Efficient gene replacement and direct hyphal transformation in *Sclerotinia sclerotiorum*

M. LEVY*, A. ERENTAL AND O. YARDEN

Department of Plant Pathology and Microbiology and The Otto Warburg Center for Agricultural Biotechnology, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel

SUMMARY

Homologous recombination is required for gene-targeted procedures such as gene disruption and gene replacement. Ku80 is part of the non-homologous end-joining DNA repair mechanism in many organisms. We identified and disrupted the *Ku80* homologue in *Sclerotinia sclerotiorum* and generated heterokaryon mutants enriched with *Ku80*-deficient nuclei (*ssku80*). Sclerotial formation and pathogenicity of *ssku80* mutants were normal on tomato fruits. The frequencies of homologous recombination in these strains were much higher than those of the wild type when transformed with a *cna1* (encoding calcineurin) replacement construct. We coupled the increase in homologous recombination with a direct BIM-LAB-mediated transformation procedure, which utilizes compressed air to assist the transforming DNA in penetrating fungal hyphae of *S. sclerotiorum*. We found this method to be efficient and reproducible, and it did not alter the fitness of the mutants. We also demonstrated the first case of direct transformation of sclerotia. Nourseothricin was introduced as a selectable marker in *S. sclerotiorum*. The tools and procedures described will improve our ability to study gene function in *S. sclerotiorum* and are most likely to be adaptable for use in other plant pathogens.

INTRODUCTION

Sclerotinia sclerotiorum is a highly successful phytopathogen that causes white mould diseases in a wide spectrum of crop plants (Boland and Hall, 1994; Purdy, 1979; Tu, 1997). During its life cycle, *S. sclerotiorum* forms sclerotia, which are asexual, pigmented, rigid resting structures (Adams and Ayers, 1979; Chet and Henis, 1975; Erental *et al.*, 2008; Le Tourneau, 1979; Willetts and Bullock, 1992). Efforts to understand the molecular basis of *S. sclerotiorum* pathogenicity can be enhanced by developing efficient and reproducible techniques for molecular manipulation of this pathogen. Recently, the sequence of the entire *S. sclerotiorum*

genome was released (<http://www.broad.mit.edu/annotation/fgi/>). The availability of this information highlights the necessity for efficient, easy and reproducible manipulation methods to facilitate functional gene analyses. Although DNA transformation protocols have been demonstrated for *S. sclerotiorum* (Rollins, 2003; Weld *et al.*, 2006), the frequency of integration of DNA via homologous recombination into the fungal genome varies.

Double-strand DNA repair in eukaryotes occurs via one of two pathways: non-homologous end joining (NHEJ) or homologous recombination. Ku70–Ku80 is a DNA binding heterodimer that forms a complex with the nucleotide kinase (XRCC4) and the DNA ligase (LIG4) to activate the NHEJ pathway for the repair of DNA double-strand breaks (DeFazio *et al.*, 2002; Jones *et al.*, 2001). The Ku-deficient mutants of *Saccharomyces cerevisiae* exhibit temperature-dependent cell-cycle arrest (Boulton and Jackson, 1996a,b). In *Arabidopsis thaliana*, Ku-deficient mutants exhibit telomere elongation (Bundock *et al.*, 2002), and in mice, Ku-deficient mutants are growth-inhibited (Nussenzweig *et al.*, 1996). Recent studies have shown that Ku-deficient mutants in filamentous fungi are deficient in NHEJ DNA repair (for a review see Krappmann, 2007). Disruption of the *Ku* gene resulted in an extremely high efficiency (50–99%) of homologous recombination in the ascomycetes *Neurospora crassa* (Ninomiya *et al.*, 2004), *Kluyveromyces lactis* (Kooistra *et al.*, 2004), *Aspergillus* species (Takahashi *et al.*, 2006a,b), *Sordaria macrospora* (Poggeler and Kuck, 2006), *Magnaporthe grisea* (Villalba *et al.*, 2007) and *Claviceps purpurea* (Haarmann *et al.*, 2008).

An efficient transformation procedure is essential for gene replacements and other gene manipulations. The standard transformation protocol in *S. sclerotiorum* is usually based on variations of protoplast preparation protocols (Harel *et al.*, 2005, 2006; Rollins, 2003). These involve using cell-wall lytic enzymes which have to be calibrated for each enzyme batch and therefore cannot be easily reproduced. One procedure recently developed for *S. sclerotiorum* that circumvents some of the problems associated with protoplast-based transformation is *Agrobacterium*-mediated transformation. However, this method relies on the efficient production of *S. sclerotiorum* ascospores, which are not always available (Weld *et al.*, 2006).

* Correspondence: E-mail: levym@agri.huji.ac.il

Fig. 1 *ssKu80* replacement construct design and verification. (a) Amplification of the two regions of *ssKu80* using pML2 as a template with primers ssKu80F (1) and Hyg-ssKu80R (2) as well as Hyg-ssKu80F (3) and ssKu80R (4). The Hyg^r cassette was amplified using pMP6 as a template and primers ssKu80-HygF (5) and ssKu80-HygR (6). All the above products were used in a fusion PCR using primers ssKu80R (1) and ssKu80F (4) to obtain the *Ku80* replacement construct and generate pML4. (b) Homologous recombination events occurring between the 5' UTR and 3' Ku regions of pML4 and the corresponding regions in the genomic DNA. Transformants were screened with the Hyg^r cassette primer pair (7 and 8). (c) Homologous recombination verification of *ssku80* mutants using primer Hyg-out (9) and primer Ku-out (10), which produces a unique 1.5-kb amplicon in the *ssku80* mutants (lanes 1, 2 and 4) while wild-type (lane 8) and ectopic transformants (lanes 3, 5, 6 and 7) produce a 1-kb amplicon (*Ku80*). Lower panel represents the control reaction using the primers of the *cna1* gene

Table 1 Nucleotide sequences of primers used in this study.

Primer	Sequence (5'–3')
Cna1F	ATGCAAGTCGATAATGCTTT
Cna1R	GTAGCTTCCAAAGTGTAGCTGAG
TubF	TGAGGGTGCTGAGCTTGTCG
TubR	GAAGACGGGGGAATGGAACC
ssKu80F	TCGGATTATCATTACACAAACGCAATC
Hyg-ssKu80R	CAGTTGAATTCGTAATCCCTGGATCCACAAG
Hyg-ssKu80F	GGGCGTACACAAATCTGGATTTCGTACTG
ssKu80R	TTGACGCTTTCAAATCTTTGCTTGAAGG
ssKu80-HygF	CTTGTTGGATCCAGGGATTACGAATCAACTG
ssKu80-HygR	CAGTACGAAATCCAGATTGTGTACGCC
HygF	GAATTCAGCGAGAGCCTGAC
HygR	GATGTTGGCGACCTCGTATT
Hyg-out	GGTCGATGCGACGCAATCGT
Ku-out	AGATGTATCGGTTAAGAGGC
NatF	ACTCTTGACGACACGGCTTAC
NatR	GTTGACGTTGGTGACCTCCAG
Nat-out	GCGGGCGTCCGCGAAGTGCA
Cna1-out	AGCTTCCAAAGTGTAGCTGAGAT

RESULTS AND EXPERIMENTAL PROCEDURES

We identified the *Ku70* and *Ku80* homologues in the *S. sclerotiorum* genome database (<http://www.broad.mit.edu/annotation/fgi/>) based on homology to the *N. crassa* Ku orthologues *Mus51* (*Ku70*) and *Mus52* (*Ku80*) (Ninomiya *et al.*, 2004). The *ssKu70* gene (SS1G_02717.1) is 2047 nucleotides long, harbours three exons, and its predicted protein shares 39% identity with the *N. crassa* protein. The *ssKu80* gene (SS1G_07128.1) is 2380 nucleotides long, harbours six exons and its predicted protein shares 44% identity with the *N. crassa* protein. To enhance the homologous recombination frequencies in *S. sclerotiorum*, we constructed an *ssKu80* gene replacement construct containing a dominant selectable marker. Based on sequence data, we designed the primers ssKu80F and ssKu80R (Table 1) to amplify *ssKu80* fragments from *S. sclerotiorum* (isolate 1980) genomic DNA. This fragment was subsequently cloned into pDrive (Qiagen, Hilden, Germany) to produce pML2 (Fig. 1a). The flanking DNA regions [approximately 1.1 kb from the 5' untranslated region (UTR) and 1.4 kb from the 3' coding sequence] of the *ssKu80* gene were amplified by PCR using pML2 as a template, with primers designed on the basis of regions adjacent to the planned fusion points with a hygromycin resistance cassette (Hyg^r) and thus also containing sequences from the Hyg^r cassette

that produce a 0.5-kb amplicon. Dashed lines represent genomic DNA, and X represents homologous recombination events. For primer sequences, see Table 1. (d) Southern blot analysis of wild-type (WT) and *ssku80* mutant DNA digested with either *EcoRV* (RV) or *BamHI* (B) and probed with a DIG-labelled DNA probe corresponding to the Hyg^r cassette.

(Fig. 1a). These primers (along with the others described below) facilitated a fusion PCR procedure (Kuwayama *et al.*, 2002). The 5'- and 3'-flanking DNA primer pairs used were ssKu80F and Hyg-ssKu80R along with Hyg-ssKu80F and ssKu80R (Table 1), respectively. The hygromycin phosphotransferase gene, a selectable marker driven by the *N. crassa cpc-1* promoter, was amplified by PCR using plasmid pMP6 (Orbach, 1994) as a template. The primers used were: ssKu80-HygF and ssKu80-HygR (Table 1; Fig. 1a), which also contained sequences from the *Ku* fusion regions. The three PCR products were mixed together in equal volumes and used as a template for the fusion PCR procedure (Kuwayama *et al.*, 2002) using primers ssKu80F and ssKu80R (Table 1; Fig. 1a). PCR conditions were as follows: initial denaturation at 94 °C for 4 min, then five cycles of 94 °C for 15 s, annealing at 50 °C for 30 s (1 °C increase in every cycle) and extension at 70 °C for 5 min, and then 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 5 min, followed by a final 10-min extension at 70 °C. The fusion PCR product was then cloned into pDrive (Qiagen) to create pML4 (Fig. 1A), which was then sequenced to verify the integrity of the construct. *S. sclerotiorum* was transformed with pML4 using the standard protoplast polyethylene-glycol-mediated transformation method (Rollins, 2003). Stable transformants were first screened for resistance in the presence of 50 mg/L hygromycin B (Hyg; Calbiochem, Riverside, CA). Purification of transformants was performed by transferring (ten rounds) the edges of the growing colonies repeatedly to fresh medium emended with 50 mg/L of the drug, resulting in enrichment of the relevant isolate with nuclei containing the selectable marker. To screen for transformants that are Hyg^r and have a *Ku80*-orthologue disruption by homologous recombination, we used specific primers to amplify a 1.5-kb region originating at the Hyg^r cassette, present in the replacement construct, and also spanning the genomic region adjacent to *ssKu80* (Fig. 1b; Table 1). These primers can amplify the 1.5-kb amplicon only if the resistance cassette has integrated into the native *ssKu80* locus. As a positive control, we used primers of the *S. sclerotiorum* serine/threonine protein phosphatase 2B catalytic subunit (calcineurin)-encoding gene *cna1* (Table 1) (Harel *et al.*, 2006). For rapid mutant screening by PCR, a simple high-throughput DNA extraction protocol from small samples of fungal hyphae was used. Mycelial plugs of *S. sclerotiorum* (wild type or mutants) were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) plates for 3–5 days and then three plugs (5 mm diameter) were harvested, frozen in liquid nitrogen and lyophilized overnight. Plugs were then ground, homogenized in the presence of 300 µL 0.5 M NaOH and centrifuged in a microcentrifuge for 10 min. An aliquot (5 µL) of the supernatant was transferred to a fresh tube and neutralized by adding 45 µL of 100 mM Tris, pH 8.0. A 1-µL aliquot of the DNA solution was then immediately used for PCR-based analysis. As shown in Fig. 1(c), mutants in which homologous recombination had

occurred produced the expected 1.5-kb amplicon in a PCR procedure using specific primers, while a mutant with no homologous recombination events yielded only the control amplicon, similar to the wild type (Fig. 1c). Eventually we isolated three heterokaryon strains enriched with nuclei disrupted in *ssku80* (designated *ssku80* mutants) which were used for further analyses. For Southern blot analysis, total genomic DNA was extracted from *ssku80* mutant strain 20 and the wild type, digested with either *Bam*HI or *Eco*RV and transferred to a Hybond-XL (Amersham Biosciences, UK) nylon membrane. Hybridization was performed at 42 °C under low-stringency conditions in the presence of ULTRAhyb solution (Ambion, Austin, TX). The blot was probed with a DIG-labelled (Roche, Indianapolis, IN) DNA fragment corresponding to the Hyg^r cassette. Results showed that only a single copy (represented by the 2.5-kb and 3.4-kb bands of the *Bam*HI or *Eco*RV-digested DNA, respectively) of the transforming construct had integrated into the *ssku80* mutant genome (Fig. 1d). As expected, no hybridization signal was evident in the wild-type DNA.

The *ssku80* mutants grew in a manner similar (albeit not identical) to the wild type. Hyphal elongation rates of the *ssku80* mutants in the presence of the selection drug were approximately 10% slower than for the wild type and sclerotia were more readily formed on Joham's medium (Joham, 1943) (data not shown) than on PDA (Fig. 2a). Thus, when studying sclerotium development, use of Joham's defined medium appears to be preferable. To determine whether *ssKu80* is required for fungal pathogenesis, we subjected *ssku80* mutants to a virulence assay. Tomato (*Solanum lycopersicum*) fruits were inoculated with either *ssku80* or wild-type *S. sclerotiorum* mycelial plugs, placed in humid chambers and incubated at 18 °C for 7 days. All *ssku80* mutants tested produced soft white rot and formed mature sclerotia on tomato fruits (Fig. 2b). We also quantified the virulence of *ssku80* relative to the wild type on *Arabidopsis thaliana*. Detached leaves of *A. thaliana* Col-0 ecotype were placed on 1.5% agar plates and inoculated with 5-mm-diameter mycelial plugs of either wild type or *ssku80*. Digital pictures were taken 48 h after inoculation and lesion sizes were analysed using ImageJ software (Abramoff *et al.*, 2004). We found no significant difference in the virulence of the two strains (Fig. 2c). As we expected that the *ssku80* mutants would be deficient in NHEJ, and thus more efficient in homologous recombination, we analysed the *ssku80* mutant for its homologous recombination frequency relative to the wild type. We produced a replacement construct for *cna1* (Harel *et al.*, 2006) with the nourseothricin N-acetyltransferase (*Nat1*) gene driven by the *Aspergillus nidulans gpdA* promoter (Seiler *et al.*, 2006) (Fig. 3). A 1.93-kb DNA fragment of *cna1* was first amplified from *S. sclerotiorum* genomic DNA by PCR with the primers *cna1F* and *cna1R* (Table 1) and then cloned into the pDrive vector (Qiagen) to generate pML5. The nourseothricin resistance cassette (Nou^r) was digested with the unique *Eco*RV and *Pst*I sites and

Fig. 2 Development and pathogenicity of the *ssku80* mutant. (a) *S. sclerotiorum* grown on PDA medium. *ssku80* mutants (left) and wild-type (right). Sclerotia are indicated by arrows. (b) Tomato fruits 10 days after inoculation with wild-type *S. sclerotiorum* strains (left) and *ssku80* mutants (right). Sclerotia are indicated by arrows. (c) Lesion area (mm²) of *ssku80* mutants as compared with the wild type (WT) 48 h post-inoculation of *A. thaliana* leaves. $n = 15$; t -test = 0.67; error bars represent standard deviations.

inserted into pML5 digested with the same restriction enzymes to generate pML6 (Fig. 3a). The pML6 replacement construct was used to transform *S. sclerotiorum* following the BIM-LAB-mediated DNA-transformation protocol (see below). Mutants were initially screened on PDA medium supplemented with 60 mg/L nourseothricin (Werner BioAgents, Jena, Germany) for nourseothricin resistance and then for several rounds on 40 mg/L nourseothricin. Homologous recombination events were confirmed by amplification using the specific primers Nat1-out from the *Nou^r* cassette and *cna1*-out from the genomic regions adjacent to the *cna1* gene (Table 1; Fig. 3c). The homologous recombination frequency in *ssku80* was 100% (20 of 20 transformants were positive for homologous recombination events) (Fig. 3c) whereas in the wild-type background, it was approximately 6% (two of 35). This frequency was similar to that observed in the initial *ssku80* transformation experiments (9%). We assume that the high frequency

Fig. 3 *cna1* replacement construct design and verification. (a) The *cna1* replacement construct designed with the nourseothricin selection cassette (*Nou^r*) using restriction sites to form pML6. (b) The homologous recombination event between pML6 and the *S. sclerotiorum* genomic DNA were verified using primers NatF (11) and NatR (12) from the *Nou^r* cassette. Dashed lines represent genomic DNA, and X represents homologous recombination events. (c) PCR analysis of *cna1* mutants using primer Nat-out (13) from the *Nou^r* cassette and primer *cna1*-out (14) from the genomic region adjacent to *cna1*, which produce a unique, approximately 0.5-kb amplicon (*Nat-cna1*). Lower panel represents the control reaction for the DNA extraction and PCR procedure using the primers *TubF* and *TubR* of the *S. sclerotiorum* tubulin gene that produce an approximately 0.5-kb amplicon (*tub1*). Transformant amplicons are shown in lanes 1–7; the last lane is the wild type (WT). For primer sequences, see Table 1.

of homologous integration events may be due to the presence of a high number of ku-deficient nuclei in the heterokaryon strain, which may mask the occurrence of rare ectopic integrations of transforming DNA. It is also tempting to speculate that for reasons that have yet to be determined, ku-deficient nuclei may be more receptive to transforming (and homologously integrating) DNA.

The sclerotia of the *cna1* mutant in an *ssku80* background (*ssku80-cna1*) produced here showed a phenotype similar to that of a previously described *cna1* antisense strain (Harel *et al.*, 2006) and sclerotia of the *ssku80-cna1* mutants germinated 48 h earlier than wild-type sclerotia, as indicated by secretion of oxalic acid (Harel *et al.*, 2006).

In some instances, strains produced by protoplast-based transformation exhibit impaired sclerotial formation when cultured on PDA plates (A. Erental *et al.*, unpublished data). It is conceivable that this apparent fitness cost (the underlying causes have yet to

Fig. 4 BIM-LAB-mediated transformation. (a) Apparatus description: 1. System pressure regulator. 2. Pressure measure—for compressed gas entrance. 3. Entrance pipe—feeds the machine with compressed air. 4. Pipe for compressed air. 5. Shooting pressure measure—measures the shooting pressure. 6. Shooting pressure measure—measures the liquid tank pressure. 7. Tank pressure regulator—controls the pressure in the liquid tank. 8. Bombardment pressure regulator. 9. Loading switch used for alternating between manual and automatic function. 10. On/off switch. 11. Lower compartment—contains the liquid storage tank. 12. Solution outlet—where discharged solution exits from the bombardment gun. 13. Trigger—evacuates pressurized air from the outlet. 14. Discharge solution inlet—where solution enters the pressurized compartment in the bombardment gun. 15. Controller—computerized unit that coordinates the system's function. (b) PCR analysis of *S. sclerotiorum* strains transformed using the BIM-LAB apparatus. Upper panel: strains transformed with plasmid pMP6 exhibit hygromycin resistance (Hyg^r). Lower panel: lines transformed with plasmid exhibit nourseothricin resistance (Nou^r). Control PCR performed with wild-type (WT) and plasmid (P) DNA. For primer sequences, see Table 1.

be elucidated) may be a consequence of the protoplast-mediated transformation protocols. This led us to develop an alternative transformation procedure that would be simple and reproducible, and would not reduce the fitness of PDA-cultured strains. For this purpose, we utilized BIM-LAB (Bio-Oz, Biotechnologies Ltd, Israel;

Fig. 4a), a plant inoculation apparatus (Dombrovsky *et al.*, 2005; Gal-On, 2007; Gal-On *et al.*, 1995), which uses compressed air to assist the transforming DNA in penetrating the fungal hyphae and sclerotia. To verify the ability of the BIM-LAB apparatus to produce transformation events, fungal hyphal tips growing on PDA plates were sprayed with circular plasmid DNA (0.1 µg/µL) that confers either hygromycin or nourseothricin resistance. The BIM-LAB system pressure was set at 3 bars for hyphal transformation and 2 bars for sclerotial transformation. The cartridge was adjusted to the feed opening of the spraying gun with a 5-cm-long tube which provides a volume of 5 µL per discharge. Transformation using the BIM-LAB apparatus was performed by spraying mycelial plugs of *S. sclerotiorum* cultured on PDA at 20–24 points per 9-cm Petri dish. Mycelial plugs from the sprayed loci were transferred to selection plates with either hygromycin or nourseothricin. The strains were purified as described above. Stable drug-resistant transformants that contained the respective selectable cassette (as confirmed by PCR; Fig. 4b) were thus obtained. As can be observed from the PCR analysis, not all the mutants isolated in that screen contained the selectable marker cassette (Fig. 4b; lane 2 of the Hyg and lanes 1, 2, 4 and 5 of the Nat1). Several culture dishes could be sprayed within an hour and 1–2 transformants were obtained from each plate (setting transformation efficiency at 5–10%), making this procedure highly appropriate for transformations aimed at inserting specific constructs into the fungus. As controls, we sprayed mycelium with a solution containing no plasmid. In addition, we also treated the mycelium with a plasmid solution (topical application) but with no application of pressure. No transformation events were evident in either case. Interestingly, we were also able to obtain stable *S. sclerotiorum* transformants (at a frequency of 5%) by spraying sclerotia with a plasmid solution containing the Hyg^r cassette. Sclerotia were first treated with 1% hypochlorite, washed twice with water, placed on PDA plates and then sprayed three times with plasmid solution. Sclerotia were allowed to germinate and the strains were purified, in the presence of the selection agent, as described above. To the best of our knowledge, this is the first demonstration of sclerotial transformation. We propose adapting such procedures for other sclerotium-producing fungi, especially those for which transformation protocols have not been developed (e.g. *Sclerotium rolfsii*).

DISCUSSION

The availability of efficient and reproducible gene manipulation methods such as transformation protocols and gene replacement procedures for *S. sclerotiorum* is currently very restricted. The ability to inactivate genes via homologous recombination is a powerful advantage in filamentous fungi, including *S. sclerotiorum*. In this study, we demonstrate that *S. sclerotiorum* deficient in Ku80 is more efficient in homologous recombination, as shown

previously for other organisms, including other filamentous fungi (Boulton and Jackson, 1996a,b; Bundock *et al.*, 2002; Kooistra *et al.*, 2004; Krappmann, 2007; Ninomiya *et al.*, 2004; Nussenzweig *et al.*, 1996). Although we noticed a slight reduction in hyphal growth rates and sclerotial formation on PDA, the strains were as pathogenic as the wild type on tomato fruits (Fig. 2b), and exhibited the same level of virulence on detached leaves of *A. thaliana* (Fig. 2c). We thus concluded that the *ssku80* mutants can serve as competent recipients for gene-disruption and gene-replacement procedures. Similar attributes have been reported in *Ku*-deficient mutants of other fungi (Ninomiya *et al.*, 2004; Takahashi *et al.*, 2006a,b).

We generated a reproducible direct transformation procedure that may offer an alternative to the currently used lytic enzyme mixtures, the performance of which is frequently batch-dependent. We calibrated the BIM-LAB-mediated transformation procedure, which uses compressed air to assist the DNA in penetrating the fungal cells, and we demonstrated that in addition to its reproducibility, this technique also reduces the fitness cost that is, at times, observed in our laboratory with protoplast-mediated transformants. The BIM-LAB-mediated method does not involve any carrier particles or a depressurized chamber as in the biolistic particle gun procedure and thus reduces costs and complications. It is highly likely that this procedure can be used to transform additional fungi.

We also developed a high-throughput procedure for DNA isolation from small samples of mycelium and demonstrated the effectiveness of nourseothricin as a new selectable marker in *S. sclerotiorum*.

The complete sequence of *S. sclerotiorum* is now available and target genes can be easily amplified and used for gene-replacement constructs. We believe that the tools described here, which include a modified protocol for rapid genomic DNA extraction, incorporation of an additional dominant selectable marker (nourseothricin) and the construction of gene-replacement-efficient strains, together with the calibration of a reproducible and reliable transformation procedure, will simplify and improve our ability to resolve gene function in this important phytopathogen.

ACKNOWLEDGMENTS

This research was supported by BARD, the United States–Israel Binational Research and Development Fund. O. Yarden is the incumbent of the Buck Family Chair in Plant Pathology.

REFERENCES

- Abramoff, M.D., Magelhaes, P.J. and Ram, S.J. (2004) Image processing with image. *J. Biophotonics Int.* **11**, 36–42.
- Adams, P.B. and Ayers, W.A. (1979) Ecology of *Sclerotinia* species. *Phytopathology*, **69**, 898–899.
- Boland, G.J. and Hall, R. (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* **16**, 93–108.
- Boulton, S.J. and Jackson, S.P. (1996a) Identification of a *Saccharomyces cerevisiae* Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. *Nucleic Acids Res.* **24**, 4639–4648.
- Boulton, S.J. and Jackson, S.P. (1996b) *Saccharomyces cerevisiae* Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. *EMBO J.* **15**, 5093–5103.
- Bundock, P., van Attikum, H. and Hooykaas, P. (2002) Increased telomere length and hypersensitivity to DNA damaging agents in an *Arabidopsis* KU70 mutant. *Nucleic Acids Res.* **30**, 3395–3400.
- Chet, I. and Henis, Y. (1975) Sclerotial morphogenesis in fungi. *Ann. Rev. Phytopathol.* **13**, 169–192.
- DeFazio, L.G., Stansel, R.M., Griffith, J.D. and Chu, G. (2002) Synapsis of DNA ends by DNA-dependent protein kinase. *EMBO J.* **21**, 3192–3200.
- Dombrovsky, A., Huet, H., Chejanovsky, N. and Raccach, B. (2005) Aphid transmission of a potyvirus depends on suitability of the helper component and the N terminus of the coat protein. *Arch. Virol.* **150**, 287–298.
- Erental, A., Dickman, M.B. and Yarden, O. (2008) Sclerotial development in *Sclerotinia sclerotiorum*: awakening molecular analysis of a 'dormant' structure. *Fung. Biol. Rev.* **22**, 6–16.
- Gal-On, A. (2007) Zucchini yellow mosaic virus: insect transmission and pathogenicity; the tails of two proteins. *Mol. Plant Pathol.* **8**, 139–150.
- Gal-On, A., Meiri, E., Huet, H., Hua, W.J., Raccach, B. and Gaba, V. (1995) Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus. *J. Gen. Virol.* **76**, 3223–3227.
- Haarmann, T., Lorenz, N. and Tudzynski, P. (2008) Use of a nonhomologous end joining deficient strain ($\Delta ku70$) of the ergot fungus *Claviceps purpurea* for identification of a nonribosomal peptide synthetase gene involved in ergotamine biosynthesis. *Fungal. Genet. Biol.* **45**, 35–44.
- Harel, A., Gorovits, R. and Yarden, O. (2005) Changes in protein kinase a activity accompany sclerotial development in *Sclerotinia sclerotiorum*. *Phytopathology*, **95**, 397–404.
- Harel, A., Bercovich, S. and Yarden, O. (2006) Calcineurin is required for sclerotial development and pathogenicity of *Sclerotinia sclerotiorum* in an oxalic acid-independent manner. *Mol. Plant–Microbe Interact.* **19**, 682–693.
- Joham, H.E., (1943) A nutritional study of the fungus *Sclerotium rolfsii*. MS thesis. TX: College Station.
- Jones, J.M., Gellert, M. and Yang, W. (2001) A Ku bridge over broken DNA. *Structure*, **9**, 881–884.
- Kooistra, R., Hooykaas, P.J. and Steensma, H.Y. (2004) Efficient gene targeting in *Kluyveromyces lactis*. *Yeast*, **21**, 781–792.
- Krappmann, S. (2007) Gene targeting in filamentous fungi: the benefits of impaired repair. *Fungal Biol. Rev.* **21**, 25–29.
- Kuwayama, H., Obara, S., Morio, T., Katoh, M., Urushihara, H. and Tanaka, Y. (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res.* **30**, E2.
- Le Tourneau, D. (1979) Morphology, cytology and physiology of *Sclerotinia* species in culture. *Phytopathology*, **69**, 887–890.
- Ninomiya, Y., Suzuki, K., Ishii, C. and Inoue, H. (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. *Proc. Natl Acad. Sci. USA*, **101**, 12248–12253.

- Nussenzweig, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K. and Nussenzweig, M.C. (1996) Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature*, **382**, 551–555.
- Orbach, M.J. (1994) A cosmid with a HyR marker for fungal library construction and screening. *Gene*, **150**, 159–162.
- Poggeler, S. and Kuck, U. (2006) Highly efficient generation of signal transduction knockout mutants using a fungal strain deficient in the mammalian ku70 ortholog. *Gene*, **378**, 1–10.
- Purdy, L.H. (1979) *Sclerotinia sclerotiorum*: History, diseases and symptomatology, host range, geographic distribution, and impact. *Phytopathology*, **69**, 875–880.
- Rollins, J.A. (2003) The *Sclerotinia sclerotiorum* *pac1* gene is required for sclerotial development and virulence. *Mol. Plant–Microbe Interact.* **16**, 785–795.
- Seiler, S., Vogt, N., Ziv, C., Gorovits, R. and Yarden, O. (2006) The STE20/germinal center kinase POD6 interacts with the NDR kinase COT1 and is involved in polar tip extension in a motor protein-dependent manner in *Neurospora crassa*. *Mol. Biol. Cell*, **17**, 4070–4082.
- Takahashi, T., Masuda, T. and Koyama, Y. (2006a) Enhanced gene targeting frequency in ku70 and ku80 disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*. *Mol. Genet. Genomics*, **275**, 1–11.
- Takahashi, T., Masuda, T. and Koyama, Y. (2006b) Identification and analysis of Ku70 and Ku80 homologs in the koji molds *Aspergillus sojae* and *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **70**, 135–143.
- Tu, J.C. (1997) An integrated control of white mold (*Sclerotinia sclerotiorum*) of beans with emphasis on recent advances in biological control. *Bot. Bull. Acad. Sin.* **38**, 73–76.
- Villalba, F., Collemare, J., Landraud, P., Lambou, K., Brozek, V., Cirer, B., Morin, D., Bruel, C., Beffa, R. and Lebrun, M.H. (2007) Improved gene targeting in *Magnaporthe grisea* by inactivation of MgKU80 required for non-homologous end joining. *Fungal Genet. Biol.* **45**, 68–75.
- Weld, R.J., Eady, C.C. and Ridgway, H.J. (2006) Agrobacterium-mediated transformation of *Sclerotinia sclerotiorum*. *J. Microbiol. Methods*, **65**, 202–207.
- Willettts, H.J. and Bullock, S. (1992) Developmental biology of sclerotia. *Mycol. Res.* **96**, 801–816.