Members of the NPF3 Transporter Subfamily Encode Pathogen-Inducible Nitrate/Nitrite Transporters in Grapevine and Arabidopsis

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Introduction

Nitrate transporters play an essential role in plants. Reduced nitrate together with fixed carbon provide the building blocks for amino acid and protein biosynthesis needed for plant growth, development and defense against pathogens. Nitrate transporters in roots take up nitrate from the soil (Tsay et al. 2007). When photosynthetic reductants are available, nitrate is transported to the leaf via xylem where it is reduced and assimilated (Lillo 2008). Nitrate transporters are therefore needed to load and unload xylem parenchyma for long-distance transport, to import nitrate into the cytosol and to store excess nitrate in the vacuole. Nitrate transporters also load phloem to remobilize nitrogen from older leaves (Fan et al. 2009).

Nitrate transporter families are large, vary by plant species and are not well characterized. The NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER FAMILY (NPF, formerly NRT1/PTR) members transport not only nitrate and peptides as their family name implies, but also diverse substrates such as glucosinolates and the plant hormones auxin and ABA (Krouk et al. 2010,..)

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Vitis vinifera, the major grapevine species cultivated for wine production, is very susceptible to Erysiphe necator, the causal agent of powdery mildew (PM). This obligate biotrophic fungal pathogen attacks both leaf and berry, greatly affecting yield and quality. To investigate possible mechanisms of nutrient acquisition by successful biotrophs, we characterized a candidate NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER FAMILY (NPF, formerly NRT1/PTR) member, grapevine NPF3.2, that was up-regulated in E. necator-inoculated susceptible V. vinifera Cabernet Sauvignon leaves, but not in resistant V. aestivalis Norton. Expression in Xenopus laevis oocytes and two-electrode voltage clamp measurements showed that VvNPF3.2 is a low-affinity transporter for both nitrate and nitrite and displays characteristics of NPF members from other plants. We also cloned the Arabidopsis ortholog, AtNPF3.1, and showed that AtNPF3.1 similarly transported nitrate and nitrite with low affinity. With an Arabidopsis triple mutant that is susceptible to E. necator, we found that AtNPF3.1 is up-regulated in the leaves of infected Arabidopsis similarly to VvNPF3.2 in susceptible grapevine leaves. Expression of the reporter β-glucuronidase (GUS) driven by the promoter of VvNPF3.2 or AtNPF3.1 in Arabidopsis indicated that both transporters are expressed in vascular tissue, with expression in major and minor veins, respectively. Interestingly, the promoter of VvNPF3.2 allowed induced expression of GUS in minor veins in PM-infected leaves. Our experiments lay the groundwork for investigating the manipulation of host nutrient distribution by biotrophic pathogens and characterizing physiological variables in the pathogenesis of this difficult to study grapevine disease.

Keywords: Arabidopsis thaliana • Erysiphe necator • Nitrate transporters • Powdery mildew • Vitis aestivalis • Vitis vinifera.

Abbreviations: dpi, days post-inoculation; GFP, green fluorescent protein; GUS, β-glucuronidase; NPF, NRT1/PTR family; NRT, nitrate transporter; PAD4, PHYTOALEXIN DEFICIENT4; PEN2, PENETRATION2; PM, powdery mildew; PTR, peptide transporter; qRT-PCR, quantitative real-time PCR; SAG101, SENESCENCE-ASSOCIATED GENE101; Va, Vitis aestivalis; Vv, Vitis vinifera.
Kanno et al. 2012, Léran et al. 2013). Nitrate transporters within the NPF that have been characterized by expression in Xenopus laevis oocytes mainly mediate nitrate transport with low affinity (millimolar range). However, at least one nitrate transporter, AtNPF6.3 (CHL1, AtNRT1.1), displays dual low and high affinity in oocytes (Liu and Tsay 2003), although the function of this transporter in high-affinity nitrate uptake in planta is unclear (Glass and Kotur 2013). In addition to nitrate, AtNPF6.3 transports auxin (Krouk et al. 2010). Nitrite, the product of cytosolic nitrate reduction, was shown to be transported by Cucumis sativus (cucumber) NPF3.1 (CsNrt1-L) (Sugiura et al. 2007). The complex system of characterized nitrate transporters that load and unload nitrate during long-distance transport in Arabidopsis (Dechorgnat et al. 2011, Wang et al. 2012, and references therein) represents only a small fraction of the 53 predicted NPF and seven high-affinity NRT2 transporters (Tsany et al. 2007).

Powdery mildew (PM) is caused by a biotrophic pathogen that must obtain nutrients from a living plant throughout its life cycle. In grapevine, PM is caused by the ascomycete Erysiphe necator. Both the leaf and berry of Vitis vinifera, the major species cultivated for wine production, are very susceptible to PM, greatly affecting yield and quality (Stummer et al. 2005, Gadoury et al. 2013). The contribution of individual plant nutrient transporters to fungal infection is unknown, although PM is a disease that one might expect to be enhanced by increased nitrate transport. It has long been known that overfertilization with nitrate increases the severity of mildews in many crop plants (Marschner 2012). It has also been shown that high nitrogen supply increased PM infection in several grapevine cultivars (Keller et al. 2003).

Here, to investigate possible mechanisms of nitrogen acquisition by biotrophic fungi, we functionally characterized a candidate NPF transporter, VvNPF3.2, that was up-regulated in E. necator-inoculated susceptible V. vinifera Cabernet Sauvignon leaves, but not in resistant V. aestivalis Norton in a previous transcriptomic analysis of grapevine responses to PM (Fung et al. 2008). Expression in Xenopus oocytes and two-electrode voltage clamp measurements showed that VvNPF3.2 and its previously uncharacterized Arabidopsis ortholog, AtNPF3.1 (At1g68570), are low-affinity transporters for both nitrate and nitrite and display biophysical characteristics of known NPF transporters. VvNPF3.2 and AtNPF3.1 appear to have overlapping but spatially distinct localization in major and minor veins. Interestingly, the VvNPF3.2 promoter caused inducible β-glucuronidase (GUS) expression in minor veins after PM infection. We propose that E. necator may be manipulating the host leaf into becoming a sink for nitrate.

**Results**

**An NPF transporter up-regulated by PM infection in susceptible grapevine leaves**

To identify PM-responsive transporter candidate genes that are up-regulated in PM-infected grapevine leaves at time points when the fungus could acquire nutrients from the plant, we examined previously published Vitis GeneChip data from a comparison of PM-inoculated Vitis GeneChip data from a comparison of PM-inoculated resistant Norton and susceptible Cabernet Sauvignon (Fung et al. 2008). In that study, secondary hyphae observed microscopically indicated that fungal haustoria were most probably developed and obtaining host nutrients by 24 h post-inoculation. No transporters were significantly up-regulated in the resistant cultivar Norton (data not shown). In contrast, in leaves of susceptible Cabernet Sauvignon, Arabidopsis orthologs of amino acid transporters, an oligopeptide transporter and a putative nitrate transporter were up-regulated by 24 h after inoculation. The putative nitrate transporter VvNPF3.2 (Vitis GeneChip probe set ID 1613896_at corresponding to the gene GSVIVT01025795001 in the PIN04002 genome), an NPF member, was up-regulated at 12, 24 and 48 h post-inoculation (data not shown).

To verify the transcript levels of VvNPF3.2, quantitative real-time PCR (qRT-PCR) was performed on cDNA that had been synthesized from the same RNA samples collected for the Vitis GeneChip data (Fung et al. 2008). Because nitrate metabolism in leaves changes in response to light and circadian rhythms (Lillo 2008), we chose 0, 24 and 48 h after PM inoculation as time points to compare mRNA levels for these Vitis genes. VvNPF3.2 transcript levels in PM-infected leaves were not significantly up-regulated as compared with mock-inoculated leaves immediately after infection. However at 24 h, VvNPF3.2 transcript levels were significantly greater in PM-inoculated leaves than in mock-inoculated leaves, whereas by 48 h any additional changes in VvNPF3.2 mRNA levels were masked by apparent non-specific inoculation-induced changes (Fig. 1). In Norton, differences between VvNPF3.2 mRNA levels in mock- and PM-inoculated samples were not significant at any of the time points (data not shown). These data partially confirm the results obtained by GeneChip hybridization, and suggest that VvNPF3.2 may play a role during host colonization by PM.

**Homology of the VvNPF3.2 transporter**

We isolated a full-length VvNPF3.2 cDNA from a Cabernet Sauvignon cDNA library using the PN40024 V. vinifera genome sequence as a reference. Among predicted proteins, VvNPF3.2 showed the highest amino acid sequence similarity to C. sativus CsNPF3.2, Arabidopsis AtNPF3.1, characterized below, and the uncharacterized Oryza sativa (rice) predicted protein OsNPF3.1 (Sugiura et al. 2007, Tsay et al. 2007). We compared the amino acid sequence of VvNPF3.2 with these orthologs (Supplementary Fig. S1). Although VvNPF3.2 and CsNPF3.2 show 72% amino acid sequence identity, there are pronounced differences in their N- and C-termini. In particular, VvNPF3.2 and the Arabidopsis and rice orthologs seem to be lacking a chloroplast targeting signal predicted for CsNPF3.2 (Sugiura et al. 2007). VvNPF3.2 has 58% and 53% identity to AtNPF3.1 and OsNPF3.1, respectively, as reported for the identity of CsNPF3.2 to these two (Sugiura et al. 2007). Computational prediction of subcellular localization using iPSORT (Bannai et al. 2002) predicts plasma membrane localization for


VvNPF3.2, AtNPF3.1 and OsNPF3.1. We experimentally verified the plasma membrane localization (and absence of chloroplast localization) of AtNPF3.1 and VvNPF3.2 by transient expression in Nicotiana benthamiana (Supplementary Fig. S2).

VvNPF3.2 is a low-affinity nitrate/nitrite transporter

Electrophysiological experiments were performed to determine the substrates transported by VvNPF3.2. As an NPF, it could be expected to transport nitrate, dipeptides or amino acids with low affinity. As an ortholog with very high similarity to CsNPF3.2, it could be expected to transport nitrate with high affinity. Co-transporters can be efficiently characterized by expressing cRNA in Xenopus oocytes and measuring changes in current when a substrate is added. Using two-electrode voltage clamp to control membrane voltage during the measurement, we clamped at –40 mV to prevent inducing the endogenous oocyte nitrate transporter when 10 or 20 mM nitrate was applied. To minimize the confounding contributions of other ions, we used a bath solution containing only 0.15 mM CaCl₂, mannitol and MES/Tris buffer (Huang et al. 1999). Under these conditions, oocytes injected with VvNPF3.2 cRNA had significantly more negative (inward) current than uninjected control oocytes when 1, 5, 10 or 20 mM HNO₃ was applied in the bath solution at pH 5 to give maximum transport (Fig. 2). Nitrate-incited currents were significantly more negative at pH 5 than at pH 7 (Fig. 3), suggesting that, like other characterized nitrate transporters, VvNPF3.2 is a proton-coupled nitrate transporter. Another hallmark of most NPF nitrate transporters characterized in Xenopus is their selectivity for nitrate over dipeptides and amino acids. We therefore performed selectivity experiments. Average currents in VvNPF3.2-injected oocytes at pH 5.0 were not significantly different from the control when 10 mM amino acids (P > 0.05) or dipeptides (P > 0.1–0.4) were applied (Fig. 4). However, 10 mM HNO₃-elicited currents in these batches of oocytes that were highly significantly different from uninjected control oocytes (P < 0.0001), indicating that VvNPF3.2 selectively transports nitrate.

CsNPF3.2 was reported to transport nitrite (Sugiura et al. 2007), prompting us to test nitrite as a possible VvNPF3.2 substrate. Nitrite could not be applied as HNO₂, necessitating its derivation at which currents were half-maximal (Kₘ) was calculated to be 23 ± 8 mM (n = 6, average ± SD). This low substrate affinity is consistent with VvNPF3.2 being an NPF member.

Uptake of uncharged or negatively charged substrates is usually coupled to uptake of one or more protons, resulting in uptake of a net positive charge that is recorded as a pH-dependent inward current. Indeed, VvNPF3.2-mediated currents were significantly more negative at pH 5 than at pH 7 (Fig. 3), suggesting that, like other characterized nitrate transporters, VvNPF3.2 is a proton-coupled nitrate transporter.

Nitrate-incited currents increased with increasing concentration; however, plots of measurements up to 10 mM were approximately linear, indicating that transport was not saturated. More than half of the measurements showed saturation by 20 mM HNO₃ and the concentration at which currents were half-maximal (Kₘ) was calculated to be 23 ± 8 mM (n = 6, average ± SD). This low substrate affinity is consistent with VvNPF3.2 being an NPF member.

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transport is evident at 5, 10 and 20 mM nitrite in VvNPF3.2-injected oocytes (Fig. 5). We concluded that VvNPF3.2 is a low-affinity grapevine nitrate/nitrite transporter.

**AtNPF3.1 is induced by *E. necator* in the susceptible Arabidopsis pen2-1 pad4-1 sag101-2 triple mutant and encodes a low-affinity nitrate/nitrite transporter**

To explore further general features of nutrient transporter induction by biotrophic pathogens, we investigated whether AtNPF3.1 is induced in Arabidopsis by *E. necator* during PM infection similarly to VvNPF3.2. Because wild-type Arabidopsis displays non-host resistance and cannot be colonized by *E. necator*, we used the pen2-1 pad4-1 sag101-2 triple mutant for infections. This mutant was reported to have lost non-host resistance and to have become susceptible to non-adapted fungal crop pathogens (Lipka et al. 2005). The pen2 mutation allows the fungus to penetrate the host cuticle and the *pad4* and *sag101* mutations prevent induced plant defenses subsequent to initial attempts at fungal colonization. Consistent with this, we found that *E. necator* is able to infect pen2-1 pad4-1 sag101-2 plants and reproduce asexually (Supplementary Fig. S3A). qRT-PCR was performed on samples from *pen2-1 pad4-1 sag101-2* mutant leaves inoculated with *E. necator* or mock-inoculated leaves. At 48 h post-inoculation, AtNPF3.1 was significantly induced in *E. necator*-inoculated leaves as compared with the mock-inoculated leaves (Supplementary Fig. S3B). This suggests that AtNPF3.1 in Arabidopsis and VvNPF3.2 in grapevine could function similarly during fungal colonization.

We expressed AtNPF3.1 in *Xenopus* oocytes and measured the currents resulting from nitrate and nitrite application. HNO₃ at 1, 5 and 10 mM and pH 5 caused significantly (*P < 0.05*) more current in the AtNPF3.1-injected oocytes than in the uninjected ones (Fig. 6). Four out of five oocytes showed saturation of the transporter at 10 mM HNO₃, with a *Kₘ* of 9 ± 3 mM (*n = 4, average ± SD). No significant levels of inward current were induced by 0.1 mM NO₂⁻ at pH 5 or 1 mM NaNO₂ at pH 5.35 in injected oocytes as compared with controls (data not shown). In contrast, 5, 10 and 20 mM NaNO₂ induced inward currents in injected oocytes that were highly significantly different from controls (Fig. 7). We concluded that AtNPF3.1 is also a low-affinity nitrate/nitrite transporter.

**Expression patterns of VvNPF3.2 and AtNPF3.1**

To study VvNPF3.2 and AtNPF3.1 expression in planta, stable transgenic Arabidopsis plants expressing promoter:GUS
Fig. 6 AtNPF3.1 transports nitrate with low affinity. Each oocyte was exposed to increasing concentrations of HNO₃ at pH 5.0 and a holding potential of −40 mV. Results are averages of difference currents ± SE. For each concentration, 8–9 AtNPF3.1-expressing oocytes and 5–6 uninjected oocytes, isolated from at least three different Xenopus frogs, were measured. Asterisks denote significant differences in current levels between uninjected and AtNPF3.1-injected oocytes as determined by Student’s t-test (**P < 0.05); ns, not significant.

![Graph](image1)

**Fig. 7** AtNPF3.1 transports nitrite with low affinity. Results are averages of difference currents ± SE at pH 5.35 and a holding potential of −40 mV. For each concentration, 9–10 AtNPF3.1-expressing and six uninjected oocytes, isolated from two different Xenopus frogs, were measured. Asterisks denote significant differences in current levels between uninjected and AtNPF3.1-injected oocytes as determined by Student’s t-test (***P < 0.005; ******P < 0.000005); ns, not significant.

![Graph](image2)

constructs were generated in the Col-0 and pen2-1 pad4-1 sag101-2 backgrounds. In 2- to 4-week old Arabidopsis plants, VvNPF3.2pro:GUS was strongly expressed in petioles and large veins of both expanding and expanded leaves, with slight expression in the main root, suggesting an involvement of VvNPF3.2 in long-distance transport (**Fig. 8A; Supplementary Fig. S4A, C**). AtNPF3.1pro:GUS was strongly expressed in expanded leaves along minor veins, and is clearly not expressed in midveins, petiole or seedling roots (**Fig. 8B; Supplementary Fig. S4B, D**). Expression was limited to smaller veins and became stronger as leaves aged, perhaps indicating that AtNPF3.1 functions in nitrogen remobilization.

We further used VvNPF3.2pro:GUS-expressing pen2-1 pad4-1 sag101-2 triple mutants to test for changes in VvNPF3.2 expression in response to *E. necator*. In **Fig. 1**, we showed that VvNPF3.2 mRNA increased by 24 h after *E. necator* infection of Cabernet Sauvignon leaves. As shown in **Fig. 8D**, VvNPF3.2pro:GUS activity steadily increased after triple mutant Arabidopsis leaves were inoculated with *E. necator* as compared with the mock-inoculated control (**Fig. 8C**), indicating that the promoter of VvNPF3.2 was responsive to *E. necator* in this heterologous system. By 5 days post-inoculation (dpi), the PM-inoculated midveins stained more strongly than the mock-inoculated midveins and more than at 0 dpi (**Fig. 8D**). At 10 dpi, the GUS activity had even expanded to the minor veins (**Fig. 8D**), possibly suggesting redirection of nitrogen distribution by *E. necator*. Induction of AtNPF3.1pro:GUS expression by *E. necator* in transgenic Arabidopsis was not tested because the mature leaf displayed a high level of GUS activity in the absence of infection.

**Discussion**

In this report we describe the cloning and characterization of the grapevine transporter VvNPF3.2 and its previously uncharacterized Arabidopsis ortholog, AtNPF3.1, the only Arabidopsis representative of the NPF3 subfamily (Lérán et al. 2013). Our measurements of currents in response to nitrate by two-electrode voltage clamp in *Xenopus* oocytes expressing VvNPF3.2 and AtNPF3.1 indicate that both proteins transport nitrate with low affinity, that VvNPF3.2 does not transport dipeptides and that VvNPF3.2 nitrate-responsive currents are dependent on low external pH. These results are consistent with transport activities that have been reported for most of the predicted Arabidopsis NPF members characterized in *Xenopus*.
HNO₂ is a weak acid with a pKₐ of ~23 mM and 9 mM, respectively) and the availability of nitrate, these transporters could transport nitrate in the plant. Nitrate in the xylem sap of decapitated plants can be found at concentrations of 1–20 mM (Glass and Siddiqi 1995), varying with season of the year and time of day. Xylem sap of pressurized V. vinifera cv Riesling leaves was reported to contain 0.5–3 mM nitrate depending upon soil and fertilization (Peuke 2000). It is possible that VvNPF3.2 and AtNPF3.1 can be switched between low-affinity and high-affinity transport by post-translational modifications, similarly to AtNPF6.3 (Li and Tsay 2003), or that they transport untested substrates with higher affinity, similarly to AtNPF2 members, low-affinity nitrate transporters that also transport glucosinolate defense compounds with high affinity (Nour-Eldin et al. 2012). In that study, AtNPF3.1 did not transport glucosinolates.

The high homology of VvNPF3.2 to CsNPF3.2 and the inward currents seen with VvNPF3.2- and AtNPF3.1-expressing Xenopus oocytes in response to millimolar nitrate raises the question of what biological function low-affinity nitrate transport may have in planta. The transport of nitrate, the product of nitrite reduction, has been less studied than the more easily measured long-distance low-affinity xylem nitrate transport. Until recently, nitrite was usually considered to be toxic, present only in very low micromolar amounts and able to cross membranes by diffusion (Heber and Heldt 1981). Because HNO₂ is a weak acid with a pKₐ value of 3.4, a significant portion of nitrite would be protonated under acidic apoplastic conditions (pH ~5–5.5) and at millimolar concentrations would permeate the membrane without the action of a transporter. Nevertheless, expression of the CsNPF3.2 gene in rice permitted vigorous growth on 30 mM KNO₃ or 20 mM KNO₂ as sole sources of nitrogen (Sustiprijatno et al. 2006), suggesting that VvNPF3.2 could have a protective function in overfertilized soils. The similarity of induction times for nitrite and nitrate uptake and the interaction of the two uptake systems suggest they may use the same transporters in some plants (Aslam et al. 1992), while evidence for distinct high-affinity nitrate and nitrite transporters in Arabidopsis was recently reported (Kotur et al. 2013). In studies of NPF transporters, nitrite is usually not tested as a substrate, and it is possible that dual nitrate/nitrite transport is a more common feature of this transporter class.

**Localization of VvNPF3.2 and AtNPF3.1**

While the affinity of a transporter for a substrate and its availability defines what can be transported, where and when the transporter is expressed mainly determines its function. The very low VvNPF3.2pro:GUS and absent AtNPF3.1pro:GUS expression in Arabidopsis roots in our experiments indicates that probably neither transporter takes up nitrate or nitrite from the soil. Other reports support the lack of AtNPF3.1 root expression in 1- (Zhao et al. 2010) and 2-week old Arabidopsis (Tsay et al. 2007). Both studies showed moderate to high expression of AtNPF3.1 in stem and leaf tissue, consistent with our results. However, slightly older roots than those used in our experiments showed moderate microarray expression (Zhao et al. 2010).

The vascular expression we showed for VvNPF3.2pro:GUS and AtNPF3.1pro:GUS has been reported for other NPF nitrate transporters. AtNPF7.2 (AtNRT1.8), a long-distance transporter that is expressed in xylem parenchyma of stems, leaves and also roots (Li et al. 2010), is found in major veins, similarly to VvNPF3.2pro:GUS. Expression of AtNPF2.13 (AtNRT1.7), which was proposed to remobilize nitrate, increases in minor veins of leaves as they age (Fan et al. 2009), similarly to AtNPF3.1pro:GUS. With expression in N. benthamiana, we have shown that AtNPF3.1 is located in the plasma membrane, befitting a role in loading or unloading nitrate in plant cells along the vasculature. Interestingly, expression of VvNPF3.2pro:GUS in E. necator-infected Arabidopsis strengthened between 0 and 10 dpi in major veins and even expanded into minor veins near the end of this period. Transporter expression in main leaf veins in the first days of infection would be expected to move nitrate from stems or from storage in petioles. By 10 d, the vast increase in fungal hyphae should cause reduced photosynthesis and local release of nitrate from vacuoles and catalyzed proteins, necessitating more local distribution of nitrate.

Biotrophs need to manipulate host metabolism and nutrient distribution in order to thrive (Chen et al. 2010). It has been reported that the nutrient demands of the fungus during PM infection affect the source–sink relationship in the plant, i.e. nutrients that would normally go to young leaves or developing seeds are instead exported to the fungus (Ayres et al. 1996, Hall and Williams 2000, Chandran et al. 2010). The ability of E. necator similarly to up-regulate VvNPF3.2 in grapevine leaves and AtNPF3.1 in susceptible Arabidopsis leaves suggests that the response of a susceptible plant to this pathogen generally involves increased nitrate or nitrite transport. Because E. necator and Golovinomyces orontii (PM of Arabidopsis) appear to lack genes encoding inorganic nitrate transporters, nitrate reductases and nitrite reductases (Spanu et al. 2010), it is most likely that the plant increases nitrate transport to leaves, reduces inorganic nitrate and provides the fungus with ammonium, amino acids and peptides. Consistent with this hypothesis, other V. vinifera transporters up-regulated 24 and 48 h after PM infection include orthologs of the amino acid transporters AtGAT1 and AtLHT1, and the oligopeptide transporter AtOPT4 (Fung et al. 2008). A proteomic study showing induction of glutamine synthetase and alanine aminotransferases in PM-infected V. vinifera leaves by 48 h post-inoculation (Marsh et al. 2010) also suggests increased organic nitrogen synthesis.

In conclusion, we anticipate that the Arabidopsis pen2-1 pad1-1 sag101-2 triple mutant will be a useful tool to elucidate the effect of nitrate transport on the susceptibility of grapevine to PM or other pathogens under different nutrient and environmental conditions. In particular, the effect of VvNPF3.2 and AtNPF3.1 on susceptibility to PM and abiotic stress, nitrate...
accumulation, growth and development could be tested with relative ease in Arabidopsis as compared to grapevine.

**Materials and Methods**

**RNA isolation and qRT-PCR**

To verify transcriptional regulation of VvNPF3.2 and VaNPF3.2, we used cDNA synthesized as described from the same RNA used in the Vitis GeneChip hybridization (Fung et al. 2008). To quantify transcripts of AtNPF3.1, total RNA was extracted from Arabidopsis pen2-1 pad4-1 sag101-2 leaves with Trizol (Invitrogen) at 0, 24 and 48 h after inoculation with *E. necator* or from mock-inoculated triple-mutant leaves. cDNA was synthesized from 1 μg of total RNA using an oligo(dT)15 primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). qRT-PCR was performed with an ABI 7500 system (Applied Biosystems) and SYBR GREEN PCR Master Mix. Transcript levels were normalized using mRNA levels of the internal standards for grapevine and Arabidopsis, respectively. Primers used are listed in Supplementary Table S1.

**Constructing transgenic Arabidopsis**

**VvNPF3.2- and AtNPF3.1-promoter GUS lines**

The AtNPF3.1 promoter from Arabidopsis Col-0 (2,025 bp upstream from the start codon) and the VvNPF3.2 promoter from *V. vinifera* Cabernet Sauvignon genomic DNA (2,303 bp upstream from the start codon) were cloned into the Gateway entry vector pDONR201 and subcloned into binary vector pYXT1 containing the GUS reporter gene. Transgenic Arabidopsis promoter–GUS lines were generated by the floral dip method (Clough and Bent 1998) using *Arabidopsis* promoter–GUS lines were soaked in 10 mM MgCl₂ with 100 μM acetoxy-syringone (Sigma-Aldrich) and adjusted to an OD₆₀₀ of 0.25–0.3. Agrobacterium was incubated for 2 h at room temperature and infiltrated into N. benthamiana leaves with a 1 ml needleless syringe. The *N. benthamiana* plants were placed in an E-7/2 reach-in growth chamber (Controlled Environments Ltd.) under a 16 h light/8 h dark cycle at 25°C, 70% relative humidity. Tissues were collected 3 d after infiltration for Western blot and confocal microscopy. Plant tissues were viewed directly under a Zeiss LSM 510 META NLO two-photon point-scanning confocal system mounted on an Axiovert 200M inverted microscope with a 40/1.2 C-Apochromat water immersion objective. Yellow fluorescent protein fluorescence was excited by a 514 nm argon laser. Sample fluorescence was detected using a 500–550 nm band-pass emission filter.

**Xenopus oocytes and two-electrode voltage clamp**

VvNPF3.2 cDNA (1,815 bp with BamHI and EcoRI flanking restriction enzyme sites) and AtNPF3.1 cDNA (1,791 bp with Apal and Xhol flanking restriction enzyme sites) were generated by PCR and cloned into the Xenopus expression plasmid pOO2 (Ludewig et al. 2002). Capped cRNA was synthesized by in vitro transcription with a mMESSAGE mMACHINE® SP6 Kit (Ambion, Inc.) according to the manufacturer's instructions. Oocytes were isolated, maintained and injected as described (Pike et al. 2009). Two-electrode voltage clamp measurements with additions of nitrate as HNO₃ or of amino acids and dipeptides were conducted in a bath solution containing 230 mM mannitol, 0.15 mM CaCl₂ and 10 mM MES/Tris at pH 5.5 (Huang et al. 1999) or pH 5.0 as noted, 2–5 s after injection with VvNPF3.2 or AtNPF3.1 cRNA. For measurements involving nitrite, the pH was 5.35 and buffering with MES/Tris was reduced to accommodate the buffering effect of sodium glutamate. To control for the effect of added sodium, bath solutions with sodium glutamate were alternated with bath solutions containing equimolar sodium nitrite. Difference current values were obtained by subtracting current levels before substrate addition from current values after addition.

**Accession numbers**

AtNPF3.1, At1g68570; CsNPF3.2 (CsNitr1-L), Z69370; OsNPF3.1, Os06g15370; VvNPF3.2, KF649633.
Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


