Discrimination among Cultivars of Cabbage Using Randomly Amplified Polyorphic DNA Markers

Rogério L. Cansian
Laboratory of Biotechnology, Department of Biological Sciences, URI, Av. 7 de Setembro, 1621, Erechim, CEP 99700-000, Rio Grande do Sul, Brazil

Sergio Echeverri-garay1
Institute of Biotechnology, University of Caxias do Sul, C.P. 1352, Caxias do Sul, CEP 95001-970, Rio Grande do Sul, Brazil

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Abstract. Randomly amplified polyorphic DNA (RAPD) markers were used to discriminate among 16 commercial cultivars of cabbage (Brassica oleracea L. Capitata Group). A set of 18 decamer primers was selected from 100 random sequences and used to characterize cultivars and to evaluate distances. The selected primers produced 105 (54%) polymorphic bands ranging in size from 100 and 2500 base pairs, out of a total of 195 bands, which allowed for discrimination of all cultivars. Similarity indices between cultivars were computed from RAPD data, and ranged from 0.72 to 0.87 with an average of 0.82. Unweighted pair-group method with arithmetic average (UPGMA) cluster analysis revealed two groups, one formed by two cultivars recommended for summer cropping, and the other by 14 cultivars. This large group was additionally divided into two subgroups. RAPD analysis provides a quick and reliable alternative for the identification of cabbage cultivars and for determination of the relationships among them.

Cabbage is one of the most important commercial crops of Brassica oleracea. Many open-pollinated cultivars and F1 hybrids are commercialized around the world. In southern Brazil, a typical subtropical region, cultivars recommended for both tropical and temperate regions are commercialized, and new cultivars are continuously being released. Traditional cultivar identification in cabbage, as in other crops, is based on a laborious evaluation of phenological and morphological characteristics. Morphological identification is difficult because visual traits, especially quantitative traits, can vary with environment. Furthermore, many cultivars are morphologically similar because of the convergent improvement toward an ideal phenotype, making their identification more difficult. As an alternative, seed proteins, isozymes, fatty acids, and other biochemical markers have been used for the identification of cultivars and phenetic analysis in several Brassica species (Arús et al., 1982; White and Law, 1991; Wills et al., 1979). The main drawback of such biochemical markers is that their expression can also be influenced by environmental conditions.

In recent years, DNA-based molecular markers have been used for cultivar identification in many plant species (Gepts, 1993). Studies of B. oleracea using DNA-based markers, including restriction fragment length polymorphisms (RFLPs) and RAPDs, have provided information regarding genetic relationships between B. oleracea and other species of the same genus (Demeke et al., 1992; Ren et al., 1995) and the diversity that exists within B. oleracea germplasm pools (dos Santos et al., 1994; Farnham, 1996; Kresovich et al., 1992; Nienhuis et al., 1993). Genebank management is particularly troublesome in Brassica species because of variation in life cycles, growth form, reproductive habit, and polulation systems that demand expensive seed regeneration protocols on a recurring basis. The identification of plant cultivars has become increasingly important with the requirement of Plant Breeders Rights (PBR) to demonstrate distinctiveness, uniformity, and stability (Plant Varieties Journal, 1991) for each new cultivar. In this context, DNA-based markers have been used as cultivar and genotype fingerprinting tools in Brassica (Hu and Quirós, 1991), Kresovich et al., 1992; Mailer et al., 1994). Despite the economic importance of cabbage, limited work has been reported on the identification and evaluation of genetic relationships between cultivars or germplasm bank entries for this crop. Most of the studies using molecular markers in Brassica have included only two to eight cabbage entries (Farnham, 1996; Kresovich et al., 1992; Nienhuis et al., 1993; Song et al., 1988). A molecular characterization of 24 French cabbage cultivars (Margale et al., 1995) showed that RAPD data gave valuable information for the analysis of genetic variability and the management of germplasm banks. However, we know of no study on the molecular characterization of commercial cabbage cultivars currently grown in Brazil. The objectives of this research were to identify the most important commercial cultivars of cabbage used in southern Brazil, and to determine the relationships among them.

Material and Methods

Sixteen commercial cultivars of cabbage were used for analysis (Table 1). Although this is a limited number of individual lines, it is a thorough representation of the commercial cabbage crop in South Brazil. These 16 cultivars represent >95% of all the cabbage seed market, and almost 100% of the cultivated area. ‘Early Jersey Wakefield’, ‘Fuyutoyo’, and ‘Louco de Verão’ account for >70% of the production in South Brazil (data supplied by the Secretary of Agriculture of Rio Grande do Sul State).

Fifty seeds of each entry were sown, one seed per cell in a 1 soil : 1 sand : 1 vermiculite mixture, in 120-cell seedling trays. Seedlings were grown in a greenhouse from April to May 1998, at which time leaves were sampled. Equal amounts (0.5 g) of leaf tissue from each of 10 plants were combined to make composite leaf samples of each entry. The composite samples were initially evaluated by comparing the amplification products of five independent mixtures of five, 10, 20, and 30 plants of the open-pollinated cultivars Early Jersey Wakefield and Early Round Dutch. In all cases, 10 plants were enough to ensure the same amplification patterns among replicates. The composite samples were frozen in liquid nitrogen, ground, and mixed with a mortar and pestle, and immediately used for DNA extraction or stored at −20 °C.

DNA was extracted from 1.5 g of leaf powder using the hexadecyltri-methyl-ammonium bromide (CTAB) protocol described by Doyle and Doyle (1987), and triplicate extractions were made for each entry. The DNA pellets were dissolved in 200 μL TE (10 mm Tris-HCl, 1 mm ethylene-diaminetetraacetic acid, disodiumsalt (EDTA), pH 8.0), and DNA content of all samples was measured at 260 nm using a spectrophotometer. To check for degradation of the DNA, the samples were loaded into 0.8% agarose gels in 1× TBE (tris/borate/EDTA, pH 8.0), submitted to electrophoresis, stained with ethidium bromide, and observed under ultraviolet (UV) illumination. The DNA used for the amplification was of high molecular weight with little degradation and free of RNA as indicated by UV spectra and gel electrophoresis.

Of the 100 primers of kits A, B, W, X, and Y, obtained from Operon DNA Technologies (Alameda, Calif.), 18 decamer oligonucleotides that showed consistent banding patterns and amplification were chosen for use. The polymerase chain reaction DNA amplification protocol was a variation of that reported
by Williams et al. (1990). Reactions were performed in a 25-µL volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 0.25% Triton-X-100, 1.25 mM of each dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology), 30 ng of single decamer primer, 60 to 80 ng of genomic DNA, and 1.5 units of Taq DNA polymerase (Pharmacia LKB Biotechnology). DNA amplification was performed using a thermal cycler (model PTC100; MJ Research, Watertown, Mass.). The thermal cycle used was 94 °C (1 min), then 45 cycles of 94 °C (1 min), 35 °C (1 min), and 72 °C (2 min), and finally 72 °C for 3 min. A negative control including all components except genomic DNA was included in all thermal cycle runs.

Following amplification, the RAPD products (20 µL) were loaded in 1.5% agarose gels in 1× TBE buffer and separated by electrophoresis. After electrophoresis, the amplification products were stained with ethidium bromide and photographed under UV light. The amplification products were stained with ethidium bromide and photographed under UV light. The thermal cycle used was 94 °C (1 min), then 45 cycles of 94 °C (1 min), 35 °C (1 min), and 72 °C (2 min), and finally 72 °C for 3 min. A negative control including all components except genomic DNA was included in all thermal cycle runs.

Results and Discussion

Initially, the 100-decamer sequences were tested against Louco de Verão and Fuyutoyo. These cultivars were chosen because they exhibited the greatest differences in pattern with the two primers arbitrarily tested (OPA-01 and OPA-02) in a first amplification. Eighteen primers were selected for the identification of cultivars and evaluation of their relationships, based on the total number of bands, the number of polymorphisms, and their reproducibility in three independent amplifications.

Using the 18 selected primers, 195 RAPD bands were scored, ranging in size from 100 and 2500 bp (Table 2). Between four and 18 bands were scored per primer, which is within the range previously reported in Brassica (Farnham, 1996; Hu and Quiros, 1991; Kresovich et al., 1992; Thorman et al., 1994). Of the total bands scored in this study, 105, or 54%, were polymorphic. This percentage of polymorphic bands is lower than that reported in the above-cited studies, which was ~70%. This difference can be attributed to the genotypes evaluated, the primers used, and to the selection of scorable bands in the present study (Feireira and Grattapaglia, 1996).

An example of the patterns obtained by the RAPD analysis of cabbage cultivars is shown in Fig. 1. Although there was a high number of shared bands between cultivars, the 16 cultivars included in this study could be differentiated by combining the information supplied by several reactions. The smallest difference, 21 bands, was found between ‘4-Estações’ and ‘Early Round Dutch’, and the largest difference, 44 bands, was between ‘Kenzan’ and ‘Louco de Verão’. At least six primers were necessary to uniquely characterize all the cultivars. Although the number of primers needed to distinguish among cultivars in other botanical varieties of B. oleracea (Hu and Quiros, 1991) and other plant species has been variable (Feireira and Grattapaglia, 1996), the present results are within the expected range (three to eight primers) for the identification of 16 cultivars.

The ability to differentiate all tested cultivars by RAPD bands suggests that this technique could be practical for the identification of cabbage cultivars, as it is for the identification of other Brassica like broccoli, cauliflower (B. oleracea L. Botrytis Group) (Hu and Quiros, 1991), and rapeseed (B. napus L.) (Maier et al., 1994), and collard (B. oleracea L. Acaaphala Group) cultivars (Farnham, 1996), as well as phenotypically similar accessions of cabbage (Phippen et al., 1997). Moreover, the quick and inexpensive RAPD technique provides a level of resolution equivalent to RFLP for the identification of cultivars within Brassica oleracea (dos Santos et al., 1994; Thorman et al., 1994). Despite the differences between the genetic relationships obtained with RAPDs and RFLPs analysis (Thorman et al., 1994), both techniques give useful information for breeding programs.

Table 1. Type of line, source, and origin of cabbage cultivars compared by RAPD analysis.

| Entry              | Type of line | Seed Origin | Commercialized in Brazil by Season | Head characteristics |
|--------------------|--------------|-------------|-----------------------------------|----------------------|
| Midori             | Hybrid       | Japan       | Tokia Seed Co.                    | All year             | Flat                 |
| Early Jersey Wakefield | OP Cultivar  | Brazil      | Isla Seed Co.                     | Summer               | Heart shape          |
| Louco de Verão     | OP Cultivar  | Brazil      | Isla Seed Co.                     | Summer               | Round                |
| Saikō              | Hybrid       | Japan       | Agroflora Seeds                  | Winter               | Round                |
| Scarlet O’Hara     | Hybrid       | Japan       | Tokia Seed Co.                    | Winter               | Red, small, heart shape |
| 4-Estações         | OP Cultivar  | USA         | Feltrin Seed Co.                  | All year             | Flat                 |
| Puyutuyo           | Hybrid       | Japan       | Sakata Seed Co.                   | All year             | Flat                 |
| Kenzan             | Hybrid       | Japan       | Kyowa Seed Co.                    | Winter/spring        | Flat, early hybrid   |
| Mammoth Red Rock   | OP Cultivar  | USA         | Isla Seed Co.                     | All year             | Red, small, round    |
| Astra              | Hybrid       | Brazil      | Asgrov Seed Co.                   | All year             | Flat                 |
| Brunswick          | OP Cultivar  | USA         | Isla Seed Co.                     | All year             | Flat                 |
| Early Round Dutch  | OP Cultivar  | USA         | Isla Seed Co.                     | All year             | Flat                 |
| Black Head         | OP Cultivar  | USA         | Agroflora Seeds                   | All year             | Round, early variety |
| Japanese           | Hybrid       | Japan       | Isla Seed Co.                     | Winter/spring        | Flat                 |
| Early Flat Dutch   | OP Cultivar  | USA         | Isla Seed Co.                     | All year             | Flat                 |
| Shinsei            | Hybrid       | Japan       | Tohoku Seed Co.                   | Winter/spring        | Flat                 |

Table 2. List of decamer oligonucleotides utilized as random primers, their sequence, the total number of fragments amplified, and polymorphic fragments amplified among the cultivars.

| Primer identification | Sequence (5’ to 3’) | No. of amplified fragments | No. of polymorphic fragments |
|-----------------------|---------------------|---------------------------|-----------------------------|
| OPA-01                | CAGGCCCTTCT         | 6                         | 4                           |
| OPA-02                | TGCGAGCTGT          | 11                        | 3                           |
| OBP-11                | GTAAGCCGTT          | 14                        | 10                          |
| OBP-12                | CTTGAGGCA           | 6                         | 1                           |
| OBP-20                | GGACCTTAC           | 4                         | 3                           |
| OPW-03                | GTCCGGAGGTG         | 12                        | 8                           |
| OPW-05                | GGCCTGATAAG         | 16                        | 10                          |
| OPW-06                | AGGCCTGAGT         | 15                        | 9                           |
| OPW-07                | CTGGACGTCGA         | 7                         | 1                           |
| OPW-08                | GACTGCTTCT          | 18                        | 12                          |
| OPW-10                | TCGCATCCTT          | 12                        | 6                           |
| OPW-13                | CACAGCGACA          | 10                        | 6                           |
| OPW-15                | ACACCGGAAC          | 16                        | 6                           |
| OPX-01                | CGCTGAGCTAAG        | 12                        | 4                           |
| OPX-06                | AGCCAGGAGG          | 11                        | 5                           |
| OPX-15                | CAGCACGACCG         | 10                        | 5                           |
| OPX-18                | GACTAGGTGGG         | 5                         | 1                           |
| OPY-08                | AGCGACGACG          | 10                        | 9                           |

Total 195 105
The similarity of the cultivars, estimated by Jaccard’s coefficient, is depicted in the UPGMA dendrogram (Fig. 2). The 16 cultivars of cabbage fell into two clusters at the 0.73 level of similarity. One was formed by two cultivars, ‘Louco de Verão’, originated in a Brazilian breeding program, and ‘Early Jersey Wakefield’, a historically important European cultivar. The other cluster can be separated into two subclusters at the 0.77 level of similarity, one formed by five cultivars, and the other by the remaining nine. Comparing morphological characteristics and groupings depicted by UPGMA, some coincidences can be observed. The two cultivars that form the first group are recommended for summer planting in Brazil, whereas all the cultivars within the large group are recommended for winter and spring planting. ‘Brunswick’ and ‘Early Flat Dutch’ are two old open-pollinated (OP) European cultivars with characteristic flat heads and large leaves clustered together. The three red cabbage cultivars fell within the same group, two of them within the same subgroup. However, no correlations were observed between quantitative characters, such as head volume, head weight, and productivity (Fracaro et al., 1999), and the groups formed by the phenetic analysis of RAPD data.

Similarity indices among cabbage cultivars ranged from 0.72 to 0.87 with a mean of 0.82. Similar values were obtained by Kresovich et al. (1992) in studying the relationships among eight cabbage cultivars. However, 0.87 is a high value when compared with those obtained by Farnham (1996) among collard entries and Mailer et al. (1994) among rapeseed cultivars. Although all the cultivars and hybrids used in this study had different origins, some shared parentage can be expected as the same germplasm may have been used in different breeding programs. Several reports, based on morphological and molecular data, demonstrated that *Brassica oleracea* Capitata Group has a large genetic base, as compared with broccoli, cauliflower, and other botanical varieties of *B. oleracea*.

We have shown that RAPD markers can be used for the identification of commercial cabbage cultivars. The application of this technique may alleviate some of the confusion of cultivar identity associated with morphological characteristics and multiple cultivar registrations. These results also indicate that RAPDs can be used to determine the relationships among cultivars, and provide important information for cabbage breeding programs.

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