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Retro Transposon-Based Marking from Potato Monoploids Utilized As a Part of Somatic Hybridization

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Abstract – IRAP (Inter-Retrotransposon Amplified Polymorphism) is a PCR (polymerase chain reaction) based marker technique that uses the proximity of LTR (long terminal repeat) regions of adjacent retrotransposons to generate markers by use of outward facing primers that anneal to LTR target sequences. The LTR regions tend to be highly conserved because they contain sequences, essential for expression. New insertions lead to polymorphism in IRAP banding patterns. Our primers were designed to target the LTR of potato Tst1 retrotransposons to detect head-to-head orientation. The potato population under investigation consisted of regenerants following electrofusion of genetically distinct pairs of monoploids (2n=1x=12). Both somaclones from unfused parental protoplasts and somatic hybrids from actual fusions were generated. The IRAP procedure led to amplification of over 78 bands, of these 2 were unique and clearly distinguished the somatic hybrids from the somaclones among the regenerated plants. IRAP produced more bands when compared with AFLP. However, polymorphic banding patterns, which could be associated with activation of retrotransposons, were low (4%) suggesting that the Tst1 retrotransposon was inactive during the protoplast-to-plant process. Fragment analysis revealed high sequence similarities to distantly related Solanaceae implying that this is an ancient retrotransposon.

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INTRODUCTION

The complexity of tetraploid genetic segregation and the restricted genetic base of Solatium tuberosum have hampered progress in conventional potato breeding. Chase (1963) proposed ploidy reduction of S. tuberosum cultivate followed by breeding at the reduced ploidy level and eventual reconstruction of synthetic tetraploids. Potato monoploids representing the lowest ploidy (2n = = 12) can be generated either by prickle pollination (Uijtewaal et al. 1987J; van Breukelen et al. 1977) or anther culture (Loughet al. 2001). One advantage of monoploids is that their derivation presuppose Of elimination of all lethal or deleterius alleles, by passage of a heterozygous diploid genome through the "monoploid sieve" (Wenzel et al. 1979). The combination of different viable alleles from the fusion of independently extracted monoploids from genetically distinct heterozygous diploid selections should result in improved hybrids through heterosis (Johnson et al. 2001).

The first protoplast isolation was achieved by Klecker in 1892 from the leaves of *Stratioles aloides* by mechanical release of protoplasts into hypertonic solution. More systematic study of protoplasts began in the second half of the 20th century (Davey et al. 2005). A comprehensive review of somatic hybridization of the potato was published by Orczyk et al. in 2003 and methods for overcoming hybridization barriers in this species were described in considerable details by Jansky (2006). This review followed the recent status of somatic hybridisation in the potato and related problems.

BACKGROUND

Dense genetic maps, together with the efficiency and accuracy of their construction, are integral to genetic studies and marker assisted selection for plant breeding. High-throughput multiplex markers that are robust and reproducible can contribute to both efficiency and accuracy. Multiplex markers are often dominant and so have low information content, this coupled with the pressure to find alternatives to radio-labelling, has led us to adapt the SSAP (sequence specific amplified polymorphism) marker method from a ^{33}P labelling procedure to fluorescently tagged markers analysed from an automated ABI 3730 *xl* platform. This method is illustrated for multiplexed SSAP markers based on retrotransposon insertions of pea and is applicable for the rapid and efficient generation of markers from genomes where repetitive element sequence information is available for primer design. We cross-reference SSAP markers previously generated using the ^{33}P manual PAGE

system to fluorescent peaks, and use these high-throughput fluorescent SSAP markers for further genetic studies in *Pisum*.

SOMATIC HYBRID IDENTIFICATION

Morphological characteristics were shown a good preliminary guideline for somatic hybrid identification. Differences in leaves, flowers, growth habit and other features served as identification markers. Somatic hybrids had a morphology ntermediate between fusion parents and morphology near to one parent (Musmeci et al. 2005, Szczerbakowa et al. 2005, Trabelsi et al. 2005, Shi et al. 2006, Borgato et al. 2007, Fock et al. 2007, Przetakiewicz et al. 2007, Thieme et al. 2008, Greplov ▪ et al. 2008). The hybrid plants originating from the one fusion combination were different in some characteristics (Szczerbakowa et al. 2005, Przetakiewicz et al. 2007). Some somatic hybrids outperformed the cultivated parent (Nouri-Ellouz et al. 2006, Bidani et al. 2007). However, the symmetry of hybrids was impossible to predict from morphology type (Trabelsi et al. 2005).

Commonly used methods for hybrid identification became ploidy level determination using flow-cytometry (Oberwalder et al. 1997) or chromosome counting. Chromosome numbers of somatic hybrids were the sum of fused partners (Shi et al. 2006, Borgato et al. 2007) or less than the sum (Kim-Lee et al. 2005, Szczerbakowa et al. 2005). Thus for precise identification of hybrids, it was appropriate to complete with more exact methods. The magnetic cell sorter (MACS) technique was utilized for the selection of heterokaryons by Borgato et al. (2007) and then completed with DNA analysis. Cytogenetic identification of interspecific somatic hybrids was recently used (Tarwacka et al. 2009). To investigate the precise chromosomal composition in somatic hybrids, genomic *in situ* hybridization (GISH) was exerted (Escalanta et al. 1998, Dong et al. 2005, Lovene et al. 2007). This method can unambiguously confirm the origin of chromosomes in hybrid plants and the contribution of each parent (Horsman et al. 2001). GISH (Wolters et al. 1994) and FISH (fluorescence *in situ* hybridization; Srebniak et al. 2002) are able to show intra or inter-genomic translocation and chromosome rearrangements in somatic hybrids.

METHODOLOGY

Plant material : The potato population used consisted of parental monoploids and regenerants, including both somaclones and somatic hybrids, resulting from electrofusion of protoplasts (Lightbourn and Veilleux 2007). Four genetically distinct pairs of anther-derived monoploids (2n = l.v =12) were used, mostly derived from an adapted population (Haynes 1972) of the diploid primitive potato Solatium phureja. but also including several complex diploid hybrids involving S. chacoense, S. tuberosum.
and S. stenotomum (Pa/ and Veilleux 1997). Before S-SAP analysis, somaclones were distinguished from somatic hybrids by amplifying microsatelliles with simple sequence repeat (SSR) primer pairs (Ashkenazi et al. 2001). For a comparison of S-SAP sequence specificity, we used genomic DNA from related Solanaceae (Datura sp.. Petunia sp., tomato (Solatium lycopersicum 'Money Maker*, Nicotiana tabacum 'Sam sun')) and from Arabidopsis t ha liana ecotype Col-0 in Southern blots with S-SAP-derived potato sequences as probes.

DNA was prepared from 10–15 g young leaf tissue (approx. 5–6 leaves) from the following pea accessions obtained from the JIC germplasm collection: JI15, JI73, JI281, JI399, JI813, JI1194, JI1201, JI2822, JI3253 (cv. Cameor), JI3108 (cv. Terese), JI992 (cv. Torsdag), JI2025 cv. Bohatyr. For the zygosity testing of parental accessions cv. Waverex, cv. Avola (four replicates of each), and their reciprocal F_1 hybrids (six from each) DNA was prepared from a single leaf using an adaptation to a rapid mini-preparation method modified to omit the phenol extraction as follows: a single leaf frozen with liquid N_2, within a 1.5 ml micro-fuge tube was ground to a fine powder using a 1 ml plastic pipette tip (rounded and sealed at the tip); 400 µl of extraction buffer (500 mM NaCl, 100 mM Tris, 50 mM EDTA, pH 8.0) was added to the powder and grinding continued, followed by addition of 20 µl of 20% w/v SDS and mixed; extraction of DNA was carried out with the addition of 400 µl chloroform:isoamyl alcohol (24:1) and after thorough mixing was centrifuged for 15 min at 16,000 g; the upper aqueous phase was removed to a fresh tube and the DNA was precipitated with 800 µl of cold absolute ethanol and centrifuged again for 10 min at 16,000 g; the pelleted DNA was washed with 1 ml of 70% ethanol and then air dried, resuspended in 50 µl of TE pH 8.0 (10 mM Tris-HCl, 1 mM EDTA), stored at 4°C. The F_2 population from the cross JI15 × JI399 has been described previously.

Extraction, cloning, and sequencing of S-SAP and IRAP fragments : The same PCR amplification procedures, using modified Cy5] fluorophore primers for analysis on the ALFexpress. were followed to produce target bands with a manual adjustable-height nucleic acid sequencing system (C.B.S.

Fluorescent samples and data collection : All 16 primer combinations were tested on 12 pea lines, using the triplex reaction ^{6FAM}PPT/PPT/Taq+2 and carried out in duplicate. The lines selected included, JI15, JI281, JI399, JI813, JI1194, and JI1201, the parental lines from RI (recombinant inbred) mapping populations that had been previously run with the ^{33}P manual PAGE system and polymorphic markers mapped. ^{HEX}PPT was used to test two primer combinations: Taq+TG, and Taq+AA for amplicon size confirmation. The F_1 zygosity experiments with cv. Avola, cv. Waverex and their F_1 hybrids made use of the triplex reaction with ^{HEX}PPT/PPT/Taq+TG. Amplification of the 92 F_2 individuals and parental

lines from the cross JI15 × JI399 was carried out with two primer combinations: ^{6FAM}PPT/PPT/Taq+CA and ^{HEX}PPT/PPT/Taq+AT.

RESULTS

The most common SSAP method in pea uses a *Taq*I restriction digest, with corresponding adapter ligation, followed by PCR amplification using a specific primer, and a Taq adapter primer. To capture *PDR1* insertions conveniently it is necessary to have two bases of selection at the 3'-end of the Taq adapter primer. In all there are 16 possible primer combinations generating amplicons in the range ca. 100 – 1300 nt. The ^{33}P radio-labelled SSAP method exploits the phosphorylation of the 5'-end of the sequence-specific primer in a kinase reaction. For the automated system a fluorescent tag, 6-FAM or HEX, was attached at the 5'-end of the sequence-specific primer during its synthesis (SIGMA-ALDRICH). Fluorescently labelled primers were HPLC-purified to generate a homogeneous length distribution

To investigate pre-existing polymorphism among monoploids and identify new polymorphisms generated during the process of protoplast fusion, callus growth, and regeneration, we examined 3 marker systems (AFLP, S-SAP, and
IRAP) for their discriminator)' ability using the ALFexpress.
The number of scorable bands per run was high in all 3 systems (90, 146, and 157, respectively), and phenograms depicting relationships among monoploid parents, somatic hybrids, and somaclones were generally as expected, with he greatest GD between parental monoploids and hybrids occurring between parental types (data not shown). However, repeated ALFexpress experiments revealed unacceptable variation in the number of scorable bands (90, 146)
and a general lack of reproducibility, which limits the use of the system. Hence, we isolated and cloned polymorphic bands from IRAP and S-SAP to confirm their identity and determine their utility for genotypic differentiation.

CONCLUSION

We have developed a fluorescent marker assay for SSAPs that retains the useful features of previously used ^{33}P method, but has improved the accuracy of marker calling and has provided useful approximations to amplicon size. In addition it has increased the number of available markers and given the ability to recognise amplicons more sensitively and with codominant marker potential. The high-throughput method described has so far used two fluorescent tags simultaneously but there is potential for at least four, this cuts down the run cost per sample per capillary. The triplex primer method, that incorporates both labelled and unlabelled specific primers, removes the need for pre-amplifications and extensive dilution series before electrophoresis on the ABI genetic analyser. The use of software for marker peak analysis reduces the error in scoring data conferring a major benefit for genetic data analysis. Isolation of fluorescent amplicons for further characterisation and development is not immediately convenient. However here we have shown using a small scale diversity analysis that there is good transferability of the method between manual PAGE and fluorescence where band to peak patterns concur. We have also shown that using peak area values the fluorescent method can distinguish the heterozygous from homozygous classes, providing the potential for a high-throughput codominant marker system.

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