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RESEARCH PAPER

Regulation of secondary wall synthesis and cell death by NAC transcription factors in the monocot *Brachypodium distachyon*

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Abstract

In several dicotyledonous species, NAC transcription factors act as master switches capable of turning on programmes of secondary cell-wall synthesis and cell death. This work used an oestradiol-inducible system to overexpress the NAC transcription factor *BdSWN5* in the monocot model *Brachypodium distachyon*. This resulted in ectopic secondary cell-wall formation in both roots and shoots. Some of the genes upregulated in the process were a secondary cell-wall cellulose synthase (*BdCESA4*), a xylem-specific protease (*BdXCP1*) and an orthologue of *AtMYB46* (*BdMYB1*). While activation of *BdMYB1* may not be direct, this study showed that *BdSWN5* is capable of transactivating the *BdXCP1* promoter through two conserved binding sites. In the course of *Brachypodium* development, the *BdXCP1* promoter was observed to be active in all types of differentiating tracheary elements. Together, these results suggest that *Brachypodium SWNs* can act as switches that turn on secondary cell-wall synthesis and programmed cell death.

Key words: *Brachypodium distachyon*, cell death, monocot, NAC transcription factor, secondary wall, xylem.

Introduction

The evolution of xylem was a key innovation that allowed land plants to colonize dry habitats. The tracheary elements have thick secondary cell walls that allow them to withstand the negative pressures necessary to elevate groundwater to aboveground organs (Sperry, 2003). The energetic cost of this transport system is minimal since mature tracheary elements undergo programmed cell death (Fukuda, 2000). In addition to tracheary elements with discontinuous secondary cell walls that can facilitate organ elongation and water movement, fibres with more uniform secondary walls are frequent in organs that require additional support (Zhong and Ye, 2009).

The main components of the angiosperm secondary cell wall are cellulose, xylan, and lignin. Specific cellulose synthases, together with additional proteins that have yet to be identified, are responsible for secondary cell-wall cellulose biosynthesis (Endler and Persson, 2011). The differentiation of xylem vessels also involves a mechanism of programmed cell death (Bollhöner *et al*., 2012). Hydrolytic enzymes, which accumulate in the vacuole in parallel with cell-wall synthesis, are released when the membrane is ruptured, resulting in autolysis of the cell contents. Among the *Arabidopsis* enzymes involved in this process are XCP1 and XCP2, two closely related cysteine proteases specific to tracheary elements (Avci *et al*., 2008).

The network of transcription factors that regulate the process of xylem differentiation has been extensively studied, particularly in *Arabidopsis* (Caño-Delgado *et al*., 2010). Seven VND genes from the NAC family of transcription factors

are expressed during xylem vessel development (Kubo *et al.*, 2005). Overexpression of either VND6 or VND7 results in ectopic transdifferentiation of many different cell types into vessel elements with their characteristic pattern of secondary cell-wall deposition (Kubo *et al.*, 2005). A large number of putative downstream targets of VND6 and VND7 have been identified (Ohashi-Ito *et al.*, 2010; Zhong *et al.*, 2010b; Yamaguchi *et al.*, 2011). In addition to transcription factors, some of the direct targets are genes involved in the synthesis of cellulose or in programmed cell death, such as *XCP1*.

Both VND7 and VND6 can bind to secondary wall NAC-binding elements (SNBEs), which in some cases encompass the previously identified tracheary element-regulating *cis*-element (TERE) (Ohashi-Ito *et al.*, 2010; Zhong *et al.*, 2010b; Yamaguchi *et al.*, 2011). The SNBE consensus sequence was identified as WNNYBTNNNNNNNAMGNHW using SND1, a NAC transcription factor expressed in fibres which is closely related to VNDs and shares many of their targets (Zhong *et al.*, 2010b). It has been proposed that secondary wall NACs (SWNs) first appeared to regulate the differentiation of tracheids in early vascular plants and latter diversified to regulate other cell types with secondary walls, such as fibres (Zhong *et al.*, 2010a).

The regulation of secondary cell-wall synthesis has an obvious interest for the manipulation of biomass production in second-generation biofuel crops (Demura and Ye, 2010). Some of the more promising species for biomass production are members of the grass family, which includes the model *Brachypodium distachyon* (Mur *et al.*, 2011). The programme of secondary cell-wall formation is expected to be somewhat similar in grasses and dicots, as the composition of their secondary walls is quite comparable, in contrast with the large divergence found in primary walls. However, some important differences have been found, such as a higher proportion of *p*-hydroxyphenyl (H) units in grass lignin (Vogel, 2008). Additionally xylan contains more arabinose side chains in grasses than in dicots and it is unclear whether both groups use the same synthesis mechanism (Faik, 2010; Scheller *et al.*, 2010). Another open question is whether grasses use different sets of genes to build xylans in primary and secondary walls.

The transcriptional regulation of secondary wall biosynthesis in grasses is very poorly understood (Handakumbura and Hazen, 2012). Coexpression analyses in rice and barley suggested the possible involvement of SWNs in this process (Christiansen *et al.*, 2011; Ruprecht *et al.*, 2011). In addition, it has been recently shown that rice and maize SWNs are expressed in xylem and fibres and that, when overexpressed in *Arabidopsis*, they can induce the formation of ectopic secondary walls (Zhong *et al.*, 2011).

This work reports that inducible overexpression in *B. distachyon* of the NAC transcription factor *BdSWN5* was sufficient to activate secondary cell-wall formation. Among the genes upregulated in the process were a secondary cell-wall cellulose synthase and *BdXCP1*, an orthologue of *AtXCP1*. This study also found that the *BdXCP1* promoter is normally active in differentiating tracheary elements and that BdSWN5 can directly regulate *BdXCP1* transcription by binding two conserved SNBE sites. These findings establish that NAC transcription factors can work as secondary cell-wall master switches in monocots and can be used to study and manipulate this process.

Materials and methods

Phylogeny

Sequences were aligned with CLUSTALW and phylogenetic trees obtained with MEGA5 (Tamura *et al.*, 2011). Trees were constructed by neighbour joining using Poisson distances and bootstrap values estimated from 500 replicates.

Genetic constructions

Brachypodium genomic DNA or cDNA was amplified with PrimeSTAR HS (Takara) using primers detailed in Supplementary Table S1 (available at *JXB* online). Coding sequences and promoter regions were cloned respectively in pENTR/D and pENTR5' (Invitrogen). Bd*SWN* sequences obtained from cDNA were submitted to Genbank (accession numbers JQ693422–JQ693429). Final constructs were assembled using LR Clonase II (Invitrogen). For overexpression in tobacco, the destination vector was pMDC32 (Curtis and Grossniklaus, 2003). For promoter–reporter constructs, the intron-containing glucuronidase entry clone pEN-L1-SI*-L2 was used (Karimi *et al.*, 2007). This clone was combined with promoter entry clones into pERV2, a double recombination vector obtained by cloning a *Hin*dIII–*Sal*I fragment from pH7m24GW,3 (Karimi *et al.*, 2007) into pIPKb001 (Himmelbach *et al.*, 2007).

For oestradiol-inducible expression in *Brachypodium*, pERV1 plasmid was constructed using as a backbone pIJB2, a plasmid kindly provided by Oumaya Bouchabké-Coussa (Institut Jean-Pierre Bourgin, INRA) with the *Sfi*I fragment from pIPKb002 (Himmelbach *et al.*, 2007) inserted into p7U, conferring Basta resistance (DNA Cloning Service). A PCR fragment containing the LEXA promoter was amplified from pMDC7 (Curtis and Grossniklaus, 2003) and cloned between the *Spe*I and *Hin*dIII sites of pIJB2. The actin intron from pIPKb003 was then cloned between the *Hin*dIII and *Avr*II sites in the previous construct. Finally, the XVE coding sequence was amplified from pMDC7 and cloned between the *Psp*XI and *Spe*I sites. An entry clone of *BdSWN5* with its intron was recombined with this vector.

The *BdXCP1* 297-bp promoter fragment in pENTR5' was used as template to obtain mutated variants by megaprimer PCR (Sarkar and Sommer, 1990). To introduce a mutation at the SNBE1 site, a megaprimer was amplified with XSNBE1R1 and PENTR5L3. In the second amplification, PENTR5R3 was added to obtain a final product that included *att*L4 and *att*R1 sites. SNBE2 mutations were introduced by using XSNBE2L1 and PENTR5R3 to produce a megaprimer, adding PENTR5L3 in the second round. Double mutants were obtained by applying the two procedures sequentially. The PCR products were used directly in LR recombination reactions and the final constructs sequenced. Constructs were introduced into *Agrobacterium tumefaciens* EHA105 (pMDC32) or AGL1 (other plasmids).

Tobacco transformation

Nicotiana tabacum cv. Petit Havana was grown in soil for 4–6 weeks before infiltration with a suspension of transformed *A. tumefaciens* cells in infiltration media (10 mM $MgSO_4$, 10 mM MES at pH 5.5, 0.5% glucose, 100 μM acetosyringone). The OD_{600} of the suspension was 0.1 for overexpression and 0.05 per construct for transactivation.

For overexpression experiments, infiltrated sections were harvested after 5 days and clarified with 70% ethanol and lactic acid. For transactivation experiments, two discs (1 cm diameter) were harvested after 2 days and GUS activity against 4-MUG was measured

(Kim *et al*., 2006). All experiments were repeated at least twice with similar results.

SYTOX staining

Leaf discs (1 cm) were incubated in 1 µM SYTOX Orange (Invitrogen) for 15 minutes under vacuum (80 kPa). After several washes, discs were observed on a Leica MZ16 fluorescence stereomicroscope equipped with a GFP Plus filter (460–500 nm excitation and 510 nm longpass barrier filter).

Brachypodium *growth and transformation*

B. distachyon plants (Bd21-3) were grown under a 16/8 light/dark cycle at 24/20 °C and 50–60% humidity. *Agrobacterium*-mediated transformation was carried out as previously described (Vogel and Hill, 2008) with minor modifications. Briefly, immature seeds were sterilized with 2% bleach and embryos were excised and placed on callus induction media (CIM). Calli were maintained at 26 °C in the dark and subcultured twice before transformation. *Agrobacterium* cultures scraped from 2-day-old YT plates were resuspended in liquid CIM with 200 µM acetosyringone and the OD_{600} adjusted to 0.6. About 90 calli per construct were incubated in *Agrobacterium* suspension for 5 minutes and placed for 5 days on empty plates with sterile filter paper and 750 µl of liquid CIM. Calli were transferred to CIM plates with 150 mg l^{-1} Timentin for 1 week and then for 2 weeks at a time to CIM plates with 150 mg l^{-1} Timentin and either 5 mg l^{-1} ammonium glufosinate (oestradiol-inducible) or 40 mg l^{-1} hygromycin (double recombination). After 6 weeks of selection, calli were moved to regeneration media and incubated at 24 °C in 16 h days. Regenerating plantlets were transferred to 50 ml glass culture tubes containing media with 0.5 × MS salts with vitamins, 3% sucrose, 0.7% activated charcoal, 0.2% phytagel, and 0.05% MES at pH 5.7. After transfer to soil, plants carrying T-DNA insertions were identified by PCR using Phire Plant Direct PCR Kit (Thermo Scientific) and the primers detailed in Supplementary Table S1.

Induction assay

Brachypodium seeds were sterilized with 2% bleach and placed in 250 ml flasks containing 100 ml of 0.25 × MS salts, 0.5 g l^{-1} MES at pH 5.8 and 1% sucrose. Flasks were placed in a rotary shaker at 125 rpm under a 16/8 light/dark cycle at 24/20 °C. After 4 h of light, flasks were kept in the dark for 44 h. Seedlings were induced by adding 100 µl of 10 mM β-oestradiol in DMSO or mock induced with 100 µl of DMSO. After 16 h in the same conditions, root tips (approx. 8 µm) and aerial parts were collected separately for RNA extraction.

Gene expression

RNA was extracted using a Plant Total RNA kit (Sigma). cDNA was synthesized using the Transcriptor First Strand cDNA synthesis Kit (Roche). End-point PCR was carried out for 40 cycles with MyTaq (Bioline). Real time PCR was performed for 40 cycles on the Mini Opticon Real-Time PCR system (Bio-rad) using SensiMix SYBR No-Rox Kit (Bioline). Amplification efficiency and C_t values were calculated with LinRegPCR (Ruijter *et al*., 2009). Statistical analysis was done with REST (Pfaffl *et al*., 2002) using *UBC18* (*Bradi4g00660*) and *SamDC* (*Bradi5g14640*) as references. Primers and annealing temperatures are detailed in Supplementary Table S1.

Electrophoretic mobility shift assay

*Bd*SWN coding regions were introduced into pET300/NT-DEST (Invitrogen) and used to transform *Escherichia coli* BL21 DE3 (Novagen). Cell cultures (200 ml) were grown to an OD_{600} of 0.7–1.0 and induced with 1 mM IPTG for 6 h at 24 °C. Cells were harvested and frozen at –80 °C. Proteins were extracted with B-PER Reagent (Pierce) with 10% glycerol, 300 mM NaCl, 5 mM imidazole, and Halt Protease Inhibitor Cocktail EDTA-free (Pierce). Purification was carried out with Dynabeads His-Tag Isolation and Pulldown Kit (Novex). Probes and competitors were amplified by PCR or obtained by annealing complementary primers (Supplementary Table S1). Labelling was incorporated with 5'-end biotin-labelled primers. Electrophoretic mobility shift assays were performed using the LightShift Chemiluminescent EMSA Kit (Pierce). Labelled promoter fragments (10 fmol) or labelled SNBE motifs (20 fmol) were incubated with 150 or 300 ng of protein, respectively, for 20 min in 10 µl of binding buffer (10 mM Tris at pH 7.5, 100 mM KCl, 1 mM DTT, 100 ng l^{-1} poly dI-dC, 0.05% Nonidep P-40). DNA was separated by polyacrylamide gel electrophoresis on 0.5 × Tris-borate-EDTA gels and electroblotted onto Byodine B nylon membranes (Pierce).

β-*Glucuronidase histochemical staining*

Tissue samples were incubated in 90% acetone at 4 °C for 20 minutes and transferred to staining buffer (100 mM sodium phosphate at pH 7, 10 mM EDTA, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.1% Triton-X, and 1 mM X-gluc) and placed under vacuum for 10 minutes. After incubation at 37 °C for approx. 4 hours, samples were cleared in 70% ethanol and lactic acid.

Results

The Brachypodium *genome includes eight* SWN *genes that are expressed in developing organs*

A phylogenetic analysis of NAC transcription factors in the *Brachypodium* genome (*Brachypodium* Genome Initiative, 2010) revealed the presence of eight *SWN* genes (Fig. 1A). Six of these genes (*SWN1* to *SWN6*) are orthologues of *Arabidopsis VND* genes and two (*SWN7* and *SWN8*) are orthologues of *SND*/*NST* genes. The phylogeny shows that at least four clades were already differentiated in the last common ancestor or monocots and dicots. The coding regions of all eight genes were amplified from cDNA and sequenced, allowing the correct annotations to be determined (Supplementary Table S2).

The expression level of all eight genes was tested by reverse trancription-PCR in developing roots, leaves, and internodes (Fig. 1B). All the genes were detected in internodes, with *SWN3*, *SWN4*, *SWN6*, and *SWN7* reaching their highest level of expression in this organ. On the other hand, expression levels in leaves were generally low or undetectable, with the exception of *SWN5* and *SWN8*. Expression in roots was also generally lower than in other organs with the exceptions of *SWN1* and *SWN2*.

Brachypodium SWN *genes can induce ectopic cell-wall formation in tobacco*

The eight *Brachypodium SWN* genes were transiently expressed in tobacco leaves via infiltration with *Agrobacterium* cultures carrying overexpression constructs. The *SWN* genes were under the control of two copies of the CaMV 35S promoter. After five days of co-cultivation, expression of all of the *SWN* genes had resulted in the ectopic formation of spiral or annular cell-wall thickenings in mesophyll and epidermal cells (Fig. 2). The percentage of cells with this type of

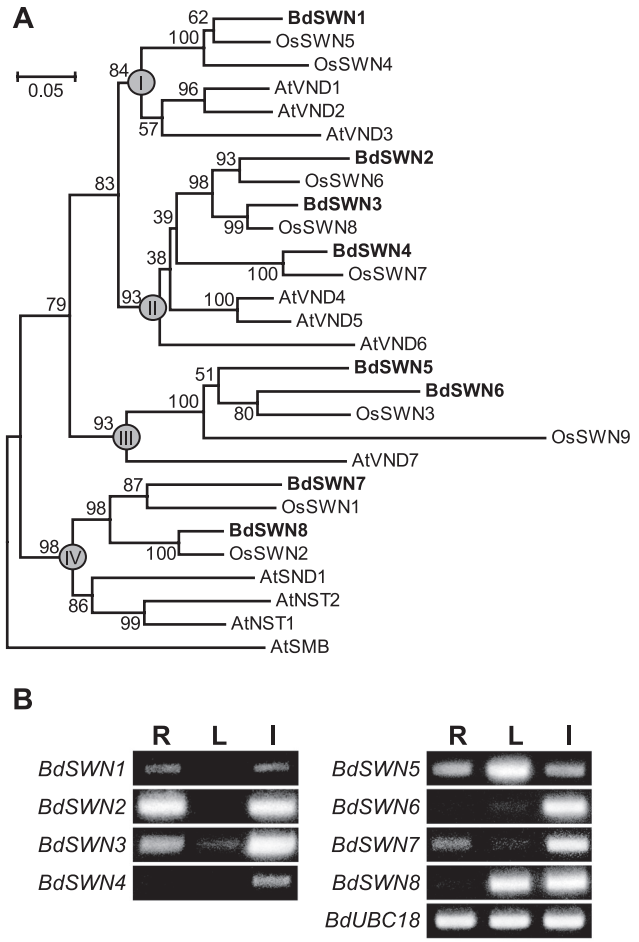

Fig. 1. (A) Phylogenetic tree of SWN proteins from *Brachypodium*, rice, and *Arabidopsis*. Annotations for *Brachypodium* and rice genes can be found in Supplementary Table S1. Bootstrap values are shown at branch nodes. Circles indicate clades differentiated in the last common ancestor of monocots and dicots. (B) Reverse transcription-PCR analysis of *BdSWN* expression in developing roots (R), leaves (L), and internodes (I). *UBC18* was used as a control. Similar results were observed with a second biological replicate.

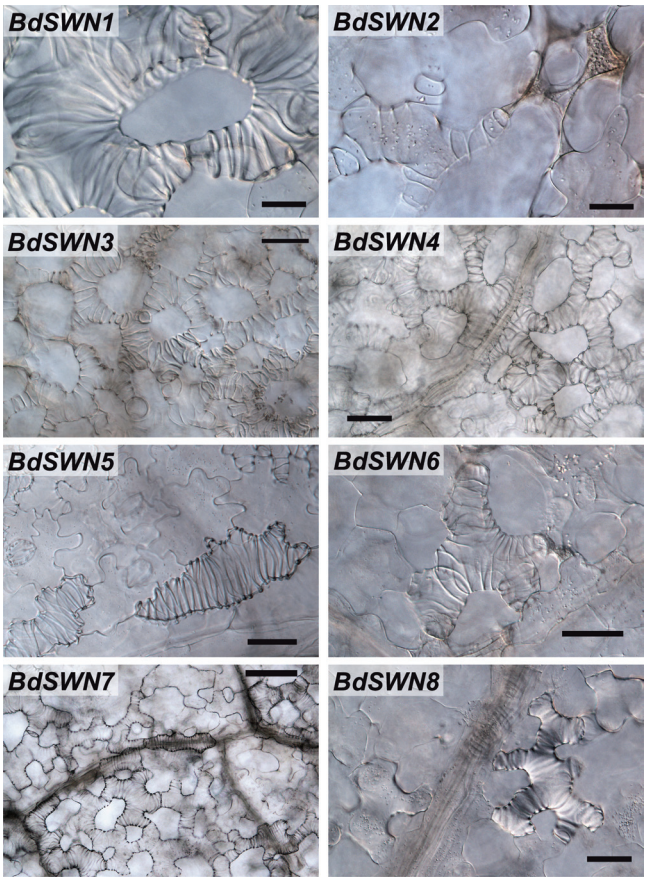

Fig. 2. Ectopic cell-wall formation in tobacco leafs induced by overexpression of different Bd-*SWN* genes. Bars = 100 μm (*SWN7*), 50 μm (*SWN3*, *SWN4*, and *SWN5*), and 25 μm (all others) (this figure is available in colour at *JXB* online).

response was particularly high in the cases of *SWN1*, *SWN3*, *SWN4*, and *SWN7*.

Transient expression of some *SWN* genes also resulted in extensive cell death after 5 days of cocultivation, as evidenced by loss of chlorophyll (Fig. 3A) and membrane permeabilization revealed by SYTOX staining (Fig. 3B). This response was consistently stronger when expressing genes in clades I and II (*SWN1*, *SWN2, SWN3*, or *SWN4*). On the other hand, transient expression of genes in clade IV (*SWN7* and *SWN8*) did not result in additional cell death beyond that caused by *Agrobacterium* infiltration. In some cases, cells died before any visible wall thickenings had been formed, resulting in the collapse of thin-walled mesophyll cells (Fig. 3C). This was particularly common when *SWN2* was transiently expressed, with the number of collapsed cells an order of magnitude above the number of cells with ectopic wall thickenings. Tobacco infiltrations were also performed with overexpression constructs for *SWN1–SWN6* that included the native introns within the coding region. Only in the case of *SWN5* was the intensity of the response increased, both for cell-wall formation and cell death (data not shown). *SWN5* is the only *BdSWN* gene with a single intron.

Inducible overexpression of SWN5 *is sufficient to cause ectopic cell-wall formation in* Brachypodium

The oestradiol-inducible transactivation system based on the XVE chimeric transcription factor was used to obtain *Brachypodium* lines with inducible overexpression of *BdSWN5*. The original vector is not suitable for grasses due to the G10-90 promoter resulting in low expression levels (Zuo *et al.*, 2006). In order to use this system in *Brachypodium*, the XVE transcription factor was placed under the control of the maize ubiquitin promoter in pIPKB002 (Fig. 4A). Additionally the actin intron from pPIKB003 was introduced in the 5'-untranscribed region after the O_{LexA}-46 promoter to increase the expression level. *BdSWN5* was selected as a phylogenetic orthologue of *AtVND7* that showed a strong effect in tobacco when the native intron was included. This intron was also included in the inducible overexpression construct.

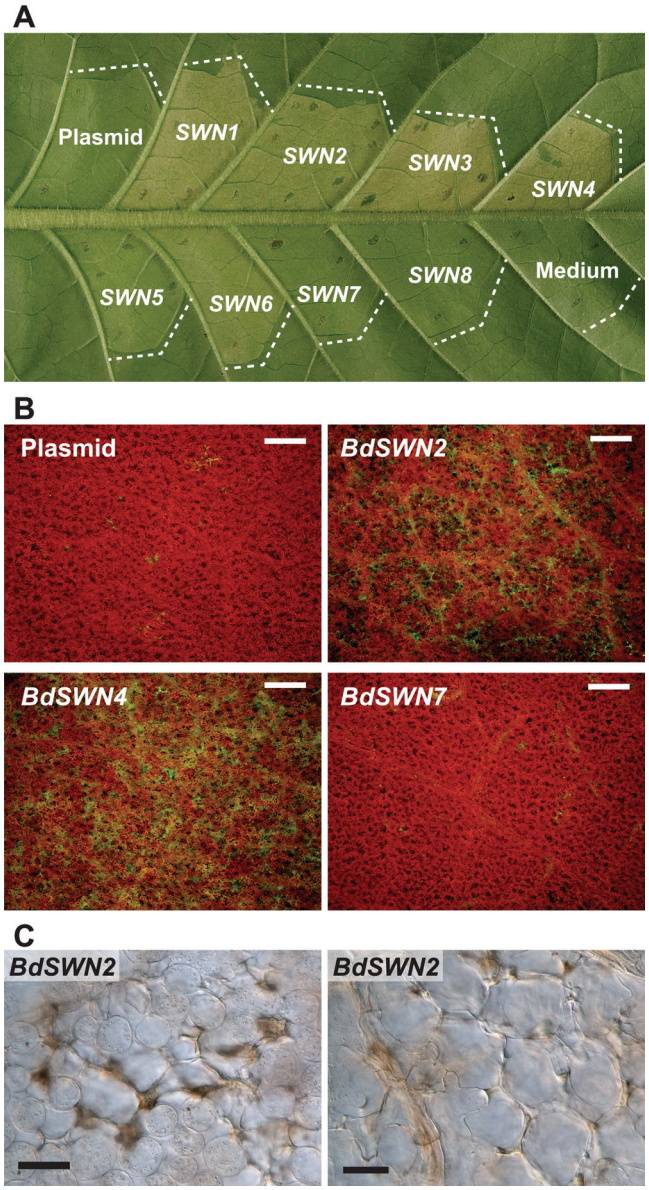

Fig. 3. Cell death in tobacco leafs induced by overexpression of different *BdSWN* genes. (A) Tobacco leaf infiltrated with *Agrobacterium* carrying an empty plasmid or different *SWN* overexpression constructs, as well as with infiltration medium. (B) SYTOX staining of non-viable cells (green) in the abaxial side of leafs transformed with different constructs. (C) Cell collapse caused by *SWN2* overexpression in the palisade and spongy mesophylls. Bars = 300 μm (B) and 50 μm (C).

Approximately 90 individual calli were transformed and 13 T_0 plants were regenerated from 10 independent calli. Plant growth was normal and most plants were fertile. To identify the lines with the strongest response seeds from nine independent lines (T_1 generation) were germinated on plates with 10 μM β-oestradiol and grown for 5 days. Five lines showed a very strong response with the development of the majority of the seedlings arrested shortly after germination, before the root had reached 5 mm (Fig. 4B). These seedlings showed accelerated vascular development as well as extensive ectopic cell-wall formation in roots, coleoptiles, and leaves, but not the coleorhiza (Fig. 4C and D). In addition, roots tended to curl and the root epidermal cells, which did not show ectopic wall formation, became partially detached from the cortex (Fig. 4C). Three other lines showed a milder response with most seedlings showing normal or partially reduced elongation, but with ectopic wall deposition in many cells, particularly in the root cortex and coleoptile. No phenotype was observed when wild-type seeds were germinated in the same plates.

Screening of T_1 plants from line 3, one of the lines with a strong response, indicated the presence of a single T-DNA locus, with an insertion in six out of 10 plants. Plant 3–4 was then identified as homozygous when all 26 T_2 descendants showed presence of the insertion. Pooled seeds from these T_2 plants were grown for 2 days in liquid culture in the dark before addition of β-oestradiol. In comparison with mock-induced seedlings, *SWN5* expression severely slowed down seedling development. After 24 hours of induction, ectopic cell-wall depositions in annular or scalariform patterns were visible in the coleoptile parenchyma of induced seedlings (Fig. 4E), but not in mock-induced seedlings (Fig. 4F). No changes were seen in the coleoptile epidermis or in developing leaves, in contrast to what had been observed in seedling germinated on oestradiol plates.

In the roots, addition of β-oestradiol resulted in an acceleration of vascular development with all types of xylem vessels differentiating simultaneously very close to the root tip, including the central metaxylem (Fig. 4G). In mock-induced seedlings, cell-wall thickenings in the central metaxylem started to appear more than 1 cm away from the tip after the cells had elongated to a length of approx. 350 μm (Fig. 4H). In addition, ectopic cell-wall formation was observed in the root cortex of induced seedlings starting before elongation (Fig. 4I). In mature cortex cells, the pattern of wall deposition was similar to that of pitted xylem vessels (Fig. 4J). Older parts of the roots, more than 1 cm away from the tip, did not show any changes, suggesting that only differentiating cells are capable of responding to *SWN5* expression.

SWN5 *overexpression can activate genes involved in secondary cell-wall synthesis and cell death*

To identify genes activated by *SWN5*, real-time PCR was used to compare expression levels in induced and mock-induced seedlings 16 h after induction (Fig. 4K). Seedlings were grown in liquid as described in the previous section. *UBC18* and *SamDC* were chosen as reference genes as their expression is stable in different organs and growth conditions (Hong *et al.*, 2008). As expected, *SWN5* expression was upregulated in response to β-oestradiol in both roots and aerial parts. *BdCESA4* (*Bradi2g49912*) was selected as a putative secondary wall cellulose synthase since it is the only *Brachypodium* orthologue of *OsCESA4* (Supplementary Fig. S1A), a rice gene required for the synthesis of secondary cell walls (Tanaka *et al.*, 2003). *BdCESA1* (*Bradi2g34240*) was selected as a putative primary wall cellulose synthase as an orthologue of both *OsCESA1* and *AtCESA1* (Supplementary

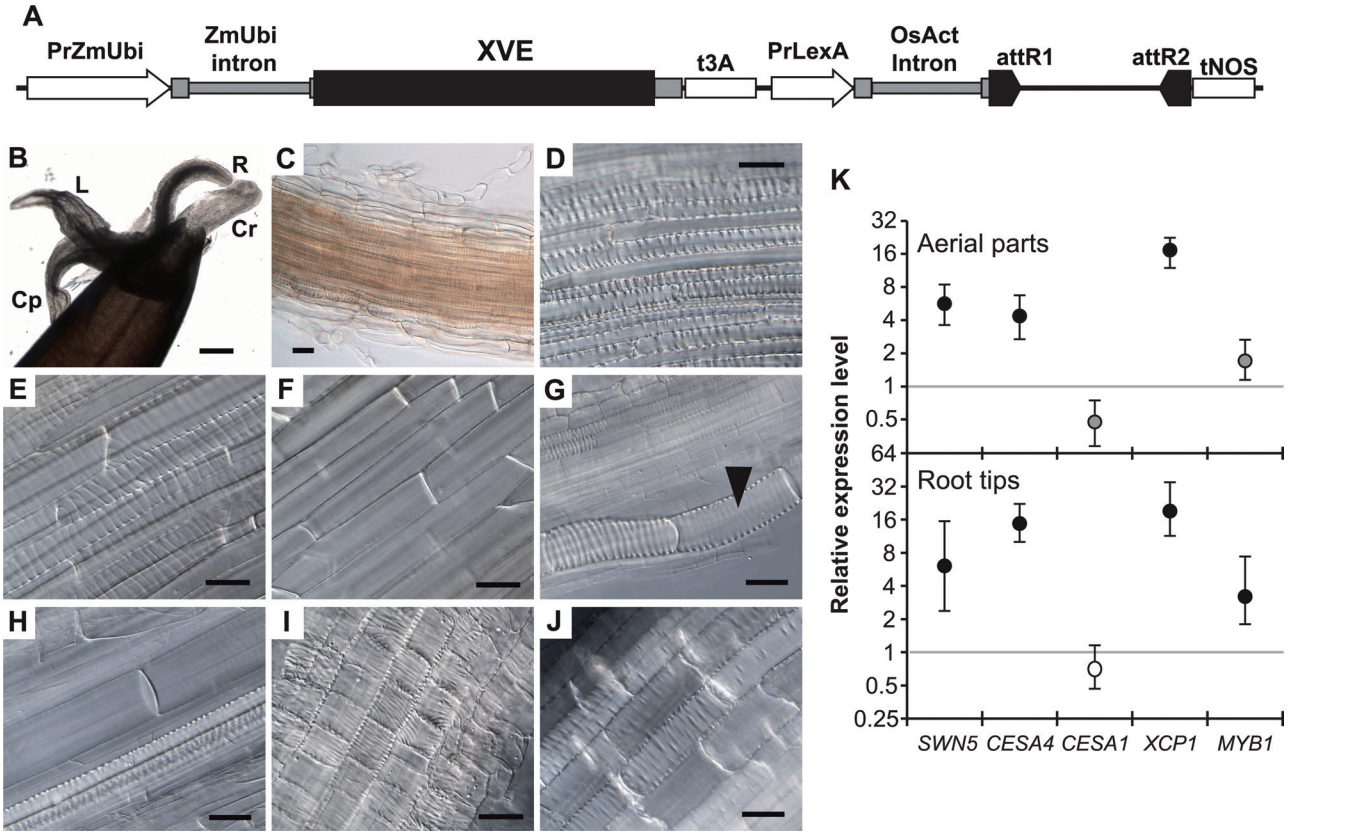

Fig. 4. Inducible overexpression of Bd*SWN5* in *Brachypodium*. (A) Plasmid used for oestradiol-inducible overexpression. (B) Arrested development of a 5-day-old transformed seedling from line 6 germinated in media with 10 μM oestradiol (Cp, coleoptile; Cr, coleorhiza; L, leaf; R, root). (C) Root of a transformed seedling from line 9 grown in the same media. (D) Leaf epidermis of a transformed seedling from line 8 grown in the same media. (E) Coleoptile parenchyma of a transformed seedling from line 3 grown in liquid 24 h after addition of oestradiol. (F) Coleoptile parenchyma of a similar seedling 24 h after mock induction. (G) Root central metaxylem (arrowhead) differentiating less than 2 mm from the tip in an induced seedling. (H) Root central metaxylem differentiating approx. 12 mm from the tip in a mock-induced seedling. (I) Root cortex in the transition zone of an induced seedling. (J) Mature root cortex in an induced seedling. Bars = 400 μm (B) and 25 μm (all others). (K) Relative expression level of selected genes in induced and mock-induced seedlings grown in liquid. Root tips (approx. 8 mm) and aerial parts were collected separately 16 h after induction. Error bars are standard errors as calculated by REST software from three biological and two technical replicates. *UBC18* and *SamDC* were used as references. Ratios that are significantly different from 1 are indicated in black ($P < 0.005$) or grey ($P < 0.02$) (this figure is available in colour at *JXB* online).

Fig. S1A). The expression pattern of *OsCESA1* strongly suggests its involvement in rice primary wall cellulose synthesis (Ruprecht *et al.*, 2011). *BdSWN5* strongly upregulated *BdCESA4* expression in both roots and aerial parts, 15-fold and 4-fold respectively. In contrast, expression of *BdCESA1* was reduced or unchanged.

BdXCP1 (*Bradi2g39320*) was identified as the single orthologue of the xylem-specific cysteine proteases *AtXCP1* and *AtXCP2* (Supplementary Fig. S1B). Finally *BdMYB1* (*Bradi4g06317*) was chosen as the orthologue of *AtMYB46* and *AtMYB83* (Supplementary Fig. S1C), both of them direct targets of *AtSWNs* (McCarthy *et al.*, 2009; Zhong *et al.*, 2010b; Yamaguchi *et al.*, 2011). The expression of both genes was found to be increased in seedlings treated with β-oestradiol, although *BdXCP1* upregulation was much higher, reaching 19-fold in roots and 17-fold in aerial parts.

Transactivation of BdXCP1 *promoter by BdSWN5 is mediated by SNBE sites*

To identify the binding sites of Bd*SWN5*, this study first tested whether this transcription factor was able to transactivate *BdMYB1* and *BdXCP1* promoter fragments (2.3 kb and 1.5 kb respectively). These fragments were placed in front of an intron-containing glucuronidase gene to generate reporter constructs. The effector construct for *SWN5* included the native intron (*SWN5i*), since this intron seemed to enhance expression in tobacco. Tobacco leaves were coinfiltrated with *Agrobacterium* cultures transformed with effector and reporter plasmids and glucuronidase activity was measured after 2 days.

There was no increase in reporter activity from the *BdMYB1* promoter–reporter construct when coinfiltrated with *SWN5* effector (Fig. 5B). However, SWN5 was able to

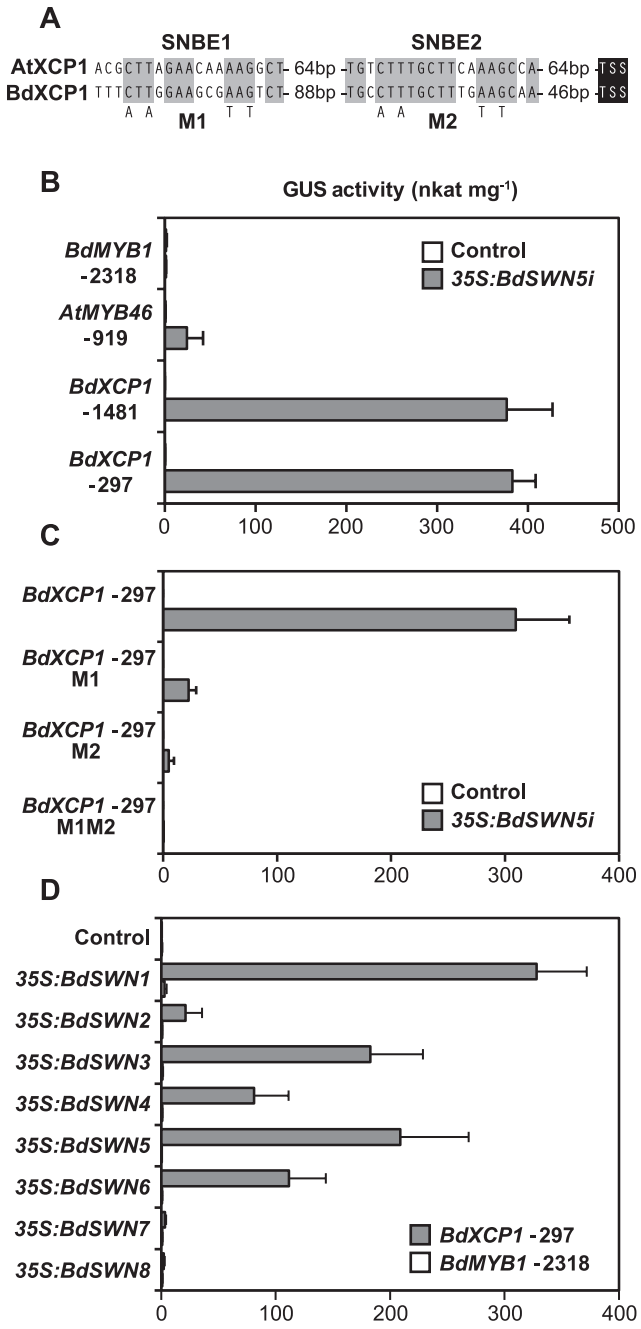

Fig. 5. Transactivation of *Brachypodium* promoters by *Bd*SWN transcription factors. (A) Alignment of SNBE motifs in the *AtXCP1* and *BdXCP1* promoters. Conserved nucleotides are boxed in grey. Nucleotide changes introduced in mutated sites are shown below the alignment. TSS = transcription start site. (B–D) Transactivation assays of promoter fragments: (B) reporter constructs were infiltrated on their own (white) or together with an effector construct with a 2×35S promoter driving the expression of *BdSWN5* (grey); (C) wild-type and mutated fragments of *BdXCP1* promoter; M1 and M2 mutations are indicated in part A; (D) *BdMYB1* (white) and *BdXCP1* (grey) promoters with eight Bd*SWN* effectors. Error bars indicate SD.

transactivate a reporter construct that included an *AtMYB46* promoter fragment, orthologous to *BdMYB1*. On the other hand, GUS activity increased to 377 pkat mg^{-1} when the *BdXCP1* promoter–reporter construct was coinfiltrated with the SWN5 effector. A similar result was observed with a truncated reporter construct that contained only the 297 bp upstream of the *BdXCP1* start codon. This promoter fragment includes two SNBE sites which are highly conserved in sequence and position when compared to the *AtXCP1* promoter (Fig. 5A).

To determine if the SNBE sites were required for the activation of the 297-bp *BdXCP1* promoter fragment, the two sites were mutated independently and simultaneously. A total of four point mutations were introduced in each site at highly conserved positions in the SNBE motif (Fig. 5A). Mutation of SNBE1 and SNBE2 resulted in 93% and 98% reductions in activation respectively, suggesting that both sites are required for full activation (Fig. 5C). Mutation of both sites simultaneously abolished transactivation by SWN5.

The reporter construct with the 297-bp *BdXCP1* promoter fragment was coinfiltrated with effector constructs for the eight *BdSWN* genes without introns (Fig. 5D). Expression of all the genes resulted in activation of the *BdXCP1* promoter, but at very low levels in the case of *SWN7* and *SWN8*. Expression of the remaining genes resulted in much larger increases in reporter activity, with *SWN1* consistently giving the highest level. When the same experiment was repeated with the *BdMYB1* promoter, the activation level was undetectable for all of the *BdSWN* genes (Fig. 5D), with the possible exception of *SWN1* ($P = 0.073$).

*BdSWN5 can bind to SNBE sites in the Bd*XCP1 *promoter*

To test if SWN5 activates the *XCP1* promoter though direct binding to SNBE sites, electrophoretic mobility shift assays were performed. Purified SWN5 protein slowed down the migration of a 142-bp fragment of the *XCP1* promoter that included both SNBE sites (Fig. 6A). Two shifted bands were clearly visible, possibly corresponding to the occupation of one or both SNBE sites. An unlabelled wild-type fragment or a fragment with a mutated SNBE1 site (M1) were able to compete with the probe for binding to SWN5. A fragment where SNBE2 was mutated (M2) was a much less effective competitor even at twice the ratio of the wild-type competitor, suggesting a higher binding affinity toward the SNBE2 site than toward the SNBE1 site. The ability to compete was almost abolished when both SNBE sites were mutated (M1M2). Finally, isolated SNBE motifs in 35-bp promoter fragments (S1 and S2) were able to compete for binding to SWN5, with SNBE2 a more effective competitor than SNBE1.

When isolated motifs were used as probes, binding of SWN5 protein was also detected (Fig. 6B). Unlabelled wild-type motifs could compete effectively, but not those with four point mutations. Consistent with the results observed with the larger fragment, unlabelled SNBE2 could outcompete labelled SNBE1, while unlabelled SNBE1 was less effective against labelled SNBE2. The higher affinity of SWN5 for SNBE2 fits with the transactivation results obtained with mutated motifs (Fig. 5C), strengthening the case for direct activation. The

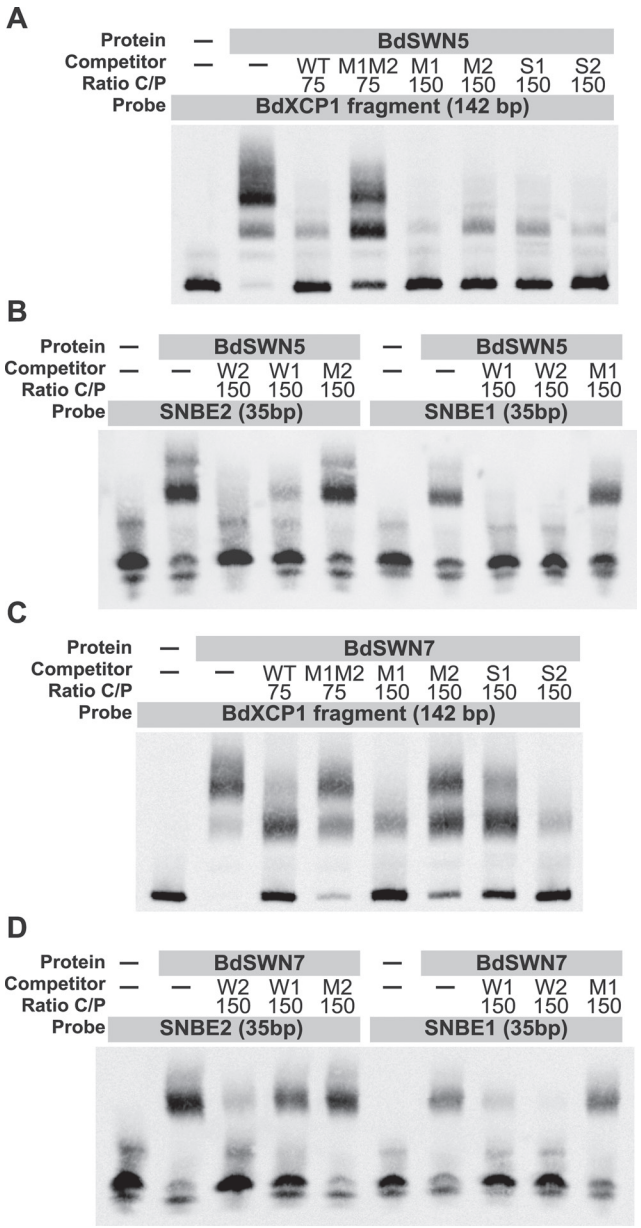

Fig. 6. Electrophoretic mobility shift assays showing binding of BdSWN proteins to *BdXCP1* promoter fragments. (A) A 142-bp *XCP1* promoter fragment in the presence of SWN5 protein and a number of different competitors: unlabelled wild-type fragment (WT), mutated fragments (M1, M2, M1M2, see Fig. 4), and isolated SNBE1 and SNBE2 motifs (S1, S2). (B) *XCP1* SNBE motifs in the presence of SWN5 protein and a number of different competitors: wild-type and mutated SNBE1 (W1 and M1), and wild-type and mutated SNBE2 (W2 and M2). (C) *XCP1* promoter fragment in the presence of SWN7 protein; legend as in A. (D) *XCP1* SNBE motifs in the presence of SWN7 protein; legend as in B.

same experiments were repeated with SWN7 protein since it had shown a reduced capacity to activate the *XCP1* promoter in transactivation assays (Fig. 5D). The results obtained with SWN7 were quite similar to those observed for SWN5 both when using as probe the 142 bp *XCP1* promoter fragment (Fig. 6C) and when using isolated motifs (Fig. 6D). However, the binding affinity of SNW7 toward SNBE1 appeared to be lower than in the case of SWN5, as shown by the poor competition of the M2 fragment or the SNBE1 motif against labelled wild-type fragment (Fig. 6C). This lower affinity was confirmed in the competition of the SNBE1 motif against labelled SNBE2 motif, as well the lower degree of shift observed with labelled SNBE1 (Fig. 6D).

BdXCP1 *promoter directs expression to developing xylem tracheary elements*

The same reporter construct containing a 1.5-kb *BdXCP1* promoter fragment used in transactivation assays was employed for stable transformation of *Brachypodium* plants with a promoter–reporter construct. Of a total of 16 independent lines examined, 15 showed a common expression pattern (Fig. 7). In roots, expression was detected in all types of xylem tracheary elements, as they progressively differentiated, starting

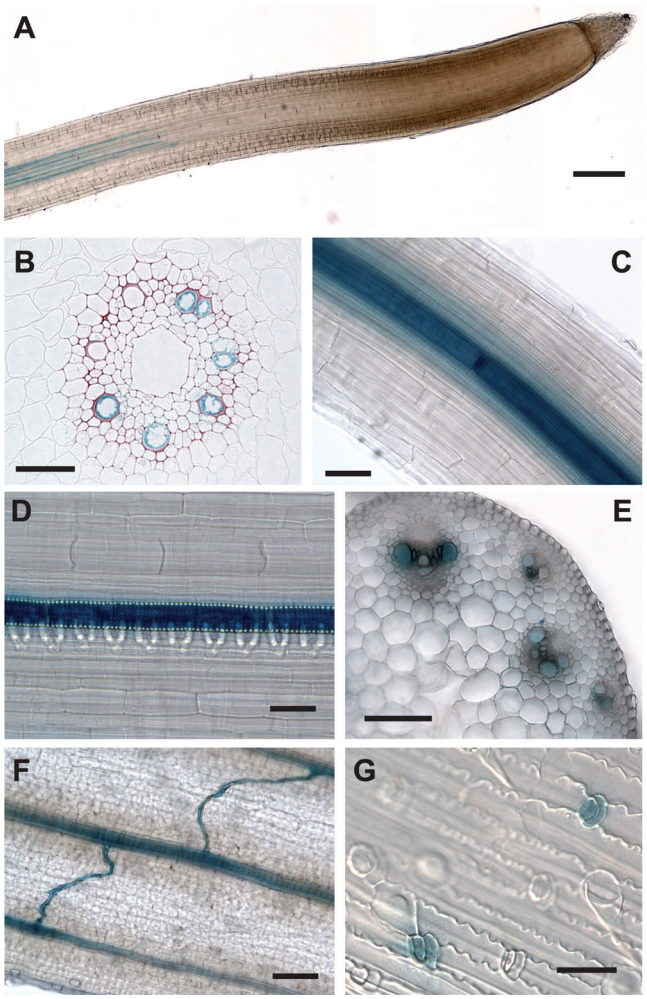

Fig. 7. Expression of β-glucuronidase driven by *BdXCP1* promoter in transformed *Brachypodium* plants. Samples were taken from 6-day-old roots (A–C), 15-day-old internodes (D and E), 6-day-old leaves (F), and 15-day-old leaves (G). Bars = 200 μm (A), 100 μm (E and F), 50 μm (C), and 25 μm (all others).

with protoxylem (Fig. 7A), followed by peripheral metaxylem (Fig. 7B) and later by central metaxylem (Fig. 7C). Staining was also evident in developing xylem of internodes, but not in developing fibres (Fig. 7D and E). In leaves, expression was observed in both longitudinal and commissural vascular bundles (Fig. 7F). Additionally, staining was observed in short leaf epidermal cells with the appearance of cork-silica cell pairs (Fig. 7G).

Discussion

The role of NAC transcription factors as key regulators of secondary cell-wall synthesis and programmed cell death has been established in several dicotyledonous species (Zhong *et al*., 2010a; Wang and Dixon, 2012). The present results now demonstrate that inducible overexpression of a *SWN* transcription factor in *Brachypodium* is sufficient to activate the programme of secondary cell-wall synthesis, establishing that SWNs can act as master switches in monocots.

This study has shown that overexpression of *BdSWN5* leads to accelerated xylem development and deposition of ectopic cell walls in different *Brachypodium* organs (Fig. 4). The patterns of ectopic cell-wall depositions are reminiscent of tracheary elements. Among the genes that are upregulated by SWN5 are *BdCESA4*, a secondary cell-wall cellulose synthase, and *BdXCP1*, a cysteine protease possibly involved in cell death (Fig. 4). Transactivation of the *XCP1* promoter by SWN5 depends on the presence of two conserved SNBE motifs, as proven by the effect of point mutations in conserved positions (Fig. 5). Electrophoretic mobility shift assays further show that SWN5 can bind to both motifs whether isolated or paired as in the genome (Fig. 6). Finally, a *XCP1* promoter fragment that includes these motifs directs expression of a reporter gene to developing xylem tracheary elements (Fig. 7). It thus appears that a direct target of BdSWNs is activated just as cells undergo the processes of secondary wall deposition and cell death. It is also possible that some epidermal cells share parts of this programme (Fig. 7G).

It thus seems likely that *SWN* genes already functioned as master switches of secondary cell-wall synthesis in the last common ancestor of monocots and dicots and that this role has been preserved in both lineages. Furthermore, the binding specificity of SWN proteins and therefore the motifs present in their target promoters appear to have been conserved. This can explain the activation of both cell-wall deposition and cell death programmes in tobacco leaves through transient expression of *BdSWN* genes (Figs. 2 and 3). Ectopic cell-wall deposition has also been observed in *Arabidopsis* when overexpressing rice and maize *SWNs* (Zhong *et al*., 2011).

Despite the conserved function of SWNs in monocots and dicots, the regulatory network of secondary walls has probably diverged after 140 million years of separate evolution (Chaw *et al*., 2004). In *Arabidopsis*, the direct activation of *AtMYB46* and *AtMYB83* by several *At*SWN transcription factors has been demonstrated both *in vitro* and *in vivo* (McCarthy *et al*., 2009; Zhong *et al*., 2010b; Yamaguchi *et al*., 2011). In *Brachypodium*, overexpression of *BdSWN5* also upregulates RNA levels of *BdMYB1*, an orthologue of *AtMYB46* and *AtMYB83* (Fig. 4). However, this study was unable to detect transactivation of the *BdMYB1* promoter by SWN5, even though this *Brachypodium* transcription factor is capable of transactivating the *AtMYB46* promoter (Fig. 5B). Transactivation of the *BdMYB1* promoter was also undetectable with the remaining BdSWNs, suggesting the lack of direct activation (Fig. 5D). A recent study showed that SWN proteins from rice and maize could transactivate a promoter with a tandem arrangement of SNBE motifs taken from rice and maize orthologues of *BdMYB1* (Zhong *et al*., 2011). It is unclear, however, if this artificial arrangement reflects the activity of the native promoter.

The results presented here can also shed some light on the functional specialization of different SWN genes. Phylogenetic analysis suggests the existence of four clearly differentiated clades in the last common ancestor of monocots and dicots (Fig. 1A). Genes from all four of these clades were capable of inducing ectopic cell-wall thickenings in transiently transformed tobacco leaves (Fig. 2). In *Arabidopsis*, overexpression of the three genes from clade I does not result in any phenotype (Kubo *et al*., 2005), while genes from the other three clades are capable of activating the deposition of secondary cell walls (Kubo *et al*., 2005; Mitsuda *et al*., 2005; Zhong *et al*., 2006). In this aspect, *Brachypodium* seems more similar to poplar, where genes from all four clades induce ectopic wall thickenings in both poplar and *Arabidopsis* (Ohtani *et al*., 2011). The pattern of cell-wall deposition caused by transient expression of *BdSWNs* is similar for all of the genes. This is also the same result obtained with poplar *SWNs* and again different from *Arabidopsis*, where overexpression of different *SWNs* can result in different patterns (Kubo *et al*., 2005; Ohashi-Ito *et al*., 2010). In any case, the pattern of ectopic wall deposition is clearly affected by the previous identity of the cell, as shown by the different effects of *BdSWN5* expression in coleoptiles and roots (Fig. 4E and J). This had previously been observed for *AtSND1* (Zhong *et al*., 2006; Ohashi-Ito *et al*., 2010) and poplar *SWNs* (Ohtani *et al*., 2011).

Overexpression of *BdSWN* genes in tobacco can also activate programmed cell death (Fig. 3). Interestingly, the cell death and the cell-wall synthesis programmes appear to be partially independent since they are activated with different intensities by different transcription factors. While *SWN4* expression results in both extensive cell-wall deposition and cell death, *SWN7* expression appears to activate primarily cell-wall synthesis and *SWN2* expression mainly results in cell death with no previous cell-wall deposition. It would be reasonable to conclude that SWN7 and SWN2 are activating different sets of genes, possibly due to differences in binding sequences or preferred motif arrangements.

The two *Brachypodium* proteins in clade IV, SWN7 and SWN8, are poor inducers of cell death in tobacco and they also show a very weak capacity to transactivate the *BdXCP1* promoter (Fig. 5D). This is unlikely to be caused by a weak activation domain, since SWN7 is very effective inducers of cell-wall synthesis in tobacco (Fig. 2). Alternatively, it could

indicate the conservation of a promoter motif or motif arrangement specific to programmed cell death between the two species. Although in electrophoretic mobility shift assays SWN7 can bind the two SNBE motifs in the *BdXCP1* promoter, it appears to bind SNBE1 with lower affinity than SWN5 (Fig. 6). Simultaneous binding to both SNBE sites seems to be required for strong activation of the *XCP1* promoter, as shown by the effect of point mutations in either of the sites (Fig. 5C). Therefore a low binding affinity for SNBE1 could partly explain the low transactivation observed with SWN7.

In poplar, SWN proteins from clade IV are not very efficient at transactivating the *PtXCP1* promoter, especially when compared to proteins in clades II and III (Ohtani *et al*., 2011). Similarly the *AtXCP2* promoter can be directly activated by clade II and clade III proteins, but not by the three *Arabidopsis* clade IV proteins (Zhong *et al*., 2010b). The situation for *AtXCP1* appears to be more complex, with results that depend on the system used (Ohashi-Ito *et al*., 2010; Zhong *et al*., 2010b). To explain the lack of *AtXCP1* expression in fibres, repression by an additional regulatory mechanism has been suggested (Bollhöner *et al*., 2012). It is possible that this mechanism acts by blocking SNBE motifs since an artificial promoter with SNBEs from *AtXCP1* can be efficiently activated by AtSND1 in transfected protoplasts (Zhong *et al*., 2010b), but is not active in *Arabidopsis* fibres (McCarthy *et al*., 2011).

The results presented here suggest that the lower efficiency of clade IV proteins at activating genes involved in cell death could be conserved in monocots. Unlike in *Arabidopsis*, this apparent functional specialization may not be linked to expression in different cell types as maize and rice genes from clades II, III and IV are expressed indistinctly in fibres and tracheary elements (Zhong *et al*., 2011). Since the reporter construct suggests that *BdXCP1* is not expressed in fibres (Fig. 7E), it is possible that a repression mechanism is also active in monocots.

The use of inducible systems capable of activating secondary cell-wall synthesis has generated a wealth of information regarding the transcriptional regulation of this complex developmental process in *Arabidopsis* (Ohashi-Ito *et al*., 2010; Zhong *et al*., 2010b; Yamaguchi *et al*., 2011). The present study has now extended this approach to the monocots, using the model species *Brachypodium dystachion*, an important step to understand the regulation of secondary cell-wall synthesis in grasses and to acquire the tools that will be necessary to improve biomass crops. The results indicate that much of what has been learned in *Arabidopsis* will still be relevant, but point also to some possible differences.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Primers used in this work.

Supplementary Table S2. Annotation of *Brachypodium* and rice genes.

Supplementary Fig. S1. Phylogenetic trees of genes selected for real-time PCR analysis.

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