Role of Site-Specific Allele Replacement into SvHK1 Locus in the Study of S. vesicarium Resistance to Dicarboximide and Phenylpyrrole Fungicides

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ABSTRACT

*Stemphylium vesicarium* is the fungal agent of pear Brown Spot and its resistance to dicarboximide fungicides has been a known concerning phenomenon since the 1990s. Previous molecular studies correlated dicarboximide resistance class with single aminoacid substitutions observed in a two-component histidine kinase (HK1), corresponding to single nucleotide polymorphism (SNPs) in the nucleotidic sequence of *SvHK1* gene. The goal of this ongoing study is to define the role of SNPs in *SvHK1* sequence on dicarboximide resistance by the replacement of the S allele with S+, R1 or R2 alleles. This result will indirectly increase the possibility of quantify, prevent and manage the iprodione and fludioxonil resistance risk in field.

INTRODUCTION

Brown spot of pear (BSP), a fungal disease caused by *Stemphylium vesicarium*, is the most important pear fungal pathogen in Italy and other European countries. Preventive applications of fungicides are needed from petal fall to fruit ripening to control the disease. In the early 1990s, problems of BSP control with dicarboximide fungicides were reported in some area of Northern Italy. Previous studies established iprodione *in vitro* resistance response classes: S (sensitive), S+ (low resistance), R1 (moderate resistance), R2 (high resistance). Cross-resistance to fludioxonil was only detected in R2 phenotype (Alberoni et al. 2008). Molecular analysis on several field isolates of *S. vesicarium* correlated observed phenotypes with single aminoacid substitutions in a two-component histidine kinase, corresponding to single nucleotide polymorphisms (SNPs) in the nucleotidic sequence of *SvHK1* gene. This gene, is predicted to encode a 1,329 amino acid protein. In S+ and R1 phenotype isolates, different single amino acid substitution were observed in the first amino acid repeat domain (F267L and...
L290S, respectively). For the R2 isolates, the exchanges T765R or Q777R were located within the histidine-kinase domain (Alberoni et al. 2010).

The goal of this ongoing study is to define the role of known SNPs in SvHK1 sequence on dicarboximide resistance by the replacement of S allele with S+, R1 or R2 alleles.

MATERIALS AND METHODS

Fungal cultures, media and growth conditions
A monoconidial culture (WT) was obtained from a field S. vesicarium population. Sensitivity of WT to iprodione and fludioxonil was assessed performing growth inhibition assays and molecular analysis as reported in Alberoni et al. (2010).

S. vesicarium strains were cultured at: 23°C and 12 h of photoperiod on V8 juice agar. For C-TAB DNA extraction, flasks containing 50 ml of Czapek dox were inoculated with 10 small plugs of colonized plugs and incubated at 23°C in the dark and 100 rpm on orbital shaker. WT was grown for 7 days while ΔSvHK1 (2, 3, 4, 5, 6, 14, 33, 42, 46, 48, 50) lines were collected after 15 days post inoculum (dpi).

DNA extraction
A modified microwave-based DNA extraction method (Dörnte & Kües 2013) was developed in our lab (unpublished data) and allowed to quickly extract DNA colony mycelium belonging to WT or SvHK1 knock-out putative mutant strains.

Genomic DNA was purified from mycelium collected harvested by filtration from liquid culture, processed following a modified C-TAB method developed in our lab (unpublished data) from the extraction protocol described by Henrion et al. (1994).

Alleles replacement strategy and construction of the linear disruption vector
The KOSvHK1 disruption vector (Figure 1) was designed in order to obtain SvHK1 knock-out mutants. Allele replacement step (Figure 2) will be carried out on ΔSvHK1 background protoplasts by cotransformation of one of the complementation vectors [S+SvHK1, R1SvHK1, R2SvHK1-I (T765R allele), R2SvHK1-II (Q777R allele)] together with the Geneticine resistance cassette.

To generate KOSvHK1 linear construct, specific primers were designed to amplify the upstream (primers 1 and primer 2) and downstream (primers 3 and primer 4) SvHK1 flanking regions, required for targeted homologous recombination (Figure 1). Hygromycin phosphotransferase coding gene (hph) was cut with SmaI (Thermo Scientific, Waltham, USA) from pAN7-1 vector. UP and DOWN fragments were fused with the hph gene by Fusion PCR technique, using as primers the tails of Primer 2 and Primer 3, complementary to the 5’ and 3’ hph regions, respectively. Fusion PCR product was used as template to obtain KOSvHK1
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**Figure 1** Knock-out scheme. Homologous recombination leads to integration of KOSvHK1 in *S. vesicarium* WT genome.

**Figure 2** Complementation scheme. Homologous recombination leads to site specific integration of complementation vector, carrying one of resistance alleles. Geneticine resistance cassette will be cotransformed.

vector sequence by Nested PCR (Figure 1). The obtained amplicon was cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, USA). The plasmidic DNA was extracted from the culture of an *E. coli* positively transformed colony by the “Wizard® Plus Sv Miniprep DNA purification System” (Promega Corporation, Madison, USA). Miniprep
product was analysed by Sanger sequencing to confirm the correctness of insert (KOSvHK1) and then used as template for further Nested PCR reaction. The KOSvHK1 amplicon, purified by “Gel/PCR Extraction & Purification Kit” (Fisher Molecular Biology, Trevose, USA), was used to transform protoplasts of *S. vesicarium* WT strain.

**Fungal transformation**

WT protoplasts formation was obtained by enzymatic lysis of cell walls of hyphae from young mycelium. A PEG-mediated *S. vesicarium* protoplasts transformation protocol was developed in our lab (unpublished data) by getting ideas from those described for transformation of other fungal plant pathogen (Cho et al. 2013; Ruiz-Díez 2002; Van Nguyen et al. 2012). TB3 agar was used as regeneration medium and a selective overlay containing the selective hygromycin B (Sigma-Aldrich, Milano, Italy) concentration allowed to obtain emerging hyg B resistant colonies, after two days of growth at 23°C and 12 h photoperiod. Tranformants were collected and transferred to 30 mm V8 plates supplemented with 200 ug/ml of hyg B.

**Screening of putative knock-out SvHK1 mutants**

DNAs of WT and all 56 hyg B resistant colonies were extracted by the microwave-based method. In order to quickly screen for site-specific insertion of KOSvHK1 in *SvHK1* locus, these templates were used to amplify internal fragments of *SvHK1* (primers: SvHK1_int_for and SvHK1_int_rev) and *hph* (primers: hph_int_for and hph_int_rev primers) (Figure 1). gDNA was purified from WT and monoconidial strains, obtained from interesting mutants lines, by C-TAB-based extraction method. High quality DNAs were used as templates for further PCR analysis. Amplification of the entire *SvHK1* locus was performed using primer 5 and primer 6, respectively located at the 5’ and 3’ of *SvHK1* flanking region sequences contained in KOSvHK1 (Figure 1). The expected length of PCR product is 5579 bp for WT strain and 4461 bp for transformant originated by a complete and site-specific gene replacement.

**Personal on-line BLAST search for SvHK1 homology in contigs database**

*In silico* analysis was conducted on the contigs database of *S. vesicarium* WT de-novo draft genome by the Personal BLAST Server, released by BMR Genomics (Padua, Italy). *SvHK1* gene sequence from Sv563 isolate, a strain sensitive to dicarboximide and phenylpyrrole fungicides (Alberoni et al. 2010), was used as query input in BLAST analysis and is currently available in NCBI GeneBank (EU711371.1).

**RESULTS**

*SvHK1* gene sequence similarity results on *S. vesicarium* WT contigs database

Search of sequence similarity of *SvHK1* ORF in the WT whole genome contigs dataset leads to individuate contig00427 as subject result with highest similarity features.
Fungal transformation-mediated gene disruption and selection of *SvHK1* knock-out lines

Fusion PCR and Nested PCR techniques were used to build the KOSvHK1 linear construct of 3194bp, containing hygromycin B resistance gene in order to replace *SvHK1* by two homologous recombination events.

Protoplasts obtained by transformation protocol were able to regenerate their cell wall and transformants emerging colonies overcome the Hyg B amended selective overlay. Fifty-six putative *SvHK1* knock-out lines were picked up and transferred on V8 plates amended with opportune hyg B concentration.

Eleven interesting lines were individuated looking to preliminar PCR-screening results conducted on all putative knock-out mutants. Amplification of the *hph* internal fragment and the *SvHK1* internal fragment were repeated on gDNAs of WT and the monoconidial strains of the eleven transformants. The expected internal 1580 bp fragment of the *hph* gene was amplified from lines Δ*SvHK1*-2, 3, 4, 5. Δ*SvHK1*-5 showed the lacking of the *SvHK1* 550bp PCR product. Moreover, the entire *SvHK1* locus was amplified from WT and mutants quality templates, and the expected shift of products length (1118 bp), due to different size of *SvHK1* and *hph* gene, clearly occurred in the sample Δ*SvHK1*-5.

Characterization of Δ*SvHK1*-5 phenotype

Δ*SvHK1*-5 was cultured on plates of V8 juice agar amended or not with 175 µg/ml of hyg B. In both of cases, the *SvHK1*-5 null mutant line showed an altered hyphal development, with reduced and asymmetric radial growth and a not uniform presence of conidia in the colony. These features remain unchanged in *SvHK1*-5 monoconidial culture (Figure 3).
DISCUSSION AND PERSPECTIVES

PCR assays results indicate a complete and site specific insertion of KOSvHK1 in the genome of ΔSvHK1-5, leading to the S allele deletion in the SvHk1 locus. The promising mutant monoconidial strain, exhibiting altered morphology and slower mycelial growth if compared to WT, is currently under assay with Southern Blotting analysis to define the unicity of insertion. Data collected by in silico analysis suggest the presence of SvHK1 sequence in contig00427 only. Southern Blotting is still needed to characterize genome organization. The lack of SvHK1 gene expression will be confirmed by Real Time PCR studies and the null mutant will be transformed with linear complementation vectors (Figure 2). Complemented strains will be tested for the expected acquired resistance level to dicarboximide and phenylpyrrole fungicides. Assessment of the role of SNP mutations in SvHK1 sequence in S. vesicarium resistant phenotypes to these compounds will allows us to develop a RealTime PCR assay to quickly determine resistant allele-frequency in monitored populations. The results obtained so far will increase the possibility of quantify, prevent and manage the iprodione and fluudioxonil resistance risk in the field.

REFERENCES


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