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Glutelin protein fraction as a tool for clear identification of Amaranth accessions

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ABSTRACT

In order to simplify the identification of amaranth accessions in gene banks or seed laboratories, a comprehensive method based on band position and relative band intensity data from the glutelin patterns of the chip microfluidic electrophoresis was developed. Chip electrophoresis protein fraction patterns were compared with the patterns obtained by the classical SDS-PAGE method. Fifty-nine Amaranth accessions (*Amaranthus australis, Amaranthus cannabinus, Amaranthus deflexus, Amaranthus retroflexus, Amaranthus tuberculatus, Amaranthus wrightii* and 53 unknown accessions of the grain species *Amaranthus caudatus, Amaranthus cruentus* and *Amaranthus hypochondriacus*) were analysed. Detailed pattern description of each group is provided here in the form of simplified pattern codes in the glutelin polymorphic area, enabling the identification of hybrid accessions and wild species. Inflorescence type and colour, weight of a thousand seeds, and seed colour were tested as additional phenotypic markers. The clustering within the grain amaranths group was related only to the different inflorescence types generally used to discriminate amaranth species. Statistical analysis of pattern similarities resulted in the segregation of the cultivated grain species, the monoecious wild species, and the dioecious wild species into three separate clusters.

1. Introduction

Amaranthus L., a genus of approximately 75 species of world-wide distribution, includes grain amaranths (*Amaranthus caudatus*, *Amaranthus hypochondriacus* and *Amaranthus cruentus*), pigweeds and waterhemps (Sauer, 1967). Due to the variation of the morphological characters and nomenclature disorder caused by repeated misapplication of names, accurate classification of amaranth genetic resources is not always possible (Costea et al., 2001; Transue et al., 1994).

For the preliminary identification of *Amaranthus* species, number, thickness, orientation and density of branches of an inflorescence can be used (Costea et al., 2001). *A. hypochondriacus* usually has stiff inflorescences, while *A. cruentus* has inflorescences with lateral branches and the inflorescences of *A. caudatus* are usually drooping (Standley, 1949). Some of these characteristics, however, can vary with the age of an individual plant and environmental variables (Sauer, 1950). In spite of the fact that there are several methods of identification based on amaranth morphology (Costea and DeMason, 2001; Costea and Tardif, 2003), this kind of identification can be performed only on adult plants – any pre-seeding analysis or identification of hybrids is not possible.

Attempts to explain amaranth taxonomy by isozymes (Chan and Sun, 1997) and DNA analysis (Lee et al., 2008; Xu and Sun, 2001) were partially successful, but there is a need to develop a quick and comprehensive method for amaranth accession identification in gene banks or seed laboratories, focused on differences between wild and grain species and hybrid genotypes.

The seed storage proteins are the direct product of the genetic system and therefore they can serve as markers of a genome and indicate genotypic specificity (Shougui et al., 2006). Analysis of

Abbreviations: Ak, commercially packed seeds from Czech Republic; Au, *A. australis*; BG, branched inflorescence type composed of many glomerules; Cn, *A. cannabinus*; CRI, Crop Research Institute in Prague; D, drooping inflorescence type; De, *A. deflexus*; ED, extremely dense inflorescence type; Ic, seeds from India produced by organic farming; Io, seeds from India produced by conventional farming; LD, lateral dense inflorescence type; LL, lateral lax inflorescence type; M, commercially packed seeds from Hungary; MW, molecular weight; NIRs, near-infra red spectrometry; P, commercially packed seeds from Czech Republic; Re, *A. retroflexus*; SDe, stiff dense inflorescence type; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SL, stiff lax inflorescence type; SD, Standard deviation; Tu, *A. tuberculatus*; U, commercially packed seeds from Ukraine; USDA-ARS, NCRPIS – United States Department of Agriculture-Agriculture Research Service, North Central Regional Plant Introduction Station; Wr, *A. wrightii*; WTS, weight of a thousand seeds.

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sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) patterns is routinely used to solve or verify taxonomic data based usually on morphological observations (Drzewiecki et al., 2003). The studies performed by several authors (Gorinstein et al., 2003; Juan et al., 2007; Marcone, 2002) showed that the SDS-PAGE of seed storage proteins can be used in the investigation of amaranth taxonomy, but methods for exact identification of amaranth genotypes of unknown origin still have not been developed. Other studies were performed by the analysis of patterns of amaranth protein fractions of a small number of grain amaranth varieties (Drzewiecki et al., 2003; Gorinstein et al., 1991), but any differences between different amaranth genotypes were not observed (Gorinstein et al., 2003). Electrophoretic patterns of protein fractions of wild species have not been researched yet. The statistical calculation of the protein similarities in these previous studies was based only on the band position data, even though Zheleznov et al. (1997) already mentioned the importance of the band intensities.

One of the reasons for the lack of more detailed studies of protein patterns and for their minor extension to the routine laboratory work might be the fact that SDS-PAGE performed on classical polyacrylamide gels is a time-consuming method, providing sometimes unclear protein patterns. Fortunately, complex automated chip electrophoretic laboratory systems have been developed recently. They can be used routinely even in small laboratories and provide a quick and simple approach to analyze protein patterns.

The objective of our research was to study differences between the protein fraction patterns (albumins, globulins, prolamins and glutelins) of amaranth genotypes obtained by chip microfluidic electrophoresis. We developed a comprehensive and authentic method for distinguishing amaranth pure line accessions from hybrids and for identification of unknown genotypes, applicable in routine practice in the gene banks or any laboratory of seed producers. Our results were compared with the classical SDS-PAGE protein patterns and with the preliminary morphological characterization of plants from the experimental field. The objective of our statistical analysis was to dermine not only the position but also the intensity of each band of the protein patterns to assess the differences between grain and several wild species.

2. Experimental

2.1. Plant material

We analysed 59 amaranth genotypes from The Crop Research Institute in Prague, Czech Republic and USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) at Iowa State University. In this collection, there were 47 accessions of unknown origin; 6 were wild species and 6 were commercially packed seeds coming from Ukraine ('U'), Hungary ('M'), Czech Republic ('Ak', 'P') and from India ('Io' – organic farming, 'Ic' – conventional farming). Investigated accessions are listed in Table 1.

2.2. Field trials

Forty-seven genotypes were grown during 2007–2009 in Prague, Czech Republic (50°05′ north, 14°20′ east, 340 m above sea level), in a randomised block design with three replicates. Every genotype was grown for 2 years. Each plot consisted of six 15 m rows. Distance between rows was 0.25 m. During the trials, selected traits were evaluated. Samples for protein determination were harvested by hand from the third or fourth row. Protein determinations were performed from the three replications combined. The commercially packed seeds and weedy species were not used in the field experiments (cultivation of the weedy species is prohibited due to weed infestation).

2.3. Morphological characterization

The inflorescence and seed colour of the accessions were described according to the Czech National Descriptor List for *Amaranthus* sp. The weight of a thousand seeds (WTS) was measured. For better identification of the amaranth accessions, we divided the inflorescence types of the collection into seven groups according to their basic morphology: stiff dense (SDe), stiff lax (SL), lateral dense (LD), lateral lax (LL), drooping (D), branched composed of many glomerules (BG) and extremely dense (ED).

2.4. Protein content determination

The protein content was measured by near-infra red spectrometry (NIRs) using an Antaris II FT-NIR Analyzer Series spectrometer (Thermo Electron Corporation, USA). Two calibration curves were used for separate protein content analysis of the black and pale seeds. The calibrations were performed using TQ software 7.2.0.161 processing the data from previous measurements by the Kjeldahl method (Czech State Norm 56 0512-12, 1995) in a Kjeltec automatic analyzer (Kjeltec 2300, Foss Tecator, Sweden) with the protein-nitrogen coefficient set to 6.25. The results of spectrometry analysis were processed by MACROS BASIC 7.3 and OMNIC software 7.3.

2.5. Separation of the protein fractions for electrophoretic analysis

Ten seeds from each genotype were selected randomly, crushed and placed in a 2 ml microtube. The protein fractions were extracted by adding 100 μl of solvent (distilled water for albumins, 0.5 M NaCl for globulins, cold 60% ethanol for prolamins), vortexing and centrifuging by 10,000 × *g* for 15 min (Universal 32R Hettich Centrifugen, Germany). The separation procedure was performed three times for each fraction, but only the supernatants from the first washes of each fraction were saved. The second and the third wash of each fraction was performed in order to completely remove the previous fraction. In the case of prolamins, after the first addition of the solvent, the tubes were vortexed and chilled to 4 °C for 4 h; after that the procedure was performed as in the case of albumins and globulins. Separated amaranth albumins, globulins, prolamins and seed pellets (glutelins) were lyophilized in a freeze dryer (Christ, Germany) for 24 h at −58 °C and 0.018 mBar. The lyophilized solid samples were mixed with 100 μl of extraction solution (0.0625 M Tris–HCl pH 8.8, 5% (w/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromphenol blue) by vortexing several times in 1.5 ml tubes. The tubes were allowed to stand at 4 °C for 3 h. After this extraction time, the tubes were centrifuged at 12,000 × *g* for 15 min and the supernatants were heated in boiling water for 2 min.

2.6. Protein separation by SDS-PAGE

Electrophoresis was performed with the Hoefer SE 600 vertical unit (Hoefer, USA). The polyacrylamide gel preparation (180 × 160 × 0.75 mm, 10% (w/v) resolving gel, 4% (w/v) stacking gel; electrode Tris-glycine-SDS buffer of pH 8.3) and the electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) with some minor changes: the gel was loaded with 30 μl of albumins, globulins, prolamins and 20 μl of glutelin extracts. The run was carried out at 45 mA per gel until the bromphenol blue moved to the bottom of the gel (about 3 h). The gels were stained with a solution of 0.1% (w/v) Coomasie Brilliant Blue (CBB) R250, 50% (w/v) methanol, 10% (w/v) acetic acid, 0.02% (w/v) bromphenol blue salt. Destaining was performed with a solution of 25% (w/v) denatured alcohol and 3.5% (w/v) acetic acid. The gels were soaked in a solution of: 45% (w/v) denatured alcohol and 3% (w/v) glycerol then dried and stored between cellophane sheets.