Molecular Population Genetic Analysis Differentiates Two Virulence Mechanisms of the Fungal Avirulence Gene *NIP1*

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Deletion or alteration of an avirulence gene are two mechanisms that allow pathogens to escape recognition mediated by the corresponding resistance gene in the host. We studied these two mechanisms for the *NIP1* avirulence gene in field populations of the fungal barley pathogen *Rhynchosporium secalis*. The product of the avirulence gene, *NIP1*, causes leaf necrosis and elicits a defense response on plants with the *Rrs1* resistance gene. A high *NIP1* deletion frequency (45%) was found among 614 isolates from different geographic populations on four continents. *NIP1* was also sequenced for 196 isolates, to identify DNA polymorphisms and corresponding *NIP1* types. Positive diversifying selection was found to act on *NIP1*. A total of 14 *NIP1* types were found, 11 of which had not been described previously. The virulence of the *NIP1* types was tested on *Rrs1* and *rrs1* barley lines. Isolates carrying three of these types were virulent on the *Rrs1* cultivar. One type each was found in California, Western Europe, and Jordan. Additionally, a field experiment with one pair of near-isogenic lines was conducted to study the selection pressure imposed by *Rrs1* on field populations of *R. secalis*. Deletion of *NIP1* was the only mechanism used to infect the *Rrs1* cultivar in the field experiment. In this first comprehensive study on the population genetics of a fungal avirulence gene, virulence to *Rrs1* in *R. secalis* was commonly achieved through deletion of the *NIP1* avirulence gene but rarely also through point mutations in *NIP1*.

Additional keywords: leaf scald of barley, neutral theory, non-synonymous mutations, synonymous mutations.

Because plants are often attacked and infected by pathogens, many different forms and levels of plant-pathogen interactions have evolved. One type of interaction takes place between plant resistance genes and pathogen avirulence genes as described by Flor's gene-for-gene model (1955). Resistance genes permit recognition of pathogen elicitors encoded by the avirulence genes that activate the plant defense system. Deleted or altered avirulence genes allow pathogens to escape recognition by

host plants, infection takes place, and virulent pathogens are said to overcome resistance. New resistance gene alleles allow plants to counter adapt. This antagonistic coevolution is expected to leave traces at the molecular level. Indeed, several plant resistance genes have been shown to be under positive diversifying selection (Bergelson et al. 2001). However, no information on adaptive evolution of avirulence genes is available.

The inactivation of avirulence genes can evolve through several mechanisms, including deletion of the entire gene, non-synonymous point mutations, frameshift mutations, or transposon insertions. Mechanisms affecting regulation of gene expression may also be involved. In the tomato pathogen *Cladosporium fulvum*, the *Avr9* avirulence gene is lacking from all strains virulent on host cultivars carrying the Cf-9 resistance gene (van Kan et al. 1991). In this same pathogen, virulence on Cf-2 tomato plants occurs as a consequence of the production of truncated AVR2 proteins due to different types of mutations (Luderer et al. 2002). Virulence on Cf-4 plants was gained through point or frameshift mutations leading to unstable AVR4 isoforms of the elicitor (Joosten et al. 1997). Avirulence gene inactivation by insertion of a transposable element was reported for *Avr2* (Luderer et al. 2002) and for the avirulence gene *Avr-Pita* of the rice pathogen *Magnaporthe grisea* (Kang et al. 2001).

A fungal avirulence gene has been identified in *Rhynchosporium secalis* (Oudem.) J. J. Davis, the causal agent of leaf scald of barley (*Hordeum vulgare* L.). This avirulence gene, termed *AvrRrs1* or *NIP1*, interacts with the *Rrs1* resistance gene of barley (Rohe et al. 1995). The product of *AvrRrs1* is a necrosis-inducing peptide, named *NIP1* (Wevelsiep et al. 1991), that stimulates a H⁺-ATPase (Wevelsiep et al. 1993). Thus, *NIP1* functions both as a toxin and an elicitor. Two other necrosis-inducing peptides, *NIP2* and *NIP3* (Wevelsiep et al. 1991), have not been found to elicit a resistance response (Hahn et al. 1993). Incompatible interactions with *Rrs1* barley cultivars are not accompanied by a hypersensitive response, but PR protein synthesis is induced (Hahn et al. 1993; Zareie et al. 2002). After spore germination and cuticle penetration, a few epidermal cells collapse, and fungal growth is stopped. In compatible interactions, collapse of many more epidermal cells is followed by the collapse of mesophyll cells, which results in visible lesions (Lehnackers and Knogge 1990). Fungal strains lacking *NIP1* or strains with either one of two specific point mutations in *NIP1* were shown to overcome *Rrs1* resistance (Rohe et al. 1995). A study on Finnish *R. secalis* isolates found two other isoforms (Kilby and Robinson 2001). Another isoform was reported from *R. secalis* isolated from Atlas 46 in Canada (Johnson et al. 1996).

Preliminary note: Throughout this paper, virulence is used as an antonym for avirulence and is defined as "the genetic ability of a pathogen race to overcome genetically determined host resistance (...) and cause a compatible (disease) interaction" (Shaner et al. 1992).

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Because *R. secalis* may cause significant yield losses when conditions are conducive for disease development (Shipton et al. 1974), many efforts for breeding resistant barley varieties have been undertaken. Several major resistance genes were identified (Goodwin et al. 1990). However, none of these genes have been cloned, and some of them may be alleles at one locus (Bjørnstad et al. 2002; Habgood and Hayes 1971). For most barley cultivars, it is not possible to know which resistance gene or genes they contain. Turk is an exception and is thought to contain the *Rrs1* resistance gene (symbol as in Søgaaard and von Wettstein-Knowles [1987] and Bjørnstad et al. [2002], originally *Rh3* [Dyck and Schaller 1961]). The gene was introgressed into cultivar Atlas, which already contained *Rh2* (Dyck and Schaller 1961). The newly obtained cultivar, Atlas 46 (*Rrs1* and *Rh2*) was released in California, but less than 10 years later it was fully susceptible to the local *R. secalis* population (Houston and Ashworth 1957). Turk's *Rrs1* gene was also introduced into Turk NIL, a near-isogenic line developed in the cultivar Ingrid as part of an effort to produce a set of differentials containing known resistance sources (Bjørnstad et al. 2002).

In this manuscript, we describe how the *R. secalis*-barley pathosystem was used to better understand avirulence gene evolution and the mechanisms leading to virulence and resistance gene breakdown. In particular, the mechanisms leading to inactivation of the *NIP1* avirulence gene in natural field populations were examined. First, the deletion frequencies of *NIP1*, *NIP2*, and *NIP3* were quantified in different *R. secalis* populations around the world. Then, the *NIP1* locus was sequenced for a global collection of 196 isolates to determine the degree of polymorphism existing at this locus, to identify point mutations that inactivate the avirulence gene, and to test whether this gene is under positive diversifying selection. *NIP1* isoforms were inferred from the DNA sequence data, and their global distribution was determined. The virulence of isolates carrying these isoforms was assessed on *rrs1* and *Rrs1* barley cultivars. Finally, we looked at the effect of selection imposed by the presence of *Rrs1* on *NIP1* in a natural *R. secalis* population in a field experiment.

RESULTS

Occurrence of the *NIP1*, *NIP2*, and *NIP3* genes.

Deletion frequency of *NIP1*, *NIP2*, and *NIP3* was assessed in 21 *R. secalis* populations by hybridization of *NIP* probes to Southern blots containing genomic *R. secalis* DNA (Table 1). Overall, the *NIP1* gene was deleted in 45% of the 614 isolates tested. In the four Ethiopian populations, *NIP1* was deleted in all isolates. On the other hand, *NIP1* was present in all the isolates collected in Norway and in four of the eight Australian populations (RS96AUS2, 3, 4, and 7). Isolates with and without *NIP1* coexisted in 11 populations, with the percentage of isolates missing *NIP1* ranging from 3 to 87%.

Overall, the *NIP2* gene was found in 92% of the 544 isolates tested and was present in all the isolates from 14 of the 21 populations. In a Swiss population (RS99CH1) collected

Fig. 1. Sliding window plot of the nucleotide diversity, π , across *NIP1* in 49 haplotypes. Window size was 100 and step size 10. The vertical bars delimit the different regions of *NIP1*. A, 5' flanking region (878 bp); B, 5' untranslated region (89 bp); C, signal peptide (66 bp); D, remainder of exon 1 (171 bp); E, intron (66 bp); F, exon 2 (9 bp); and G, 3' flanking region (245 bp).

Table 1. Number and percentage of *Rhynchosporium secalis* isolates with (+ and % +) and without (–) *NIP1*, *NIP2*, and *NIP3* genes in 21 populations

Country	Population	NIP1			NIP2			NIP3		
		+	–	% +	+	–	% +	+	–	% +
Australia	RS96AUS2	36	0	100	35	1	97	36	100	
	RS96AUS3	33	0	100	33	0	100	33	100	
	RS96AUS4 ^a	26	0	100	26	0	100	26	100	
	RS96AUS5	32	1	97	24	9	73	33	0	100
	RS96AUS6R, X ^a	20	15	57	34	1	97	35	0	100
	RS96AUS6BR ^b	0	2	0	2	0	100	2	0	100
	RS96AUS7	31	0	100	10	21	32	31	0	100
	RS96AUS8	5	23	17	28	0	100	28	0	100
Ethiopia	RS01ET1	0	28	0	26	0	100	26	0	100
	RS01ET2	0	13	0	12	0	100	13	0	100
	RS01ET3	0	31	0	28	0	100	29	0	100
	RS01ET4	0	28	0	21	0	100	22	0	100
Norway	RS90sNO	36	0	100	36	0	100	35	0	100
Switzerland	RS99CH1 ^c	8	12	40	0	12	0	17	1	94
	RS99CH2	12	16	43	17	0	100	26	0	100
	RS99CH5	7	20	26	22	0	100	26	0	100
	RS99CH6	16	17	48	13	0	100	30	0	100
	RS00CH	6	39	13	42	1	98	42	1	98
	RS01CH1	27	5	84	27	0	100	27	0	100
	RS01CH2	23	15	61	31	1	97	29	0	100
California	RS88CAR	17	13	58	31	0	100	31	0	100
Total		336	278	55	498	46	92	577	2	100

^a Isolates collected from barleygrass.

^b Isolates collected from bromegrass.

^c Isolates collected from rye.

from rye, all isolates lacked *NIP2*, and in two Australian populations, RS96AUS5 and RS96AUS7, 27 and 68% of the isolates, respectively, lacked *NIP2*. In four populations, single isolates had a *NIP2* deletion. The *NIP3* gene was found in all but two of the 579 isolates examined. One of the isolates lacking *NIP3* was collected from rye in Switzerland, whereas the other one was also from Switzerland but was collected from barley.

To test whether gene deletion was independent among genes, linkage disequilibrium tests were applied. Neither two-locus tests for linkage disequilibrium nor a multilocus analysis were significant, and no disequilibrium could be detected. Therefore, gene deletion was independent among the *NIP1*, *NIP2*, and *NIP3* genes.

Variation in the NIP1 gene.

A 1,524-bp long DNA sequence including *NIP1* was sequenced for 168 isolates. These sequences were deposited in GenBank (accession number AY507678 to AY507845). Among

Fig. 2. Distribution of d_N/d_S ratios for 32 pairwise comparisons among 15 *NIP1* haplotypes into classes, where d_N and d_S are the number of nucleotide substitutions that cause amino acid replacements or are silent, respectively, divided by the number of possible substitutions of each type. $d_N/d_S > 1$ indicates comparisons in which amino acid substitutions are more frequent than predicted by neutral substitution rates.

NIP1 mature protein																																																																											
Type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60															
1 ^a	D	R	C	R	Y	T	L	C	D		G	A	L	K	A	V	S	A	C	L		H	E	S	E	S	C	L	V	P	G		D	C	C	R	G	K	S	R	L	T		L	C	S	Y	G	E	G	G	N	G		F	Q	C	P	T	G	Y	R	Q	C											
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cating that positive diversifying selection is acting on the *NIP1* gene. A neutral model ($\ln = -381.08$) that allows ω to be either 0 (purifying selection) or 1 (neutral selection) fitted the data significantly less well than a selection model ($\ln = -369.81$) that includes an additional category of sites with ω estimated from the data ($2\Delta\ln = 22.54$, d.f. 1, $P < 0.0001$). Out of 57 codons, 96% were neutral whereas 4% were in a category with $\omega = 43.2$, indicating that the codons are under different selection pressures. This model yielded a very high value for ω , which may be due to the very small number of synonymous mutations in our data. This same reason probably explains why more complex ML models could not be fitted satisfactorily to the data.

Codon usage bias.

Since only one synonymous mutation was found in *NIP1*, and therefore, purifying selection may be suspected to operate on synonymous codons, several parameters related to codon usage bias were estimated. Codon bias index values ranged from 0.520 to 0.592, with an average value of 0.553 over 57 codons. Overall G+C content was 0.523, 0.580 at the second position, and 0.458 at the third position. The effective number of codons, which may range from 20 (only one codon used per amino acid) to 61 (all synonymous codons are equally used), was 38.9. All these estimates of codon usage were intermediate between random codon usage and maximum codon bias and did not show an obvious codon usage bias.

Variation in NIP1.

The DNA sequence coding for *NIP1* could be sequenced unambiguously for 196 isolates. In addition to the seven previously known NIP1 types (Johnson et al. 1996; Kilby and Robinson 2001; Rohe et al. 1995), 11 new types were identified (Table 2). Some presented new combinations of previously known amino acid changes (types 9 and 11; Table 2), whereas all others presented new mutations. All mutations were point mutations. In type 7, a mutation (AG $\rightarrow$ AA) at the acceptor splice site located at the boundary between the intron and the second exon most likely results in aberrant splicing of the pre-mRNA. In type 17, there was a point mutation that destroyed the start codon. Even though no NIP1 may be expressed from these two types, the amino acid translation was indicated in Table 2 for comprehensiveness. Some sites seemed to be particularly variable (e.g., amino acid 21 of the mature protein, which is located in loop II of the protein [van't Slot et al. 2003]) whereas other regions of the protein were conserved (e.g., amino acids 11 to 20, which represent the C-terminal half of loop I and β -strand 2, or amino acids 31 to 41, which form β -strands 3 and 4 separated by a β -turn).

Among 196 isolates, none were found that carried NIP1 types 3 to 6 (Table 3). Types 1 and 2 were the most common, followed by 11 and 13. Type 1 was found in every region, while type 2 was found in every region except in the Middle East. Type 1 was the most common type in Scandinavia and California, whereas type 2 was the most common type in Australia. Types 11 and 12 were shared between Middle-East and

Table 3. Distribution of NIP1 types and gene diversity in *Rhynchosporium secalis* from five regions

		NIP1 type ^a															N	Gene diversity
Region	Population	1	2	7	8	9	10	11	12	13	14	15	16	17	18			
Australia	RS96AUS1		1													1	0.31	
	RS96AUS2		3													3		
	RS96AUS3	2	5													7		
	RS96AUS4 ^b	1	6													7		
	RS96AUS5	1	6													7		
	RS96AUS6 ^b	1	7								1					9		
	RS96AUS7		7													7		
	RS96AUS8		3													3		
	RS96AUS10	2	1													3		
	RS96AUST	1	1													2		
	Subtotal	8	40	0	0	0	0	0	0	0	1	0	0	0	0	49		
Middle-East	RS02JO1							8								8	0.71	
	RS02JO2							8		1				7		16		
	RS02SY1									4						4		
	RS02SY4							1		1			3			5		
	RS02SY6 ^c	1						2	2	2			1			8		
	RS97TR									2						2		
	Subtotal	1	0	0	0	0	0	19	2	10	0	0	4	7	0	43		
Scandinavia	RS96FIF	4	3													7	0.59	
	RS96NO1N1	15				7	1									23		
	RS96NO1NK	4				2			2							8		
	RS96SW							1								1		
	Subtotal	23	3	0	0	9	1	1	2	0	0	0	0	0	0	39		
California	RS88CAR	18	4	0	2	0	0	0	0	0	0	0	0	0	0	24	0.40	
Western Europe	RS97FR		1	1						2						4	0.77	
	RS97DE		3	4								7				14		
	RS97GB	1	6									3				10		
	RS99CH1 ^d			3						3					1	7		
	RS01CH1			1						5						6		
	Subtotal	1	10	9	0	0	0	0	0	10	0	10	0	0	1	41		
Total		51	57	9	2	9	1	20	4	20	1	10	4	7	1	196	0.82	

^a NIP1 types 3, 4, 5, and 6 were not found in this survey.

^b Isolates collected from *Hordeum leporinum* (barleygrass).

^c Isolates collected from *Hordeum spontaneum*.

^d Isolates collected from *Secale cereale* (rye).

Scandinavian populations and type 13 between Middle-East and Western European populations. The other types were restricted to single populations (types 8, 10, 14, 17, and 18) or regions (types 7, 9, 15, and 16). One isolate collected from rye in Switzerland and one from barley grass in Australia carried NIP1 types (14 and 18) not found in any other isolates. Distribution of NIP1 isoforms did not differ strikingly between barley and rye or wild grasses populations.

Gene diversity gave a measure of variation within regions (Table 3). All gene diversity values were significantly different from each other, as shown by *t*-tests. Australia and California were the two regions with the lowest gene diversities, 0.31 and 0.40, respectively. In Australia, only two NIP1 types were found, if we exclude the population collected from barley

grass (RS96AUS6) contributing NIP1 type 14. In California, only NIP1 type 8 was found in addition to the two most common types 1 and 2. Gene diversities in the populations from Scandinavia, Western Europe, and the Middle East (0.59, 0.77, and 0.71, respectively) were higher than those found in Australia and California.

Pathogenicity tests.

A total of 41 *R. secalis* isolates representing 14 different NIP1 isoforms were inoculated on *rrs1* (Atlas and Ingrid) and *Rrs1* (Atlas 46 and Turk NIL) barley cultivars. Inoculations with the control isolates UK7 (*AvrRrs1*) and AU2 (*avrRrs1*) were conducted in each of the four trials, and disease scores were consistent across trials for both isolates on all four barley

Table 4. Disease scores of 41 *Rhynchosporium secalis* isolates representing 14 NIP1 types on the four barley cvs. Atlas, Atlas 46, Ingrid, and Turk NIL

NIP1 type	Trial	Isolate	Disease score ^a				N ^b
			Atlas	Atlas 46	Ingrid	Turk NIL	
1	I	UK7	0.07 ± 0.07	0.00 ± 0.00	4.36 ± 0.36	0.00 ± 0.00	55
	II	UK7	0.71 ± 0.19	0.00 ± 0.00	5.00 ± 0.00	0.07 ± 0.07	55
	III	UK7	0.07 ± 0.07	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
	IV	UK7	0.00 ± 0.00	0.00 ± 0.00	4.86 ± 0.10	0.00 ± 0.00	58
2	I	AU1	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
3	I	AU3	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.07	0.15 ± 0.15	54
4	I	AU2	5.00 ± 0.00	5.00 ± 0.00	4.80 ± 0.20	5.00 ± 0.00	57
	II	AU2	4.73 ± 0.12	4.50 ± 0.34	4.73 ± 0.27	5.00 ± 0.00	56
	III	AU2	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	56
	IV	AU2	5.00 ± 0.00	5.00 ± 0.00	4.93 ± 0.07	5.00 ± 0.00	57
7	II	RS97DE742	0.71 ± 0.22	0.69 ± 0.17	4.86 ± 0.14	5.00 ± 0.00	55
	I	RS97DE773	0.29 ± 0.13	0.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	56
	IV	RS97FR787	0.07 ± 0.07	0.00 ± 0.00	3.57 ± 0.27	4.14 ± 0.14	58
8	I	RS88CA27	3.86 ± 0.33	4.71 ± 0.13	3.43 ± 0.36	4.43 ± 0.25	56
	II	RS88CA27	4.71 ± 0.16	5.00 ± 0.00	5.00 ± 0.00	4.93 ± 0.07	57
9	II	RS96NO1N1B11	0.43 ± 0.17	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	55
	II	RS96NO1N1G43	0.14 ± 0.10	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
10	I	RS96NO1NKD23	0.07 ± 0.07	0.00 ± 0.00	4.93 ± 0.07	0.00 ± 0.00	54
	I	RS96NO1N1B41	0.07 ± 0.07	0.00 ± 0.00	3.00 ± 0.36	0.00 ± 0.00	55
	IV	RS96NO1N1B41	0.14 ± 0.14	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	58
11	IV	RS02JO2H2	0.07 ± 0.07	0.00 ± 0.00	4.50 ± 0.14	0.07 ± 0.07	58
	IV	RS02SY6-12a	0.08 ± 0.08	0.00 ± 0.00	4.50 ± 0.15	0.00 ± 0.00	49
	IV	RS02SY6-20a	0.00 ± 0.00	0.00 ± 0.00	4.09 ± 0.25	0.00 ± 0.00	48
	I	RS96SWF32	0.00 ± 0.00	0.00 ± 0.00	3.93 ± 0.21	0.53 ± 0.27	58
12	III	RS02SY6-14a	0.00 ± 0.00	0.00 ± 0.00	4.71 ± 0.13	0.07 ± 0.07	56
	III	RS02SY6-30a	0.07 ± 0.07	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	55
	II	RS96NO1NKG11	0.36 ± 0.17	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	55
	I	RS96NO1NKI11	0.07 ± 0.07	0.00 ± 0.00	4.53 ± 0.13	0.00 ± 0.00	59
13	II	RS01CH1A14a.1	0.29 ± 0.16	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
	I	RS01CH1B3a	0.29 ± 0.22	0.00 ± 0.00	4.73 ± 0.15	0.00 ± 0.00	53
	II	RS01CH1B9a	0.00 ± 0.00	0.00 ± 0.00	4.71 ± 0.16	0.00 ± 0.00	56
	II	RS97FR784	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
	I	RS97FR785	0.00 ± 0.00	0.07 ± 0.07	4.92 ± 0.08	0.00 ± 0.00	56
	II	RS97TR737	2.43 ± 0.63	0.00 ± 0.00	4.36 ± 0.20	0.00 ± 0.00	56
15	I	RS97DE684	0.00 ± 0.00	0.00 ± 0.00	4.69 ± 0.24	0.00 ± 0.00	50
	II	RS97DE711	1.00 ± 0.32	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
	II	RS97GB693	1.07 ± 0.32	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	59
16	III	RS02SY4-41a	0.21 ± 0.15	0.00 ± 0.00	5.00 ± 0.00	0.07 ± 0.07	56
	III	RS02SY4-58a	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	56
	IV	RS02SY6-28a	0.07 ± 0.07	0.29 ± 0.19	4.73 ± 0.14	5.00 ± 0.00	52
17	IV	RS02JO2A1	0.00 ± 0.00	0.29 ± 0.29	4.29 ± 0.16	4.57 ± 0.14	57
	III	RS02JO2C3	0.00 ± 0.00	0.00 ± 0.00	3.93 ± 0.37	4.36 ± 0.13	56
	IV	RS02JO2D3	0.00 ± 0.00	0.07 ± 0.07	4.54 ± 0.14	4.47 ± 0.17	57
	IV	RS02JO2E9	0.00 ± 0.00	0.07 ± 0.07	5.00 ± 0.00	4.93 ± 0.07	58
Deletion	III	RS01ET1A10a	0.00 ± 0.00	0.00 ± 0.00	3.79 ± 0.35	4.57 ± 0.17	56
	III	RS01ET1A1b	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.00	4.79 ± 0.11	57
	III	RS88CA14	0.36 ± 0.36	0.36 ± 0.25	4.36 ± 0.29	4.29 ± 0.24	56
	IV	RS88CA157	0.00 ± 0.00	0.08 ± 0.08	2.00 ± 0.43	4.69 ± 0.13	52
H ₂ O	I	H ₂ O	0.14 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	56
	II	H ₂ O	0.86 ± 0.14	0.46 ± 0.14	0.71 ± 0.13	0.57 ± 0.14	55
	III	H ₂ O	0.20 ± 0.11	0.07 ± 0.07	0.27 ± 0.15	0.00 ± 0.00	60
	IV	H ₂ O	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	51

^a Disease scores (DS) according to Reitan et al. 2002, shown as averages (± standard error). DS ≤ 2 indicates a resistant reaction; 2 < DS ≤ 3, moderately resistant; 3 < DS ≤ 4, moderately susceptible; and DS > 4, susceptible. DS > 3 are in bold.

^b N is the number of plants scored.

cultivars (Table 4). This indicates that results were reproducible and that all trials were successful. AU2 and RS88CA27 (type 8) were the only two isolates able to cause disease on Atlas or on Atlas 46. All isolates could infect Ingrid, except AU3, which may have lost pathogenicity through repeated in vitro transfers. The two isolates virulent on Atlas 46 were able to infect Turk NIL. Additionally, isolates carrying NIP1 type 7 or 17 and isolates lacking NIP1 were also able to overcome the resistance in Turk NIL. One isolate carrying NIP1 type 16 was able to infect Turk NIL, while the other two isolates carrying the same NIP1 type were not. However, this isolate died in silica gel storage, preventing any verification of this result.

Field experiment.

A field experiment was conducted in Chico and Davis, California, with two adjacent plots planted to Atlas and Atlas 46 to examine the selection effect of *Rrs1* on the *R. secalis* population. DNA samples were extracted from potentially infected leaf material, and amplification of the mating type idiomorphs was used to detect the presence of *R. secalis* DNA. Among the leaves collected from Atlas 46 in Chico, *R. secalis* DNA could be detected in only one out of 33 samples. From leaves collected at Davis, on the other hand, *R. secalis* DNA could be amplified from 30 and 50 samples collected from Atlas and Atlas 46, respectively (Table 5). The NIP1 gene could be amplified from 28 samples from Atlas but from none of the samples from Atlas 46. This significant difference ($\chi^2 = 71.79$, $P < 0.001$) indicates that deletion of the NIP1 gene in *R. secalis* growing on Atlas 46 was the cause of virulence on the *Rrs1* barley cultivar.

DISCUSSION

The two main mechanisms leading to virulence at the NIP1 locus in *R. secalis* were examined. We assessed deletion frequency and diversity at the molecular level for this fungal avirulence gene. From sequence data, amino acid sequences were inferred, and pathogenicity of the different types found was tested. We found that some of these isoforms were restricted to certain geographical regions. These data may contribute to a better understanding of the mechanisms (deletions and alteration of avirulence genes by point mutations) that lead to resistance breakdown in agroecosystems.

Deletion of NIP1 was much more frequent than that of NIP2 and NIP3. These latter two genes encode proteins that, like NIP1, induce necrosis in barley but, according to current knowledge, do not function as elicitors (Hahn et al. 1993). Therefore, the high deletion frequency observed for NIP1 may be attributed to its elicitor function and to the selection of *R. secalis* isolates lacking NIP1 by cultivation of *Rrs1* barley varieties.

NIP1 deletion frequency varied considerably among the populations studied, suggesting a heterogeneous use of *Rrs1* by breeders. In Ethiopia, all isolates lacked NIP1. Because of the high gene diversity found in these populations with restric-

tion fragment length polymorphisms (RFLP) (P. Zaffarano, C. C. Linde, and B. A. McDonald, *unpublished data*), a founder effect can be excluded. Therefore, we hypothesize that the widespread NIP1 deletion is due to a past or present widespread distribution of the *Rrs1* resistance gene. The Ethiopian barley landraces were shown to be distinct from those of other countries (Bjørnstad et al. 1997), which implies that the *R. secalis* populations are also likely to be different. Ethiopian *R. secalis* strains were virulent on Ethiopian landraces (Yitbarek et al. 1998), but Californian isolates were avirulent on these same landraces (Webster et al. 1980), supporting the distinctness of the Ethiopian population of *R. secalis*.

On the other hand, populations were found in which all isolates had NIP1, and even more interesting, populations were found in which isolates with NIP1 coexisted with isolates lacking the gene. This coexistence indicates that the fitness cost of NIP1 deletion cannot be very high, otherwise isolates with NIP1 would rapidly be positively selected and NIP1 would become fixed. This hypothesis was corroborated by experiments showing only a slight decrease in virulence of a mutant strain in which the NIP1 gene had been replaced through homologous recombination (W. Knogge, *unpublished data*). In contrast, replacement of NIP3 resulted in a much greater loss of virulence (W. Knogge, *unpublished data*). The Californian population, which contains isolates with and without NIP1, was collected from a barley composite cross (McDonald et al. 1989). There, some of the parental cultivars may have contained *Rrs1*, which may still be present in some of the barley plants, creating a heterogeneous selection pressure that could have maintained the polymorphism in the *R. secalis* population.

Deletion of NIP1 was the only mechanism found for virulence in the California field experiment with naturally infected Atlas and Atlas 46. All of the samples from Atlas 46 were lacking NIP1. In this same area, a population had been collected in 1988, and as mentioned above, some isolates were already lacking NIP1. This suggests that isolates without NIP1 may already have been present in this region and increased in frequency on Atlas 46.

The study of the second mechanism for gaining virulence, i.e., alteration of the avirulence gene by point mutations, required an analysis of DNA polymorphism. Often, nucleotide diversity is not constant across the different regions of a gene. Because introns do not have any apparent function, they are expected to evolve more rapidly and, consequently, to show a higher nucleotide diversity than exons or 5' flanking regions (MacIntyre 1985). Analysis of variation in the NIP1 gene showed that nucleotide diversity was not constant across the different gene regions and was unexpectedly about four times higher in the region coding for the main part of the mature protein than in any other region. This increased variation in the sequence coding for the main part of mature NIP1 hints at positive diversifying selection. However, tests for neutrality of mutations did not detect selection acting on the whole DNA sequence, but these tests are conservative (Ford 2002). On the

Table 5. Polymerase chain reaction amplification of the mating type idiomorphs (*MAT1-1*, *MAT1-2*) and of the NIP1 gene in 80 DNA samples^a

MAT1 amplicon	N ^b	Atlas		Atlas 46	
		NIP1 absent	NIP1 present	NIP1 absent	NIP1 present
None	39				
MAT1-1	3	0	3	0	0
MAT1-2	49	2	23	24	0
MAT1-1 and MAT1-2	28	0	2	26	0
Total	80 ^c	2	28	50	0

^a Obtained from diseased leaf material collected from two barley cultivars, Atlas and Atlas 46, in Davis, California.

^b Number of DNA samples analyzed for MAT1 amplification.

^c Total excluding samples for which MAT1 did not amplify.

other hand, the evolutionary pathway method showed that there was an excess of nonsynonymous mutations in the sequence coding for the mature NIP1. The ML method confirmed that *NIP1* is under positive diversifying selection.

The neutral theory of molecular evolution proposes that the vast majority of mutations are neutral or deleterious (Kimura 1983). The recent accumulation of sequence data allowed a test of this theory, and the proportion of genes under positive diversifying selection may be larger than predicted by the neutral theory (Ford 2002). An increasing number of genes or proteins have been shown to be under positive selection (Wolfe and Li 2003). Most of them are involved in male or female reproduction, defense systems, or immunity or in evading defense systems or immunity. In this latter group, many proteins from pathogenic microbes are found that are possibly used as targets by the host immune or defense system. Most relevant to this study, several plant resistance genes were shown or are suspected to evolve in response to positive selection (Stahl and Bishop 2000). A report on cell wall degrading enzymes produced by pathogens and their host plant-produced inhibitors indicated that both types of protein are under positive selection and illustrates the arms race going on between plant and pathogens (Stotz et al. 2000). The coevolution between these enzymes and their inhibitors could be similar to the coevolution between resistance genes and their corresponding avirulence genes.

Since selection does not act on genotypes but rather on phenotypes and since *NIP1* encodes a protein with two functions—NIP1 is an elicitor and a toxin—one may ask which one of these two functions is driving positive diversifying selection. One hypothesis is that positive selection is acting to slightly modify the protein, thus preventing its recognition by plant receptors without impeding its toxin function. Does this hypothesis hold when confronted with the actual knowledge on NIP1 mode of action?

The plant receptor to which NIP1 binds has not yet been isolated, but a high-affinity NIP1-binding site was recently detected in membranes of barley, irrespective of the resistance genotype (K. A. E. van't Slot and W. Knogge, *unpublished data*). Furthermore, NIP1 has been shown to stimulate the activity of the H⁺-ATPase in plasma membranes from resistant and susceptible plants (Wevelsiep et al. 1993). The two virulent NIP1 types 3 and 4 carry two amino acid alterations that turn them into ineffective elicitors (compatible interaction), but these two types also do not activate the H⁺-ATPase and do not cause leaf necrosis (Fiegen and Knogge 2002). It was therefore suggested that both NIP1 functions are mediated through a single receptor and that the signaling pathway branches downstream to lead to the two functions of the protein (Knogge et al. 1999). The receptor is therefore most likely not encoded by the *Rrs1* resistance gene (Fiegen and Knogge 2002), and a model assuming direct binding of the elicitor to a receptor encoded by the resistance gene is unlikely. More complex models partly inspired by the “guard hypothesis” (Van der Biezen and Jones 1998) have been proposed (van't Slot 2002). These findings of the linkage between the elicitor and the toxin functions do not support the hypothesis of positive diversifying selection acting to modify NIP1 to avoid its recognition by the plant while simultaneously retaining its toxin function.

Among the many amino acid mutations observed in NIP1, only a few (probably three, one for each of the three virulent types, excluding types 7 and 17, which most likely gain virulence through nonexpression) lead to virulence on *Rrs1* barley cultivars, and it is plausible to believe that these mutations were retained by positive diversifying selection. However, all the other mutations did not affect virulence. These mutations

may have been successful at evading recognition in earlier host populations (including wild barley progenitors), but in the meantime, new cultivars were bred with other recognition strategies. Knowledge of the effect of the mutation in the virulent type 8 and of the other mutations on the toxicity of the different NIP1 isoforms may increase understanding of the ecological meaning of positive diversifying selection. A better understanding of the biochemical pathways in which NIP1 is involved may also shed more light on the selection process.

All but two of the isolates could not infect Atlas but were virulent on Ingrid. This could be explained by the fact that Atlas contains the resistance gene *Rh2* (Goodwin et al. 1990) and that these isolates could not overcome this resistance. In our study, the reaction of Atlas upon inoculation with isolate UK7 was scored as incompatible. This contradicts previous data that considered UK7 as being virulent on Atlas (Lehnackers and Knogge 1990). However, the Atlas seed used in the present study was not obtained from the same source and the Atlas and Atlas 46 accessions may be heterogeneous. UK7 was virulent on Ingrid, which is thought not to contain any resistance genes. In general, the near-isogenic cultivar pair Ingrid-Turk NIL seemed more appropriate to test for the ability of the different NIP1 types to overcome *Rrs1* resistance, since Turk NIL probably contains only this resistance gene (Bjørnstad et al. 2002). In this respect, isolates carrying NIP1 types 5 and 6, which were avirulent on Atlas and Atlas 46 (Kilby and Robinson 2001), should probably also be inoculated on Ingrid and Turk NIL.

In addition to isolates carrying NIP1 isoforms 3 and 4, four classes of isolates were able to avoid detection by the *Rrs1* resistance gene: the isolates with *NIP1* deletions and those with NIP1 types 7, 8, or 17. Virulence of types 7 and 17 is most likely due to the nonexpression of NIP1 in the isolates carrying this type. Two isolates with NIP1 type 7 were previously collected from Atlas 46 (Johnson et al. 1996). Virulence of NIP1 type 8 may be attributed to a mutation from tyrosine to phenylalanine at amino acid 44, which is located in loop III of the protein in the same region as the inactivating mutation in type 4 at amino acid 45 (Rohe et al. 1995; van't Slot et al. 2003).

The only virulent NIP1 types that were found in this study were types 7, 8, and 17. Even though types 3 and 4 were previously observed in Australia (Rohe et al. 1995), we did not find these types among the 49 Australian isolates we sequenced. Isolates carrying the virulent type 7 were found in different Western European countries, including France, Germany, and Switzerland. Isolates carrying NIP1 type 8 were found only in the Californian population, and isolates carrying type 17 were found only in one of the Jordanian populations (RS02JO2). Since each of the virulent NIP1 types was restricted to a population or at most to a region, we can hypothesize that virulence arose independently three times.

Variation in NIP1 types within regions was measured with gene diversity. Gene diversity was the lowest in Australia. Diversity for isozyme and RFLP loci were also lower in Australian than in Scandinavian *R. secalis* populations (Goodwin et al. 1993; Salamati et al. 2000), and it was suggested that the low diversity in the Australian population is the result of a founder event (Salamati et al. 2000). The low diversity in the Californian population may be due to the small spatial scale over which isolates were collected and to the small sample size compared with other regions.

NIP1 types 1 and 2 were by far the most common types found. However, type 2 stimulates the H⁺-ATPase less than type 1 and causes only chlorotic (instead of necrotic) lesions, indicating a weaker toxicity (Fiegen and Knogge 2002). If higher toxicity is correlated with higher fitness, one would

expect isolates carrying type 1 to have a fitness advantage over isolates carrying type 2 and, therefore, to be more frequent. This was not the case in the regions surveyed. Overall both types were equally common. In two regions (Scandinavia and California), type 1 was more frequent, whereas type 2 was more frequent in two other regions (Australia and Western Europe). Our interpretation is that either a higher toxicity does not confer higher fitness or the fitness advantage is too small to lead to detectable differences in frequency.

Gain of virulence on *Rrs1* cultivars was achieved in two ways: through deletion of *NIP1* and through point mutations in this avirulence gene. Virulence due to altered *NIP1* seems to have arisen independently at least three times, in California, Western Europe, and Jordan. However, these mutations were observed in only 18 out of 196 isolates (9%). The finding of positive diversifying selection acting on *NIP1* suggests that diversifying selection operating at resistance gene loci in plants leads to diversifying selection for corresponding avirulence gene loci in plant pathogens. On the other hand, deletion of *NIP1* appears to be a more common mechanism for gaining virulence, with 45% of the isolates lacking *NIP1*. This high proportion may be due to the irreversible character of deletions, and the high frequency suggests that there is either a low or no fitness cost associated with *NIP1* deletion. In addition, if mutated *NIP1* proteins that lost the elicitor function are also

nonfunctional toxins, the deletion mechanism may offer an advantage over the point mutation mechanism, since the production of a mutated *NIP1* would involve the cost of synthesizing the protein but no benefit.

MATERIALS AND METHODS

Origin of *R. secalis* isolates.

R. secalis isolates collected in Australia, Ethiopia, Finland, Norway, Switzerland, and California were described previously (Table 6). Populations were collected from naturally infected barley fields along one or more transects or using a hierarchical sampling strategy (McDonald et al. 1999). Additional Australian isolates were collected from barley grass (*Hordeum leporinum* Link) on plants bordering two of the barley fields (populations 4 and 6). In Norway in the early 1990s, isolates were collected from different fields and locations at random. In 1997, single isolates were collected from fields in France, Germany, Great Britain, and Turkey from different barley cultivars. A hierarchical collection was made in 1996 from an unknown barley variety in Sweden. In Jordan, two hierarchical collections were made 2 km apart, in Al-Raba in 2002. Three collections were made in the north of Syria in 2002. The first population was collected in a barley field near the village of Batajik in the Al Bab-Aleppo province, and the second col-

Table 6. Country of origin, host species, host cultivar, and sampling scheme of the *R. secalis* populations used in the present study

Country	Population	Code ^a	Host	Cultivar	Sampling	Reference or source
Australia	1	RS96AUS1	Barley	Clipper	Hierarchical	McDonald et al. 1999
	2	RS96AUS2	Barley	Clipper	Hierarchical	McDonald et al. 1999
	3	RS96AUS3	Barley	O'Connor	Hierarchical	McDonald et al. 1999
	4	RS96AUS4	Barleygrass		Random	From plants bordering RS96AUS3 field
	5	RS96AUS5	Barley	Schooner	Hierarchical	McDonald et al. 1999
	6	RS96AUS6R, X	Barleygrass		Transects	From plants bordering RS96AUS5 field
	7	RS96AUS7	Barley	Arapollis	Hierarchical	McDonald et al. 1999
	8	RS96AUS8	Barley	Stirling	Hierarchical	McDonald et al. 1999
	9	RS96AUS10	Barley	Yerong	Transects	McDonald et al. 1999
	10	RS96AUST	Barley	Tantagara	"Hot-spot"	McDonald et al. 1999
Ethiopia	1	RS01ET1	Barley	Local barley landrace (LR)	Hierarchical	Linde et al. 2003
	2	RS01ET2	Barley	Local barley LR	Hierarchical	Linde et al. 2003
	3	RS01ET3	Barley	Local barley LR	Hierarchical	Linde et al. 2003
	4	RS01ET4	Barley	Local barley LR	Hierarchical	Linde et al. 2003
Finland		RS96FIF	Barley	Kymppi	Hierarchical	Salamati et al. 2000
France		RS97FR	Barley	Systel, Marilor, Maeva	Random	J. Speakman
Germany		RS97DE	Barley	Danilo, Caribe, Rocca	Random	J. Speakman
Great Britain		RS97GB	Barley	Maris Otter, Gaelic, Pipkin	Random	J. Speakman
Jordan	1	RS02JO1	Barley	Unknown	Hierarchical	K. Meles
	2	RS02JO2	Barley	Unknown	Hierarchical	K. Meles
Norway	1	RS90sNO	Barley	Unknown	Random	S. Salamati
	2	RS96NO1N1	Barley	Tyra	Hierarchical	Salamati et al. 2000
	3	RS96NO1NK	Barley	Tyra	Hierarchical	Salamati et al. 2000
Sweden		RS96SW	Barley	Unknown	Hierarchical	S. Salamati
Switzerland	1	RS99CH1	Rye	Danko	Hierarchical	B.A. McDonald
	2	RS99CH2	Barley	Unknown	Hierarchical	Linde et al. 2003; Collection 1
	3	RS99CH5	Barley	Mixture of 2 two-row varieties	Hierarchical	Linde et al. 2003; Collection 2
	4	RS99CH6	Barley	Two-row variety	Hierarchical	Linde et al. 2003; Collection 3
	5	RS00CH	Barley	Unknown	Hierarchical	Linde et al. 2003; Collection 4
	6	RS01CH1	Barley	Baretta	Transects	Linde et al. 2003; Collection 5
	7	RS01CH2	Barley	Unknown	Transects	Linde et al. 2003; Collection 6
Syria	1	RS02SY1	Barley	Rihane	Transects	B. A. McDonald and C. C. Linde
	2	RS02SY4	Barley	Arabi Aswad	Transects	B. A. McDonald and C. C. Linde
	3	RS02SY6	<i>Hordeum spontaneum</i>		Random	B. A. McDonald and C. C. Linde
Turkey		RS97TR	Barley	Unknown	Random	J. Speakman
California, U.S.A.		RS88CAR	Barley	CC II and V	Hierarchical	McDonald et al. 1989

^a A code is assigned to each population. The first two letters of the code (RS) stand for the organism, *R. secalis*, and the two numbers following describe the year of the collection. Then, two or three letters stand for the country where the collection was made, and the last number indicates the different populations collected in that particular year and country. For instance, RS96AUS3 refers to the third *R. secalis* population collected in 1996 in Australia.

lection was made from the local barley landrace Arabi Aswad near the village of Al Jurn Al Aswad in the Raqqa province; both are located along transects. The third population was collected from *Hordeum spontaneum* growing in a field planted to cumin near the village of Aim Issa. In Switzerland, population 1 was collected from an organic rye field. Populations were grouped into five regions (Table 3). Isolations were made from plant leaves, as described in McDonald and associates (1999), and DNA was extracted as described in that paper or as described in Linde and associates (2003). A subset of these populations (Table 1) was used to detect the presence of the three *NIP* genes, and another partially overlapping subset (Table 3) was used to study the variation in the *NIP1* gene. Isolates identified as distinct genotypes with RFLP were chosen from these populations for sequencing.

Hybridization of the *NIP1*, *NIP2*, and *NIP3* genes.

For each gene, a probe was generated with polymerase chain reaction (PCR). The probes were labeled with ^{32}P , using nick translation (Invitrogen Corporation, Carlsbad, CA, U.S.A.) and were hybridized to Southern blots with DNA of *R. secalis* isolates. Primers designed by Kilby and Robinson (2001) were used to amplify the *NIP1* gene. For *NIP2* and *NIP3*, primers were designed based on the sequences of these two genes (W. Knogge, unpublished data). Each PCR mix (final volume 60 μl) contained 0.5 μM of each primer, 100 μM of each nucleotide, 6 μl of 10 $\times$ PCR buffer (500 mM KCl, 15 mM MgCl_2 , and 100 mM Tris-HCl [pH 9.0]), and 1.5 U of *Taq* DNA polymerase (Amersham Biosciences, Europe GmbH, Otelfingen, Switzerland). The following cycling conditions were employed: initial denaturation for 2 min at 96°C, followed by 30 cycles of denaturation for 1 min at 96°C, annealing for 1 min at 64°C, and elongation for 1 min 30 s at 72°C, followed by a final elongation step of 10 min at 72°C. The PCR products were purified using a multiscreen PCR plate MANU 030 PCR (Millipore, Billerica, MA, U.S.A.). When a probe did not bind to any DNA fragment of a particular isolate, the gene in question was considered to be deleted in this isolate only after ensuring that other probes bound to this DNA digest. Otherwise, this datum point was excluded.

For all populations except the one from Norway, the data were clone-corrected with RFLP data collected previously (McDonald et al. 1999, B. A. McDonald unpublished data, P. Zaffarano, unpublished data and). Seven single-locus probes and one multicopy probe were used to detect clones (McDonald et al. 1999). We applied tests of linkage disequilibrium in Popgene version 1.32 (Yeh et al. 1999). A two-locus linkage disequilibrium test (Weir 1979) was used to test for linkage disequilibria between pairs of loci, and a multilocus test based on the number of heterozygous loci between two randomly chosen isolates in a population (Brown et al. 1980) was applied.

Variation in the *NIP1* gene.

Based on the sequence of *NIP1* (U20363) in the GenBank sequence database, primers were designed to amplify and sequence four fragments spanning the entire known sequence (Table 7). PCR conditions were as above, except for the annealing temperature, which was 65°C, and a 1-min elongation

step. Some of the purified PCR products were sequenced using a Beckman Coulter CEQ 2000 dye terminator cycle sequencing kit with both the forward and reverse primers on a Beckman Coulter CEQ 2000 automated sequencer (Beckman Coulter, Fullerton, CA, U.S.A.), according to the manufacturer's instructions. Amplicons were purified on Sephadex G-50 fine DNA grade resin columns on a multiscreen HV plate (Millipore). Other PCR products were sequenced with ABI PRISM BigDye Terminator v3.0 and 3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.). Each reaction contained 1 μl ready reaction mix, 1.5 μl 5 $\times$ reaction buffer (400 mM Tris-HCl, pH 9.0, 10mM MgCl_2), 1 μM primer, 3 to 10 ng PCR product, and water up to 10 μl . The cycle sequencing conditions were according to the manufacturer's instructions. Prior to Sephadex purification, 10 μl ddH $_2\text{O}$ and 2 μl 2.2% sodium dodecyl sulfate were added to the reactions, and samples were heated at 98°C for 5 min. These samples were analyzed on a 3100 ABI automated sequencer.

Sequence analysis.

For each isolate, sequences obtained with forward and reverse primers were aligned and edited manually with Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). The same software was used to align sequences from different isolates. DnaSP version 3.53 (Rozas and Rozas 1999) was used to calculate nucleotide diversity and to perform tests of neutral evolution.

The 1,524-bp-long sequence including the *NIP1* gene was divided into seven distinct regions. The 5' flanking region ranged from sites -967 to -90. The 5' untranslated region was defined as starting at the most 5' transcription start site (-89) mentioned in Rohe and associates (1995). The signal peptide (sites 1 to 66), the remainder of the first exon (67 to 237), the intron (238 to 303), and the second exon (304 to 312) were as described previously (Rohe et al. 1995). The entire region 3' of the last exon (313 to 557) was called the 3' flanking region, since no polyadenylation signal and therefore no 3' untranslated region could be identified.

Tests of neutral selection.

Different approaches were used to test for neutral selection. First, Tajima's D (Tajima 1989) and Fu and Li's F^* and D^* test statistics (Fu and Li 1993) were applied. These tests are based on differences between estimates of nucleotide polymorphism. The three test statistics were calculated for all sequences and also separately for each of the five regions.

Second, methods based on comparisons of synonymous (d_s) and nonsynonymous (d_N) substitution rates were used. Only the part of the first exon coding for the mature *NIP1* was used for this analysis, since the signal peptide showed too little polymorphism for a meaningful statistical analysis and the second exon was only three codons long and conserved. The analyses were conducted with the 13 haplotypes found among the studied isolates and with two sequences obtained from isolates AU2 and AU3 (Rohe et al. 1995), representing two haplotypes not found in this study. The ratio between d_N and d_s , ω , indicates which selection pressure is acting on the gene under consideration. If purifying selection removes nonsynonymous mutations, ω will be <1. If nonsynonymous mutations are neu-

Table 7. Oligonucleotides used for PCR amplification of the *NIP1* gene

Fragment	Forward primer 5'→3'	Reverse primer 5'→3'
1	NIP1_21F AATTCGACGCTGCTCATTGG	NIP1_441R CGCACCCAGAACGGCCAAGC
2	NIP1_380F AGCCTTGACAGCAGGAGCAC	NIP1_802R CCTGTGGATGGGCAATGG
3	NIP1_801F GGGTTCCAGGATAATTAAGCC	NIP1_1200R AGCCATTGCCACCTTCAC
4	NIP1_1117F AGCTCTCAAAGCTGTTTCCG	NIP1_1558R GCCACGATAGATAGTATATGAATTGC

tral, they will be fixed at the same rate as synonymous mutations and ω will be $=1$ (Kimura 1983). If nonsynonymous mutations are beneficial, they will be retained by positive diversifying selection and ω will be >1 . First, we used an evolutionary pathway method to estimate d_S and d_N , and computed these values according to Nei and Gojobori (1986) with the Jukes and Cantor correction (1969) for all pairwise comparisons and over all sequences with MEGA2 (Kumar et al. 2001). A t -test was conducted with overall d_N and d_S and their bootstrap variance (Hughes and Nei 1988), to test whether ω is greater than one. Since this test is prone to type I error (Zhang et al. 1997), the critical value was raised to $t > 2.2$, which, even if biased, corresponds to $P < 0.05$ (Bishop et al. 2000). Second, we used a ML method to calculate d_S and d_N . Using the CODEML program in the PAML package (Yang 1997), three codon-based models were applied to the data: i) a one-ratio model that assumes one category for ω , which was then either estimated from the data or fixed at one (all mutations are neutral), ii) a neutral model in which substitutions are either neutral or deleterious (ω can assume two values, either 0 or 1), and iii) a selection model that includes a third category of advantageous substitutions (ω can assume three values, 0, 1, or a value estimated from the data). Tests of positive diversifying selection were constructed by comparing twice the log likelihood difference between two models with a χ^2 distribution. Each model was run using a neighbor-joining phylogeny as well as two heuristic ML trees calculated in PAUP 4.0 (D. L. Swofford, Sunderland, MA, U.S.A.). Since the results were very similar among the three trees, only those for the tree with the best goodness-of-fit are shown.

Codon usage bias.

To estimate the extent of codon bias in *NIP1*, measures of codon usage, such as the codon bias index (Morton 1993), G+C content at second and third positions as well as overall, and the effective number of codons (Wright 1990) were estimated in DnaSP.

Variation in *NIP1*.

Amino acid sequences of the mature *NIP1* were deduced from the DNA sequences, and *NIP1* types were numbered. The distribution of these *NIP1* types was examined across the populations studied, grouped into five geographical regions, and gene diversity (Nei 1973) was calculated for each region. To test for significant statistical differences in gene diversity among regions, bootstrap analyses were performed with the Resampling Stats software package (version 5.02; Resampling Stats, Inc., Arlington, VA, U.S.A.) to calculate standard deviations. For each bootstrap replication, a random sample of 24 isolates (the sample size of the smallest region, California) was taken from each region, and gene diversity was calculated. This was repeated 10,000 times, and the mean gene diversity and its standard deviation were calculated for each region and compared among each other with t -tests applying a Bonferroni correction.

Pathogenicity tests.

Isolates. The pathogenicity of 35 isolates representing 11 *NIP1* types and of four isolates lacking *NIP1* was tested on two isogenic pairs of barley cultivars, Atlas Cih0 4117 and Atlas 46 Cih0 7323 and Ingrid and Turk NIL (supplied by Å. Bjørnstad, Agricultural University of Norway, Ås, Norway). Whenever possible, three isolates were chosen to represent each *NIP1* type (Tables 4 and 6). *NIP1* type 18 was represented by only one isolate, which could not be recovered from silica gel storage. *NIP1* type 14 was represented by a single isolate that was not present

in our isolate collection and could not be tested. *NIP1* types 8 and 10 were also represented by only one isolate each, and therefore pathogenicity tests for these isolates were repeated twice. Due to limited greenhouse space and resources, the experiment was split into four trials. Each trial always included a negative control (inoculation with water) and positive controls. Four isolates, UK7, AU1, AU2, and AU3, were used as positive controls. Isolates UK7 and AU1 (both *AvrRrs1*) are virulent on *rrs1* cultivars only (Rohe et al. 1995). They showed the same reaction in the first trial, and therefore, only UK7 was used as a positive control in the following trials. Isolates AU2 and AU3 (both *avrRrs1*) are virulent on both *rrs1* and *Rrs1* cultivars (Rohe et al. 1995). Isolate AU3 was used only in the first trial, since it sporulated only weakly later on. The tested isolates were taken from silica gel storage and plated on Difco lima bean agar (Becton, Dickinson and Co., Sparks, MD, U.S.A.) plates amended with kanamycin (50 mg/liter). One colony was transferred to a fresh lima bean agar plate. About two weeks later, 2 ml of sterile water was added to the plate, and spores were scraped off with a microscope slide. A total of 100 μ l of the spore solution was transferred to a NUNC cryovial filled with sterile silica gel for storage. The rest of the spore suspension was used to inoculate three lima bean agar plates. Two weeks later, water was added to these plates, and spores were again scraped away with a microscope slide. The spore suspension was filtered through two layers of cheesecloth and was centrifuged for 2 min at $3,000 \times g$. The pellet was resuspended in fresh water and was centrifuged a second time. Spore concentration was measured with a Thoma cell hemacytometer and was adjusted to 10^6 spores per milliliter.

Inoculations. One-liter pots were filled with Rasenerde Top Dressing (sand, compost, perlite, white peat, mineral fertilizer; Ricoter AG, Aarberg, Switzerland) and were planted with five seeds of the same cultivar. For each isolate, three pots were planted with each cultivar. The plants were grown in the greenhouse with a 14-h day at 17°C and a 10-h night at 15°C . Humidity was set at 60%. Inoculations were conducted when the second leaves of the plants were fully developed. A few drops of Tween 20 were added to 60 ml of spore suspension, and this mixture was sprayed onto six pots of five plants each placed on the rotating table of a compressed air inoculation machine. For 48 h after inoculation, 100% humidity was ensured by covering the plants with plastic tents, with a humidifier connected to each tent. Two weeks after inoculation, disease symptoms were assessed on the second leaf using a 0 to 5 scale (Reitan et al. 2002). Disease scores below 2 were classified as resistant reactions, between 2 and 3 as moderately resistant, between 3 and 4 as moderately susceptible, and above 4 as susceptible.

Field experiment.

In 2003, a field experiment was conducted in California at two locations, Davis and Chico. At each location, one plot was planted with the barley cultivar Atlas and another plot with the cultivar Atlas 46. Normally, isolations of *R. secalis* are made from diseased green leaves. However, an unusually dry year resulted in the plants drying out sooner than expected. As a result, diseased leaves were collected at the end of the growing season. *R. secalis* did not sporulate satisfactorily on this very dry material, and therefore, a PCR-based method was used to detect *R. secalis*. DNA extraction was performed directly from leaf material from a total of 176 leaf pieces, with DNeasy plant mini kit (Qiagen, Venlo, The Netherlands). When possible, leaf pieces with recognizable and distinct scald lesions were selected. Multiplex PCR of the mating type idiomorphs (Linde et al. 2003) was used to detect the presence of *R. secalis* DNA. Then, PCR of the *NIP1* gene with Kilby and

Robinson's primers (2001) was conducted, to detect the presence of this gene. To confirm the absence of *NIP1* in the samples for which no amplicon was detected, a second PCR was conducted with a lower annealing temperature (50 instead of 63°C), and a third PCR was conducted with primers NIP1_21F and NIP1_1558R (Table 7). All PCR reactions included positive (DNA from known isolates) and negative (water and barley DNA) controls.

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