Molecular characterisation and polymorphism of \textit{MinLm1}, a minisatellite from the phytopathogenic ascomycete \textit{Leptosphaeria maculans}

Abstract A sequence-characterised amplified region marker was identified in the phytopathogenic fungus \textit{Leptosphaeria maculans}, which generated a single-banding pattern corresponding to six alleles showing size polymorphism between \textit{L. maculans} field isolates. The size polymorphism was due to 2–7 tandem repeats of the 23-bp motif 5' TCTTACTTACATACACCTCCC 3'. The repeated sequence, termed \textit{MinLm1}, shares many features specific to minisatellites, e.g. a very strong G/C strand asymmetry, the presence of 6-bp direct repeats at both ends of the sequence and its occurrence in a region rich in microsatellites such as (CT)n, (ATG)n, (GTG)n and (CAT)n. \textit{MinLm1} shows a very high degree of conservation of the bases from one repeat to another and from one isolate to another (percent match range: 99.6–100%), whatever their geographical or temporal relatedness. \textit{MinLm1} is a single-locus minisatellite located on chromosomes sized 2.79 Mb and 2.48 Mb, of \textit{L. maculans} isolates a.2 and H5, respectively. In agricultural populations of \textit{L. maculans}, two alleles of \textit{MinLm1} were prevalent, corresponding to 2x and 5x repeats of the core motif. Differences in allele frequencies were observed in some cropping conditions, suggesting that \textit{MinLm1} is an informative marker for epidemiological studies of the pathogen.

Keywords \textit{Leptosphaeria maculans} · Phoma lingam · Minisatellite · Tandem repeats

Introduction

Eukaryotic genomes are known to contain repetitive and highly polymorphic DNA sequences. Of these, tandemly repeated sequences consist of arrays of two to several thousand basic motifs, which are arranged in a head-to-tail fashion and are usually considered as corresponding to non-coding DNA (Weising et al. 1995). Such tandem repeats are usually discriminated according to their length and copy number of the basic element. For example, microsatellites consist of very short (1–10 bp), basic motifs which are repeated a few times only to give 20–100 bp arrays at each locus; and they are very abundantly distributed over many genomic loci (Hancock 1999). Minisatellites, in contrast, consist of longer motifs (10–60 bp). Depending on the organism from which they originate, they show a variable degree of repetition at a given locus, to give repeat blocks of 0.1–30 kb (Armour et al. 1999). Minisatellites were originally reported to occur at numerous loci in genomes and to form “families” with related sequences, as exemplified by the human GC-rich minisatellites described by Jeffreys et al. (1985), which share the common short core sequence, GGGCAGGAXG. However, the recent availability of large-scale sequencing data facilitates a reconsideration of the former common assumptions about the structure and putative role of minisatellites (Vergnau and Denoeud 2000). The analysis of such data demonstrated that: (1) depending on the alleles analysed, a very low number of repeats can nevertheless correspond to a minisatellite, (2) there are no clear-cut families of minisatellites, even though they are often characterised by a high GC content and a strong strand asymmetry, (3) minisatellites are much more common than previously thought, even in prokaryotes (Vergnau and Denoeud, pers. comm.), therefore being likely to play significant structural or functional roles in genomes, (4) minisatellites can be found over the whole length of chromosomes and (5) homologous sequences may be scattered throughout the genomes, but identical
tandem repeats are not commonly found scattered in the genomes (Amarger et al. 1998). However, their genomic location shows a strong bias toward telomeres in some, but not all eukaryotes (Vergnaud and Denoeud 2000). Finally, one emerging feature of mini- and microsatellites is their usual occurrence as intermingled sequences in a particular stretch (Weising et al. 1995). This feature, along with the accumulation of point mutations within repeat units may result in sequences which are termed “cryptically simple”, in which the repeat structure is more or less concealed (Hancock 1999).

In contrast to animal or plant species where very numerous minisatellites have been described, few fungal minisatellite sequences are cloned and available for large-scale analyses of individuals (epidemiology, population genetics, etc.). To date, only five fungal minisatellites are available, described in yeasts (Andersen and Nilsson-Tilgiren 1997; Haber and Louis 1998), in one model filamentous ascomycete, Podospora anserina (named PaMini; Hamann and Osiewacz 1998) and in the phytopathogenic filamentous fungus Botrytis cinerea (termed MSB1; Giraud et al. 1998). No structural relationships are evident between these minisatellites; e.g. MSB1 is an AT-rich minisatellite, whereas PaMini contains numerous poly(GT) stretches (Giraud et al. 1998; Hamann and Osiewacz 1998). Chromosomal location of the fungal minisatellites described to date also appears to be highly variable, as at least two yeast minisatellites are telomeric (Haber and Louis 1998), whereas MSB1 is a single locus minisatellite located in one intron of the ATP synthase gene (Giraud et al. 1998).

Leptosphaeria maculans is a filamentous ascomycete causing stem canker or blackleg, one of the most damaging diseases of oilseed rape (Brassica napus and B. rapa) world-wide. Both the importance of the disease and the complexity of the pathosystem necessitated that population genetics studies were performed, in order to clarify particular points on the biology and etiology of the pathogen. The most important aspects were to clarify the role of sexual reproduction in the life cycle, the level of dissemination of ascospores and the true relationship between the primary leaf infections, which appear in early autumn in continental Europe, and the causal individual strains of the damaging symptoms on stems, crown canker, that develop from May to early July. For such studies there is a need for easy-to-use PCR-based markers that can easily discriminate individual isolates on a large scale.

In the course of building a genetic map of L. maculans, we identified a randomly amplified polymorphic DNA (RAPD)-based marker showing size polymorphism between the parental isolates and a multi-allele profile when used to characterise field isolates. In this paper, we describe the characterisation of what appears to be the first minisatellite described in L. maculans, MinLm1. Sequence and size polymorphism of MinLm1 are investigated both in world-wide and in specific field collections of isolates; and the results support the usefulness of the minisatellite for population genetic studies of L. maculans.

Materials and methods

L. maculans isolates

The a2 (Mat") and H5 (Mat") isolates of L. maculans were obtained from single ascospores isolated from diseased B. napus stubble (Gall et al. 1994; Ansan-Melayah et al. 1995). In vitro crosses between these two isolates were performed as described by Gall et al. (1994) and allowed us to recover progeny consisting of 10 complete tetrads and 88 random ascospores (Balesdent et al. 2001). The field isolates analysed here originated from four collections maintained at INRA, Versailles, and represent scales of sampling ranging from a single plant to a world-wide collection (Table 1): (1) the collection “Versailles 98-99” consisted of 64 isolates obtained from three plants, from one single experimental field at Versailles during the 1998–1999 growing season (Table 1), (2) the Centre Techniques des Oléagineux Métropolitains (CETIOM) collection contained 91 French isolates collected in one single field at Saint Florent (central France) during the 1997–1998 growing season. For these first two collections, isolates were further classified as a function of the time of sampling [Autumn and Winter (leaf-level sample), or June (stem canker sample)] and the B. napus cv. they were isolated from [Capitol (resistant cv.) and Lipton (susceptible cv.) for the CETIOM sample; Capitol for the Versailles sample]. (3) 170 isolates of the IMAScore collection, corresponding to European Union (EU)-wide samples (Volke 1999) and (4) selected isolates from the IBCN collection, comprising isolates from all continents (Rouxel and Seguin-Swartz 1995; Voigt et al. 1998; Pouparta et al. 2000). All of these isolates were classified as belonging to the Tox" species of the L. maculans species complex, using biological traits (toxin production, mating ability) and molecular tools [internal transcribed spacer-RFLP and repetitive element-based PCR fingerprints (Rep-PCR)] (Balesdent et al. 1998; Jedryczka et al. 1998; Volke 1999). In the case of the Versailles collection, inter-simple sequence repeats (ISSR) fingerprinting of isolates (Balesdent et al. 1999) was performed to ensure that only different individuals were analysed. All fungal cultures were maintained on V8-juice agar medium. Long-term storage was ensured by storing agar plugs, collected from actively growing cultures, in sterile mineral oil at 4°C.

In addition, 65 DNA samples extracted from leaf lesions as described below were analysed and are hereafter termed “Versailles 2000” sample.

DNA extraction

Genomic DNA was extracted from freeze-dried mycelium (Balesdent et al. 1998) using the DNeasy plant mini kit (Qiagen, Courtaboeuf, France), according to the recommendations of the manufacturer. For Southern blotting of restricted genomic DNA, DNA was extracted from freeze-dried mycelium, in accordance with the protocol of Balesdent et al. (1998).

In addition, fungal DNA was directly extracted from infected leaves in the field as follows: infected leaves of 65 plants of B. napus cvs Goeland and Westar (susceptible cultivars) were collected in October 2000 in an experimental field at Versailles. Leaf discs, 1 cm in diameter and centred on leaf lesions, were cut out with a cork borer and individually placed in 1.2 ml-rack polypyrrole tubes, along with one 3-mm tungsten carbide bead (Qiagen). The samples (1 lesion/plant) were frozen in liquid nitrogen and ground twice for a total of 3 min at 30 pulses/s, using the Retsch MM300 mixer mill (Retsch, Haan, Germany). DNA was extracted using the DNeasy 96 plant kit (Qiagen) and recovered in a final volume of 100 μl. PCR were performed with the undiluted DNA eluate as a template. DNA extracted from uninfected, greenhouse-grown plants of cvs Goeland and Westar was used as a PCR control.