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The wheat SnRK1α family and its contribution to *Fusarium* toxin tolerance

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ABSTRACT

Deoxynivalenol (DON) is a mycotoxin produced by phytopathogenic *Fusarium* fungi in cereal grain and plays a role as a disease virulence factor. TaFROG (*Triticum aestivum Fusarium* Resistance Orphan Gene) enhances wheat resistance to DON and it interacts with a sucrose non-fermenting-1 (SNF1)-related protein kinase 1 catalytic subunit α (SnRK1α). This protein kinase family is central integrator of stress and energy signalling, regulating plant metabolism and growth. Little is known regarding the role of SnRK1α in the biotic stress response, especially in wheat. In this study, 15 wheat (*Triticum aestivum*) SnRK1α genes (*TaSnRK1αs*) belonging to four homoeologous groups were identified in the wheat genome. *TaSnRK1αs* are expressed ubiquitously in all organs and developmental stages apart from two members predominantly detected in grain. While DON treatment had either no effect or downregulated the transcription of *TaSnRK1αs*, it increased both the kinase activity associated with SnRK1α and the level of active (phosphorylated) SnRK1α. Down-regulation of two *TaSnRK1αs* homoeolog groups using virus induced gene silencing (VIGS) increased the DON-induced damage of wheat spikelets. Thus, we demonstrate that *TaSnRK1αs* contribute positively to wheat tolerance of DON and conclude that this gene family may provide useful tools for the improvement of crop biotic stress resistance.

1. Introduction

Deoxynivalenol (DON) is a mycotoxin commonly produced by phytopathogenic *Fusarium* fungi in cereal grain and is harmful to human and animal health [1,2]. DON is also a *Fusarium* virulence factor, facilitating the spread of the fungi within plant tissue [3,4]. Resistance to DON is an important component of cereal resistance to the economically important Fusarium Head Blight (FHB) disease; this disease is caused when *Fusarium* fungi attack the plant inflorescence, reducing yield and contaminating grain with DON [5]. Breeding for FHB and mycotoxin resistance is a goal of many researchers and breeding companies; the usage of economically costly chemicals has shown only moderate success in disease control [6–8]. The success and efficiency of breeding programmes will be determined by our understanding of the host-pathogen relationship and associated defence responses.

DON resistance can be achieved via cellular detoxification processes. For example, UDP-glycosyltransferases (UGTs) have been shown to convert DON to less toxic DON-3-O-glucoside and overexpression of wheat *UGT* (*TaUGT3*), barley *UGT* (*HvUGT13248*), and Brachypodium *UGT* (*Bradi5gUGT03300*) increased DON tolerance in transgenic plants [9–13]. Other cereal genes potentially associated with cellular detoxification processes have been shown to directly affect DON tolerance in wheat. For example, gene silencing of the wheat ABC transporter, *TaABCC3.1,* or a wheat cytochrome P450, *TaCYP72A*, resulted in enhanced susceptibility to DON [14,15]. Enhanced DON resistance has also been achieved via overexpression of a novel wheat gene, namely the *Triticum aestivum Fusarium* resistance orphan gene (*TaFROG)*; this gene contributes to both DON and FHB resistance in wheat [16]. The gene showed little to no basal expression in wheat but was activated in response to DON or DON production by *F. graminearum* [16]. TaFROG has no known protein domains but interacts with a NAC-like transcription factor (TaNACL-D1) and an evolutionarily-conserved sucrose non-fermenting-1 (SNF1) related protein kinase 1 catalytic subunit α (TaSnRK1α1-A) [16,17]. *TaNACL-D1* also contributes to FHB resistance [17]. We hypothesized that, like TaFROG, TaSnRK1α might play a role in DON resistance.

The SNF1 protein kinase family includes the yeast SNF1, the mammalian AMP-activated protein kinase (AMPK) and the plant SNF1-

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related protein kinases 1 catalytic subunit α (SnRK1α). This evolutionarily-conserved protein kinase family has a central role in regulating energy homeostasis by activating energy-producing pathways and inhibiting energy-consuming pathways under starvation and energy-depleting stress conditions [18–22]. It has been proposed that SnRK1α has a role in integrating environmental and endogenous signals to modulate plant growth, development and survival [20,23–27]. There is growing evidence that SnRK1α plays an important role in plant defence mechanisms against herbivores, fungi, bacteria and viruses [28,29]. For example, in *Solanaceae*, overexpression of *SnRK1α* enhanced viral resistance, while plants carrying antisense constructs became more susceptible to geminivirus infection [30,31]. SnRK1α in rice confers broad-spectrum disease resistance: SnRK1α-overexpressor plants are more resistant to pathogens whose lifestyle is hemibiotrophic (*Xanthomonas oryzae pv. oryzae* and *Pyricularia oryzae*) and necrotrophic (*Cochliobolus miyabeanus* and *Rhizoctonia solani*), whereas SnRK1α-silenced plants were more susceptible [32,33]. Additionally, SnRK1α overexpression in rice boosted the jasmonate-mediated defence response and therefore it was suggested that SnRK1α is involved in basal plant immunity [33]. Nevertheless, the exact mechanisms involving SnRK1α in plant defence remain to be detailed and are probably very diverse.

In this study, we demonstrate the role of SnRK1α in wheat resistance to the *Fusarium* mycotoxin DON. Bread wheat (*Triticum aestivum*) SnRK1α genes (*TaSnRK1αs*) were identified and classified phylogenetically. Gene expression studies were conducted to determine the effect of DON treatment on the expression of *TaSnRK1α* genes in wheat heads. The effect of the mycotoxin on the kinase activity of TaSnRK1αs was evaluated by measuring AMARA peptide kinase activity and the phosphorylation state at the conserved activation loop threonine residue. A reverse genetic study using virus induced gene silencing (VIGS) assessed the contribution of *TaSnRK1αs* to DON resistance on wheat ears.

2. Materials and methods

2.1. Data source, sequence retrieval and phylogeny of SnRK1α

SNF1 and AMPKα protein sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) whereas the three Arabidopsis and Rice SnRK1α protein sequences [34,35], were collected from the genome release Tair10 in the TAIR database (https://www.arabidopsis.org/index.jsp) or from *Oryza sativa* v7 in Phytozome v12.1 database (https://phytozome.jgi.doe.gov/pz/portal.html), respectively (all corresponding proteins ID are listed in Supplementary Table S1). To identify bread wheat SnRK1α proteins (TaSnRK1αs), full length protein sequences of Arabidopsis and rice SnRK1α were used for a BLAST search with BLASTp against the local database of wheat (high confidence proteins, IWGSC RefSeq v1.1 annotation) to retrieve all protein sequences with ≥70% amino acid identity. Using InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search), protein sequences were analysed for protein domains and selected to have at least two out of the three characteristic protein domains of SnRK1α (protein kinase domain (IPR000719), ubiquitin-associated domain (IPR015940) and the kinase associated domain 1 (KA1 = CTD) (IPR001772)). Protein sequences containing the NAF protein domain typical of SnRK3s (also known as CBL-interacting kinases [36,37]) were removed. Full length protein sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with default settings and the resulting multiple sequence alignment was used to build a Neighbor-Joining tree with evolutionary distances computed using the JTT matrix-based method with MEGA7 [38]. The phylogenetic tree was annotated and coloured with iTOL v3 [39]. Prediction of protein molecular weight was done using Expasy tool (https://web.expasy.org/compute_pi/).

2.2. Plant material and growth conditions

Spring wheat cultivar (cv.) CM82036, kindly provided by Prof. Hermann Buerstmayr (IFA-Tulln, Austria), was used in this study. This wheat cultivar carries at least two QTL (*Qfhs.ndsu-3BS* and *Qfhs.ifa-5A*) that confer resistance to FHB and DON [40,41]. Wheat seeds were germinated for 3 days on moistened filter papers (Whatman No.1) in Petri plates at 20 °C in the dark. Germinated seedlings were transferred into 3 l pots containing John Innes No.2 compost (Westland Horticulture, Dungannon, UK) mixed with fertiliser (3 g/3 l pot) (Osmocote Exact standard, Everris, Netherlands). Plants were grown under contained glasshouse conditions, 25/18 °C for a 16/8 h light-dark photoperiod.

2.3. Plant material for Gene expression and protein activity studies

Plants were cultivated as described above. DON treatment and sample collection were conducted as described by [16], except two central spikelets of the heads were treated with 20 µl of either 0.02% (v/v) Tween 20 (mock) or 16.87 mM DON in 0.02% Tween 20 (v/v) for gene expression experiment. RNA or total protein were extracted from one pooled sample per treatment (representing a pool of 4 heads from individual plants) per trial. Both gene expression and protein activity experiments comprised 3 independent trials.

2.4. DNA, RNA extraction and cDNA synthesis

DNA was extracted from wheat leaf tissue using the E.Z.N.A.® High Performance (HP) DNA Mini Kit (OMEGA) according to the kit instruction. Wheat spikelet total RNA was extracted with a CTAB (hexadecyltrimethylammonium bromide) / PVP (Polyvinylpyrrolidone) RNA extraction buffer as described previously [42] and DNAse-treated using the TURBO DNA-free TM kit (Ambion Inc., USA) following the manufacturer's instructions. The RNA quality and yield were examined with electrophoresis through an agarose gel and measured with the ultraviolet absorbance using a NanoDrop 1000 (Thermo Scientific). Reverse transcription (RT) of total RNA was conducted as previously described by [43]. Each RT reaction was performed using 1 µg of RNA with 100 U of M-MLV RT (Invitrogen) according to the kit instructions. The RT reaction was diluted with nuclease-free water to a total volume of 100 µl.

2.5. Virus-induced gene silencing (VIGS)

Silencing of *TaSnRK1α* genes in wheat was carried out using the VIGS technique. VIGS was conducted as described previously, with some modifications [14]. The barley stripe mosaic virus (BSMV)-derived VIGS vectors used in this study consisted of the wild type BSMV ND18 *α*, *β*, γ tripartite genome [44,45]. Two independent, non-overlapping gene fragments were used for VIGS of *TaSnRK1α* genes. Both fragments were PCR-amplified (primers listed in Supplementary Table S2) from the C-terminal part of *TaSnRK1α1-A*. VIGS target sequences were chosen to preferentially target *TaSnRK1α1-A* and its homoeologs (*TaSnRK1α1-B* and *TaSnRK1α1-D*) and were based on the previously cloned *TaSnRK1α1-A* gene sequence which codes for an interacting protein of the DON resistance protein TaFROG [16]. The specificity and silencing efficiency of the constructs was predicted using siRNA finder si-Fi (labtools.ipk-gatersleben.de/index.html). PCR products of the silencing fragments were cloned into *Not*I/*Pac*I – digested γ RNA vector pSL038-1 [45]. A BSMV γ RNA construct containing a 185 bp fragment of the barley phytoene desaturase (*PDS*) gene served as a positive control for VIGS as previously described [45]. Vectors containing the BSMV *α* and γ genomes, the γ RNA genome encoding silencing fragments of *TaSnRK1α1-A* (BSMV:S1 or BSMV:S2), or *PDS* were linearized with *Mlu*I. The vector carrying the BSMV *β* genome was linearized using *Spe*I. Capped *in vitro* transcripts were prepared from the linearized

plasmids using the mMessage mMachine T7 *in vitro* transcription kit (AM1344, Ambion, USA) following the manufacturer's protocol.

Inoculation with BSMV constructs were performed at the growth stage 47 [46] by rub-inoculation of the flag leaf and following the protocol described by [47]. A 1:1:1 ratio mixtures of the BSMV *in vitro* transcripts (BSMV α and β and either γ RNA (BSMV:00), BSMV:S1 or BSMV:S2) were used for the rub inoculation. At mid-anthesis (growth stage 65), DON treatment was conducted as described by [16]. To assess the effect of the gene silencing, 24 h after DON treatment, one spikelet above the treated spikelets was sampled for RNA extraction and subsequent gene expression analysis. The number of damaged (discoloured and necrotic) spikelets (including treated spikelets) was assessed 14 days after DON treatment. In total, two trials were carried out, each including positive and negative controls. For BSMV:00 (empty vector), BSMV:S1 and BSMV:S2, each trial included 10–20 biological replicates (individual head from 5 to 10 plants) per treatment combination.

2.6. Quantitative reverse transcriptase PCR analysis

Quantitative real-time PCR (qRT-PCR) analysis was conducted using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). qRT-PCR primers (Supplementary Table S2) used in this study were designed using Primer3web (http://primer3.ut.ee/ [48]. Each reaction contained 1.25 µl of a 1:5 (v/v) dilution of cDNA, 0.2 µM of each primer and 1X Fast SYBR® Green Master Mix (Applied Biosystems, 4,385,612) in a total reaction volume of 12.5 µl. PCR conditions were: 1 cycle of 20 s at 95 °C; 40 cycles of 1 s at 95 °C and 20 s at 60 °C; and a final cycle of 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C for the dissociation curve. All qPCR analyses were conducted in duplicate (each using cDNA generated from independent reverse transcriptions). Data acquisition and analysis were performed using the QuantStudio Software V1.3 (Applied Biosystems). The threshold cycle (Ct) values obtained by qPCR were used to calculate the relative gene expression using the formula $2^{-(\text{Ct target gene} - \text{Ct average of housekeeping genes})}$ as described previously [49].

2.7. Preparation of protein extracts

Crude protein extracts were prepared from wheat spikelets for AMARA kinase activity measurement and western blot analysis. Total soluble protein was extracted from 500 mg of tissue ground using liquid N_2 in a pestle and mortar with 2.5 ml of ice-cold homogenisation buffer (100 mM Tricine-NaOH (pH = 8.2), 25 mM sodium-fluoride, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM benzamidine, 5 mM dithiothreitol (DTT), 1x protease inhibitor cocktail (Sigma P9599), phosphatase inhibitors (PhosStop, Roche) and 2% (w/v) insoluble polyvinylpyrrolidone. Homogenate was centrifuged at 13,000 *g* at 4 °C. Supernatant (1.5 ml) was desalted using PD-10 columns (GE Healthcare) pre-equilibrated with homogenisation buffer. Eluent was supplemented with protease inhibitor cocktail and 1 µM okadaic acid before freezing in liquid N_2. Protein concentrations were determined using the Pierce™ 660 nm Protein Assay (Thermo scientific) following the manufacturer's instructions.

2.8. AMARA peptide kinase assay

SnRK1α kinase activity was assayed as described previously, with modifications [50]. Assays were carried out in a final volume of 25 µl in microtiter plate wells at 30 °C. The assay medium was 40 mM HEPES-NaOH (pH = 7.5), 5 mM $MgCl_2$, 200 µM ATP containing 12.5 kBq [γ-^{33}P]ATP (PerkinElmer), 4 mM DTT, 0.5 µM okadaic acid, 1x protease inhibitor cocktail and 200 µM AMARA peptide (AMARAASAAALARRR) (Isca Biochemicals, UK). AMARA peptide is a synthetic substrate for SnRK1α in which the minimal recognition motif for phosphorylation is present (ϕ-x-basic-2x-S-3x-ϕ, where ϕ is a hydrophobic residue) [51,52]. Assays were started with the addition of 5 µl plant extract into the assay buffer and stopped after 6 min by transferring 10 µl to P81 phosphocellulose squares (Millipore) immersed immediately in 1% (v/v) phosphoric acid. The basic amino acid residues of the phosphorylated peptide substrate provided adhesion to the phosphocellulose paper. These were washed four times with 800 ml 1% (v/v) phosphoric acid then immersed in acetone for 15 min, air-dried and transferred to vials containing 5 ml Ecoscint™A scintillation liquid (National diagnostics). ^{33}P incorporation into AMARA peptide was counted using liquid scintillation analyser (Tri-Carb 2900 TR). AMARA kinase activity within each SnRK1α extract was assessed four times (individual reactions) and for each SnRK1α extract one reaction was conducted replacing AMARA peptide with water to determine basic radioactivity. Six squares were not washed for estimating the maximum radioactivity. The AMARA kinase activity was then calculated as nmol ^{33}P incorporated into AMARA peptide per mg protein per minute.

2.9. Western blot analysis

Protein extracts from wheat spikelets were used for western blot analysis to detect SnRK1α proteins and phospho-SnRK1α (pSnRK1α) proteins. 12.5 µg proteins were separated in a Bolt™ 8% Bis-Tris polyacrylamide gel (Invitrogen) using a Bolt MES SDS running buffer (Life Technologies) and according to manufacturers' instructions. After protein transfer to nitrocellulose membranes using an iBlot gel transfer system (Life Technologies), SnRK1α was detected using a monoclonal antibody (Abmart) at a 1/2000 dilution produced against synthetic peptides (5′- EKGRLQEEEARRFF-3′) derived from the conserved protein kinase domain of TaSnRK1α and a secondary anti-mouse horseradish peroxidase antibody (#62-6520, Invitrogen) at a 1:20,000 dilution. Phospho-SnRK1α proteins were detected using a monoclonal antibody against the phospho-Thr-172 (#2535, Phospho-AMPKα (Thr172) (40H9), Cell Signalling Technology) at a 1/1000 dilution and a secondary anti-rabbit horseradish peroxidase antibody (#7074, Cell Signalling Technology) at a 1:7500 dilution. Immunoreactive bands were detected using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare). Following electrochemiluminescence assay, emitted signal was imaged with the Fusion-FX (Vilber Lourmat). Ponceau S staining (G-Biosciences) of blots was performed and band intensities were quantified using FIJI software [53]. On each immunoblot, densities of SnRK1α or pSnRK1α bands were measured and their relative densities were obtained after normalisation with the amount of protein loaded estimated with the Ponceau S staining images.

2.10. Statistical analysis

All statistical analyses were performed using SPSS statistics version 24 software (IBM). The statistical significance of differences was determined by comparing individual treatments using the Mann-Whitney U test or using Kruskal-Wallis test in the case of gene expression analyses.

3. Results

3.1. Identification and phylogenetic analysis of bread wheat SnRK1α proteins

Bread wheat or common wheat (*Triticum aestivum*) has a complex genome allohexaploid (AABBDD, 2n = 6x = 42 chromosomes) with three sub-genomes designated A, B and D. The latest annotation of the wheat cv. Chinese Spring genome (IWGSC RefSeq v1.1; [54]) was searched for genes encoding SnRK1α proteins based on similarity with known plant SnRK1α proteins and the presence of the characteristic SnRK1α functional domain. This identified 16 genes encoding putative SnRK1α proteins, but thereafter one was excluded

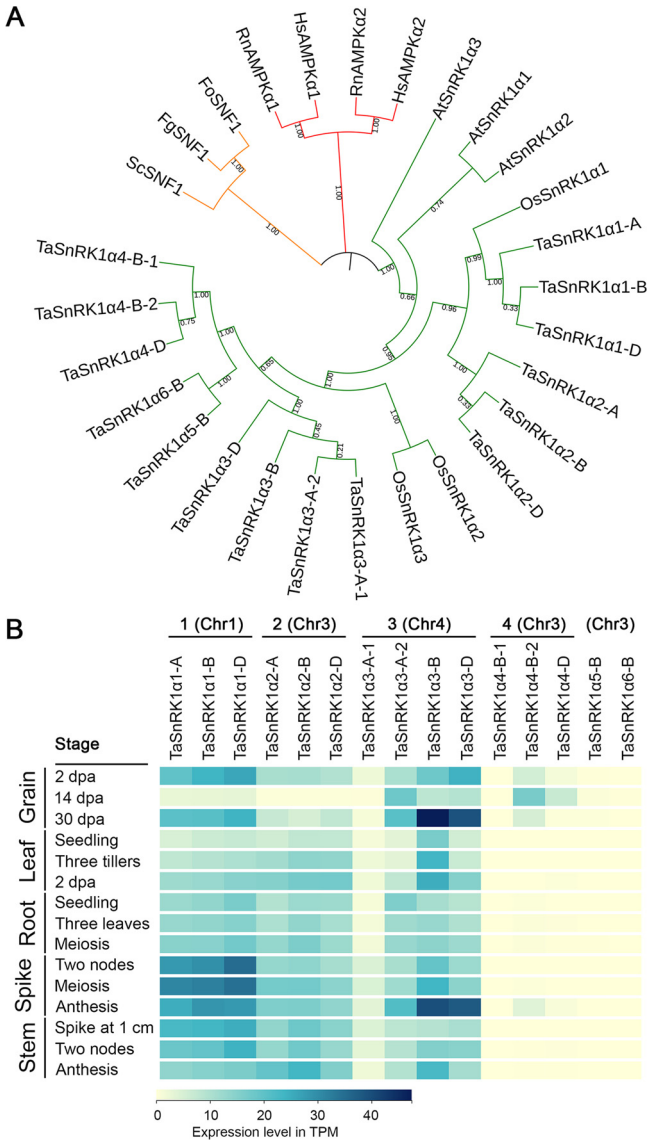

Fig. 1. Phylogeny and expression profile of wheat SnRK1α proteins. (A) The Neighbor-Joining tree was constructed with MEGA7 using full length amino acid sequences of 3 fungi SNF1s (yellow branches), 4 animals AMPKαs (red branches) and 21 plant SnRK1αs (green branches). The percentage of replicate in the bootstrap test (1000 replicates) are shown next to the branches. Organisms: *Saccharomyces cerevisiae* (Sc), *Fusarium graminearum* (Fg), *Fusarium oxysporum* (Fo), *Rattus norvegicus* (Rn), *Homo sapiens* (Hs), *Arabidopsis thaliana* (At), *Oryza sativa* (Os) and *Triticum aestivum* (Ta). (B) Heat map of the expression profiles of wheat *SnRK1α* (*TaSnRK1α*) genes at different developmental stages and tissues of cultivar Chinese Spring. The chromosome (Chr) location and the four groups of homoeologs (1–4) are indicated above *TaSnRK1α1* genes number. TPM (Transcripts Per kilobase Million) values representing the level of expression are indicated. Data were extracted from Wheat Expression Browser [92] and correspond to the experiment [56] (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(TraesCS3D02G491800.1) because the encoded protein did not include the Prosite signatures of a functional SnRK1α kinase domain (Protein kinases ATP-binding region signature (PS00107) or the serine/threonine protein kinases active-site signature (PS00108). The 15 wheat SnRK1α (*TaSnRK1α*) genes are presented in Supplementary Table S1, while Supplementary Table S3 details their chromosome location, splice variants, predicted molecular weights and their characteristic functional SnRK1α domain composition. We used an unambiguous nomenclature to name each TaSnRK1α similar to that of AMPK and Arabidopsis SnRK1αs [35], including the homoeology and the nature of the chromosome. Based on homoeolog analysis using the Ensembl Plants resource [55], *TaSnRK1αs* were found to form 4 groups of homoeologs. This clustering agreed with the results obtained via phylogenetic analysis that was conducted to gain an insight into the evolutionary relationships of TaSnRK1α proteins. For the latter, the full-length amino acid sequences and of those of SNF1s, AMPKαs and SnRK1αs from Arabidopsis (AtSnRK1α) and rice (OsSnRK1α) were used to construct a Neighbor-Joining tree (Fig. 1A). Three of the 4 groups represent triads of the A, B, and D genome homoeologs; these are on chromosomes 1 (*TaSnRK1α1-A*, *TaSnRK1α1-B* and *TaSnRK1α1-D*), 3 (*TaSnRK1α2-A*, *TaSnRK1α2-B* and *TaSnRK1α2-D*) and 4 (*TaSnRK1α3-A-1*, *TaSnRK1α3-B* and *TaSnRK1α3-D*), with chromosome 4 also encoding an additional homoeolog (*TaSnRK1α3-A-2*). The fourth group comprises homoeologs located on chromosome 3B and 3D (*TaSnRK1α4-B-1*, *TaSnRK1α4-B-2* and *TaSnRK1α4-D*). Finally, two *TaSnRK1α* genes (*TaSnRK1α5-B* and *TaSnRK1α6-B*) that formed a distinct cluster are present on chromosome 3B. As expected, TaSnRK1α proteins clustered with rice SnRK1αs (Fig. 1A), indicating an independent evolution of monocotyledon SnRK1αs compared to Arabidopsis SnRK1αs, as previously shown [35].

Using publicly available RNAseq data for wheat [56], we analysed the developmental and tissue-specific expression profile of *TaSnRK1αs* and found that homoeolog groups 1 to 3 are expressed ubiquitously in different wheat organs and at different developmental stages (Fig. 1B). Overall, within those groups, each homoeolog exhibits a similar pattern and level of expression, apart from *TaSnRK1α3-A-1*, which has a lower expression level than the others from group 3. Group 4 homoeologs presented a different profile, with two of the three members being expressed specifically in the grain, and the third, *TaSnRK1α4-B-1* along with *TaSnRK1α5-B* and *TaSnRK1α6-B* having very low expression in all tissue and developmental stages (≤ 0.6 TPM (Transcripts Per kilobase Million)).

3.2. DON treatment has either no effect or downregulates the transcription of TaSnRK1α genes

Previous studies within our laboratory identified a DON-responsive gene in spikes that codes for an interacting protein of TaSnRK1α1-A [16]. Illustrated from *in silico* analysis in Fig. 1B, *TaSnRK1α1-A* and homoeologs *TaSnRK1α1-B and TaSnRK1α1-D* (hereafter *TaSnRK1α1*) are expressed in spike as their closest phylogenetic groups (*TaSnRK1α2-A*, *TaSnRK1α2-B*, *TaSnRK1α2-D*; hereafter *TaSnRK1α2*) and (*TaSnRK1α3-A-2*, *TaSnRK1α3-B* and *TaSnRK1α3-D*, hereafter *TaSnRK1α3*). Thus, we designed distinct qRT-PCR assays for these three groups and used these to assess the effect of DON treatment on their transcription in flowering spikes of the wheat cv. CM82036 at 1–3 days post-toxin treatment. qRT-PCR analyses confirmed that both *TaSnRK1α1, TaSnRK1α2* and *TaSnRK1α3* were expressed in spikes (Fig. 2A-C). DON treatment did not affect the expression of *TaSnRK1α3* (Fig. 2C) and induced a downregulation relative to mock treatment at 2 days post-inoculation (dpi) or for all time points for *TaSnRK1α1* and *TaSnRK1α2*, respectively (Fig. 2A-B).

3.3. DON treatment enhanced TaSnRK1α activity

The effect of DON on the amount and kinase activity of TaSnRK1αs in wheat heads was assessed. To test TaSnRK1α activity, we used the AMARA peptide kinase activity assay. AMARA peptide is a substrate used to detect plant SnRK1α activity [57] and was successfully used before to detect SnRK1α activity in different wheat tissues [26,58]. While the AMARA peptide kinase activity measured in *A. thaliana* is attributed to SnRK1αs [59,60], AMARA peptide can be a substrate for SnRK2 proteins [60]. Thus, we also assessed the amount of TaSnRK1α in wheat heads via western blot analysis using two antibodies: one

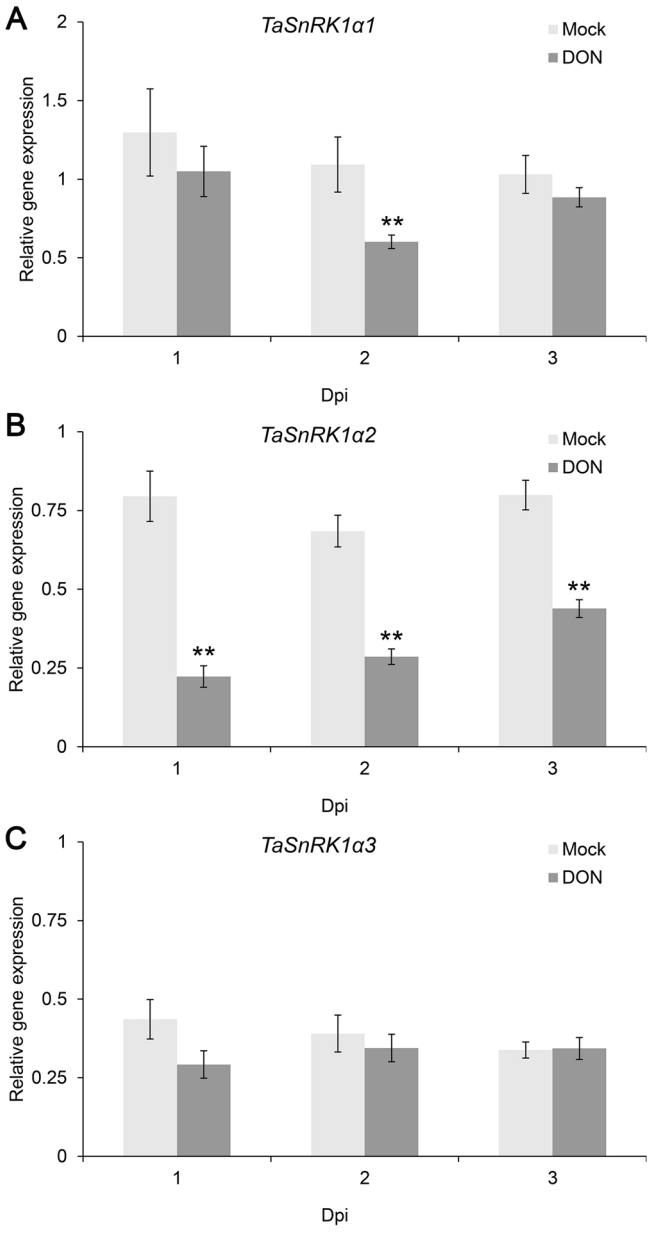

Fig. 2. *TaSnRK1α1, TaSnRK1α2* and *TaSnRK1α3* gene expression levels in wheat heads after treatment with DON. Wheat spikelets were treated with either DON or Tween-20 (mock) and harvested from 1 to 3 days post inoculation (dpi). (A) *TaSnRK1α1,* (B) *TaSnRK1α2* and (C) *TaSnRK1α3* gene expression levels was assessed via qRT-PCR. The *TaPP2AA3* and *TaYLS8* genes were used as internal reference to calculate the relative gene expression using the equation $2^{-(\text{Ct target gene} - \text{Ct average of housekeeping genes})}$. Data represent the mean of three independent trials (each includes two technical replicates per treatment from a pooled of 4 biological sample) and error bars represent ± SEM. Significant differences are indicated with an asterisk (**, $P < 0.01$).

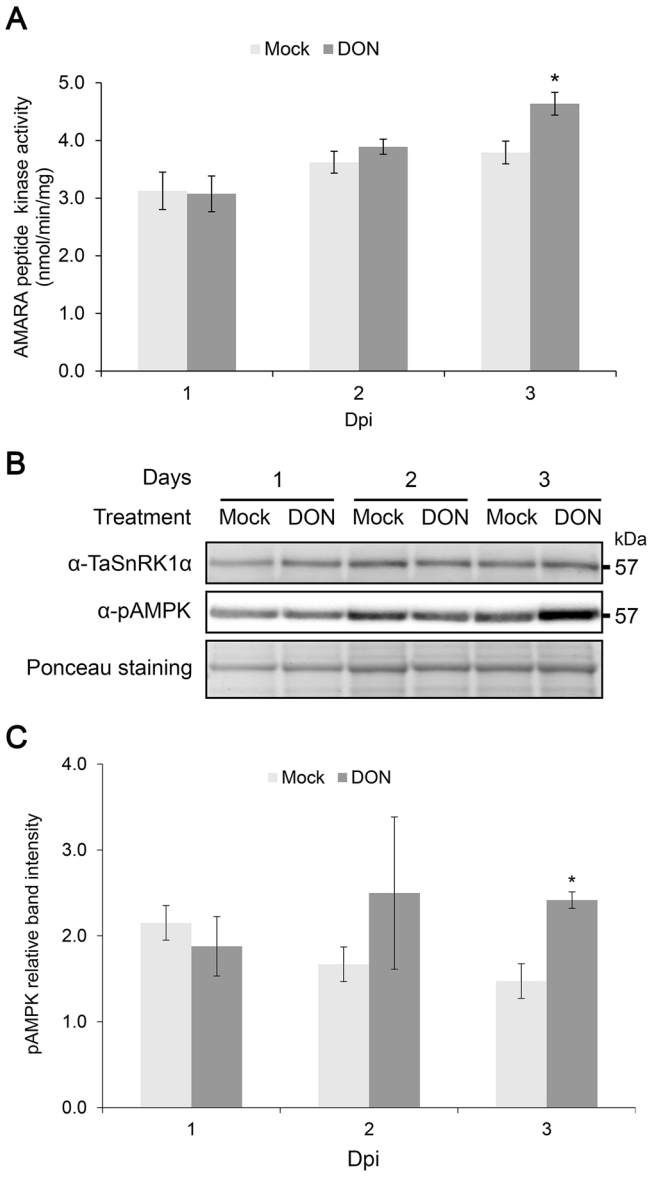

Fig. 3. Kinase activity and TaSnRK1α phosphorylation state in wheat heads after treatment with DON. Protein isolated from wheat spikelets treated with either DON or Tween-20 (mock) from 1 to 3 days post inoculation (dpi) were used to measure (A) AMARA peptide kinase activity and (B, C) phosphorylation state of TaSnRK1α activated on the conserved Threonine residue (Thr-172). (A) AMARA peptide kinase activity was estimated using the AMARA peptide as substrate. (B) TaSnRK1α and phosphorylated TaSnRK1α were detected by western blot using specific antibodies anti-TaSNRK1α (α-TaSnRK1α) or anti-phospho-AMPKα (α-pAMPK) antibodies, respectively. Images correspond to the region contained between the 50 and 60 kDa molecular weight markers. Using molecular weight standards, the size of the protein band detected with α-TaSnRK1α and α-pAMPK antibodies was estimated at 57 kDa. (C) Quantitative level of phosphorylated TaSnRK1α was measured by the relative band intensity obtained with α-pAMPK antibody and normalized with total protein visualization using Ponceau S staining. Data represent the mean of three independent trials (each includes four technical replicates per treatment from a pooled sample). Error bars represent SEM. Significant differences are indicated with an asterisk (*, $P < 0.05$).

raised against a peptide from the protein kinase catalytic domain and Phospho-Thr-172-AMPKα antibody (α-pAMPK) specific to activated phosphorylated TaSnRK1α (pTaSnRK1α) [61–63]. Based on epitope locations and protein sequence similarity, both antibodies are predicted to detect all TaSnRK1αs (Supplementary Fig. S1). As expected, in all wheat tissues tested and for both antibodies a similar band was detected at 57–59 kDa (Supplementary Fig. S2), corresponding to the majority of predicted TaSnRK1α protein sizes (Supplementary Table S3). Several other proteins were detected and may correspond to smaller TaSnRK1α, TaSnRK1α splice variants (Supplementary Table S3) or unspecific binding to other wheat proteins. But for quantification purposes, we focused on one band with a molecular weight of 57–59 kDa as this was previously attributed to wheat SnRK1αs [26] and because TaFROG was shown to interact with the 57 kDa TaSnRK1α1-A [16].

Compared to mock treatment, DON increased the AMARA peptide kinase activity in spikelets at 2 and 3 dpi, with a significant difference

($P = 0.029$) of 18% for the latest time point (Fig. 3A). The increase of AMARA peptide kinase activity was also coincident with an increase in the level of activated TaSnRK1α observed. While the amount of TaSnRK1α proteins did not change over time in wheat spikelets treated or not with DON (Fig. 3B and Supplementary Fig. S3), the toxin treatment led to an increase in the amount of pTaSnRK1α at 3 dpi (Fig. 3B-C). Thus, we concluded that DON treatment of wheat spikelets enhances activated TaSnRK1α and also AMARA kinase activity which is most likely due to TaSnRK1α activity.

3.4. *TaSnRK1α genes contribute to DON tolerance in wheat spikes*

Virus-Induced Gene Silencing (VIGS) was used to determine if reducing *TaSnRK1αs* transcript levels altered the phenotypic response to DON in the toxin resistant cv. CM82036. Gene silencing was achieved using two non-overlapping constructs, BSMV:S1 or BSMV:S2, designed based on *TaSnRK1α1-A* gene sequence and predicted to efficiently silence *TaSnRK1α1* and potentially the closest phylogenetic group *TaSnRK1α2* (Fig. 1A). BSMV:00, the empty vector, served as a negative control. The efficacy of gene silencing was assessed via qRT-PCR assays that targeted either *TaSnRK1α1*, *TaSnRK1α2* or the outgroup *TaSnRK1α3*. In gene-silenced plants compared to control plants (BSMV:00), expression of *TaSnRK1αs* was reduced by 53–58% and 25–37% for *TaSnRK1α1* and *TaSnRK1α2*, respectively (Fig. 4A-B). On the contrary, no significant silencing of the outgroup *TaSnRK1α3* was observed (Fig. 4C). Therefore, VIGS reduced the transcript levels of *TaSnRK1α1* and, to a lesser extent, *TaSnRK1α2*. Using the same plant materials, the effect of the gene silencing on TaSnRK1α activity was evaluated by measuring AMARA peptide kinase activity. In silenced plants, the AMARA peptide kinase activity was reduced compared to control plants for each independent VIGS trial (Fig. 4D and Supplementary Fig. S4). This indicates that reducing *TaSnRK1α1 and TaSnRK1α2* transcript levels decreased AMARA peptide kinase activity which is most likely and mainly the result of TaSnRK1α activity. DON treatment induces systemic premature bleaching of spikelets [64], and the effect of VIGS on this phenotype was assessed by quantifying the level of DON-damaged spikes on the heads from the VIGS trial at 14 days post-toxin treatment (Fig. 4E). Gene silencing of *TaSnRK1αs* with either BSMV:S1 or BSMV:S2 led to more DON-induced damage of spikelets than observed for control plants treated with BSMV:00 (Fig. 4E). Quantification of damaged spikelets showed that silencing of *TaSnRK1αs* resulted in a 69 and 106% increase in the number of DON-damaged spikelets compared to the control (BSMV:00) for BSMV:S1 and BSMV:S2, respectively (Fig. 4F). These results indicate that *TaSnRK1α* genes play a role in the toxin tolerance response.

4. Discussion

Resistance to FHB disease is complex; many quantitative trait loci (QTL) have been identified that can enhance resistance to FHB or DON [65–68]. The underlying genes remain elusive, and their identification will undoubtedly enhance our understanding of FHB resistance and its effective deployment in the field. The recently identified *TaFROG* orphan gene has been shown to contribute to both FHB and DON resistance. This gene did not co-locate with a known QTL for FHB resistance and the molecular mechanisms underlying its role are not yet known [16]. TaFROG is a highly intrinsically disordered protein without known protein domains and was shown to interact with a wheat SnRK1α (TaSnRK1α1) and a NAC-like transcription factor (TaNACL-D1) [16,17]. Like TaFROG, TaNACL-D1 contributes to FHB resistance and further experimentation is needed to determine if this is due to positive effects on DON tolerance (although the trend was for it to have a positive effect on DON tolerance, its impact was not significant) [17].

In this study, our main aim was to investigate the contribution of wheat SnRK1αs to DON tolerance, but, first, given the increasing evidence regarding the importance of this gene family in stress tolerance [28,29,33], we took advantage of the latest bread wheat genome annotation (IWGSC RefSeq v1.1) to investigate SnRK1α diversity in this important crop. We retrieved 15 *SnRK1α* genes from the genome that code for proteins with predicted characteristic functional domains of SnRK1αs (protein kinase domain, ubiquitin-associated domain and the

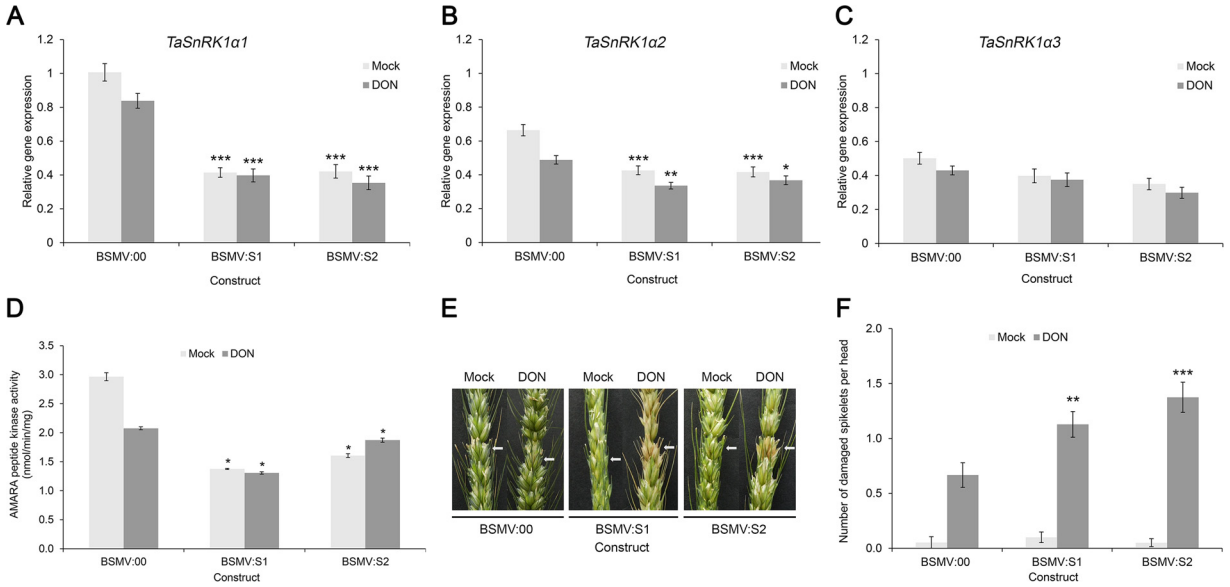

Fig. 4. Effect of *TaSnRK1α* silencing on DON wheat head tolerance. Virus-induced gene silencing (VIGS) was employed to silence *TaSnRK1α* genes in wheat heads. VIGS constructs used were empty vector (BSMV:00) or independent constructs targeting *TaSnRK1αs* (BSMV:1 and BSMV:2). Following VIGS treatment of the flag leaves, at mid-anthesis two central spikelets were treated with either Tween-20 (mock) or DON. Gene silencing of *TaSnRK1αs* in wheat heads was quantified by qRT-PCR analysis using primers specific to (A) *TaSnRK1α1*, (B) *TaSnRK1α2* and (C) *TaSnRK1α3* genes. The *TaPP2AA3* and *TaYLS8* genes were used as internal reference to calculate the relative gene expression using the equation $2^{-(\text{Ct target gene} - \text{Ct average of housekeeping genes})}$. (D) Effect of *TaSnRK1α* gene silencing on AMARA peptide kinase activity. Visualization (E) and quantification (F) of the DON-damaged spikelets. Data represent the mean of two independent trials (each includes 10–20 biological replicates per treatment) except for (D) where the mean represent of four technical replications per treatment from a pooled biological sample from one representative VIGS trial. Error bars indicate SEM and significant differences are indicated with an asterisk (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

kinase associated domain 1) [35,69]. Rice, maize and Brachypodium each appear to have three SnRK1αs [35,70–72]. The larger diversity of wheat SnRK1αs (TaSnRK1αs) can be partially explained by the hexaploid nature of the genome and the existence of sub-genome-specific homoeologs for most wheat SnRK1αs. Gene duplication events might also explain the expansion of TaSnRK1αs in the wheat genome. Our analysis of the evolutionary relationship of wheat, Arabidopsis and rice SnRK1α proteins indicates that wheat and rice SnRK1αs clustered in a group distinct to those from Arabidopsis. This suggests, as described by [35,72], that *Poales* SnRK1αs evolved and diversified independently from *Brassicaceae*. Based on gene expression patterns and phylogeny, some plant SnRK1αs were previously described to be specific to cereals (named SnRK1b/B) and shown to be expressed mostly in seeds [73,74]. Similarly, our analysis indicated that two *TaSnRK1αs* (*TaSnRK1α4-B-2 and TaSnRK1α4-D*) are predominantly expressed in seeds. On the other hand, many other *TaSnRK1αs* (groups *TaSnRK1α1–3*) are ubiquitously expressed and others (*TaSnRK1α3-A-1, TaSnRK1α4-B-1, TaSnRK1α5-B and TaSnRK1α6-B*) have very low expression in all tissues.

DON is not only harmful for plants but also for animal and human health. The cytotoxicity of DON is closely related to intracellular reactive oxygen species (ROS) production, the ribotoxic stress response, and the induction of apoptosis that can lead to immunosuppression [75–78]. Analysis of the effect of DON on *TaSnRK1αs* gene expression in the FHB/DON resistant wheat cv. CM82036 revealed a significant down-regulation in gene expression at one time point for *TaSnRK1α1* and at all three time points for *TaSnRK1α2* genes, while *TaSnRK1α3* genes were unaffected by the toxin. This suggests that *TaSnRK1αs* expression is not greatly affected by DON. Most reports indicate that *SnRK1α* genes are not regulated by environmental factors or nutrient status, except that Arabidopsis *AtSnRK1α2* and maize *ZmSnRK1α1–3* expression was induced by the sugar trehalose or in dark conditions, respectively [79,80]. DON increased the activity of TaSnRK1α proteins and enhanced AMARA peptide kinase activity in wheat heads. Most of the calcium-independent phosphorylation of the synthetic AMARA peptide is associated with SnRK1α activity and thus it is a good indicator of this protein activity [60]. Immunodetection of phosphorylated TaSnRK1α proteins supported the AMARA peptide kinase activity results that showed more phosphorylated TaSnRK1α (active form) present in DON-treated tissue. Additionally, silencing of *TaSnRK1α1-2* in wheat heads resulted in a decrease in AMARA peptide kinase activity, supporting the link between the kinase activity and SnRK1α. The possibility that the enhanced kinase activity in response to DON is a component of the defence response was validated by the VIGS experiment where gene silencing of *TaSnRK1α1-2* enhanced the phytotoxic effects of the toxin. This is the first time that plant SnRK1αs have been associated with DON or toxin defence mechanisms in plants.

it is possible that *TaSnRK1α* has a function similar to its human orthologue AMPKα. Previously, It has been shown that a rapid phosphorylation and dephosphorylation occurred on a ß subunit of AMPK kinase complex after macrophages were treated with DON [81]. In intestinal epithelial cells, DON treatment increases pAMPKα (pThr172) similarly to pTaSnRK1α, and induces autophagy in an IKK complex- and AMPKα-dependent way to protect intestinal homeostasis against DON by reducing ROS levels and maintaining the cellular stress response [82]. In wheat, a high concentration of DON infiltrated in stem tissues has been shown to increase hydrogen peroxide levels [83]. Gene expression studies have identified genes encoding ROS scavenging enzymes to be up-regulated in response to DON [84,85]. Autophagy is linked to tolerance of oxidative stress in Arabidopsis [86] and there is evidence that Arabidopsis SnRK1α (KIN10) controls the expression of autophagy-related (*ATG*) genes and positively regulates autophagy [87,88]; therefore, it is tempting to speculate that, like the AMPKα in animal cells, SnRK1α may help to protect plant tissue against DON by reducing ROS level via autophagy. Programmed cell death (PCD) is another potential mechanism where SnRK1α might contribute to DON tolerance. There are several studies demonstrating that DON induces PCD in plant tissue [83,89] and the SnRK1 complex has been implicated in PCD [90,91].

In recent years it has become very clear that SnRK1α is an important player in defence mechanisms against a variety of pathogens [28,29]. In our study, using virus induced gene silencing, SnRK1αs immunodetection and kinase activity, we provide the first evidence that SnRK1αs contribute to plant tolerance to the fungal mycotoxin DON. Future work will assess whether SnRK1αs consequently also contribute to plant FHB resistance and the molecular mechanisms behind the mycotoxin tolerance. As TaSnRK1α1-A interacts with TaFROG [16], and because both contribute to DON tolerance, it's likely that these proteins serve the same biological pathway in response to the toxin.

In conclusion, wheat SnRK1α is a large and diverse family. Based on gene expression, TaSnRK1α kinase activity and *TaSnRK1αs* gene silencing we demonstrated that wheat SnRK1αs play a role in the *Fusarium* toxin response and tolerance. Future studies on SnRK1α functional specification/redundancy and upstream/downstream signalling components will complete our understanding of the role of SnRK1 in plant defence and may uncover interesting tools for crop improvement to prevent disease losses.

Author contributions

A. Perochon and F. M. Doohan conceived and designed the project. A. Perochon, F. M. Doohan, N. G. Halford, M.J. Paul supervised the experiments. A. Perochon, K. B. Malla and Z. Váry performed the experiments and the data analysis. A. Perochon, Z. Váry. and F. M. Doohan wrote the manuscript with contributions from other authors.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2019.110217.

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