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Silencing against the conserved NAC domain of the potato *StNAC103* reveals new *NAC* candidates to repress the suberin associated waxes in phellem

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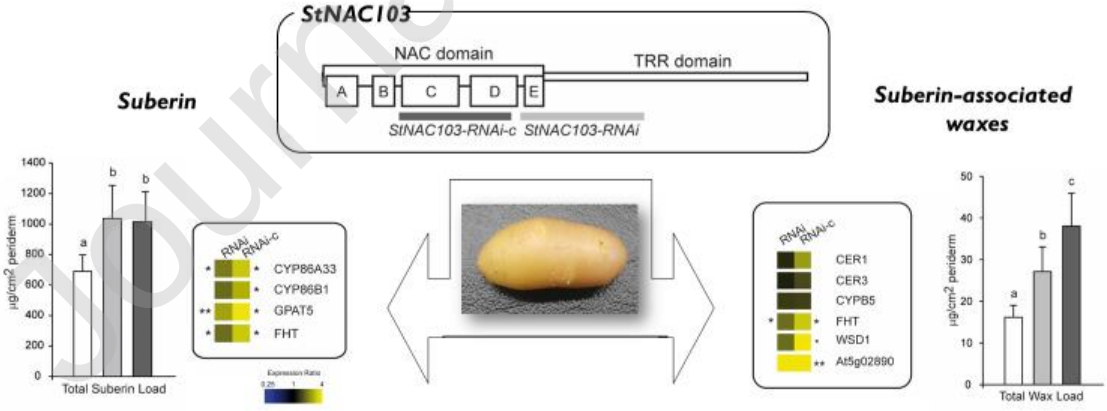
Graphical Abstract



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Highlights

- . Conserved silencing to the NAC domain of *StNAC103* increases suberin and wax load
- . Conserved silencing has stronger effects on waxes than *StNAC103* specific silencing
- . Conserved silencing is correlated with upregulation of genes related with waxes
- . *StNAC103* silencing boosts lipid-regulators and inhibits polyamine enzymes

Abstract

Both suberin and its associated waxes contribute to the formation of apoplastic barriers that protect plants from the environment. Some transcription factors have emerged as regulators of the suberization process. The potato *StNAC103* gene was reported as a repressor of suberin polyester and suberin-associated waxes deposition because its RNAi-mediated downregulation (*StNAC103-RNAi*) over-accumulated suberin and associated waxes in the tuber phellem concomitantly with the induction of representative biosynthetic genes. Here, to explore if other genes of the large *NAC* gene family participate to this repressive function, we extended the silencing to other *NAC* members by targeting the conserved NAC domain of *StNAC103* (*StNAC103-RNAi-c*). Transcript profile of the *StNAC103-RNAi-c* phellem indicated that *StNAC101* gene was an additional potential target. In comparison with *StNAC103-RNAi,* the silencing with *StNAC103-RNAi-c* construct resulted in a similar effect in suberin but yielded an increased load of associated waxes in tuber phellem, mainly alkanes and feruloyl esters. Globally, the chemical effects in both silenced lines are supported by the transcript accumulation profile of genes involved in the biosynthesis, transport and regulation of apoplastic lipids. In contrast, the genes of polyamine biosynthesis were downregulated. Altogether these results point out to *StNAC101* as a candidate to repress the suberin-associated waxes.

Abbreviations: ABA, abscisic acid; ADC, arginine decarboxylase; AP2/ERWEBP, apetala2/ehtylene-responsive element binding proteins; APRT, adenine phosphoribosyl transferase; ASFT, aliphatic suberin feruloyl transferase; AtNAP, NAC-like activated by AP3/PI; bHLH, basic helix loop helix; CD2/ANL2, cutin deficient 2/anthocyaninless 2; FHT, fatty ω-hydroxyacid/fatty alcohol hydroxycinnamoyl transferase; GC-FID, gas chromatography-flame ionization detector; GC-MS, gas chromatography-mass spectrometry; HD-ZIPIV, homeodomain leucine zipper; KCS, β-ketoacyl-CoA synthase; LTP, lipid transfer protein; NAC, NAM, ATAF and CUC2; PI4Kγ5, phosphatidylinositol 4-kinase; RD26, responsive to dessication 26; SPDS,

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spermidine synthase; SPMS, spermine synthase; TRR, transcription regulatory region, VLCFA, very long chain fatty acids; WOX4, WUSCHEL-related HOMEOBOX; WRI1, Wrinkled1.

Keywords: Suberin, suberin-associated waxes, periderm, phellem, NAC transcription factors, *Solanum tuberosum*

1. Introduction

The periderm is a protective organ consisting of three different tissues: the phellem, the phellogen or cork cambium and the phelloderm. The phellem is the external tissue responsible of the protection against dehydration and pathogens [1]. The protective function of the phellem is achieved by the deposition of suberin and associated waxes in the cell wall providing a hydrophobic barrier in the apoplast. Suberin, also named aliphatic suberin, is an insoluble polyester mainly composed by oxygenated fatty acid derivatives (such as ω-hydroxy-fatty acids, α,ω-diacids) and in minor amounts by fatty acids, primary alcohols, glycerol and ferulic acid [2–5]. Suberin-associated waxes are the organic solvent-soluble extract mostly consisting of linear very long chain fatty acid derived compounds, including alkanes, primary alcohols, fatty acids and feruloyl esters of primary alcohols [6]. This fraction has been proposed to play a significant role in the apoplastic barrier of potato tuber phellem as the removal of wax fraction increased the water permeability by a factor of 100 [6]. Suberin deposition is located between the plasma membrane and the primary cell wall and is suggested to be bound to the latter by a lignin-like polymer, also known as the suberin polyphenolic domain, mainly consisting of hydroxycinnamic acids [3,5]. Suberin deposition occurs naturally in external and internal tissues to prevent water, ion and gas movement, such as in root and shoot periderm, cotton fibres, abscission zones, endodermis, exodermis/hypodermis and bundle sheaths of monocots [4,5]. Suberin deposition can also be induced by wounding to protect the healing tissue from water loss and pathogens [7].

The first reported transcription factor with ability to induce suberin deposition ectopically in leaf and activation of biosynthetic genes of suberin was *AtMYB41* [8], a stress responsive gene only detectable under ABA (abscisic acid), desiccation and salt treatments [9]. Recently, new MYB transcription factors have been described as regulators of suberin biosynthesis. Specifically, overexpression of apple *MdMYB93* in *Nicotiana benthamiana* leaves correlated with strong accumulation of suberin and induction of genes involved in suberin synthesis [10]. Moreover,

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Arabidopsis seeds of the *myb9* and *myb107* mutants showed significant reduction in suberin accumulation and downregulation of the corresponding suberin biosynthetic genes [11]. Additional evidences of Myb107 role in suberin regulation were reported by Gou et al. [12] by probing the interaction of this transcription factor with genes involved in suberin synthesis. The cork oak QsMYB1, putative ortholog of AtMYB68, was proposed as a master regulatory factor of cork formation and differentiation, due to its ability to bind to lignin and suberin biosynthetic genes [13]. In contrast, a repressor of suberin and associated waxes from the NAC family, *StNAC103*, was reported in potato [14]. The *StNAC103* gene is expressed in the tuber phellem and is also induced by wound-healing and ABA. The knockdown of *StNAC103* (*StNAC103-RNAi*) was correlated with an increased load of suberin and associated waxes, a modification of the chemical composition of both components and a strong induction of some genes related with suberin and wax accumulation [14].

NAC proteins constitute one of the largest families of plant-specific transcription factors found in land plants [15] and they have been suggested to contribute to the adaptation of plants to the terrestrial environment [16]. In general, NAC proteins share a well-conserved N-terminal domain and a diversified C-terminal, designed as NAC domain and transcription regulatory region (TRR), respectively [15,17]. The NAC domain is divided in five subdomains: two involved in the binding of DNA (C and D), one participating in the dimerization (A) and two implicated in the functional diversity (B and E), whereas the TRR domain can either activate or repress transcription [17]. NAC transcription factors have been involved in tolerance to abiotic stress, defence, development, senescence, auxin signalling and secondary cell wall biosynthesis [15]. Several *NAC* genes have been defined as master transcriptional switches for xylem differentiation, and together with *MYB* genes, regulate lignin biosynthesis in harmony with plant development and environmental adaptation [18]. A total of 110 *NAC* genes coding for 135 proteins are found in potato [19]. *StNAC103* belongs to the subgroup *NAC-c*, which contains genes responsive to ABA and abiotic stress such as *StNAC017* and *StNAC030* [19] and genes involved in organ development, like *StNAC044*, the putative ortholog to *CUC3* [20]. Interestingly, the Arabidopsis ortholog of *StNAC103* is *ANAC058*, whose overexpression provides hypersensitivity to exogenously applied ABA, a germinating-inhibiting compound [21]. The fact that suberin-deficient seeds display the same phenotype concerning ABA hypersensitivity [22] is consistent with a repressive role of ANAC058 in suberization. It has been suggested that the enhanced sensitivity to ABA can be explained by the impaired apoplastic barrier in the seed, which results in a higher permeability to water and ABA [22].

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Here, to further understand the involvement of *NAC* genes in the apoplastic barrier regulation, we report the extended silencing, by targeting the conserved NAC domain of *StNAC103* (*StNAC103-RNAi-c*), and their effects in potato tuber phellem. *StNAC103-RNAi-c* lines and *StNAC103* specifically silenced lines (*StNAC103-RNAi*) [14] show similar amounts and composition of suberin, whereas stronger accumulation of the suberin-associated waxes in the potato tuber phellem is observed in *StNAC103-RNAi-c*. Transcript accumulation analyses of different NAC genes and the putative targets of StNAC103 are presented, providing a further detail of molecular mechanisms underlying *StNAC103* silencing.

2. Materials and Methods

2.1. Plant Material, potato tissues and RNA extraction

Potato plants (*Solanum tuberosum* Group Tuberosum) cv. Desirée were *in vitro* propagated as described previously by Serra et al. [23]. To produce tubers, *in vitro* plants were transferred to soil and grown for 3 months in a walk-in chamber. The periderm (skin) of potato tuber was manually dissected using sterile scalpels and immediately frozen in liquid nitrogen. Total RNA was extracted using guanidine hydrochloride [24] and the RNA fraction was loaded on an RNeasy MinElute (Qiagen) column to perform DNAse digestion and further purify the RNA. RNA integrity and concentration was analysed by formamide-formaldehyde denaturing agarose gel electrophoresis and a Nanodrop spectrophotometer, respectively.

2.2. Cloning, binary vector construction and plant transformation

Synthesis of first-strand cDNA was carried out using 2 µg of DNAse digested RNA, oligo(dT)18 primers and Superscript III reverse transcriptase (Invitrogen). The design of primers used to amplify and clone the regions for *StNAC103* silencing was performed using the information available at that time from the potato Expressed Sequence Tag assembly (TC143904) and the putative orthologs of cork oak (EE743827) [25], Arabidopsis (At3g18400) and Petunia x hybrid (GI:21389167). The conserved silencing (*StNAC103-RNAi*-c) was triggered using a conserved fragment of 229 bp in the NAC domain. Amplification products were first cloned into the pENTR/D TOPO vector (Life Technologies) and then transferred into the binary destination vector pBIN19RNAi [23] using LR clonase II (Invitrogen). Sequence of the primers used for the cloning are reported in Table S1. *Agrobacterium tumefaciens* (GV2260) was transformed with the recombinant plasmids according to Höfgen and Willmitzer [26] and afterwards potato leaves were infected with these *A. tumefaciens* cells. Kanamycin-resistant plants were regenerated *in vitro* by organogenesis following the protocol of Banerjee et al. [27].

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2.3. Real-time PCR analysis

cDNA synthesis was performed using the high capacity cDNA reverse transcription kit (Life Technologies) and random primers. Real-time PCR was done in an optical 96-well plate with an ABI PRISM 7300 Sequence Detector System (Applied Biosystems) synthesis. The gene-specific PCR primers were designed with Primer 3 [28] and checked with Primer Express 3.0 (Applied Biosystems). PCR reactions contained 1 x Power SYBR Green Master Mix reagent (Applied Biosystems), 300 nM of the respective primers and 5 µl of a 25-fold dilution of the corresponding cDNA in a final volume of 20 µl. Wild-type plants were used to standardize the results and APRT, encoding an adenine phosphoribosyl transferase, was used as a housekeeping gene [29,30]. The amplification conditions for all PCRs were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After amplification, a dissociation step was performed to confirm the presence of a single amplicon. All data were processed and analysed with 7300 SDS 1.3.1 software (Applied Biosystems). For microfluidics quantitative PCR (qPCR; Fluidigm), 1.25 µl of the synthesized cDNA was pre-amplified and then purified with exonuclease treatment. Pre-amplified and purified cDNAs were diluted to 1:5 and used for real-time qPCR amplification by the BioMark™ system (Fluidigm). The primer specificity for each amplification reaction also was confirmed by a dissociation curve. Data collection and analysis was performed using Fluidigm Real-Time PCR analysis software 3.0.2 (Fluidigm). The amplification efficiency was determined for each primer pair by performing standard curves with a 5-fold dilution series (1/1, 1/5, 1/25, 1/125, 1/625) of template. The geometric mean of two validated housekeeping genes, APRT and Cyclophilin [29,30], was used as reference to normalize data.

The mRNA abundance was calculated as relative transcript abundance = $(\text{Etarget})^{\Delta\text{Ct target (control-sample)}}/(\text{Ereference})^{\Delta\text{Ct reference (control-sample)}}$ [31]. The controls used were a mix with equal amounts of different biological replicates of untransformed lines. The absence of genomic DNA contamination was confirmed by non-retrotranscriptase controls (RT-), whereas the lack of environmental contamination was checked with non-template controls (NTC). Sequence of primers used during Real-time PCR is shown in Table S1. MeV [32] was used for expression to generate a heatmap.

2.4. Suberin and wax chemical analyses

For aliphatic suberin and wax chemical analyses, the periderm membranes were isolated. Periderm membranes are obtained by enzymatic digestion of suberin-free cell walls as detailed by Schreiber et al. [6]. Hence, although designated as periderm, this tissue consists of the phellem or cork. Briefly, tuber skin was separated from the inner flesh with a cork-borer and

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treated with a 2% (v/v) cellulase and a 2% (v/v) pectinase solution. When digestion was completed, the remaining phellem membranes were washed, dried and stored at room temperature. All membranes were obtained from tubers after 39 days' storage at room temperature to permit the periderm maturation.

First the wax fraction was extracted from 1-2 dry isolated periderms (2-6 mg dry weight) using a chloroform and methanol (1:1 v/v) solution for 18 h. 2 µg C24 alkane was added to the solution as internal standard. The periderm was then removed and rinsed two times with new chloroform: methanol solution. The cleaning solutions were mixed together with the overnight extract and the solvent evaporated to dryness. The residue obtained was then used for wax analysis without further purification. Aliphatic suberin was depolymerised by transesterification immersing the dry wax-free periderms in ~10% of boron trifluoride in methanol (BF3/MeOH) and incubating the samples at 70ºC for 16-18 hours in a teflon-sealed screw-cap tube [33]. Then, 10 µg of C32 alkane were added as internal standard and the periderm removed. The methanolysate was transferred to a new vial containing 2 ml of saturated $NaHCO_3$ aqueous solution. The aqueous: methanol phase was then extracted two times with chloroform and, after phase separation, the chloroform extract was rinsed with ultrapure water. The organic phase was dried with anhydrous sodium sulphate powder and the solvent evaporated to dryness. The dry residues from both chloroform/methanol extracts (waxes) and the monomers obtained after depolymerisation (suberin) were derivatized using N, O-bis (trimethylsilyl) trifluoroacetamide and derivative products were identified by GC-Mass Spectrometry (MS) and quantified by GC-Flame Ionization Detector (FID).

GC-MS analyses were performed with a selective mass detector with ion trap (Trace GC 2000 series coupled to a Thermo Scientific Polaris Q mass spectrometer), using a BPX-5 capillary column (30 m length, 0.25 mm i.d., 0.25 µm film thickness (SGE, Australia & Pacific Region)) with a split/splitless injector in the splitless injection mode (splitless time 1 min), with the injector temperature at 280ºC and using helium as the carrier gas at a constant flow rate of 1 ml/min. The oven temperature program for wax analyses was as follows: initially the temperature was 120ºC, then increasing by 15ºC min^{-1} up to 240ºC, hold 1 min at this temperature, then increasing by 3ºC min^{-1} up to 340ºC and hold for 26 minutes. For suberin, the oven temperature was initially set at 140ºC, then increasing by 3ºC min^{-1} up to 310ºC, and finally hold for 3.3 min at 310ºC. Electron impact ionization was operated at 70 electronvolts and the transfer line was held at 290ºC. The ion source was set at 225ºC. MS acquisition was carried out in full-scan mode, with a scan range of 50-700 amu (atomic mass unit). For each peak, the mass spectrum was compared with data reported in literature [34–36].

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GC-FID analyses were performed in a Shimadzu GC-2010 Plus, using a TRB-1ht capillary column (30 m length, 0.25 mm i.d., 0.1 µm film thickness, Teknokroma), with a split/splitless injector in the splitless injection mode (splitless time 1 min), and using helium as the carrier gas at a constant flow rate of 1 ml/min. The injector temperature was 280ºC. The oven temperature program for wax analyses was as follows: initially the temperature was 120ºC, then increasing by 15ºC min^{-1} up to 240ºC, then by 3ºC min^{-1} up to 380ºC and hold for 5.33 minutes. The detector temperature was maintained at 380ºC. For suberin trimethylsilylderivatives obtained after N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatisation, the initial oven temperature program started at 140ºC, followed by 3ºC min^{-1} increases up to 310ºC, where it was held for 5 min. The detector temperature was maintained at 320ºC. Chromatograms were processed using GC Solution software (version 2.41) from Shimadzu.

2.5. Statistical analyses

Results from wild-type and transformed periderms were statistically analysed using one-way Analysis of Variance (ANOVA). One-way ANOVA was performed to assess differences among means. When variances were found to be heterogeneous using Levene's test, Welch modification was used to robustly assess differences among means. Posthoc analysis was used to compare groups showing statistical differences, using Tukey HSD test if there is homogeneity of variances, and Games-Howell test when variances are heterogeneous.

3. Results

3.1. Transgenic RNAi potato lines show repression of StNAC103 and other NAC members in the phellem

To determine the contribution of the NAC family regulators to suberization, we performed silencing using the conserved NAC domain of *StNAC103* to trigger the mechanism of RNA interference. These lines were named as *StNAC103-RNAi-c* (Fig. 1A). Transcript abundance of *StNAC103* was evaluated in these lines and compared with those obtained with gene specific silencing reported as *StNAC103-RNAi* [14], in which the highly variable region corresponding to the TRR domain was targeted (Fig. 1A). The three *StNAC103-RNAi-c* lines showing the lowest *StNAC103* expression (Fig. 1B) were selected for further analyses together with the two lines with the *StNAC103-RNAi* construct previously characterized [14]. In all these lines, *StNAC103* was strongly downregulated, with transcript abundances 3 to 15 times lower than wild-type lines.

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To uncover the *StNAC* family members putatively silenced in *StNAC103-RNAi-c*, we performed BLASTN analyses against the potato transcript database using as a query the region used for producing the *StNAC103-RNAi-c* (Table I) and the *StNAC103-RNAi* (Table II) lines. We found 26 putative targets of *StNAC103-RNAi-c* silencing construct, including *StNAC103* and one gene not annotated as *NAC* (Table I). Amongst the identified genes, even some of them displayed a complete match in 21 consecutive nucleotides with the *StNAC103-RNAi-c* construct, others presented one or two mismatches (Fig. S1). In contrast, only two *NAC* genes other than *StNAC103* were identified as putative targets of the *StNAC103-RNAi* region, and they contained 1 or 2 mismatches to the silencing construct (Table II).

Transcript accumulation of all 26 genes showing significant similarity to the silencing sequences (Tables I and II) was analysed in the phellem of *StNAC103-RNAi* and *StNAC103-RNAi-c* lines (Fig. 2). We confirmed again that *StNAC103* transcript accumulation was similar in both types of silencing lines. In addition, *StNAC072*, *StNAC017* and *StNAC096*, which were predicted as silenced only in the *StNAC103-RNAi-c* lines, showed a decrease in transcript levels in both silencing lines, suggesting that their repression is a consequence of *StNAC103* downregulation. *StNAC032* exhibited lower gene expression in *StNAC103-RNAi-c* lines and slight downregulation in *StNAC103-RNAi*. In contrast, *StNAC101* showed a strong reduction in transcript accumulation specifically in *StNAC103-RNAi-c* lines, suggesting that it was a direct target of the hairpin RNAi together with *StNAC103*.

To further characterize all the genes showing significant similarity to the silencing constructs, *in silico* expression analyses were performed using RNA-seq data reported for different potato tissues and conditions (Fig. S2) [37,38]. Only *StNAC103* was preferentially expressed in the potato tuber peel (including phellem), whereas *StNAC101*, *StNAC032*, *StNAC072*, *StNAC017* and *StNAC096* were also expressed in a large variety of tissues and conditions in addition to potato tuber peel. Thus, based on their transcript abundance, a putative role of these genes in potato suberin is possible.

Overall, considering all these results, the *StNAC103-RNAi-c* hairpin presumably targeting *StNAC101* and *StNAC103* causes the phenotype described below.

3.2. Wide silencing has a stronger effect on suberin-associated waxes in potato phellem

Total suberin content was measured in the phellem, showing a total load 1.5 times higher in both *StNAC103-RNAi* and *StNAC103-RNAi-c* than in wild-type tissues (Fig. 3A), in agreement with higher accumulation of primary alcohols, ω-hydroxyacids, diacids and ferulic acid (Fig. 3B). When inspecting the single monomer abundances included in each substance class, no major

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differences were detected between *StNAC103-RNAi* and *StNAC103-RNAi-c* lines (Fig. S3B). Therefore, *StNAC103-RNAi-c* showed a phenotype nearly identical to *StNAC103-RNAi* concerning suberin content. In contrast, when suberin-associated waxes were quantified in the tuber phellem, *StNAC103-RNAi-c* resulted in a stronger phenotype than *StNAC103-RNAi*, showing twice the total wax load of wild-type lines. These levels were about one third higher than those of the *StNAC103-RNAi* lines (Fig. 4A). When the main waxes compounds were analysed in detail, it was apparent that the levels of alkanes and feruloyl esters of primary alcohols were all significantly higher in the *StNAC103-RNAi-c* phellem than in *StNAC103-RNAi* (Fig. 4B). Notably, alkane content was three times more abundant in *StNAC103-RNAi-c* lines and twice in *StNAC103-RNAi* than wild-type lines (Fig. 4C). In contrast, feruloyl esters were only more abundant in *StNAC103-RNAi-c*, being nearly equally abundant in *StNAC103-RNAi* and in wild-type lines. The precise feruloyl esters that increased in the *StNAC103-RNAi-c* phellem were the major compounds, specifically C23, C28 and C30 feruloyl esters (Fig. 4D). Other waxes compounds (primary alcohols and fatty acids) were also analysed in detail and no important differences among lines were detected (Fig. S3A).

3.3. NAC silencing alters regulatory genes and biosynthetic genes involved in suberin, waxes and polyamines

To determine which genes were transcriptionally altered in *StNAC103-RNAi* and *StNAC103-RNAi-c* as a consequence of gene silencing, we analysed the transcript abundance pattern of some genes involved in the biosynthetic pathway of suberin and associated waxes, including the synthesis of very long chain fatty acids (VLCFA) and feruloyl-CoA, the formation of suberin monomers and waxes compounds, and their transport to the apoplast. Overall, all these steps were induced in transgenic lines compared to wild-type, excepting those genes involved in feruloyl-CoA accumulation (Fig. 5). The *KCS (*β-ketoacyl-CoA synthase) gene, involved in VLCFA biosynthesis [23], was upregulated in *StNAC103-RNAi-c* lines. The orthologs to *CER1*, *CER3* and *CYPB5*, which are related with the biosynthesis of alkanes [39-40], were not differentially expressed between silenced and wild-type lines. Regarding *FHT,* which is involved in the biosynthesis of feruloyl esters of primary alcohols and ω-hydroxyacids in both waxes and suberin [41–43], was upregulated in both silenced lines. Other genes related with waxes, such as the *At5g02890* [44] and *WSD1* [45], were clearly upregulated in *StNAC103-RNAi-c* but not in *StNAC103-RNAi*. Concerning suberin genes, *CYP86A33* and *GPAT5* [46, 47] showed upregulation in both silenced lines, and *CYP86B1* [48] only in *StNAC103-RNAi-c*. Also, two LTPs (lipid transfer proteins), putatively involved in the transport of waxes and suberin monomers across the cell wall [49], were only upregulated in *StNAC103-RNAi-c*. In addition, there are two *BAHD* genes

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annotated as benzoyltransferases with unknown function that are preferentially induced in potato peel [37,38] and upregulated in *StNAC103-RNAi-c*.

Transcript accumulation of regulatory genes that could control the phellem composition was also analysed (Fig. 6). These included transcription factors related to the synthesis of acyl-lipids, cutin and waxes (*Wrinkled1 or WRI1* and *Cutin deficient 2/Anthocyaninless 2 or CD2/ANL2*) [50–52], controlling cambium activity (*WUSCHEL-related HOMEOBOX or WOX4*) [53] and cell differentiation (*ALCATRAZ*) [54]. As shown in Fig. 6, *WOX4* did not show significant differences in any of the silenced lines compared to the wild-type, whereas *ALCATRAZ* and *CD2/ANL2* were upregulated in both *StNAC103-RNAi-c* and *StNAC103-RNAi* lines and *WRI* was upregulated only in *StNAC103-RNAi-c*. All these results agree with the induction of suberin and wax biosynthetic genes and with the higher amounts of suberin and waxes detected in *StNAC103* silencing lines.

Since a *NAC* family member represses polyamine biosynthesis [55] and polyamine levels increased in a suberin-deficient potato mutant [41], we also analysed the expression pattern of some polyamine biosynthetic genes. The genes encoding enzymes that produce putrescine, spermine and spermidine from arginine (arginine decarboxylase, spermine synthase and spermidine synthase; ADC, SPMS and SPDS, respectively) were downregulated in the transgenic lines compared to wild-type, and the level of repression was similar in *StNAC103-RNAi-c* and *StNAC103-RNAi* (Fig. 6). Altogether suggests a cross-talk between suberin and polyamine biosynthesis and reinforces a putative role of polyamines in phellem formation.

4. Discussion

StNAC103-RNAi-c lines showed an obvious increase of suberin and associated waxes in the potato tuber phellem in comparison to the wild type. Similar effects on suberin amount and composition were also observed in *StNAC103-RNAi* lines [14] (Fig. 3), but the boost in suberin-associated waxes was higher in *StNAC103-RNAi-c*, specially enhancing the alkanes and feruoyl esters content (Fig. 4). Since NACs are transcription factors, the chemical changes observed in knockdown lines were compared with the gene expression of biosynthetic genes of suberin and suberin-associated waxes (Fig. 5). KCS enzyme yielding to VLCFAs [23] was only upregulated in *StNAC103-RNAi-c* lines suggesting that it may trigger a higher accumulation of VLCFA to be channelled to the downstream pathways, hence contributing to the boost in wax amount of *StNAC103-RNAi-c* lines. Concerning genes involved in suberin biosynthesis, the similar upregulation in both silenced lines is consistent with the analogous increase in suberin load in both transgenic lines. Regarding wax genes, the orthologs to the *WSD1* and *At5g02890* [44,45] were only upregulated in *StNAC103-RNAi-c* lines again in agreement with the higher

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accumulation of suberin-associated waxes in these lines. Concerning FHT, it synthesizes feruloyl esters to be incorporated mainly in suberin (96% of the apoplastic lipids [46]) but also in waxes [41], and as a consequence, the *FHT* transcript accumulation cannot be used as a readout to evaluate the solely increase of feruloyl esters in waxes of *StNAC103-RNAi-c.* In agreement, *FHT* was upregulated in both transgenic lines consistent with the increase in suberin of ferulic acid together with ω-hydroxyacids and primary alcohols in *StNAC103-RNAi* and *StNAC103-RNAi-c*. The genes related with the biosynthesis of alkanes such as *CER1*, *CER3* and *CYPB5* [39,40], were not correlated with the boost of alkanes in both silencing lines. But interestingly, the upregulation in both silenced lines of the transcription factor *CD2/ANL2*, not detected in wild-type samples (Fig. 6), may explain the alkanes increase because *cd2/anl2* mutants display a strong reduction in alkanes of cuticular waxes and a global drop of cutin monomers [51]. Even, the overall increase of suberin monomers in silenced lines also can be due to the upregulation of *CD2/ANL2*. Finally, two *LTP* genes, that may be involved in the transport of suberin and waxes compounds through the apoplast [49], were only upregulated in *StNAC103-RNAi-c* lines and therefore, they could also promote a higher accumulation of suberin-associated waxes in these lines. As a whole, *StNAC103* downregulation was correlated with a strong upregulation of the aliphatic metabolism associated to the suberin polyester and waxes biosynthesis, while the phenolic metabolism was not affected (Fig. 5). In the same line of evidence, differential regulation of aliphatic and phenolic metabolisms was also observed during wound-induced suberization in potato tuber [56].

Three out of the four regulatory genes analyzed in the phellem of the silencing lines showed statistically significant differences. As explained above, the gene *CD2/ANL2* was strongly upregulated in both *StNAC103* transgenic lines in agreement with the increase of suberin and wax compounds. It has been reported that *CD2/ANL2* is a class IV homeodomain Leucine zipper (HD-ZIP IV) whose mutation in addition to the effects in cutin and waxes, gives rise to an increase in cuticle water permeability [51,57] and also lower cell wall extensibility [58]. *ALCATRAZ* is a basic helix loop helix (bHLH) transcription factor involved in fruit dehiscence by promoting cell differentiation [59] and was also upregulated in both *NAC* silencing lines. The activation of *ALCATRAZ* in the phellem of silencing lines could be related to the regulation of processes that also occurs in the abscission zone such as cell wall modification or the formation of apoplastic barriers [60]. *WRI1* was upregulated only in *StNAC103-RNAi-c* lines and this result is consistent with the higher accumulation of waxes because *WRI* is a transcription factor of the *AP2/ERWEBP (Apetala2/Ehtylene-responsive element binding proteins)* class involved in the induction of fatty acid biosynthetic genes [50,61,62] and the accumulation of the most abundant cutin monomers

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[52]. Regarding the genes involved in polyamines biosynthesis (*ADC*, *SPMS* and *SPDS)*, they are similarly downregulated in both silencing lines. Interestingly, the putative ortholog of *ANAC072* in trifoliate orange, *PtrNAC72*, is able to repress the biosynthesis of putrescine by binding to the promoter of *PtADC* (arginine decarboxylase) [55]. In potato phellem, *StNAC072*, the putative ortholog of *ANAC072*, is similarly downregulated in both silencing lines like the polyamines biosynthetic genes, suggesting that *StNAC072* does not have a repressive role in potato or that other transcription factors are responsible for polyamine repression.

The specific increase in suberin-associated waxes in the *StNAC103-RNAi-c* phellem, suggests co-silencing of other *NAC* genes that may repress the biosynthesis of waxes compounds by targeting some of the genes involved in wax biosynthesis, *KCS* or *LTPs (lipid transport protein)*. This stronger phenotype was explained because to trigger RNA interference in these lines we used a region of the conserved NAC domain, which according to BLASTN analyses was found in 26 genes (Table I). *StNAC057* was the most similar gene after *StNAC103* to both silencing constructs, but surprisingly it was not downregulated neither using an RNAi construct containing a fragment of the TRR domain nor the one containing a region of the NAC domain. In contrast, other genes with less similarity, such as *StNAC072*, *StNAC017* and *StNAC069,* showed a clear downregulation in both types of silencing lines (Fig. 2). The lack of significant similarity of these three genes with the sequence used for silencing *StNAC103-RNAi* lines suggests that they are not directly downregulated by the artificial RNAi but probably indirectly downregulated due to *StNAC103* silencing. Regarding *StNAC032*, although there is downregulation in *StNAC103-RNAi-c* phellem in comparison to the wild-type, the transcript levels in *StNAC103-RNAi* are similar to both the wild-type and the *StNAC103-RNAi-c,* thus it is unclear if the gene is directly downregulated by *StNAC103-RNAi-c*. Conversely, *StNAC101* is downregulated only in *StNAC103-RNAi-c,* rendering it the preferred candidate to participate in the biosynthesis of suberin-associated waxes.

In contrast to *StNAC103*, *StNAC101* did not have a preferential expression in the potato tuber peel. Instead, it showed a broad expression in several organs, tissues and conditions of potato plants, including the tuber peel (Fig. S2) [37,38]. Hence, despite the role of *StNAC101* is not probably restricted to the potato tuber phellem, it can participate in the repression of suberin-associated waxes in potato tuber phellem.

StNAC101 is highly induced by abiotic stress and ABA treatments [19], similarly to *StNAC103* [14]. The putative Arabidopsis ortholog of *StNAC101* is *ANAC072* (also named *responsive to dessication* 26, *RD26),* however it is worth emphasizing that this gene has two orthologs in

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potato: *StNAC072* and *StNAC101* [19]. *ANAC072* is also strongly induced by dehydration, ABA and high-salinity and it was suggested that the capacity of *ANAC072* to provide tolerance to drought was achieved by detoxification of toxic aldehydes [63]. It has also been proposed that *ANAC072* has a central role in ABA signalling and desiccation stress [64]. Recently it has been shown that *RD26* controls multiple catabolic genes to rearrange metabolism giving rise to senescence [65].

The ortholog of *StNAC032* in Arabidopsis, *ANAC029/AtNAP (*NAC-like activated by AP3/PI*),* was involved in leaf senescence by stomata closure as a result of inhibiting the ABA pathway [66,67]. The *NAC* genes downregulated unambiguously in both types of silencing are *StNAC072*, *StNAC017*, and *StNAC096*, and their putative Arabidopsis orthologs are *ANAC072* and *ANAC055*, *ANAC100* and *ANAC078*, respectively [19]. As explained above, *ANAC072* was associated with abiotic stress [63], similarly to *ANAC055* and this drought tolerance provided by *ANAC055* is concomitant with proline accumulation and activation of the corresponding biosynthetic gene [68]. In contrast, very little is known about *ANAC100*, besides the fact that it is targeted by miRNA164 [69] and that it can bind to the promoters of some genes involved in the degradation of chlorophyll and leaf senescence [70]. Concerning *StNAC096*, its putative ortholog in Arabidopsis, *ANAC078*, displays many different functions, including regulation of flavonoid biosynthesis under high light [71], regulation of the proteasomes [72,73], and interaction with an atypical type II phosphatidylinositol 4-kinase (PI4Kγ5), to modulate cell proliferation and leaf development by negatively regulating auxin synthesis [74]. As a result, the decrease in the transcript level of *StNAC103*, *StNAC101*, *StNAC032*, *StNAC072*, *StNAC017* and *StNAC096* can have different physiological and molecular effects, in most cases related to ABA and abiotic stress, two factors inducing suberin deposition. ABA and dehydration have been long associated to the suberization process in potato tuber phellem [75,76]. More recently, it has been widely reported that abiotic stresses such as drought and salt stress induce suberin accumulation in roots [4] and activate both suberin and wax biosynthesis [77], which in turn provides increased tolerance to drought and higher water use efficiency [78–80]. It has also been reported that ABA induces activation of suberin genes [75,81–85], leading to suberin deposition [85,86], which in endodermis only occurs when the ABA signalling pathway is active [85]. In agreement with this, biosynthesis of suberin-associated waxes is also induced by salt stress in Arabidopsis taproots that already contained periderm [87]. The fact that suberin and their associated waxes share fatty acid precursors [23,88] suggests that a similar ABA regulation trend is expected also for waxes. Conversely ethylene decreases suberin biosynthesis and it is suggested to trigger degradation of suberin lamellae in Arabidopsis endodermis, evidencing that plasticity of suberin

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is necessary to allow a better adaptation of roots to different soil environments [85]. Taken together, these observations support again a strong connection between ABA/abiotic stress and the suberization process and that NAC repressors aid to the proper regulation of suberin-associated waxes in the apoplastic barrier. However, the precise contribution of each *NAC* gene in the process is still unclear and deserves further investigation.

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Figure legends

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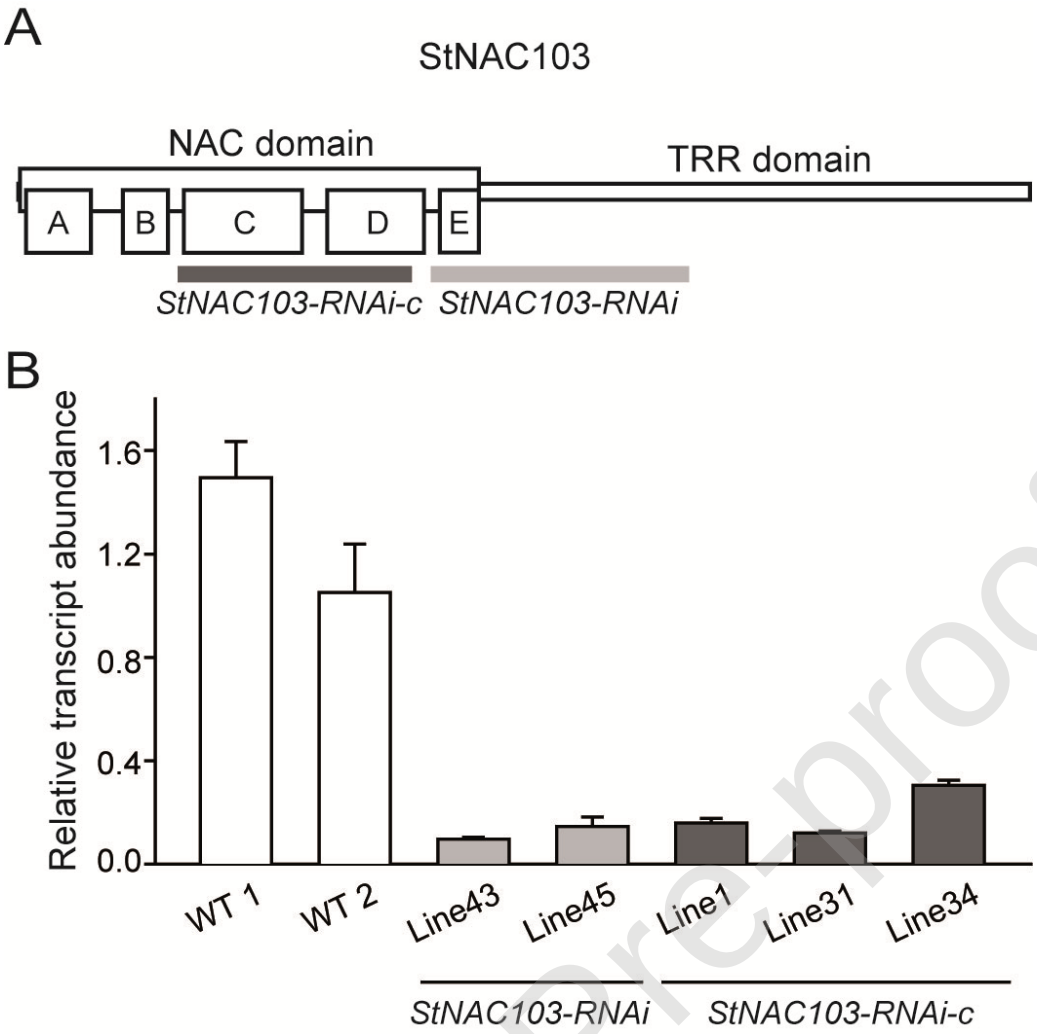

Fig. 1. *StNAC103* silencing in potato tuber phellem. (A) Scheme of the constructs designed to *StNAC103* silencing (*StNAC103-RNAi* targets the variable TRR region and *StNAC103-RNAi-c* the conserved NAC domain, specifically subdomains C and D). (B) *StNAC103* relative transcript abundance in potato tuber phellem of wild-type and silencing lines. Transcript amounts were expressed relative to the reference gene. Relative mRNA expression levels were expressed as mean ± SD of three replicates.

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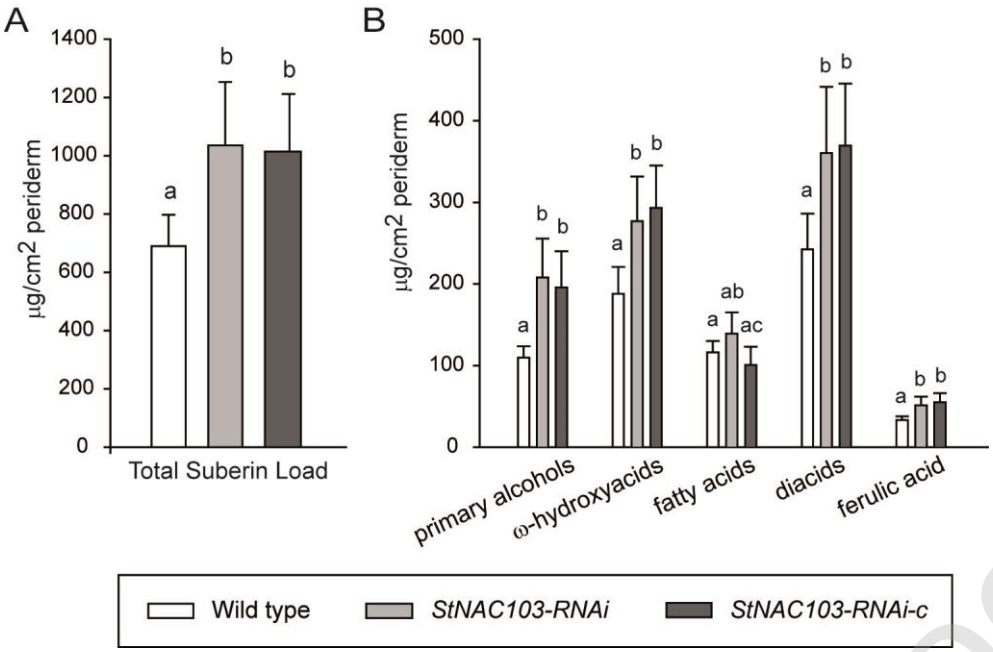

Fig. 2. Effects of *StNAC103* silencing in the transcript abundance of the *NAC* genes predicted as targets of *StNAC103-RNAi-c* in tuber phellem. Results were obtained by microfluidics qPCR and were represented as relative transcript abundance for wild-type, *StNAC103-RNAi* and *StNAC103-RNAi-c* lines. Values are the mean ±SD of biological replicates of the wild-type (n=4) and two independent transformation events including each a minimum of four biological replicates for *StNAC103-RNAi* (line 43 n=5, line 45 n=4) and for *StNAC103-RNAi-c* (line 1 n=4, line 31 n=4). Statistical significant differences ($P < 0.05$) are indicated by lowercase letters. Arrows indicate genes downregulated in at least one transgenic line.

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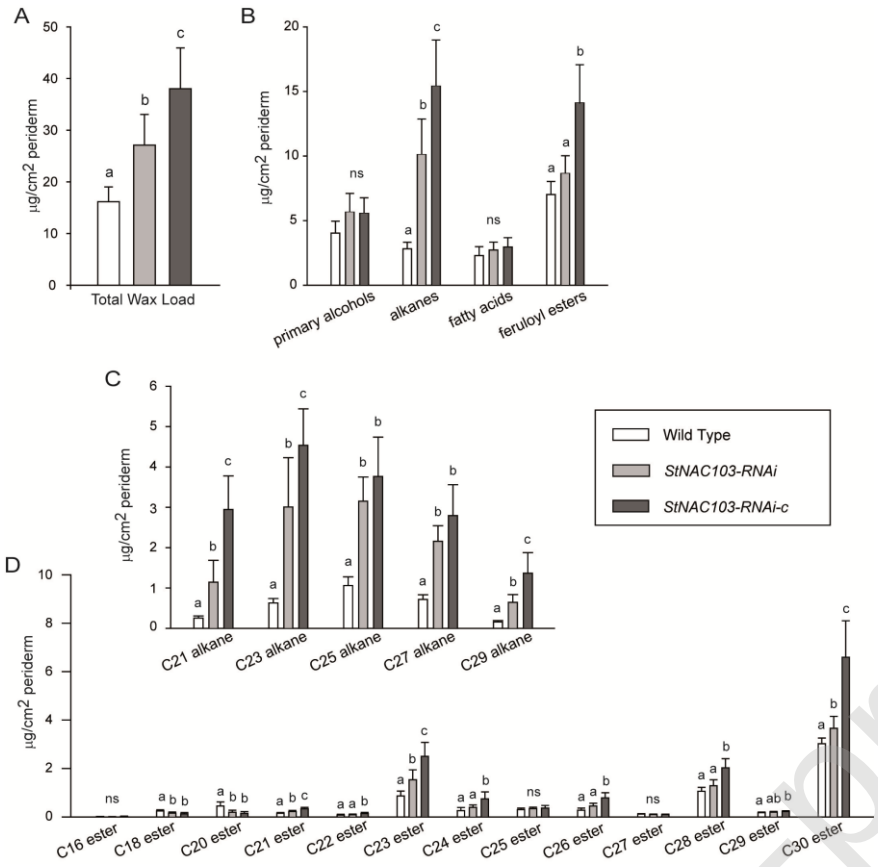

Fig. 3. Effects of *StNAC103* silencing in suberin chemical composition of potato tuber phellem. (A) Total suberin amount in tuber phellem of wild-type and silencing lines shown as the amount per surface area from 39-day-stored tubers. (B) Composition of suberin substance classes in wild-type and silenced lines. Values are the mean ± SD of n biological replicates of wild-type (n=5), two independent transformation events for *StNAC103-RNAi* (n=4, n=3) and three for *StNAC103-RNAi-c* (n=4, n=4, n=4). Statistically significant differences ($P < 0.05$) are indicated by lowercase letters.

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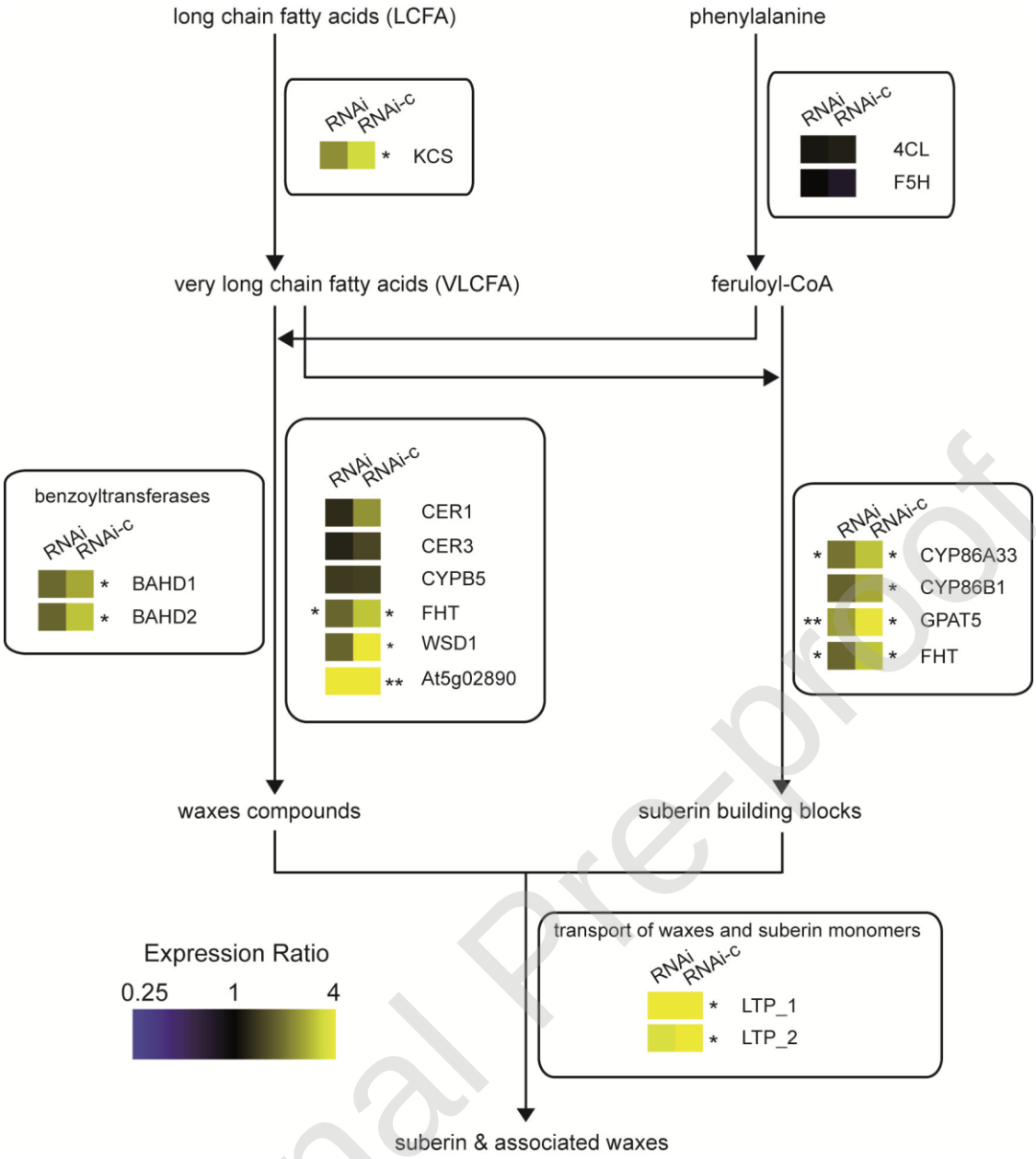

Fig. 4. Effects of *StNAC103* silencing in wax chemical composition of potato tuber phellem. (A) Total wax load in tuber phellem of wild-type and silencing lines shown as the amount per surface area from 39-day-stored tubers. (B) Total amount of different wax substance classes of tuber phellem in wild-type and silencing lines. (C) Profile of alkanes and (D) feruloyl esters of primary alcohols. Values are the mean ± SD of n biological replicates of wild-type (n=5), two independent transformation events for *StNAC103-RNAi* (n=4, n=3) and three for *StNAC103-RNAi-c* (n=4, n=4, n=4). Statistical significant differences ($P < 0.05$) are indicated by lowercase letters.

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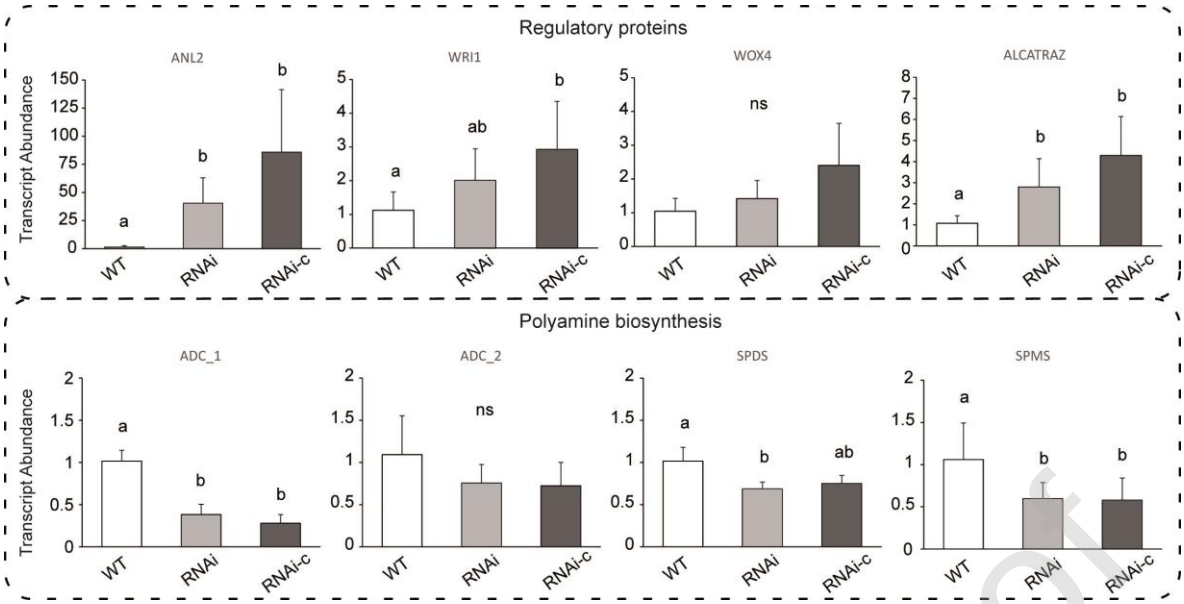

Fig. 5. Heatmap of expression values for suberin and wax related genes in tuber phellem of *StNAC103-RNAi* and *StNAC103-RNAi-c* lines. Units are fold change values of RTA (relative transcript abundance) in *StNAC103-RNAi* and *StNAC103-RNAi-c* divided by RTA values in wild-type. These values were obtained by microfluidics qPCR and the values come from the mean ±SD of two independent transformation events and a minimum of four biological replicates (wild-type: n=4; *StNAC103-RNAi* line 43 n=5, line 45 n=4; *StNAC103-RNAi-c* line 1 n=4, line 31 n=4). Significant differences are denoted with one asterisk ($P < 0.05$) or two asterisks ($P < 0.01$). Most genes are annotated according the Arabidopsis orthologs, excepting *FHT* and *CYP86A33* because its role in potato is known.

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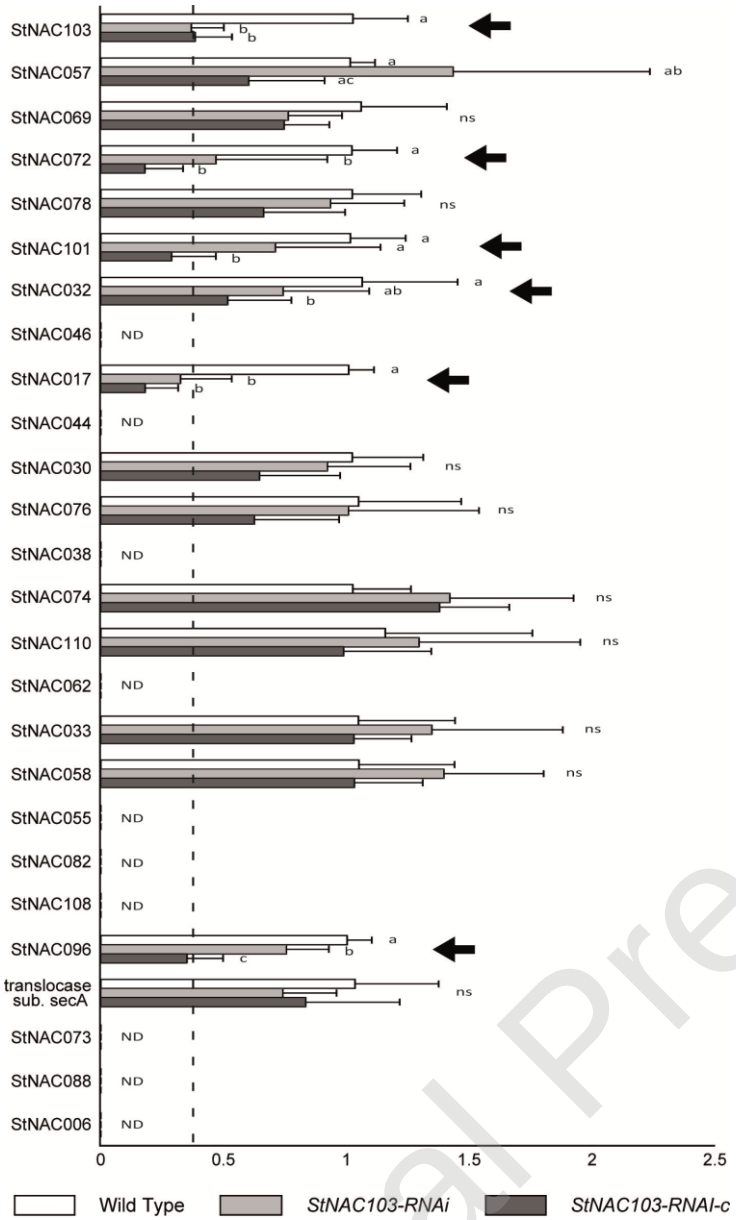

Fig. 6. Effects of *StNAC103* silencing in the transcript abundance of regulatory and polyamine biosynthetic genes in tuber phellem. Results were obtained by microfluidics qPCR and were represented as relative transcript abundance for wild-type, *StNAC103-RNAi* and *StNAC103-RNAi-c* lines. Values are the mean ±SD of the wild-type (n=4) and two independent transformation events for *StNAC103-RNAi* (line 43 n=5, line 45 n=4) and for *StNAC103-RNAi-c* (line 1 n=4, line 31 n=4). Statistically significant differences ($P < 0.05$) are indicated by lowercase letters.

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Table I. List of the putative targets of *StNAC103-RNAi-c* construct. Results of the BLASTN analysis in potato transcript database using the *StNAC103-RNAi-c* construct as a query.

Table II. List of the putative targets of *StNAC103-RNAi* construct. Results of the BLASTN analysis in potato transcript database using the *StNAC103-RNAi* construct as a query.

Table I. Putative genes co-silenced with the *StNAC103-RNAi-c* construct.

Gene identifier	Gene name	NAC subgroup	Mismatch in consecutive 21nt fragment	Blastn e-value (RNAi sequence as a query)
PGSC0003DMG400005384	*StNAC103*	NAC-c	0	1.00E-125
PGSC0003DMG400028779	*StNAC057*	NAC-c	0	2.00E-64
PGSC0003DMG400012403	*StNAC069*	NAC-c	0	2.00E-33
PGSC0003DMG400019294	*StNAC072*	NAC-f	0	1.00E-12
PGSC0003DMG400029593	*StNAC078*	NAC-c	0	2.00E-11
PGSC0003DMG400015342	*StNAC101*	NAC-f	0	3.00E-07
PGSC0003DMG400002824	*StNAC032*	NAC-f	0	2.00E-05
PGSC0003DMG400031266	*StNAC046*	NAC-i	0	1.00E-03
PGSC0003DMG400001338	*StNAC017*	NAC-c	1	3.00E-20
PGSC0003DMG400009920	*StNAC044*	NAC-c	1	2.00E-14
PGSC0003DMG400019523	*StNAC030*	NAC-c	1	4.00E-13
PGSC0003DMG400014845	*StNAC076*	NAC-c	1	6.00E-12
PGSC0003DMG400000887	*StNAC038*	NAC-q-TNAC	1	8.00E-05
PGSC0003DMG400022134	*StNAC074*	NAC-b	1	1.00E-03
PGSC0003DMG400019092	*StNAC110*	NAC-n	1	2.00E-02
PGSC0003DMG400007130	*StNAC062*	NAC-e	1	2.00E-02
PGSC0003DMG400032555	*StNAC033*	NAC-f	1	3.20E-01
PGSC0003DMG400033047	*StNAC058*	NAC-c	2	9.00E-14
PGSC0003DMG400026079	*StNAC055*	NAC-a	2	5.00E-05
PGSC0003DMG400004740	*StNAC082*	NAC-a	2	2.00E-02
PGSC0003DMG400004332	*StNAC108*	NAC-e	2	2.00E-02
PGSC0003DMG400031071	*StNAC096*	NAC-j	2	8.10E-02
PGSC0003DMG400006051	*translocase sub secA*	-	2	8.10E-02

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PGSC0003DMG400039898	*StNAC073*	NAC-m	2	3.20E-01
PGSC0003DMG400025031	*StNAC088*	NAC-f	2	3.20E-01
PGSC0003DMG400021474	StNAC006	NAC-e	2	3.20E-01

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Table II. Putative genes co-silenced with the *StNAC103-RNAi* construct.

Gene identifier	Gene name	NAC subgroup	Mismatch in consecutive 21nt fragment	Blastn e-value (RNAi sequence as a query)
PGSC0003DMG400005384	StNAC103	NAC-c	0	1.00E-122
PGSC0003DMG400028779	StNAC057	NAC-c	1	1.00E-03
PGSC0003DMG400009920	StNAC044	NAC-c	2	6.10E-02

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