I. INTRODUCTION

Leaf rust is considered as one of the most important foliar diseases of wheat worldwide and it occurs almost everywhere where wheat is grown (Dehne and Oerke, 1998; Cherukuri *et al*., 2005). Leaf rust causes significant yield losses every year. Severe wheat yield losses due to damage by leaf rust can range from 30 to 50% (McIntosh *et al*., 1995). The disease can reduce yield by 1% for every 1% increase in the infection level (Khan *et al*., 1997). The fungal pathogen *Puccinia triticina* Eriks. (synon. *P. recondita* Roberge ex Desmaz. f.sp. *tritici* Eriks. and E. Henn) largely known to cause leaf rust on both bread wheat (*Triticum aestivum* L) and durum wheat (*Triticum turgidum* ssp. *durum*) occurs almost everywhere where its host wheat is grown. On the other hand, the fungus *P. tritici-duri* V.-Bourgin, which causes leaf rust specifically on durum wheat (*Triticum turgidum* ssp. *durum*), was reported to be present in the Mediterranean region where durum wheat is traditionally cultivated and the alternate host, *Anchusa italica*, commonly grows (Ezzahiri *et al*., 1992). However, information on the occurrence of *P. tritici-duri* in other durum wheat producing regions of the world is generally lacking. Unless otherwise indicated, in this thesis, the name *P. triticina* denotes the fungal pathogen which commonly causes leaf rust on both bread and durum wheat.

Use of host resistance is the most economical (Kolmer, 1996) and environment-friendly method of controlling wheat leaf rust. Since fungicides are not readily available to small-scale farmers in developing countries like Ethiopia or due to the fact that their use in low input systems is not economically justifiable, use of leaf rust resistant cultivars remains to be the only practical and effective method of controlling the disease. However, host resistance may not always be readily available for use against leaf rust in the agronomically superior wheat cultivars, demanding wheat breeders and pathologists to look for resistance genes in the cultivated and wild forms of wheat.

According to McIntosh and Brown (1997) breeding for host resistance should be pre-emptive or anticipatory in approach so that cultivars to be developed should have adequate level of resistance against future pathotypes. Maximum success in this approach

can be realized through use of proper knowledge of the virulence of the pathogen genotypes and host resistance genes that occur in a given wheat-growing area. Annual virulence surveys, practices of gene postulation, and genetic analysis would greatly help to understand the status of the current host – pathogen interaction thereby making it possible to predict the appearance of new virulence combinations in the future (McIntosh, 1992; McIntosh and Brown, 1997). For instance, when the current situation indicates that the wheat cultivars grown in an area posses a single gene for resistance or when wheat cultivars are of similar genetic background for leaf rust resistance, it would be imperative to expect mutation to take place in the pathogen population (Statler, 1985; Steele *et al*., 2001) to form a new rust pathotype in the near future.

Wheat breeders and pathologists developed wheat differential near-isogenic lines (NILs) for specific leaf rust resistance genes to facilitate studies made to determine virulence phenotypes and relative frequency of pathotypes (Kolmer, 1996). Virulence analysis, with the help of NILs, enabled wheat pathologists and breeders to have a better understanding of the structure of the pathogen population. From virulence surveys, it becomes possible to determine the relative prevalence and distribution of virulence phenotypes or races, to assess the effectiveness of host resistance genes against the prevalent pathogen races, to detect the formation of new pathotypes, and to monitor their subsequent spreads over time and space. Long *et al*. (1998) identified 62, 42, and 51 virulence phenotypes (or races) among 681, 683, and 701 *P. triticina* isolates in the years 1993, 1994, and 1995, respectively in the United States. Twenty-one virulence phenotypes were identified in 253 *P. triticina* isolates in the South Atlantic States of the US in 1999 (Kolmer, 2002). McCallum and Seto-Goh (2002) also identified 38 virulence phenotypes from 394 *P. triticina* isolates collected from leaf rust infected wheat plants in Canada.

Gene postulation is the most widely used method to identify genes for leaf rust (*Lr*) resistance in various wheat cultivars or lines. Many researchers have employed this technique for identifying *Lr*-genes in a group of wheat genotypes (Wamishe and Milus, 2004). For instance, Statler (1984) identified genes *Lr*1, 2a, 2c, 10, 17, and 18 in 25 hard

red spring wheats; McVey and Long (1993) postulated genes *Lr*1, 2a, 3, 3ka, 9, 10, 11, 14a, 16, 17, 18, 24, 26, and 30 in 86 hard red winter wheat lines; Kolmer (2003) postulated *Lr*1, 2a, 9, 10, 11, 18, and 26 in a group of 35 soft red winter wheat cultivars and 17 breeding lines; and Wamishe and Milus (2004) postulated genes *Lr*1, 2a, 2c, 3, 3ka, 9, 10, 11, 14a, 18, 20, 23, 24, and 26 to be present in 116 wheat lines.

Gene postulation is advantageous over genetic analysis in that it provides the opportunity for quick identification of the probable race-specific seedling leaf rust resistance genes in a large group of wheat lines (Kolmer, 2003) and generates information on both the number and type of leaf rust resistance genes possibly present in wheat cultivars under study. Despite the fact that genetic analyis for leaf rust resistance is a more accurate method of assessing resistance genes in wheat cultivars, it takes much longer time as it involves crossing between susceptible and resistant parents and testing the segregating populations (Oelke and Kolmer, 2004).

Postulation of genes depends on the principle of gene-for-gene interaction (Flor, 1971) between the host line and pathogen isolates to determine the most probable resistance genes in wheat cultivars tested. Presence of race-specific resistance genes can be postulated based on phenotypic expressions in the form of infection types (ITs) as the wheat lines are infected with a series of pathogen isolates/races (Wamishe *et al*., 2004; Kolmer, 2003). The ITs produced on NILs, containing specific resistance genes, are the basis for comparison with the ITs of target wheat cultivars. The reaction of different target wheat cultivars can be compared with the reaction of NILs. For instance, when a wheat cultivar and a NIL are inoculated with the same pathogen isolate or race, the wheat cultivar with similar reaction type with the NIL is postulated to have the gene present in that specific NIL.

Once the resistance status of currently grown wheat cultivars is characterized through virulence analyis and gene postulation, the next practice would be the identification of new sources of resistance in the cultivated or wild forms of wheat. Then the transfer of target resistance gene(s) into wheat cultivar of interest follows. Use of several resistance

genes in a single cultivar through gene pyramiding technique is of paramount importance as the additive effects of several genes gives the cultivar a wider base of leaf rust resistance (Roelfs *et al*., 1992).

So far, more than 50 wheat leaf rust resistance genes have been described (Feuillet *et al*., 2003) and many of these genes originated from the wild forms of wheat, including *Lr*38, which was transferred to wheat from *Agropyron intermedium* (Wienhues, 1966, 1973). Such leaf rust resistance genes have been transferred to wheat with the objective of enhancing the resistance of existing wheat cultivars (Kolmer, 1996). Other leaf rust resistance genes include *Lr*9 which was transferred to common wheat from *Triticum umbellulatum* (Soliman *et al*., 1963), *Lr*19, *Lr*24, and *Lr*29 from *Agropyron elongatum* (Sharma and Knott, 1966; Browder, 1973; Sears, 1973), *Lr*28 and *Lr*35 from *Triticum speltoides* (McIntosch *et al*., 1982; Kerber and Dyck, 1990), *Lr*32 and *Lr*39 from *Triticum tauschii* (Kerber, 1987; Gill and Raupp, 1987), and *Lr*50 from *Triticum timopheevii* subsp. *armeniacum* (Brown-Guedira *et al*., 1997).

Use of PCR-based molecular markers for the identification of leaf rust resistance genes will help to reduce the time, effort and expense of their detection and use in wheat genotypes through marker-assisted selection (MAS) and pyramiding of major genes into wheat cultivars of interest (Gupta *et al*., 1999; Naik *et al*., 1998; Seyfarth *et al*., 1999). According to Gupta *et al*. (1999), PCR-based molecular markers were identified for 20 specific leaf rust resistance genes in wheat and this practice is becoming increasingly important. Recently, additional molecular markers were developed for the genes *Lr*16 (McCartney *et al*., 2005), *Lr*21 (Huang and Gill, 2001), *Lr*34 (Suenaga *et al*., 2003) *Lr*39 (Raupp *et al*., 2001), *Lr*46 (William *et al*., 2003), *Lr*50 (Brown-Guedira *et al*., 2003), *Lr*51 (Helguera *et al*., 2005), and *Lr*52 (Hiebert *et al*., 2005). Some of the leaf rust resistance genes have been cloned and sequenced. For instance, a completely linked RAPD marker with the leaf rust resistance gene *Lr*9 was cloned and sequenced after which a sequence-tagged-site (STS) marker was developed (Schachermayr *et al*., 1994). A closely linked RAPD marker with the leaf rust resistance gene *Lr*28 was also cloned and sequenced to develop a STS marker (Naik *et al*., 1998). Similarly, a completely

linked RFLP marker with the leaf rust resistance gene *Lr*35 was cloned and sequenced to develop a STS marker (Seyfarth *et al*., 1999).

A more detailed research is expected in the foreseeable future to study the molecular basis of virulence/avirulence combinations in the host – pathogen systems. So far, only little has been done on the molecular tagging of genes in pathotypes responsible for virulence. *P. triticina* is not an exception. Recently, Thara *et al.* (2003) identified a number of *in planta* induced fungal genes from susceptible wheat leaves four days after infection with *P. triticina*. Twenty-five out of 40 cDNA ribosomal protein genes were likely to be of fungal origin. Zhang *et al.* (2003) identified both wheat host genes that were differentially regulated across a range of time-points during compatible (susceptible) interactions along with *P. triticina* fungal genes were expressed *in planta*. *P. triticina* is one of the fungi on which the Fungal Genome Initiave has placed a special focus (Birren *et al*., 2003). The genome size of *P. triticina* was estimated to be 90 Mb and comprised 19 chromosomes. A genetic map is being developed using AFLPs, several cDNA libraries have been developed at different stages of the fungus. Approximately 20,000 ETS have been sequenced generating ~4500 unique sequences. Such more refined works targeting at the understanding of the host – pathogen interaction at molecular level will facilitate the detection of genes involved in infection processes and hence will contribute in developing better management practices against the disease.

Relationships between virulence of *P. triticina* isolates and molecular polymorphism have been studied previously (Kolmer, 2000, 2001; Kolmer *et al*., 1995; Kolmer and Liu, 2000; Kosman *et al*., 2004; Park *et al*., 2000). The magnitude of correlation values varied greatly across the above findings. For instance, reasonably high correlation between virulence and molecular polymorphism was reported by Kolmer (2000) and Kolmer *et al*. (1995) with *r* values of 0.53 and 0.58, respectively. On the other hand, Park *et al*. (2000) observed only little correlation between virulence and RAPD polymorphisms of *P. triticina* isolates.

The present study on the genetic analysis of in the wheat – *P. triticina* was undertaken following two major approaches, classical and molecular. In the classical approach, we studied the virulence of isolates from Ethiopia and Germany on different wheat cultivars; the isolates were identified as physiologic races of the pathogen; and leaf rust resistance genes were postulated for their presence in different wheat cultivars. In the molecular approach, genetic diversity of leaf rust isolates collected from both countries was studied and microsatellite (simple sequence repeat, SSR) markers were identified for use in detecting the presence or absence of the leaf rust resistance gene *Lr*38 in wheat. The major objectives of the study were therefore (1) to determine the molecular genetic diversity of leaf rust isolates collected from Ethiopia and Germany; (2) to characterize leaf rust resistance in the host – pathogen interaction through virulence analysis and gene postulation; and (3) to map the leaf rust resistance gene *Lr*38 using SSR markers.

II. MATERIALS AND METHODS

2.1 Organisms

2.1.1 *Puccinia triticina* isolates

The following monopustule isolates of *P. triticina* Eriks. were used in the study.

- *P. triticina* isolates for molecular diversity, virulence and gene postulation studies (Table 1)
- Isolate DZ7-24 for genetic linkage analysis of SSR markers with the leaf rust resistance gene *Lr*38.

2.1.2 Plants

The following bread wheat (*Triticum aestivum* L.) cultivars/lines were used in this study.

- Cultivar Monopol for developing, multiplying, and maintaining monopustule isolates of *Puccinia triticina* Eriks.
- Thatcher-derived near-isogenic lines (NILs) supplied by Dr V. Lind, Aschersleben were used for virulence analysis and gene postulation (Table 2)
- Wheat cultivars for virulence analysis and gene postulation (Table 3)
- Cultivar Kubsa as a susceptible parent
- The NIL for *Lr*38 (RL6097) as a resistant parent
- F_2 and F_3 plants and the two parents (RL6097 and Kubsa): for mapping the *Lr*38 with SSR markers (Table 4).

2.2 Developing monopustule isolates

The Ethiopian *P. triticina* isolates were collected from farmers' wheat fields in the main cropping season (summer) of 2003. The collection was made in 10 km intervals along the roadsides. The German *P. triticina* isolates were kindly provided by Dr Kerstin Flath, Federal Biological Research Center for Agriculture and Forestry (BBA), Kleinmachnow, Berlin.

The seeds of cultivar Monopol, with no known leaf rust resistance gene described, were sown to plastic pots (7 x 7 x 8 cm) filled with seedling substrate (Klasmann-Deilmann GmbH, Geeste, Germany). Watering plants was done as required by plants. No additional fertilizer was applied to plants.

When seedlings became 10 - 15 days old, leaves were harvested and cut into segments of 5 - 7 cm long. The leaf segments were placed on 5 % water agar as described by Mebrate and Cooke (2001) containing benzimidazole (0.035 % w/v) and streptomycin sulphate (0.05 % w/v). The leaf segments were sparsely inoculated with bulk spores of each isolate using a cotton swab moistened with sterile distilled water. The inoculated leaf segments were kept under moist and dark condition for 24 hrs. Then, the leaf segments were incubated in a chamber adjusted to 20 °C and continuous white light for 7 to 9 days. When symptoms fully develop, a single pustule was isolated by a moistened cotton swab and inoculated to new leaf segments for multiplication. A total of 43 monopustule isolates of *P. triticina* were developed (Table 1).

2.3 Grouping of *P. triticina* isolates

To have more insight into the molecular diversity of the pathogen isolates, 3 groups were made based on the region of collection. As leaf rust samples from Ethiopia were collected from regions largely differing in environmental conditions and production systems (Bechere *et al*., 2000), isolates from Ethiopia were divided into two sub-regions. The German isolates were not subdivided into groups.

The first region consisted of 17 isolates of *P. triticina* (167-176, WBR14, Bonn1, Bonn2, WBR1, K-B1, Mon1-p10, 77WxR, WBR7, WBR5, WBR4, WBR10, WBR12, K-B2, WBR2, WBR3, and WBR6) collected from Germany. Bread wheat is the major type of wheat grown in Germany. South & Southeastern Ethiopia formed the second region with 12 isolates (E-30, E-33, E-35, E-43, KK, PPRC2-1, PPRC2-3, PPRC3-1, PPRC3-3, Bekoji, Dodolla, and E-4). This region is known for its bread wheat production. According to Ensermu *et al*. (1998), about 75 % of Ethiopia's bread wheat area is located

Table 1. *P. triticina* isolates, origin, host cultivar, year of collection, and purpose in this study.

Isolate	Origin[1]	Host cultivar[2]	Year of collection	Purpose[3]
Ak9-3	Akaki, CE	Pavon-76, BW	2003	a,b
Alemgena	Alemgena, CE	Gerardo, DW	2003	a, b
Bekoji	Bekoji, SSE	Galama, BW	2003	a, b
CD	Chefe Donsaia, CE	Israel, BW	2003	a, b
Dodolla	Dodolla, SSE	Na, BW	2003	a
DZ7-21	Debre Zeit, CE	Arendeto, DW	2003	a, b
DZ7-23	Debre Zeit, CE	Na, DW	2003	a, b
DZ7-24	Denkaka-Debre Zeit, CE	Na, BW	2003	a, b, c
E-4	Goffer Estate Farm, SSE	Na, BW	2003	a
E-8	Sama Senbet, CE	Pavon-76, BW	2003	a
E-16	Tefki, CE	Kubsa, BW	2003	a, b
E-19	Keta-Tulubollo, CE	Na, BW	2003	a
E-22-2	Wolliso-Goro, CE	Kubsa, BW	2003	a
E-23	Wolliso, CE	Israel, BW	2003	a
E-30	Shenshicho, SSE	N/a, BW	2003	a, b
E-33	Durame, SSE	Dashen, BW	2003	a, b
E-35	Butajira, SSE	N/a, BW	2003	a, b
E-43	Womber Godeti, SSE	Gerardo, DW	2003	a, b
E-52	Gogecha-Akaki, CE	Pavon-76, BW	2003	a
KK	Kersana Kondaltiti, SSE	Pavon-76, BW	2003	a, b
Minjar1	Minjar, CE	Pavon-76, BW	2003	a, b
Minjar2	Minjar-Arerti, CE	Pavon-76, BW	2003	a, b
PPRC2-1	Lemo, SSE	Katar, BW	2003	a, b
PPRC2-3	Lemo, SSE	Katar, BW	2003	a, b
PPRC3-1	Adaali, SSE	Abola, BW	2003	a, b
PPRC3-3	Adaali, SSE	Abola, BW	2003	a, b
Bonn1	Bonn, Germany	Dekan, BW	2004	a
Bonn2	Bonn, Germany	Munk, BW	2004	a, b
K-B1	Lower Saxony, Germany	Denver, BW	2003	a
K-B2	Lower Saxony, Germany	Dekan, BW	2003	a, b
Mon1-p10	Mainz, Germany	Monopol, BW	2003	a, b
167-176	Germany	Na	Before 1990	a, b
77WxR	Germany	Na	Before 1990	a, b
WBR1	Germany	Na	Before 1990	a
WBR2	Germany	Na	Before 1990	a, b
WBR3	Aschersleben, Germany	Borenos, BW	2001	a, b
WBR4	Bonn, Germany	Dekan, BW	2003	a, b
WBR5	Bonn, Germany	Dekan, BW	2003	a
WBR6	Bonn, Germany	Drifter, BW	2003	a, b
WBR7	Bonn, Germany	Drifter, BW	2003	a, b
WBR10	Mainz, Germany	Punch, BW	2003	a, b
WBR12	Mainz, Germany	Dekan, BW	2003	a
WBR14	Mainz, Germany	Monopol, BW	2003	a, b

[1] CE: Central Ethiopia; and SSE: South & South-East Ethiopia.
[2] BW: bread wheat; DW: durum wheat; and Na: specific variety name not available
[3] a: Isolates used in the molecular diversity study; b: isolates used in virulence analysis and gene postulation; and c: isolate used in mapping *Lr*38 with SSR markers.