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RESEARCH PAPER

Unique features of the grapevine VvK5.1 channel support novel functions for outward K+ channels in plants

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Abstract

Grapevine (*Vitis vinifera* L.), one of the most important fruit crops, is a model plant for studying the physiology of fleshy fruits. Here, we report on the characterization of a new grapevine Shaker-type K^+ channel, VvK5.1. Phylogenetic analysis revealed that VvK5.1 belongs to the SKOR-like subfamily. Our functional characterization of VvK5.1 in *Xenopus* oocytes confirms that it is an outwardly rectifying K^+ channel that displays strict K^+ selectivity. Gene expression level analyses by real-time quantitative PCR showed that *VvK5.1* expression was detected in berries, roots, and flowers. In contrast to its *Arabidopsis thaliana* counterpart that is involved in K^+ secretion in the root pericycle, allowing root to shoot K^+ translocation, *VvK5.1* expression territory is greatly enlarged. Using *in situ* hybridization we showed that *VvK5.1* is expressed in the phloem and perivascular cells of berries and in flower pistil. In the root, in addition to being expressed in the root pericycle like AtSKOR, a strong expression of *VvK5.1* is detected in small cells facing the xylem that are involved in lateral root formation. This fine and selective expression pattern of *VvK5.1* at the early stage of lateral root primordia supports a role for outward channels to switch on cell division initiation.

Keywords: Flowers, new physiological properties, new tissue location sites, phloem, potassium Shaker channel, root lateral primordium, root to shoot translocation.

Introduction

Potassium (K^+) plays an important role in many plant physiological processes. This ion is the most abundant cation in the cytosol, with a concentration ranging between 100 mM and 200 mM (Ache *et al.*, 2001; Wada *et al.*, 2008). K^+ ions are involved in basic functions such as the neutralization of non-diffusible negative charges, the control of cell membrane polarization, electrical signalling, and osmoregulation (Sharma *et al.*, 2013; Dreyer *et al.*, 2014; Véry *et al.*, 2014). Indeed, K^+ is involved in many integrated functions at the whole-plant level such as cellular expansion/elongation and division (Sano et al., 2007, 2009), the control of guard cell turgor allowing stomatal movements (Hosy *et al.*, 2003; Lebaudy *et al.*, 2008*b*), maintenance of cytosolic pH homeostasis, and the setting of the membrane potential (Maathuis, 2009; Marschner, 2012). In plants, the first molecular actors involved in K^+ translocation and distribution are the Shaker K^+ channels. These channels, expressed at the cell plasma membrane, dominate the membrane K^+ conductance in most cell types and are implicated in the control of long-distance K^+ transport (Sharma *et al.*, 2013; Véry *et al.*, 2014). More precisely, K^+ Shaker channels are involved in a number of physiological functions that require sustained large-scale K^+ fluxes. Furthermore, they drive the inwardly or outwardly rectifying K^+ fluxes of the plasma membrane according to the membrane potential, allowing the inward or outward K^+ translocation of the cell (Sharma *et al.*, 2013). For instance, in *Arabidopsis thaliana* roots, the inward Shaker channel AKT1 and the outward channel SKOR are involved in K^+ uptake from the soil (Lagarde *et al.*, 1996; Hirsch *et al.*, 1998; Xu *et al.*, 2006) and the secretion of K^+ into the xylem sap, allowing K^+ translocation to the shoots (Gaymard *et al.*, 1998).

Shaker K^+ channels are multimeric proteins formed by the assembly of four Shaker gene products, known as subunits (Dreyer *et al.*, 1997, 2004). Within this tetrameric structure, the four subunits can either be provided by a single Shaker gene (e.g. a homomeric Shaker channel), or by different Shaker genes (e.g. a heteromeric Shaker channel) (Lebaudy *et al.*, 2008*a*, 2010). Each subunit has a hydrophobic core comprising six transmembrane domains, including a pore domain between the fifth and sixth transmembrane domains as well as a voltage sensor domain (the S4 transmembrane domain) (Zimmermann and Sentenac, 1999). The N- and C-terminal regions are cytoplasmic. The large C-terminal region begins just after the end of the sixth transmembrane domain (S6) and successively contains a C-linker domain, a cyclic nucleotide-binding domain (CNBD), an ankyrin domain, and a K_{HA} domain (Ehrhardt *et al.*, 1997; Sharma *et al.*, 2013; Nieves-Cordones *et al.*, 2014; Véry *et al.*, 2014).

In grapevine, K^+ plays an essential role in the initiation and control of the massive fluxes that are necessary for berry loading during its maturation (Cuéllar *et al.*, 2013; Nieves-Cordones *et al.*, 2019, Zhu *et al.*, 2019). Moreover, in the context of environmental changes, K^+ ion accumulation increases during grape ripening, leading to an excessive neutralization of organic acids (Cuéllar *et al.*, 2010; 2013; Rogiers *et al.*, 2017; Nieves-Cordones *et al.*, 2019). This results in grape berries with low acidity at harvest, yielding wines with poor organoleptic properties and low potential ageing. This is a major concern for grape production, although information on the molecular determinants that control berry K^+ accumulation during its ripening, as well as acidity in grape berry, is still fragmentary. This lack of information hinders wine producers from making sustainable choices to help face future challenges.

Here, we report on the characterization of the grapevine Shaker K^+ channel VvK5.1. Based on our phylogenetic analysis, we show that this Shaker belongs to the SKOR subgroup of the Shaker channel family. Furthermore, electrophysiology analysis reveals that the VvK5.1 channel displays classical slowly activating outward K^+ currents upon depolarizing voltage pulses. In grapevine, *VvK5.1* is mainly expressed in three organs: the flowers, the grape berry phloem, and the roots. In the flowers, there is a strong expression of this channel in the stigmas and in the transmitting tract. In the grape berry phloem, the expression of *VvK5.1* continuously increases after post-veraison and during grape ripening. In the roots, *VvK5.1* expression is detected in the pericycle parenchyma cells, which is reminiscent of the role that the *A. thaliana* SKOR channel plays in secreting K^+ into the xylem sap. We also detected an intense expression of *VvK5.1* in the lateral root primordium. Finally, the roles played by the VvK5.1 channel in these three organs are discussed.

Materials and methods

Plant material

Four-year-old grapevines (*Vitis vinifera* cv. Cabernet Sauvignon) were potted in 70 litre plant containers and grown in field conditions. Plant watering was carried out by a controlled drip-irrigation system. The plant water status was checked by measuring leaf water potential (Ψ) at dawn (Cuellar *et al.*, 2010), which remained close to –0.2 MPa throughout grape maturation. All organs were collected at fruit set, with the exception of berry samples, which were harvested at different stages of grape ripening. For *VvK5.1* root expression analysis, root samples were harvested from 2-month-old rooted canes grown in 'perlite'. All collected samples were immediately frozen in liquid nitrogen, crushed, and used for RNA extraction.

Cloning of the VvK5.1 cDNA subunit

First-strand cDNAs were generated from total RNA extracted from ripe berries using SuperScript III RT polymerase (Invitrogen 18080-051). Full-length *VvK5.1* Shaker subunit cDNA (2466 bp) was then amplified using VvK5.1-V2-F1 and VvK5.1-V2-R1 specific primers (see Supplementary Table S1 at *JXB* online) prior to cloning into the pDONR207 entry vector using a Gateway strategy (Invitrogen).

Functional characterization of VvK5.1

VvK5.1 cRNAs produced using the mMessage mMachine T7 ultra transcription Kit (Invitrogen) were injected (15 ng in 14 nl) using a microinjector (Nanoliter2010, WPI, https://www.wpiinc.com) into *Xenopus laevis* oocytes. As a control, the same volume (14 nl) of water was injected. After injections, oocytes were kept at 20 °C in ND96 solution (pH 7.4; 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, and 50 μg ml^{-1} gentamicin). Membrane currents were recorded 2–4 d after injections with a two-microelectrode voltage-clamp amplifier (GeneClamp 500B; Axon Instruments, http://www.moleculardevices.com) and a Digidata 1322A interface (Axon Instruments), using pipettes filled with 3 M KCl. Recordings were visualized and saved on a pClampex 10 (Axon Instruments: Molecular Devices Corp., Sunnyvale, CA, USA). During recordings, oocytes were maintained in percolating solutions of 100 (K100), 50 (K50), 10 (K10), 1 (K1), or 0.1 (K0.1) mM potassium gluconate supplemented respectively with 0, 50, 90, 99, or 99.9 mM sodium gluconate. Each of these solutions contained 1 mM $CaCl_2$, 2 mM $MgCl_2$, 31 mM sorbitol, and 5 mM HEPES (pH 6.5 and 7.4) or 5 mM MES (pH 5.0 and 5.5). Furthermore, either 10 mM TEA (tetraethyl ammonium) or 10 mM Ba^{2+} was added to the K10 solution at pH 7.4 in order to test channel inhibition. Two voltage-clamp protocols were applied with 8 s voltage pulses, one from −110 mV to +40 mV and the other from −100 mV to + 50 mV (in −15 mV steps for each protocol). Each step started with a holding potential of −40 mV, then a 250 ms pulse at −100 mV was conducted before the different voltage steps. After each pulse of 4.5 s, the voltage returned to −100 mV for 2 s before returning to the holding potential.

Analyses were performed with the pClampfit 10 software program (Axon Instruments). Current–voltage (I/V) curves were obtained by plotting the steady-state currents at the end of the activating voltage pulses against the corresponding applied membrane potentials.

Total RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from grapevine samples using the Spectrum plant total RNA kit (Sigma Aldrich) and quantified with the Quant-iT Ribogreen RNA reagent (Invitrogen). After DNase I treatment (Invitrogen), first-strand cDNAs were synthesized with SuperScript III reverse transcriptase (Invitrogen) and used as a template for the RT-qPCR experiments. All steps were carried out according to the manufacturer's instructions. RT-qPCR analyses were performed on the Lightcycler480 system using TB Green Premix Ex Taq (TAKARA) with 20 ng of total RNA in 10 µl. Reactions were performed in triplicate using two independent biological samples. All amplification plots were analysed with Lightcycler480 software, using a threshold of 0.25 to obtain C_T (cycle threshold) values. The *VvK5.1* expression level was then normalized using the corresponding C_T values of the control housekeeping gene *VvEf1-alpha* (Terrier *et al.*, 2005; Nieves-Cordones *et al.*, 2019). Standard curves were obtained by successive dilution with known quantities of *VvK5.1* and *VvEf1-alpha* cDNA amplicons. Specific primers for amplifying *VvK5.1* (VvK5.1-V2-qP-3'-F1 and VvK5.1-V2-qP-3'-R1; Supplementary Table S1) and *VvEf1-alpha* (EF1-F/EF1-R; Cuéllar *et al.*, 2010) were designed using the PRIMER3 website (http://primer3.ut.ee/) and are provided in Supplementary Table S1.

Tissue localization of VvK5.1 mRNA by in situ hybridization

Fresh grapevine organs (flowers, roots, and ripe berries) from plants grown in well-irrigated conditions were harvested, fixed in 4% paraformaldehyde solution (Bio-Rad), dehydrated, embedded in paraffin, and cut into 11 µm thick sections. VvK5.1 RNA sense and antisense probes were synthetized using the specific VvK5.1 primers (T7P-VvK5.1-V2-up/VvK5.1-V2-down and VvK5.1-V2-up/T7P-VvK5.1-V2-down; Supplementary Table S1) and labelled with UTP-digoxigenin during the transcription step (MAXIscript T7 transcription kit; Invitrogen). An 18S rRNA probe was used as positive control. Samples were hybridized overnight with VvK5.1 RNA probes and then incubated for 1 h with an anti-digoxigenin antibody conjugated to alkaline phosphatase (1/500 dilution; Roche, http://www.roche.com). Hybridization signals were revealed using the vector blue kit III (Vector Laboratories, http://www.vectorlabs.com; positive signal is blue). *In situ* hybridization experiments were independently repeated twice.

The DAPI (Sigma-Aldrich) nuclear counterstain was used to visualize the cell nucleus. DAPI [0.001% in 1× phosphate-buffered saline (PBS)] was applied onto hybridized tissues for 10 min in the dark. Slides were then observed with an Eclipse Ni-E microscope at the ×4, ×10, and ×20 objective magnifications (Nikon, https://www.microscope.healthcare.nikon.com), and images were taken with a Nikon DS-Ri2 camera using the NIS elements software. *In situ* hybridization pictures were taken in white light or DIC (differential interference contrast) mode. For fluorescent pictures, DAPI was excited at 350 nm in order to recover the emitted signal between 450 nm and 490 nm (blue).

Localization of VvK5.1 activity in planta

A 1.4 kb region upstream of the *VvK5.1* gene (XP_010660282.1) ATG codon was amplified through two successive PCR runs using VvK5.1-prom-Fw1/VvK5.1-prom-Rv1 and VvK5.1-prom-Fw2/VvK5.1-prom-Rv2 primers (Supplementary Table S1), and inserted into the pGWB3 destination Gateway vector (Nakagawa *et al.*, 2007) containing the β-glucuronidase (GUS) gene. *Arabidopsis thaliana* plants (Col ecotype) were transformed using the floral dip method (Clough and Bent, 1998). Transformants were selected on hygromycin, and homozygotes were recovered in the next generation. GUS activity was detected according to Lagarde *et al.* (1996), and slides were observed using an Eclipse Ni-E microscope (Nikon, https://www.microscope.healthcare.nikon.com).

Results

Molecular cloning of VvK5.1 *cDNA*

A 2466 bp cDNA was cloned by RT-PCR from the total RNA of post-veraison *V. vinifera* (cv. Cabernet Sauvignon) using the VvK5.1-V2-F1/VvK5.1-V2-R1 specific primers (Supplementary Table S1). The deduced VvK5.1 polypeptide contains 821 amino acids and shares similarity with other plant Shaker potassium channels. The hydrophobicity profile predicts that VvK5.1 displays the typical structural regions of a Shaker subunit with six membrane-spanning domains (S1–S6) flanked by N- and C-terminal cytosolic sequences of variable lengths. The N-terminal domain is very short, in contrast to the large C-terminal sequence that successively contains a C-linker domain, a CNBD, an ankyrin domain, and a K_{HA} domain (Ehrhardt *et al.*, 1997; Sharma *et al.*, 2013; Nieves-Cordones *et al.*, 2014). The six-transmembrane domain includes a well-defined pore region between the fifth and sixth transmembrane segments, which contains the hallmark TxxTxGYGD of highly K^+-selective channels. Identification of the closest relatives of the grapevine VvK5.1 channel by phylogenetic analysis revealed that VvK5.1 is highly similar to the SKOR channels already characterized in several dicotyledonous plants (Fig. 1). These channels belong to the outwardly rectifying K^+ channel subgroup of the Shaker family. The VvK5.1 sequence displays 76.5, 75.2, 71.6, and 66.5% amino acid sequence identities (ASI) throughout its entire protein length with CmSKOR1 from melon (*Cucumis melo*), CusSKOR1 from cucumber (*Cucumis sativa*), *A. thaliana* AtSKOR, and AtGORK, respectively.

The grapevine channel VvK5.1 mediates outwardly rectifying K^+ currents in Xenopus laevis *oocytes*

Heterologous expression of the VvK5.1 channel in *X. laevis* oocytes induced outwardly rectifying currents upon depolarizing voltage pulses (Fig. 2), which were never observed in control oocytes injected with water. These time-dependent slowly activating currents

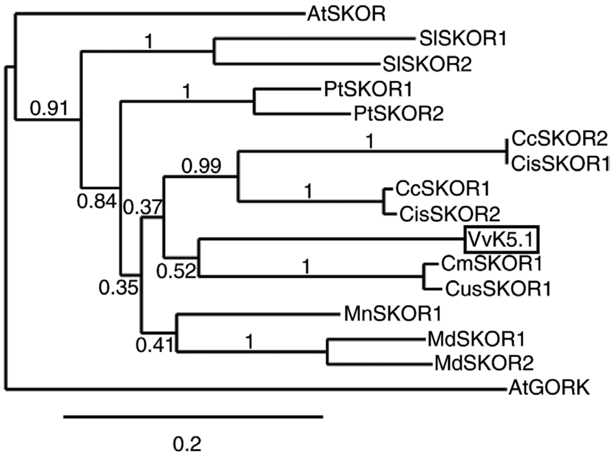

Fig 1. The phylogenetic relationships of VvK5.1. An unrooted phylogenetic tree was constructed with 16 polypeptide Shaker K^+ channel sequences belonging to the outwardly rectifying potassium channel subfamily. VvK5.1 (XP_010660282.1) is the grapevine Shaker channel. Other sequenced species close to VvK5.1 are AtSKOR (At3g02850) and AtGORK (At5g37500) from *Arabidopsis thaliana*; SlSKOR1 (XP_004240037.1) and SlSKOR2 (XP_004250206.1) from tomato (*Solanum lycopersicum*); CcSKOR1 (XP_006421368.1) and CcSKOR2 (XP_006427880.1) from clementine (*Citrus clementina*); CisSKOR1 (XP_006464550.1) and CisSKOR2 (XP_015389397.1) from orange tree (*Citrus sinensis*); CmSKOR1 (XP_008460504.1) from melon (*Cucumis melo*); CusSKOR1 (XP_004140369.2) from cucumber (*Cucumis sativa*); MnSKOR1 (XP_010108959.1) from blackberry (*Morus notabilis*); MdSKOR1 (XP_008343075.1) and MdSKOR2 (XP_008381509.1) from apple (*Malus domestica*); and PtSKOR1 (XP_006372521.1) and PtSKOR2 (XP_002305894.2) from poplar (*Populus trichocarpa*). Bootstrap values are reported next to the nodes of the tree. Branch length is proportional to the evolutionary distance between the outward rectifying potassium channels of different dicotyledon species.

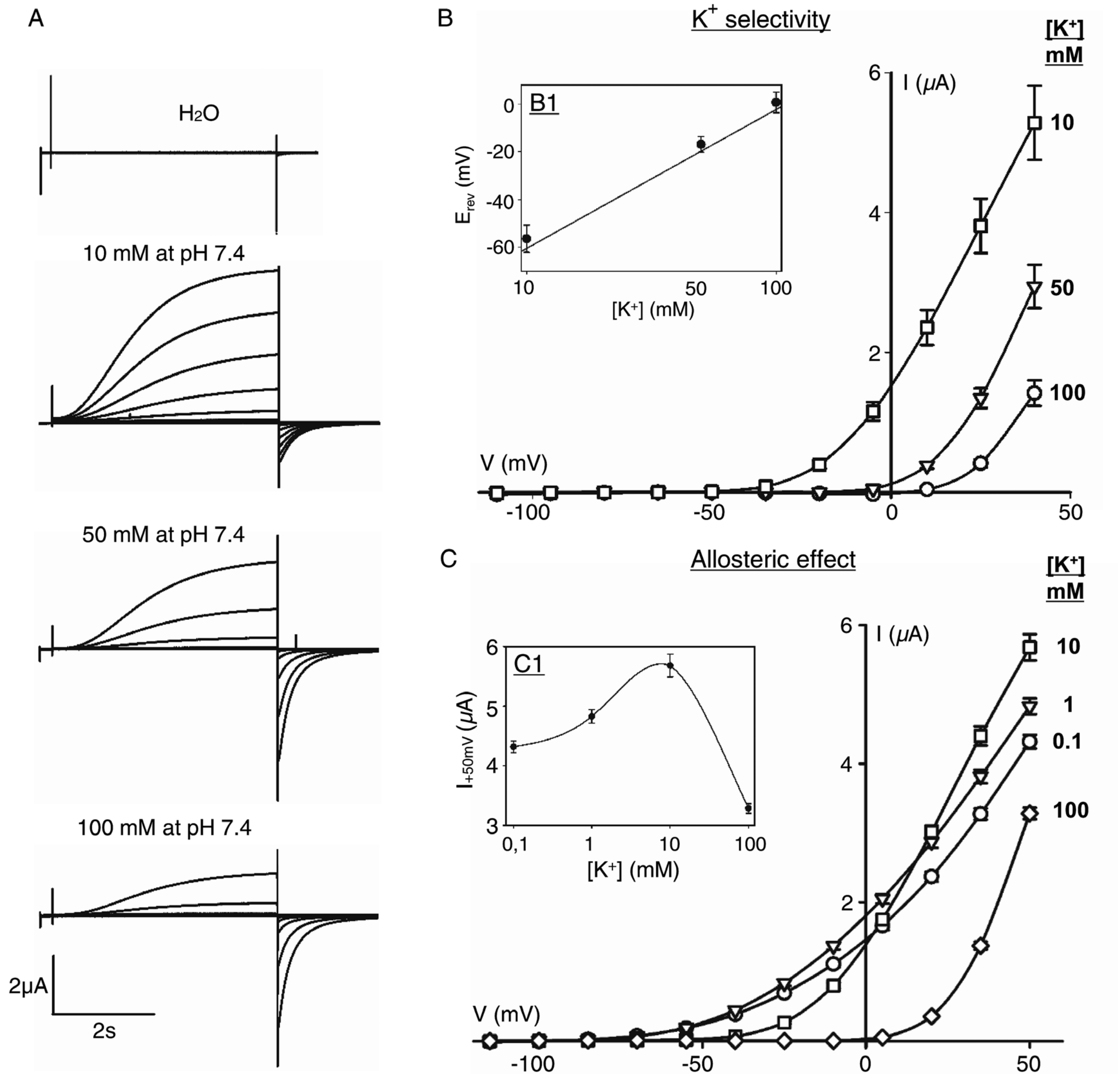

Fig 2. Functional characterization of the grapevine channel VvK5.1 by heterologous expression in *Xenopus laevis* oocytes. (A) Representative current traces in response to voltage–clamp pulses from –110 mV to +40 mV that depend on varying external K^+ concentrations at pH 7.4 as indicated. Time-dependent outwardly rectifying currents are activated upon depolarization. (B) Current–voltage curves for mean currents mediated by VvK5.1 that depend on K^+ concentrations; *n*=10 ±SE. Note the shift in the activation potential at the reduced external K^+. (B1) VvK5.1 reversal potentials (E_{rev}) were determined from tail current analysis, indicating a shift in dependence on external K^+ concentrations as shown; *n*=10 ±SE. The line in the insert corresponds to the predicted Nernst potentials of K^+. Tail currents were analysed at voltage pulses ranging from –110 mV to +40 mV, following activation of the channel at +30 mV. (C) Current–voltage curves for mean currents mediated by VvK5.1 that depend on K^+ concentrations; *n*=8 ±SE. (C1) The maximal currents at +50 mV decreased between 10 mM and 0.1 mM K^+, despite the increase in driving force.

showed sigmoidal activation kinetics (Fig. 2A). Current amplitudes increased with lower external K^+ levels according to the driving force for K^+ efflux. Current–voltage curves (Fig. 2B) plotted from the steady-state currents at the end of the voltage pulses from –110 mV to +40 mV, as shown in Fig. 2A, revealed strict outward rectification of these K^+-dependent currents. The activation potential displays a shift depending on external K^+ concentration allowing, at lower K^+ concentrations, an efflux of K^+ at more hyperpolarized potentials. Further analyses of reversal potentials (E_{rev}) when plotting tail currents from –110 mV to +40 mV after activation of the channel at +30 mV showed a shift towards more hyperpolarized potentials when the external K^+ concentration was decreased. This shift of around –56 mV corresponds to the predicted Nernst potential of K^+ for a 10-fold change in the K^+ gradient (Fig. 2B, insert B1), proving the K^+ selectivity of the channel.

Further analyses of the dependence of the VvK5.1 channel on external K^+ revealed a decrease in the maximal currents at depolarized potentials when K^+ decreases below 10 mM (Fig. 2C). At concentrations of 1 mM or 0.1 mM K^+, current amplitudes at positive potentials decreased even though the channel was activated at more hyperpolarized potentials, suggesting an allosteric regulation (Fig. 2C1). This kind of regulation has already been observed for other voltage-gated channels, such as the *A. thaliana* SKOR channel (Gaymard *et al.*, 1998). Dependence of the VvK5.1 current amplitudes on external pH was tested at four different pH values at 10 mM K^+ (Fig. 3A). No significant differences were observed in solutions adjusted to pH 7.4 or to pH 6.5. However, further acidification of the external solution to pH 5.5 or pH 5.0 reduced the VvK5.1 current, suggesting the regulation of channel permeation by protons. This pH dependence has also been observed for the *A. thaliana* outward rectifier GORK and SKOR (Ache *et al.*, 2000; Lacombe *et al.*, 2000), and is in contrast to the pH dependence of plant inward rectifiers such as KAT1 (Brüggemann *et al.*, 1999) or VvK1.2 (Cuéllar *et al.*, 2013). Finally, pharmacological properties of the VvK5.1 channel were tested by external perfusion with the known K^+ channel blockers, BaCl and TEA (Fig. 3B). In both cases, the current amplitudes were largely inhibited, and maximal currents were respectively reduced by 40% (10 mM BaCl, n=11) and 75% (10 mM TEA, n=5). Altogether, our functional characterization of the grapevine channel VvK5.1 in oocytes demonstrates typical properties of plant outwardly rectifying voltage-gated K^+ channels.

VvK5.1 *expression patterns*

The spatial expression patterns of the *VvK5.1* gene were investigated by RT-qPCR on total RNA extracted from roots, stems, leaves, tendrils, and stalks (all at fruit set), and from flowers and berries during their development. Unexpectedly, our results showed that *VvK5.1* transcripts are mainly expressed in three organs: the roots, the flowers, and the berries, with expression levels ranging between 450 copies ng^{-1} and 550 copies ng^{-1} of total RNA (Fig. 4A). During grape berry development, *VvK5.1* expression remains consistently very

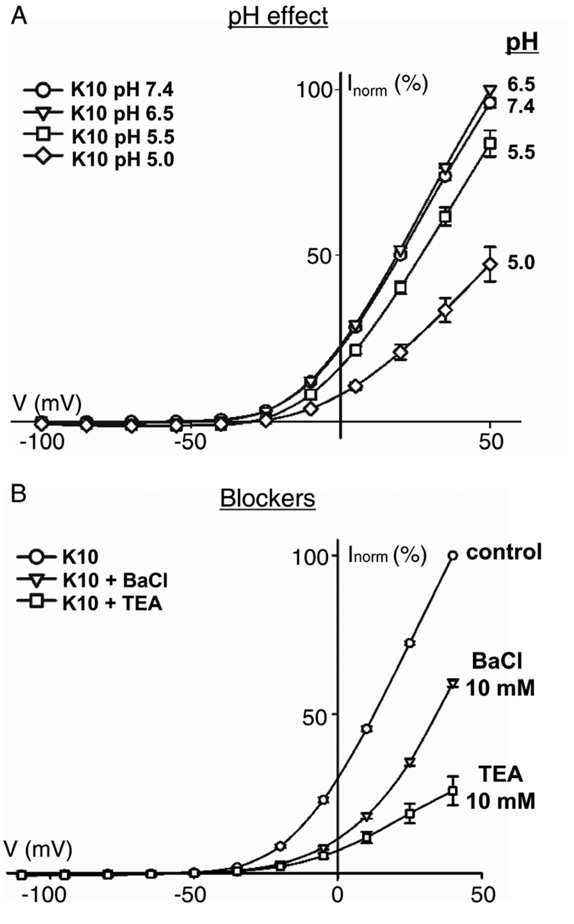

Fig 3. Functional properties of the grapevine channel VvK5.1 when expressed in *Xenopus laevis* oocytes. (A) Dependence of VvK5.1-normalized mean currents upon external pH in 10 mM K^+; n=8 ±SE. Normalization was conducted by setting the currents at +50 mV in the standard solution (pH 6.5) to 100%. The current is reduced upon acidification of the external bath solution. (B) Inhibition of VvK5.1-mediated normalized currents by 10 mM BaCl (n=11 ±SD) or TEA (n=5 ±SD) in an external solution of 10 mM K^+ at pH 7.4. Inhibition is shown as a percentage of control currents (n=16).

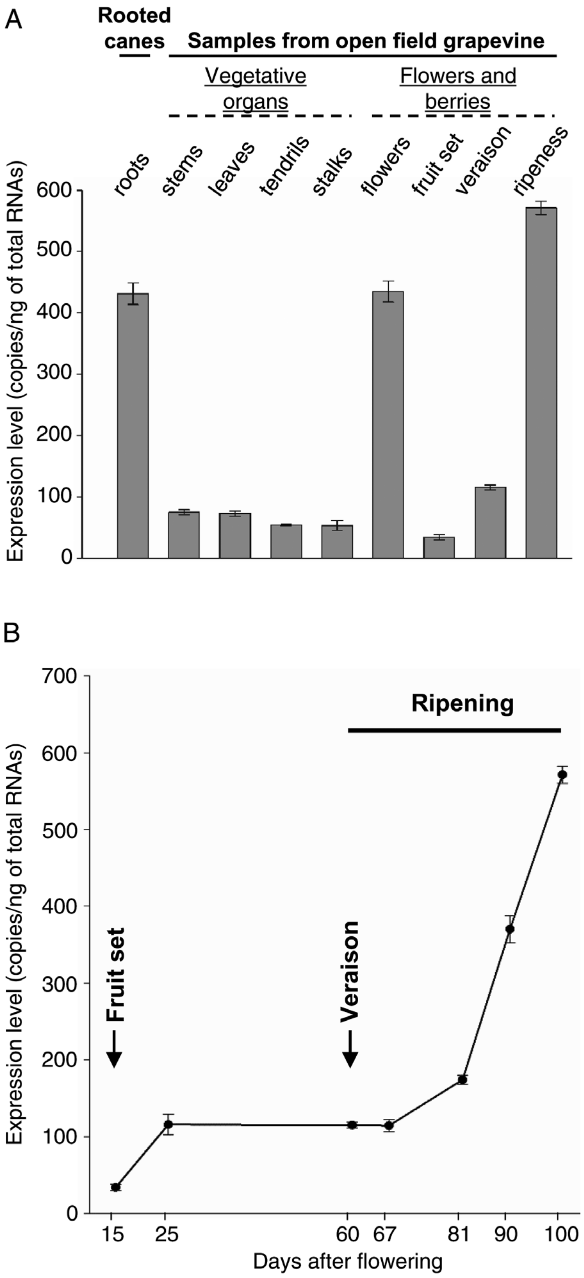

Fig 4. *VvK5.1* transcript levels in grapevine organs and during berry development. RT-qPCR was performed on first-strand cDNAs synthesized from total RNAs of different organs. (A) *VvK5.1* transcript levels in roots from rooted canes, or in vegetative organs (stems, leaves, tendrils, and stalks) collected at fruit set (15 d after flowering), flowers, or in berries at three different developmental stages (fruit set, veraison, and ripeness). Vegetative organs, flowers, and berries were collected from grapevines grown in open field conditions under standard irrigation. (B) *VvK5.1* transcript levels of berries collected at different developmental stages in field conditions. The fruit set, veraison, and ripening phases are indicated. Note that VvK5.1 expression suddenly and strongly increased in grape berries at veraison. The mean values and SE of two biological replicates are presented.

low until the berry post-veraison period (Fig. 4B). From this stage onward, the accumulation of *Vv5.1* transcripts is strongly accelerated, increasing by up to 5-fold at ripeness.

In order to identify the exact localization of *VvK5.1* expression at the tissue level, *in situ* hybridization experiments were performed on flowers, ripe berries, and roots (Figs 5–7). In flowers, VvK5.1 signals were detected in the transmitting tract and in the stigmas (Fig. 5B, C), while in the ovule, the signal was detected in the nucellus (Fig. 5E, F).

In ripe berries, strong *VvK5.1* expression was located in the phloem of the central and peripheral vascular bundles (Fig. 6B, C, E, H). It is worth noting that this *VvK5.1* expression was observed throughout the sieve tube system, which is composed of companion cells and enucleated sieve tubes (Fig. 6H, I, K, L). In addition, *in situ* hybridization results showed that the *VvK5.1* gene is expressed in the epicarp cells (Fig. 6F).

In roots, *VvK5.1* expression was detected in the vascular cylinder (Fig. 7B, C). *In situ* hybridization signals were found in the root pericycle and to a lesser extent in the phloem. In the pericycle, we observed heterogenous *VvK5.1* signals distributed between two different cell types: large parenchyma cells (LPCs) and small cells (SCs) of the pericycle. The SCs were always located near the xylem pole (Fig. 7), as revealed by cell nucleus labelling with DAPI (Fig. 7F). In this zone, the high number of observed nuclei indicates the presence of numerous cells. However, previous research has shown that the lateral root primordia arise from localized cell divisions into a pericycle zone located in the proximity of the xylem (Casero *et al.*, 1989; Dubrovsky *et al.*, 2000; Hochholdinger and Zimmermann, 2008), and so the strong expression of *VvK5.1* in this zone suggests that this Shaker could be involved in primordia formation (Fig. 7E, F, H, I). To examine this hypothesis, we analysed the tissue-specific activity of the *VvK5.1* promoter by histochemical analysis of GUS staining (blue) in transgenic plants of *A. thaliana* expressing a *VvK5.1 promoter::GUS* fusion construct. As shown in Fig. 8, *GUS* expression (blue colour) is observed in *A. thaliana* root cells beginning at the two-cell stage (Fig. 8B). Subsequently, further oriented divisions followed by cell enlargement give rise to an organized organ, the root primordium, which develops its own root apex and root cap, and grows through the parent root cortex to emerge at the root surface as a lateral root (Fig. 8C–G). This experiment confirms the involvement of the outward Shaker channel VvK5.1 in the lateral root formation. To date, no involvement of any outward K^+ channels in lateral root primordia formation has been described.

Discussion

Two outward Shaker K^+ channels have been identified and extensively characterized in the plant model *A. thaliana* (Gaymard *et al.*, 1998; Hosy *et al.*, 2003). The first one, GORK, encodes the major voltage-gated outwardly rectifying K^+ channel of the guard cell membrane, which mediates guard cell K^+ release, allowing stomatal closure (Ache *et al.*, 2000; Pilot *et al.*, 2001; Szyroki *et al.*, 2001; Hosy *et al.*, 2003; Lebaudy *et al.*, 2008*b*). The GORK channel is also expressed in the root hairs, where it could play a role in physiological processes that involve K^+ efflux from the roots (Demidchik, 2014). Furthermore, its expression has been reported in the phloem, and its role in the electrical propagation of action potentials has recently been deciphered (Cuin *et al.*, 2018). In contrast, the second *A. thaliana* K^+ channel, SKOR, is mainly expressed in the root

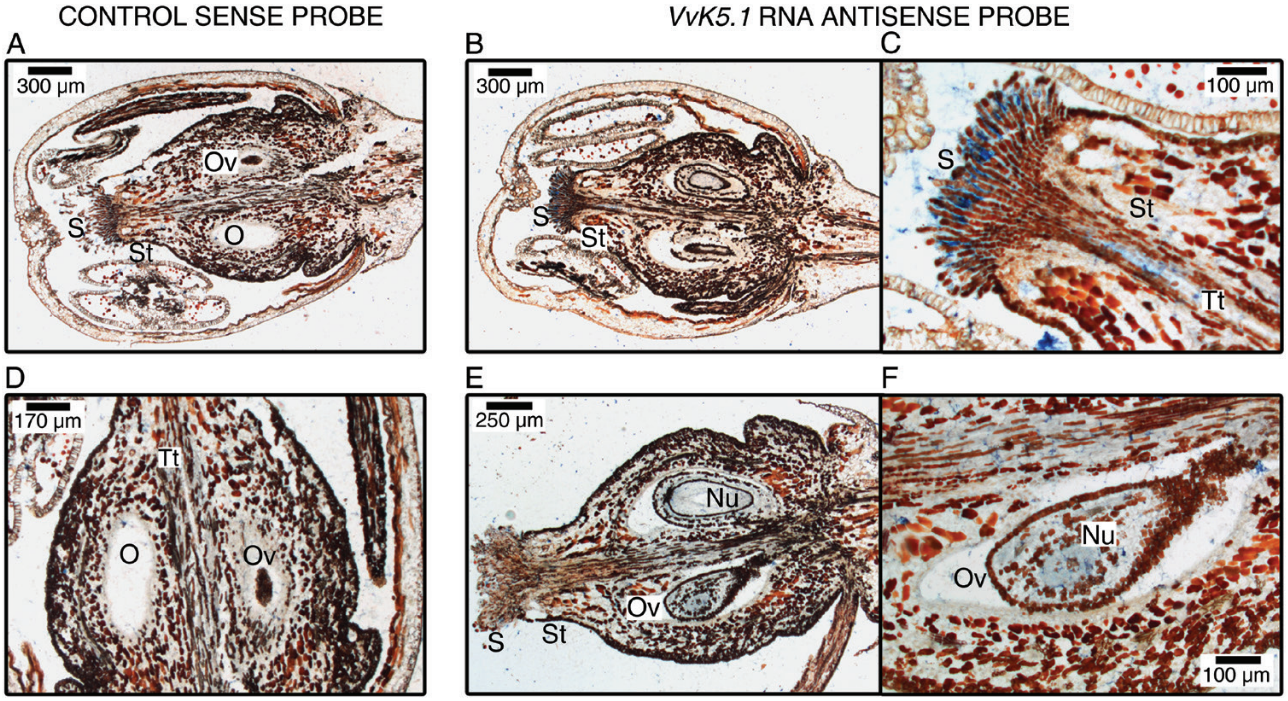

Fig 5. *In situ* localization of VvK5.1 transcripts in flowers. Longitudinal sections of flowers were hybridized with a VvK5.1 RNA sense probe as negative control (left column: A, D) or antisense probe (two right columns: B, C, E, F). Sections hybridized with the sense probe did not show any signal. Sections hybridized with the *VvK5.1* antisense probe showed positive blue signals in the stigmas, the transmitting tract (B and C), and the ovule (E, F). In the ovule, blue signal was observed in the nucellus (F). Nu, nucellus; O, ovary; Ov, ovule; S, stigmas; St, style; Tt, transmitting tract.

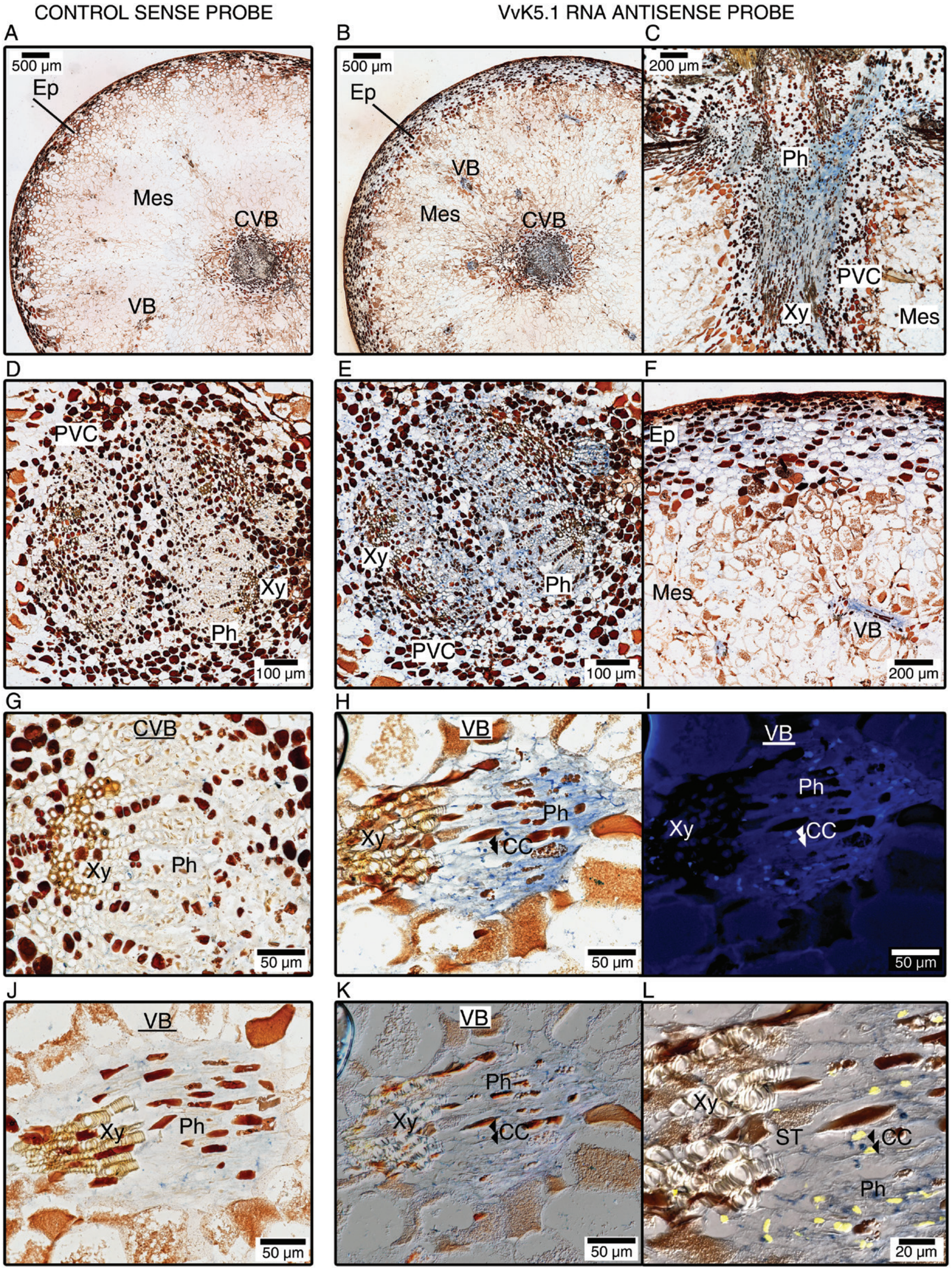

Fig. 6. *In situ* localization of *VvK5.1* transcripts in berries during ripening. Longitudinal and equatorial sections were hybridized with the *VvK5.1* RNA sense probe as negative control (left column: A, D, G, J) or *VvK5.1* RNA antisense probe (right column: B, C, E, F, H, I, K, L). Sections probed with sense probes did not show any significant signal. Sections hybridized with RNA antisense probe showed positive blue signals in ripening berries. Intense blue signals were specifically found in the phloem (B, C, E), perivascular cells (B, C, E), and, to a lesser extent, in the epicarp cells (F). DAPI staining was performed after *in situ* hybridization of a longitudinal section revealed a vascular bundle (H), in order to identify companion cells via their nucleus and to distinguish them from enucleated phloem sieve tubes. The section stained with DAPI was observed by fluorescence microscopy to localize cell nuclei (I), and by DIC microscopy to visualize the cell walls (K). A zoomed composite picture (L) merging (H), (I), and (K) was constructed using Image J to help localize phloem companion cells (CCs) and enucleated sieve tubes (STs). As an example, the locations of two CCs are indicated by double arrows in (H), (I), (K), and (L). Note that these two cells display a positive blue signal after *in situ* hybridization (H and L). CC, companion cells; CVB, central vascular bundle; Ep, epicarp; VB, vascular bundle; Mes, mesocarp; Ph, phloem; PVC, perivascular cells; ST, sieve tubes; Xy, xylem.

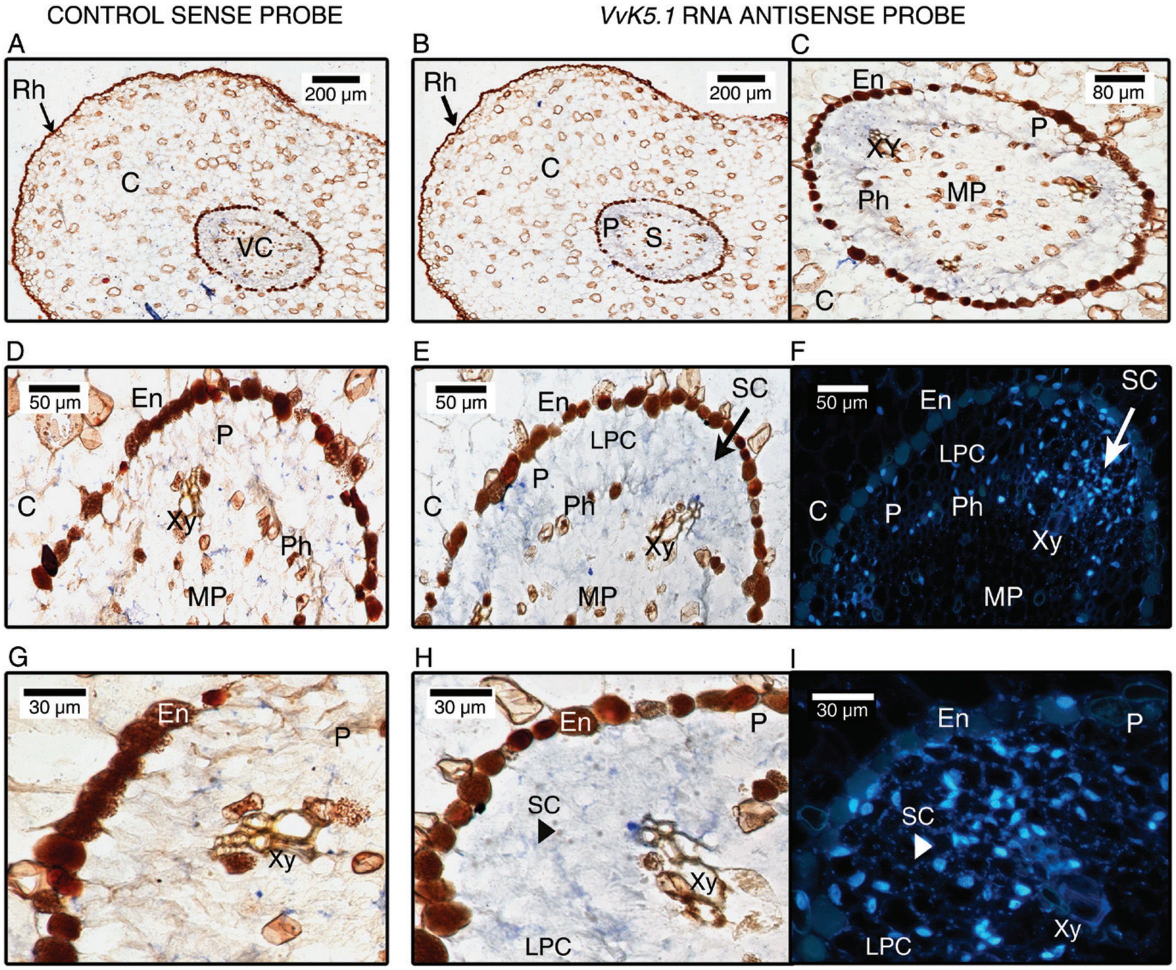

Fig. 7. *In situ* localization of VvK5.1 transcripts in roots of rooted canes. Equatorial sections were hybridized with *VvK5.1* RNA sense probe as negative control (left column: A, D, G) or with *VvK5.1* RNA antisense probe (two right columns: B, C, E, F, H, I). Sections hybridized with *VvK5.1* sense probe did not show any blue staining at the different magnifications (A, D, G). In contrast, positive signals were observed in the stele with *VvK5.1* RNA antisense probe (B, C, E, H). The blue signals were located in the phloem in the small (SC) and large parenchyma cells (LPC) of the pericycle (C, E, H). A weaker signal was also detectable in the phloem. (E) and (H) were observed by fluorescence microscopy after DAPI staining to visualize nuclei and the different cell density of the pericycle (F, I). Rh, rhizodermis; C, cortex; En, endoderm; P, Pericycle; PM, medullary parenchyma; Xy, xylem; Ph, phloem; SC, small cells; LPC, large parenchyma cells.

pericycle and xylem parenchyma cells, and is involved in K^+ secretion into the xylem sap (Gaymard *et al.*, 1998; Nguyen *et al.*, 2017; Long-Tang *et al.*, 2018). The grapevine channel VvK5.1 belongs to the SKOR-like Shaker channel group (Fig. 1). Although the closest VvK5.1 relative is the CmSKOR1 melon channel (*C. melo*, with 76.5% ASI), which is primarily expressed in the root pericycle and is essentially involved in the translocation of K^+ from the root to the shoot like its *A. thaliana* counterpart SKOR (Long-Tang *et al.*, 2018), the wide-ranging expression profile of the *VvK5.1* gene (which is highly linked to the channel's functional properties) strongly indicates that VvK5.1 could be involved in different processes.

The VvK5.1 channel in flowers

In contrast to the ancestral wild grapevine *V. vinifera* subsp. *sylvestris*, which is a dioecious plant, the cultivated grapevine (*V. vinifera*) used for wine and grape production have hermaphroditic flowers. The main method of pollination in these domesticated vines is self-fertilization. However, in an inflorescence, the percentage of ovaries yielding fruit is strongly variable, and is affected by different factors including environmental changes such as light, temperature, and water stress (Lecourieux *et al.*, 2017; Fábián *et al.*, 2019), but also vineyard management and grape variety features (Dry *et al.*, 2010). Indeed, low fruit set can limit crop yield, resulting in losses to grape and wine production. In grapevine, as for all flowering plants, the fertilization process begins with pollen hydration, once the pollen has landed on the stigma. This hydration stage is a key step, since pollen grains need to absorb water for germination. Germination is then initiated at the surface of the stigmatic papilla cells, and the pollen produces a pollen tube that grows in the intercellular space through the style, where it enters into the transmitting tract and delivers the two sperm cells to the ovule for the typical double fertilization of plants (Dresselhaus and Franklin-Tong, 2013; Doucet *et al.*, 2016). The pistil is the site of pistil–pollen interaction and the cooperative processes that regulate pollen hydration, germination, pollen tube growth, and thereby fertilization. Potassium ions are required at each step (Campanoni and Blatt, 2007; Michard *et al.*, 2009).

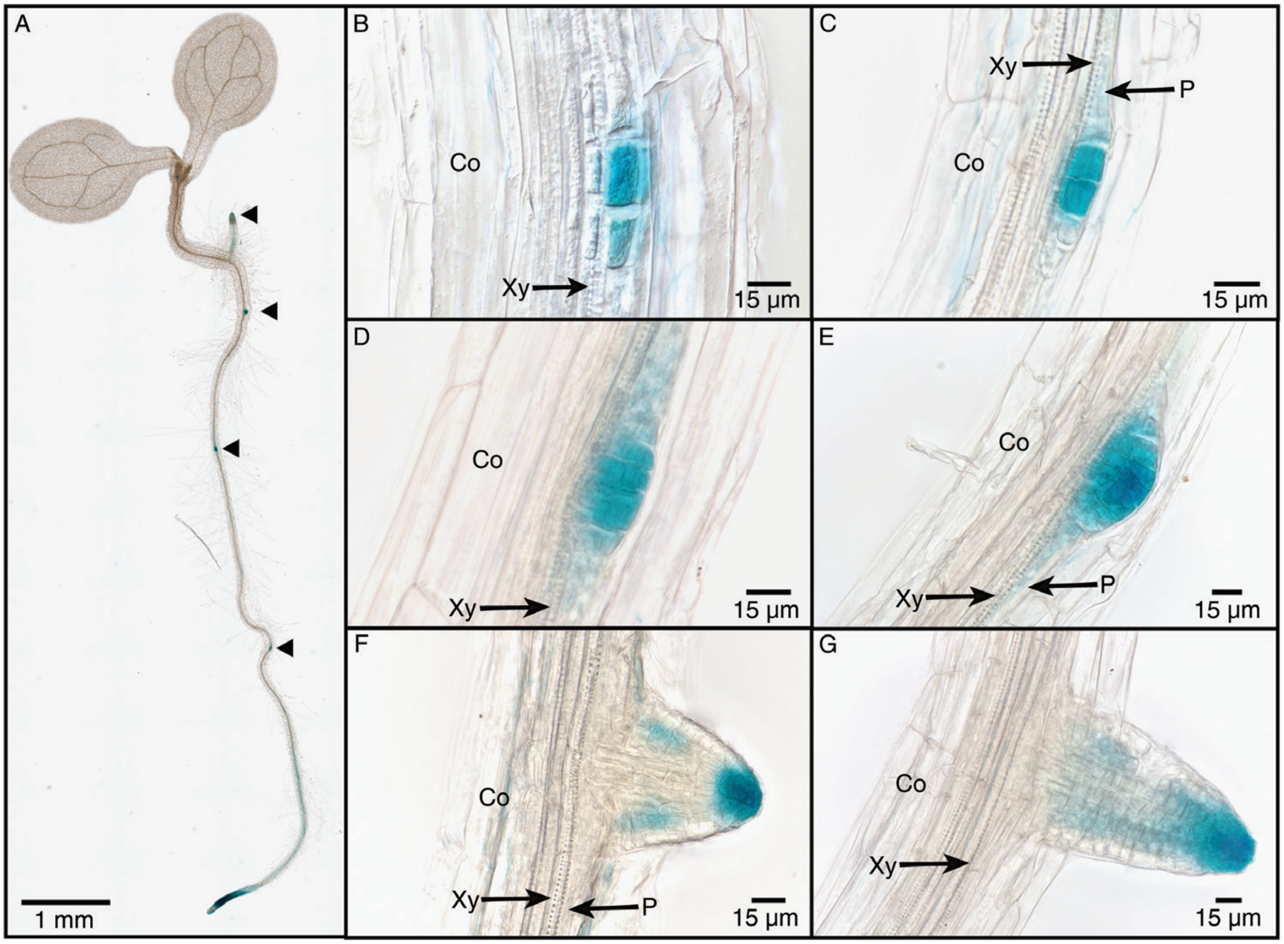

Fig. 8. Lateral root primordium-specific activity of the VvK5.1 promoter. Tissue-specific activity of the VvK5.1 promoter was investigated by histochemical analysis of GUS staining (blue colour) in transgenic Arabidopsis seedlings expressing *GUS* under control of the VvK5.1 promoter region. This activity was observed in 6- (A) and 10-day- (B–G) old seedlings grown *in vitro* in a growth chamber (16 h light photoperiod, 140 µM photons m^{-2} s^{-1}, 21 °C ,and 70% humidity during both light and darkness) on half-strength Murashige and Skoog (MS/2) medium, supplemented with hygromycin (25 mg l^{-1}). (A) A full view of the whole plantlet. Lateral primordia locations are indicated by arrows. (B–G) A coordinated cell division programme produces the root primordium, shown from the two-cell stage (B) to the stage where the lateral root primordium emerges at the root surface (G). Note that in each stage of the cell division programme, an intense blue colour is present within the most central cells of the primordium, which are known to undergo further cell divisions. In the stages where the primordium emerges at the root surface (F and G), the most intense blue colour is located within the root apex and the root cap.

Here, we demonstrated that the Shaker channel VvK5.1 is only expressed in the stigma and transmitting tract, and not in pollen grains (Fig. 5; Supplementary Fig. S1). Therefore, the channel is likely to be involved in the dialogue between pistil and pollen. VvK5.1 is a voltage-gated outwardly rectifying K^+ channel activated under membrane depolarization that drives K^+ secretion into the intercellular space. We propose that this K^+ efflux could trigger water secretion from stigmatic papilla cells for use in pollen hydration, thus enabling germination. Subsequently, the pollen tube will grow down through the style. Pollen tube development is known to be very fast and highly polarized, with pronounced oscillations in growth rate (Zheng *et al.*, 2018). This extensive growth by the pollen tube requires the influx of water and solutes into the cell before accumulating in the expanding vacuole. To sustain this growth rate, there are efficient ion transport systems at the pollen plasma membrane that have been identified and extensively studied. Among these systems, the inward K^+ Shaker channel SPIK (Mouline *et al.*, 2002; Zhao *et al.*, 2013) is specifically expressed in pollen as well as pollen tubes, and its disruption results in impaired pollen tube growth (Mouline *et al.*, 2002). The tandem-pore K^+ channel AtTPK4 is also involved in this process (Becker *et al.*, 2004), and the cation/proton exchangers CHX21 and CHX23 have been shown to be essential for pollen tube guidance toward the ovule (Lu *et al.*, 2011). Another study using the loss-of-function triple mutant *chx17chx18chx19* in *A. thaliana* revealed a mutant pollen altered at different levels including pollen tube growth, which is probably affected by the absence of AtCHX19 (Padmanaban *et al.*, 2017). In contrast, information on K^+ transport systems expressed in the pistil remains fragmentary. The growth of the pollen tube is dependent on K^+ availability, and, when the K^+ concentration is too low (preventing K^+ influx into the cell), a total absence of tube growth is observed (Mouline *et al.*, 2002). By relying on its functional features, the outward K^+ VvK5.1 channel (which is expressed in the transmitting tract cells) drives K^+ efflux and is probably involved in the secretion of K^+ into the intercellular space of these cells. Thus, driving K^+ secretion in the apoplast should make K^+ more available, enabling the necessary K^+ influx into the pollen tube for its development.

Involvement of the VvK5.1 channel in the berry phloem

Veraison is a key step in berry development, occurring at the onset of ripening. During this stage, long-distance transport from the xylem and phloem vasculatures towards the berries is profoundly restructured. The xylem becomes non-functional (Keller *et al.*, 2006; Chatelet *et al.*, 2008*a*, *b*; Choat *et al.*, 2009; Knipfer *et al.*, 2015) and berry loading is fed by a directional flow of water, sugar, and nutrients driven by massive K^+ fluxes from the phloem. Since *VvK5.1* expression precisely arrives in the berry starting at veraison and continues to increase throughout the grape ripening period (Fig. 4B), it is tempting to assume that this channel takes part in the necessary reorganization of the transport mechanisms for berry loading. The fact that this *VvK5.1* expression is strictly located in the berry phloem and the perivascular cells supports this idea (Fig. 6).

In flowering plants, the phloem pipe is comprised of files of sieve elements and companion cells. During phloem development, the nuclei and vacuoles in the sieve elements are degraded, leading to enucleated phloem sieve tubes that have lost their capacity for transcription. The enucleated sieve tubes then become dependent on an association with the neighbouring nucleated companion cells in order to allow the plasma membrane of these sieve elements to remain functional. In the berry phloem, we identified *VvK5.1* expression in both the enucleated phloem sieve tubes and the companion cells (Fig. 6H–L). This suggests that the *VvK5.1* mRNAs identified within the sieve tube were probably delivered through the sieve plate pores via an associated companion cell, and do not belong to the large population of mRNAs that serve as mobile systemic signalling agents.

At veraison, K^+ transport in the berry switches from the symplastic to the apoplastic mode (Zhang *et al.*, 2006), meaning that ions, water, and solutes must cross plasma membranes at least twice before accumulating within the flesh cell vacuoles. Due to this, we and others have invested in the identification and characterization of K^+ transport systems involved in grape berry loading during its maturation. Indeed, the weakly rectifying K^+ Shaker channel VvK3.1, expressed in the berry phloem, is involved in the massive K^+ efflux from the phloem cell cytosol to the berry apoplast (Nieves-Cordones *et al.*, 2019). By switching to its non-rectifying mode, VvK3.1 drives the K^+ efflux that allows K^+ ions to move down their transmembrane concentration gradient. This is a major challenge for grapevine, as the fruit is energetically limited due to the disappearance of stomata after veraison (Blanke *et al.*, 1999), and the additional energy stored in the transmembrane K^+ gradient, known as the K^+ battery, also allows sucrose retrieval under energy-limited conditions (Gajdanowicz *et al.*, 2011; Dreyer *et al.*, 2017; Nieves-Cordones *et al.*, 2019). At the same time, the inwardly rectifying K^+ channel VvK1.2, which is localized in the plasma membranes of perivascular and flesh cells and is strongly activated by its interactions with specific VvCIPK/VvCBL pairs (Cuéllar *et al.*, 2013), drives the rapid absorption of K^+ into these cells to keep the apoplastic K^+ concentration at low levels (0.1–1 mM) (Ache *et al.*, 2001; Nieves-Cordones *et al.*, 2019). Thus, the phloem stream flux towards the sink should be triggered but also retained as long as the transmembrane gradient of K^+ is maintained at the phloem plasma membrane. The VvK5.1 Shaker channel is an outward rectifying channel that is voltage dependent and only open under membrane depolarization. However, during berry loading, massive K^+ fluxes hyperpolarize the plasma membrane potential, which explains why the outward channel VvK5.1 is probably not involved in the phloem unloading. In contrast, this channel is both K^+ selective and K^+ sensing, similar to the GORK and SKOR channels in *A. thaliana* (Gaymard *et al.*, 1998; Ache *et al.*, 2000, Johansson *et al.*, 2006). According to the behaviour of outward K^+ channels, the VvK5.1 current amplitude decreases when external K^+ concentrations increase (Fig. 2B), except in the 0.1–1 mM external K^+ concentration range. At these concentrations, which correspond to the expected apoplastic concentrations during phloem unloading, the current amplitudes decrease even though the channel is activated at more hyperpolarized potentials, strongly indicating an allosteric regulation. As shown in Fig. 2C, at external concentrations of 0.1 mM or 1 mM K^+, activation of the VvK5.1 channel occurs at a voltage of approximately −65 mV, whereas when the external K^+ concentration is of the order of 100 mM, the channel opening takes place at a positive voltage. In this context, in which a low apoplastic K^+ concentration is maintained in the berry by storage of K^+ in the flesh cells, we propose that the VvK5.1 channel should intervene in the repolarization of the plasma membrane. In plants, as in animals, this repolarization is proposed to be induced by K^+ (Cuin *et al.*, 2018). Recently, Cuin *et al.* (2018) expanded the role of the outward Shaker channels in *A. thaliana* by studying the involvement of voltage-gated ion channels in the propagation of action potentials. By directly recording action potentials from the midvein of Arabidopsis leaves, the authors demonstrated that the outward rectifying channel GORK is involved in the control of the membrane potential via the repolarization phase. A similar role can be observed for the VvK5.1 channel at the sites of phloem unloading, in which the voltage is driven back to its resting potential. There may be an identical role for VvK5.1 in the perivascular cells that does not exclude its involvement in K^+ secretion into flesh cell apoplasts before being stored in the vacuoles of these cells.

A dual role for VvK5.1 in the root

The third organ in which *VvK5.1* is significantly expressed is the root. In this organ, *VvK5.1* expression is mainly detected in the pericycle, which is clearly composed of two cell types (Fig. 7): the LPCs and many SCs of the pericycle. The expression of *VvK5.1* in the LPCs is reminiscent of that of the SKOR channel in the Arabidopsis root pericycle and xylem parenchyma, and clearly indicates that the VvK5.1 channel should be involved in K^+ secretion in the xylem sap, thus playing a major role in K^+ translocation from the root to the shoot as previously described for SKOR-like channels (Gaymard *et al.*, 1998; Nguyen *et al.*, 2017; Long-Tang *et al.*, 2018). On the other hand, it is worth noting that the intense signal detected in the SCs does not match any known localization of SKOR expression, or that of any other outward Shaker channel. These SCs are precisely located in the protoxylem pole pericycle, and the large number of cells as revealed by the number of nuclei detected in this zone is indicative of an intense cell division process. Previous reports have established that the lateral roots are formed from pericycle cells located near the xylem pole (Casero *et al.*, 1989; Dubrovsky *et al.*, 2000; Hochholdinger and Zimmermann, 2008). These cells begin a coordinated programme of cell division and differentiation in order to produce a root primordium. The SCs, designated as lateral root founder cells, undergo this cell division programme with three successive phases. During the first phase, a single-cell layered primordium containing ~10 cells is produced. In the second phase, the most central cells undergo cell division leading to a two-cell layered primordium. In the third phase of lateral root formation, the central cells undergo several rounds of division that give rise to a primordium with an ellipsoid shape. This primordium grows through the different cell layers of the main root before emerging from the root surface. This process is perfectly illustrated in Fig. 8, where the different steps of lateral root formation are shown in the *A. thaliana* root expressing the GUS reporter gene under the control of the *VvK5.1* promoter. This confirms the involvement of the VvK5.1 channel in establishing the lateral roots that shape the root architecture. K^+, the most abundant inorganic cation in plants, is also essential to their existence. Specifically, it is involved in various physiological processes, including osmotically driven functions such as cell movement, regulation of stomatal aperture, cell expansion in growing tissues, and long-distance phloem transport. K^+ channels also play a central role in plant growth and development, by driving K^+ fluxes into or out of the cells. In particular, their roles in cell division and cell elongation/expansion have been investigated using tobacco BY-2 protoplast cultures (Sano *et al.*, 2007, 2009), demonstrating that the activation of the outward channel could be used as a switch to induce cell division. In parallel, these studies revealed that K^+ uptake and the increase in cell K^+ content are necessary for cell elongation in relation to cytoplasmic pH regulation. In the context of these studies, we propose here that the K^+ efflux driven by the VvK5.1 channel could allow the commencement and maintenance of the cell division programme that enables lateral root primordium formation.

Even though the functional properties of VvK5.1 are reminiscent of those of classical outward K^+ Shaker channels, our results demonstrate overall that this channel has acquired a unique expression profile of its own. Some of our observed tissue-specific *VvK5.1* expression patterns are to be expected, including in the LPCs of the pericycle, whereas other patterns are completely novel, such as the *VvK5.1* expression in the lateral root primordium. It is tempting to assume that these particular tissue-specific types of expression confer VvK5.1 with new properties that are perfectly adapted to the needs of grapevine. Nevertheless, the physiological relevance of these evolutionary differences indicates that this biological diversity must be further investigated, since these differences are likely to have important roles in plant physiological functions.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primers used in this study.

Fig. S1. *In situ* localization of VvK5.1 transcripts in flowers, showing pollen.

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