QUANTIFICATION OF FLAX RESISTANCE TO POWDERY MILDEW BY THE RANDOM AMPLIFIED POLYMERIC DNA (RAPD)

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Abstract

Eight flax cultivars were evaluated for powdery mildew (PM) resistance under field conditions in 2001/2002 and 2002/2003 growing seasons. The tested cultivars could be divided into two distinct groups. The first group included the resistant cultivars Dakota, Wilden and Williston Brown, while the second one included the susceptible cultivars Cortland, Linore, C.I. 2008, Giza 7, and Giza 8. The difference in disease severity was highly significant ($p< 0.01$) between any cultivar belonging to the first group and any cultivar belonging to the second group. The tested cultivars were analyzed with a random decamer primer using the polymerase chain reaction (PCR). The primer succeeded in amplifying a total of 20 DNA fragments for the 8 cultivars that were analyzed. Data for PM ratings and amounts of DNA fragments were entered into a computerized stepwise multiple regression analysis. Using the predictors supplied by stepwise regression, a two-factor model constructed to predict PM severity. This model showed that PM severity differences were due largely to the DNA fragments no_s. 11 and 13, (699 and 872 bp) which accounted for 97.04% of the variation in severity ratings. These results indicate that RAPD analysis may provide a supplementary assay to field tests to distinguish quantitatively between PM resistant or susceptible genotypes.

INTRODUCTION

Powdery mildew (PM) of flax (*Linum usitatissimum* L.) is caused by the obligate parasite *Oidium lini* Škoric. This fungus is found on flax in Egypt only in its imperfect (conidial) stage. The pathogen infects all the aboveground flax organs including stems, leaves, flowers, and capsules. PM occurs annually in all flax production areas in Egypt (Mansour, 1998).

Significant negative correlations were found between disease intensity ratings and agronomic traits (Aly *et al.*, 1994). Currently, all commercially grown flax cultivars are susceptible to the disease, although field observations indicate that some experimental lines are more susceptible than others (Aly *et al.*, 2001).

Foliar application of fungicides has become the only commercially available management practice for controlling the disease and minimizing associated losses in seed and straw yield (Aly *et al.*, 1994 and Mansour, 1998). Complete dependence on fungicides for the disease control carries risks for the producers in that accurate coverage and distribution of fungicides may not be achieved and there are potential problems with correct timing of application. Furthermore, increasing concern for the environment will likely mean greater regulation of pesticide usage (Pearce *et al.*, 1996).

Use of cultivars with PM resistance can resolve all these problems. Currently, field evaluation is the only reliable method to distinguish flax genotypes with PM resistance. However, the precision of field evaluation of genetic resistance is adversely affected by environmental variation and heterogeneous levels of natural inoculum. In addition, field evaluation is expensive and time consuming.

Therefore, another reliable method, either alternative or complementary to field evaluation, is required for the identification of flax genotypes with PM resistance.

Genetic diversity among genotypes of plants can be evaluated with seed proteins and isozymes markers (Gepts, 1993). However, a large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. This limits the use of isozymes, which may also lack adequate level of polymorphism (Tatineni *et al.*, 1996). Molecular genetic markers have developed into powerful tools to analyze genetic relationships and genetic diversity. Restriction fragment length polymorphisms (RFLPs) can be used, but they are costly and time-consuming. Random amplified polymorphic DNA (RAPD) is a useful technique to evaluate taxonomic identity and kinship (Hadrys *et al.*, 1992). RAPDs were shown to provide a level of resolution equivalent to RFLPs for determining genetic relationships among *Brassica oleracea* L. genotypes (Dos Santos *et al.*, 1994) and among *B. napus* L. breeding lines (Hallden *et al.*, 1994). The technical simplicity and speed of RAPD methodology is a principal advantage (Gepts, 1993). Recently, Ashry *et al.* (2002) used RAPD analysis to detect PM resistance in flax.

In the present study, RAPD analysis was employed to develop a regression model to predict PM resistance in selected flax genotypes.

MATERIALS AND METHODS

Evaluation of flax genotypes for PM resistance:

Experiments were conducted in 2001/2002 and 2002/2003 growing seasons at Giza Agricultural Research Station. Experiment consisted of a randomized complete block design of five replicates (blocks). Plots of 2x3 (6 m^2) consisted of ten rows spaced 20 cm apart. Seeds of each genotype (Dakota. Wilden, Williston brown, Cortland, Linore, C.I.2008 Giza7 and Giza8) were sown by hand at a rate of 70 g/plot. Planting dates were in the first week of December. Disease severity was rated visually in the last week of April (Nutter *et al.*, 1991).

DNA isolation and RAPD analysis:

DNA was isolated from 500 mg of healthy fresh leaves of each sample using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by using "Gene Quanta" system, Pharmacia Biotech. The purity of the DNA for all samples were between 90-97%. Concentration was adjusted to 6 ng/µl for all samples using TE buffer (pH 8.0).

Polymerase chain reaction (PCR) mixture was prepared with PCR bead tablets (Amessham Pharmacia Biotech.), which contained all the necessary reagents except the DNA template and the 10-mer primers.

The kits of Amessham Pharmacia Biotech were tested including 6 primers; however, only the Primer d (CCCGTCAGCA)-3 gave positive reactions.

Thirty ng of each DNA extracted sample and 5 µl of the 10-mer random primer (15 ng/ml) were added to a PCR bead tablets. The total volume was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II Biometra: Denaturing at 95°C for 5 min and 45 cycles each consisted of the following steps: Denaturing at 95°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min. Final extension was carried out at 72°C for 5 min and held at 4°C.

Five µl of 6X tracking buffer (manufactured by Qiagen) was added to 25 µl of the amplification product.

Amplification product analysis:

The amplified DNA (15 µl) for all samples was electrophoresed using the electrophoretic unit WIDE mini-sub-cell GT (Bio-Rad) on 1% agarose containing 0.5 µg/ml ethidium bromide at 75 constant voltage, and determined with UV transilluminator.

Gel analysis:

Gel was scanned for molecular weight (bp) and amount (%) of bands by the gel documentation system AAB (Advanced American Biotechnology, Fullerton, CA, 92631). The different molecular weights of bands were determined against DNA standard (G317 A. Promega Inc., USA) with molecular weights 1000, 750, 500, 300, 150 and 50 bp.

Statistical analysis

Analysis of variance (ANOVA) of the field data was performed with the MSTAT-C statistical package (A Microcomputer Program for the Design, Management, and Analysis of Agronomic Research Experiments, Michigan State Univ., USA). Least significant difference (LSD) was used to separate the genotypes into susceptible and resistant classes. Field and RAPD analysis data were analyzed by a stepwise multiple regression using a computerized program. In this analysis, PM severity (dependent variable) was predicted by using the amounts (%) of bands as predictors (independent variables).

RESULTS

Evaluation of flax cultivars for PM resistance (Table 1) revealed that the tested cultivars could be divided into two distinct groups. The first group included the resistant cultivars Dakota, Wilden, and Williston Brown, while the second group included the susceptible cultivars Cortland, Linore, C.I. 2008, Giza 7 and Giza 8. The difference in disease severity was highly significant between any cultivar in the first group and any cultivars in the second group. Within the resistant group, the differences were nonsignificant. However, within the susceptible group, Giza 7 was significantly less susceptible than Cortland or C.I. 2008.

Table 1. Reaction of eight flax cultivars to powdery mildew under field conditions at Giza in 2001/2002 and 2002/2003 growing seasons.

Cultivar	Disease severity [a] (%)
Dakota	19.67
Wilden	25.11
Williston Brown	27.58
Cortland	99.48
Linore	95.26
C.I. 2008	100.00
Giza 7	87.26
Giza 8	93.46

LSD = 8.06 (P = 0.05)
LSD = 11.19 (P = 0.01)
[a] Disease severity was the percentage of infected leaves per plant in a random sample of ten plants per plot. Each value was the mean of two growing seasons.

A total of 20 DNA fragments were amplified for the 8 cultivars that were analyzed (Fig. 1 and Table 2). No single cultivar was amplified for all the 20 fragments. The amplified fragments for each cultivar ranged from 3 to 5, and no single fragment was common to all the cultivars (Table 2). Each cultivar, except Dakota, was characterized by unique fragment(s). For example, Giza 8 was characterized by the unique bands no$_s$. 3, 10, and 12. Band No. 18 was unique to Wilden.

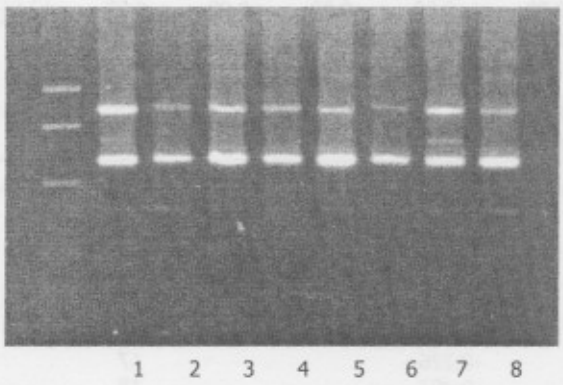

Fig. 1. RAPD banding patterns of flax cultivars obtained by a random decamer primer and electrophoresed on agarose gel. Lanes from left to right were DNA marker (unnumbered lane), Giza 8 (1), Giza 7 (2), Dakota (3), Cortland (4), Wilden (5), Linore (6), C.I. 2008 (7) and Williston Brown (8).

Pearson correlation coefficient was calculated to measure the degree of association between PM severity and the amount (%) of each separated fragment (Table 3). However, no single DNA fragment was satisfactorily correlated with PM severity.

Fragment No. 13 may be a noticeable exception because it was negatively correlated with PM severity ($r = -0.646$, $p < 0.10$).

Table 2. DNA banding patterns for flax cultivars obtained by RAPD analysis and electrophoresed on agarose gel, as indicated by the gel documentation system.

DNA fragment		Flax cultivar							
no.	bp	Giza 8	Giza 7	Dakota	Cortland	Wilden	Linore	C.I. 2008	Williston Brown
1	156	14.49[a]	17.92	0.00	0.00	0.00	0.00	0.00	0.00
2	224	0.00	0.00	0.00	21.13	0.00	0.00	0.00	0.00
3	429	19.78	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	431	0.00	28.45	0.00	0.00	0.00	0.00	38.01	37.43
5	433	0.00	0.00	35.49	14.06	29.31	37.90	0.00	0.00
6	596	27.51	0.00	0.00	0.00	37.66	0.00	0.00	32.25
7	603	0.00	0.00	42.42	0.00	0.00	0.00	27.83	0.00
8	610	0.00	35.68	0.00	31.06	0.00	0.00	0.00	0.00
9	613	0.00	0.00	0.00	0.00	0.00	34.67	0.00	0.00
10	692	9.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00
11	699	0.00	0.00	0.00	0.00	0.00	0.00	9.35	0.00
12	862	28.33	0.00	0.00	0.00	0.00	0.00	0.0	0.00
13	872	0.00	0.00	22.09	0.00	17.30	0.00	24.81	22.65
14	883	0.00	0.00	0.00	32.11	0.00	0.00	0.00	0.00
15	888	0.00	17.95	0.00	0.00	0.00	0.00	0.00	0.00
16	898	0.00	0.00	0.00	0.00	0.00	14.78	0.00	0.00
17	1102	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.67
18	1112	0.00	0.00	0.00	0.00	15.72	0.00	0.00	0.00
19	1173	0.00	0.00	0.00	10.63	0.00	0.00	0.00	0.00
20	1260	0.00	0.00	0.00	0.00	0.00	12.65	0.00	0.00

[a] Amount (%) of the designated DNA fragment.

Table 3. Relationship between powdery mildew severity [a] on eight flax cultivars and DNA content [b] of healthy leaves from these cultivars.

no. [c]	r[d]	no.	r
1	0.357	11	0.344
2	0.339	12	0.273
3	0.273	13	-0.646[e]*
4	0.043	14	0.339
5	-0.350	15	0.205
6	-0.514	16	0.293
7	-0.276	17	-0.447
8	0.407	18	-0.474
9	0.293	19	0.339
10	0.273	20	0.293

[a] The percentage of infected leaves per plant in a random sample of ten plants per plot.
[b] Amount of DNA (%).
[c] no. of DNA fragment.
[d] Pearson correlation coefficient, which measure the degree of association between disease severity and the designated DNA fragment.
[e] Pearson correlation coefficient is significant at $P < 0.10$ (*).

Data for PM ratings and amounts of DNA fragments were entered into a computerized stepwise multiple regression analysis. The analysis constructed a predictive model by adding predictors (in these case amounts DNA fragments) to the model in order of their contribution to R^2. The analysis was effective in eliminating those variables with little or no predictive value by incorporating into the model only those variables that made a satisfactorily significant contribution to R^2 value of the model (Podleckis *et al.*, 1984). Using the predictors supplied by stepwise regression, a two-factor model was constructed to predict PM severity (Table 4). This model showed that PM severity differences were due largely to the DNA fragments No_s. 11 and 13, which accounted for 97.04% of the variation in severity ratings.

Table 4. Stepwise regression model that describes the effect of some DNA fragments (X) on severity [a] (Y) of powdery mildew on flax.

Stepwise regression model	R^{2b}	F. value
$Y = 93.209 - 3.299X_{13} + 9.479X_{11}$	97.04%	81.96**

[a] Disease severity is the percentage of infected leaves per plant in a random sample of ten plants per plot.
[b] Coefficient of determination. Relative contribution of the predictors X_{13} and X_{11} to R^2 are 41.73 and 55.31%, respectively. F. value is significant at $P < 0.01$ (**).

DISCUSSION

The conventional method for evaluating flax cultivars for PM resistance is to evaluate them under field conditions. Experience with flax PM showed that field evaluation has its potential limitations. Under field conditions, susceptibility of cultivars to PM may be obscured by the nonhomogeneous distribution of the natural inoculum. In some years, susceptible cultivars may escape from infection due to the lack of inoculum or the prevalence of unfavorable environmental conditions. In addition, field tests are expensive and time-consuming. Thus, a new method should be developed to evaluate resistance of flax genotypes to PM. RAPD analysis is a reasonable candidate to achieve this goal for the following reasons: This analysis is independent from the pathogen. It evaluates genetic diversity among flax genotypes independent from environmental variation. Electrophoretic patterns of the amplified DNA can be obtained rapidly and with small amounts of tissues. Therefore, large number of single plant selections can be tested without sacrificing the plant.

The utility of the electrophoretic data depends on the method for statistical analysis. Multiple regression was a logical choice for construction of a predictive model, but the complex nature of banding patterns warranted a method to eliminate bands with no predictive value.

Stepwise regression is the best variable selection procedure because it eliminates, from the model, any variable whose contribution to predictive ability is statistically insignificant (Draper and Smith, 1981 and Podleckis *et al.*, 1984).

In the present study, primer {d(CCCGTCAGCA)-3} succeeded in amplifying a reasonable number of DNA fragments and the stepwise regression model they generated proved effective in predicting PM severity from banding patterns. The model accounted for 97.04% of the explained "model" variation in PM severity.

The most common technique for selection of PM-resistant flax cultivars has been through ratings of visible foliar symptoms. The time and effort involved in these selection tests have limited plant breeders in selecting PM-resistant genotypes. RAPD analysis such as that described here may provide a supplementary assay to field tests to distinguish between PM resistant or susceptible genotypes quantitatively.

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التعبير الكمى عن مقاومة أصناف الكتان للبياض الدقيقى بإستعمال تقنية التضاعف العشوائى لمناطق متباينه فى الحمض النووى دى إن إيه

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قيمت ثمانية أصناف من الكتان وذلك من حيث المقاومة لمرض البياض الدقيقى، تحت ظروف الحقل، خلال موسمى ٢٠٠١/٢٠٠٢ و ٢٠٠٢/٢٠٠٣، بمحطة البحوث الزراعية بالجيزة. انقسمت الأصناف إلى مجموعتين محددتين. المجموعة الأولى اشتملت على الأصناف المقاومة داكوتا وويلدن وويلستون براون، فى حين اشتملت المجموعة الثانية على الأصناف القابلة للإصابة كورتلاندولينور والصنف ٢٠٠٨ وجيزة ٧ وجيزة ٨. كان الفرق فى شدة الإصابة عالى المعنوية بين أى صنف يقع فى المجموعة الأولى وأى صنف يقع فى المجموعة الثانية. استعمل بادئ عشوائى لإجراء تفاعل البوليميريز المتسلسل على الحمض النووى دى.إن.إيه، المستخلص من الأصناف الثمانية. نجح البادئ المستخدم فى إحداث تضاعف لإجمالى عشرين قطعة من الحمض النووى للأصناف الثمانية. أمكن باستخدام أسلوب الانحدار المتعدد المرحلى التوصل إلى نموذج رياضى لوصف العلاقة بين شدة المرض (متغير تابع) وكميات الحمض النووى التى حدث لها تضاعف (متغير مستقل). أظهر هذا النموذج أن ٩٧,٠٤% من التباين فى شدة المرض من الممكن أن يعزى إلى تأثير قطعتى الحمض النووى ١١ و ١٣. تدل نتائج الدراسة الحالية على انه من الممكن استخدام تقنية التضاعف العشوائى لمناطق متباينة من الحمض النووى دى.إن.إيه كوسيلة مكملة لاختبارات الحقل، للتفرقة الكمية بين أصناف الكتان المقاومة أو القابلة للإصابة بالبياض الدقيقى.