Analyzing Genetic Diversity for Virulence and Resistance Phenotypes in Populations of Stem Rust (*Puccinia graminis* f. sp. *secalis*) and Winter Rye (*Secale cereale*)

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ABSTRACT

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Stem rust (*Puccinia graminis* f. sp. *secalis*) leads to considerable yield losses in rye-growing areas with continental climate, from Eastern Germany to Siberia. For implementing resistance breeding, it is of utmost importance to (i) analyze the diversity of stem rust populations in terms of pathotypes (= virulence combinations) and (ii) identify resistance sources in winter rye populations. We analyzed 323 single-uredinial isolates mainly collected from German rye-growing areas across 3 years for their avirulence/virulence on 15 rye inbred differentials. Out of these, 226 pathotypes were detected and only

56 pathotypes occurred more than once. This high diversity was confirmed by a Simpson index of 1.0, a high Shannon index (5.27), and an evenness index of 0.97. In parallel, we investigated stem rust resistance among and within 121 heterogeneous rye populations originating mainly from Russia, Poland, Austria, and the United States across 3 to 15 environments (location-year combinations). While German rye populations had an average stem rust severity of 49.7%, 23 nonadapted populations were significantly ($P < 0.01$) more resistant with a stem rust severity ranging from 3 to 40%. Out of these, two modern Russian breeding populations and two old Austrian landraces were the best harboring 32 to 70% fully resistant plants across 8 to 10 environments. These populations with the lowest disease severity in adult-plant stage in the field also displayed resistance in leaf segment tests. In conclusion, stem rust populations are highly diverse and the majority of resistances in rye populations seems to be race specific.

Stem rust (*Puccinia graminis* Pers.:Pers. f. sp. *secalis* Eriks. & E. Henn.) of winter rye (*Secale cereale* L.) is a devastating disease in areas with continental climate. The minimum temperature for the infection is 15°C and the optimum temperature for reproduction is 26°C at high light intensity (Obst und Gehring 2004). Uredinia with brick-red uredospores occur after flowering mainly on stems but also on leaf sheaths, leaves, and spikes. During host maturity, telia of black teliospores appear on infected tissue. Stem rust epidemics in rye are capable to reduce grain yield considerably. Solodukhina und Kobylansky (2000) reported losses of thousand-grain weight by up to 36% in Russia.

Winter rye is an important cereal crop with a total acreage of 5.3 million hectares worldwide in 2014 (FAO 2016). About 75% of this acreage is located in Central and Eastern Europe, namely Belarus, Germany, Poland, the Russian Federation, and Ukraine. Rye is the only cross-pollinating crop among the small-grain cereals with a strong self-incompatibility system. In Germany, about 40% of the total rye acreage is grown by open-pollinated population cultivars, the remainder by hybrid cultivars. Both types of cultivars, however, represent highly heterogeneous stands due to the outcrossing nature of rye and because the hybrids are composed of several lines (Geiger

and Miedaner 2009). Open-pollinated population cultivars are produced by random mating with selection. Selection, however, cannot focus on individual genotypes, like in self-pollinated species, but aims to change gene frequencies such that desirable genotypes predominate the population (Acquaah 2012). The resulting cultivars are consisting of many, genetically different and highly heterozygous individuals that are produced by panmictic intermating (Schnell 1982). Commercial hybrid cultivars in rye constitute a cross between a single-cross seed parent and a two-line synthetic as pollen parent ($A \cdot B \times \text{Syn } C, D$; Geiger and Miedaner 2009) and are, therefore, fully segregating in commercial stands. Resistance evaluation in such genetically variable types of cultivars must include a representative number of plants that are individually rated for their disease severity. In outcrossing species, like winter rye, genotypic variance within populations is always larger than that between populations. In ergot resistance in winter rye, for example, 70% of genetic variation was found within populations compared with 30% between populations (Mirdita et al. 2008).

The first stem rust epidemic occurred in winter rye in 1992 in Northern and Eastern Germany (Obst und Gehring 2004). In the federal state of Brandenburg, stem rust is recognized today as the economically most important disease of winter rye. In 2003, stem rust infections were also observed in breeding nurseries in South-Western Germany and reoccurred irregularly since then. Stem rust can be controlled by fungicides. However, the application is rather late in growth period and, therefore, sprayings cannot be combined with the control of other diseases like leaf rust (*Puccinia recondita* Roberge ex Desmaz). Growing of resistant cultivars can reduce the need for fungicides and is an economically and environmentally

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friendly strategy to control stem rust. In organic farming, resistant cultivars are the only means for control.

Race-specific resistance to stem rust in rye was already described in the early 20th century (Mains 1926). The resistance genes (*R*) interact directly or indirectly with corresponding avirulence (*Avr*) gene products of the pathogen in a gene-for-gene relationship (Flor 1971). This reaction is highly specific and often elicits rapid and localized cell death by hypersensitivity. A single mutation in the pathogen, however, can render an *R* gene ineffective. Some rye-derived *R* genes to stem rust have long been used in wheat breeding, e.g., *Sr27*, *Sr31*, *Sr36*, that were introgressed into wheat via wheat-rye translocation lines involving rye chromosome 1R (Luig and Watson 1976; Mago et al. 2005; Purnhauser et al. 2011; Singh et al. 1994). In rye as a crop, however, reports on stem rust resistance are scarce. Tan et al. (1976) found six independent race-specific *R* genes in only four rye inbred lines demonstrating the richness of stem rust resistances in rye. This type of resistance is easy to handle for the breeder, but notorious for low durability (for reviews Stuthman et al. 2007; Miedaner 2016). Causes are the genetic variation of the pathogen via sexual and asexual recombination, migration, and directional selection toward virulence by using single *R* genes in homogeneous cultivars, like in wheat, barley, and oats (McDonald and Linde 2002). Rye, however, is cross-pollinating and individual population cultivars may contain plants with all levels of resistance. Thus, selection pressure by monogenic resistances might be lower than in self-pollinating crops.

Alternatively, quantitative resistance is available delaying rust development (“slow rusting”) although a susceptible infection type is given (Kolmer 2013). This type of resistance must be expressed in the adult-plant stage to be effective in the field. Quantitative resistance has been suggested to be generally more durable than race-specific resistance (Stuthman et al. 2007) and was detected in practically all pathosystems, including stem rust of wheat (Hare and McIntosh 1979). Although the genetic basis of quantitative resistance is largely uncharacterized in the cereal/stem rust pathosystem, first studies indicated that it is polygenically inherited (Knott 1982). Genomic selection has, therefore, been suggested to select efficiently for quantitative rust resistances (Rutkoski et al. 2011). For rye, no report on quantitative resistance is available.

For the analysis of pathotypes as well as race-specific resistance, a differential set of inbred lines and corresponding single-uredinial isolates is essential, but was until now not available in rye. In modern German germplasm of winter rye, nothing is known on stem rust resistance. Resistance sources have been reported mainly from some Russian and U.S. populations (Tan et al. 1976; Solodukhina and Kobylansky 2000, 2007). Solodukhina and Kobylansky (2007) described a resistant Russian population from the 1930s (“Derjavinskaya 29”) where 85% of individual plants within the population were totally resistant to stem rust. Other rye populations displayed 0.1 to 18% resistant plants in their study. Some of these resistances could be also attributed to single, race-specific resistance genes. Similarly Tan et al. (1976) described *R* genes from nonadapted rye sources (‘Kenya’, ‘Gator’, ‘Wrens’, ‘Elbon’ × ‘Gator’). The agronomic performance and climatic adaptation of such genetic resources is usually poor under the intensive growing conditions in Germany with average grain yields of 5.5 to 6.1 t ha⁻¹ in 2012 to 2014 (FAO 2016). However, they could be a starting point for resistance breeding by introgression into elite material. Moreover, rye can readily serve as donor of new *R* genes to stem rust resistance for both wheat and triticale.

The evolutionary potential of a pathogen population is reflected in its population genetic structure (McDonald and Linde 2002). To develop a rational approach for breeding durable resistance the amount of variation in host and pathogen populations must be analyzed. The higher the genetic variation of stem rust populations the more likely race-specific resistances are becoming ineffective. Because all recent rye germplasm in Germany is highly susceptible there should be only a low selection pressure to the pathogen. In

view of this, our hypothesis was that the stem rust population should not be too diverse with most isolates having only a few virulences. This is also the cause why we concentrated on foreign rye germplasm (genetic resources).

The specific objectives were to (i) develop a differential set of rye inbred lines for evaluating avirulence/virulence combinations, (ii) analyze frequency of pathotypes and virulence complexity of rye stem rust populations, (iii) identify rye germplasm with stem rust resistance among nonadapted populations from a world-wide collection, (iv) analyze the genetic variation within resistant outcrossing rye populations, and (v) test resistances found in adult-plant stage additionally in detached leaf segment test in the seedling stage to investigate the type of resistance. To reach these goals, we analyzed 323 single-uredinial isolates of rye stem rust collected across Germany in 3 years and 121 winter rye populations by inoculation trials across 3 to 15 environments (location × year combinations); material with higher resistance was tested in more environments than susceptible populations. This study is the first comprehensive analysis of diversity of both pathotypes in the stem rust pathogen and resistances in the rye host, respectively.

MATERIALS AND METHODS

Sampling procedure and establishment of stem rust isolates.

Isolates were collected randomly in the years 2011 to 2013 from infected rye stems that were sent by breeders and plant protection services of the federal states of Germany. Spores were scraped off from infected stems, suspended in a 0.1% agar solution and brushed directly on detached leaf segments of a susceptible hybrid cultivar (‘Palazzo’, KWS LOCHOW, Bergen, Germany) for multiplication. Leaf segments were placed upside down in petri dishes containing water agar (6 g liter⁻¹ of agar, Carl Roth, Karlsruhe, Germany), benzimidazol (35 mg liter⁻¹, Carl Roth), and silver nitrate (1.5 mg liter⁻¹, Carl Roth). To induce infection, the plates were incubated at 100% relative humidity and 20°C in the dark for 24 h. Afterward, they were maintained at 20°C with continuous light. Single-uredinial isolates were established by diluting spores collected from infected leaves on a susceptible genotype and subculturing of individual developing uredospore pustules as described above. In total, 323 single-uredinial isolates were analyzed. Out of them, 312 isolates originated from 11 federal states of Germany, five isolates from Poland, and six isolates from Russia. We do not partition the samples according to their origin because sampling size was highly varying ranging from 1 (Saxonia-Anhaltina and Berlin) to 98 (Hessia). To maintain the isolates, spores were harvested from inoculated leaf segments, placed in 0.2-ml tubes, and dried on Silica Gel Orange (Carl Roth) in the refrigerator (6°C) for 4 to 4.5 h. Afterward, they were stored at -80°C for about 1.5 years.

Development of a detached leaf segment test. Detached leaf segments (3 cm long) of 8-day-old primary leaves of each of 50 rye inbred lines were placed upside down in square plastic dishes with 12 compartments containing water agar (6 g liter⁻¹ of agar, Carl Roth) complemented by benzimidazol (35 mg liter⁻¹, Carl Roth) and silver nitrate (1.5 mg liter⁻¹, Carl Roth). Inoculum was prepared by mixing 1.5 ml of a 0.1% agar solution with 0.8 mg of uredospores. The suspension was evenly distributed with a fine brush on the detached leaf segments. Afterward, plates were incubated as mentioned above. Twelve to fourteen days after inoculation, leaf segment reactions were assessed. For calculating means from the infection type, the Stakmann et al. (1962) scale was converted to a numeric scale such that symbols ‘0’ = 0, ‘0;’, ‘;’, and ‘1’ were unified to 1, ‘2’ = 2, ‘3’ = 3, ‘4’ = 4. Infection types 0 to 2 were interpreted as incompatible (resistant/avirulent) and infection types 3 to 4 as compatible (susceptible/virulent). For each entry, three leaf segments were tested. When they did not react similarly, the test was repeated. For controlling the success of infection, a leaf segment of the highly susceptible standard cultivar Palazzo was included in each compartment. The test was evaluated only when

this standard cultivar showed a compatible infection type of 3 to 4. From the 50 rye inbred lines, only those showing a differential reaction with the isolates were chosen. Finally, 15 inbred lines from the private company KWS LOCHOW GMBH (Bergen, Germany) were selected for the differential set.

Evaluating virulence frequency and diversity. Virulence structure of the stem rust population was described by the frequency of virulent isolates on 15 differentials and their virulence complexity in the seedling stage. Isolates from the growing seasons 2011, 2012, and 2013 were inoculated on the 15 differentials during the winter months following the respective growing season and the percentage of virulent isolates was determined. Differentials displaying frequencies of virulent isolates of 0 to 10% were classified as highly resistant, 11 to 20% as resistant, 21 to 50% as moderately resistant, and >50% as susceptible (Felsenstein und Jaser 2000). The number of differential lines that an isolate is capable of infecting is defined as virulence complexity (Welz 1986).

Virulence diversity was described by the Simpson (S) and Shannon (H) indices calculated as

$$S = 1 - \sum (n_i^2 - n_i) / (N^2 - N)$$

$$H = - \sum P_i \times \ln P_i / \ln R$$

where N is the number of tested isolates, n_i the number of observed isolates of the i th pathotype, P_i the frequency of the i th pathotype, and R the total number of pathotypes in the sample (Müller et al. 1996). A virulence phenotype is a specific combination of virulences. Both indices account for the number of different pathotypes (richness) in the population. The higher the value the more different pathotypes were found.

The evenness index (EH) is based on the Shannon index (H) and is estimated as

$$EH = H / \ln R, \text{ with } H = - \sum P_i \times \ln P_i$$

where P_i is the frequency of the i th pathotype and R is the total number of pathotypes in the sample. If pathotypes occur in similar frequencies $EH = 1$, if they occur in highly variable frequencies the value will be 0.

Rye populations and field testing. This study comprised in total 122 winter rye populations including six German (Conduct, Recrut as released varieties, Firmament, Lautenbacher, Lichtkornroggen, Rolipa as specialty ryes bred for organic farming) and one Polish (Amilo) standard population grown under organic farming conditions. The other populations originated from Russia ($n = 47$ populations), Austria ($n = 12$), United States ($n = 12$), Poland ($n = 9$), and 15 other countries with one to five populations each (Supplementary Table S1). One population ('Florida Black' from the United States) was left out because it did not survive the winter in 2 out of 3 years. They largely differed in origin, age, and breeding status (landraces, improved landraces, old cultivars, modern breeding material). Entries were arranged according to α -lattice designs with three replicates in 3 years (2012, 2013, and 2014). Five trial sites were used: Berlin-Dahlem (DAL, latitude 52°44'94", longitude 13°27'68", 45 m above sea level [a.s.l.], 600 mm annual precipitation, 8.8°C mean annual temperature), Dahnsdorf (DAN, 52°09'98", 12°65'89", 77 to 85 m a.s.l., 526 mm precipitation, 8.5°C mean annual temperature), Petkus (PET, 51°98'69", 13°34'84", 145 m a.s.l., 600 mm precipitation, 8.4°C mean annual temperature), Bad Vilbel (VIL, 50°18'05", 8°74'11", 106 to 142 m a.s.l., 705 mm precipitation, 9.4°C mean annual temperature), Stuttgart-Hohenheim (HOH, 48°70'97", 9°20'46", 245 m a.s.l., 643 mm precipitation, 10.1°C mean annual temperature). To test the maximum number of populations with restricted budget we used the following procedure: In 2012, we tested seven standard populations and 63 entries in DAL, DAN, and HOH. Thirty of these entries, including the standards, were additionally tested in PET and VIL. In

2013, the seven standards and the eleven most resistant entries from 2012 were retested at all five locations. Additionally, 52 new populations were tested at DAL, DAN, and HOH, 12 of them also in PET and VIL. Finally, in 2014 the seven standards and 23 most resistant populations from previous years were retested. So each population was tested in at least three locations and, when sufficiently resistant, in maximal 15 environments. As an environment we define a location-year combination. The standards were tested in all 15 environments. At each location, each entry was grown in three-row microplots of 0.5 to 1 m length and 0.625 m width. To avoid the long-strawed, nonadapted rye entries (up to 220 cm tall) from falling into each other due to lodging, we used a chessboard-like design, i.e., each plot with a rye entry was bordered by four plots of similar size that were planted with a lodging resistant triticale cultivar ('Tulus', NORDSAAT, Halberstadt, Germany). We consider triticale as a nonhost for *P. graminis* f. sp. *secalis* and indeed, we never found stem rust infections in triticale during the experiment. This additionally reduces interplot interference caused by drifting of spores during inoculation or secondary distribution of spores.

Inoculum production, inoculation, and resistance assessment in field trials. Inoculation was performed with a mixture of five to six isolates of *Puccinia graminis* f. sp. *secalis* that were pretested for their virulence on a differential set of 15 inbred lines (Supplementary Table S2). The isolates were able to infect 3 to 10 of the differential lines. Uredospores were produced on 7-day-old seedlings of a susceptible rye hybrid by spraying the primary leaves with a 0.1% agar-uredospore suspension. The plants were kept for 24 h at 21°C and 100% humidity in the dark and for the remainder time at 21°C, 10.000 Lux and 16 h light in a climate chamber with maximal humidity. After 12 days, the uredospores were shaken off in glass petri dishes and stored on glycerin in the refrigerator (6°C) for up to 3 months. Isolates were separately propagated and mixed in equal amounts prior to inoculation. Inoculation was done at EC59-65 (end of heading till mid of flowering) by applying a 0.1% agar suspension with 120 mg of spores per 100 m² by a micro-sprayer (Micron Ulva, Bromyard Industrial Estate, Bromyard, Herefordshire HR7 4HS, U.K.).

Stem rust severity was assessed at early-dough stage (EC80-84), when the uredinia and telia were clearly visible on the stems. The stem segment between the second leaf from top (F-1) and the next internode was visually assessed as percentage of the stem covered by rust. We assessed 15 randomly chosen stems per plot individually, each stem from a different plant.

From the 15 single ratings per plot mean stem rust severity was calculated. All computations were based on these plot means. We performed a two-stage analysis following standard procedures after Cochran and Cox (1957). The phenotypic data of each environment were firstly analyzed separately by the PLABSTAT (Utz 2015) software package. Because of a low genetic differentiation as indicated by low repeatabilities, PET12 and PET14 were excluded from the further analyses of variance (Supplementary Table S3). Repeatability (Rep) estimate is calculated using the following formula: $Rep = \sigma_g^2 / (\sigma_g^2 + \sigma^2/R)$, where σ_g^2 denotes the genotypic variance, σ^2 the error variance, and R is the number of replications. Basically, it is a heritability on basis of one location and usually given on a 0 to 1 scale.

The adjusted entry means from each environment were used in a second step to estimate variance components using Proc MIXED procedure of SAS v.9.1.3 (SAS Institute, Cary, NC) based on the following linear model: $y_{ij} = \mu + g_i + e_j + (ge)_{ij} + \varepsilon$, where y_{ij} was the phenotypic observation for the i th genotype at the j th environment, μ was an intercept term, g , e denote the effects of genotype and environment, respectively, ge the genotype $\times$ environment interaction effects, and ε the error effects. All main effects were considered as random for this analysis. Heritability (h^2) on an entry-mean basis was estimated from the variance components as the ratio of genotypic to phenotypic variance following the formula (Fehr 1987): $h^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{ge}^2/E + \sigma^2/ER)$, where σ_g^2 denotes the

genotypic variance, σ^2_{ge} the genotype $\times$ environment interaction variance, and σ^2 the error variance, E , and R are the numbers of environments and replications, respectively. Heritability estimates are given on a 0 to 1 scale. Mean stem rust severities of the rye entries were compared with the susceptible population cultivar 'Recrut' using Dunnett-Hsu test by Proc MIXED procedure of SAS v.9.1.3 (SAS Institute).

Isolating rye stem rust from barberry species. To find out whether common barberry, *Berberis vulgaris*, serve as alternate host to stem rust of rye in Germany, 20 samples infected with aecia were collected in different locations of Saxony-Anhalt and Saxony in 2014 and 2015. In total, seven samples were tested according to the method described by Hale (2012). Pots with seed of the rye cultivar Palazzo (KWS LOCHOW GMBH, Bergen, Germany) and the standard susceptible cultivars Cartago (wheat) and Igri (barley) were cultivated in a rust-free environment in the greenhouse. The 10 days old seedlings were laid down in plastic boxes and misted with water. After putting a metal screen on top, the barberry leaves were arranged aecia side down on the screen, covered with a paper towel and moistened lightly. The boxes were incubated in the dark at high humidity. After 48 h, masses of ejected aeciospores could be observed on the surface of the seedling leaves. The pots were placed upright and maintained at 15 to 17°C and moderate humidity. Ten days after inoculation the daily inspection of the plants started.

RESULTS

Development of a leaf segment test. For designing a preliminary differential set, 50 inbred lines preselected in adult-plant stage in the field were tested in detached leaf segments with 69 stem rust isolates in 2011. The most discriminating 15 inbred lines were selected (Fig. 1) and further tested in the two following years by all collected isolates. A line was substituted when it was highly susceptible to the majority of isolates.

The differential lines were infected by 0.3 to 80% of 323 isolates tested (Fig. 1). The frequencies for most differentials were rather consistent across years although in each year newly collected isolates were tested. Only in 2013, substantially more isolates were detected that could infect two of the highly resistant differentials, D25 and D30. A total of 7 out of the 15 differential genotypes got infected by more than 50% of the tested isolates at least in one year, suggesting a low resistance level of the differentials to stem rust. For three genotypes (D26, D31, and D36) virulences were found only rarely, but no genotype remained totally disease free across all isolates. The pattern of virulence/avirulence combinations in the

differential set (Fig. 2) clearly demonstrates race-specific resistances in seedling stage. Twelve isolates being virulent to 4 to 10 differentials were selected for their optimal discrimination ability.

Virulence diversity and virulence complexity of the German stem rust population. Pathotypes were tested with the described differential set of 15 inbred lines. We found 226 pathotypes (= virulence combinations) among 323 single-uredinal isolates across 3 years. Out of these, only 56 pathotypes occurred more than once. This high diversity was confirmed by a Simpson index of 1.0, a high Shannon index (5.27), and an evenness index of 0.97. Virulence complexity ranged from 0 to 11 in the 3 years (Fig. 3). The most frequently occurring pathotypes were virulent on five to six inbreds across years.

Isolating rye stem rust from barberry species. Four cultures of rye stem rust were obtained from seven barberry samples tested on seedlings of the rye cultivar Palazzo. No cultures of wheat or barley stem rust could be obtained. This indicates that the sexual stage of rye stem rust occurs under natural conditions in Germany.

Testing rye populations in adult-plant stage. Inoculations were successful at all 15 environments, however, the mean stem rust severity ranged between 8 and 58% (Fig. 4). In 2014, severity was lower than in the years before at all locations. Inoculations resulted, however, in high repeatability estimates in all environments ranging from 0.75 to 0.93 with the exception of PET12 (0.41) and PET14 (0.28) illustrating a good genotypic differentiation in 13 out of 15 environments. Similarly, the analysis of variance across environments revealed a highly significant genotypic differentiation among all tested rye populations and among those tested at more than five environments as well (Table 1). The small contribution of genotype $\times$ environment interaction and error variances resulted in a high entry-mean heritability estimate. The tested German rye populations ranged from 46 to 54% stem rust severity across 15 locations and were, thus, considered as susceptible. Mean stem rust severity of the entries ranged from 3.3 to 62.3%.

Stem rust severity was calculated relative to the mean of the seven standard cultivars to compensate for the differing mean infection levels at the individual environments and the nonorthogonal testing (Fig. 5). The distribution of the populations was considerably skewed toward susceptibility demonstrating that stem rust resistance was rare among the tested entries and only a few populations were superior compared with the standard susceptible cultivar 'Recrut' (Table 2). Due to the genetic heterogeneity of rye populations, stem rust severity was estimated by rating plants individually (Fig. 6). The best populations had 23 to 70% totally resistant plants, i.e., without any visible symptom. Additionally, 16 to 26% of plants were only less infected (<9%) within these populations.

Fig. 1. Frequency of isolates (%) of *Puccinia graminis* f. sp. *secalis* virulent on the 15 most discriminating differential lines; isolates originated from a collection of 69, 41, and 213 isolates in 2011, 2012, and 2013, respectively, and were tested at the seedling stage in leaf segments (N = sample size).

Comparison of adult-plant and seedling resistance. The populations that were most resistant in adult-plant stage in the field also had a lower frequency of susceptible seedlings (infection type 3 and 4) in the detached leaf segment test (Fig. 7). Most of the populations that were moderately resistant in the field (18 to 40% stem rust severity), however, were highly susceptible (>70% susceptible plants) in the seedling stage.

DISCUSSION

The aim of this study was to analyze in parallel the diversity within populations of both components of the rye/stem rust pathosystem, *Secale cereale* and *P. graminis* f. sp. *secalis*. The evolutionary potential of a pathogen and the diversity within the

host gene pool are important parameters to optimize the future resistance breeding strategy (McDonald and Linde 2002). For analyzing stem rust diversity, it was necessary to develop a differential set of rye inbred lines and a set of differentiating stem rust isolates. The latter were also used for testing rye populations for their resistance in the field and in the laboratory. As all tested German populations were highly susceptible to stem rust, new resistance sources were searched for in rye populations from 14 European countries and from the United States, Argentina, Uruguay, Iran, and India. Altogether, these experiments should allow to draw sound conclusions on the chances for resistance selection in breeding populations.

Development of a differential set. The basis of the differential set were 50 inbred lines selected at the breeding company KWS

	Isolate											
Line	121-1	121-5	94-1	85-2	39-4	106-4	44-1	71-1	3h-3	86-1	39-2	43-1
D26	0	2	0	3	0	0	2	0	0	1	0	0
D31	1	0	1	1	0	0	3	0	2	1	0	0
D36	3	3	0	2	0	0	2	3	0	1	1	0
D30	1	0	0	2	0	0	1	3	3	3	0	0
D35	2	2	0	2	0	0	1	0	3	3	0	4
D48	2	2	3	3	0	3	2	2	0	3	3	1
D45	0	3	0	2	3	3	2	2	2	3	3	3
D44	3	2	1	3	3	2	3	1	2	2	3	3
D47	2	3	2	3	0	3	3	1	2	2	3	3
D43	0	2	3	2	3	2	2	3	3	3	3	3
D25	1	1	3	2	3	3	1	3	3	3	0	3
D41	0	2	3	0	4	0	3	3	4	0	3	4
D37	3	3	2	2	0	2	3	3	3	3	0	4
D46	3	3	3	3	0	3	3	2	3	2	3	3
D33	2	2	2	3	4	3	3	3	4	4	4	4
VC	4	5	5	6	6	6	7	7	8	8	8	10

Fig. 2. Avirulence/virulence combinations (0 to 2 is considered incompatible/avirulent, 3 to 4 is compatible/virulent reaction) of the final differential set with 12 isolates of *Puccinia graminis* f. sp. *secalis* and 15 rye differential lines; VC = virulence complexity referring to the number of differential rye lines that can be infected by the respective isolate.

Fig. 3. Frequency of isolates of *Puccinia graminis* f. sp. *secalis* that were able to infect 0 to 15 differential rye lines (= virulence complexity) split into samples of *n* = 69, 41, and 213 isolates collected in 2011, 2012, and 2013, respectively, and tested at the seedling stage in leaf segments (*N* = sample size).

LOCHOW for their adult-plant resistance in the field. The origin of the inbreds were nonadapted rye populations from Eastern Europe (B. Schmiedchen, *personal communication*). We could see in all tested lines clear race-specific reactions on leaf segments (Fig. 2). Therefore, those lines with best differentiation of the stem rust isolates were selected. This illustrates that selection by practical breeders favored race-specific resistances because they are easy to recognize and to handle. The new differential set is the basis for all following experiments. By the use of self-fertile rye inbred lines we avoid any heterogeneity within the genotypes and maintain them by selfing. Both features are not available in self-incompatible rye germplasm (e.g., open-pollinating cultivars).

Genetic variation of pathotypes. High diversity was found for pathotypes among isolates of stem rust sampled across 3 years in Germany. In total, 323 analyzed single-uredinial isolates represented 226 pathotypes. This high diversity resulted in a maximal Simpson index of 1.0. Results from each of the 3 years are not comparable because of the highly differing number of isolates analyzed. The high diversity in pathotypes might be explained by the long-term cultivation of rye, missing recent resistance selection, migration of stem rust spores from Eastern Europe, and sexual or parasexual recombination. Rye is cultivated in Central Europe at least since the Celtic occupation as early as the 8th to 5th centuries B.C. (Sencer and Hawkes 1980), its cultivation in Eastern Europe even dating back to the early bronze age (Körber-Grohne 1987). The high frequency of less virulent isolates might be a result of the missing resistance breeding to stem rust in rye, at least since World War II. When commercially grown cultivars are highly susceptible, as in our case, all pathotypes can regularly reproduce, even those without any virulence. Indeed, in 2013, the year with the largest sample size, about 2% of the isolates could not infect any of the differential lines and >20% could grow on only one or two differential lines (Fig. 3). Sexual recombination could also explain this high diversity of pathotypes. The alternate host, barberry, can be found in many parts of Germany in natural hedges. In the framework of this study four out of seven barberry leaves with aecia gave rise to rye stem rust colonies illustrating frequent sexual recombination in Germany. Accordingly, Berlin et al. (2012) could demonstrate an important role of barberry for the variation of *P. graminis* f. sp. *secalis* in Sweden by molecular markers. Sexual recombination and the largely missing host-driven selection would sufficiently explain a high diversity of the pathogen as in other parts of the world (Jin 2011). Both explanations are supported by the fact that most of the pathotypes occurred only once. Roelfs and Groth (1980) already demonstrated that a sexually derived wheat stem rust population had a higher frequency of distinct pathotypes than a asexually propagated population. The Simpson index of diversity in their

study was 0.974 for the sexual population, an estimation that is similar high like ours (1.0). Sexual recombination leads frequently to new combinations of pathotypes and because of the missing selection by matching resistance genes in the host all isolates can freely reproduce asexually on winter rye. Also in Sweden, each isolate of rye stem rust occurred only once out of a sample of 69 isolates analyzed by 11 markers (Berlin et al. 2012). The authors refer to this high genetic diversity and found only a low significance of the index of association as expected when sexual reproduction occurs. On this background it is, however, astonishing that, on average, in our study rye stem rust isolates could infect five to six differential lines simultaneously. And such isolates were found with varying frequencies in all 3 years. Even isolates infecting ≥10 out of 15 differential lines occurred, although only rarely in 2012 (6%) and 2013 (2%). These more complex pathotypes might be remnants from the past, when probably rye landraces with some percentages of resistant plants were grown and/or originate from migration events of isolates with diverse virulence combinations followed by sexual recombination (McDonald and Linde 2002). Winter rye was the most frequently grown staple food in Germany from at least 500 A.D. until the 1960s and it is still an important cereal in Poland, Belarus, and Russia (FAO 2016), where stem rust epidemics occur regularly.

Results obtained here for stem rust are illustrating an even higher diversity than those of an earlier study on German leaf rust populations in rye (Miedaner et al. 2012). In leaf rust of rye (*P. recondita*), 317 pathotypes were found among 827 isolates. The frequency of unique pathotypes was lower for leaf rust than in stem rust (38% versus 75%), but Simpson and evenness indices were similar. Both findings are in sharp contrast with the composition of rust populations in wheat. For leaf rust in wheat, only a few number of pathotypes was found across Europe with four pathotypes representing 64% of the total population (Park and Felsenstein 1998; Mesterházy et al. 2000). Accordingly, a high rate of clonal

TABLE 1. Estimates of variance components and entry-mean heritability for stem rust severity (%) of 121 rye populations evaluated across 3 to 15 environments after inoculation by *Puccinia graminis* f. sp. *secalis*^a

Parameter	df	Variance component
Source of variance		
Environment (E)	12	121.32**
Genotype (G)	120	142.47**
E × G	497	37.77**
Error	953	14.78
Heritability		0.97

^a ** Indicates significance at *P* < 0.01.

Fig. 4. Mean stem rust severity (%) of all tested self-incompatible rye populations (*n* = 30 or 70 per location) after inoculation by *Puccinia graminis* f. sp. *secalis* at five field locations in 3 years.

reproduction was postulated for wheat leaf rust in France (Goyeau et al. 2007) and all of Europe (Kolmer et al. 2013). Recurrent mutations were postulated to be the most likely source of virulence diversity in leaf rust (Kolmer et al. 2013). In stripe rust of wheat, only two pathotypes are dominating European populations since 2011 (Hovmöller et al. 2015). This difference to our rye stem rust data are most probably caused by both the intensive resistance breeding in European wheat using race-specific resistance genes and the homogeneity of cultivars of the self-pollinating wheat. In contrast, in the cross-pollinated winter rye only heterogeneous types of cultivars are available.

In conclusion, host-specific selection by heterogeneous rye cultivars to leaf and stem rust seems to be much weaker than in homogeneous wheat cultivars and allows leaf and stem rust populations to maintain a high diversity of pathotypes including isolates with only a low complexity. Genetic variation of leaf and

stem rusts in rye is enhanced by a regular sexual reproduction and possibly migration from the large rye-growing areas of Eastern Europe where resistances occur in breeding populations.

Resistance donors. All tested German populations were highly susceptible to stem rust. Also the frequency distribution of the nonadapted populations was skewed toward susceptibility (Fig. 5) illustrating that on population level stem rust resistances are rare. They have been described previously in Russian populations (Solodukhina und Kobylansky 2007) and rye populations from Southern United States used for grazing ('Gator', 'Wrens', and 'Elbon' × 'Gator') (Tan et al. 1976). Also one old Austrian landrace was described as stem rust resistant ('Lungauer Tauern') (BAES 2015). Therefore, we searched mainly in rye populations from these countries and some other Eastern European countries for resistance donors and added some exotic accessions. Indeed, from the 10 most resistant populations, five were from Russia, two from Austria, and each of one from the United States, Hungary, and Argentina. The Russian entries named "Hy" are originating from crosses among different populations and were selected for rust resistances by the Agricultural Research Institute of non-Chernozem Zone near Moskow. We could confirm for the first time their high resistance level under Central European conditions. Caused by the out-crossing nature of rye and its strong genetic self-incompatibility, it is not possible to select populations consisting only of resistant plants, even the best entries still contain a proportion of susceptible plants (Fig. 6). However, Hy75/81 had 70% fully resistant plants and additionally 20% of plants with a stem rust rating of <10%. This is a result of several cycles of recurrent mass selection with a strong focus on stem rust resistance under conditions of natural infection (A. A. Goncharenko, *personal communication*). Interestingly, the old Austrian landrace 'Tiroler' was similarly resistant. The percentage of noninfected plants varied from 20 to 60% in the other resistant populations.

Speculations about inheritance of stem rust resistance. As illustrated by the differential set in the detached leaf test, the tested rye inbred lines are all showing clear race-specific interactions. Since they have been selected in a breeding nursery by their adult-plant resistance (B. Schmiedchen, *personal communication*), we

Fig. 5. Frequency distribution of mean stem rust severity (%) of 114 non-adapted rye populations relative to the average of seven susceptible standard populations (=100%) after inoculation by *Puccinia graminis* f. sp. *secalis* across environments; numbers above the columns refer to the number of rye populations in that class.

TABLE 2. Mean stem rust severity (%) of those self-incompatible rye populations significantly ($P < 0.01$) more resistant than the susceptible German standard population cultivar 'Recrut' after inoculation by *Puccinia graminis* f. sp. *secalis* in 8 to 15 environments; the performance of Recrut is given for comparison^a

	2012					2013					2014					2012–2014	2013–2014
	HOH	DAL	DAN	VIL	PET	HOH	DAL	DAN	VIL	PET	HOH	DAL	DAN	VIL	PET		
Recrut	68.50	67.23	59.63	48.37	29.53	50.84	59.59	65.31	41.26	87.43	38.81	59.78	68.86	35.22	13.33	52.91	52.04
Hy_75/81	5.41	2.18	4.47			3.52	3.76	3.81	0.18	4.38	0.00	7.23	3.51	2.00	5.00	3.50	3.34
Tiroler						2.22	1.35	4.47			0.44	4.37	5.94	3.67	5.00		3.43
Hy_2407/87						5.87	14.80	12.41			0.78	10.18	12.30	4.22	5.00		8.20
Oberkärntner						7.22	6.50	19.12			2.45	3.58	15.78	20.33	11.67		10.83
Talowskaja_29	32.85	13.14	24.13	23.23	11.95	8.41	12.72	16.34	16.67	12.21	3.22	34.55	18.10	15.78	8.33	16.78	14.63
Hy_9a/86	34.07	8.09	12.42			14.15	12.57	22.68	16.98	20.01		15.65	23.15	6.67	6.67	16.09	15.39
Hungarian_Giant						25.67	14.58	34.50			6.00	4.87	26.31	5.67	6.67		15.53
Wheeler	45.75	10.17	17.08			13.21	21.60	17.15	12.22	24.76	7.37	13.91	21.83	19.55	10.00	18.05	16.16
Talowskija						12.90	18.40	20.74			13.50	28.91	26.63	7.33	6.67		16.89
Manfredi						23.03	20.77	34.44			5.11	5.66	47.59	5.33	5.00		18.37
Wrens_Abruzzi	44.52	16.65	23.66			21.24	14.04	33.00	13.72	60.83	3.33	0.00	35.99	7.56	6.67	21.63	19.64
Gator	47.78	14.70	20.07	26.06	11.52	24.90	13.95	35.02	13.81	55.27	3.77	10.02	35.97	11.45	6.67	22.06	21.08
Elbon	40.99	23.62	10.25	14.67	5.89	14.08	16.52	43.76	15.87	49.44	1.56	23.30	33.21	9.11	6.67	20.60	21.35
Alfa						25.58	29.21	31.09			4.44	35.79	36.18	10.78	6.67		22.47
Kärntner						32.20	22.09	38.51			11.98	22.81	34.57	7.11	11.67		22.62
Zidlochowieck_r	43.17	13.16	21.62			29.34	29.64	30.01	32.37	30.07	31.00	47.53	29.53	25.11	6.67	28.40	29.13
Vjatka_2	55.81	38.67	41.32	26.39	14.42	43.53	22.91	36.16	37.60	59.55	15.00	32.69	40.60	14.78	8.33	32.52	31.12
Instituckie_Wcz	50.56	18.09	33.29	28.17	13.10	30.12	29.71	54.17	22.48	63.15	7.11	47.08	37.98	17.78	8.33	30.74	31.79
Luovskaja	55.56	36.62	33.57	29.01	14.38	52.40	36.64	50.40	18.54	74.22	14.67	24.11	44.37	7.22	6.67	33.23	32.92
Halle_Rostres						23.41	18.01	47.22	23.89	56.18							33.74
Shitomirskaja	55.54	26.72	46.26			49.44	30.97	48.90	35.33	68.38	17.44	24.05	44.50	14.45	8.33	36.18	34.18
Novinka	56.24	26.56	50.65	29.06	13.59	45.51	30.29	43.89	28.87	71.67	21.72	34.91	46.60	25.66	5.00	35.35	35.41
Abruzzi						20.64	25.65	52.44	29.48	72.23							40.09
LSD _{5%}	8.14	13.96	12.21	6.08	10.52	12.14	9.58	8.52	6.60	13.81	8.11	16.32	11.07	7.25	6.64		

^a HOH = Hohenheim, DAL = Berlin-Dahlem, DAN = Dahnisdorf, VIL = Bad Vilbel, and PET = Petkus.

can conclude that they follow the scheme of a classical monogenic inheritance with *R* genes that are expressed through the whole life time of the host. This was also substantiated by some of the resistant field-tested rye populations that reacted similarly resistant in the leaf segment test. Accordingly, we found in all rye populations displaying stem rust resistance in the field a varying percentage of fully resistant plants, i.e., plants without any visible symptoms (Fig. 6). This could not be assigned to disease escape because in the same small plots also susceptible rye stems were found.

In breeding programs, seedling tests are desirable for disease resistance screening because a large number of lines can be evaluated in a short period of time and with modest space requirements (Sun and Steffenson 2005). Most of the race-specific resistances selected in seedling stage are, however, not durable. Some of them provided at least a long-lasting resistance in the past. The rye-derived gene *Sr31* was effective in wheat against all races of stem rust from its introduction in the 1930s till 1998 when the new race *Ug99* appeared (Singh et al. 2011). In barley, *Rpg1* controlled losses to stem rust from its first use in 1942 until 1989 when the first virulent pathotype appeared in the Upper Midwest region of the United States (Sun and Steffenson 2005). In addition, rye as a cross-pollinating crop has the advantage that even in modern population and hybrid cultivars the “dirty crop” approach (Marshall and Pryor 1978) is inherent, i.e., cultivars consist from plants ranging from full resistance to high susceptibility. In leaf rust of rye, however, this did not reveal a longer durability of the tested race-specific genes (Wilde et al. 2006), but the effect of host heterogeneity on stem rust is not known. At least, in this study the populations with high frequencies of stem rust resistant plants were quite stable in their stem rust resistance across more than eight environments (Table 2). Some populations being moderately resistant in the field showed a high susceptibility in the detached leaf test with >70% susceptible plants (Fig. 7). These might be candidates for searching adult-plant resistances or quantitative resistances.

Conclusions. The high pathotype diversity of stem rust populations in Germany points to a high evolutionary potential of the pathogen to adapt to *R* genes in the host. Although the percentage of highly virulent isolates is still low compared with other rusts, they might considerably increase with the future introduction of race-specific resistances in commercial rye cultivars. This might even be favored by the heterogeneous cultivar types in rye because cultivar mixtures are known to increase virulence complexity, at least in powdery mildew (Wolfe and Finckh 1997). On the other hand, some

nonadapted rye populations seem to be a rich source of race-specific resistances and it might be useful for practical breeders to extract these genes and deploy them alone or, surely better, in varying combinations (Brown 2015). This strategy could be successful if the *R* genes last for a while, like e.g., *Sr31* for wheat stem rust originating from rye. However, with this short-term strategy always new, effective *R* genes must be available for the breeders to supplement or replace the introduced genes if they get ineffective. In the long run, the use of adult-plant resistances might be a more durable solution. Such resistances showed up in U.S. rye cultivars (e.g., Elbon, Wheeler, Wrens Abruzzi, and Gator) and some Russian populations (e.g., Zidlochowiecke Rane, Institutkie Wczesne) that displayed a compatible infection type in seedling stage but were significantly more resistant in the field than the susceptible standards. For a future knowledge-based strategy for resistance selection, it is urgently necessary to study the inheritance of both types of resistance and to test the genetic independency of

Fig. 7. Correlation between mean stem rust severity in adult-plant stage tested in the field and percentage of susceptible plants in detached leaf segment test in seedling stage tested in the laboratory for 17 populations after inoculation by *Puccinia graminis* f. sp. *secalis* in 2012 or 2013; stem rust severity of the susceptible German standard cultivar Recruit in the field is indicated by an arrow; *r* = coefficient of correlation. ** indicates significance at *P* < 0.01.

Fig. 6. Frequency of individual plants of different classes of stem rust severity within selected resistant rye populations after inoculation by *Puccinia graminis* f. sp. *secalis* compared with the susceptible German standard cultivar Recruit; all cultivars were orthogonally tested in three locations at 3 or 2 years, respectively (*n* = 185 plants for 2012 to 2014 and 135 plants for 2013 to 2014).

R genes from different sources by genetic mapping to pave the way for a more durable host resistance against these highly variable stem rust populations.

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