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Development of molecular markers for powdery mildew resistance to support breeding for high quality hops

Powdery mildew is one of the most important fungal diseases on hop (*Humulus lupulus*). In this research project molecular markers were developed to support the time-consuming and difficult work of hop resistance breeding without modifying the genome. Using the AFLP (amplified fragment length polymorphism) technique, a PCR based method, in combination with a newly established leaf resistance test system specific DNA markers could be detected associated with the presence of specific powdery mildew resistance genes. Using these markers the efficiency in selecting for powdery mildew resistant seedlings can be increased.

Descriptors: *Humulus lupulus*, breeding, marker assisted selection, powdery mildew, molecular marker

1 Introduction

Hop (*Humulus lupulus L.*) is an important ingredient in the beer-brewing process. The secondary metabolites, resins and essential oils of the hop cones impart flavour, bitterness and aroma to beers. Due to the long-term monoculture growing system hop is exposed to a high pressure of infection by several diseases and pests. Powdery mildew, caused by the fungus *Podosphaera macularis ssp. humuli* [Braun] represents the major economic risk factor in hop growing. Powdery mildew (PM) is responsible for drastic losses of yield and quality. Regular protective treatments with pesticides are indispensable for commercial hop growing. In 2002 hop growers in the Hallertau had to spend 4,9 Mio. Euro only for controlling PM. In order to prevent environmental problems and to ensure hop quality without any harmful residues of pesticides, breeding for powdery mildew resistance is the most important objective at the Hop Research Center Hüll. At current, there are two PM resistant genes available that are still effective in the German hop growing regions: the *R2* gene from the English cultivar 'Wye Target' and the *Rbu* gene from the Slovenian cv. 'Buket'. But the development of hop cultivars carrying these resistance genes while also showing broad resistance to other diseases and excellent brewing quality is a long, laborious procedure taking 10–15 years and even longer for aroma varieties. One problem in classical breeding is that so far the selection of powdery mildew resistant hop plants is based on the phenotype after artificial or natural infection with fungal spores in the greenhouse or in the field. However, this phenotypic expression of susceptibility or resistance can significantly be affected by environmental factors, by different fungal spore loads and the developmental stage of the plants themselves, leading to wrong or inconsistent resistance data. Moreover, using the classical breeding system based on phenotypic assessment it is not possible to recognize hop seedlings carrying more than one effective resistance gene which would provide a more durable disease resistance.

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Tables and Figures see Appendix

In order to meet the high demands of the hop and brewing industry it is necessary to reduce the long process of the selection and phenotypic assessments of promising breeding lines by exploiting highly reproducible, consistent and easy to use molecular markers which allow the reliable identification of desired genotypes. DNA based markers can routinely be applied for marker assisted selection (MAS) which is particularly useful in hop due to its high level of heterozygosity. In recent years, several applications of PCR (polymerase chain reaction) based methods were described in hop research (5, 6, 9, 10, 11, 12, 13, 15, 17)**.** Moreover, MAS can save labour and space for screening huge populations. The AFLP method established for the hop genome analysis in combination with a newly established PM resistance test system based on detached young leaves (16; research funded by the Wissenschaftsförderung der Deutschen Brauwirtschaft) were important preconditions to identify closely linked molecular markers for PM resistance.

2 Materials and methods

2.1 Plant material and "bulk segregant analysis"

For the development of molecular markers linked to PM resistance the bulk segregant analysis was used (8). Therefore specific crosses were made between a powdery mildew resistant individual and a highly susceptible plant. In total, six mapping populations were examined. Resistance in three mapping populations is based on the *R2* gene deriving from the English cultivar 'Wye Target' which is still effective against PM infections in the German hop growing regions. Two mapping populations were created to identify molecular markers associated with the resistance gene *Rbu* deriving from the Slovenian cultivar 'Buket'. In addition, one crossing between two resistant hop plants carrying the *R2* and the *Rbu* gene, respectively, was made to identify seedlings harboring both resistance genes (*R2* and *Rbu*).

2.2 Detached leaf assay

From approximately six to eight-week-old hops grown from seeds in the greenhouse in each case the youngest, completely unfolded pair of leaves was taken and inoculated artificially with PM spores in Petri dishes in the laboratory (16). After eight days incubation (22 °C; 12 hours light/dark cycle) the leaves of the seedlings

were assessed and compared with the reference variety (highly susceptible leaf of the variety 'Northern Brewer') and classified as resistant or susceptible. The test was repeated at least twice. A PM isolate (HU2) with defined virulence properties (*v3,vB* virulence type) was employed as an inoculum for all seedling populations which allowed to distinguish PM susceptible and resistant seedlings very easily. Furthermore, for the mapping population deriving from the crossing 'Buket' (*Rbu*) x 98/27/731 (*R2*) the English isolate E9 of the *v1,v2* virulence type was used to differentiate between individuals with resistance based on *R2* and *Rbu*, respectively.

2.3 DNA extraction and AFLP assays

Genomic DNA was extracted using 150 mg of freeze-dried, finely ground leaves, hop cones or pellets following the protocol according to Doyle and Doyle (3). DNA was diluted to a working concentration of approximately 25 ng/µl by visual comparison with a λ DNA standard on 1% agarose gels. The AFLP procedure was performed as described by Vos et al. (19). These studies included the primer combinations used in former molecular investigations in hop (5+12). As *Mse*I specific primers for the pre-selective amplification step *M00* was used without selective nucleotides. After the polymerase chain reaction (PCR) the denatured products were separated on 5% denaturing polyacrylamid gels. A 50–500 bp ladder (fluorescein labelled) was used as molecular weight marker.

2.4 Data analysis and linkage mapping

The AFLP patterns were analyzed with the computer software Cross Checker (2). The data matrix generated by this program was used as input data file for the software JoinMap® 3.0 (18), to perform linkage mapping. Linkage groups were determined using a minimum LOD (logarithm of the odds) threshold of 6.0. The map was constructed using the Kosambi mapping function (7).

3 Results and discussion

3.1 Marker development for the *R2* gene from 'Wye Target'

One hundred and twenty individuals of three full-sib mapping populations segregating for the PM resistance gene *R2* were screened for disease resistance in the laboratory using the detached leaf assay and afterwards in the greenhouse. Resistance data did not deviate significantly from a 1:1 ratio of segregation (Table 2) and thus confirmed that a single dominant gene *R2* is involved in the resistance reaction of the seedlings.

Among all 91 AFLP primer pairs tested 20 could differentiate between the DNA bulks of ten fully resistant and ten highly susceptible hop plants. Chi-square analysis revealed that most of these AFLP markers followed a Mendelian segregation. AFLP markers were re-examined on all individuals of the progeny including the parents. Seven DNA fragments inherited from the resistant parent (markers in coupling phase) were identified as putative AFLP markers linked to the PM resistance locus *R2*.

Figure 1 shows that the AFLP marker R2-L2_164 is present in all PM resistant seedlings and missing in all susceptible plants.

3.2 Linkage analysis and mapping of the *R2* powdery mildew resistance gene

Another objective was to localize the *R2* resistance gene in the hop genome. Therefore a male and female chromosome map were created from the molecular data of the crossing 84/08/24 x 98/44/49. Using 91 AFLP primer combinations 620 polymorphic fragments could be produced. Five hundred forty-nine fragments were classified as 1:1 segregating markers (*lm* x *ll* and *nn* x *np*, respectively), 414 contributed by the female (84/08/24) and 135 by the male parent (98/44/49). Ninety-seven markers were heterozygous in both parents (*hk* x *hk*) segregating 3:1. Segregation of 17 microsatellites (1, 4, 14) was also investigated in the mapping population.

Using 620 AFLPs (which include the above mentioned putative *R2* resistance markers) and 17 microsatellites, the *R2* resistance locus and the male sex locus *M* linkage analysis was performed and the male and female maps were constructed using JoinMap®3.0. Based on a LOD of six, 303 AFLP markers and all 18 SSRs (single sequence repeats) were assigned to nine female linkage groups covering 320.6 cM. In the male map 150 AFLPs and 15 SSRs were placed on eight linkage groups spanning a distance of 237.4 cM.

Located on the largest female group Gr-F1 covering 101 cM, the *R2* locus conferring PM resistance was flanked by two AFLP markers at a distance of 1.6 cM. In total, six AFLP markers are closely linked to the resistance locus *R2* at a distance of 1.7–3.8 cM (Fig. 2). This is for the first time that two markers adjacent to the *R2* resistance locus could be detected. This is also the first time that PM resistant markers could be assigned to a genetic map in hops.

3.3 Molecular markers associated with the *Rbu* gene from 'Buket'

Based on the resistance data provided by the detached leaf assay from two 'Buket' crossings DNA pools with ten resistant or susceptible individuals were produced. These DNA pools were screened with 240 AFLP primer combinations for differences in the DNA banding patterns. So far three highly promising markers for the *Rbu* gene were detected which could be found in almost all resistant seedlings from all three 'Buket' crossings. Considering the number of "false positive" individuals (hop plants which are assessed as being resistant, but are missing the marker DNA fragment in the genetic fingerprint analysis and vice versa) in the various crossings, the hit rate varies for the different resistance markers identified. The concurrence between phenotypical and genotypical PM resistance with the 'Buket' marker Rbu-279 is between 92 and 97 %, with Rbu-284 (Fig. 3) between 91,7 and 92,5 % and with Rbu-319 between 92,5 and 94,5 %.

3.4 Identifying hop seedlings carrying the combination of the two resistance genes *R2* and *Rbu*

Special interest was focused on the population generated from the cross of 'Buket' (*Rbu*) and a PM resistant male seedling derived from 'Wye Target' (*R2*). Screening for PM resistance by using the fungal isolate HU2 the progeny (131 individuals) showed a 3:1 segregation for resistance. Twenty-eight seedlings were susceptible and 103 proved to be resistant. Further testing was conducted using the E9 isolate, a PM strain with *v2* virulence. In this way seedlings carrying the *R2* gene as the only source of resistance could be detected due to their susceptibility towards the *v2* pathotype.

AFLP analysis revealed that markers for both resistance genes (*R2* and *Rbu* gene) could be detected by using one specific AFLP primer combination. Thus, it was possible to recognize seedlings

with resistance based on *R2* or *Rbu* and moreover, to identify hops within the progeny which possess both resistance genes (Fig. 4). This is the first time that the pyramiding of two resistance genes in hop could be detected by molecular markers.

4 Conclusions

Genetic markers developed within this project will significantly improve and speed up the selection step. Resistance to powdery mildew can be detected directly on the DNA level. The developmental stage of the hop plants, environmental factors and the varying infection potential of the fungal spores can be excluded as modifying factors. Utilizing these molecular resistance markers small leaves of only few milligrams are sufficient to select for resistance without the need of prior fungal infection. Moreover, in contrast to infection tests in which solely a plant can be recognized as phenotypic susceptible or resistant, using DNA markers the presence of different resistance genes can be detected in one individual. This concept of pyramiding several resistance genes in one hop variety keeps promise for a much more durable disease resistance. In this way, gene diagnosis is of outmost importance for the resistance breeding at the Hop Research Center Hüll.

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Appendix

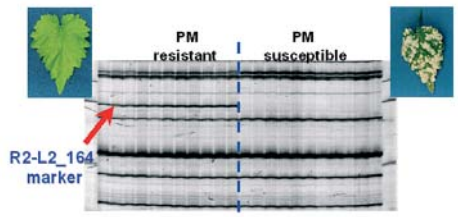

Fig. 1 AFLP marker R2-L2_164 to select hop seedlings carrying the *R2* gene

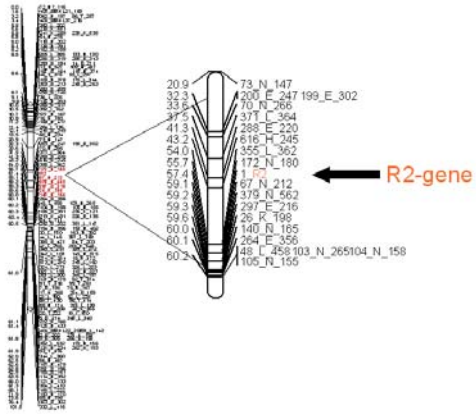

Fig. 2 Mapping of the *R2* resistance locus

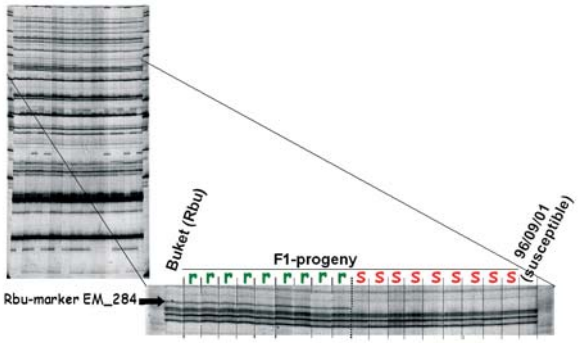

Fig. 3 AFLP marker Rbu-284 to select hop seedlings carrying the *Rbu* gene

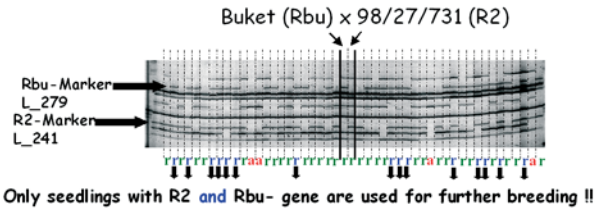

Fig. 4 Within the progeny derived from the cross of two PM resistant parents which carry only a single resistance gene (*R2* or *Rbu*) seedlings could be identified with resistance based on one or two genes (*R2* and *Rbu* in combination) by the presence of one or both resistance markers in the genetic fingerprint

Table 1 Mapping populations segregating for the resistance genes *R2* and *Rbu*

Wye Target (*R2* gene) x 96/09/01 (susceptible)
Wye Target (*R2* gene) x 93/36/02 (susceptible)
84/08/24 (*R2* gene) x 98/44/49 (susceptible)
Buket (*Rbu* gene) x 96/09/01 (susceptible)
Buket (*Rbu* gene) x 97/36/05 (susceptible)
Buket (*Rbu* gene) x 98/27/731 (R2 gene)

Table 2 Segregation of the resistance genes in the mapping populations

Progeny from the crosses	No. of seedlings (F1-Pop.)	Phenotype resistant:susceptible	expected	χ^{2-}	PM isolate
Buket (*Rbu*) x 96/09/01	120	57 : 63	1:1	0.30	HU2
Buket (*Rbu*) x 97/36/05	160	84 : 76	1:1	0.40	HU2
Buket (*Rbu*) x 98/27/731 (*R2*)	131	103 : 28	3:1	0.92	HU2+E9
Wye Target (*R2*) x 93/36/02	120	67 : 53	1:1	1.63	HU2
Wye Target (*R2*) x 96/09/01	120	58 : 62	1:1	0.13	HU2
84/008/24 (*R2*) x 98/44/049	120	67 : 53	1:1	1.63	HU2