The role of vegetative cell fusions in the development and asexual reproduction of the wheat fungal pathogen Zymoseptoria tritici

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Abstract

**Background:** The ability of fungal cells to undergo cell-to-cell communication and anastomosis, the process of vegetative hyphal fusion, allows them to maximize their overall fitness. Previous studies in a number of fungal species have identified the requirement of several signaling pathways for anastomosis, including the so far best characterized soft (So) gene, and the MAPK pathway components MAK-1 and MAK-2 of Neurospora crassa. Despite the observations of hyphal fusions’ involvement in pathogenicity and host adhesion, the connection between cell fusion and fungal lifestyles is still unclear. Here, we address the role of anastomosis in fungal development and asexual reproduction in Zymoseptoria tritici, the most important fungal pathogen of wheat in Europe.

**Results:** We show that Z. tritici undergoes self-fusion between distinct cellular structures, and its mechanism is dependent on the initial cell density. Contrary to other fungi, cell fusion in Z. tritici only resulted in cytoplasmic mixing but not in multinucleated cell formation. The deletion of the So orthologous ZtSof1 disrupted cell-to-cell communication affecting both hyphal and germling fusion. We show that Z. tritici mutants for MAPK-encoding ZtSlt2 (orthologous to MAK-1) and ZtFus3 (orthologous to MAK-2) genes also failed to undergo anastomosis, demonstrating the functional conservation of this signaling mechanism across species. Additionally, the ΔZtSof1 mutant was severely impaired in melanization, suggesting that the So gene function is related to melanization. Finally, we demonstrated that anastomosis is dispensable for pathogenicity, but essential for the pycnidium development, and its absence abolishes the asexual reproduction of Z. tritici.

**Conclusions:** We demonstrate the role for ZtSof1, ZtSlt2, and ZtFus3 in cell fusions of Z. tritici. Cell fusions are essential for different aspects of the Z. tritici biology, and the ZtSof1 gene is a potential target to control septoria tritici blotch (STB) disease.

**Keywords:** Cell-to-cell communication, Anastomosis, Vegetative growth, Melanization, Asexual reproduction

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Background
Communication is a ubiquitous primitive characteristic developed by all living species. The ability to communicate effectively may affect mating, predation, competition, dominance hierarchy, signal modalities, and survival [1–3]. This complex mechanism starts when a given organism (the sender) secretes in the environment a self-produced molecular signal (the message) that alters the behavior of another organism (the receiver) [1, 3]. Communication also happens at the cellular level. This so-called cell-to-cell communication creates a complex signaling network that involves different extracellular signals and distinct cell types that regulate several pathways [4–6]. Inter- and intraspecies cell-to-cell communication has been widely studied in fungi to address biological functions including the secretion of pheromones to attract the opposite sexual partner, the production of quorum sensing molecules controlling the expression of virulence factors or morphological changes, and the regulation of cell fusions during vegetative growth [6–9].

The fungal mycelium is formed by three integrated processes, including hyphal extension, branching, and vegetative hyphal fusion (VHF) (also known as anastomosis) [10]. In this last-mentioned process, two growing cells with identical vegetative compatibility loci engage in cell-to-cell communication, which is thought to involve the secretion of unknown diffusible molecules and results in re-direction of polarized hyphal growth toward each other. After physical contact, the cell walls are remodeled, the plasma membranes fuse, and the two interconnected cells exchange cytoplasm and organelles [11]. When the anastomosed individuals are vegetatively incompatible, the two fused cells rapidly collapse following DNA degradation by programmed cell death, or they are severely inhibited in their growth [12]. It is widely accepted that mycelial network formed through VHF facilitates the intra-hyphal communication, translocation of water and nutrients, and signal molecules, which improve the general homeostasis and spatial expansion of the fungal colony [13, 14]. In some fungi, hyphal fusion is required for pathogenicity and host adhesion [15–17]. Cell fusions can also occur between germinating conidia, the asexual spores of many fungi. The process of fusion between germinating conidia involves the formation and interaction of specialized hyphae, so-called conidal anastomosis tubes (CATs). CATs are thinner and shorter than vegetative hyphal fusions (VHFs), and its induction is dependent on nutrient deprivation and initial cell density [18]. Cell fusions may serve to improve colony establishment, as well as to increase the genetic variability by facilitating heterokaryosis and parasexual recombination [18]. Gene or chromosome transfers by cell fusions between individuals of the same or different species allow certain fungi to acquire pathogenicity or to broaden their host specificity [19–21]. Albeit non-self-anastomoses are described [22, 23], this might be a rare event in nature.

In the last decades, different studies about the molecular mechanisms underlying cell fusion identified several mutants defective in anastomosis, revealing that fungal communication and fusion are complex mechanisms that encompass several signaling pathways [6]. The best-characterized mutant is the soft (So) gene of Neurospora crassa [24]. So, it is proposed to be the scaffold protein for some mitogen-activated protein kinase (MAPK) from the cell wall integrity (CWI) signaling pathway implicated in the regulation of different fungal processes [25–27]. For instance, So contributes to septal plugging during hyphal injury or damage caused by environmental stresses [28, 29]. Nevertheless, So gene has an essential role in the hyphal anastomosis, presumably by regulating the secretion or perception of an undefined chemotransductant in an oscillatory manner with the Fus3 (orthologous to MAK-2 in N. crassa) from the MAPK pheromone response pathway, as demonstrated for N. crassa [11]. Beyond N. crassa, the So was also characterized in other model organisms, plant pathogens, and endophytic fungi [15–17, 29, 30]. Though all So mutants fail to undergo hyphal fusions, the distinct effects on pathogenicity reported for those null mutants suggest that the biological contribution of anastomosis might depend on the infection strategies developed by different fungal pathogen species.

Zymoseptoria tritici is an apoplastic pathogen with a hemibiotrophic lifestyle and considered the most damaging pathogen of wheat in Europe [31]. This fungus has the ability to undergo morphological transitions in response to the environment, switching between hyphal growth and yeast-like growth [32–34]. Hyphae formed from either germinated ascospores (sexual spore), pycnidiospores (asexual spores), or blastospores (asexual yeast-like spores produced by budding) are essential for penetrating wheat leaves through the stomata and colonization of the apoplastic space. After a long asymptomatic phase (which varies depending on the wheat genotype and fungal strain combination) [35–37], the onset of the necrotrophic phase is followed by the appearance of lesions, disintegration of host tissue, and formation of asexual fruiting bodies. Though Z. tritici is among the top 10 most studied phytopathogens [38], there is little known about vegetative cell fusion in this organism. To date, it was shown that the deletion of the β-subunit of the heterotrimeric G protein MgGpb1 or ZtWor1, a transcriptional regulator of genes located downstream of the cyclic adenosine monophosphate (cAMP) pathway, results in germ tubes that undergo extensive anastomosis [39, 40].
In this study, we aimed to determine whether vegetative cell fusions play essential biological roles in the lifestyle of *Z. tritici*. We showed that the ubiquitous ability of *Z. tritici* to undergo self-fusion was disrupted by the deletion of *ZtSof1*, affecting both hyphal and germling fusions. The characterization of mutants lacking the MAPK-encoding *ZtSlt2* or *ZtFus3* indicates a conserved role of the CW1 and pheromone response pathways on fungal anastomosis. We found that *ZtSof1* contributes to vegetative growth and is required for melanization, but not to maintain the fungal cellular integrity. We discovered that anastomoses are dispensable for pathogenicity, but they are essential for asexual fruiting body development. In the absence of cell fusions, *Z. tritici* does not undergo asexual reproduction. These findings illustrate the impact of *ZtSof1* for fungal development and the importance of vegetative cell fusions for fungal fitness.

**Results**

**Cell fusions in *Z. tritici* allow the bidirectional transfer of cytoplasmic content but do not enable multinucleated cell formation**

We co-inoculated either blastospores or pycnidiospores of both 1E4GFP and 1E4mCh, fluorescent strains onto water agar (WA—1% agar in water), a hyphal-inducing medium, to investigate the ability of *Z. tritici* to undergo self-fusions. Though *Z. tritici* produces blastospores and pycnidiospores as asexual spores instead of conidium, we used the CAT terminology to define the fusion between germinating spores. CATs formed between blastospores or pycnidiospores germlings happened at high cell density (10^7 blastospores/mL), starting after 4 h of incubation, but they were frequently observed after 17 h of incubation, resulting in cells co-expressing both fluorescent proteins (Fig. 1b). On the other hand, vegetative hyphal fusions (VHF) from germinated blastospores or pycnidiospores were noticed at 40 h after incubation (hai) only at low cell density (10^6 blastospores/mL) (Fig. 1c, d). Multiple interconnections via fusion bridges were observed in all tested morphotypes (Fig. 1b–d). The co-infection of wheat plants using either blastospores or pycnidiospores of 1E4GFP and 1E4mCh strains also resulted in VHFs on the wheat leaf surface (Additional file 1: Fig. S1). Self-fusions and cytoplasmic mixing occurred in the first 48 hai (Additional file 1a-b: Fig. S1a-b). To monitor for nucleus movement enabling multinucleated cell formation, we used the IPO323 ZtHis1-ZtGFP strain [41]. As previously reported, only one nucleus per cell was observed in this strain. No multinucleated septal compartments were observed between the two interconnected hyphae at the fusion bridges or ou-lying of the fusion points (Additional file 2: Fig. S2). These findings suggest that cell fusions culminate in cytoplasmic mixing in *Z. tritici*, but it does not lead to the generation of multinucleated cells in this uninucleated fungus.

**The mutual perception or response to genetically identical fusion partners requires the *ZtSof1* gene and different MAPK pathways**

So has an essential role in self-anastomosis [24]. To determine whether this gene plays the same role in *Z. tritici*, we identified the So orthologous (ZtSof1) in the *Z. tritici* genome (Mycgr3G74194 or Zt09_7_00503), which consists of a 3794-bp open reading frame that encodes a polypeptide of 1227 amino acid and is widely distributed within the Dothideomycetes (Additional file 3: Fig. S3). We phenotyped the ∆ZtSof1, ∆ZtSof1, and ∆ZtSof1-comp mutants generated on the 1E4 strain genetic background for the presence of interconnected individuals through anastomoses. Fusion bridges between blastospore germlings and filamentous hyphae were only observed for those strains possessing the *ZtSof1* gene, but none between ∆ZtSof1 mutant cells (Fig. 2a and b), demonstrating that *ZtSof1* plays an essential role in anastomosis between genetically identical *Z. tritici* strains.

To ensure the failure of cytoplasmic exchange on those individuals lacking the *ZtSof1* gene, we mixed blastospores of each tested strain with 1E4GFP blastospores in a 1:1 ratio and traced the spores up to 40 h. Hyphal fusions and the continuous streaming of cytoplasmic green fluorescence coming from the fusion with the 1E4GFP strain were observed for all tested combinations (Additional file 4: Fig. S4, and Additional file 5: Fig. S5), except for the combination of ∆ZtSof1 and 1E4GFP spores (Additional file 6: Fig. S6). These findings confirmed that *ZtSof1* is required in both fusion partners for a mutual recognition previous to the anastomosis.

To determine whether the CWI and pheromone response pathways also contribute to anastomoses in *Z. tritici*, we incubated the knocked-out ZtSlt2 (orthologous to *MAK-1* in *N. crassa*) and ZtFus3 (orthologous to *MAK-2* in *N. crassa*) mutants on WA plates. No hyphal fusion was observed for both mutants. Unlikely, fusion bridges were regularly found between IPO323 wild-type or ∆ZtSlt2-complemented fungal cells (Additional file 7: Fig. S7). These results indicate the recruitment of both CWI and pheromone response MAPK signaling cascades for the regulation of self-fusion in *Z. tritici*, as it has been observed in *N. crassa* [11].

**Deletion of *ZtSof1* affects growth and melanization**

To assess whether vegetative cell fusions affect fungal development, we determined the radial growth of ∆ZtSlt2, ∆ZtSof1, and ∆ZtSof1-comp on different nutritional environments inducing different morphotypes. We observed a slightly reduced growth of ∆ZtSof1 colonies when grown on WA plates, a condition that
induces hyphal growth, compared to those possessing the ZtSof1 gene (Additional file 8a: Fig. S8a). On average, the colony radii were 5.31 ± 0.12 for ΔZtKu70, 4.87 ± 0.10 for ΔZtSof1, and 5.50 ± 0.09 for ΔZtSof1-comp [radial growth (mm) ± standard error] (Additional file 8c: Fig. S8c). Nevertheless, ΔZtKu70 and ΔZtSof1-comp generated colonies with highly hyphal dense margins, while ΔZtSof1 exhibited sparse filamentations at the colony periphery (Additional file 8b: Fig. S8b). Unlikely, the ΔZtSof1 mutant grew significantly faster than the ΔZtKu70 and ΔZtSof1-comp on the nutrient-rich PDA medium, a condition that induces blastosporulation (yeast-like growth) (Additional file 8e: Fig. S8e). Over time, the relative growth rate of ΔZtKu70 and ΔZtSof1-comp were respectively 32% and 16% lower than the ΔZtSof1 mutant. No morphological differences were detected between blastospores produced by the tested strains (Additional file 8d: Fig. S8d). The data suggest that ZtSof1 mutation may affect fungal growth in a morphotype-dependent manner. Consistent with these findings, we found that the deletion of ΔZtSof1 increased blastosporulation in a nutrient-rich liquid medium. At 48 and 72 hai, the ΔZtSof1 mutant produced a significantly higher amount of blastospores than ΔZtKu70 and ΔZtSof1-comp strains (Additional file 9: Fig. S9).

Interestingly, no melanin accumulation was observed in ΔZtSof1 mutant colonies (Fig. 3), demonstrating the impact of ZtSof1 deletion on Z. tritici pigmentation. We
**Fig. 2** ZtSof1 is required for vegetative cell fusion in *Zymoseptoria tritici*. All mutant lines derived from the 1E4 strain. 

**a** Fusion bridges between blastospore germlings were observed at high cell density for ΔZtKu70 and ΔZtSof1-comp strains but not in the ΔZtSof1 mutant. Black arrows indicate CATs. 

**b** VHFs were noticed at low cell density and only for those strains possessing the ΔZtSof1 gene. White triangles point to the fusion bridges between two fused hyphal cells.

**Fig. 3** Disruption of ZtSof1 impacts the melanization of *Zymoseptoria tritici*. The defective fusion mutant was significantly less melanized than the ΔZtKu70 and ΔZtSof1-comp strains, which exhibited higher melanin accumulation over time. Bars represent standard errors of the mean gray values on at least 40 colonies. Different letters on the top of the bars indicate a significant difference among the tested strains according to the analysis of variance (ANOVA). The notch displays a 95% confidence interval of the median. Open circles represent the outlier values of each strain. Pictures shown below the bar plot represent the melanization level of ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp strains. Gray value scale (0 = black and 255 = white) is shown on the left.
postulated that the ΔZtSof1 mutant could either display cellular integrity defects and/or being susceptible to environmental stresses. We tested nine different abiotic stressors, such as temperature, oxidative, osmotic, cell wall, and cell membrane stresses. Overall, no variability in stress responses was noticed among the strains (Fig. 4). However, ΔZtSof1 formed slightly bigger colonies than those from ΔZtKu70 and ΔZtSof1-comp, corroborating the increased growth rate observed for this mutant on nutrient-rich medium (Additional file 8e: Fig. S8e). Therefore, we found no evidence that the ZtSof1 gene is involved in the maintenance of the cell wall integrity of Z. tritici.

Hyphal fusions are essential for the development of asexual fruiting bodies

We inoculated a susceptible wheat cultivar with the tested Z. tritici strains to assess the biological role of vegetative cell fusion during the pathogen lifecycle in planta. Typical symptoms caused by Z. tritici infections were visible after 11 days post-infection (dpi), and the disease progression was similar among the plants inoculated with ΔZtKu70, ΔZtSof1, or ΔZtSof1-comp strains (Fig. 5a and Additional file 10: Fig. S10). This result indicates that ZtSof1 is neither required for host penetration nor for the asymptomatic or necrotrophic phases of the fungus. The asexual fruiting bodies (pycnidia) were

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**Fig. 4** ZtSof1 is dispensable for cellular integrity. A serial dilution of blastospore suspensions of ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp strains were exposed for 5 days to nine different stress conditions, including different temperatures (18°C and 27°C), oxidative stress (0.5 and 1 mM of hydrogen peroxide (H₂O₂)), osmotic stresses (1 M sodium chloride (NaCl) and 1 M sorbitol), cell wall stresses (2 mg/mL Congo red and 10 μg/mL Calcofluor white (CFW)), and plasma membrane stress (0.01% sodium dodecyl sulfate (SDS)). The tested strains do not vary on their tolerance to different cellular stressors.
visible on plants inoculated with those strains possessing the ZtSof1 gene at 14 days post-inoculation (dpi). In contrast, plants infected with ΔZtSof1 never developed pycnidia. The failure to undergo asexual reproduction was observed in a broad range of wheat genotypes (Additional file 11: Fig. S11).

To distinguish whether pycnidium formation would be a consequence of the lack of hyphal fusions or susceptibility of ΔZtSof1 cells to defense compounds produced by the plant, we used a wheat extract agar medium to induce pycnidium formation in vitro. ΔZtSof1 aggregated into mycelial knots, which is the initial developmental stage of the pycnidium, but no asexual reproductive structures were further developed. The pycnidium-like structures formed by ΔZtKu70 or ΔZtSof1-comp strains were exuding a whitish liquid containing pycnidiospores (Fig. 5b). Next, we evaluated the pycnidium formation in planta. The wheat plants infected with 1E4GFP or 1E4GFPΔZtSof1 strains were monitored using confocal microscopy up to 12 dpi. We observed mainly host penetration, initial intercellular hyphal extension, and sub-stomatal colonization at the earlier stages of the plant infection (Additional file 12: Fig. S12 – 6 and 7 dpi). No difference was noticed in fungal development; however, hyphal fusions established during epiphytic host colonization were only detected for 1E4GFP strain. At 8 and 9 dpi, we observed that the primary intercellular hyphae surrounding the stomatal guard cells

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**Fig. 5** The role of vegetative hyphal fusions for disease progression and pycnidial development. a Susceptible wheat cultivar Drifter inoculated with ΔZtKu70, ΔZtSof1, or ΔZtSof1-comp strains were evaluated up to 21 days post-infection (dpi). All tested strains exhibited similar disease progression, including the onset of the necrotrophic phase at 11 dpi. b Pycnidia production on wheat extract agar. After 20 days of incubation, ΔZtKu70 and ΔZtSof1-comp strains produced brown pycnidium-like structures exuding a whitish liquid similar to the oozed cirrhus-containing pycnidiospores spores observed for Z. tritici-infected wheat plants. In contrast, ΔZtSof1 mutant formed mycelial knots, but those structures never developed in mature asexual fruiting bodies.
produced specialized knots from where secondary hyphae emerged and elongated (Fig. 6 and Additional file 12: Fig. S12). These secondary hyphae fused with other nearby hyphae, creating an interconnected network in the sub-stomatal cavity (e.g., for the 1E4_{GFP} strain) or they kept extending as individual hyphae (e.g., for the 1E4_{GFP}\Delta ZtSof1 mutant) (Fig. 6 and Additional file 12: Fig. S12). The combination of sub-stomatal hyphal accumulation and anastomoses generates the pre-pycnidium at 12 days, which later supports the asexual reproduction of \textit{Z. tritici}. For the \Delta ZtSof1_{GFP} mutant, the filamentous hyphae kept extending as individual hyphae, and no fusion points were observed. The lack of anastomosis stops the developmental process of the pycnidium formation. For earlier time points (6, 7, and 8 dpi), please see Additional file 12: Fig. S12.
hence, impairing the asexual cycle of the fungus. Therefore, we concluded that hyphal fusions are decisive for the pycnidal development, and the disturbance of this mechanism ceases the asexual reproduction of *Z. tritici*.

**Discussion**

The evolution of the fungal language allowed a fine-tune coordination of signal senders and receivers driving their ecological diversifications and, consequently, the regulation of complex signaling networks. Here, we explored the functional relationship of a gene involved in cell-to-cell communication and its biological contribution to the development and fitness of a fungal plant pathogen.

Vegetative cell fusion is one of the most important developmental processes of a mycelial fungal colony [10, 42]. The cytoplasmic continuity generated by cell fusion provides adaptive advantages to the interconnected mycelial network by allowing resource sharing and introduction of genetic material [19, 42, 43]. CATs forming at earlier stages of the vegetative growth have been reported in several filamentous fungi [18]. We observed that these specialized fusion bridges require a specific cell density, indicating that *Z. tritici* may induce CATs at a critical concentration of a self-produced molecule, presumably a quorum-sensing molecule. For instance, *Fusarium oxysporum* secretes and senses both α- and α-pheromone, and their perception regulates spore germination in a cell density-dependent manner [44]. On the other hand, the induction of VHF s was often observed at lower cell density, indicating that VHF s may be induced at low self-produced molecule concentration or some other environmental signal. *Z. tritici* is a pleomorphic fungus changing growth morphology according to the environment. Nutrient-limited conditions induce both hyphal growth and fusions. Plant pathogens typically experience nutrient limitations while growing on leaves, and the perception of a nutrient-limited environment may act as a stimulus to induce interconnected mycelial formation in foliar plant pathogens, as observed for the causal agent of anthracnose disease, *Colletotrichum lindemuthianum* [45]. Both germinating blastospores or pycnidiospores underwent CATs and VHF s in vitro and *in planta*, supporting our previous observation [34]. Therefore, we demonstrated that vegetative cell fusions are a ubiquitous cellular process, which may have an essential contribution to the lifestyle of *Z. tritici*.

Nucleus transfer between two encountering hyphae occurs in some fungal species [46, 47]. The consequences of genetic exchange include the formation of viable heterokaryons and the risk of introgression of pathogenic elements or virulence genes [20, 48, 49]. Unlike the majority of fungal species that form a multinucleated hyphal network [47], *Z. tritici* has only one nucleus per septal compartment [41], which raises the question of whether anastomosis would enable multinucleated cell formation in *Z. tritici*. We showed that the new cell compartment is occupied by a migrating nucleus coming from a neighboring nucleus divided by mitosis after the fusion bridge between two identical *Z. tritici* cells. However, cells containing multiple nuclei were not observed neither near nor far from the anastomosis point, indicating that *Z. tritici* may have evolved to limit the spread of genetic elements and restrict the formation of multinucleated cells. Hence, we cannot discard that multinucleated, homokaryotic, or heterokaryotic cells can be formed at low frequency in nature. These properties could impact the evolution of the pathogen, and therefore, additional studies will be needed to underlie the roles played by the genetic exchange during vegetative cell fusions in *Z. tritici*.

In the last decades, the identification of fusion-defective mutants has contributed to the understanding of the molecular mechanisms underlying cell communication and fusion, especially by unveiling the interplay of the mitogen-activated protein kinase (MAPK) pathways during chemotropic interactions [11]. MAPK pathways are involved in extracellular signal perception and regulation of diverse genes essential for mating, filamentation, pathogenicity, cell integrity, and stress responses [50–55]. Therefore, the deletion of MAPK-encoding genes results in pleiotropic phenotypes due to their involvement in multiple biological processes. Though fungal communication and fusion require the regulation of several genes [6], the cross-talk between cell wall integrity (CWI) and pheromone response, two conserved MAPK signaling pathways, is essential to produce, secrete, and sense the chemotractant molecule produced during cell fusion [11]. The deletion of *Slt2* or *Fus3* orthologous genes disrupts the signaling cascade affecting self-anastomosis in filamentous fungi. We used the *ZtSlt2* and *ZtFus3* to demonstrate the functional conservation of these MAPK pathways in *Z. tritici*. *ZtSlt2* and *ZtFus3* are known as essential genes for the pathogenicity and developmental processes of *Z. tritici*, including vegetative growth, melanization, and pycnidium formation [56, 57]. Here, we showed that both ΔZtSlt2 and ΔZtFus3 mutants were unable to undergo anastomosis. Our results indicate that cell fusions in *Z. tritici* follow the same signaling mechanism described for *N. crassa* [11, 58], where the signal sending and receiving are coordinated by genes associated with the CWI and pheromone response pathways. Besides, this is the first report of those pathways regulating cell communication in *Z. tritici*.

To evaluate the impact of vegetative cell fusion in the biology of a hemibiotrophic fungus, we used the ZtSof1 orthologous of the *N. crassa* So gene. Contrary to the MAPK-related genes, the characterization of *So
orthologs results in low pleiotropy [15, 16, 24]. Filamentous fungi lacking \(S_0\) gene were impaired in self-anastomosis [15–17, 24], including \(Z. tritici\), in which the deletion of \(ZtSof1\) abolished vegetative cell fusion. We characterized the impact of the fusion deficiency at different developmental stages of \(Z. tritici\). For instance, the \(\Delta ZtSof1\) mutant exhibited a more asymmetrical hyphal extension in a nutrient-poor medium inducing hyphal growth than the fusion-competent individuals. It was demonstrated that the direction of the nutrient distribution occurs from the central part of the mycelium to outwards, and its streaming speed is driven by anastomosis [42]. Thereby, the reduced colony extension observed for the \(\Delta ZtSof1\) may be a consequence of the irregular distribution of cytoplasmic content throughout the mycelial colony. In contrast, the induced blastosporulation and larger yeast-like colonies exhibited by the fusion-defective mutant in nutrient-rich environments may be a consequence of the disruption of the CWI pathway or its cross-talk with other MAPK pathways involved in fungal growth. The genetic relationship between vegetative growth and the \(ZtSof1\) gene remains to be elucidated.

We showed that the \(\Delta ZtSof1\) colonies do not accumulate melanin, resulting in whiter and larger colonies than those formed by \(\Delta ZtKu70\) and \(\Delta ZtSof1\)-comp strains. These findings suggest a possible trade-off between energy cost for pigment production and growth. The reduction of fungal growth caused by the higher accumulation of melanin was reported before for \(Z. tritici\) [59]. Melanins are dark-pigmented secondary metabolites often associated with the fungal cell walls. Though fungi can produce different kinds of melamins, it is suggested that melanization of \(Z. tritici\) is only controlled by the polyketide synthase (PKS) gene cluster containing catalytic enzymes and transcription regulators of the 1,8-dihydroxynaphthalene (DHN) melanin [59, 60]. It was demonstrated for plant pathogenic fungi that the deletion of CWI-associated genes inhibited pigmentation by reducing the expression of DHN melanin biosynthetic genes [61–64]. Therefore, the deletion of \(ZtSof1\) may impair the CWI-regulatory cascade and, consequently, the regulation of DHN-melanin production, resulting in the lack of pigmentation observed for both \(\Delta ZtSof1\) and \(\Delta ZtSlt2\) [56] mutants. On the other hand, the CWI, high-osmolarity glycerol (HOG), cyclic adenosine monophosphate (cAMP), and the pheromone response pathways can interact regulating melanization in a cooperative manner [65–67]. Consistent with the findings in other fungi, mutants in these signaling pathways, including \(\Delta ZtFus3\), display altered pigmentation in \(Z. tritici\) [33, 39, 56, 57, 68, 69]. Additionally, a QTL mapping study identified \(ZtSlt2\), \(ZtHog1\), \(ZtGpa1\), and \(ZtFus3\), contributing to the melanization of this fungus [60]. Thereby, we cannot discard that the deletion of \(ZtSof1\) may affect the cross-talk between CWI with other signaling cascades impairing fungal melanization. Further investigations are needed to elucidate both hypotheses.

Melanin is postulated to contribute to fungal protection against fungicide and environmental stresses in \(Z. tritici\) [59, 60], and its regulation depends on environmental cues and colony development [60], though it is not yet a fully understood mechanism. We used nine different cellular stressors to evaluate whether (i) the defect in melanin accumulation or (ii) the deletion of \(ZtSof1\), the scaffold protein for the MAPK genes from the CWI pathway, affects pathogen stress tolerance. Since the non-melanized \(\Delta ZtSof1\) mutant displayed the same degree of stress sensitivity than \(\Delta ZtKu70\) and \(\Delta ZtSof1\)-comp strains, we concluded that \(ZtSof1\) does not act as a scaffold protein for all CWI pathway functions. Consistent with our findings, the model fungus \(Sordaria macrospora\), the PRO40 (orthologous to \(ZtSof1\)), operates as a scaffold for the CWI-encoding genes during fungal development, hyphal fusion, and stress response, but not for growth under cell wall stress agents [25]. Our results showed that melanin accumulation of \(Z. tritici\) does not benefit fungal survival in harsh environments, at least for the stressful conditions tested in this study.

We demonstrated that VHFs are dispensable for the pathogenicity of \(Z. tritici\). The fusion-defective \(\Delta ZtSof1\) mutant displayed a similar host damage progression than those individuals possessing the gene. This finding exemplifies the distinct effects of cell fusions on fungal pathogenicity. For instance, for the soil-borne \(Fusarium oxysporum\), VHF-impaired mutants exhibited only a slightly reduced virulence, whereas for the necrotrophic plant pathogen \(Alternaria alternata\), VHFs are necessary for the full virulence of the fungus [15, 16]. Though the deletion of \(ZtSof1\) is not essential for host penetration, colonization, or for the onset of the necrotrophic phase \textit{per se}, it is during hyphal accumulation in the sub-stomatal cavity that the fusion defect impacts \(Z. tritici\) fitness. The infection process during wheat colonization was microscopically detailed before for \(Z. tritici\) [70]; however, the study did not explore the contribution of VHFs. Here, we showed that the primary intercellular hyphae surrounding the stomatal guard cells produced specialized knots from where secondary hyphae emerge and elongate to fuse with other adjacent hyphae. Hence, the preliminary hyphal network creates the basis for a symphogenous development that builds the concave-shaped pycnidial wall of the mature pycnidium. On the other hand, the inability to undergo anastomosis ceases the development of the asexual fruiting bodies at this stage, and therefore abolishes fungal reproduction. The accumulation of hyphae observed in the sub-stomatal
chamber by the ΔZtSof1 mutant confirms that a specific signal triggers sub-stomatal hyphal aggregation independent of the putative chemoattractant molecule secreted by the fungus to induce hyphal fusion. In line with this hypothesis, a recent study showed that the disruption of the transcription factor ZtStuA impairs hyphal aggregation, resulting in the lack of pycnidium formation in vitro and in planta [68]. Therefore, we suggest that ZtStuA acts regulating genes necessary for the pre-pycnidium stages (e.g., hyphal aggregation) [68], while ZtSof1 controls the hyphal fusions required for the formation of the mature pycnidium. The initiation of the asexual sporulation may depend on the regulatory genes, such as ZtBrlA2 and ZtFlbC, as described by Tiley et al. Moreover, the inability of ΔZtFus3 to undergo VHFs and produce pycnidia [57] confirms the interplay between the CWI and pheromone response pathways during hyphal fusion, a developmental process essential for the asexual reproduction of Z. tritici. Fleissner et al. [24] demonstrated that the deletion of So in N. crassa also affects female fertilization, blocking the sexual reproduction of the mutant. Further experiments need to be performed to address this question for Z. tritici, but, likely, the ZtSof1 gene may also play a crucial role in the sexual reproduction of this pathogen.

Conclusion
The characterization of the ZtSof1 gene demonstrated its fundamental role in fungal biology. Beyond the impact of ZtSof1 for self-fusion, we show the contribution of this gene for fungal development, including vegetative growth and melanization. Besides, we demonstrated that VHFs are dispensable for pathogenicity, but essential for pycnidial development, and these mechanisms might be controlled by the interplay between the CWI and the pheromone response pathways. Our data show how cell fusion affects Z. tritici fitness and provides a new gene target to control septoria tritici blotch (STB) disease.

Methods
Strains and growth conditions
The Swiss Z. tritici strain ST99CH_1E4 (abbreviated as 1E4), described by Zhan et al. [71], and mutant lines derived from this strain were used in this study. 1E4 strains expressing cytoplasmic GFP (1E4GFP) or mCherry (1E4mCh) were provided by Andrea Sanchez-Vallet after being generated by Sreedhar Kilaru and Gero Steinberg. The knocked-out ΔZtSls2 [56] and ΔZtFus3 [57] mutants were provided by Marc-Henri Lebrun (National Institute of Agricultural Research – INRA, France). Because the MAPK mutants were generated in the genetic background of IPO323 [72], this strain was also used as a control. The strain IPO323 ZtHis1-ZtGFP [41] was provided by Gero Steinberg (Exeter University). Each strain was stored in glycerol at −80 °C until required and then recovered in yeast-sucrose broth (YSB) medium (10 g/L yeast extract, 10 g/L sucrose, 50 μg/mL kanamycin sulfate; pH 6.8) incubated at 18 °C for 4 days.

Plant infection to obtain fluorescent pycnidiospores
Wheat seedlings from the susceptible wheat cultivar Drifter were grown for 16 days in the greenhouse at 18 °C (day) and 15 °C (night) with a 16-h photoperiod and 70% humidity. Blastospore suspensions of 1E4GFP or 1E4mCh were obtained after 4 days of growth in the YSB medium. Spore suspensions were adjusted to a final concentration of 10⁹ blastospores/mL in 30 mL of sterile water supplemented with 0.1% (v/v) Tween and applied to run-off using a sprayer, and the plants were kept for 3 days in sealed plastic bags, followed by 21 days in a greenhouse. Leaves with pycnidia were harvested and transferred to a 50-mL Falcon tube containing sterile water and gently shaken to harvest the pycnidiospores. Fluorescent pycnidiospores were used to assess the cell fusion events during in vitro and in vivo growth. Pycnidiospore suspension-tagged GFP or mCherry were also adjusted to a final concentration of 10⁶ pycnidiospores/mL, and a new batch of plants was inoculated as described above. Plants co-infected by both 1E4GFP and 1E4mCh strains were used to observe VHFs on the wheat leaf surface.

Characterization of cell fusion events in vitro and in vivo
The ability of Z. tritici to undergo cell fusions was evaluated using blastospores and pycnidiospores of 1E4GFP and 1E4mCh. Cell concentrations were adjusted to 3.3 × 10⁷ blastospores/mL or 3.3 × 10⁶ blastospores/mL to induce CATs or VHFs, respectively. Three hundred microliters of each morphotype and fluorescence was plated on WA to create a ratio of 1:1 and to provide a final concentration of 10⁸ blastospores/mL or 10⁶ blastospores/mL. A section of about 1 cm² of agar was aseptically cut and placed on a microscope slide. The mixing of both cytoplasm contents through CATs or VHFs was checked up to 40 hai using a Leica DM2500 fluorescence microscope with LAS v.4.6.0 software. GFP excitation was at 480/40 nm and detected at 507/30 nm, respectively, whereas mCherry was excited at 580/20 nm and detected at 632/60 nm. VHFs during spore germination on wheat leaf surfaces were obtained via confocal images using a Zeiss LSM 780 inverted laser scanning microscope with ZEN Black 2012 software. An argon laser at 500 nm was used to excite GFP fluorescence and chloroplast autofluorescence, while mCherry excitation was at 588 nm. The emission wavelength was 490–535, 624–682, and 590–610 nm for GFP, chloroplast autofluorescence, and mCherry, respectively. Plants co-inoculated with blastospores or
Plasmid constructions and transformations

Primers used for cloning, sequencing, and knock-out confirmations are listed in Table S1. DNA assemblies were conducted with the In-Fusion HD Cloning Kit (Takara BIO) following the manufacturer’s instructions. The plasmid constructions to generate the three mutants in the 1E4 genome background and used in this study (ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp) are described in Additional file 13: Fig. S13. The pES1-ΔZtSof1 construction was also used to knock out the ZtSof1 gene in the 1E4GFP genome background, enabling the visualization of the GFP-tagged mutant during host infection.

Z. tritici 1E4 strain was transformed by Agrobacterium tumefaciens-mediated transformation (ATMT) according to Meile et al. [73]. The knock-out of the target genes was verified by a PCR-based approach using a forward primer specific to the upstream sequence of the disrupted gene and a reverse primer specific to bind in the resistance cassette (Additional file 14: Table S1). We determined the copy number of the transgene by quantitative PCR (qPCR) on genomic DNA extracted with the DNeasy Plant Mini Kit (Qiagen). We used qPCR target gene as the selection marker and the 18S rDNA as the reference gene (Additional file 14: Table S1). Lines with a single insertion were selected for further experiments.

Phenotypic characterizations

For all phenotypic analyses, ΔZtKu70 was considered the wild-type (WT) strain. To pinpoint the role of the ZtSof1 gene on the vegetative cell fusion, we added blastospore suspension cells of ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp to a final concentration of 10^6 blastospores/mL or 10^7 blastospores/mL into WA and incubated at 18 °C to induce CATs or VHFs, respectively. For the MAPK ΔSlt2 and ΔFus3, IPO323 and ΔSlt2-complemented strains, WA plates were inoculated only at a final concentration of 10^6 blastospores/mL. Cell fusion events were monitored up to 40 hai by light microscopy. Because fusion bridges were not observed between individuals lacking ΔZtSof1, we mixed 150 μL of 10^6 blastospores/mL of ΔZtKu70, ΔZtSof1, or ΔZtSof1-comp strains with the same concentration of 1E4GFP blastospores in a ratio of 1:1 to confirm the failure of cytoplasmic streaming. At least 50 spores of each sample combination were monitored.

To test for altered fungal growth, we used PDA (39 g/L potato dextrose agar, 50 μg/mL kanamycin sulfate) or WA media to induce blastospore or hyphal growth, respectively. Two hundred microliters of spore suspension of ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp was plated at a final concentration of 2 × 10^4 blastospores/mL on each plate in the 1E4 genome background and used in this study (ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp) are described in Additional file 13: Fig. S13. The pES1-ΔZtSof1 construction was also used to knock out the ZtSof1 gene in the 1E4GFP genome background, enabling the visualization of the GFP-tagged mutant during host infection.

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independent PDA plates amended with the mentioned stresses and incubated at 18 °C. Colony phenotypes were assessed by digital images taken at 5 dpi.

Virulence assay and pycnidium formation in vitro
Seeding, greenhouse and plant growth conditions, inoculum preparation, and plant inoculation followed the procedures described by Meile et al. [73]. To estimate the percentage of leaf covered by lesions (PLACL) and pycnidium formation, we harvested the second leaves of Drifter plants inoculated with ΔZtKu70, ΔZtSof1, or ΔZtSof1-comp strains at 8, 10, 11, 12, 14, 16, and 21 dpi. Leaves were mounted on a paper sheet, scanned with a flatbed scanner, and analyzed using automated image analysis [77]. Data analysis and plotting were performed using the ggplot2 package [75].

The defect in pycnidium formation was confirmed by plating blastospores of ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp strains onto wheat extract agar medium (50 g/L blended 21-day-old wheat leaves cultivar Drifter, 10 g/L agar) and incubated under UV-A light (16:8 light:dark cycle) up to 40 days at 18 °C, following the protocol optimized by Tiley et al. [68].

Confocal laser scanning microscopy of infected wheat leaves
To assess the impact of ZtSof1 deletion on fungal fitness during host colonization, we inoculated wheat plants with 1E4GFP and other two independent GFP-tagged ΔZtSof1 mutants. Infected leaves were harvested at 6, 7, 8, 9, 10, 11, and 12 dpi and checked for developmental stages of asexual fruiting bodies. Microscopy was conducted using Zeiss LSM 780 inverted laser scanning microscope with the software Mega6. An argon laser at 500 nm was used to excite GFP fluorescence and chloroplast autofluorescence with an emission wavelength of 490–535 nm and 624–682 nm, respectively. Analyses, visualization, and processing of image z-stacks were performed using ImageJ software [74].

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12915-020-00838-9.

Additional file 1: Figure S1. Vegetative hyphal fusion occurs during epiphytic growth on wheat leaves. Co-infection of wheat plants with blastospores (A) or pycnidiospores (B) from the 1E4 strain expressing either the cytoplasmic green fluorescent protein (GFP) or the red fluorescent protein (mCherry) resulted in hyphal fusions and cytoplasmic exchange after 48 hours of infection. Hyphal fusion during epiphytic colonization may assist the fungus to create an interconnected network supporting its establishment on the leaf surface before host penetration.

Additional file 2: Figure S2. Hyphal fusion does not lead to the generation of multinucleated cells in Zymoseptoria tritici. Blastospores of the IPO323 ZtHis1-ZtGFP strain, which has the GFP as a fluorescent marker labeling the nucleus, were plated on WA plates at a final concentration of 10⁵ blastospores/mL and incubated at 18°C. After 72 hours of incubation, none septum containing more than one nucleus was observed neither at hyphal bridges nor distant of the fusion point. Black triangles indicate the septal compartment containing only one nucleus at the fusion bridges.

Additional file 3: Figure S3. Scheme showing the phylogenetic relationship of the So gene within Ascomycete species. The so gene sequence from Neurospora crassa (XM_958983.3) was blasted against the Z. tritici genome (https://genome.igi.doe.gov/Mycgr3/Mycgr3home.html) to identify its orthologous in this fungus. The Z. tritici So orthologous protein sequence was used for a Blastp analysis against the NCBI database (National Center of Biotechnology Information). Blast searches at expected value homology cut-off of 1e⁻¹⁰ were included as positive. A dataset containing So orthologous proteins of different Ascomycete species were used for phylogenetic analysis. Protein sequences were aligned using the Aliview program [78]. The best-fit model of amino acid evolution was the LG+G, determined by Mega6 software [79]. Amino acid sequences were aligned using Muscle, followed by maximum likelihood (ML) phylogeny reconstruction using 1,000 bootstraps and performed with the software Mega6 [79]. (A) The illustration demonstrates the ZtSof1 gene locus and its protein sequence containing the Atrophin 1, WW, and Phox domains. Comparison of ZtSof1 protein sequence with its orthologs showed 53% identity with Epichloë fusca; 54% identity with Neurospora crassa and Sordaria macrospora; 55% identity with Fusarium oxysporum; 60% identity with Aspergillus oryzae; and 63% identity with Alternaria brassicicola. (B) The alignment of the WW protein-protein interaction domain, including the PPLP motif of 41 different fungal species. Red boxes surround the two conserved tryptophan residues spaced by 22 amino acids apart. (C) Phylogenetic analysis grouped the orthologs of the ZtSof1 gene onto three groups based on fungal Classes (Dothideomycetes, Sordariomycetes, and Chaetothyriomycetes) together with Eurotiomycetes, independently whether they were parasites, mutualists or saprotrophs. Three members of Basidiomycetes were used as an out-group to root the tree.

Additional file 4: Figure S4. Cytoplasmic streaming between ΔZtKu70 and the GFP-tagged 1E strain. Blastospores of ΔZtKu70 and 1E4GFP were co-inoculated on water agar (WA) plates, a hyphal fusion-inducing condition. After 40 hours of incubation, fusion bridges were observed between ΔZtKu70 and 1E4GFP strains. The detection of the green fluorescent protein in the cytoplasm of the recipient hypha ΔZtKu70 confirms the cytoplasmic streaming between the two fused individuals (panel 1). Black asterisks point to the non-fluorescent ΔZtKu70 spore before hyphal fusion. White triangle indicates the fusion point between the ΔZtKu70 and 1E4GFP strains.

Additional file 5: Figure S5. Cytoplasmic streaming between ΔZtSof1-comp and GFP-tagged 1E4 strain. Blastospores of ΔZtSof1-comp and 1E4GFP were co-inoculated on water agar (WA) plates, a hyphal fusion-inducing condition. After 40 hours of incubation, fusion bridges were observed between ΔZtSof1-comp and 1E4GFP strains. The detection of the green fluorescent protein in the cytoplasm of the recipient hypha ΔZtSof1-comp confirms the cytoplasmic streaming between the fused individuals (panel 1). Black asterisks point to the non-fluorescent ΔZtSof1-comp spore before hyphal fusion. White triangles indicate the fusion points between the ΔZtSof1-comp and 1E4GFP strains.

Additional file 6: Figure S6. Co-inoculation of ΔZtSof1 and GFP-tagged 1E4 strain confirms the failure of the ΔZtSof1 mutant to undergo hyphal fusion. Blastospores of ΔZtSof1 and 1E4GFP were co-inoculated on water agar (WA) plates, a hyphal fusion-inducing condition. After 40 hours of incubation, fusion bridges were observed between ΔZtSof1-comp and 1E4GFP strains. The detection of the green fluorescent protein in the cytoplasm of the recipient hypha ΔZtSof1-comp confirms the cytoplasmic streaming between the fused individuals (panel 1). Black asterisks point to the non-fluorescent ΔZtSof1-comp spore before hyphal fusion. White triangles indicate the fusion points between the ΔZtSof1-comp and 1E4GFP strains.

Additional file 7: Figure S7. MAPK-encoding ZtSlt2 and ZtFus3 genes are required for anastomosis in Zymoseptoria tritici. (A) Hyphal fusions were regularly found in the wild-type strain (IPO323). The deletion of
ZtSof1 impact the vegetative growth in a morphotype-depending manner in Zymoseptoria tritici. A nutrient-poor medium (WA), inducing hyphal growth, and a nutrient-rich medium (PDA), inducing blastosporulation, were used to assess the effect of ZtSof1 deletion on fungal radial growth. (A) The ΔZtSof1 mutant exhibited a similar colony morphology than ΔZtKu70 and ΔZtSof1-comp colonies, whereas the ΔZtSof1 mutant exhibited only a few filamentous at the colony periphery. Dashed squares point to the localization of microscope images. (C) Thought no morphological differences were observed for the tested ZtKu70 strains, the fusion defective ΔZtSof1 mutant had a slight, but significant reduction of its radial growth (mm) compared to ΔZtKu70 and ΔZtSof1-comp when grown on a nutrient-limited medium. At least 40 colonies of each tested strains were evaluated. Two and three stars indicate a p-value <0.005 and <0.0005, respectively. (D) No morphological differences were noticed for the blastospores produced by ΔZtKu70, ΔZtSof1, or ΔZtSof1-comp strains. (B) ΔZtSof1 mutant grew faster and had higher radial growth over time than the ΔZtKu70 and ΔZtSof1-comp strains when incubated on PDA. Bars represent standard errors of the radial growth (mm) of at least 40 colonies. Different letters on the top of the bars indicate a significant difference among the tested strains according to the Analysis of Variance (ANOVA). The notch displays a 95% confidence interval of the median. Open circles represent the outlier values of each strain. Pictures shown below the bar plot illustrate the colony sizes of ΔZtKu70, ΔZtSof1, and ΔZtSof1-comp strains.

Additional file 10: Figure S10. Percentage of leaves covered by lesions (%L) for each of the wild-type (WT) strains and the mutants after growth on fresh wheat leaf for 14 days. All WT strains reached an interconnected network in the substomatal cavity. Unlike, the second hyphae of the ΔZtSof1 mutant kept extending as individual filaments. No anastomosis was observed until this developmental stage. For later time points (9 and 12 dpi), please see Figure 6.

Additional file 11: Figure S11. ΔZtSof1 and ΔZtKu70 strains do not vary in pathogenicity, except for the failure of the ΔZtSof1 mutant to undergo asexual reproduction. Five different winter cultivars infected with ΔZtKu70 or ΔZtSof1 strains were, respectively, evaluated at 14- and 21 days post-inoculation (dpi) for host damage and pathogen reproduction. We used five different winter cultivars of wheat (Triticum aestivum L.) based on their susceptibility or resistance to Zymoseptoria tritici. The resistant cultivar, Drifter, was inoculated with the fluorescent 1E4 GFP (wild-type) strain. At 6 dpi, the epiphytic filamentous hyphae penetrate the host tissue through stomatal openings. At 7 dpi, the fungus initiated the intracellular hyphal colonization of the substomatal chamber. The filamentous surrounding the stomatal guard cells produce specialized knots from where secondary hyphae emerge and germinate. Up to this point, none morphological difference of hyphal extension or intracellular hyphal colonization was noticed between 1E4-WT and ΔZtSof1-comp strains. At 8 dpi, the secondary hyphae fuse with another nearby hypha (represented by black circles) in the 1E4-WT strain, creating an interconnected network in the substomatal cavity. Unlike, the second hyphae of the ΔZtSof1 mutant kept extending as individual filaments. No anastomosis was observed until this developmental stage. For later time points (9 and 12 dpi) please see Figure 6.

Additional file 12: Figure S12. Confocal microscopy images and schematic demonstration of hyphal penetration, substomatal colonization, and initial stages of pycnidial development. Susceptible wheat cultivar Drifter was inoculated with the fluorescent 1E4-WT (wild-type) and ΔZtSof1-comp strains and monitored by confocal microscopy at different days post-infection (dpi). At 6 dpi, the epiphytic filamentous hyphae penetrate the host tissue through stomatal openings. At 7 dpi, the fungus initiated the intracellular hyphal colonization of the substomatal chamber. The filamentous surrounding the stomatal guard cells produce specialized knots from where secondary hyphae emerge and germinate. Up to this point, none morphological difference of hyphal extension or intracellular hyphal colonization was noticed between 1E4-WT and ΔZtSof1-comp strains. At 8 dpi, the secondary hyphae fuse with another nearby hypha (represented by black circles) in the 1E4-WT strain, creating an interconnected network in the substomatal cavity. Unlike, the second hyphae of the ΔZtSof1 mutant kept extending as individual filaments. No anastomosis was observed until this developmental stage.

Abbreviations
VHF: Vegetative hyphal fusion; CAT: Conidial anastomosis tube; MAPK: Mitogen-activated protein kinase; CWI: Cell wall integrity; ZtKu70: Zymoseptoria tritici heterodimeric regulator of the NHEJ pathway; ZtSof1: Zymoseptoria tritici soft gene

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Authors' contributions
CSF and JGC conceived the research and experimental designs. CSF conducted the experiments, performed the data analysis, and wrote the manuscript with JPC. MMZ collaborated on the acquisition and analysis of the data. All authors approved the final version of the manuscript.
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