction of 16 *H. chilense* accessions to *P. recondita*, *P. hordei* and *P. striiformis*. They found that *H. chilense* accessions differed in the mechanism of resistance to inappropriate rusts. The level of early abortion of sporelings and the frequency of plant-cell necrosis associated with the infection sites varied among accessions. On some *H. chilense* accessions they found very few appressoria formed by *P. hordei* and other leaf rust species over stomata (Rubiales & Niks, 1992b). This reaction was considered as a case of avoidance (Rubiales & Niks, 1992a). Percentages of germ tubes forming an appressorium on some *H. chilense* accessions could be only a third to a tenth of the appressorium formation on other *H. chilense* accessions or on cultivated barley (*H. vulgare*) and wheat (*Triticum aestivum*). The observed low appressorium formation was neither associated with

differences in spore deposition or germination nor with a low stoma frequency and suggested either disorientation of germ tube growth over the leaf surface or failure to recognise stomata by germ tubes.

Subsequently, Rubiales and Niks (1996) tried to determine the mechanism responsible for the failure of appressorium formation on some *H. chilense* accessions. Observations on germ tube development indicated that low appressorium formation was not due to poor orientation of the germ tubes towards stomata, but to poor recognition of the guard cells that is manifested as lack of differentiation of appressoria. Overgrowth of stomata on accessions with high avoidance seemed either to be due to the absence of an effective appressorium inducing structure or to failure of the germ tube to perceive the inducing structure. These authors presented evidence that extensive wax covering of the guard cells obscures the stomatal features that normally trigger appressorium formation. Rubiales and Niks (1996) reported also that avoidance is associated with a high stoma density on the abaxial side of the leaves and with longer stomatal pores. They suggested that accessions with an extensive wax covering compensate for reduction in gas exchange per stoma by a larger total stomatal pore area. The same authors found few accessions with intermediate avoidance level. The lack of intermediate types suggested a simple Mendelian inheritance of the avoidance trait, or that the avoidance accessions may represent a distinct ecotype of *H. chilense*.

The role of the wax layer and stomatal cell dimensions on the avoidance trait

Several authors suggested that the wax layer plays a role in the orientation of germ tube growth across the leaf towards the stomata. Lewis and Day (1972) have implicated the wax crystal lattice of the cuticle in directing germ tube growth. Wynn and Staples (1981) reported that germ tubes failed to adhere to leaves without epicuticular wax and as a consequence failed to grow directionally. Rubiales and Niks (1996) observed reduction of appressorium formation after wax removal in the accession H1 of *H. chilense*, in the wheat cv. Little Club and in the barley line L94, also suggesting such a role for wax crystals in the orientation of germ tube growth. Jenks and Ashworth (1999), on the other hand, hypothesised that the wax structure, covering the leaf surface, may disorient fungal hyphae growth across plant surfaces. However, the wax crystals,

observed on *H. chilense* (Chapter 3), are not arranged according to a regular lattice, making it less likely that they trigger the orientation of germ tube growth. Indeed, in Chapter 3 of this thesis, we demonstrate that the wax layer does not contribute to nor hamper the orientation of the germ tube growth towards stomata.

Orientated growth of the germ tubes seems to start only upon contact with the epidermal cell junctions and development of lateral growings (Chapter 3). These results are in accordance with those of Read et al. (1997) who hypothesised that the cell junctions guide germ tubes to grow perpendicularly to the main vein of the leaf, increasing the probability of germ tubes to encounter stomata which are organised in rows parallel to the main vein.

Edwards and Bowling (1986) suggested that pH gradients could guide *Uromyces vicia-fabae* germ tubes to stomata. Gradients of pH have been found around closed, but not open, stomata of dicotyledonous species. Cessation of the active H^+ efflux by the cells of the stomatal complex, upon stomatal closure, appears to be the cause of establishment of the pH gradient. In Chapter 3, no evidence was found of positive attraction of the germ tubes of *P. hordei* by stomata of *H. chilense*. Germ tubes could pass a stoma even at a very short distance (<1μm) without changing direction towards that stoma. Maybe the different arrangement of the epidermal and stomatal cells on the monocotyledonous *H. chilense*, when compared with that on the dicotyledonous plant species, interferes with the occurrence and perception of this stimulus by the fungus *P. hordei*. On the other hand, *P. hordei* could just be not responding to this kind of stimulus.

Several observations support the hypothesis that extensive wax covering of the guard cells obscures the stomatal features that normally trigger appressorium formation. Rubiales and Niks (1996) investigated the stomatal guard cell ultrastructure of three *H. chilense* accessions and of wheat cv. Little Club. The three *H. chilense* accessions had contrasting avoidance levels. The stomata of the high avoidance accessions had a very extensive wax covering, obscuring the stomatal opening. When the stomata of the low avoidance accession and of the Little Club were open, the aperture was clearly visible, but this was not the case for the high avoidance accessions. There were no obvious differences between the *H. chilense* accessions and cv. Little Club in the wax covering of the leaf surface away from the stomata. In Chapter 3 it was shown that in high

avoidance accessions the removal of the wax layer allows appressoria to develop over stomata, which would otherwise be overgrown. Contrary to the work of Rubiales and Niks (1996), removal of the wax on the low avoidance accessions did not reduce the appressorium differentiation. These authors presumed that these results were due to poor germ tube orientation. As no effect of the wax layer on the orientation of germ tubes was observed by us in Chapter 3, the differences between our results and the results of Rubiales and Niks (1996) on appressorium differentiation are possibly due to a technical artefact. It remains an open question what could explain these differences in results.

By analysis of a segregating F_2, the extensive wax coverage of the guard cells was associated with avoidance (Chapter 7). Scanning electron microscopy confirmed that plants with a high avoidance level showed a more extensive wax coverage of the stomatal complexes than plants with a low level of avoidance. However, on plants with intermediate levels of avoidance this association was not so obvious. Plants with intermediate levels of avoidance could possess either stomata highly or barely covered by wax crystals. This indicates that in addition to wax quantity on the stomatal complex also other features, like maybe wax composition, modify the avoidance level.

In Chapter 3 no significant differences were observed in the stomatal cell dimensions between stomata overgrown and stomata at which an appressorium was differentiated. Therefore, it is unlikely that dimension of cells and spacing of cell junctions are responsible for the stomata overgrowth on the avoidance accessions. We conclude that the overgrowth of stomata by the rust germ tube is mainly due to the more extensive wax covering of the stomatal apparatus on the high avoidance accessions.

Some authors (Staples et al., 1983; Allen et al., 1991a; Collins & Read, 1997; Read et al., 1997) working with artificial membranes and several cereal rust fungi suggested that physical features of the host alone are sufficient to induce appressorium formation. Read et al. (1997) argued that it is unclear which precise topographical features are involved in appressorium induction of cereal rust fungi *in vivo*. The spacing and dimensions of the grooves on the artificial membranes were not of the same order of magnitude as those of the guard cell junctions and stomatal apertures. In addition, the percentage of germ tubes, which differentiated into appressoria, was on the artificial membranes lower than when germ tubes encounter stomatal complexes *in vivo* (Read et

al., 1997). An intriguing question to be addressed in future is: "what are the precise features of the stoma in cereals that induce the appressorium differentiation of leaf rust fungi?"

Occurrence of the avoidance character in nature and association with other traits

Rubiales and Niks (1996), studying a collection of *H. chilense* accessions, concluded that the lack of intermediate avoidance accessions suggests a simple Mendelian inheritance of the avoidance trait or, alternatively, that the avoidance accessions may represent a distinct ecotype of *H. chilense*. In Chapter 2, we analysed an extended collection of *H. chilense* accessions that overlapped with those studied by Rubiales and Niks (1996). We found a continuous distribution of the percentage of appressorium formation on these accessions. Many accessions had an intermediate level of avoidance. Indeed, our intermediate avoidance accessions were mainly those that were not included in the study of Rubiales and Niks (1996). Based on these observations a simple Mendelian inheritance of avoidance was no longer expected. The polygenic inheritance of the avoidance character was definitely confirmed by the segregation in a F_2 population described on Chapter 7.

The avoidance was indeed associated with a distinct ecotype of *H. chilense*. The results reported in Chapter 2 indicated the existence of three rather well-defined morphological and molecular ecotypes or subspecific taxa in *H. chilense*. High avoidance appears to be associated with one of these particular ecotypes collected mainly from humid places. However, Von Bothmer et al. (1980, 1995) in spite of the large variation found, preferred to keep *H. chilense* as one polymorphic taxon, since they observed an overlapping in all morphological characters studied in this species. The cluster composition based on AFLP fingerprints, described on Chapter 2, confirms the cluster composition based on morphological traits, but suggested clearly distinct groups. This supports the idea to recognise distinct ecotypes or subspecific taxa in *H. chilense*.

It was remarkable, that, based on AFLP polymorphisms, the genetic distance between *H. chilense* accessions from the two extreme ecotypes was as large as the

genetic distance between the non-crossable cultivated barley and diploid wheat (Chapter 4). This indicates the existence of an extreme amount of genetic variation in *H. chilense*.

The association of high avoidance with a particular ecotype or subspecific taxon of *H. chilense* may suggest explanations for the evolution of avoidance in nature: avoidance is confined to a particular cluster of *H. chilense* accessions that occur in humid environments. This indicates that the avoidance character of *H. chilense* might have evolved to protect the plants against rust pathogens. Under very low humidity conditions rust development is meagre (Wahl, 1970). This implies that in humid habitats, selection pressure imposed by rust fungi is higher than in dry habitats. However, it is unknown to us what rust species, if any, occur in substantial amounts on *H. chilense* in natural habitats. Alternatively, high avoidance could merely be a consequence of morphological features that evolved in the particular morphotype as a response to selection imposed by its habitat. In Chapters 3 and 7 the evident role of the wax layer on the avoidance character was demonstrated. Extensive wax covering of stomatal guard cells prevents appressorium formation over them. The filamentous wax structures at the entrance of Stika spruce stomata have been proposed as a means to help keep water out of the intracellular air spaces by partially blocking the stomatal entrance (Jeffree et al., 1971). However, an extensive leaf wax layer is normally associated with environments with reduced water availability or high radiation (Jenks & Ashworth, 1999). In conclusion, the association of avoidance with humid habitats (Chapter 2), where no special provisions for water efficiency are required, favoured the hypothesis that the extensive wax covering of stomata, causing the avoidance, resulted from the selection due to a high rust pressure, rather than avoidance being a 'side-product' of the adaptive extensive wax covering.

The putative subspecific taxon with a particular high level of avoidance was further characterised, among other traits, by a greater number of stomata/cm^2 on the abaxial leaf epidermis (Chapter 2). This association is of special interest, since accessions with an extensive wax covering (high avoidance level) may compensate for any reduction in gas exchange per stoma with a greater total stomatal density. Rubiales and Niks (1996), suggested that this association between avoidance and stoma density on the abaxial epidermis may either be due to pleiotropy, to genetic linkage between genes for avoidance and for stoma density or both traits may just be associated by

chance over ecotypes. Results from Chapter 6 indicated that avoidance and stomata density are not genetically associated. Both traits showed a more or less continuous distribution in a segregating F_2 population indicating quantitative inheritance of the traits. There was no significant correlation between avoidance and stoma density on the abaxial epidermis of the leaf (Chapter 6). QTL analysis based on the AFLP marker linkage map of *H. chilense* (Chapter 5), revealed that the genetic control of avoidance and stoma density was indeed polygenic. The detected QTLs for avoidance were different from the QTLs for stoma density. This indicates that the association between avoidance and the stoma density, detected in the *H. chilense* collections (Chapter 2), was probably not due to pleiotropic effects of genes, nor to close linkage. This implies that both traits have jointly evolved in the particular ecotype, where the reduced stomata conductance due to extensive wax covering may have resulted in selection for higher stoma density.

Future research and practical applications

Till now *H. chilense* has produced fertile amphiploid hybrid offspring with cultivated tetraploid and hexaploid wheat and with *Agropyron cristatum* (Martín & Chapman, 1977; Martín et al., 1998; 1999). Especially in wheat, where addition and substitution lines of *H. chilense* chromosomes have been developed, the introgression of traits from *H. chilense* can be attempted.

The avoidance of *H. chilense* is not only effective to *P. hordei* but also to wheat leaf rust (*P. triticina*) (Rubiales & Niks, 1992b). *P. triticina* and *P. hordei* need similar topographical stimuli to differentiate appressoria on artificial membranes (Collins & Read, 1997). It is therefore not surprising that excessive wax covering of stomata causes avoidance of both rust fungus species. Therefore, transfer of the genes conferring avoidance of *P. hordei* in *H. chilense* to wheat, might confer avoidance of the wheat leaf rust in that crop.

In planning the transfer of genes for avoidance to wheat, attention must be given not only to the percentage of explained phenotypic variability but also to the additive effects of the detected QTLs for avoidance. The QTLs with the largest and most

reproducible effects are the prime candidates for marker assisted introduction into wheat breeding lines.

The three detected QTLs for avoidance explained in total 77% of the avoidance difference between the two parental lines (Chapter 7). The additive effects of the single QTLs were 6.1, 6.2 and 15.0 % more germ tube/stoma encounters that did not result in appressorium differentiation. These three QTLs were located on three different chromosomes 1, 3 and 5, respectively. Since the QTL located on chromosome 5 has an effect on avoidance more than twice as big as the effect of the other two QTLs, future research should concentrate on the introgression of *H. chilense* chromosome 5 in a wheat background.

The feasibility to introduce the avoidance character in wheat is hard to assess now. It should be verified whether avoidance is expressed in addition lines of the *H. chilense* H7 accession on Chinese Spring wheat cv. for chromosome 5 H^{ch}. Such addition lines are already being developed (Antonio Martín, pers. com.). Chromosome addition lines of *H. chilense* on breadwheat have already been used to transfer resistance to cereal root-knot nematode (*Meloidogyne naasi*) to wheat (Person-Dedryver et al., 1990). The development of substitution lines from the interesting addition lines should be the next step to incorporate the genes for avoidance to modern wheat cultivars. Unfortunately, the avoidance in tritordeum seems not to be expressed, even if the *H. chilense* parent has a high level of avoidance (Rubiales & Niks, 1992a). This suggests that the levels of avoidance of the respective addition/substitution lines on Chinese Spring might not be elevated as well.

In wheat, avoidance could be used in addition to the other two types of defence mechanisms against rust fungi. The other two types of defence mechanisms available in wheat are the hypersensitivity resistance genes (*Lr* genes, McIntosh et al., 1998) and genes for non-hypersensitive prehaustorial resistance (e.g. *Lr34*, Rubiales & Niks, 1995). Combination of these three types of defence in the same cultivar might confer a more general and durable protection.

A defence mechanism is considered durable when it remains effective for a long period of time. Avoidance may be a more durable alternative mechanism to the generally ephemeral hypersensitive resistance to leaf rust fungi. Its effectiveness against different leaf rust fungus species (*P. hordei*, *P. triticina*, *P. recondita* and *P. recondita*

agropyrina) confers it a wide range of action. However, to some other rust fungi that have been tested, avoidance is not effective (e.g. *P. striiformis*, Rubiales & Niks, 1992b). Right now, is very hard to predict the durability of this mechanism since no leaf rust fungus species that co-evolved with *H. chilense* is available for research. In the case such a rust species would be identified and avoidance would still be effective to that rust, then we can conclude that avoidance is a durable defence mechanism.

The AFLP marker map of *H. chilense* reported on Chapter 6 will allow mapping of genes or quantitative trait loci for other interesting agronomic traits of *H. chilense* segregating between H1 and H7 accessions. Resistances to several other wheat pathogens (*P. striiformis*, *P. graminis* and *Fusarium culmorum*) (Rubiales & Niks, 1992b; Rubiales et al., 1996) are among these interesting traits. In addition, a more dense linkage marker map with a larger number of co-dominant markers will also provide a more accurate location for the avoidance QTLs. Furthermore, the development and analysis of recombinant inbred lines (RILs), from this F_2 population, may contribute to a more precise genetic analysis of *H. chilense* morphological and agronomic interesting traits.

An interesting comparison with a novel method of genetic analysis, not requiring a segregating population, could be feasible in the available *H. chilense* collection. Screening the *H. chilense* accessions with a large number of molecular markers may identify QTLs for several interesting agronomic traits, directly from the germplasm, without the use of segregating populations, as described by Parminder et al. (1996). This method will have the advantages that no segregating population has to be developed, the results are not related to a single cross and it can be used for screening breeding material. Results obtained with the F_2 segregating population could be use to confirm the precision of this method of genetic analysis.

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Summary

Hordeum chilense is a perennial species occurring in Chile and Argentina. This wild barley species shows a very wide range of variation of morphological and agronomic characters and crosses easily with other members of the *Triticeae* tribe.

H. chilense is one of the five wild barley species in which avoidance of rust fungi as been reported. The avoidance mechanism to rust fungi is characterised by stomata overgrowth by the fungal germ tube, with no appressorium formation or penetration of the stomata, resulting in early failure of the infection process. This avoidance is an interesting mechanism of defence against rust infection, especially when it could be transferred to cultivated cereal species.

In this thesis the genetic basis of this avoidance mechanism and associations of avoidance with other characters in *H. chilense* were established.

We tested the hypothesis that the avoidance character occurs in a certain morphologically and molecularly distinct ecotype of *H. chilense*. A *H. chilense* collection of 88 accessions was characterised for morphological and agronomic traits, level of avoidance of *Puccinia hordei*, habitat of origin and AFLP fingerprint. Cluster analysis using both morphological/agronomic and AFLP fingerprint data suggested three distinct clusters of accessions. High avoidance was typical to the accessions of one of these three clusters. The accessions in the cluster with the higher levels of avoidance had been collected in humid habitats. This putative subspecific taxon was further characterised by shorter and wider spikes, more erect culms, a shorter uppermost internode until flag leaf and a greater amount of stomata density on the abaxial leaf side. We conclude that *H. chilense* consists of at least three rather well defined morphologically and genetically distinct subspecific taxa, one of which has a very high level of avoidance of barley leaf rust.

We studied the effect of the cuticular wax layer on the orientation of germ tube growth and on appressorium differentiation of *P. hordei*. Several orientation parameters and appressorium differentiation of *P. hordei* germ tubes were measured on *H. chilense* leaves with and without the wax layer.

Removal of the cuticular wax layer did not result in poor and also not in better germ tube orientation. Evidence was obtained that epidermal cell junctions rather than the wax crystals provided the landmarks to guide germ tubes along the transverse axis of the leaf. On high avoidance accessions the removal of the wax layer allowed

appressoria to develop over stomata that would otherwise be overgrown. This suggests that the overgrowth of stomata on *H. chilense* leaves by *P. hordei* germ tubes is mainly due to the wax covering of the stomatal apparatus.

A molecular map of the wild barley *H. chilense* would greatly facilitate to map and efficiently transfer agronomic traits from *H. chilense* to cereal genomes. As a first step towards a map construction, we evaluated AFLP markers in *H. chilense* using diploid wheat (*Triticum monococcum*) and cultivated barley (*H. vulgare*) as references. *H. chilense* showed a higher percentage of polymorphisms than diploid wheat. It was remarkable that, based on AFLP markers generated by 12 *Eco*RI/*Mse*I primer combinations, the cultivated barley was more similar to diploid wheat than to *H. chilense*. Even more surprisingly, the genetic distance between the interfertile *H. chilense* accessions (H1 and H7) was almost as large as the genetic distance between the non-crossable cultivated barley and diploid wheat. *Eco*RI/*Mse*I AFLP fingerprints revealed more polymorphism than *Pst*I/*Mse*I AFLP fingerprints for all species tested and were chosen for generating the first AFLP linkage map of *H. chilense*.

The mapping population consisted of 100 F_2 plants derived from a cross between two genetically distinct *H. chilense* accessions that were contrasting for the level of avoidance, H1 and H7. The constructed map contained 443 AFLP markers, on nine long and ten shorter linkage groups, covering a genetic distance of 714 cM. Nine of these linkage groups were assigned to *H. chilense* chromosomes using a set of *H. chilense* in *T. aestivum* addition and substitution lines. Strong clustering of AFLP markers was observed at putative centromeric regions. A skeletal map with a uniform distribution of markers was extracted from this linkage map. This skeletal map was applied to detect and map QTLs underlying avoidance and stoma density on the abaxial leaf epidermis.

Three QTLs were detected for avoidance and three other QTLs for stoma density. Both traits segregated independently in the F_2.

Avoidance on *H. chilense* is effective to barley leaf rust, wheat leaf rust and rye leaf rust. As *H. chilense* shows good crossability with several cultivated cereals avoidance of leaf rusts may be introgressed into these cultivated cereals, especially wheat. Addition and substitution lines of *H. chilense* in Chinese Spring wheat cv. are

already available or being developed as a first step towards transfer of the genes governing the avoidance trait.

Samenvatting

Hordeum chilense is een overblijvende plantensoort die voorkomt in Chili en Argentinië. Het is een wilde verwant van gerst, en toont een rijke variatie aan morfologische en landbouwkundige eigenschappen en is goed kruisbaar met andere soorten binnen de tribus van de *Triticeae.*

H. chilense behoort tot één van de vijf wilde gerstesoorten waarin vermijding van roestschimmels is gevonden. Deze vermijding van infectie door roestschimmels wordt gekenmerkt door het overgroeien van de huidmondjes op het blad door de kiembuizen, zonder dat er appressoriumvorming optreedt en zonder dat de huidmondjes binnengedrongen worden. Daardoor mislukt de infectiepoging in een zeer vroeg stadium van het infectieproces. Deze vermijding is een interessant mechanisme van afweer van de plant tegen de roestschimmel, vooral wanneer het overgebracht zou kunnen worden naar graansoorten.

In dit proefschift werden de overerving van deze vermijding en de associaties van deze vermijding met andere eigenschappen in *H. chilense* vastgesteld.

We toetsten de hypothese dat de vermijding vóórkomt in een bepaald morfologisch en moleculair onderscheidbaar ecotype van *H. chilense.* Een *H. chilense* collectie, bestaande uit 88 accessies, werd gekarakteriseerd voor morfologische en landbouwkundige eigenschappen, niveau van vermijding van de dwergroestschimmel *Puccinia hordei,* biotoopkenmerken van de plaats waar de accessie werd verzameld, en AFLP profiel. Clusteranalyse van de morfologische/landbouwkundige eigenschappen en AFLP-profielen wees uit, dat de accessies uiteenvielen in drie clusters. Een hoog niveau van vermijding was typisch voor de accessies behorend tot één van deze drie clusters. Deze accessies waren verzameld in tamelijk waterrijke habitats. Dit vermoedelijke subspecifieke taxon werd verder gekenmerkt door kortere en bredere aren, vertikaler groeiende stengels, een kortere afstand van de bovenste knoop tot het vlagblad , en een groter aantal huidmondjes per oppervlakte-eenheid op de epidemis van de onderzijde van het blad. We concludeerden dat *H. chilense* bestaat uit drie morfologisch en genetisch tamelijk duidelijk verschillende subspecifieke taxa, waarvan één een erg hoog niveau van vermijding van de gerst dwergroestschimmel vertoont.

We bestudeerden vervolgens het effect van de cuticula-waslaag op de orientatie van de groei van de kiembuizen van de roest *P. hordei,* en op de appressoriumdifferentiatie door deze schimmel. Verscheidene parameters die de

orientatie en appressoriumvorming van *P. hordei* kiembuizen kwantificeren werden gemeten op *H. chilense* bladeren, al dan niet na verwijdering van de waslaag van het bladoppervlak.

Verwijdering van de waslaag resulteerde niet in een slechtere of betere orientatie van de kiembuizen dwars op de lengterichting van het blad. Er werden aanwijzingen gevonden dat de kiembuizen de plaatsen waar epidermiscelwanden aan elkaar grenzen, als kenmerk gebruiken om zich op te oriënteren en niet de kristallen van de cuticulaire waslaag. Op de accessies met een hoog niveau van vermijding resulteerde verwijdering van de waslaag in de vorming van appressoria over huidmondjes, die normaliter overgroeid zouden worden. Dit wijst er sterk op, dat de overgroei van huidmondjes van *H. chilense* door de *P. hordei* roestschimmelkiembuizen vooral veroorzaakt wordt door bedekking van de huidmondjes door een waslaag.

Een moleculaire merker-kaart van *H. chilense* is zeer nuttig om de genetica van landbouwkundig interessante eigenschappen vast te stellen, en de verantwoordelijke genen efficiënt te incorporeren in de chromosomen van granen. Als een eerste stap voor de constructie van een dergelijke kaart onderzochten we de mate van AFLP-merker polymorfisme in *H. chilense*, en gebruikten daarbij diploide tarwe (*Triticum* monococcum) en cultuurgerst (*H. vulgare*) als referentie. *H. chilense* vertoonde een hoger niveau van polymorfisme dan diploide tarwe. Het was opmerkelijk dat, gebaseerd op AFLP-merkers gegenereerd door 12 *Eco*RI/*Mse*I primer-combinaties, gecultiveerde gerst meer gelijkenis vertoonde met diploide tarwe dan met *H. chilense*. Nóg opmerkelijker was het feit dat de perfect kruisbare *H. chilense* accessies (H1 en H7) genetisch bijna even sterk van elkaar verschilden als cultuurgerst verschilde van diploide tarwe, die volstrekt niet kruisbaar met elkaar zijn. Voor alle drie getoetste soorten vertoonden *Eco*RI/*Mse*I AFLP profielen meer polymorfisme dan profielen gegenereerd met *Pst*I/*Mse*I primercombinaties. Eerstgenoemde primercombinaties werden daarom gebruikt om de eerste AFLP moleculaire merkerkaart van *H. chilense* te construeren.

De karterings-populatie bestond uit 100 F_2 planten uit de kruising tussen accessies H1 en H7. Deze twee accessies verschillen genetisch sterk sterk van elkaar, en bovendien vertonen ze een groot verschil in niveau van vermijding. De geconstrueerde kaart bestond uit 443 AFLP merkers, en telde negen lange en 10 kortere

koppelingsgroepen. De totale genetische lengte van de kaart was 714 cM. Met behulp van een set van additie- en substitutielijnen van *H. chilense* chromosomen in *T. aestivum* kon van negen van de koppelinggroepen worden vastgesteld met welke chromosomen ze correspondeerden. De AFLP-merkers vertoonden een sterke clustering in wat vermoedelijk de centromeer-regio's van de chromosomen waren. Een vereenvoudigde merkerkaart werd verkregen door van merkers die nauw- of totaal gekoppeld waren, de meeste weg te laten. Deze vereenvoudigde kaart werd gebruikt om de posities vast te stellen van QTLs die het niveau van vermijding en de huidmondjesdichtheid op de onderkant van het blad bepalen.

Drie QTLs werden gevonden voor niveau van vermijding, en drie andere QTLs voor huidmondjesdichtheid. Deze eigenschappen splitsten onafhankelijk van elkaar uit in de F_2.

De vermijding door *H. chilense* is effectief tegen zowel de dwergroest van gerst, de bruine roest van tarwe als die van rogge. Aangezien *H. chilense* goed kruisbaar is met verscheidene graansoorten, kan vermijding van bruine-roestsoorten in principe overgebracht worden in granen, met name in tarwe. Additie- en substitutielijnen van *H. chilense* in de tarwecultivar 'Chinese Spring' zijn in ontwikkeling en deels al beschikbaar als een eerste stap naar introductie van de genen die verantwoordelijk zijn voor de vermijdingseigenschap.

Resumo

Hordeum chilense Roem. & Schult. é uma espécie perene autóctone do Chile e da Argentina. Esta cevada selvagem apresenta uma grande variabilidade morfológica e agronómica, cruzando-se facilmente com outros membros da tribo *Triticeae*.

H. chilense é uma das cinco espécies de cevada selvagem em que foi descrito o mecanismo de evitação (do inglês "avoidance") a determinados fungos do grupo das ferrugens. Este mecanismo de evitação caracteriza-se por um crescimento sobre o estoma do tubo germinativo do fungo, sem formação de appressório ou penetração no estoma, resultando numa paragem prematura do processo de infecção. Este mecanismo é uma interessante defesa contra os fungos das ferrugens, especialmente quando possa ser transferido para cereais cultivados.

Nesta tese foram estabelecidas a base genética deste mecanismo de evitação e as associações da evitação com outros caracteres em *H. chilense*. Testou-se a hipótese da evitação ocorrer num determinado ecótipo de *H. chilense* bem defenido morfológica e molecularmente. Uma colecção de 88 acessões de *H. chilense* foi descrita a nível morfológico e agronómico, capacidade de evitação do fungo *Puccinia hordei*, habitat de origem e perfil de marcadoresde AFLP (Amplified Fragment Length Polymorphism). A análise de agrupamentos, usando dados morfológico-agronómicos e o perfil de marcadores AFLPs, sugeriu a existência de três grupos distintos de acessões. Níveis elevados de evitação eram característicos das acessões de um destes agrupamentos. As acessões com altos níveis de evitação foram recolhidas em habitats húmidos. Este hipotético táxone subespecífico era, igualmente, caracterizado por espigas curtas e largas, um entrenó superior curto até à folha bandeira e uma grande densidade de estomas na superfície abaxial da folha. Concluimos então que a espécie *H. chilense* é constituida por pelo menos três táxones subespecíficos bem defenidos morfológica e molecularmente, apresentando um dos táxones um elevado nível de evitação ao fungo da ferrugem da folha da cevada.

Paralelamente, o efeito da camada de ceras cuticulares na orientação do crescimento do tubo germinativo e na diferenciação do apressório do fungo *P. hordei* foi estudado. Vários parâmetros de orientação e de diferenciação do apressório do fungo *P. hordei* foram medidos em folhas de *H. chilense* com e sem a camada de ceras cuticulares.

A remoção das ceras cuticulares não afectou significativamente a orientação do tubo germinativo. Foram obtidas evidências de que as junções das celulas epidérmicas são as responsáveis pela orientação do tube germinativo ao longo do eixo transversal da folha. Nas acessões com elevados níveis de evitação, a remoção das ceras cuticulares permitiu que os apressórios se desenvolvessem sobre os estomas que de outro modo seriam sobrecrescidos. Isto sugeriu que o sobrecrescimento dos estomas nas folhas de *H. chilense*, pelos tubos germinativos do fungo *P. hordei*, é principalmente devido à cobertura do aparelho estomático pelas ceras cuticulares.

Um mapa molecular da cevada selvagem *H. chilense* viria facilitar enormemente a localização e melhorar a transferência de características agronómicas de *H. chilense* para os genomas de cereais cultivados. Como primerio passo para o desenvolvimento de um mapa, avaliou-se a técnica dos marcadores AFLP em *H. chilense*, usando trigo diploide (*Triticum monococcum*) e cevada cultivada (*H. vulgare*) como referências. A espécie *H. chilense* apresentou uma maior percentagem de polimorfismos do que o trigo diploide. Foi extraordinário que a cevada cultivada tenha surgido mais semelhante ao trigo diploide do que ao *H. chilense*, usando marcadores AFLP gerados por 12 combinações de primers *Eco*RI/*Mse*I. Ainda mais surpreendente foi o facto de que a distância genética entre acessões interférteis de *H. chilense* (H1 e H7) ter sido quase tão grande como a distância genética entre as espécies não cruzáveis, a cevada cultivada e o trigo diploide. Os perfis de AFLPs baseados em *Eco*RI/*Mse*I revelaram mais polimorfismo do que os perfis baseados na combinação *Pst*I/*Mse*I em todas as espécies testadas, sendo consequentemente escolhidos para gerar o primeiro mapa de ligamento de *H. chilense* baseado em AFLPs.

A população de mapeamento consistiu em 100 plantas F_2 derivadas de um cruzamento entre duas acessões de *H. chilense* geneticamente distintas e com níveis contrastantes de evitação (H1 e H7). O mapa obtido contém 443 marcadores AFLP, distribuidos por 9 grupos longos e 10 grupos mais curtos, cobrindo um total de 714cM. Nove destes grupos de ligamento foram atribuídos a cromossomas de *H. chilense* usando um conjunto de linhas de adição e de substituição de *H. chilense* em *T. aestivum*. Um forte agrupamento de marcadores AFLP foi observado nas hipotéticas regiões centroméricas. Um sub-mapa, com uma distribuição uniforme de marcadores, foi extraido do mapa de ligamento. Este sub-mapa foi utilizado para detectar e mapear

QTLs (Quantitative Trait Loci) controlando o mecanismo de evitação e a densidade de estomas na superficie abaxial da folha.

Três QTLs foram detectados para a evitação e três outros QTLs para a densidade de estomas. Ambas características segregavam independentemente na F_2.

A evitação em *H. chilense* é efectiva contra a ferrugem da folha da cevada, trigo e centeio. Como o *H. chilense* pode ser facilmente cruzado com várias espécies de cereais cultivados, a evitação à ferrugem das folhas pode ser introgredida nestas espécies cultivadas, especialmente em trigo. Como primeiro passo para a transferência dos genes que controlam o mecanismo de evitação, linhas de adição e de substitutição de *H. chilense* na cv. "Chinese Spring" de trigo estão já disponiveis ou a ser desenvolvidas.

Acknowledgements

The last four-years-and-something of a gypsy existence have been full of sun and a lot of rain...Full of smiles at midday light and a sad moment over here and over there....Full of incredible books and delicate music, and above all full of new knowledge. I learned a lot about "avoidance" but also about people. It would never have been possible to add the last letter to this thesis without the help, support, advice and encouragement of a lot of people. I am deeply thankful to all of you.

I own special thanks to my promotors, Piet Stam and Antonio Martín, and co-promotor, Rients Niks, which were the central fulcrum of this thesis.

To my Dutch promotor, Piet, thanks for all your patience when teaching me how to solve the mysteries of this incredible world of mapping. All your kindness and cleaver suggestions were priceless to finish this thesis.

To my Spanish promotor, Antonio, you were one of the first ones to help me build up this dream. Your enchantment by the wild barley *Hordeum chilense* was contagious and soon we were writing a project for my PhD thesis. All your bright ideas, even from the distant sunny Córdoba, formed always a very important part of my work.

To my co-promotor, Rients, since the classroom of Aula Dei, in Zaragoza, your great knowledge on plant-pathogen interactions amazed me. That was the first time I ever heard about avoidance, and after some years, in collaboration with the research group from Córdoba, we ended up working together. I learnt from you how complex and fascinating may be the research on plant-pathogen interactions. Your constructive criticisms about my work, often difficult to deal with, and prompt help in the endless process of transforming my "Portuguese poetry" into a more scientific writing will be something I will never forget...

Two other persons have given an incredible contribution to this thesis, Diego Rubiales and Pim Lindhout, and for that I am very thankful to them. Diego, I inherited your research on avoidance and your love for microscopes and I am deeply grateful to all the ideas and knowledge that you shared with me. Life in the avoidance world would have been much more complicated without your precious contributions. The large experience on mapping resistance characters and broad vision on plant-pathogen interactions of Pim always opened my eyes to what was happening on the other side of the fence. Your remarks and comments were always the fine tuning of my work.

I am grateful to all the people in my previous lab in Córdoba that encouraged me to come to the Netherlands, especially to my previous supervisors, Ana Torres and José Ignácio Cubero.

It was a pleasure for me to be a member of the "resistance working group" in the Plant Breeding Laboratory. Discussions within this group were always helpful for my research and they extended my knowledge about plant-pathogen interactions. Also the friendly atmosphere of this big "resistance family" contribute to make me feel at home in this big 'unacclimatized' swimming pool that the Netherlands is... Many, many thanks to all the resistance members, Pim, Rients, Theo, Guusje, Ron, Ralph, Viviane, Corine, Marieke, Maarten, Fien, Petra, Yuling, Leandra, Sanwen, Sylvestre, Iris, Elaine, Ronald and Wei Li.

I am thankful also to Ies Bos and Jaap Buntjer for all their precious help in solving my statistical problems in the data analysis, and to Dr. Ramana for all his suggestions on chromosomal studies and his kind encouragement on my microscope work.

I would like to thank the technicians of the Plant Breeding Laboratory for all their help and teachings, especially Fien and Petra that supervised my first trembling steps with AFLPs. Also in the first part of my thesis in Córdoba, I could count on the precious help of Carmen and Ana that guided me through the secrets of *H. chilense* histology.

Many thanks to Annie Marchal and Letty Dijker-Lefers for all their help in various administrative matters. Finally, after all these years, I hope I can handle a fax machine without cold sweat...

I would also like to thank all the people from Unifarm for taking care of my plants all these years. They are quite resistant but they would never survive to the Dutch weather without the contribution especially of Anton, Mart, Henk and Michel.

Then, I cannot forget my two dear colleagues, companions of fate, and friends, Corine and Luisa. For all the long, long hours spent together in the penthouse of the Plant Breeding Laboratory, for all the support and encouragement in the hard times, and all the laughing and crazy cabarets that we have taken into scene in our two-room theatre. For all the threshold mails with tea and oranges and very unhealthy cookies in attach, my deepest, deepest thanks.

My thanks go also to all my other colleagues and friends from the Plant Breeding Laboratory. Specially to Tae-ho and Hans for always being happy to help me with their computer skills that saved me form several fatal errors...to Haider, for all his patience with my exploding UV light bulb and several consecutive hours of classical music in the lab...to Vivianne, for the many "pdf" troubles I create... to Tytti, Maarten, Ronald and Carolina for all the small personal and professional fruits that they offered me from this immense tree, on this very special corner, that is the Plant Breeding Laboratory.

But life does not end with work. Outside the Department a great group of friends made my life much more pleasant in the "Centre of the World". The south European group (Diego, Susana, Asun, Leyre, Matteo, Andrea, Pierre, Paula, Francesc, Laura, Sergio and Michael) with the uncountable delicious dinners and philosophical discussions were always the perfect medicine for an exhausted "avoidance" head. The Magriços & Co. in Wageningen (Susana, Célio, Luisa, Rob, Sónia, Sara, Graça among others), or spread around the world (Rita, Zé António, Angjelina) or in Portugal (Pedro, Luisa, Paulo, Catarina) always gave to my days a very Portuguese flavour. The beautiful Portuguese cuisine and wines, the unforgettable parties in the Portuguese embassy at the Centre of the World and the many many emails will always have a special place in my memories. I realise that I will need the Portuguese grammar-book by now, but if it weren't for you I probably would be applying for an intensive Portuguese course... And to all the rest of the friends that I end up meeting in this very special passing place, that are still around or that already moved to another corner of this world (Lucia, Jacaranda, Alejandro, Magda, Paloma, Neil, Nick), my deepest thanks for all the support and encouragement.

And last but not the least, I would like to thank my family and specially my parents for accepting and supporting my "irrequietos" gypsy genes all these years.

A todos vocês o meu mais profundo bem hajam.

Carlota

Curriculum vitae

Maria Carlota Morais e Cunha Vaz Patto was born on February 20th 1972 in Oliveira do Hospital, Portugal. In 1989 she entered the Instituto Superior de Agronomia at the Technical University of Lisbon, Portugal. From 1983 to 1984 she performed research on genotype and sowing date influence on the *Lupinus mutabilis* Sweet architecture, integrated on the European project "Adaptation of *Lupinus mutabilis* Sweet to European soil and climate conditions", for her graduation thesis, at the Department of Botanical and Biological Engineering from the Instituto Superior de Agronomia. In September 1994 she graduated, obtaining her Diploma de Licenciatura in Agronomic Engineering with majors in Plant Breeding. From October 1994 till July 1995 she attended a postgraduate course on Plant Breeding at the Mediterranean Agronomic Institute of Zaragoza (IAMZ), International Center for Advanced Mediterranean Agronomic Studies (CIHEAM), Spain. From October 1995 till January 1997 she performed research for her MSc thesis on the subject "Use of molecular markers for localisation of agronomic valuable genes and for *Vicia faba* systematic" at the Centro de Investigación y Desarrollo Agrario (CIDA), Córdoba, Spain. In June 1997 she graduate obtaining a MSc degree conferred by International Center for Advanced Mediterranean Agronomic Studies (CIHEAM), in IAMZ, Zaragoza, Spain. From January 1997 she started a four years PhD research project which is described in this thesis. The first six months were spend at the Instituto de Agricultura Sostenible (IAS), CSIC, Córdoba, Spain and the last four years at the Laboratory of Plant Breeding, Department of Plant Sciences of the Wageningen University.

The research presented in this thesis was carried out at the Laboratory of Plant Breeding, Wageningen University, The Netherlands, in close collaboration with the Instituto de Agricultura Sostenible (IAS), CSIC, Córdoba, Spain. The research was financed by PRAXIS XXI, Fundação para a Ciência e a Tecnologia, Lisboa, Portugal, Research fellowship number BD/9124/96.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG.

Vaz Patto, M.C. (2001)

The genetics and mechanism of avoidance of rust infection in *Hordeum chilense*.
Thesis Wageningen University - with references – with summaries in English, Dutch and Portuguese. Laboratory of Plant Breeding, Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands.

Key words: Avoidance, *Hordeum chilense*, wild barley, *Puccinia hordei*, rust fungi, thigmotropic responses, epicuticular waxes, appressorium, AFLP fingerprinting, genetic linkage map.

Printed by Ponsen & Looijen BV, Wageningen, The Netherlands.