Conservation of the *Ustilago maydis* effector See1 in related smuts

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Abbreviations: Pep1, Protein essential for penetration 1; Cmu1, Chorismate mutase 1; Pit2, Protein involved in tumors 2; Tin2, Tumor inducing 2; See1, Seedling efficient effector 1; SGT1, suppressor of the G2 allele of *skp1*; MAPK, Mitogen activated protein kinase; RT-qPCR, Reverse transcription quantitative real-time polymerase chain reaction.

Ustilago maydis is a biotrophic fungus that induces formation of tumors in maize (*Zea mays* L). In a recent study we identified See1 (Seedling efficient effector 1) as an *U. maydis* organ-specific effector required for tumor formation in leaves. See1 is required for *U. maydis* induced reactivation of plant DNA synthesis during leaf tumor progression. The protein is secreted from biotrophic hyphae and localizes to the cytoplasm and nucleus of plant cell. See1 interacts with maize SGT1, a cell cycle and immune regulator, interfering with its MAPK-triggered phosphorylation. Here, we present new data on the conservation of See1 in other closely related smuts and experimental data on the functionality of See1 ortholog in *Ustilago hordei*, the causal agent of barley covered smut disease.

Ustilago maydis is a biotrophic basidiomycetous fungal pathogen that establishes compatibility with its host maize, causing tumors on all aerial parts.^{1,2} To establish biotrophy and reprogram the host cells metabolically, *U. maydis* secretes effector molecules to overcome the basal PAMP-triggered plant defense response.³ Once biotrophy is established, *U. maydis* manipulates the host cell by causing massive changes in primary and secondary metabolism to deviate the plant resources for its own growth benefit.⁴ *In silico* analysis of the *U. maydis* genome predicted around ~550 proteins that are secreted and likely function as effectors targeting diverse plant cell compartments.^{3,5} Many of these potential effector genes are arranged in clusters, from which several are important in virulence.^{1,6} Till date, only few *U. maydis* effectors have been functionally characterized, Pep1 (that inhibits plant peroxidase), Cmu1 (that suppresses salicylic acid synthesis), Pit2 (that inhibits apoplastic cysteine proteases), Tin2 (that targets anthocyanin biosynthesis) and the organ-specific effector See1.⁷⁻¹³

Leaf tumor formation is a particular hallmark in *U. maydis* infection that commonly does not take place in other monocot smut fungi, with the exception of *Ustilago esculenta* that infects wild rice causing stem galls with total destruction of the flowering tissue.¹⁴ To infect all maize aerial organs and interact with developmentally distinct host tissues, *U. maydis* deploys different sets of genes.^{15,16} Parallel transcriptome profiling during seedling

infection revealed that *U. maydis* alters the host gene expression most dramatically in leaves than tassels; suggesting that the tumor induction in leaves might implicate major host developmental reprogramming.¹⁷ *U. maydis* secreted proteins also showed an organ-specific regulation indicating that some effectors act depending upon the colonized organ in host.¹⁷ These organ-specific effectors act as a second line of virulence after the initial immune suppression tackled by the core effectors.^{16,17} *U. maydis* organ-specific effector mutants exhibited a quantitative reduction in virulence presenting fewer and smaller tumors indicating that tumor formation results from the activity of organ-specific effectors which not only manipulate defense responses but also cell cycle control.¹⁶

We recently showed that See1, one of the previously identified organ specific effectors of *U. maydis*, initiates tumor formation and expansion only in vegetative maize tissues.¹³ Over-expression of See1 is sufficient to induce tumor formation in vegetative tissues including the tassel peduncle supporting its role in triggering tumor development.¹³ The expression profile of *see1* shows perfect organ specificity, supporting its requirement only in maize leaves but not in floral tissues.¹³ See1 is translocated from biotrophic hyphae to the maize cell cytoplasm and nucleus where it interacts with maize SGT1 interfering with its MAPK-induced phosphorylation.^{13,18} SGT1 is a highly conserved eukaryotic

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protein involved in several processes including kinetochore assembly, protein ubiquitination and host cell response to pathogen attack.^{19,20} In recent years, SGT1 has been identified as a conserved hub that is target of several bacterial effector proteins acting as a part of the defense signaling cascade against bacterial pathogens.²¹⁻²³ It is possible that See1 acts with maize SGT1 not only to shut down the defense signaling but also to activate the host cell cycle, a pre-requisite for tumor development which involves cell enlargement and increased cell division.^{24,25} Hence, a combination of immune suppression and nutrient re-channeling, particularly facilitating acquisition of sucrose, could trigger an uncontrolled cell proliferation ultimately resulting in plant tumors.¹³

In a previous study noted by Schilling *et al.*¹⁶ we hypothesized that *U. maydis* might have acquired the ability to cause tumors in leaves by gaining novel functions and accelerated divergence from the closely related species. By genome blast analysis we now

determined if See1 is conserved in other related smut species, whose sequences are currently available in the public databases. This revealed See1 orthologues in smut species including *Ustilago hordei*, *Pseudozyma hubeiensis*, *Sporisorium reilianum* and the dicot smut *Melanopsichium pennsylvanicum*. All orthologous proteins harbor an N terminal signal peptide sequence (SignalP 4.1, <http://www.cbs.dtu.dk/services/SignalP>).²⁶ suggesting that these proteins are secreted (Fig. 1, Multiple sequence alignment was performed based on the amino acid sequences of See1 orthologues using Praline, <http://www.ibi.vu.nl/programs/pralinewww/>). However, comparative analysis of the See1 orthologues revealed relatively low amino acid conservation: *U. maydis* See1 showed 34% total amino-acid identity with *U. hordei*, 49.4% with *P. hubeiensis*, 46.7% with *S. reilianum* and 31% with *M. pennsylvanicum* (Table 1). This shows that the See1 protein is overall highly diversified presenting only one conserved block in the central region (amino acids 38 to 91 in the See1 protein) that has above 50% identity within the tested smuts.(Table 1, Fig. 1).

Within the conserved region, 17 (out of 54) amino acids are identical among all smuts analyzed (Fig. 1). Such highly conserved amino acids might have a structural or functional significance for See1 function. Analysis of *U. maydis* See1 protein to identify functional sites by the Eukaryotic Linear Motif Resource (<http://elm.eu.org/>) showed a hypothetical BRCA like domain (amino acid positions 41 to 45) present in the conserved region of the protein. The BRCA domain (named after the C-terminal domain of a breast cancer susceptibility protein) is mainly found in proteins involved in cell cycle checkpoint and functions in response to DNA damage.²⁷

This amino acid region is also predicted as a putative NLS signal as per the NLS database (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi).²⁸ and may be involved in the translocation of the protein into the nucleus as shown previously for the See1 effector.¹³ The region is present with a considerable gap after the predicted signal peptide (amino acids 1 to 21) that follows the characteristic motifs for translocated effectors, generally present after 12–17 amino acids after the signal peptide.²⁹

In *U. hordei*, 3 amino acids within the predicted BRCA motif,

Figure 1. Amino acid sequence alignment of See1 orthologues from different smuts. Alignment sequences of the full length proteins from *U. maydis* (UmSee1), *S. reilianum* (SrSee1), *P. hubeiensis* (PhSee1), *M. pennsylvanicum* (MpSee1) and *U. hordei* (UhSee1) were obtained from the public databases. Sequence alignment was generated by the multiple sequence alignment program Praline using default parameters and represents the color scheme of the alignment for amino acid conservation. The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. The range of conserved sequences is depicted by different colors as shown. A predicted N-terminal signal peptide (amino acids 1–21) indicated by a blue rectangle. All the orthologues show a conserved region (See138–91) which shows maximum homology between the sequences. A putative BRCA-like motif is present in the See1 protein which is shown by the black line box.

Table 1. Amino acid sequence analysis of See1 orthologues from the various smuts and the homology of conservation between the full sequence and the conserved region sequence in the See1 protein as compared to the Umsee1

Species	Full sequence		Conserved region (a.a. 38–91)	
	Sequence identity with <i>U.maydis</i> See1 (%)	Sequence similarity with <i>U.maydis</i> See1 (%)	Sequence identity with <i>U.maydis</i> See1 (%)	Sequence similarity with <i>U.maydis</i> See1 (%)
<i>U. hordei</i>	34.0	43.1	55.6	68.5
<i>P. hubeiensis</i>	49.4	65.3	66.7	87.0
<i>S. reilianum</i>	46.7	61.7	63.0	83.3
<i>M. pennsylvanicum</i>	31.0	45.5	51.9	74.1

namely ‘IKS’ corresponding to positions 40–42 in *U. maydis* See1, are absent in the conserved region (Fig. 1). This observation tempted us to further investigate if the ortholog from *U. hordei* could functionally complement the SG200 Δ see1 mutant phenotype in *U. maydis*. For this purpose, the *U. hordei* See1 along with its native promoter or *U. maydis* See1 promoter was cloned and re-introduced into the SG200 Δ see1 mutant background. Interestingly, neither SG200 Δ see1_{pUh-UhSee1}, nor SG200 Δ see1_{pUm-UhSee1} were able to complement the SG200 Δ see1 phenotype (Fig. 2), indicating that the *U. hordei* See1 is not a functional homolog of *U. maydis* See1. This shows that *U. hordei* See1 does not functionally complement SG200 Δ see1. A possible reason for this might be the absence of (₄₀IKS₄₂) within putative BRCA domain and predicted NLS in *U. maydis* see1. Additionally, the *U. hordei* see1 also lacks the C-terminal region of the protein, which is known to be acting as an effector domain in other phytopathogenic fungi.³⁰

We tested whether the “IKS” motif in See1 is responsible for its function. To this end, a mutant version of See1 (*Umsee1*^{mut}) carrying a mutation in the putative BRCA domain (₄₀IKS₄₂ exchanged to ₄₀AAA₄₂) was generated and tested for complementation of the *U. maydis* see1 knockout strain. The resulting *U. maydis* strain was not able to fully

Figure 2. (A) Disease symptoms caused by SG200 Δ see1Uhsee1 under the native *U. hordei* promoter in comparison with the wild-type progenitor strain SG200 and SG200 Δ see1. The *U. hordei* See1 ortholog did not show a complementation of the knockout phenotype of reduction in leaf virulence. Maize seedling leaves were scored at 12 dpi. SG200: Virulent *U. maydis* progenitor strain, SG200 Δ see1: Umsee1 deletion mutant, SG200 Δ see1 Uhsee1: complemented knockout strain with *see1* from *U. hordei* under native promoter (B) Representation of typical symptoms of infection caused by the strains described in (A) at 12 dpi. (C) Quantification of infection symptoms on maize seedlings at 12 dpi with the Uhsee1 under Umsee1 promoter in comparison to the wild-type and *see1* deletion mutant. (D) Representation of typical symptoms of infection caused by the strains described in (C) at 12 dpi. SG200: Virulent *U. maydis* strain that causes wild-type symptoms. Δ see1: *see1* deletion mutant. SG200 Δ see1-UmP Uhsee1: SG200 Δ see1 complemented with *see1* from *U. hordei* under *U. maydis* *see1* promoter. s.i: single integration and m.i: multiple integration. n= number of plants infected.

Figure 3. Complementation of the SG200Δsee1 Umsee1 with the mutated BRCA domain with the amino acids IKS substituted by AAA. Quantification of infection symptoms on maize seedlings (12 dpi). SG200: Virulent *U. maydis* strain that causes wild-type symptoms. Δsee1: see1 deletion mutant. Δsee1-Umsee1^{mut}: SG200Δsee1 complemented with a construct containing the see1 from *U. maydis* but with the residues 40IKS₄₂ in the BRCA like domain mutated to "AAA." (B) Example of typical symptoms of infection caused by the strains described in (A), 12dpi. n= number of plants infected.

well to SG200Δsee1_pUm-UhSee1, which indicates that along with the putative BRCA, additional factors may contribute to the virulence function of See1 effector. Hence, a detailed analysis of the domain mapping in the See1 protein will be a subject of further investigation.

U. maydis See1 is an organ-specific effector important for tumor formation and expansion in leaves, a characteristic of infection that is not present in other smuts, including *U. hordei*. In this line, one might speculate that deletion of a conserved sequence motif reflects that this effector is simply not required in *U. hordei*. Another hint in this direction is the C-terminal deletion of 25 amino acids in *U. hordei* See1 (Fig. 1). The central question asked from these observations is, how an organ-specific effector like See1 might have evolved in smut species, and if/how its functionality is related to host jumps and/or fungal lifestyle, respectively. This hypothesis emerges from the finding of the homologous motif being well conserved in *M. pennsylvanicum*. Despite the species and particularly its host are evolutionary quite unrelated to *U. maydis* the BRCA domain is seen to be present. It will therefore be interesting to test whether See1 is functional in *M. pennsylvanicum*. This fungus is known to have undergone a host jump from monocots to dicots among the Ustilaginales causing a similar infection pattern like *U. maydis* including the formation of tumors.³¹ Future studies of See1 orthologues from different smut species, addressing both their transcriptional regulation and the protein function, will be performed to gain further insight into the role of See1 like effectors in modulation of host cell cycle and the induction of plant tumors.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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restore tumor formation when *Umsee1*^{mut} was expressed under the native *see1* promoter (Fig. 3). However, symptoms observed were slightly increased compared to the *see1* knockout strain as

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