Uniwersytet im. Adama Mickiewicza w Poznaniu
Wydział Biologii

ROZPRAWA DOKTORSKA

mgr Karolina Izbiańska

Funkcje nadtlenoazotynu w odporności liści ziemniaka (*Solanum tuberosum* L.) na *Phytophthora infestans* (Mont.) de Bary

Functions of peroxynitrite in resistance of potato leaves (*Solanum tuberosum* L.) to *Phytophthora infestans* (Mont.) de Bary

Promotor: prof. UAM dr hab. Magdalena Arasimowicz-Jelonek
Promotor pomocniczy: dr hab. Jarosław Gzyl

Poznań 2018

Promotorowi
Pani Prof. UAM dr hab. Magdalenie Arasimowicz-Jelonek
Serdecznie dziękuję za nieocenione wsparcie naukowe i zaufanie w pracy badawczej,
za wielką życzliwość, inspirujące rozmowy
oraz wszelką pomoc na każdym etapie realizacji niniejszej rozprawy

Promotorowi pomocniczemu
Panu Dr hab. Jarosławowi Gzylowi
Serdecznie dziękuję za pomoc w rozwiązywaniu problemów badawczych,
liczne dyskusje oraz rady udzielone podczas wykonywania doświadczeń laboratoryjnych

Dziękuję wszystkim osobom, które pomogły przy realizacji niniejszej rozprawy doktorskiej.
W szczególności pragnę podziękować:

Wszystkim pracownikom i doktorantom Zakładu Ekofizjologii Roślin za przyjazną atmosferę pracy i wszelką pomoc przy wykonywaniu doświadczeń laboratoryjnych.

Pragnę też serdecznie podziękować Pani Prof. dr hab. Jolancie Floryszak-Wieczorek, za okazaną życzliwość, cenne wskazówki i sugestie podczas powstawania niniejszej pracy, oraz umożliwienie realizacji części zadań badawczych w Katedrze Fizjologii Roślin Uniwersytetu Przyrodniczego w Poznaniu

Badania wykonano w ramach:

OPUS 2011/01/B/NZ9/00243, tytuł projektu: „Poszukiwanie nowych źródeł syntezy i funkcjonowania tlenku azotu w odporności liści ziemniaka na *Phytophthora infestans*" Projekt finansowany przez Narodowe Centrum Nauki

OPUS 2014/13/B/NZ/02177, tytuł projektu: „Identyfikacja i analiza funkcjonalna zdarzeń metabolicznych zależnych od tlenku azotu u *Phytophthora infestans* (Mont.) de Bary"
Projekt finansowany przez Narodowe Centrum Nauki

Spis treści

STRESZCZENIE ROZPRAWY DOKTORSKIEJ

W odpowiedzi na atak patogenicznego mikroorganizmu w komórkach roślinnych dochodzi m.in. do generowania tlenku azotu (NO), co może prowadzić do formowania innych reaktywnych form azotu (RFA), spośród których największe znaczenie biologiczne przypisywane jest nadtlenoazotynowi ($ONOO^-$). Wiedza dotycząca funkcjonalności tej pochodnej NO u roślin, szczególnie w interakcji roślina – patogen, pozostaje bardzo ograniczona, stąd nadrzędnym celem pracy doktorskiej było zweryfikowanie udziału nadtlenoazotynu oraz poznanie funkcjonalnych modyfikacji wywołanych za pośrednictwem tej reaktywnej formy azotu w odporności liści ziemniaka na *Phytophthora infestans* (Mont.) de Bary. Doświadczenia prowadzono na liściach ziemniaka (*Solanum tuberosum* L.) odmian skrajnie różniących się odpornością na *P. infestans*, lęgniowca będącego sprawcą zarazy ziemniaka. Układ badawczy pozwolił zatem określić, na ile zdarzenia metaboliczne zależne od $ONOO^-$ towarzyszą aktywacji skutecznych odpowiedzi obronnych w odporności typu ETI indukowanej przez efektory patogena (ang. *Effector-Triggered Immunity*), a na ile odpowiedziom obronnym w odporności określanej jako bazowa PTI (ang. PAMP-*Triggered Immunity*; PAMP-*Pathogen-Associated Molecular Patterns*), która zaangażowana jest w rozpoznawanie konserwatywnych czynników pochodzących od patogena.

Eksperymenty przeprowadzone w pierwszym etapie badań wykazały, że głównym źródłem biosyntezy NO w liściach ziemniaka jest reduktaza azotanowa (NR), a zależna od NR nadprodukcja NO wraz z akumulacją anionorodnika ponadtlenkowego prowadzi do formowania $ONOO^-$ w odpowiedzi na *P. infestans*. Wykorzystując precyzyjne metody detekcji nadtlenoazotynu stwierdzono różnice międzyodmianowe w kinetyce generowanego $ONOO^-$. Odmiana odporna charakteryzowała się wczesnym i okresowym formowaniem tej RFA, natomiast w odmianie podatnej podwyższony poziom $ONOO^-$ obserwowano zdecydowanie później tj. dopiero w pierwszej dobie po inokulacji. Poinfekcyjna akumulacja nadtlenoazotynu korelowała w czasie ze wzrostem ekspresji peroksydazy tioredoksyny (*TPx*), wskazując na potencjalne zaangażowanie produktu *TPx* w kontrolę endogennego poziomu $ONOO^-$ u obu odmian ziemniaka.

W oparciu o sekwencyjne traktowanie liści odmiany podatnej donorem $ONOO^-$ i *P. infestans* stwierdzono, że podwyższony poziom $ONOO^-$ we wczesnych godzinach po inokulacji sprzyja ograniczeniu kolonizacji tkanek rośliny-gospodarza przez patogen m.in. na drodze szybszej i bardziej efektywnej akumulacji mRNA dla *PR-1* oraz *PR-2*, uznawanych za kluczowe markery odpowiedzi obronnej rośliny na stres biotyczny.

Poinfekcyjny charakter generowania nadtlenoazotynu odzwierciedlał akumulację nitrowanych białek w liściach obu odmian ziemniaka. Stąd, w odmianie odpornej obserwowano wczesną i przejściową akumulację modyfikowanych *via* $ONOO^-$ białek. Ponadto analizy nitroproteomu liści odmiany odpornej wykazały akumulację białek podobnych do subtylizyny (SBT5.3, SBT1.7) w komórkach otaczających strefę reakcji nadwrażliwości. W toku dalszych analiz nad poszukiwaniem związków modyfikowanych *via* $ONOO^-$ wykazano, że inokulacja liści *P. infestans* prowadzi do nitrowania kwasów nukleinowych tj. RNA i mRNA. W oparciu o detekcję 8-nitroguaniny, po raz pierwszy w świecie roślin udokumentowano, obecność nitro-RNA oraz nitro-mRNA. Analiza porównawcza pomiędzy genotypem odpornym a podatnym wskazała na okresowy, istotnie wyższy poziom nitrowanego RNA oraz mRNA w inokulowanych liściach odmiany odpornej. Ponadto, zastosowanie zmiataczy endogennego $ONOO^-$ prowadziło do obniżenia poziomu nitrowanego mRNA oraz ograniczenia liczby komórek wykazujących reakcję TUNEL-pozytywną.

Podsumowując uzyskane wyniki należy stwierdzić, iż wczesna i okresowa, akumulacja $ONOO^-$ w liściach ziemniaka odgrywa istotną rolę w odporności na *P. infestans,* zarówno poprzez selektywne nitrowanie białek, jak i kwasów nukleinowych, zaangażowanych w regulację zasięgu programowanej śmierci komórek ziemniaka oraz ekspresji genów *PRs*.

SUMMARY OF DOCTORAL THESIS

As a reaction to the attack of pathogenic microorganism plant cells generate nitric oxide (NO), which may lead to the formation of other reactive nitrogen species (RNS), among which the greatest biological significance is attributed to peroxynitrite ($ONOO^-$). As far as plants are concerned the knowledge related to the functionality of this NO-derivative, particularly during a plant-pathogen interaction remains very limited, hence, the overriding aim of the doctoral thesis was to verify participation of $ONOO^-$ and the recognition of the functional modifications caused by the RNS in the resistance phenomenon of potato leaves to *Phytophthora infestans* (Mont.) de Bary. Experiments were conducted on the potato (*Solanum tuberosum* L.) leaves of cultivars, whose resistance radically differs to *P. infestans*, an oomycete pathogen causing late blight disease. Thus, the research system allowed to determine to what extent $ONOO^-$-dependent metabolic events accompany effective defense responses in effector-triggered immunity (ETI) as well as the extent of the defense during pathogen-associated molecular patterns (PAMPs) that trigger immunity (PTI) which is involved in the recognition of conserved pathogen-derived factors.

Experiments carried out in the first stage of the study showed that nitrate reductase (NR) is the main source of NO biosynthesis in potato leaves. Moreover, NR-dependent NO overproduction together with the accumulation of superoxide anion leads to the formation of $ONOO^-$ in response to *P. infestans*. The adoption of precise $ONOO^-$ detection methods, has led to recognizing the contrasting kinetics of $ONOO^-$ generation in both potato cultivars. The resistant genotype was characterized by the early and transient formation of $ONOO^-$ whereas in the susceptible one, an elevated level of $ONOO^-$ was observed much later, i.e. after the first 24 hours after the inoculation. The postinfectious $ONOO^-$ accumulation was correlated in time with thioredoxin peroxidase (*TPx*) gene expression, potentially implicating TPx in the control of endogenous $ONOO^-$ in both potato cultivars.

The next step of the study subjected the susceptible potato leaves to the sequential treatment with the $ONOO^-$ donor and *P. infestans* it has revealed that an elevated level of $ONOO^-$ in the early stages after inoculation is conducive to slowing down colonization of the host tissues by the pathogen, mainly *via* a faster and stronger up-regulation of *PR-1* and *PR-2* mRNA level, which are considered to be key markers of the plant's defense against the pathogen.

The post-infectious nature of the $ONOO^-$ formation reflected the accumulation of nitrated proteins in the leaves of both potato cultivars. Hence, an early and transient accumulation of the $ONOO^-$-modified proteins was observed in the resistant genotype.

Moreover, the analyses of nitroproteome in leaves of the resistant cultivar have indicated the accumulation of subtilisin-like proteins (SBT5.3, SBT1.7) in the cells which surrounded the hypersensitive reaction zone. In the course of further analysis to find compounds modified *via* $ONOO^-$, it was revealed that the inoculation of leaves by *P. infestans* leads to nucleic acids nitration, i.e. RNA and mRNA. Based on the 8-nitroguanine detection, the presence of nitro-RNA and nitro-mRNA was documented, for the first time, in plants. The comparative analysis has indicated a transient and significantly higher level of nitrated RNA and mRNA in the leaves of resistant genotype after inoculation. Furthermore, the use of endogenous $ONOO^-$ scavengers has led to lowering the level of nitrated mRNA and reducing the number of cells exhibiting a TUNEL-positive reaction.

To summarise the obtained results, it should be noted that the early and transient accumulation of $ONOO^-$ in the potato leaves plays a crucial role in the resistance to *P. infestans,* both by the selective nitration of proteins as well as nucleic acids involved in the regulation of the programmed cell death range and the expression of *PR* genes.

WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Funkcje nadtlenoazotynu w odporności liści ziemniaka (*Solanum tuberosum* L.) na *Phytophthora infestans* (Mont.) de Bary

Publikacja 1.

Floryszak-Wieczorek J., Arasimowicz-Jelonek M., **Izbiańska K.** (2016) The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*. *Plant Physiology and Biochemistry,* 108: 468–477 (**IF=2,928; 35 pkt MNiSW**).

Publikacja 2.

Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K**., Gzyl J., Jelonek T. (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*. *Plant Pathology,* 65: 754–766 (**IF=2,383; 35 pkt MNiSW**).

Publikacja 3.

Izbiańska K., Floryszak-Wieczorek J., Gajewska J., Meller B., Kuźnicki D., Arasimowicz-Jelonek M. (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans*. *Frontiers in Plant Science*, 9: 672 (**IF=3,678; 40 pkt MNiSW**).

WPROWADZENIE

Szybka aktywacja szlaków przekazywania sygnałów, wyzwalająca szeroki wachlarz odpowiedzi obronnych, jest jedną z pierwszych reakcji uruchamianych przez roślinę w odpowiedzi na atak patogena. Dzięki receptorom błonowym PRR (ang. *Pattern Recognition Receptors*), rozpoznającym tzw. wzorce molekularne powiązane z patogenami PAMP (ang. *Pathogen-Associated Molecular Patterns*) dochodzi do uruchomienia odporności określanej jako bazowa lub podstawowa – PTI (ang. PAMP-*Triggered Immunity*), która może przejawiać się m.in. wybuchem tlenowym, depozycją kalozy, bądź indukcją genów związanych z patogenezą (Świątek i Śliwka, 2011). Z kolei, rozpoznanie czynników awirulencji patogena specyficznych względem danego gospodarza, tzw. efektorów, prowadzi do uruchomienia odporności indukowanej przez efektory – ETI (ang. *Effector-Triggered Immunity*). Istotą odporności typu ETI jest aktywacja roślinnych genów odporności *R*, których produkty, białka R, są wewnątrzkomórkowymi receptorami specyficznie oddziałującymi z produktami genów awirulencji patogenów. W efekcie tego oddziaływania dochodzi do uruchomienia szlaku transdukcji sygnału, który prowadzi do reakcji nadwrażliwości (ang. *Hypersensitive Response*, HR) (Świątek i Śliwka, 2011).

Badania ostatnich 20 lat wykazały, że w inicjowaniu i koordynowaniu zdarzeń obronnych, kluczową rolę odgrywa tlenek azotu (NO) – cząsteczka sygnałowa, która dzięki prostocie budowy i właściwościom fizykochemicznym może oddziaływać na wiele procesów wewnątrzkomórkowych. Na potwierdzenie, sygnałową funkcję NO udokumentowano empirycznie, zarówno w odporności roślin typu PTI, jak i w odporności indukowanej przez efektory ETI (m.in. Leitner i in., 2009; Schlicht i Kombrink, 2013; Bellin i in., 2013; Trapet i in., 2015; Floryszak-Wieczorek i Arasimowicz-Jelonek, 2016). Dowiedziono również, że wzmożone generowanie NO, obserwowane już w pierwszych minutach po rozpoznaniu patogena, może prowadzić do formowania innych reaktywnych form azotu (RFA), co istotnie wpływa na stan redoks komórki i dalszą sekwencję uruchamianych zdarzeń metabolicznych (Floryszak-Wieczorek i Arasimowicz-Jelonek, 2016).

Spośród wszystkich RFA największe znaczenie biologiczne, poza NO, przypisywane jest nadtlenoazotynowi ($ONOO^-$). Pod względem biochemicznym $ONOO^-$ jest silnym utleniaczem oraz związkiem nitrującym, który powstaje w wyniku gwałtownej reakcji pomiędzy NO, a anionorodnikiem ponadtlenkowym ($O_2^{\cdot -}$). Ze względu na krótki czas półtrwania obu cząsteczek macierzystych (NO – 3-5 sekund; $O_2^{\cdot -}$ – kilka milisekund), formowanie nadtlenoazotynu wymaga równoczesnego tworzenia obu reagujących ze sobą rodników, obecnych w tych samych strukturach komórkowych (Arasimowicz-Jelonek

i Floryszak-Wieczorek, 2011). Jak dotąd, mechanizm działania nadtlenoazotynu został stosunkowo dobrze poznany tylko w odniesieniu do komórek zwierzęcych i ludzkich, u których cząsteczka ta, poprzez charakterystyczne reakcje nitrowania białek, kwasów tłuszczowych oraz kwasów nukleinowych, wiązana jest głównie ze stresem nitro-oksydacyjnym, który towarzyszy stanom patofizjologicznym o różnym podłożu.

W świecie roślin wyższych, wiedza dotycząca pochodnych NO, pozostaje ciągle bardzo fragmentaryczna. Chociaż obecność *in vivo* $ONOO^-$ została udokumentowana w kilku układach eksperymentalnych (m.in. Chaki i in., 2009; Gauples i in., 2011; Arasimowicz-Jelonek i in., 2012; Corpas i Barroso, 2014; Krasuska i in., 2014; Floryszak-Wieczorek i Arasimowicz-Jelonek, 2016; Gzyl i in., 2016; Krasuska i in., 2017), to jednak rola tej molekuły u roślin pozostaje niewyjaśniona. Niemniej jednak, pierwsze prace doświadczalne, w których zastosowano podejście farmakologiczne dostarczyły dowodów, iż w przeciwieństwie do komórek zwierzęcych nadtlenoazotyn nie jest tak toksyczny dla komórek roślinnych. Na potwierdzenie, ekspozycja komórek soi na stężenie 1 mM egzogennego $ONOO^-$ nie spowodowała ich śmierci, podczas gdy stężenie nadtlenoazotynu 1000 razy niższe (1 μM) okazało się być letalne dla komórek zwierzęcych (Delledonne i in., 2001). Udokumentowano ponadto, że poddanie roślin *Arabidopsis* działaniu wysokiego stężenia $ONOO^-$ (3 mM), nie powoduje śmierci komórek (pomimo obserwowanego wzrostu nitrowanych białek) zarówno u formy dzikiej, jak i mutantów linii PrxII E z obniżoną ekspresją *PrxII E*, wykazującą aktywność reduktazy nadtlenoazotynu (Romero-Puertas i in., 2007). Dowiedziono również, że wzbogacenie komórek korzeni łubinu w egzogenny $ONOO^-$ (1 mM) podczas stresu kadmowego, wręcz sprzyjało ich żywotności (Arasimowicz-Jelonek i in., 2012). Dostępne dane literaturowe wskazują, że $ONOO^-$ może być formowany także w warunkach fizjologicznych, jako nieodłączny element metabolizmu zdrowych komórek, ponieważ organizmy roślinne są ewolucyjnie wyposażone w szerokie spektrum mechanizmów unieczynniających nadmiar $ONOO^-$ (m.in. Romero-Puertas i in., 2007; Arasimowicz-Jelonek i Floryszak-Wieczorek, 2011). Najnowsze badania opublikowane przez Gzyl i in. (2016) wykazały, że selektywne nitrowanie wolnych reszt tyrozynowych w białkach *via* $ONOO^-$ stanowi ważny mechanizm regulujący aktywność białek w komórkach korzeni siewek soi, zarówno w warunkach fizjologicznych, jak i w trakcie działania stresu kadmowego. Również Serrano i in. (2012) stwierdzili, iż to właśnie nadtlenoazotyn, poprzez nitrowanie białek, funkcjonuje jako cząsteczka sygnałowa w aktywacji programowanej śmierci komórek (PCD), w wywołanej reakcji samoniezgodności pyłku *Olea europaea* L. Stosunkowo niedawno udokumentowano, że nitrowanie kwasów tłuszczowych *via* $ONOO^-$ prowadzi także u roślin, do generowania biologicznie aktywnych

nitrolipidów, które mogą uczestniczyć w transdukcji sygnału (m.in., jako endogenne źródło NO), w odpowiedzi *Arabidopsis thaliana* na różne abiotyczne czynniki stresowe (Mata-Pérez i in., 2016).

Powyższe wyniki badań wskazują, iż generowanie $ONOO^-$ w organizmach roślinnych jest nie tylko markerem stresu nitrozacyjnego, ale poprzez selektywne nitrowanie biocząsteczek, może stanowić ważny modulator szlaków sygnałowych, bądź metabolicznych, uruchamianych zarówno w procesach rozwojowych, jak i w reakcji roślin na różne stresy środowiskowe. Należy podkreślić, że wiedza dotycząca roli $ONOO^-$ i związków modyfikowanych za pośrednictwem nadtlenoazotynu, szczególnie w interakcji roślina – patogen, jest ograniczona. Do momentu podjęcia badań, będących celem niniejszej rozprawy, obecność *in vivo* $ONOO^-$ w odpowiedzi na stres biotyczny, udokumentowano w zaledwie dwóch układach eksperymentalnych (Saito i in., 2006; Gauples i in., 2011; Bellini i in., 2016).

Zaraza ziemniaka, powodowana przez mikroorganizm z grupy *Chromista*, gromady *Oomycetes* – *Phytophthora infestans* (Mont.) de Bary, jest najważniejszą, pod względem ponoszonych strat ekonomicznych, chorobą ziemniaka (*Solanum tuberosum* L.). Towarzyszy uprawom ziemniaka na całym świecie, przynosząc obniżenie lub całkowitą utratę plonu i generując wysokie koszty ochrony chemicznej upraw. Jak wiadomo, drugi komponent badanej interakcji – ziemniak, to gatunek o niezmiernie istotnym znaczeniu gospodarczym w skali globalnej, gdyż po ryżu, pszenicy i kukurydzy jest najważniejszą rośliną uprawną na świecie. Obecnie łączny koszt ochrony chemicznej przed *P. infestans* oraz strat przez nią powodowanych jest szacowany, w samej tylko Europie, na ponad miliard euro rocznie. Co więcej, stosowane fungicydy nie gwarantują stuprocentowej skuteczności w ochronie przed zarazą oraz nie pozostają obojętne dla środowiska. W celu zmniejszenia zakresu ochrony chemicznej konieczna jest hodowla odpornych na *P. infestans* odmian ziemniaka. Do tej pory, zidentyfikowano kilkadziesiąt *R* genów odporności na zarazę, wprowadzonych do odmian uprawnych z różnych dzikich gatunków *Solanum* (Rodewald i Trognitz, 2013). Niestety patogen przejawia znaczące zdolności do przełamywania odporności uwarunkowanej mechanizmem ETI opartym o białka R. Wynika to z niestabilności genomu *P. infestans* oraz możliwości rozmnażania płciowego form kojarzeniowych A1 i A2, co prowadzi finalnie do zmian w populacji i powstawania nowych, często bardziej agresywnych ras patogena (Raffaele i in., 2010). Wobec braku odmian zdecydowanie odpornych na ten patogen oraz w związku z uodparnianiem się izolatów *P. infestans* na komercyjnie stosowane fungicydy, niezwykle ważne stają się wszelkie próby poznania oraz wzmocnienia naturalnej odporności rośliny-gospodarza.

Stąd, nadrzędnym celem przedstawionej rozprawy doktorskiej było zweryfikowanie udziału nadtlenoazotynu oraz poznanie funkcjonalnych modyfikacji wywołanych za pośrednictwem tej reaktywnej formy azotu, w odporności liści ziemniaka na zarazę powodowaną przez *Phytophthora infestans*.

Przyjęto, że cel ten zostanie osiągnięty poprzez realizację 3 głównych zadań badawczych.

(1) Określenie źródła biosyntezy NO oraz monitorowanie generowania NO i $O_2^{\cdot -}$ wraz z $ONOO^-$ w liściach ziemniaka inokulowanych *P. infestans*, przy wykorzystaniu wysoce precyzyjnych technik pomiarowych, dających obraz generowania RFA w czasie rzeczywistym.

(2) Poszukiwanie korelacji pomiędzy akumulacją $ONOO^-$, a poziomem ekspresji genów kodujących kluczowe markery odporności roślin, tj. PR-1, PR-2 (beta-1,3-glukanazę) oraz PR-3 (chitynazę).

(3) Detekcję oraz identyfikację związków modyfikowanych za pośrednictwem $ONOO^-$ w ziemniaku oraz wytypowanie tych modyfikacji, które mają istotny wpływ na odporność liści względem patogena.

Wszystkie doświadczenia prowadzono na odciętych liściach ziemniaka (*Solanum tuberosum* L.), odmian skrajnie różniących się odpornością na hemibiotroficzny patogen *Phytophthora infestans* (izolat 1.3.4.7.10.11; MP 946). Wykorzystana w doświadczeniach odmiana podatna Bintje nie posiada *R*-genów odporności. Natomiast, odmiany odporne posiadają w swym genomie zidentyfikowane *R*-geny odporności tj. *R1* i *R2-like* (Bzura – Gebhardt i in., 2004; Plich i in., 2015) oraz *R3a*, *R3b*, *R4*, *Rpi-Smira1* i *Rpi-Smira2* (Sarpo Mira – Rietman i in., 2012) i wykazują reakcję nadwrażliwości względem MP 946. Wobec powyższego, układ badawczy pozwolił określić, na ile zdarzenia metaboliczne zależne od $ONOO^-$ towarzyszą aktywacji skutecznych odpowiedzi obronnych w odporności typu ETI, a na ile odpowiedziom obronnym podczas PTI.

OMÓWIENIE WYNIKÓW

Niewątpliwie dużą przeszkodą w wyjaśnieniu funkcji sygnałowej oraz regulacyjnej NO/$ONOO^-$ w odporności roślin, jest brak w pełni rozpoznanych źródeł syntezy NO. Najczęściej wyróżnia się trzy główne możliwości powstawania NO w organizmach roślinnych, tj.: szlak zależny od L-argininy z udziałem potencjalnej syntazy tlenku azotu typu ssaczego (NOS-*like*), szlak zależny od azotynów z udziałem reduktazy azotanowej (NR) oraz nieenzymatyczną drogę przekształcania azotynów do NO (m.in. Gupta i in., 2011; Rőszer, 2014; Jeandroz i in., 2016). **Wobec powyższego, pierwszym etapem pracy doktorskiej było uzyskanie odpowiedzi na pytanie, co odpowiada za syntezę NO w infekowanych patogenem liściach ziemniaka?** Przeprowadzone doświadczenia wykazały, że wczesne i wzmożone generowanie NO, obserwowane w liściach ziemniaka w odpowiedzi na *P. infestans*, było ściśle skorelowane w czasie, zarówno ze wzrostem poziomu transkryptu genu *NR*, jak i zwiększeniem aktywności enzymatycznej kodowanego białka **(Publikacja 1, Fig. 3 A-B)**. Niezależne zastosowanie specyficznych inhibitorów syntezy NO wykazało istotne, poinfekcyjne blokowanie generowania tej molekuły w odpowiedzi na wolframian (inhibitor NR) oraz nieznaczne hamowanie przez podanie L-NAME (ester metylowy L-N^G-nitroargininy; inhibitor syntazy tlenku azotu – NOS-*like*). Tym samym uzyskane wyniki wykazały, że reduktaza azotanowa jest głównym źródłem NO w liściach ziemniaka inokulowanych *P. infestans* **(Publikacja 1, Fig. 2)**. Podobnie, jak opisano wcześniej w bulwach ziemniaka infekowanych *P. infestans* (Yamamoto i in., 2003).

Chociaż stosunkowo dawno, Alamillo i Garcia-Olmedo (2001) zasugerowali już udział $ONOO^-$ w zamieraniu komórek podczas reakcji nadwrażliwości, obserwowanej w odpowiedzi *Arabidopsis* na awirulentny szczep *Pseudomonas syringae,* to jednak obecność *in vivo* tej RFA odnotowano dotychczas jedynie we wczesnych etapach odpowiedzi komórek tytoniu BY-2 na elicytor INF1, pochodzący z *P. infestans* (Saito i in., 2006) oraz liści *Arabidopsis thaliana* na awirulentny szczep bakterii *Pseudomonas syringae* pv *tomato* (Gaupels i in., 2011; Bellini i in., 2016). **Chcąc zatem sprawdzić, jak kształtuje się status metaboliczny NO w liściach ziemniaka inokulowanych *P. infestans*, w kolejnym etapie badań przeprowadzono analizę generowania NO i $O_2^{\bullet -}$ – cząsteczek macierzystych nadtlenoazotynu, jak również zbadano poziom samego $ONOO^-$.** Wykorzystując wysoce specyficzny fluorochrom Cu-FL (czułość detekcji NO na poziomie 100 pikomoli), stwierdzono dwufazowy przebieg generowania NO w infekowanych liściach odmiany odpornej. Pierwszy, wczesny wybuch NO, odnotowano pomiędzy 1 a 3 godziną po inokulacji

(hpi), drugi, nieco słabszy, w 24 hpi. W odmianie podatnej generowanie NO było zdecydowanie niższe i opóźnione w czasie, w stosunku do odmiany odpornej, osiągając maksimum w 6 hpi **(Publikacja 2, Fig. 2)**. W odniesieniu do anionorodnika ponadtlenkowego stwierdzono od 1 hpi stopniowy wzrost generowania w obu odmianach **(Publikacja 2, Fig. 3)**.

Różnice międzyodmianowe odnotowano także w kinetyce formowania $ONOO^-$, która była skorelowana w czasie z akumulacją NO i $O_2^{\cdot -}$. Posługując się dwoma wysoce czułymi metodami ilościowej detekcji $ONOO^-$ **(Publikacja 2, Fig. 1a-b oraz Publikacja 3, Fig. 1)** wykazano poinfekcyjne generowanie $ONOO^-$ w liściach obu genotypów ziemniaka. Przy czym odmiana odporna, podobnie jak w przypadku NO, charakteryzowała się wczesnym (do 6 hpi) i wzmożonym formowaniem tej RFA **(Publikacja 2 i Publikacja 3)**. Ponadto rejestracja generowania $ONOO^-$ w kolejnych dobach po inokulacji wykazała obecność drugiego, słabszego „wybuchu" tej RFA w 72 hpi **(Publikacja 3, Fig. 1A)**. W przypadku odmiany podatnej istotnie wyższą akumulację nadtlenoazotynu odnotowano dopiero w 24 hpi **(Publikacja 2 i Publikacja 3)**. Równocześnie przeprowadzona analiza cytochemiczna wykazała, iż wzmożone generowanie $ONOO^-$ było zlokalizowane przede wszystkim w komórkach epidermy oraz miękiszu palisadowym i gąbczastym **(Publikacja 2, Fig. 1c-f)**. Obecność $ONOO^-$ obserwowano również w zdrowych, nieinfekowanych liściach obu odmian ziemniaka, jakkolwiek była ona ograniczona tylko do nielicznych komórek parenchymatycznych **(Publikacja 2, Fig. 1g-h)**.

W celu utrzymania homeostazy redoks w komórce roślinnej narażonej na stres biotyczny, wczesnemu generowaniu RFA oraz reaktywnych form tlenu (RFT) powinna towarzyszyć aktywacja systemu antyoksydacyjnego. Z szeregu roślinnych antyutleniaczy na szczególną uwagę, w kontekście poinfekcyjnego formowania nadtlenoazotynu w liściach ziemniaka, zasługują peroksydazy tioredoksyn (TPx). **Stąd, w kolejnym etapie badań przeprowadzono analizę ekspresji genu peroksydazy tioredoksyny (*TPx*), którego produkt, jak wskazuje najnowsza literatura, może stanowić bardzo istotny element mechanizmów kontrolujących endogenny poziom $ONOO^-$ (Ferrer-Sueta i in., 2018).** W rezultacie wykazano, że zwiększenie puli $ONOO^-$ poprzez traktowanie donorem $ONOO^-$ (3-morfolino-sydnonimina, SIN-1) zdrowych liści obu odmian ziemniaka, istotnie indukowało ekspresję *TPx* już w pierwszych godzinach po potraktowaniu **(Publikacja 2, Fig. S1)**. Co więcej, wzmożona akumulacja $ONOO^-$, w odpowiedzi na *P. infestans*, była skorelowana w czasie z istotnym zwiększeniem ekspresji *TPx* u obu odmian ziemniaka **(Publikacja 2, Fig. 4 a-b)**.

Ogólnie wiadomo, że w zainfekowanych tkankach akumulacja białek związanych z patogenezą (PRs; ang. *Pathogenesis-Related Proteins*) stanowi efektywną linię obrony uruchamianą przez rośliny w odpowiedzi na atak patogenów. Już pierwsze prace eksperymentalne dotyczące metabolizmu NO u roślin wykazały, iż podanie donora $ONOO^-$ powoduje w liściach tytoniu wzrost ekspresji genu kodującego białko PR-1, uznawane za kluczowy marker odpowiedzi obronnej rośliny (Durner i in., 1998). **Zatem, w celu określenia potencjalnego udziału nadtlenoazotynu w uruchamianiu odpowiedzi obronnych, w kolejnym etapie badań, wykorzystano wyłącznie liście odmiany podatnej, które traktowano donorem $ONOO^-$ w postaci SIN-1.** Uzyskane dane wykazały, że egzogenny $ONOO^-$ indukuje ekspresję *PR-1* oraz *PR-2*, począwszy od 3 godziny po potraktowaniu **(Publikacja 2, Fig. S2)**. Ponadto sekwencyjne traktowanie liści odmiany podatnej donorem $ONOO^-$ i *P. infestans* powodowało szybszą i wzmożoną indukcję genów kodujących białka PR skorelowaną z ograniczoną kolonizacją tkanek rośliny-gospodarza **(Publikacja 2, Fig. 6 i Fig. 5)**. Zwiększonej ekspresji *PR-1*, *PR-2* oraz *PR-3* nie zaobserwowano pod wpływem sekwencyjnego oddziaływania zmiatacza $ONOO^-$ i *P. infestans*, co jednoznacznie potwierdziło udział tej RFA w regulacji ekspresji genów kodujących białka PR (**Publikacja 2, Fig. 6**).

Selektywne nitrowanie biocząsteczek *via* $ONOO^-$ może być ważnym mechanizmem regulatorowym, wpływającym na wewnątrzkomórkowe szlaki przekazywania sygnałów zależne od NO. Należy jednak podkreślić, że zjawisko nitrowania biocząsteczek za pośrednictwem $ONOO^-$ u roślin jest nowym, zasygnalizowanym jedynie obszarem badań w metabolizmie NO, wymagającym weryfikacji eksperymentalnej. **Stąd też kolejną istotną kwestią podjętą w niniejszej pracy było uzyskanie odpowiedzi na pytanie, czy i w jakim stopniu akumulacja nitrowanych za pośrednictwem $ONOO^-$ związków w liściach ziemniaka, sprzyja uruchamianiu skutecznych odpowiedzi obronnych względem *P. infestans*?**

Reakcją charakterystyczną i jednocześnie najlepiej rozpoznaną dla $ONOO^-$ jest nitrowanie wolnych reszt tyrozynowych w białkach, prowadzące do zmiany ich struktury i aktywności katalitycznej. W odniesieniu do roślin, dostępne dane literaturowe wskazują, iż najczęstszą konsekwencją tego procesu jest utrata funkcji modyfikowanego białka (Kolbert i in., 2017). Niemniej jednak, w przypadku zwierząt eksperymentalnie dowiedziono, że nitrowanie tyrozyny może prowadzić również do wzmocnienia aktywności katalitycznej białka (Yeo i in., 2015). Przeprowadzone analizy nitroproteomu wykazały, że liście odmiany podatnej podlegały silnemu nitrowaniu w następstwie infekcji, co może świadczyć

o niekontrolowanej nadprodukcji RFA, prowadzącej do stresu nitrozacyjnego. Wśród białek, które zidentyfikowano jako podlagające wzmożonemu nitrowaniu w trakcie rozwoju choroby (w 24 i 48 hpi), były białka zaangażowane w podstawowe procesy metaboliczne takie jak translacja, fotosynteza czy glikoliza **(Publikacja 2, Fig. 7b i Tab. 2)**. Podobnej tendencji nie obserwowano w przypadku odmiany odpornej w 24 i 48 hpi. Wręcz przeciwnie, odnotowano obniżoną akumulację nitrowanych białek związanych z odpowiedziami obronnymi, w tym: katalazę, peroksydazę anionową, S-transferazę glutationową oraz białka z grupy PR (beta-1,3-glukanazę oraz chitynazę) **(Publikacja 2, Fig. 7a i Tab. 2)**.

Szczególnie istotne znaczenie w kontekście uruchamiania skutecznych odpowiedzi obronnych, w badanym układzie eksperymentalnym, może mieć nitrowanie beta-1,3-glukanazy należącej do rodziny białek PR-2. Jak powszechnie wiadomo, enzym ten odpowiedzialny jest za hydrolizę glukanów, będących głównym składnikiem ściany komórkowej grzybopodobnych lęgniowców. Zatem precyzyjna regulacja aktywności beta-1,3-glukanazy może potencjalnie przekładać się na ograniczenie porażenia rośliny przez *P. infestans*. Powyższa obserwacja stała się podstawą do dalszych dociekań naukowych nad funkcjonalnością tej potranslacyjnej modyfikacji, w regulacji aktywności kluczowych białek odporności roślin i zaowocowała projektem badawczym PRELUDIUM 12 pt. „Analiza jakościowa i funkcjonalna nitrowanej beta-1,3-glukanazy w odporności liści ziemniaka na *Phytophthora infestans* (Mont.) de Bary". Przeprowadzone dotychczas badania, z wykorzystaniem donora nadtlenoazotynu oraz rekombinowanej beta-1,3-glukanazy, jednoznacznie potwierdzają, że enzym ten podlega nitrowaniu. Natomiast efektem tej reakcji jest hamowanie aktywności katalitycznej białka, co może mieć bardzo istotne znaczenie w inaktywacji *P. infestans*. Otrzymane wyniki zostały zaprezentowane podczas międzynarodowej konferencji dotyczącej biologii NO u roślin (Izbiańska i in., 2018; *7th Plant Nitric Oxide International Meeting*; Nicea, Francja).

Obok wspomnianych powyżej zmian, w profilu nitroproteomu liści odmiany odpornej stwierdzono pojawienie się dodatkowego prążka (B13), który zawierał białka podobne do subtylizyny o aktywności proteaz serynowych (SBT5.3 i SBT1.7) **(Publikacja 2, Fig. 7a i Tab. 2)**. Dodatkowy eksperyment obejmujący punktową inokulację liści, a tym samym umożliwiający precyzyjną ocenę zmian w strefie reakcji nadwrażliwości i jej okolicy, wykazał podwyższoną akumulację nitrowanych białek, zidentyfikowanych jako proteazy podobne do subtylizyny (tj. SBT5.3 i SBT1.7), jedynie w strefie okalającej HR. Ponadto akumulacja wspomnianych nitrowanych proteaz korelowała z hamowaniem ich aktywności proteolitycznej **(Publikacja 3 Fig. 5A-D, Fig. S1 oraz Tab. S1)**. Dowiedziono wcześniej, że

subtylizyny mogą pełnić funkcję enzymów egzekutorowych – kaspaz (Fernández i in., 2015), dlatego obecnie wnioskuje się, że nitrowanie tych białek i w konsekwencji hamowanie ich aktywności proteolitycznej, może mieć szczególnie istotne znaczenie w kontroli zasięgu aktywnego zamierania komórek ziemniaka. Ponadto, analiza ilościowa ogólnej puli nitrowanych białek, przy wykorzystaniu specyficznych testów ELISA, potwierdziła, że wzmożona akumulacja nitrowanych białek ściśle korelowała w czasie z tempem generowanego $ONOO^-$ w infekowanych liściach obu odmian ziemniaka **(Publikacja 3, Fig. 4A-B)**. Należy podkreślić, że w odmianie odpornej wzrost ogólnej puli nitrowanych białek odnotowano zarówno w trakcie pierwszego (1-6 hpi), jak i po drugim wybuchu $ONOO^-$ (72 hpi) **(Publikacja 3, Fig. 4A)**. Uzyskane wyniki wykazały także obecność nitrowanych białek w zdrowych, nieinfekowanych liściach obu odmian ziemniaka. Zatem stanowi to kolejny dowód na to, że zjawisko nitrowania *via* $ONOO^-$ jest fizjologiczną modyfikacją potranslacyjną białek **(Publikacja 2, Fig. 7)**.

W toku dalszych analiz nad poszukiwaniem związków modyfikowanych *via* $ONOO^-$ wykazano, że inokulacja liści *P. infestans* prowadziła również do nitrowania RNA oraz mRNA. Nitrowanie puli RNA i mRNA *via* $ONOO^-$ zostało wykazane na podstawie analizy poziomu akumulacji 8-nitroguaniny, specyficznego markera nitrowania nukleotydów. Należy podkreślić, że jest to pierwsze doniesienie literaturowe, dokumentujące obecność tej modyfikacji w organizmach roślinnych (Publikacja 3, Fig. 3). W przypadku odmiany odpornej stwierdzono stukrotnie wyższy poziom nitrowanego RNA, począwszy od pierwszej godziny po inokulacji, przy czym podobną tendencję obserwowano także w przypadku puli izolowanego mRNA. Według najnowszych badań, modyfikacje mRNA mogą stanowić jeden z mechanizmów regulacji ekspresji genów, a tym samym wpływać na poziom kodowanych przez nie białek (Gilbert i in., 2016; Chmielowska-Bąk i in., 2018). Dalsze analizy wykazały, że odnotowany w odmianie odpornej, wysoki poziom nitrowanego RNA i mRNA towarzyszył aktywnemu zamieraniu jąder komórek liści atakowanych przez *P. infestans*, co wykazała reakcja TUNEL dając wynik pozytywny. W ten sposób potwierdzono, że w komórkach podlegających programowanej śmierci, czyli podczas HR dochodzi do akumulacji nitrowanych biocząsteczek tj. białek (**Publikacja 3, Fig. 5a)** oraz kwasów nukleinowych **(Publikacja 3, Fig. 3C,E i Fig. 2a)**. Natomiast w odmianie podatnej, istotny wzrost poziomu nitrowanego RNA/mRNA stwierdzono dopiero w późniejszych stadiach rozwoju choroby **(Publikacja 3, Fig. 3D,F)**. Na podstawie uzyskanych wyników można zatem wnioskować, że nitrowanie RNA/mRNA u roślin jest nie tylko markerem uszkodzeń kwasów nukleinowych wywołanych przez akumulację $ONOO^-$, ale może

stanowić wysoce selektywny proces potranskrypcyjnej regulacji ekspresji genów zaangażowanych w uruchamianie i/lub regulację śmierci komórek podczas HR. Na potwierdzenie, zastosowanie zmiataczy endogennego $ONOO^-$ wyraźnie ograniczało liczbę komórek zamierających aktywnie, a jednocześnie obniżeniu ulegał poziom nitrowanego mRNA **(Publikacja 3, Fig. 2b-c).** Uzyskane wyniki potwierdzają zatem hipotezę zaproponowaną przez Alamillo i Garcia-Olmedo (2001), że nadtlenoazotyn, obok NO i RFT, uczestniczy w programowanej śmierci komórek podczas reakcji nadwrażliwości.

WNIOSKI

Badania nad poznaniem funkcji nadtlenoazotynu w odporności liści ziemniaka na *Phytophthora infestans* prowadzą do następujących konkluzji:

1) Reduktaza azotanowa stanowi główne źródło biosyntezy NO w liściach ziemniaka.

2) Generowanie tlenku azotu oraz anionorodnika ponadtlenkowego w miejscu bezpośredniego kontaktu patogena z tkanką liścia prowadzi do formowania nadtlenoazotynu, jednakże kinetyka i natężenie tej reakcji jest uzależnione od genotypu ziemniaka. Odmiana odporna charakteryzowała się wczesnym i okresowym generowaniem $ONOO^-$, któremu towarzyszył istotny wzrost akumulacji NO i $O_2^{\cdot -}$. Z kolei w odmianie podatnej wzmożone generowanie $ONOO^-$ obserwowano dopiero od pierwszej doby po infekcji.

3) Analiza ekspresji genu peroksydazy tioredoksyny na poziomie akumulacji transkryptu wskazała, że TPx w liściach ziemniaka może pełnić rolę modulatora poziomu $ONOO^-$.

4) Poinfekcyjna akumulacja nitrowanych białek, w liściach obu odmian ziemniaka odzwierciedlała charakter generowania $ONOO^-$. Stąd, wczesna i przejściowa akumulacja modyfikowanych *via* $ONOO^-$ białek w odmianie odpornej, wskazuje na możliwość okresowej zmiany funkcjonalnej tych białek. Natomiast wzrost puli nitrowanych białek odnotowany w kolejnych godzinach po inokulacji w odmianie podatnej, w połączeniu z nadprodukcją RFA, odzwierciedla stres nitro-oksydacyjny sprzyjający rozwojowi choroby.

5) Przeprowadzona równolegle analiza nitroproteomu w komórkach otaczających strefę HR wykazała podwyższoną ekspresję białek podobnych do subtylizyny (SBT5.3, SBT1.7), co korelowało z hamowaniem ich aktywności proteolitycznej w badanej strefie. Uzyskane wyniki wskazują zatem, że akumulacja nitrowanych proteaz może mieć

szczególnie istotne znaczenie w kontroli zasięgu reakcji nadwrażliwości poprzez wygaszenie ich funkcji egzekutorowej w odpowiedzi typu HR.

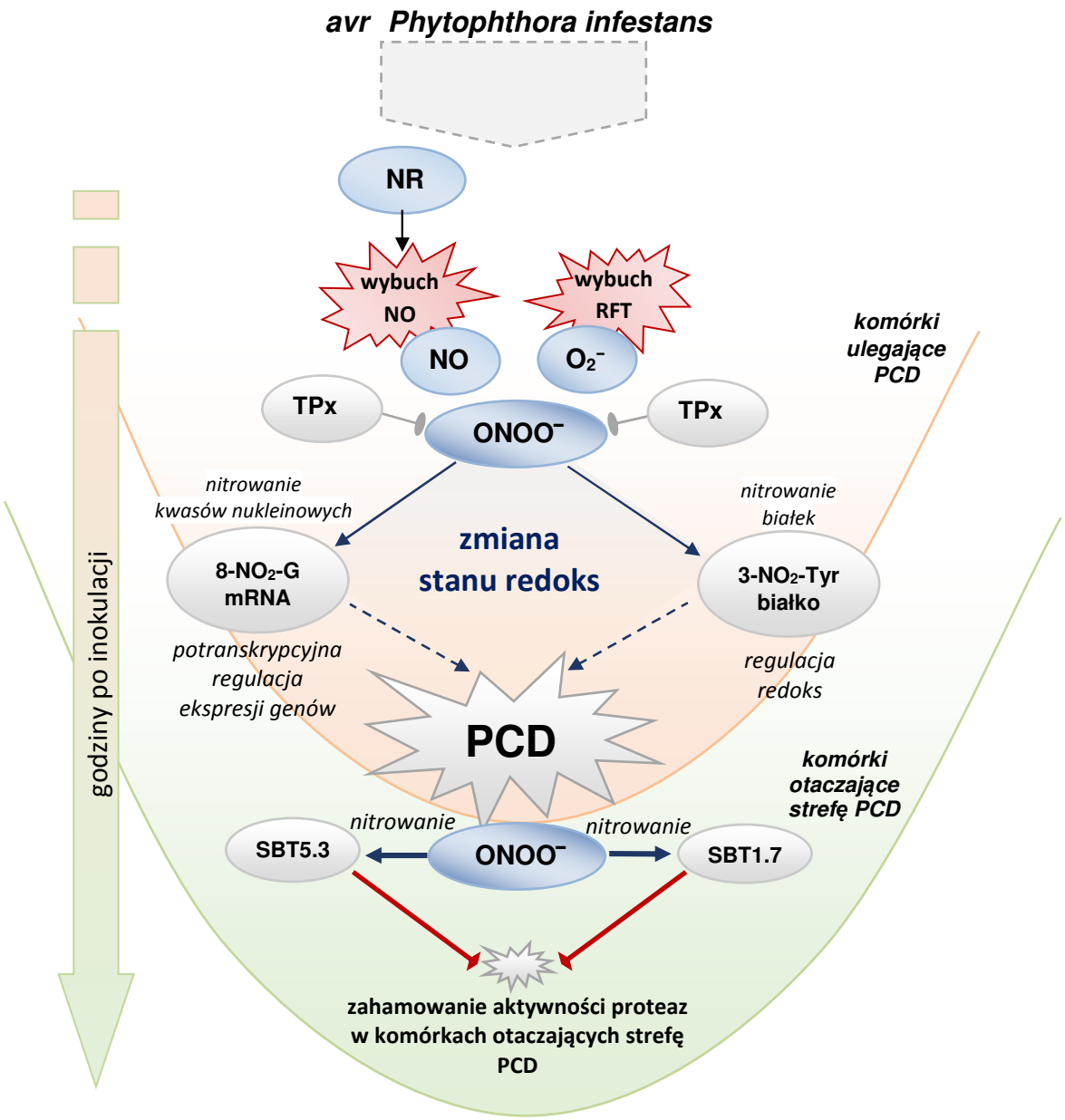
6) Udokumentowano po raz pierwszy w świecie roślin, że kwasy nukleinowe tj. RNA i mRNA podlegają nitrowaniu *via* $ONOO^-$. Analiza porównawcza pomiędzy genotypem odpornym, a podatnym wskazała na okresowy, istotnie wyższy poziom nitrowanego RNA oraz mRNA w inokulowanych liściach odmiany odpornej. Eliminacja $ONOO^-$ za pośrednictwem zmiatacza, skutkowała istotnie obniżonym poziomem mRNA oraz reakcją TUNEL negatywną w komórkach liści ziemniaka odmiany odpornej, co wskazuje, że nitrowanie mRNA może być procesem wysoce selektywnym, wpływającym na potranskrypcyjną regulację ekspresji genów zaangażowanych m.in. w programowaną śmierć komórki podczas HR.

7) Obok wyszczególnionych powyżej nitrowanych biocząsteczek o istotnym znaczeniu defensywnym, również sekwencyjne traktowanie liści odmiany podatnej donorem $ONOO^-$ i *P. infestans* powodowało szybszą i wzmożoną indukcję genów kodujących białka PR-1, PR-2 i PR-3, co korelowało z ograniczoną kolonizacją tkanek rośliny-gospodarza.

PODSUMOWANIE

Na podsumowanie przeprowadzonych badań stwierdza się, iż wczesna i okresowa, akumulacja $ONOO^-$ w liściach ziemniaka w odpowiedzi na *P. infestans,* wywołuje selektywne nitrowanie białek oraz kwasów nukleinowych, zaangażowanych w regulację zasięgu programowanej śmierci komórek ziemniaka oraz indukcję ekspresji genów *PRs*, przez co sprzyja uruchamianiu skutecznych odpowiedzi obronnych związanych z odpornością (Ryc. 1).

Wyniki niniejszej rozprawy doktorskiej mają znaczenie nie tylko poznawcze, ale mogą również posłużyć jako punkt wyjścia do badań nad nowymi mechanizmami ograniczającymi rozwój patogenów *via* NO. Szczególnie istotne znaczenie w kontekście uruchamiania skutecznych odpowiedzi obronnych może mieć poznanie i charakterystyka funkcjonalnych celów nitrowania mRNA. Modyfikacje mRNA mogą bowiem wpływać na poziom ekspresji określonych białek i tym samym stanowić jeden z mechanizmów regulacji ekspresji genów, co w konsekwencji przyczyni się do lepszego zrozumienia interakcji roślina – patogen oraz może być przydatne m.in. w hodowli odpornościowej posługującej się celowanymi modyfikacjami genetycznymi.



Rycina 1. Skonstruowana w oparciu o uzyskane wyniki, sekwencja zdarzeń metabolicznych z udziałem nadtlenoazotynu w odporności liści ziemniaka na *P. infestans* **(Publikacja nr 3, zmodyfikowane)**.

LITERATURA

1. **Alamillo JM**., Garcıa-Olmedo F. (2001). Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. Plant Journal. 25: 529–540.
2. **Arasimowicz-Jelonek M.**, Floryszak-Wieczorek J. (2011) Understanding the fate of peroxynitrite in plant cells–from physiology to pathophysiology. Phytochemistry, 72: 681–688.
3. **Arasimowicz-Jelonek M**., Floryszak-Wieczorek J., Deckert J., Rucinska-Sobkowiak R., Gzyl J., Pawlak-Sprada S., Abramowski D., Jelonek T., Gwózdz EA. (2012). Nitric oxide implication in cadmium-induced programmed cell death in roots and signaling response of yellow lupine plants. Plant Physiol. Biochem. 58: 124– 134.
4. **Bellin D**., Asai S., Delledonne M., Yoshioka H. (2013).Nitric oxide as a mediator for defense responses. Mol. Plant Microbe Interact. 26: 271–277.
5. **Bellin D**., Delledonne M., Vandelle E. (2016). Detection of peroxynitrite in plants exposed to bacterial infection. Methods Mol. Biol., 1424: 191–200.
6. **Chaki M**., Valderrama R., Fernández-Ocaña AM., Carreras A., López-Jaramillo J., Luque F., Palma JM., Pedrajas JR., Begara-Morales JC., Sánchez-Calvo B., Gómez-Rodríguez MV., Corpas FJ., Barroso JB. (2009). Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. J. Exp. Bot. 60: 4221–4234.
7. **Chmielowska-Bąk J**., Izbiańska K., Ekner-Grzyb A., Bayar M., Deckert J. (2018). Cadmium stress leads to rapid increase in RNA oxidative modifications in soybean seedlings. Front Plant Sci. 8: 2219.
8. **Corpas FJ**., Barroso JB. (2014). Peroxynitrite ($ONOO^-$) is endogenously produced in *Arabidopsis* peroxisomes and is overproduced under cadmium stress. Ann. Bot. 113: 87–96.
9. **Delledonne M**., Zeier J., Marocco A., Lamb C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. PNAS,USA. 98: 13454–13459.
10. **Durner J**., Wendehenne D., Klessig DF. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc. Natl. Acad. Sci. USA. 95: 10328–10333.
11. **Fernández MB**., Daleo GR., Guevara MG. (2015). Isolation and characterization of a *Solanum tuberosum* subtilisin-like protein with caspase-3 activity (StSBTc-3). Plant Physiol. Biochem. 86: 137–146.
12. **Ferrer-Sueta G**., Campolo N., Trujillo M., Bartesaghi S., Carballal S., Romero N., Alvarez B., Radi R. (2018). Biochemistry of peroxynitrite and protein tyrosine nitration. Chem. Rev. 118: 1338−1408.

13. **Floryszak-Wieczorek J**., Arasimowicz-Jelonek M. (2016). Contrasting regulation of NO and ROS in potato defense-associated metabolism in response to pathogens of different lifestyles. PLoS One. 11(10): e0163546.
14. **Gaupels F**., Spiazzi-Vandelle E., Yang D., Delledonne M. (2011). Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response. Nitric Oxide. 25: 222–228.
15. **Gebhardt C**., Ballvora A., Walkemeier B., Oberhagemann P., Schuler K. (2004). Assessing genetic potential in germplasm collections of crop plants by marker-trait association: a case study for potatoes with quantitative variation of resistance to late blight and maturity type. Mol. Breed. 13: 93–102.
16. **Gilbert WV**., Bell T.A., Schaening C. (2016). Messenger RNA modifications – form, distribution, and function. Science. 352: 1408–1412
17. **Gupta KJ**., Fernie AR., Kaiser WM., van Dongen JT. (2011). On the origins of nitric oxide. Trends Plant Sci. 16: 160–168.
18. **Gzyl J**., Izbiańska K., Floryszak-Wieczorek J., Jelonek T., Arasimowicz-Jelonek M. (2016). Cadmium affects peroxynitrite generation and tyrosine nitration in seedling roots of soybean (*Glycine max* L.). Environ. Exp. Bot. 131: 155–163.
19. **Izbiańska K.**, Gajewska J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Peroxynitrite-mediated posttranslational regulation of β-1,3-glucanase activity in potato leaves inoculated with *Phytophthora infestans.* 7th Plant Nitric Oxide International Meeting, Nice (France) 24 – 26/10/2018
20. **Jeandroz S**., Wipf D., Stuehr DJ., Lamattina L., Melkonian M., Tian Z., Zhu Y., Carpenter EJ., Wong GK., Wendehenne D. (2016) Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. Sci. Signal. 1: 9 (417).
21. **Kolbert Z**., Feigl G., Bordé Á., Molnár Á., Erdei L. (2017). Protein tyrosine nitration in plants: present knowledge, computational prediction and future perspectives. Plant Physiol. Biochem. 113: 56–63.
22. **Krasuska U**., Andrzejczak O., Staszek P., Borucki W., Gniazdowska A. (2017). meta-Tyrosine induces modification of reactive nitrogen species level, protein nitration and nitrosoglutathione reductase in tomato roots. Nitric Oxide. 68: 56–67.
23. **Krasuska U**., Ciacka K., Bogatek R., Gniazdowska A. (2014). Polyamines and nitric oxide link in regulation of dormancy removal and germination of apple (*Malus domestica* Borkh.) embryos. J. Plant Growth Regul. 33:590–601.
24. **Leitner M**., Vandelle E., Gaupels F., Bellin D., Delledonne M. (2009). NO signals in the haze: nitric oxide signalling in plant defence. Curr. Opin. Plant Biol., 12: 451–458.
25. **Mata-Pérez C**., Sánchez-Calvo B., Padilla MN., Begara-Morales JC., Luque F., Melguizo M., Jiménez-Ruiz J., Fierro-Risco J., Peñas-Sanjuán A., Valderrama R., Corpas FJ., Barroso JB.

(2016). Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in *Arabidopsis*. Plant Physiol. 170: 686–701.

26. **Plich J**., Tatarowska B., Lebecka R., Sliwka J., Zimnoch-Guzowska E., Flis B. (2015). R2-like gene contributes to resistance to *Phytophthora infestans* in polish potato cultivar Bzura. Am. J. Potato Res. 92: 350–358.
27. **Raffaele S**., Win J., Cano LM., Kamoun S. (2010). Analyses of genome architecture and gene expression reveal novel candidate virulence factors in the secretome of *Phytophthora infestans*. BMC Genomics. 11: 637.
28. **Rietman H**., Bijsterbosch G., Cano LM., Lee HR., Vossen JH., Jacobsen E., Visser RG., Kamoun S., Vleeshouwers VG. (2012). Qualitative and quantitative late blight resistance in the potato cultivar Sarpo Mira is determined by the perception of five distinct RXLR effectors. Mol Plant Microbe Interact. 25: 910-919.
29. **Rodewald J**., Trognitz B. (2013). Solanum resistance genes against *Phytophthora infestans* and their corresponding avirulence genes. Mol.Plant Pathol. 14: 740–757.
30. **Romero-Puertas MC**., Laxa M., Mattè A., Zaninotto F., Finkemeier I., Jones AM., Perazzolli M., Vandelle E., Dietz KJ., Delledonne M. (2007). S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. Plant Cell. 19: 4120–4130.
31. **Rőszer T**. (2014). Biosynthesis of nitric oxide in plants. In eds. Mohammad NK., Mohammad MF., Corpas JF.Nitric oxide in plants: metabolism and role in stress physiology. Springer, New York, 17-32.
32. **Saito S**., Yamamoto-Katou A., Yoshioka H., Doke N., Kawakita K. (2006). Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. Plant Cell Physiol. 47: 689–697.
33. **Schlicht M**., Kombrink E. (2013). The role of nitric oxide in the interaction of *Arabidopsis thaliana* with the biotrophic fungi, *Golovinomyces orontii* and *Erysiphe pisi*. Front. Plant Sci. 4, 351.
34. **Serrano I**., Romero-Puertas MC., Rodrıguez-Serrano M., Sandalio LM., Olmedilla A. (2012). Peroxynitrite mediates programmed cell death both in papillar cells and in self-incompatible pollen in the olive (*Olea europaea* L.). J. Exp. Bot. 63: 1479–93.
35. **Świątek M**., Śliwka J. (2011). Przegląd badań nad regulacją ekspresji genów głównych odporności roślin na patogeny. Biuletyn Instytutu hodowli i aklimatyzacji roślin, 262: 89–101.
36. **Trapet P**., Kulik A., Lamotte O., Jeandroz S., Bourque S., Nicolas-Francès V., Rosnoblet C., Besson-Bard A., Wendehenne D. (2015). NO signaling in plant immunity: a tale of messengers. Phytochemistry, 112: 72–79.
37. **Yamamoto A**., Katou S., Yoshioka H., Doke N., Kawakita K. (2003). Nitrate reductase, a nitric oxide-producing enzyme: induction by pathogen signals. J. General Plant Pathol. 69: 218–229.

38. **Yeo W**.-S., Kim YJ., Kabir MH., Kang JW., Kim KP. (2015). Mass spectrometric analysis of protein tyrosine nitration in aging and neurodegenerative diseases. Mass Spectrom. Rev. 34: 166e183.

OŚWIADCZENIA WSPÓŁAUTORÓW

Publikacja 1.

Floryszak-Wieczorek J., Arasimowicz-Jelonek M., Izbiańska K. (2016) The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*. *Plant Physiology and Biochemistry,* 108: 468–477 (IF=2,928; 35 pkt MNiSW).

Poznań, 17.12.2018

Prof. dr hab. Jolanta Floryszak-Wieczorek

Katedra Fizjologii Roślin

Uniwersytet Przyrodniczy w Poznaniu

Ul. Wołyńska 35

60-637 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską
stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 1) Jolanta Floryszak-Wieczorek, Magdalena Arasimowicz-Jelonek, Karolina Izbiańska (2016) The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans,*
Plant Physiology and Biochemistry, 108: 468–477

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- opracowaniem koncepcji badań i planowaniem eksperymentów,
- interpretacją uzyskanych wyników i przygotowaniem publikacji.

Swój wkład oceniam na 30%

Poznań, 17.12.2018

Prof. UAM dr hab. Magdalena Arasimowicz-Jelonek

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską

stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 1) Jolanta Floryszak-Wieczorek, Magdalena Arasimowicz-Jelonek, Karolina Izbiańska (2016) The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*
Plant Physiology and Biochemistry, 108: 468–477

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- udziałem w opracowaniu koncepcji i planowaniu badań,
- opieką merytoryczną w trakcie realizacji badań,
- oznaczaniem aktywności reduktazy azotynowej (NiR),
- udział w oznaczaniu zawartości azotanów,
- analizą statystyczną wyników,
- udziałem w interpretacji wyników i przygotowaniu publikacji.

Swój wkład oceniam na 30%

Poznań, 17.12.2018

Mgr Karolina Izbiańska

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

Oświadczam, że w publikacji:

(Publikacja 1) Jolanta Floryszak-Wieczorek, Magdalena Arasimowicz-Jelonek, Karolina Izbiańska (2016) The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*
Plant Physiology and Biochemistry, 108: 468–477

mój wkład był związany z:

- prowadzeniem uprawy ziemniaka
- przygotowaniem zawiesiny zarodników *P. infestans*,
- inokulacją materiału roślinnego,
- zbiorem materiału do analiz,
- udziałem w oznaczaniu poziomu tlenku azotu (NO),
- oznaczaniem aktywności reduktazy azotanowej (NR)
- przeprowadzeniem eksperymentu z wykorzystaniem inhibitorów enzymów odpowiedzialnych za generowanie NO,
- izolacją RNA oraz analizą ekspresji genów,
- udziałem w analizie statystycznej wyników.

Swój wkład oceniam na 40%

Izbiańska Karolina

Publikacja 2.

Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Izbiańska K., Gzyl J., Jelonek T. (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*. *Plant Pathology,* 65: 754–766 (IF=2,383; 35 pkt MNiSW).

Poznań, 17.12.2018

Prof. UAM dr hab. Magdalena Arasimowicz-Jelonek

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską

stanowiących podstawę rozprawy doktorskiej

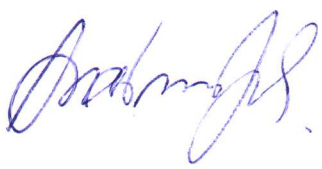
W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 2) Magdalena Arasimowicz-Jelonek, Jolanta Floryszak-Wieczorek, Karolina Izbiańska, Jarosław Gzyl, Tomasz Jelonek (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*
Plant Pathology, 65: 754–766

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- opracowaniem koncepcji i planowaniem badań,
- opieką merytoryczną w trakcie realizacji badań,
- interpretacją wyników i przygotowaniem publikacji.

Swój wkład oceniam na 20%



Poznań, 17.12.2018

Prof. dr hab. Jolanta Floryszak-Wieczorek

Katedra Fizjologii Roślin

Uniwersytet Przyrodniczy w Poznaniu

Ul. Wołyńska 35

60-637 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską
stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 2) Magdalena Arasimowicz-Jelonek, Jolanta Floryszak-Wieczorek, Karolina Izbiańska, Jarosław Gzyl, Tomasz Jelonek (2016) Implication of peroxynitrite in defenceresponses of potato to *Phytophthora infestans*
Plant Pathology, 65: 754–766

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- udziałem w opracowaniu koncepcji i planowaniu badań,
- udziałem w interpretacji wyników i przygotowaniu publikacji.

Swój wkład oceniam na 20%

Poznań, 17.12.2018

Mgr Karolina Izbiańska

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

Oświadczam, że w publikacji:

(Publikacja 2) Magdalena Arasimowicz-Jelonek, Jolanta Floryszak-Wieczorek, Karolina Izbiańska, Jarosław Gzyl, Tomasz Jelonek (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*
Plant Pathology, 65: 754–766

mój udział obejmował:

- traktowanie roślin donorem nadtlenoazotynu (SIN1) oraz jego zmiataczem (ebselen)
- przygotowanie zawiesiny zarodników *P. infestans*,
- inokulację materiału roślinnego,
- wyznaczenie indeksu chorobowego,
- zbiór materiału do analiz,
- oznaczanie ilościowe poziomu nadtlenoazotynu, tlenku azotu i anionorodnika ponadtlenkowego,
- cytochemiczne obrazowanie nadtlenoazotynu,
- izolację RNA oraz analizę ekspresji genów,
- udział w analizie nitroproteomu,
- udział w interpretacji wyników.

Swój wkład oceniam na 40%

Izbiańska Karolina

Poznań, 17.12.2018

dr hab. Jarosław Gzyl

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską

stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 2) Magdalena Arasimowicz-Jelonek, Jolanta Floryszak-Wieczorek, Karolina Izbiańska, Jarosław Gzyl, Tomasz Jelonek (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*
Plant Pathology, 65: 754–766

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- opieką merytoryczną w trakcie realizacji badań,
- udziałem w analizie nitroproteomu,
- udziałem w interpretacji wyników.

Swój wkład oceniam na 10%

Poznań, 17.12.2018

dr hab. Tomasz Jelonek

Katedra Użytkowania Lasu

Uniwersytet Przyrodniczy w Poznaniu

Ul. Wojska polskiego 71A

60-6 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską

stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 2) Magdalena Arasimowicz-Jelonek, Jolanta Floryszak-Wieczorek, Karolina Izbiańska, Jarosław Gzyl, Tomasz Jelonek (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*.
Plant Pathology, 65: 754–766

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- przeprowadzeniem testów statystycznych.

Swój wkład oceniam na 5%.

Jelonek

Publikacja 3.

Izbiańska K., Floryszak-Wieczorek J., Gajewska J., Meller B., Kuźnicki D., Arasimowicz-Jelonek M. (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans*. *Frontiers in Plant Science*, 9: 672 (IF=3,678; 40 pkt MNiSW).

Poznań, 17.12.2018

Mgr Karolina Izbiańska

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

Oświadczam, że w publikacji:

(Publikacja 3) Karolina Izbiańska, Jolanta Floryszak-Wieczorek, Joanna Gajewska, Barbara Meller, Daniel Kuźnicki, Magdalena Arasimowicz-Jelonek (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans*
Frontiers in Plant Science, 9: 672

mój udział obejmował:

- prowadzenie uprawy ziemniaka,
- traktowanie roślin donorem nadtlenoazotynu (SIN1) oraz jego zmiataczem (ebselen),
- udział w przygotowaniu zawiesiny zarodników *P. infestans*,
- udział w inokulacji materiału roślinnego,
- udział w zbiorze materiału do analiz,
- izolację RNA oraz mRNA,
- opracowanie metody i analizę poziomu akumulacji 8-nitroguaniny w puli RNA oraz mRNA z wykorzystaniem testów ELISA,
- analizę ilościową ogólnej puli nitrowanych białek,
- oznaczanie ilościowe poziomu nadtlenoazotynu,
- analizy nitroproteomu,
- detekcję programowanej śmierci komórek metodą TUNEL,
- analizę statystyczną danych,
- udział w interpretacji uzyskanych wyników oraz przygotowaniu publikacji.

Swój wkład oceniam na 55%

Izbiańska Karolina

Poznań, 17.12.2018

Prof. dr hab. Jolanta Floryszak-Wieczorek

Katedra Fizjologii Roślin

Uniwersytet Przyrodniczy w Poznaniu

Ul. Wołyńska 35

60-637 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską
stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 3) Karolina Izbiańska, Jolanta Floryszak-Wieczorek, Joanna Gajewska, Barbara Meller, Daniel Kuźnicki, Magdalena Arasimowicz-Jelonek (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans* Frontiers in Plant Science, 9: 672

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- udziałem w opracowaniu koncepcji i planowaniu badań,
- udziałem w interpretacji wyników i przygotowaniu publikacji.

Swój wkład oceniam na 10%

Poznań, 17.12.2018

Mgr Joanna Gajewska

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską

stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 3) Karolina Izbiańska, Jolanta Floryszak-Wieczorek, Joanna Gajewska, Barbara Meller, Daniel Kuźnicki, Magdalena Arasimowicz-Jelonek (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans* Frontiers in Plant Science, 9: 672

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- udziałem w zbiorze materiału do analiz,
- udziałem w izolacji RNA i białek do testów ELISA

Swój wkład oceniam na 5%

Gajewska

Poznań, 17.12.2018

Mgr inż. Barbara Meller

Katedra Fizjologii Roślin

Uniwersytet Przyrodniczy w Poznaniu

Ul. Wołyńska 35

60-637 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską
stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 3) Karolina Izbiańska, Jolanta Floryszak-Wieczorek, Joanna Gajewska, Barbara Meller, Daniel Kuźnicki, Magdalena Arasimowicz-Jelonek (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans*
Frontiers in Plant Science, 9: 672

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- przygotowaniem zawiesiny zarodników *P. infestans*, punktową inokulacją liści wraz ze zbiorem materiału do analiz, których wyniki zostały zamieszczone na rycinie 5.

Swój wkład oceniam na 5%

Meller Barbara

Poznań, 17.12.2018

Mgr inż. Daniel Kuźnicki

Katedra Fizjologii Roślin

Uniwersytet Przyrodniczy w Poznaniu

Ul. Wołyńska 35

60-637 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską
stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 3) Karolina Izbiańska, Jolanta Floryszak-Wieczorek, Joanna Gajewska, Barbara Meller, Daniel Kuźnicki, Magdalena Arasimowicz-Jelonek (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans* Frontiers in Plant Science, 9: 672

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- histochemiczną detekcją martwych komórek na liściach ziemniaka z wykorzystaniem błękitu trypanowego.

Swój wkład oceniam na 5%

Daniel Kuźnicki

Poznań, 17.12.2018

Prof. UAM dr hab. Magdalena Arasimowicz-Jelonek

Zakład Ekofizjologii Roślin

Wydział Biologii UAM w Poznaniu

Ul. Umultowska 89

61-614 Poznań

OŚWIADCZENIE

dotyczące udziału w pracach wspólnych z mgr Karoliną Izbiańską
stanowiących podstawę rozprawy doktorskiej

W związku z zamiarem włączenia przez mgr Karolinę Izbiańską publikacji:

(Publikacja 3) Karolina Izbiańska, Jolanta Floryszak-Wieczorek, Joanna Gajewska, Barbara Meller, Daniel Kuźnicki, Magdalena Arasimowicz-Jelonek (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans* Frontiers in Plant Science, 9: 672

do Jej rozprawy doktorskiej oświadczam, że mój wkład w powstanie powyższej publikacji był związany z:

- opracowaniem koncepcji i planowaniem badań,
- opieką merytoryczną w trakcie realizacji badań,
- interpretacją wyników i przygotowaniem publikacji.

Swój wkład oceniam na 20%

KOPIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Publikacja 1.

Floryszak-Wieczorek J., Arasimowicz-Jelonek M., Izbiańska K. (2016) The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*. *Plant Physiology and Biochemistry,* 108: 468–477 (IF=2,928; 35 pkt MNiSW).

Plant Physiology and Biochemistry 108 (2016) 468–477

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*

Jolanta Floryszak-Wieczorek [a, *], Magdalena Arasimowicz-Jelonek [b], Karolina Izbiańska [b]

[a] Department of Plant Physiology, Poznan University of Life Sciences, Wolynska 35, 60-637, Poznan, Poland
[b] Department of Plant Ecophysiology, Adam Mickiewicz University, Umultowska 89, 61-614, Poznan, Poland

ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form
10 August 2016
Accepted 11 August 2016
Available online 18 August 2016

Keywords:
Nitrate and nitrite
Nitrate reductase
Nitric oxide
Nitrite reductase
Non-enzymatic NO formation
Phytophthora infestans, potato

ABSTRACT

In contrast to the in-depth knowledge concerning nitric oxide (NO) function, our understanding of NO synthesis in plants is still very limited. In view of the above, this paper provides a step by step presentation of the reductive pathway for endogenous NO generation involving nitrate reductase (NR) activity and nitrite implication in potato defense to *Phytophthora infestans*. A biphasic character of NO emission, peaking mainly at 3 and then at 24 hpi, was detected during the hypersensitive response (HR). In avr *P. infestans* potato leaves enhanced *NR* gene and protein expression was tuned with the depletion of nitrate contents and the increase in nitrite supply at 3 hpi. In the same time period a temporary down-regulation of nitrite reductase (*NiR*) and activity was found. The study for the link between NO signaling and HR revealed an up-regulation of used markers of effective defense, i.e. Nonexpressor of *PR* genes (*NPR1*), thioredoxins (*Thx*) and *PR1*, at early time-points (1–3 hpi) upon inoculation. In contrast to the resistant response, in the susceptible one a late overexpression (24–48 hpi) of *NPR1* and *PR1* mRNA levels was observed. Presented data confirmed the importance of nitrite processed by NR in NO generation in inoculated potato leaves. However, based on the pharmacological approach the potential formation of NO from nitrite bypassing the NR activity during HR response to *P. infestans* has also been discussed.

1. Introduction

Nitric oxide (NO) is a reactive and simple atomic structure molecule affecting a broad spectrum of physiological and patho-physiological processes in every living organism, including plant cells. Despite the increasing body of knowledge regarding its complex and numerous functions, NO production is always a matter of controversy. Nitric oxide is synthesized in each living cell, but its level is modulated by various endogenous and exogenous stimuli. It may not be excluded that the origin of constitutive NO production in healthy plants differs from the source of the post-stress boosted NO generation.

The biosynthetic pathways of NO in plants may be classified as either oxidative or reductive in operation. The oxidative route involves mammalian-type L-arginine-dependent nitric oxide synthase (NOS)-like activity. The NOS-like activity has been detected in various plant organs, including leaves, roots and nodules, as well as cell cultures (Cueto et al., 1996; Ribeiro et al., 1999; Foissner et al., 2000; Tun et al., 2001). Although homolog genes of mammalian NOS have been identified recently in the genome of photosynthetic organisms including the marine green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Foresi et al., 2010), there is no evidence that higher plants have retained this gene (Hancock, 2012). A recently performed *in silico* search for NOS homologs within 1087 sequenced transcriptomes of land plants revealed no typical NOS sequences, including species, in which the NOS-like activity has been detected (Jeandroz et al., 2016). Polyamines and hydroxylamine are other candidates involved in the oxidative route of NO synthesis in plant cells, activated under normoxic conditions (Tun et al., 2006; Rumer et al., 2009; Wimalasekera et al., 2011).

The reductive pathway towards NO synthesis is dependent on nitrite as a primary substrate and may occur practically within the whole cell environment, including the cytoplasm, mitochondria, chloroplasts, peroxisomes as well as the apoplast (Rőszer, 2014). Up to date assimilatory nitrate reductase (NR) has been considered as the hub enzyme for NO synthesis *via* the reductive route. Under physiological conditions the enzyme reduces nitrate to nitrite at the expense of NAD(P)H, but is also able to catalyze 1-electron transfer

* Corresponding author.
E-mail address: florysza@up.poznan.pl (J. Floryszak-Wieczorek).

http://dx.doi.org/10.1016/j.plaphy.2016.08.009
0981-9428/

from NAD(P)H to nitrite, resulting in NO formation (Planchet and Kaiser, 2006). Stöhr et al. (2001) suggested the presence of a plasma membrane bound nitrite: NO-reductase (NI-NOR), which was found to be insensitive to cyanide and unique to NR. The NI-NOR, identified only in tobacco roots and not in leaves, reduced nitrite to NO using reduced cytochrome *c* as an electron donor. NO generation of NI-NOR was comparable to the nitrate reducing activity of a root-specific NR (Stöhr et al., 2001). Nitrite reduction to NO may also occur in the mitochondrial inner membrane. The mitochondrial respiratory chain is able to reduce nitrite to NO at complex III (cytochrome *bc*1) and complex IV (cytochrome-c oxidase); however, this mechanism results in mitochondrial NO generation in cells exposed to hypoxia (Igamberdiev et al., 2010; Gupta and Igamberdiev, 2011; Castello et al., 2006). What is noteworthy, nitrite reductase activity, optimally operative under O_2 limitation, has been assigned to the molybdopterin enzyme family including xanthine oxidoreductase and aldehyde oxidase (Weidert et al., 2014), but there is little evidence of its major involvement in plant systems.

An alternative route of NO production involves non-enzymatic NO formation. This type of chemical NO release might occur at the acidic pH of the apoplast, in the presence of ascorbic acid and other reductants (Yamasaki et al., 1999; Bethke et al., 2004; Wang and Hargrove, 2013). Moreover, there is *in vitro* evidence that simultaneous exposure of carotenoids to nitrite and light resulted in NO generation (Cooney et al., 1994).

Several lines of evidence suggested that NR activity is the core source for NO signal production by plants in response to both abiotic and biotic stress factors. The induction of an NR-dependent route of NO synthesis was documented during osmotic stress (Kolbert et al., 2010), water stress (Sang et al., 2008), hypoxia (Benamar et al., 2008; Blokhina and Fagerstedt, 2010) as well as a response to pathogen or elicitor treatment (Yamamoto-Katou et al., 2006; Salgado et al., 2010). Importantly, the *Arabidopsis* mutant *nia1nia2* lacking NR and constantly producing less NO was more susceptible to bacterial or fungal pathogens (Modolo et al., 2005, 2006; Perchepied et al., 2010; Rasul et al., 2012). In addition, Shi and Li (2008) using NR-deficient mutants documented that NO synthesis in response to *Verticillium dahliae* toxins is mostly the origin of the NR pathway and the contribution of the NOS-system appeared to be secondary under pathophysiological conditions.

The mutant or transgenic plants having an altered NO production, while being informative for studying NO-related signaling, are usually different from the wild type plants in terms of many physiological and biochemical features. Thus, direct effects due to an impaired NO synthesis are difficult to distinguish from those caused by metabolic alteration (Leitner et al., 2009). NR deficiency leads to impaired nitrogen assimilation and in consequence affects primary and secondary metabolism. As it was indicated by Modolo et al. (2006), the Arabidopsis *nia1nia2* mutant showed reduced levels of nitrites and amino acids (excluding L-Arginine). These findings revealed a significant role of NR in providing the substrates for NO synthesis both through oxidative and reductive pathways (Salgado et al., 2006). What is more, when NR-deficient (*nia1nia2*) mutants were treated with L-Arginine the total amino acid content increased, whereas *Arabidopsis thaliana* plants were unable to potentiate NO emission and induce hypersensitive response (HR) to *P. syringae* (Oliveira et al., 2009). In turn, when nitrite was supplied into NR-deficient leaves, NO synthesis and HR response to *P. syringae* were recovered (Modolo et al., 2006). In general, the available data revealed a direct effect of NR activity on the delivery of substrates for NO synthesis and an indirect effect on the establishment of plant resistance to the pathogen.

In conclusion, almost 20 years of extensive studies evidencing NO as a fundamental signaling molecule in plant organisms have left a considerable information gap concerning the NO synthesis. Although NOS-like and NR activities are supposed to be the two major enzymatic sources of NO production in plants, the nitrite can be converted into NO by NR-independent route as well (Modolo et al., 2005; Chen et al., 2014). For this reason in the presented paper we focused on the reductive pathway for NO generation involving NR activity and nitrite implication in potato defense to *Phytophthora infestans*. In particular, a combined parallel analysis has been conducted concerning both NR and NiR gene expression with protein activities, nitrate reduction with nitrite leaf tissue concentration during avr *P. infestans* response providing NO overproduction. Finally, looking for the link between pathogen challenged NO generation and HR response we compared the expression of different defense markers, i.e. *NPR1*, *Thx* and *PR1*, in resistant and susceptible potato leaves upon inoculation.

2. Materials and methods

2.1. Plant material

Sterile potato plants (*Solanum tuberosum* L.) of cv. Bintje – (lacking *R* genes) highly susceptible to isolate 1.3.4.7.10.11. *Phytophthora infestans* and cv. Bzura – (carrying *R* genes derived from *S. demissum*) highly resistant and incompatible to 1.3.4.7.10.11. *P. infestans* were used in the experiments. Potato plants from *in vitro* tissue culture were transferred to soil and they were grown in a growth chamber with 16 h of light (180 μmol $m^{-2} \cdot s^{-1}$) at 18 ± 2 °C and 60% humidity for 4 weeks.

2.2. Pathogen culture and inoculation with P. infestans

Phytophthora infestans (Mont.) de Bary (1.3.4.7.10.11., isolate MP946), virulent for 'Bintje' and avirulent for 'Bzura', was kindly obtained from the Plant Breeding and Acclimatization Institute, Research Division at Młochów, Poland. Isolate MP946 triggered hypersensitive pointed cell death in the 'Bzura' genotype identified as TUNEL-positive. Potato plants were inoculated by spraying leaves with 5 ml of the oomycete zoospore suspension at a concentration of 2.0 × 10^5 per 1 ml of water and then were kept overnight at 100% relative humidity and 18 °C and afterwards they were transferred to a growth chamber.

2.3. Measurement of nitric oxide generation

The NO-FL fluorescence from extracts of potato leaves challenged with *P. infestans* was assayed spectrofluorimetrically using a selective nitric oxide sensor (CuFL) similar as described Lim et al. (2006). The copper-complex of FL (2-{2-Chloro-6-hydroxy-5-[2-methylquinolin-8-ylamino)methyl]-3-oxo-3H-xanthen-9-l}benzoic acid) was prepared as 1 mM water stock solution according to the manufacturer's instructions (Strem Chemicals). Leaf tissue (500 mg of fresh weight) was homogenized in 2 ml of 10 mM potassium-phosphate buffer (pH 7.0) and centrifuged at 21,000 × g for 30 min at 4 °C. Then, 100 μl of supernatant was used for NO assay by adding CuFL to the final concentration of 2 μM. After 30 min of incubation in darkness at 25 °C the fluorescence intensity was determined with the Fluorescence Spectrometer Perkin Elmer LS50B (UK) equipped with microtiter plates with 96 wells using 488 nm and 516 nm for excitation and emission, respectively. All data (F) are normalized with respect to the emission of CuFL (F_{CuFL}). [CuFL] = 2 μM. Each value was expressed as NO-FL fluorescence intensity [F/F_{CuFL}].

2.4. Inhibition of NO-synthesizing enzymes

To determine NO generation routes, specific inhibitors of reductive and oxidative NO sources were separately added to the incubation medium, i.e. tungstate (TG), vanadium (V), N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and aminoguanidine (AG). To block NR activity, 0,1 mM Na_2WO_4 or 0,1 m $MVOCl_2$ were used in *in vitro* assays. NOS-like routes of NO synthesis were affected by the addition of 5 mM AG or/and 5 mM L-NAME. Additionally, 1 mM carboxyPTIO was taken to scavenge NO in the CuFL-based fluorimetric approach.

2.5. Assays of enzymes activity

2.5.1. Nitrate reductase activity

The activity of NR was determined using a spectrophotometric assay (Corzo and Niell, 1991). Leaf tissue (250 mg of fresh weight, approx. 5 leaf discs) were placed in a 25-ml conical flask in 5 ml of incubation mixture, containing 100 mM phosphate buffer (pH 7.5), 100 mM KNO_3, 2% 1-propanol (v/v). After samples were incubated at 30 °C for 1 h, 1 ml of 1% sulphanilamide in 1 M HCl (v/v) and 1 ml of 0.01% N-(1-naphtyl)ethylenediamine dichloride (v/v) were added. After 15 min of incubation, optical density was measured at 540 nm. Enzyme activity was expressed as the production of nmol $NO_2 \times h^{-1} \times g^{-1}$ FW and was calculated according to the standard curve prepared in the range of 5–30 nM of NO_2.

2.5.2. Nitrite reductase activity

The activity of NiR was determined using a spectrophotometric assay according to Orea et al. (2001). Leaf tissue (250 mg of fresh weight) was ground in liquid nitrogen and homogenized with 2 ml of 100 mM Trs-HCl pH 8.0 containing 1 mM EDTA, 3% BSA, 0,1% Triton-X-100, 1 mM DDT, 1 mM PMSF. Homogenates were centrifuged at 15 000 × *g* for 15 min. Obtained supernatants were used directly for NiR measurements. The assay mixture contained 200 mM Tris-HCl (pH 8.0), 6 mM KNO_2, 20 mM methyl viologen and crude extract (100 μl). The reaction was started by addition of $Na_2S_2O_4$ (150 mg/ml). After samples were incubated at 40 °C for 20 min, 1 ml of 1% sulphanilamide in 1 M HCl (v/v) and 1 ml of 0.01% N-(1-naphtyl)ethylenediamine dichloride (v/v) were added. After 15 min of incubation, optical density was measured at 540 nm. Calculations were made subtracting the sample absorbance from that of the reference. Enzyme activity was expressed as the production of μmol $NO_2 \times h^{-1} \times g^{-1}$ FW.

2.6. Gene expression measurements

The RNA was isolated from 150 mg of frozen leaf tissue using TriReagent® (Sigma) according to the method of Chomczynski and Sacchi (1987). The obtained RNA was purified with the use of a Deoxyribonuclease I Kit (Sigma). For the reverse transcription 1 μl of RNA from every experimental variant was processed with a RevertAid™ Reverse Transcriptase Kit (Thermo Scientific) according to the manufacturer's instructions. Real-time PCR was performed on a Rotor Gene 6000 Thermocycler (Corbett Life Sciences). The reaction mixture contained 0.1 μM of each primer, 1 μl of 5 × diluted cDNA, 10 μl of the Power SYBR® Green PCR Master mix (Applied Biosystems) and DEPC-treated water to the total volume of 20 μl. The real-time PCR reaction conditions included an initial 5-min denaturation at 95 °C, followed by 55 cycles consisting of 10 s at 95 °C, 20 s at 53 °C and 30 s at 72 °C. Primers for genes *(NR, NiR, PR1, NPR1, Thx)* transcript accumulation are presented in the Suppl. Tab. 1. The data were normalized to the reference genes encoding the elongation factor and 18S rRNA. All used primers were designed using Primer-BLAST (Ye et al., 2012). The C_t values were determined with the use of a Real-time PCR Miner (Zhao and Fernald, 2005) and the relative gene expression was calculated with the use of efficiency corrected calculation models presented by Pfaffl (2001).

2.7. Quantification of total nitrate pool

The concentration of nitrate content was determined by the colorimetric assay proposed by Cataldo et al. (1975). Leaf tissue (250 mg of fresh weight) were ground in liquid nitrogen and then homogenized in 2 ml of distilled water. After 15-min thermal denaturation of samples (100 °C), the homogenates were centrifuged at 15,000 × g for 30 min. To the supernatant volume (100 μl), 400 μl of 5% salicylic acid in concentrated H_2SO_4 were added and incubated for 20 min at RT. Further, to raise the pH above 12, 9.5 ml of 2 M NaOH were pipetted into each sample. Optical density was measured at 410 nm (Jasco V-780). Nitrate concentration was calculated according to the standard curve prepared in the range of 5–30 mg $NO_3 \times g^{-1}$ FW.

2.8. Quantification of total nitrite pool

The concentration of nitrite content was determined by chemiluminescence using a Sievers® Nitric Oxide Analyzer NOA 280i (GE Analytical Instruments, USA) according to the procedure proposed by Corpas et al. (2008). The detection of NO_2^- was based on the reductive decomposition of nitroso compounds by an iodine/triiodide mixture at RT. Leaf tissue (250 mg of fresh weight) was homogenized in Tris–HCl 0.1 M buffer pH 7.5 (1:4, w/v) containing 100 μM DTPA, 1 mM EGTA, 1 mM PMSF, 0.1 mM neocuproine, 0.25% (v/v) Triton X-100 and centrifuged at 10,000 × g for 10 min. The supernatants were served for analysis by the injections of 30 μl of supernatant to a purge chamber of the NOA containing 8 ml of glacial acetic acid, 300 μl of antifoam agent (30:1, v:v, Sigma-Aldrich) and 300 μl of 200 μM $CuSO_4$ at RT. Obtained data were calculated according to the standard curve prepared in the range of 0–80 pM of $NaNO_2$.

2.9. Statistical analysis

All results are based on three independent experiments, each with at least three biological replicates. For each experiment, means of the obtained values were calculated along with standard deviations. The analysis of variance was performed (ANOVA) and the mean values were compared by Tukey's test ($\alpha = 0.05$).

3. Results

3.1. NO generation in potato resistance

Using the NO-selective fluorescent probe copper-complex of FL an enhancement of NO generation during 48 hpi was observed in *avr P. infestans* inoculated resistant potato, when compared to the mock inoculated (control) potato leaves (Fig. 1A). A biphasic character of NO emission, peaking mainly at 3 and then at 24 hpi, was detected. However, the first peak of NO burst was much more pronounced than the subsequent one. In contrast, susceptible potato infected with *P. infestans* did not reveal marked changes in the NO status after inoculation (Fig. 1B).

3.2. The effect of inhibitors on NO emission in HR response

In order to recognize the source of NO production at 3 hpi during HR response we applied *in vitro* different inhibitors related to the inactivation of the NO reductive or oxidative pathway,

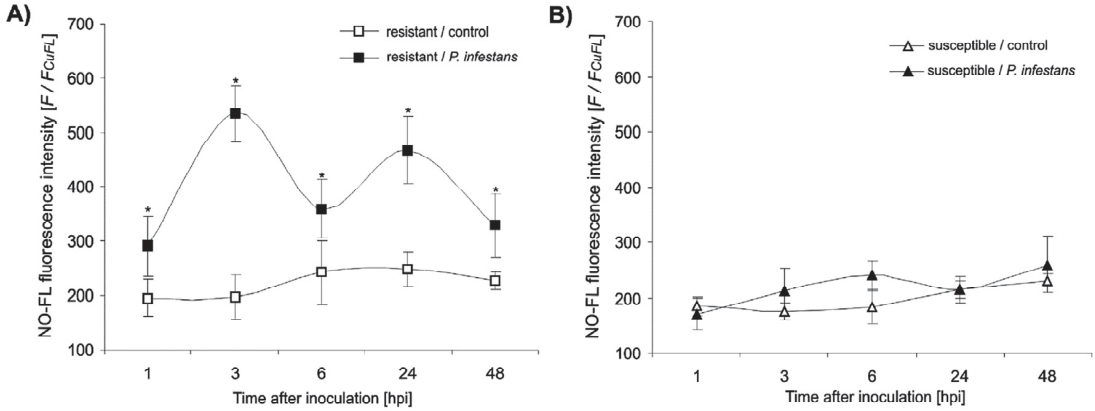


Fig. 1. Nitric oxide generation in potato leaves of resistant 'Bzura' **(A)** and susceptible 'Bintje' **(B)** genotype challenge inoculated with avirulent and virulent isolate of *P. infestans*. The NO-FL fluorescence from extracts of potato leaves challenged with *P. infestans* was determined as described in Materials and methods. All data (F) are normalized with respect to the emission of CuFL (F_{CuFL}). [CuFL] = 2 μM. Each value was expressed as NO-FL fluorescence intensity [F/F_{CuFL}]. * significantly different from mock-inoculated (control) potato leaves, $P < 0.05$. Values represent the average of data ± SD of three independent experiments.

respectively (Fig. 2). To this end, an avr inoculated leaf extract exhibiting NO emission by 66% higher than the control, was independently supplied with the following inhibitors: tungstate (TG), vanadium (V) and aminoguanidine (AG) with L-NAME. NO-Fl fluorescence of the medium was strongly inhibited by tungstate (52%) and vanadium (48%), while the reduction by aminoguanidine with L-NAME was smallest (8%) when compared with the avr inoculated leaf extract without an inhibitor. In turn, 1 mM cPTIO at more than 70% effectively scavenged the NO production.

To further determine the nitrite impact on NO emission, the avr inoculated potato leaf extract (at 3 hpi) was supplied with 50 μM NO_2^- (Fig. 2). The extract supplemented with exogenous nitrite showed an enhanced production of NO, approx. 30% higher than the pathogen augmented without nitrite. In turn, a 17% reduction of NO emission was found when the avr inoculated leaf extract was supplied first with tungstate and then nitrite. Although it is worth noting that an addition of nitrite restored NO production significantly higher than the NO level found in the avr inoculated leaf extract plus tungstate, but deprived of nitrite.

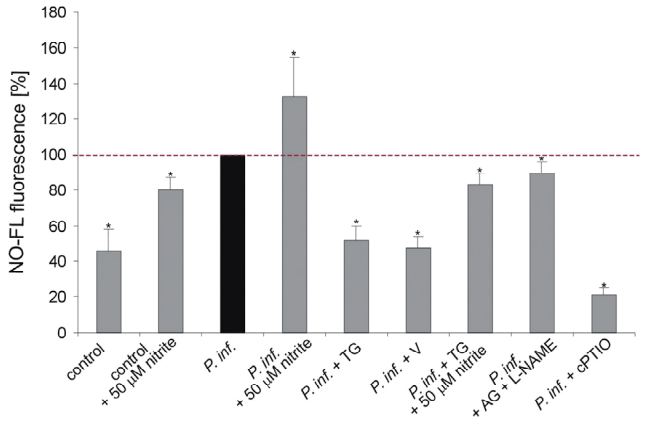


Fig. 2. Nitric oxide generation in non-inoculated and inoculated resistant potato leaves (at 3 hpi) analyzed *in vitro* with the following inhibitors: 0.1 mM TG (Tungstate), 0,1 mM V (Vanadium), 5 mM L-NAME, 5 mM AG (Aminoguanidine) or 1 mM carboxyPTIO, used separately or in combination; NO emission was also monitored in medium supplied with 50 μM NO_2 plus TG or deprived of it. * significantly different from *P. infestans* inoculated potato leaves, $P < 0.05$. Values represent the average of data ± SD of at least three independent experiments.

Both findings strongly suggest that potato leaves were not able to effectively produce NO by the oxidative pathway and most of NO was derived from the reduction of nitrite by NR-dependent or independent routes.

3.3. Combined analysis of NR–dependent route in potato resistance to *P. infestans*

In order to confirm the NO origin in potato defense we explored the activity and gene expression of NR together with the nitrate endogenous pool in leaves of potato cultivars susceptible and resistant to *P. infestans*. When analysing the *NR* gene expression it was found that except for the first rise of *NR* transcript accumulation (2.5-fold) at 3 hpi, elicited only by avr *P. infestans*, the next up-regulation of *NR* (2-fold increase) was commonly noted at 24 hpi in both potato interactions with the oomycete pathogen (Fig. 3A).

In turn, obtained data revealed significant differences in NR activity between incompatible and compatible responses to the pathogen (Fig. 3B, C). The avirulent pathogen enhanced NR activity starting from 1 hpi, with the maximum, *ca.* 2.5-fold increase at 3 hpi, followed by its decrease after 24 hpi. In the compatible interaction NR activity significantly raised during disease development, apart from 24 hpi. It is worth noting that the constitutive level of nitrate assimilation was 2-fold lower in the compatible genotype in relation to the incompatible one.

In potato leaves of the resistant genotype the total nitrate pool rapidly decreased (almost 2-fold) after inoculation (1–3 hpi) and it was significantly lower than in the control leaves until 48 hpi (Fig. 3D). A particularly strong decline in the nitrate level was also found in *P. infestans* inoculated susceptible potato leaves during 1–6 hpi, while in the following hours it increased to the level recorded in the control (Fig. 3E).

Interestingly, an inhibition of *NiR* gene expression was observed during the first 6 hpi in both the compatible and incompatible response after pathogen treatment (Fig. 4A). Only at 24 hpi a substantial up-regulation of the mRNA level for *NiR* was found, more pronounced (4-fold increase) in HR response rather than in the susceptible one (3-fold increase). Nitrite reductase gene expression correlated in time with NiR activity, thus the avr inoculated leaves at 3 and 48 hpi revealed a strong decline in enzyme activities (Fig. 4B), tuned with the highest level (4 and 6-fold increase, respectively) of nitrite contents in the cytosol (Fig. 4D). In turn, in

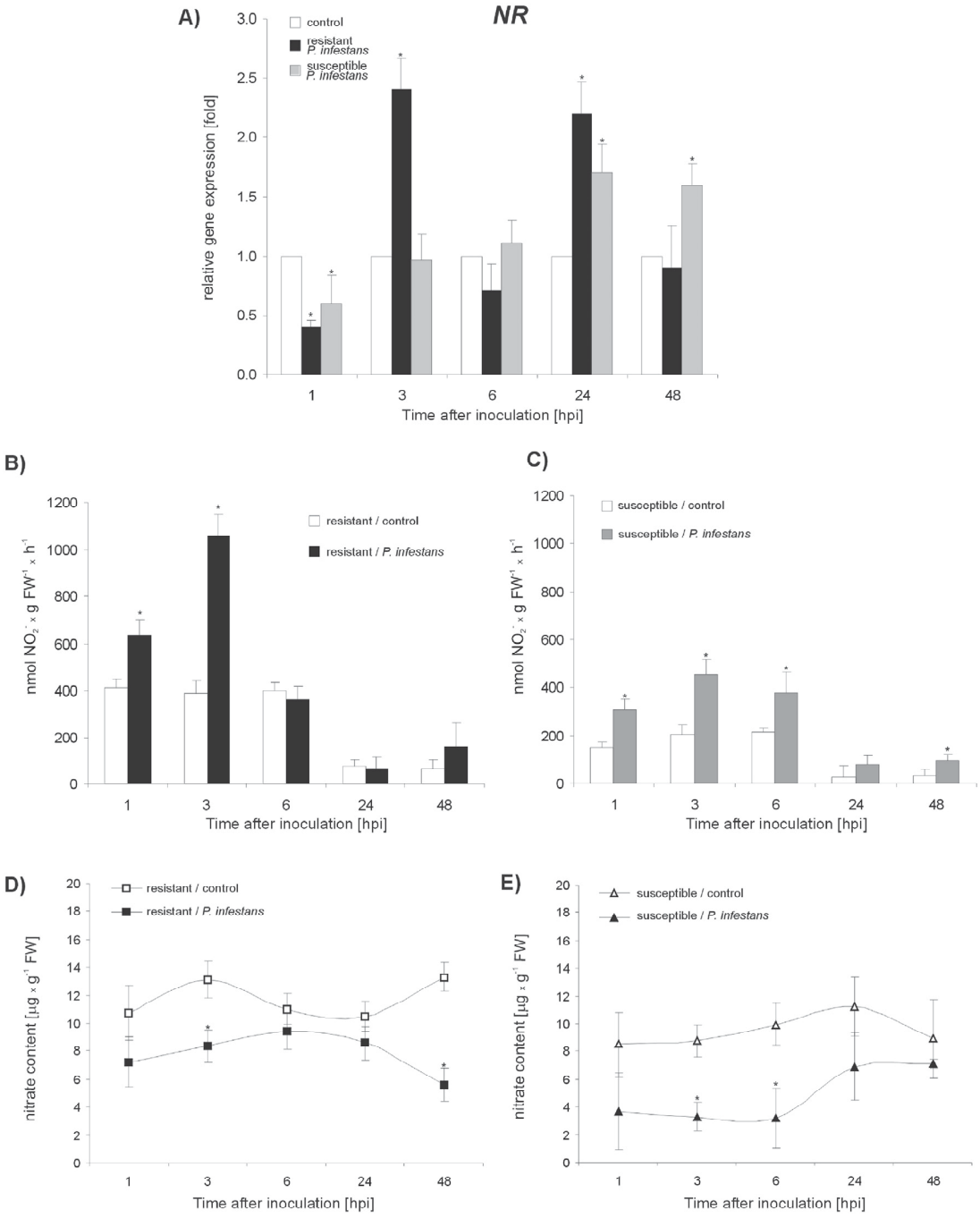


Fig. 3. The effect of *P. infestans* inoculation on nitrate reductase (NR) pathway in potato leaves. NR at the level of gene transcript accumulation in resistant and susceptible genotypes **(A)**; NR at the level of protein activity in resistant **(B)** and susceptible **(C)** genotype; total pool of nitrate in resistant **(D)** and susceptible **(E)** genotype after challenge inoculation. * significantly different from mock-inoculated (control) potato leaves, $P < 0.05$. Values represent the average of data ± SD of three independent experiments.

the susceptible response the pathogen did not affect NiR activity; however, nitrite content increased 2.5-fold at 1 hpi and was higher than in the mock inoculated leaves at the analyzed time points after challenge inoculation (Fig. 4C, E).

3.4. Indirect effect of NO on redox mediated changes tuned with immunity

In the plant-pathogen encounter *NPR1* (Nonexpressor of *PR* genes 1 – a positive co-regulator of resistance) is a key modulator of systemic responses in plants through the effect on *PR1* expression and its involvement in the signaling interaction of SA and jasmonic acid pathways (Koornneef et al., 2008). Thioredoxins (*Thx*) govern the reduction of the NPR1 oligomers release monomers that translocate to the nucleus and indirectly activate the expression of *PR* genes (Tada et al., 2008). Therefore semi-quantitative real-time RT-PCR was performed to analyse *NPR1*, *PR-1*, *Thx* genes expression upon inoculation, in order to better

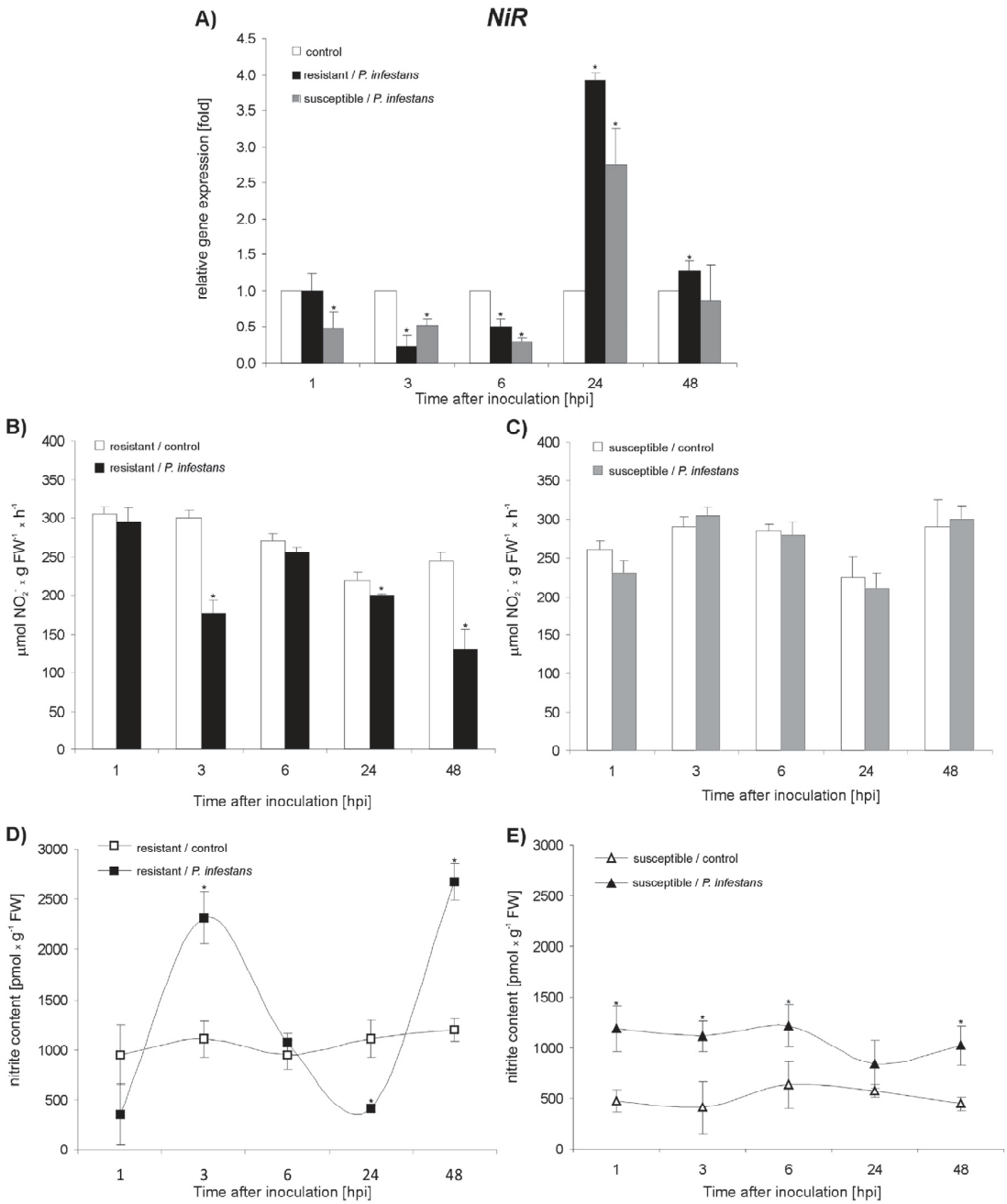


Fig. 4. The effect of *P. infestans* inoculation on nitrite reductase (NiR) pathway in potato leaves. NiR at the level of gene transcript accumulation in resistant and susceptible genotypes **(A)**; NiR at the level of protein activity in resistant **(B)** and susceptible **(C)** genotype; total pool of nitrite in resistant **(D)** and susceptible **(E)** genotype after challenge inoculation.
* significantly different from mock-inoculated (control) potato leaves, $P < 0.05$. Values represent the average of data ± SD of three independent experiments.

clarify how NO affected the SA-signaling defense pathway in potato leaves.

Early (1–3 hpi) up-regulation of *NPR1* tuned with the *PR1* gene expression was found in resistant potato (Fig. 5A, B). In contrast to the resistant response, in the susceptible one a late overexpression (24–48 hpi) of *NPR1* and *PR1* mRNA levels was observed (Fig. 5A, B). Similarly, the above-mentioned key regulators of resistance, *P. infestans* triggered *Thx* gene up-regulation mainly in the HR response from 3 to 24 hpi (Fig. 5C).

4. Discussion

In spite of the growing body of evidence for the important pathophysiological role of NO in plant defense and significant progress made in NO-dependent establishment of pathogen resistance, it is still unclear whether and under which conditions NO is overproduced by nitrite in stress. The finding that potato leaves in the presence of increased nitrite concentrations, tuned with a temporary NiR suppression, showed a boosted NO generation early

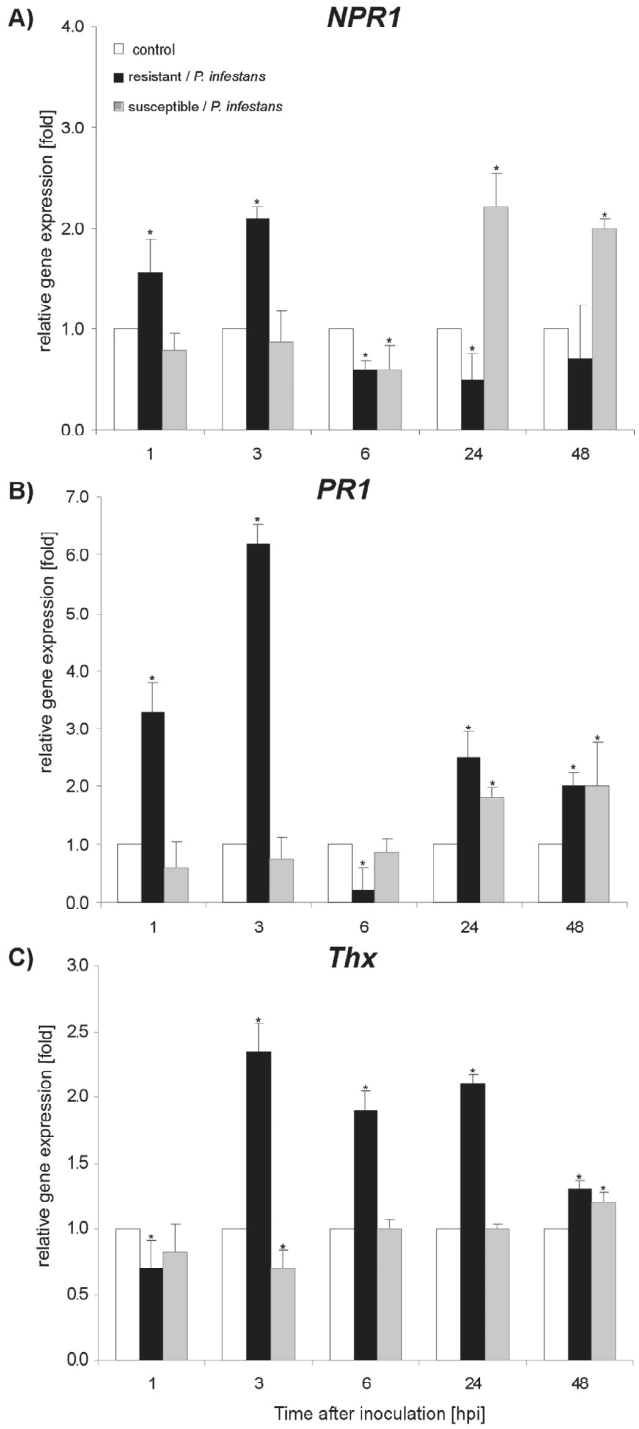


Fig. 5. The effect of *P. infestans* inoculation on gene expression of key regulators of potato immunity. Accumulation of gene transcript for NPR1 (Nonexpressor of *PR* genes 1 – a positive co-regulator of resistance) **(A)**; PR-1 (pathogen-related protein 1) **(B)**; Thx (Thioredoxins) **(C)** in resistant and susceptible genotypes. * significantly different from mock-inoculated (control) potato leaves, $P < 0.05$. Values represent the average of data ± SD of three independent experiments.

after avr *P. infestans* inoculation is new and broadens our pathophysiological knowledge.

As we found, the endogenous NO level in potato leaves displayed a time-dependent increase during HR response. The NO emission was biphasic in character, with an early, strong augmentation at 3 hpi and a weaker one at 24 hpi. However, the first transient phase of NO overproduction seems to be essential in the determination of the resistant response to the avr pathogen.

In tune with this statement, our previous experimental approach with the same genotype of potato leaves, cv. Bzura, exposed to avr and vr *P. infestans* revealed that only in an incompatible response, early NO generation led to peroxynitrite formation and together with hydrogen peroxide production, synchronized first with NADPH oxidase and then with SOD activities, induced effective HR defense responses (Abramowski et al., 2015).

The biphasic pattern of NO overproduction with the first higher wave of NO recorded at 1 hpi and the second weaker at 6–8 hpi was documented in a soybean cell suspension exposed to avr *P. syringae* (Delledonne et al., 1998). Two phases of NO production were also found in wheat and *Arabidopsis* (Kolbert et al., 2008, 2010) under osmotic stress. According to those authors, early stress-induced NO generation might play a significant role in the process of plant acclimation to stress conditions, which was not dependent on NOS-like or NR activity.

To search for NO-sources, we used the extract of avr resistant leaves (at 3 hpi) possessing *in vitro* capacity to release approx. 70% more NO than the control and supplied it with various inhibitors of NO production detected by NO-FL fluorescence. Used inhibitors affected *in vitro* NO emission. The extract treatment with an inhibitor of NR (tungstate or vanadium) or NOS-like associated pathways (L-NAME and aminoguanidine) decreased NO production by approx. 50% and 8%, respectively, in relation to the avr inoculated leaves. On this basis the putative involvement of the arginine derived NO route was considered as an insignificant origin of NO synthesis in potato leaves.

Focusing on the reductive pathway of NO synthesis in potato leaves we first analyzed both the NR transcript abundance and activity. After potato challenge with avr *P. infestans* the observed profile of NR mRNA accumulation temporarily correlated with nitrate assimilation and the emission of NO-associated fluorescence (NO-FL). The enhanced *NR* gene expression and NO burst at the early time point were tuned with a relatively high NR activity at 1–3 hpi in both potato genotypes. However, in the incompatible response an early increase in NR activity was 2-fold greater than in the compatible one. A positive correlation between NR activity and nitrate depletion was also found. *Phytophthora infestans* treatment significantly reduced nitrate concentrations in the two genotypes during the first day after inoculation.

A transient increase in the *NR* transcript and enhanced nitrite-dependent NO production (at 6 hpi) were found in an incompatible, but not a compatible, interaction in potato tubers challenged with *P. infestans* or pathogen hyphal wall components (Yamamoto et al., 2003). Moreover, NO generation (1–3 hpi) in the hypersensitive cell death induced by elicitin was distinctly diminished by the silencing of *NR* genes in *Nicotiana benthamiana* (Yamamoto et al., 2004; Yamamoto-Katou et al., 2006).

Nitrate reductase is a relatively labile protein and even at physiological conditions it may be rapidly degraded through phosphorylation at serine residue to interact with 14-3-3 proteins and undergoes proteolysis (Kaiser and Huber, 2001). Furthermore, NR is up- and down-regulated in response to various treatments and environmental stresses (Lillo et al., 2004), which modify one electron reduction of nitrite to form NO using NAD(P)H as the electron donor (Yamasaki et al., 1999; Rockel et al., 2002). In *Arabidopsis* roots H_2O_2-mediated phosphorylation by MAP kinase 6 activated NR and modified NO production (Wang et al., 2010). The results presented by Rosales et al. (2011) showed that NR activity in wheat leaves was negatively regulated by NO emitted from the NO donors (SNP and GSNO). In turn, conflicting data were found by Du

et al. (2007), who found a positive impact of exogenous NO on NR activity in *Brassica chinensis*.

The emerging evidence suggests that NR seems the only likely source of NO from nitrite with respect to plant defenses. However, it remains an unanswered issue how NR undergoes a regulatory shift from its preferential high-affinity substrate into nitrate to low-affinity substrate into nitrite with the final NO production (Gupta et al., 2011). Currently, it is believed that NR-mediated NO production under biotic stresses requires a high nitrite accumulation with the inhibition of plastidial NiR and a relatively low nitrate concentration (Gupta et al., 2011; Mur et al., 2013). Genetic evidence using antisense NiR tobacco (clone 271) also supports a link between nitrite and NO (Morot-Gaudry-Talarmain et al., 2002). However, in these transformants with a strongly reduced NiR activity, the relative increase in NO generation was much higher (100-fold rise) than the relative increase in the nitrite concentration (10-fold rise). It was postulated that nitrite was not equally distributed among subcellular compartments, and cytosolic nitrite concentration available for NR might differ from the total concentration determined in fresh leaf tissue (Morot-Gaudry et al., 2002).

Evidently, the accumulation of nitrite in cells is a basic condition to provoke NR-dependent NO production. In the presented paper mainly in avr *P. infestans* potato an enhanced NR gene and activity were tuned with the depletion of nitrate content and the transient rise in nitrite accumulation at 3 hpi. At the same time period a temporary down-regulation of *NiR* and protein activity was noted. It seems likely that the early increase in the nitrite pool was tuned with NR-dependent NO production. As it was previously stated, the first transient phase of NO overproduction seems to be pivotal in the onset of the resistant response to the avr pathogen. In turn, the next weaker NO emission (at 24 hpi) was probably not driven by NR activity. The late drastic rise in the total nitrite accumulation at 48 hpi could reflect chloroplast impairment and contribute to the HR-type potato cell death because of the toxic and deleterious nature of nitrites.

In the compatible interaction, despite a rapid 2-fold drop in the concentration of nitrate and a similar increase in nitrite accumulation, post-stress NO production was not observed. The lack of NO emission in susceptible potato was probably due to the fact that the concentration of nitrate, as an NR preferential substrate, drastically diminished in the following hours after being challenged with *vr P. infestans*. Admittedly, NR activity increased (at 1–6 hpi), but similarly as nitrite it only reached a level similar to the control resistant genotype. Moreover, at the same period after inoculation the inhibition of plastidial NiR activity was not found.

The question returns on the regulation and conditions for the reductive pathways leading to NO synthesis, while bearing in mind that probably NR is engaged in NO production only in ~1% or even less of its nitrate-reducing capacity (Rockel et al., 2002; Planchet et al., 2005).

Additionally, nitrite has emerged as an alternative source of NO. Nitric oxide can be non-enzymatically formed from nitrite in the presence of such reductants as ascorbate or reduced glutathione, which was shown both in illuminated algae suspension and in pure nitrite solution with these reductants (Mallick et al., 2000).

Although NR is the most considerable route of NO generation in plants, there is a growing body of evidence that nitrite might be converted into NO by the NR-independent route (Modolo et al., 2005, 2006). Based on experiments using the *nia1 nia2* double mutant deficient in NR with a low NO content and an impaired HR response to avr *Pseudomonas syringae*, it was shown that supplementing this mutant with nitrite restored NO production against avirulent bacteria. A slightly improved NO emission after the delivery of nitrite to the *nia1 nia2* mutant was also found in response to *Pst AvrB* using a chemiluminescence detector (Chen et al., 2014).

Notably, it has been shown here that the avr leaf extract (at 3 hpi) supplemented *in vitro* with 50 μM nitrite emitted over 30% more NO than the avr infected one. Moreover, in the same experimental approach the nitrite treatment mitigated the inhibitory effect of tungstate as well.

Relatively little is known about nitrite being a transient intermediate in the nitrate assimilation and the precursor to NO production. The nitrite-dependent NO synthesis has been implicated in ABA-induced stomatal closure (Desican et al., 2004; Bright et al., 2006). Using the transcriptome approach Wang et al. (2007) documented that nitrite can serve as a signal to control the expression of almost 900 genes equally well or even better than nitrate. Interestingly, their experimental data revealed a strong down-regulation of the *NIA1* mRNA level after 2 h of 5 or 250 μM nitrite treatments. Previously published data documented an even longer repression of *NIA1* mRNA accumulation after 6 and 24 h of nitrite treatment in *Arabidopsis* roots (Loque et al., 2003).

In Arabidopsis, NR is encoded by two genes: *NIA1* responsible for 10% of NR activity and *NIA2* accounted for 90% of the total NR activity (Wilkinson and Crawford, 1993), but *NIA1* is mainly responsible for NO production in ABA-stimulated guard cells (Bright et al., 2006) and in plant cold acclimation and freezing tolerance (Zhao et al., 2009). Southern analysis documented the presence of at least two *NR* genes (*StNR5* and *StNR6*) in potato tubers (cv. Rishiri) genome (Yamamoto et al., 2003). However both *StNR5* and *StNR6* transcripts were induced and their levels were similar after the *P. infestans* elicitor - hyphal wall components (HWC) treatment for 6 h.

We revealed that *avr P. infestans* early triggered *NR* in potato leaves while increasing nitrite concentrations to the extent that nitrite reduction cannot keep up due to *NiR* temporary suppression and this would enhance NO production. However, a further decrease in nitrite concentrations could cause a decline of NO generation (from 3 hpi).

In turn, less efficient NR activity with a constant low supply of nitrate led to a slow rate of two-step nitrate reduction without NO generation in a compatible interaction of potato leaves to vr *P. infestans*. It seems difficult to assume the maintenance of a high nitrite concentration, because the virulent pathogen did not suppress NiR activity, however, gene expression for *NiR* was down-regulated (1–6 hpi). Yet another possibility of a limited NO production managed by NR might be connected with the cytosolic NADH, which appears to be the major limiting factor apart from the nitrate/nitrite supply in infected leaves of susceptible potato.

What is noteworthy, recently we presented that early NO generation positively correlated in time with the enhanced content of S-nitrosothiols (SNOs) which was governed by GSNO reductase (GSNOR) activity in the response of potato cv. Bzura to avr *P. infestans* (Abramowski et al., 2015). Thereby, most of SNOs, e.g. GSNO or other NO-related species, should be taken into account as sources capable of mobilizing the stress-induced NO as well. Probably basal or constitutive NO production in plants exists and differs substantially from the post-stress production with respect to the NO source and its capacity to generate NO. According to many authors, early stress-induced NO generation can play a significant role in the process of plant resistance or acclimation to stress conditions (e.g. Yu et al., 2014; Simontacchi et al., 2015).

Our next findings provided evidence that the first transient phase of NO overproduction is pivotal in triggering defense responses to avr *P. infestans*. First hours after inoculation in potato responses are likely to be important for resistance, because *P. infestans* as a hemibiotrophic pathogen is in its early biotrophic phase at approx. 22 hpi in the compatible interaction, next it enters its destructive necrotrophic phase at approx. 46 hpi (Vleeshouwers et al., 2000). This is confirmed by the very early induction (at 1 hpi)

of two key transcription factors (TDF10 and TDF11), showing similarity to the molecular sensor of ROS and the WRKY to regulate the expression of PR1, PR2, PR3 and PR5 proteins, found in the incompatible interaction between potato leaves and *P. infestans* (Orłowska et al., 2012).

A positive interconnection between NO synthesis from substrates provided by NR and resistance to pathogens has been presented using various experimental approaches (Salgado et al., 2010). In our experimental proposal looking for the link between NO signaling and HR response we found that the used markers of effective defense were up-regulated, i.e. *NPR1, Thx* and *PR1* at early time-points (1–3 hpi) upon inoculation. In contrast to the resistant response, in the susceptible one a late overexpression (24–48 hpi) of *NPR1* and *PR1* mRNA levels was observed. Probably, effective defenses were stimulated too late and were too weak to protect the susceptible potato against *vr P. infestans*.

Summing up, our attempt to identify NO sources in potato resistance revealed that potato leaves reduced mainly nitrite to NO by both NR-dependent and additionally by NR-independent routes. Even though the aspect of non-enzymatic NO generation from nitrite is rather speculative, the basic outline for endogenous NO synthesis *via* NR was documented step by step in potato leaves immunity to *P. infestans*. In the future an integrated genetic and pharmacological approach will be needed to investigate in more detail how the host plant attack by *P. infestans* governs the drawback of nitrite reduction to NO production bypassing the NR activity.

Author contribution

JFW provided the idea, planned and supervised the research work, interpreted the results, wrote the manuscript. MAJ planned and carried out the experiments, participated in results interpretation and writing the manuscript. KI carried out the experiments concerning *NR* and *NiR* gene expression measurement. All authors have read and approved the manuscript.

Acknowledgements

The authors are greatly thankful to dr D. Abramowski for his help in nitrite determination.

This work was supported by the National Science Centre – grant no. NCN 2011/01/B/NZ9/00243.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.08.009.

References

Abramowski, D., Arasimowicz-Jelonek, M., Izbianska, K., Billert, H., Floryszak-Wieczorek, J., 2015. Nitric oxide modulates redox-mediated defense in potato challenged with *Phytophthora infestans*. Eur. J. Plant Pathol. 143, 237–260.

Benamar, A., Rolletschek, H., Borisjuk, L., Avelange-Macherel, M.H., Curien, G., Mostefai, H.A., Andriantsitohaina, R., Macherel, D., 2008. Nitrite-nitric oxide control of mitochondrial respiration at the frontier of anoxia. Biochim. Biophys. Acta 1777, 1268–1275.

Bethke, P.C., Badger, M.R., Jones, R.L., 2004. Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell 16, 332–341.

Blokhina, O., Fagerstedt, K.V., 2010. Oxidative metabolism, ROS and NO under oxygen deprivation. Plant Physiol. Biochem. 48, 359–373.

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., Neill, S.J., 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J. 45, 113–122.

Castello, P.R., David, P.S., McClure, T., Crook, Z., Poyton, R.O., 2006. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. Cell Metab. 3, 277–287.

Cataldo, D.A., Maroon, M., Schrader, L.E., Youngs, V.L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6, 71–80.

Chen, J., Xiao, Q., Wang, C., Wang, W.H., Wu, F.H., Chen, J., He, B.Y., Zhu, Z., Ru, Q.M., Zhang, L.L., Zheng, H.L., 2014. Nitric oxide alleviates oxidative stress caused by salt in leaves of a mangrove species, Aegiceras Corniculatum. Aquat. Bot. 117, 41–47.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Cooney, R.V., Harwood, P.J., Custer, L.J., Franke, A.A., 1994. Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. Environ. Health Perspect. 102, 460–462.

Corpas, F.J., Carreras, A., Esteban, F.J., Chaki, M., Valderrama, R., del Rio, L.A., Barroso, J.B., 2008. Localization of S-nitrosothiols and assay of nitric oxide synthase and S-nitrosoglutathione reductase activity in plants. Methods Enzymol. 437, 561–574.

Corzo, A., Niell, F.X., 1991. Determination of nitrate reductase activity in *Ulva rigida* C. Agardh by the in situ method. J. Exp. Mar. Biol. Ecol. 146, 181–191.

Cueto, M., Hernandez-Perera, O., Martin, R., Bentura, M.L., Rodrigo, J., Lamas, S., Golvano, M.P., 1996. Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. FEBS Lett. 398, 159–164.

Delledonne, M., Xia, Y., Dixon, R.A., Lamb, C., 1998. Nitric oxide functions as a signal in plant disease resistance. Nature 394, 585–588.

Desican, R., Cheung, M.K., Bright, J., Henson, D., Hancock, J.T., Neill, S.J., 2004. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J. Exp. Bot. 55, 205–212.

Du, S., Zhang, Y., Lin, X., Wang, Y., Tang, C., 2007. Regulation of nitrate reductase by nitric oxide in Chinese cabbage pakchoi (*Brassica chinensis* L.). Plant Cell Environ. 45, 134–141.

Foissner, I., Wendehenne, D., Langebartels, C., Durner, J., 2000. *In vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J. 23, 817–824.

Foresi, N., Correa-Aragunde, N., Parisi, G., Calo, G., Salerno, G., Lamattina, L., 2010. Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. Plant Cell 22, 3816–3830.

Gupta, K.J., Igamberdiev, A.U., 2011. The anoxic mitochondrion as nitrite: NO reductase. Mitochondrion 11, 537–543.

Gupta, K.J., Fernie, A.R., Kaiser, W.M., van Dongen, J.T., 2011. On the origins of nitric oxide. Trends Plant Sci. 16, 160–168.

Hancock, J.T., 2012. NO synthase? Generation of nitric oxide in plants. Period. Biol. 114 (1), 19–24.

Igamberdiev, A.U., Bykova, N.V., Shah, J.K., Hill, R.D., 2010. Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. Physiol. Plant. 138, 393–404.

Jeandroz, S., Wipf, D., Stuehr, D.J., Lamattina, L., Melkonian, M., Tian, Z., Zhu, Y., Carpenter, E.J., Wong, G.K.S., Wendehenne, D., 2016. Occurrence, structure, and evolution of nitric oxide synthase–like proteins in the plant kingdom. Sci. Signal. 9 (417) re2.

Kaiser, W.M., Huber, S.C., 2001. Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. J. Exp. Bot. 52, 1981–1989.

Kolbert, Z., Sahin, N., Erdei, L., 2008. Early nitric oxide (NO) responses to osmotic stress in pea, *Arabidopsis* and wheat. Acta Biol. Szeged 52, 63–65.

Kolbert, Z., Ortega, L., Erdei, L., 2010. Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of *Arabidopsis thaliana* L. roots. J. Plant Physiol. 167, 77–80.

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Otter, Den, Floor, C., Van Loon, L.C., Pieterse, Corné, M.J., 2008. Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant Physiol. 147, 1358–1368.

Leitner, M., Vandelle, E., Gaupels, F., Bellin, D., Delledonne, M., 2009. NO signals in the haze: nitric oxide signalling in plant defence. Curr. Opin. Plant Biol. 12, 451–458.

Lillo, C., Meyer, C., Lea, U.S., Provan, F., Oltedal, S., 2004. Mechanism and importance of post-translational regulation of nitrate reductase. J. Exp. Bot. 55, 1275–1282.

Lim, M.H., Xu, D., Lippard, S.J., 2006. Visualization of nitric oxide in living cells by a copper-based fluorescent probe. Nat. Chem. Biol. 2, 375–380.

Loque, D., Tillard, P., Gojon, A., Lepetit, M., 2003. Gene expression of the NO_3^- transporter NRT1.1 and the nitrate reductase NIA1 is repressed in Arabidopsis roots by NO_2^-, the product of NO_3^- reduction. Plant Physiol. 132, 958–967.

Mallick, N., Mohn, F.H., Rai, L.C., Soeder, C.J., 2000. Evidence for the non-involvement of nitric oxide synthase in nitric oxide production by the green alga Scenedesmus obliquus. J. Plant Physiol. 156, 423–426.

Modolo, L.V., Augusto, O., Almeida, I.M.G., Magalhaes, J.R., Salgado, I., 2005. Nitrite as the major source of nitric oxide production by *Arabidopsis thaliana* in response to *Pseudomonas syringae*. FEBS Lett. 579, 3814–3820.

Modolo, L.V., Augusto, O., Almeida, I.M.G., Pinto-Maglio, C.A.F., Oliveira, H.C., Seligman, K., Salgado, I., 2006. Decreased arginine and nitrite levels in nitrate reductase-deficient *Arabidopsis thaliana* plants impair nitric oxide synthesis and the hypersensitive response to *Pseudomonas syringae*. Plant Sci. 171, 34–40.

Morot-Gaudry-Talarmain, Y., Rockel, P., Moureaux, T., Quilleré, I., Leydecker, M.T., Kaiser, W.M., Morot-Gaudry, J.F., 2002. Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. Planta 215, 708–715.

Mur, L.A.J., Mandon, J., Persijn, S., Cristescu, S.M., Moshkov, I.E., Novikova, G.V., Hall, M.A., Harren, F.J., Hebelstrup, K.H., Gupta, K.J., 2013. Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plants. http://dx.doi.org/10.1093/aobpla/pls052.
Oliveira, H.C., Justino, G.C., Sodek, L., Salgado, I., 2009. Amino acid recovery does not prevent susceptibility to Pseudomonas syringae in nitrate reductase double-deficient Arabidopsis thaliana plants. Plant Sci. 176, 105–111.
Orea, A., Pajuelo, P., Pajuelo, E., Márquez, A.J., Romero, J.M., 2001. Characterisation and expression studies of a root cDNA encoding for ferredoxin–nitrite reductase from *Lotus japonicus*. Physiol. Plant 113, 193–202.
Orłowska, E., Fiil, A., Kirk, H.G., Llorente, B., Cvitanich, C., 2012. Differential gene induction in resistant and susceptible potato cultivars at early stages of infection by *Phytophthora infestans*. Plant Cell Rep. 31, 187–203.
Perchepied, L., Balagué, C., Riou, C., Claudel-Renard, C., Rivière, N., Grezes-Besset, B., Roby, D., 2010. Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. Mol. Plant Microbe Interact. 23, 846–860.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 2004–2007.
Planchet, E., Kaiser, W.M., 2006. Nitric oxide (NO) detection by DAF fluorescence and chemiluminescence: a comparison using abiotic and biotic NO sources. J. Exp. Bot. 57, 3043–3055.
Planchet, E., Gupta, K.J., Sonoda, M., Kaiser, W.M., 2005. Nitric oxide emission from tobacco leaves and cell suspensions: rate-limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J. 41, 732–743.
Rasul, S., Wendehenne, D., Jeandroz, S., 2012. Study of oligogalacturonides-triggered Nitric Oxide (NO) production provokes new questioning about the origin of NO biosynthesis in plants. Plant Signal. Behav. 7, 1031–1033.
Ribeiro, E.A., Cunha, F.Q., Tamashiro, W.M.S.C., Martins, I.S., 1999. Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Lett. 445, 283–286.
Rockel, P., Strube, F., Rockel, A., Wildt, J., Kaiser, W.M., 2002. Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. J. Exp. Bot. 53, 103–110.
Rosales, E.P., Iannone, M.F., Groppa, M.D., Benavides, M.P., 2011. Nitric oxide inhibits nitrate reductase activity in wheat leaves. Plant Physiol. Biochem. 49, 124–130.
Rőszer, T., 2014. Biosynthesis of nitric oxide in plants. In: Khan, M.N., Mobin, M., Mohammad, F., Corpas, F.J. (Eds.), Nitric Oxide in Plants: Metabolism and Role in Stress Physiology. Springer International Publishing, Switzerland, pp. 17–32.
Rumer, S., Gupta, K.J., Kaiser, W.M., 2009. Plant cells oxidize hydroxylamines to NO. J. Exp. Bot. 60, 2065–2072.
Salgado, I., Modolo, L.V., Augusto, O., Braga, M.R., Oliveira, H.C., 2006. Mitochondrial nitric oxide synthesis during plant-pathogen interactions: role of nitrate reductase in providing substrates. In: Lamattina, L., Polacco, J.C. (Eds.), Nitric Oxide in Plant Growth, Development and Stress Physiology. Springer Verlag, Berlin, pp. 239–254.
Salgado, I., Oliveira, H.C., Braga, M.R., 2010. Nitrate reductase-deficient plants: a model to study nitric oxide production and signaling in plant defense response to pathogen attack. In: Hayat, S., Mori, M., Pichtel, J., Ahmad, I. (Eds.), Nitric Oxide in Plant Physiology. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp. 89–101.
Sang, J., Jiang, M., Lin, F., Xu, S., Zhang, A., Tan, M., 2008. Nitric oxide reduces hydrogen peroxide accumulation involved in water stress-induced subcellular antioxidant defense in maize plants. J. Integr. Plant Biol. 50, 231–243.
Shi, F.M., Li, Y.Z., 2008. *Verticillium dahliae* toxins-induced nitric oxide production in *Arabidopsis* is major dependent on nitrate reductase. BMB Rep. 41, 79–85.
Simontacchi, M., Galatro, A., Ramos-Artuso, F., Santa-María, G.E., 2015. Plant survival in a changing environment: the role of nitric oxide in plant responses to abiotic stress. Front. Plant Sci. 6, 977.
Stöhr, C., Strule, F., Marx, G., Ullrich, W.R., Rockel, P., 2001. A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. Planta 212, 835–841.
Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., Dong, X., 2008. Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science 321, 952–956.
Tun, N.N., Holk, A., Scherer, G.F.E., 2001. Rapid increase of NO release in plant cell cultures induced by cytokinin. FEBS Lett. 509, 174–176.
Tun, N.N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Floh, E.I., Scherer, G.F., 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in Arabidopsis thaliana seedlings. Plant Cell Physiol. 47, 346–354.
Vleeshouwers, V.G., van Dooijeweert, W., Govers, F., Kamoun, S., Colon, L.T., 2000. The hypersensitive response is associated with host and nonhost resistance to Phytophthora infestans. Planta 210, 853–864.
Wang, R., Xing, X., Crawford, N., 2007. Nitrite acts as a transcriptome signal at micromolar concentrations in *Arabidopsis* roots. Plant Physiol. 145, 1735–1745.
Wang, P., Du, Y., Li, Y., Ren, D., Song, C.P., 2010. Hydrogen peroxide-mediated activation of MAP kinase 6 modulates nitric oxide biosynthesis and signal transduction in *Arabidopsis*. Plant Cell 22, 2981–2998.
Wang, X., Hargrove, M.S., 2013. Nitric oxide in plants: the roles of ascorbate and hemoglobin. Plos One 8, e82611.
Weidert, E.R., Schoenborn, S.O., Cantu-Medellin, N., Choughule, K.V., Jones, J.P., Kelley, E.E., 2014. Inhibition of xanthine oxidase by the aldehyde oxidase inhibitor raloxifene: implications for identifying molybdopterin nitrite reductases. Nitric Oxide 37, 41–45.
Wilkinson, J.Q., Crawford, N.M., 1993. Identification and characterization of a chlorate-resistant mutant of Arabidopsis thaliana with mutations in both nitrate reducatse structural genes NIA1 and NIA2. Mol. Gen. Genet. 239, 289–297.
Wimalasekera, R., Villar, C., Begum, T., Scherer, G.F., 2011. Copper amine oxidase1 (CuAO1) of *Arabidopsis thaliana* contributes to abscisic acid- and polyamine-induced nitric oxide biosynthesis and abscisic acid signal transduction. Mol. Plant 4, 663–678.
Yamamoto, A., Katou, S., Yoshioka, H., Doke, N., Kawakita, K., 2003. Nitrate reductase, a nitric oxide producing enzyme: induction by pathogen signals. J. Gen. Plant Pathol. 69, 218–229.
Yamamoto, A., Katou, S., Yoshioka, H., Doke, N., Kawakita, K., 2004. Involvement of nitric oxide generation in hypersensitive cell death induced by elicitin in tobacco cell suspension culture. J. Gen. Plant Pathol. 70, 85–92.
Yamamoto-Katou, A., Katou, S., Yoshioka, H., Doke, N., Kawakita, K., 2006. Nitrate reductase is responsible for elicitin-induced nitric oxide production in *Nicotiana benthamiana*. Plant Cell Physiol. 47, 726–735.
Yamasaki, H., Sakihama, Y., Takahashi, S., 1999. An alternative pathway for nitric oxide production in plants: new features of an old enzyme. Trends Plant Sci. 4, 128–129.
Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinforma. 13, 134.
Yu, M., Lamattina, L., Spoel, S.H., Loake, G.J., 2014. Nitric oxide function in plant biology: a redox cue in deconvolution. New Phytol. 202, 1142–1156.
Zhao, S., Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comput. Biol. 12, 1047–1064.
Zhao, M.G., Chen, L., Zhang, L.L., Zhang, W.H., 2009. Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in *Arabidopsis*. Plant Physiol. 151, 755–767.

Appendix A. Supplementary data

(http://dx.doi.org/10.1016/j.plaphy.2016.08.009)

Table S1. Sequences of primers used for the real-time PCR reaction.

Gene accession number in NCBI database	Gene	Primers
U76701.1	*NR*	Forward: ACGTCTCACCGGTAAACACC Reverse: GAAGTGGGACTGGGGTGATG
FN691930.1	*NiR*	Forward: AGAAAGCCGTTCCGTGTGAT Reverse: TCGTGGAACTGCACCAAAGT
JX428804.1	*Thx*	Forward: TGATTCGAACACCATCCTGA Reverse: CAGAGGGAGGAGGTGAACAA
AY615281.1	*NPR1*	Forward: TGGAGCCTACGCTCTTCATT Reverse: TGATTGAGGGTTCCTTACGC
NM_001247429.1	*PR1*	Forward: GAGCTGGGGACTGCAGGATGC Reverse: CCGCGTTGAGCTGGGGGAAA
X67238	*18S RNA*	Forward: GGGCATTCGTATTTCATAGTCAGAG Reverse: CGGTTCTTGATTAATGAAAACATCCT
AB061263	*ef1α*	Forward: ATTGGAAACGGATATGCTCCA Reverse: TCCTTACCTGAACGCCTGTCA

Publikacja 2.

Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Izbiańska K., Gzyl J., Jelonek T. (2016) Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*. *Plant Pathology,* 65: 754–766 (IF=2,383; 35 pkt MNiSW).

Plant Pathology (2016) **65**, 754–766 Doi: 10.1111/ppa.12471

Implication of peroxynitrite in defence responses of potato to *Phytophthora infestans*

M. Arasimowicz-Jelonek[a]*, J. Floryszak-Wieczorek[b], K. Izbiańska[a], J. Gzyl[a] and T. Jelonek[c]

[a]*Department of Plant Ecophysiology, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznan;* [b]*Department of Plant Physiology, Poznan University of Life Sciences, Wołynska 35, 60-637 Poznan; and* [c]*Department of Forest Utilization, Poznan University of Life Sciences, Wojska Polskiego 71A, 60-625 Poznan, Poland*

In this study peroxynitrite ($ONOO^-$) is proposed as an important player in defence responses during the interaction of potato (*Solanum tuberosum*) and the oomycete pathogen *Phytophthora infestans*. The potato–*avr P. infestans* model system exhibited a transient programme of boosted $ONOO^-$ formation correlated in time with the burst of nitric oxide (NO) and superoxide during the first 6 h post-inoculation (hpi). The early $ONOO^-$ over-accumulation was not accompanied by *TPx* gene expression. In contrast, the compatible interaction revealed a 24 h delay of $ONOO^-$ formation; however, an enhanced level of NO and superoxide correlated with *TPx* up-regulation was recorded within the earlier stages of pathogen infection. Peroxynitrite over-accumulation in the susceptible potato coincided with an enhanced level of protein tyrosine nitration starting from 24 hpi. Surprisingly, the nitroproteome profile of the resistant potato did not show any visible difference after inoculation, apart from one band containing subtilisin-like protease-like proteins, which appeared 48 h after pathogen attack. An additional pharmacological approach showed that treatment of the susceptible genotype with $ONOO^-$ followed by inoculation with *P. infestans* contributed to slowing down of the colonization of host tissues by the pathogen via a faster and stronger up-regulation of the key defence markers, including the *PR-1* gene. Taken together, the results obtained indicate that a precise control of emitted NO and superoxide in cooperation with thioredoxin-dependent redox sensors in sites of pathogen ingress could generate a sufficient threshold of $ONOO^-$, triggering defence responses.

Keywords: peroxynitrite, *Phytophthora infestans*, plant defence, potato, protein tyrosine nitration, reactive nitrogen species

Introduction

Recent advances in understanding of plant defence signalling pathways have shown that plants are capable of recognizing the type of invaders, and they tightly regulate complex defence mechanisms in order to fend off pathogens. Nitric oxide (NO) has been identified as a crucial signalling molecule effective in triggering plant responses against a broad range of pathogens during both pathogen-associated molecular patterns-triggered immunity and a highly specific effector-triggered immunity (Schlicht & Kombrink, 2013). This gaseous free radical is generated by plants as a local NO burst (the NO hotspots type) during the first minutes following pathogen challenge, stimulating a further sequence of defence events (Floryszak-Wieczorek *et al.*, 2007). The burst of NO, occurring rapidly after pathogen recognition in synergy with reactive oxygen species (ROS), may lead to peroxynitrite ($ONOO^-$) formation, which may function as an NO-dependent signal via protein modification by tyrosine residue nitration (Arasimowicz-Jelonek & Floryszak-Wieczorek, 2011; Vandelle & Delledonne, 2011).

From the chemical point of view, $ONOO^-$ is a reactive nitrogen species (RNS), a product of the rapid and diffusion-controlled reaction between two radicals, i.e. NO and superoxide anion (O_2^-). Within the physiological milieu $ONOO^-$ is a relatively short-lived molecule, which may readily migrate through biological membranes and interact with biotargets in the surrounding cells within the radius of one or two cells (~5–20 μm) (Szabó *et al.*, 2007). $ONOO^-$ generation seems to be spatially associated with sources of the superoxide anion, which is much shorter-lived than the NO molecule (Szabó *et al.*, 2007). It was recently documented by Corpas & Barroso (2014) in the *Arabidopsis* model that peroxisomes are endogenous sources of $ONOO^-$ in root and guard cells, where the compound is over-produced under heavy metal stress. Due to its chemical nature, $ONOO^-$ is an important biological oxidant and nitrated compound, contributing to oxidative and nitrosative stress in living cells (Arasimowicz-Jelonek & Floryszak-Wieczorek, 2011). However, the tyrosine nitration phenomenon, which has been assumed to be a reliable marker of nitro-oxidative stress, may be provoked by other cellular nitrating agents. The fate of $ONOO^-$

*E-mail: marasimowicz@wp.pl

Published online 30 October 2015

Plant Pathology

formation and action in plant cells seems to be rather different than its well-recognized equivalent in animals. First, in contrast to animal cells, $ONOO^-$ itself seems to be less destructive for plants (Delledonne *et al.*, 2001). It has been documented that $ONOO^-$ is not an essential mediator of NO/ROS induced cell death in plants (Delledonne *et al.*, 2001). Secondly, accumulating data indicate that $ONOO^-$ might be continuously formed in healthy tissues as an inevitable event of plant cell metabolism, because plant cells are adapted to detoxify its excess by a broad range of both nonenzymatic and enzymatic mechanisms including thiol-dependent peroxidases. The *Arabidopsis* peroxiredoxin IIE (PrxII E) and poplar glutathione peroxidase 5 (Gpx5) were both able to reduce $ONOO^-$ to NO^-_2 (Romero-Puertas *et al.*, 2008; Ferrer-Sueta & Radi, 2009). The enzymes were then reactivated by thioredoxin in an NADPH-consuming manner (Gross *et al.*, 2013). A similar function as that proposed for *Arabidopsis* PrxII E could also apply to 2-CysPrx (Dietz, 2011).

Although the first reports speculating on the presence of $ONOO^-$ in plants had implicated $ONOO^-$ in the hypersensitive response (HR; Alamillo & García-Olmedo, 2001), the *in vivo* formation of this RNS during biotic interactions has been demonstrated only in *Arabidopsis thaliana* challenged with *Pseudomonas syringae* pv. *tomato* (Gaupels *et al.*, 2011) and tobacco cells treated with the elicitor INF1 secreted by *Phytophthora infestans* (Saito *et al.*, 2006).

Phytophthora infestans is an oomycete pathogen causing late blight disease in solanaceous plants, particularly in potato, tomato and their wild relatives. The pathogen is a major threat to sustainable food production worldwide, as control measures and crop losses due to the disease reach multibillion-dollar amounts (Ali *et al.*, 2014). Therefore, understanding the molecular basis of resistance and susceptibility to late blight is highly relevant for breeding for disease resistance.

Although it is strongly suggested that balanced levels of ROS and RNS are required for plant immune responses, the fate of $ONOO^-$ during these events is still ambiguous. The present study focuses on the analysis of the intensity and kinetics of $ONOO^-$ formation and assessment of $ONOO^-$ importance in triggering defence responses in potato (*Solanum tuberosum*) leaves challenged by the hemibiotroph *P. infestans*.

Materials and methods

Plant materials and growth conditions

Solanum tuberosum 'Bintje' and 'Sarpo Mira' were used in the experiments. Disease-free plants of both cultivars derived from *in vitro* tissue culture were transferred to soil and grown in a growth chamber with 16 h of light (180 μmol m^{-2} s^{-1}) at 18 ± 2°C and 60% humidity for 4 weeks.

Pathogen culture

Phytophthora infestans 1.3.4.7.10.11 (MP 946) isolate virulent to Bintje and avirulent (causing hypersensitive reaction) to Sarpo Mira, was kindly supplied by the Plant Breeding and Acclimatization Institute (IHAR), Research Division at Młochów, Poland. The pathogen was grown on a cereal–potato medium with an addition of dextrose. A zoospore suspension of *P. infestans* was prepared as described by Floryszak-Wieczorek *et al.* (2007).

Plant challenge inoculation with *P. infestans*

For *P. infestans* infection, 10 leaves of each potato cultivar detached from 10 plants (i.e. one leaf per plant) were inoculated by spraying the abaxial surface of the leaves with a zoospore suspension in water (10^5 zoospores mL^{-1}). Leaves were then maintained at 100% humidity in a growth chamber until 72 h post-inoculation (hpi).

Peroxynitrite donor and scavenger treatment

To evaluate the effect of exogenous $ONOO^-$ on disease development, *PR* and *TPx* gene expression, potato leaves were sprayed with the $ONOO^-$ donor, 50 μM 3-morpholinosydnonimine (SIN-1; Calbiochem), which gradually decomposed to yield equimolar amounts of NO and O_2^-, and the $ONOO^-$ scavenger, 50 μM ebselen (Sigma). Control plants were treated with water. After 5 h of incubation leaves were gently dried and inoculated as described above.

Assessment of disease development

Disease development on potato leaves was assessed on the third day after inoculation by visually estimating the percentage of leaf area covered by late blight symptoms. Values presented in the results are means from 30 leaves (10 leaves from each of three independent experiments).

Peroxynitrite detection

The level of $ONOO^-$ was assayed according to Huang *et al.* (2007) using folic acid as the $ONOO^-$ scavenger, giving high fluorescent emission products. Leaf discs (1 g) were immersed in an incubation mixture containing a barbital buffer solution (pH 9·4) and folic acid (10^{-5} M). Fluorescence intensity of the solution was recorded at 460 nm with the excitation wavelength set at 380 nm. The standard curve was prepared with SIN-1 from 1 to 14 nM. Additionally, the fluorescent reagent aminophenyl fluorescein, APF (Sigma; excitation 495 nm; emission 515 nm) was used to localize the $ONOO^-$ production in leaf tissue. Leaves were incubated for 1 h at room temperature with 10 μM APF, prepared using loading buffer (10 mM Tris-HCl, pH 7·4). After incubation, samples were washed five times for 1 min with 10 mM Tris-HCl, pH 7·4 and leaf fragments were embedded in a mixture of 15% acrylamide/bisacrylamide stock solution. Then 70 μm-thick sections were cut under 10 mM phosphate-buffered saline (PBS) with a vibratome (Leica). Ultrathin sections were immediately studied under an LSM 510 microscope (Zeiss) equipped with standard filters and collection modalities for APF green fluorescence. Images were processed and analysed using an LSM Image Browser (Zeiss).

Nitric oxide and superoxide detection

Nitric oxide formation was detected using a fluorescent dye Cu-FL (the copper complex of FL (2-{2-chloro-6-hydroxy-5-[2-methylquinolin-8-ylaminomethyl]-3-oxo-3H-xanthen-9-l}benzoic

acid). Cu-FL was prepared as 1 mM stock solution in water according to the manufacturer's instructions (Strem Chemicals). Leaf tissue (0·5 g) was homogenized in 2 mL of 10 mM potassium phosphate buffer (pH 6·0). The extract was centrifuged at 21 000 *g* for 30 min at 4°C. Then, 100 μL of supernatant were immediately used for NO assay by adding Cu-FL to a final concentration of 2 μM. Fluorescence intensity in supernatants was determined with an LS 50B fluorescence spectrometer (Perkin Elmer) using 488 and 516 nm for excitation and emission, respectively. The level of O_2^- was assayed spectrophotometrically on the basis of the capacity of the superoxide anion radical to reduce nitroblue tetrazolium (NBT) to diformazan, according to Doke (1983).

Gene expression

RNA was isolated from 0·2 g of frozen leaf and root tissues using TriReagent (Sigma). The obtained RNA was purified with the use of a deoxyribonuclease kit (Sigma). For the reverse transcription, 1 μg RNA from every experimental variant was processed with a reverse transcription kit (Thermo Scientific Fermentas) according to the manufacturer's instructions. Real-time PCR was performed on a RotorGene 6000 thermocycler. The reaction mixture contained 0·1 μM of each primer, 1 μL of 5 × diluted cDNA, 10 μL of the Power SYBR Green PCR Master mix (Applied Biosystems) and DEPC-treated water to a total volume of 20 μL. The real-time PCR reaction conditions included an initial 5 min denaturation at 95°C, followed by 55 cycles consisting of 10 s at 95°C, 20 s at 55°C and 30 s at 72°C. The reaction was finalized by denaturation at a temperature rising from 72 to 95°C by 1°C/5 s. Reaction specificity was confirmed by the occurrence of one peak in the melting curve analysis. The data were normalized to two reference genes encoding the elongation factor (EF1α, AB061263) and 18S RNA (X67238). All primers used are presented in Table 1. The C_t values were determined with the use of REAL-TIME PCR MINER (Zhao & Fernald, 2005) and the relative gene expression was calculated with the use of efficiency corrected calculation models presented by Pfaffl (2001).

Protein extraction, SDS-PAGE and western blot

Leaves (0·25 g) were ground in liquid N_2 to a fine powder and then suspended in a ratio of 1:3 (w/v) in 50 mM Tris-HCl buffer (pH 7·6) with 2 mM EDTA, 4 mM DTT, 0·6% PVPP, 1 mM PMSF and plant inhibitor cocktail (Sigma). The crude extracts were centrifuged at 10 000 *g* for 15 min at 4°C, then the concentration of supernatant proteins was determined with the Bradford assay, using bovine serum albumen (BSA) as a standard. The solution of proteins was mixed with the Laemmli sample buffer and boiled for 10 min at 70°C. The sample of proteins (50 μg each) was separated on a gradient SDS PAGE (4–20%; Bio-Rad) and gels were stained with Coomassie Brilliant Blue G-250 or proteins were electroblotted to PVDF membranes. After transfer, membranes were blocked (5% BSA) and used for cross-reactivity assays with polyclonal antibodies against nitrotyrosine (Life Technologies) at a dilution of 1:1000. For immunodetection, the goat anti-rabbit antibody conjugated to horseradish peroxidase (Agrisera) and Lumi-Light western blotting substrate (Roche) was used. The intensity of bands was quantified using a Gel Doc system (Bio-Rad) coupled with a high-resolution camera.

Table 1 Primers used in real-time PCR

GenBank accession no.	Protein	Primers (5′–3′)	
AF043248.1	PR-3	F	ACTGGAGGATGGGCTTCAGCA
		R	TGGATGGGGCCTCGTCCGAA
AJ009932.1	PR-2	F	TTGGCCTTCTGAGGGACACCC
		R	GTGTTCCAGTCCCTCCTTTCACG
NM_001247429.1	PR-1	F	GAGCTGGGGACTGCAGGATGC
		R	CCGCGTTGAGCTGGGGGAAA
GQ180105.1	TPx	F	GAGCTTTCACTCCCACTTGC
		R	GGCATGGGTGTATTTTCCAG
JX428804.1	Thx	F	TGATTCGAACACCATCCTGA
		R	CAGAGGGAGGAGGTGAACAA
X67238	18S RNA	F	GGGCATTCGTATTTCATAGTCAGAG
		R	CGGTTCTTGATTAATGAAAACATCCT
AB061263	EF1α	F	ATTGGAAACGGATATGCTCCA
		R	TCCTTACCTGAACGCCTGTCA

Mass spectrometry (MS) and protein identification

Protein identification was performed using liquid chromatography coupled to the mass spectrometer at the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland).

The PVDF membrane fragments with protein were put in a solution of 100 mM ammonium carbonate (pH 8) and directly subjected to the standard procedure of trypsin digestion, during which proteins were reduced with 10 mM DTT for 30 min at 56°C, alkylated with iodoacetamide in darkness for 45 min at room temperature and digested overnight with 10 ng μL^{-1} trypsin. The resulting peptide mixtures were concentrated and desalted on an RP-C18 pre-column (Waters), and further peptide separation was achieved on a nano-Ultra Performance Liquid Chromatography (UPLC) RP-C18 column (Waters, BEH130 C18 column, 75 μm i.d., 250 mm long) of a nanoACQUITY UPLC system, using a 45-min linear acetonitrile gradient. The column outlet was directly coupled to the electrospray ionization (ESI) ion source of the Orbitrap Velos type mass spectrometer (Thermo), working in the regime of data-dependent MS to MS/MS switch with HCD type peptide fragmentation. An electrospray voltage of 1·5 kV was used. Raw data files were preprocessed with the MASCOT DISTILLER software (v. 2.4.2.0; MatrixScience). The obtained peptide masses and fragmentation spectra were matched to the National Center Biotechnology Information (NCBI) non-redundant database (37 425 594 sequences; 13 257 553 858 residues), with a Viridiplantae filter (1 760 563 sequences) using the MASCOT search engine (Mascot Daemon v. 2.4.0, Mascot Server v. 2.4.1; MatrixScience). The following search parameters were applied: enzyme specificity was set to semiTrypsin, peptide mass tolerance to ± 30 ppm and fragment mass tolerance to ± 0·1 Da. The protein mass was left as unrestricted, and mass values as monoisotopic with two missed cleavages being allowed. Alkylation of cysteine by carbamidomethylation was set as fixed, whilst oxidation of methionine and carboxymethylation on lysine were set as a variable modification.

Protein identification was performed using the MASCOT search engine, with the probability-based algorithm. The expected value threshold of 0·05 was used for analysis, which means that all peptide identifications had less than 1 in 20 chance of being a random match.

Statistical analysis

All the experiments included three independent experiments carried out in at least three replications. For each experiment,

means of the values obtained were calculated along with standard deviations. The analysis of variance was performed (ANOVA) and the mean values were compared by Tukey's test ($\alpha = 0{\cdot}05$).

Results

RNS and ROS generation during potato–*P. infestans* incompatible and compatible interactions

Peroxynitrite formation was quantitatively measured using the folic acid method and visualized using the fluorescence probe APF. Time-course experiments using a quantitative measurement of $ONOO^-$ revealed *c.* 3-fold induction of $ONOO^-$ production within 1 hpi in resistant potato leaves (Fig. 1a). Then, $ONOO^-$ accumulation gradually decreased, reaching the level of the control at 24 hpi. In the susceptible genotype, the pathogen induced a strong $ONOO^-$ formation (over 2-fold increase) starting from 24 hpi (Fig. 1b). Real-time imaging by fluorescence microscopy confirmed the results provided by the fluorometric method (Fig. 1c–f). Bioimaging with APF revealed that control potato leaves generated some $ONOO^-$, with green fluorescence corresponding to $ONOO^-$ restricted to cells adjacent to vascular bundles (Fig. 1g,h). The significantly enhanced peroxynitrite-dependent green fluorescence was noted in susceptible potato leaves challenge-inoculated with *P. infestans* at 48 hpi, which was localized in epidermal cells, the palisade and spongy mesophyll (Fig. 1f). Additionally, the application of ebselen, a specific $ONOO^-$ scavenger, resulted in the suppression of $ONOO^-$ generation in potato leaves, showing specificity of the assays used for $ONOO^-$ detection in the plant material (Fig. 1i).

The levels of NO and superoxide anion forming $ONOO^-$ were also modified in potato leaves inoculated with *P. infestans*. A quantitative measurement of NO using the selective Cu-FL revealed that NO increased much faster (within the first 3 h of challenge) in the resistant than in the susceptible potato (Fig. 2). A delayed NO production was found during the compatible interaction (Fig. 2b). NO accumulation significantly increased starting from 6 hpi, reaching its highest level at this time point. Additionally, an early superoxide production was observed in response to *P. infestans* in both potato cultivars (Fig. 3a,b). However, the maximum superoxide production was recorded at different time points, i.e. at 6 and 48 hpi in the resistant and susceptible potato, respectively.

Thioredoxin peroxidase and thioredoxin gene expression during potato–*P. infestans* interactions

It has been documented that thioredoxin peroxidases (TPxs) may also reduce $ONOO^-$, in addition to hydrogen peroxide or phospholipid hydroperoxides (Wood *et al.*, 2003). Based on the quantitative PCR data, it was found that *TPx* could also be indirectly engaged in the regulation of $ONOO^-$ concentration in leaves of both potato genotypes. The ONOO donor SIN-1 provoked an over 2-fold up-regulation of *TPx* gene expression starting from 1 and 3 h after pretreatment in healthy resistant and susceptible leaves, respectively (Fig. S1). Interestingly, the basal level of *TPx* was higher in the susceptible potato. Pathogen challenge resulted in a significantly induced gene expression starting from 3 and 1 hpi in both resistant and susceptible potato, respectively (Fig. 4a,b). In the resistant response, a peak of *TPx* transcript accumulation was observed at 24 hpi, following the $ONOO^-$ burst, whereas in the susceptible one, *TPx* expression reached the highest amount (*c.* 20-fold increase in comparison to mock-inoculated leaves) at 3 hpi, preceding $ONOO^-$ overproduction. The induced *TPx* expression was accompanied by *Thx* mRNA accumulation (Fig. 4c,d). The only exception was noted at 1 hpi in the susceptible potato, when a *c.* 3-fold down-regulation of the *Thx* gene expression was observed (Fig. 4d). SIN-1 did not provoke changes in the *Thx* expression profile (data not presented).

Late blight disease development on potato leaves exposed to the $ONOO^-$ donor and scavenger

To assess the effect of $ONOO^-$ on late blight disease development, potato leaves were pretreated for 5 h with 50 μM SIN-1 (susceptible potato) or 50 μM ebselen (resistant potato) and then inoculated with the oomycete pathogen. At 3 days post-inoculation (dpi), reduced disease spots were found on susceptible leaves enriched with exogenous $ONOO^-$, with the area covered by late blight at 24% (Fig. 5). In contrast, the area infected by *P. infestans* on leaves not pretreated with SIN-1 reached 35%. Pre-exposure to $ONOO^-$ scavenger and subsequent challenge inoculation did not modify the resistant potato–*P. infestans* interaction (Fig. 5).

Peroxynitrite enhanced expression of genes encoding proteins involved in disease resistance

To gain further insight into the participation of $ONOO^-$ in the activation of defence to an oomycete pathogen in potato leaves, an analysis was performed using the susceptible genotype pretreated with SIN-1, or resistant genotype pretreated with ebselen. It was found that SIN-1 provoked *PR-1* and *PR-2* up-regulation in healthy leaves (Fig. S2). Moreover, the quantitative RT-PCR data revealed that leaves enriched with $ONOO^-$ and subsequently challenge-inoculated showed an early and stronger induction of *PR-1* in comparison to H_2O-pretreated inoculated leaves (Fig. 6a). The expression of *PR-1* started to rise at 3 hpi and then gradually increased to reach the highest amount (over 7-fold increase in comparison to H_2O-pretreated inoculated leaves) at 48 hpi. The *PR-2* mRNA was effectively up-regulated already at 1 and 24 hpi in leaves sequentially treated with SIN-1 and *P. infestans*. Moreover, an over 2-fold enhancement of the *PR-3* gene expression was observed at 48 h of pathogen challenge. Leaves of resistant potato lacking $ONOO^-$ due to ebselen pretreatment did not show *PR-1* expression during the first 6 h after challenge inoculation (Fig. 6b).

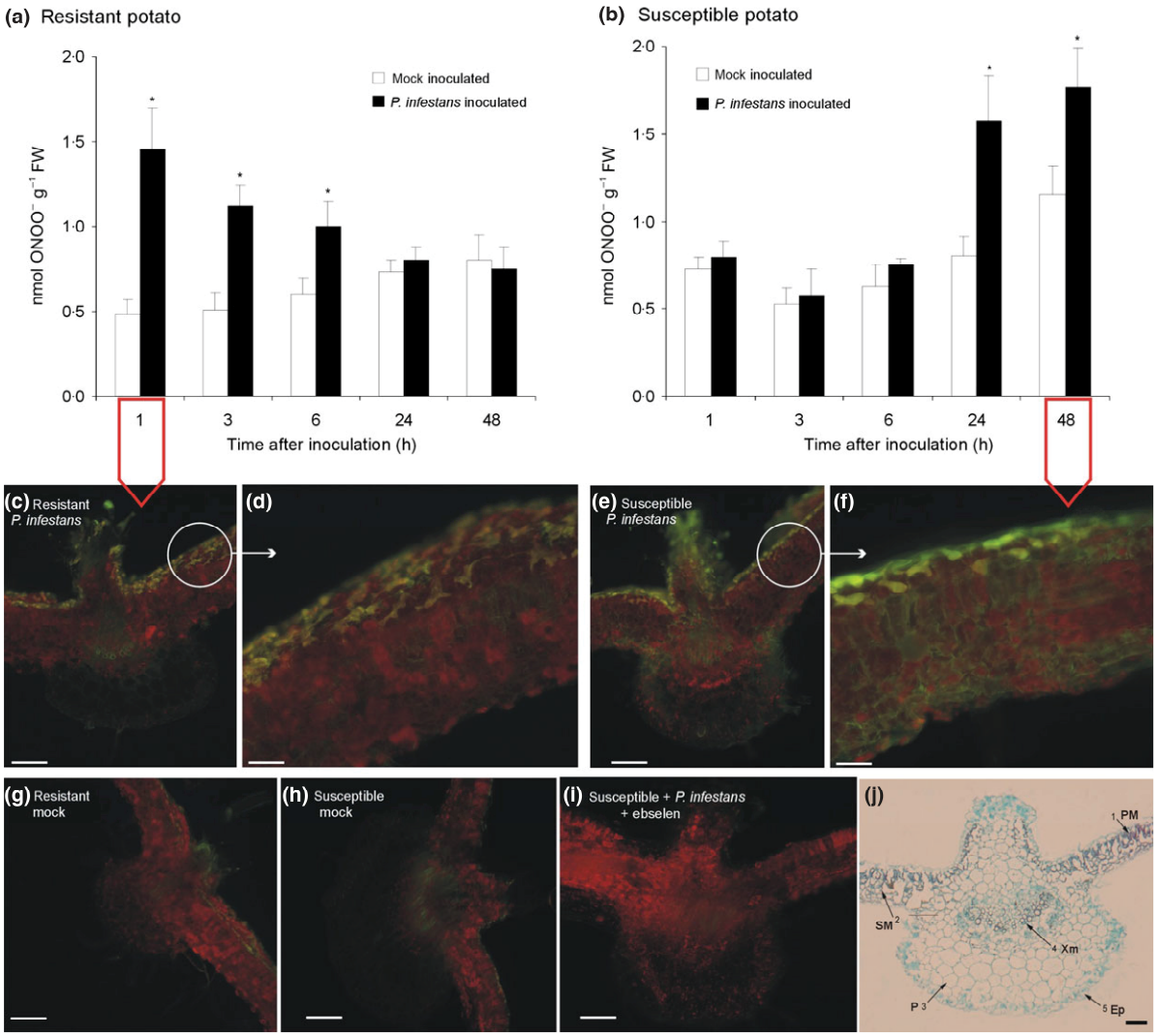


Figure 1 Peroxynitrite formation in leaves of resistant (a) and susceptible (b) potato inoculated with *Phytophthora infestans*. The analyses were performed at 1, 3, 6, 24 and 48 h after challenge inoculation. Asterisks indicate values that differ significantly from the mock-inoculated (control) potato leaves at $P < 0{\cdot}05$ (*). Bio-imaging of peroxynitrite formation with fluorescent reagent APF at 1 hpi in resistant (c, d) and at 48 hpi susceptible (e, f) potato. Leaves of mock-inoculated (control) potato (g, h); sequential treated (50 μM ebselen + *P. infestans* at 48 hpi) leaves of susceptible potato (i); leaf cross section of the susceptible potato genotype viewed under white light (j): 1 – palisade mesophyll (PM), 2 – spongy mesophyll (SM), 3 – parenchyma (P), 4 – xylem (Xm), 5 – epidermal cells (Ep). Images show general phenomena representative of three individual experiments. The green fluorescence corresponds to peroxynitrite and the red colour is due to the chlorophyll autofluorescence. Bars indicate 150 μm (c, e, g–i), 60 μm (d, f) and 200 μm (j).

Application of ebselen also affected expression of *PR-3* at 48 hpi.

Tyrosine nitration pattern during potato defence response to *P. infestans*

Tyrosine nitration protein patterns detected with an antibody against nitrotyrosine in potato leaves challenged with *P. infestans* at 24 and 48 hpi are presented in Figure 7. A total of 15 immunoreactive bands were detected in leaves mock-inoculated and inoculated with *P. infestans* of both potato genotypes. These bands were analysed by LC-MS-MS/MS after trypsin digestion, using the MASCOT search engine to analyse MS data in order to identify proteins from primary-sequence databases. The identified immunoreactive proteins are listed in Table 2, with the protein score confidence interval (CI) in almost all cases being over 99%, which indicates that the proteins were not identified by random matches of peptide mass data. Amino acid sequences of the homologous proteins identified are shown in Table S1.

The most prominent immunoreactive bands were observed during the compatible interaction. The protein bands, the intensities of which were enhanced after pathogen challenge, contained proteins involved in primary metabolism and defence responses (Table 2). Only one protein band (B6, 23 kDa) appeared *de novo* at 24 hpi and another protein band (B10, 38 kDa)

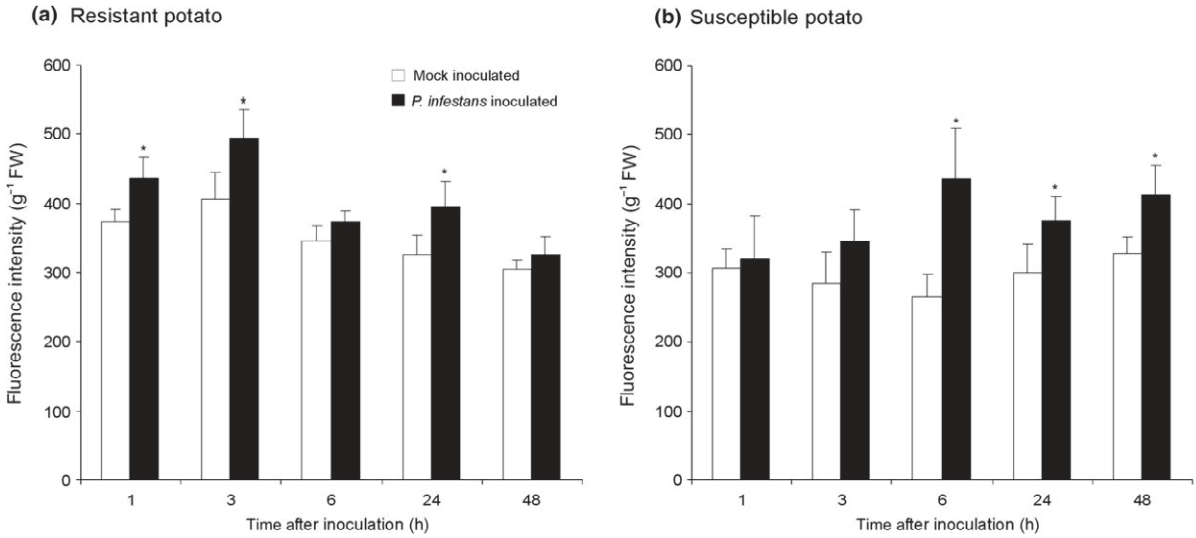


Figure 2 Nitric oxide production in leaves of resistant (a) and susceptible (b) potato inoculated with *Phytophthora infestans*. NO production was assayed spectrofluorimetrically using a selective NO sensor (Cu-FL). The analyses were performed at 1, 3, 6, 24 and 48 h after challenge inoculation. Asterisks indicate values that differ significantly from the mock-inoculated (control) potato leaves at $P < 0{\cdot}05$ (*).

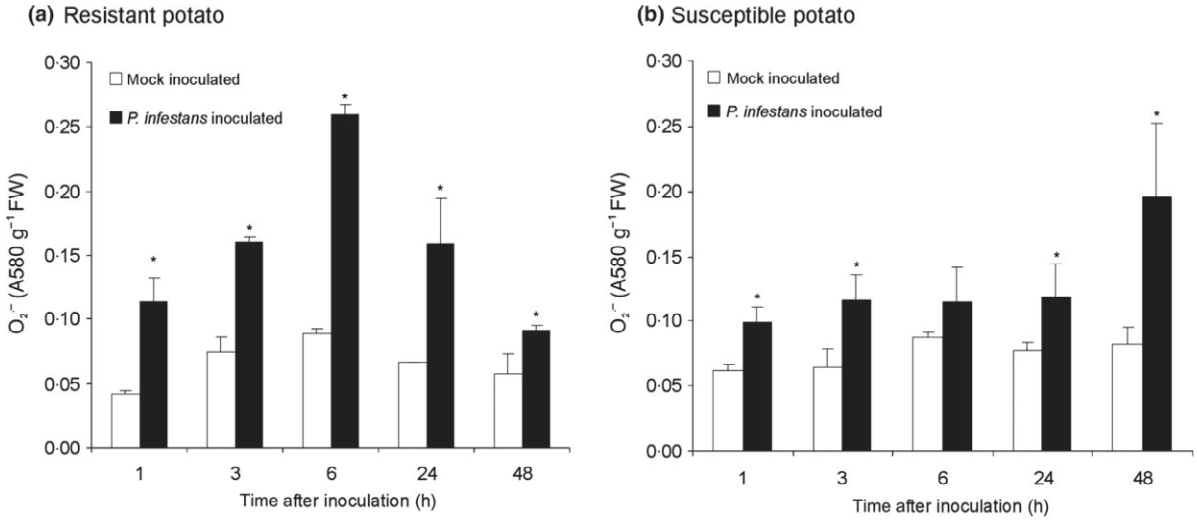


Figure 3 Superoxide production in leaves of resistant (a) and susceptible (b) potato inoculated with *Phytophthora infestans*. The analyses were performed at 1, 3, 6, 24 and 48 h after challenge inoculation. Asterisks indicate values that differ significantly from the mock-inoculated (control) potato leaves at $P < 0{\cdot}05$ (*).

appeared after 48 h of *P. infestans* challenge inoculation. The pathogen also provoked a lowered expression of one protein band (B12, 50 kDa). During the incompatible interaction, no increase was found in the total protein pool undergoing tyrosine nitration; however, one protein band (B13, 85 kDa) appeared at 48 h after pathogen attack which contained proteins involved in proteolysis (Table 2). Additionally, two bands (B5, 22 kDa; B12, 50 kDa), the intensities of which were diminished after pathogen infection, contained proteins involved mainly in defence response and photosynthesis (Table 2).

Discussion

This report presents evidence of ONOO contribution to the potato defence against the oomycete pathogen *P. infestans*. In the resistant potato genotype, *P. infestans* provoked a transient peak of $ONOO^-$ formation within the first hpi; however, the elevated generation of the RNS was maintained up to 6 hpi. The early $ONOO^-$ over-accumulation in potato leaves was coincident with the burst of NO and superoxide. Gaupels *et al.* (2011), applying a leaf disk assay with the HKGreen-2 fluores-

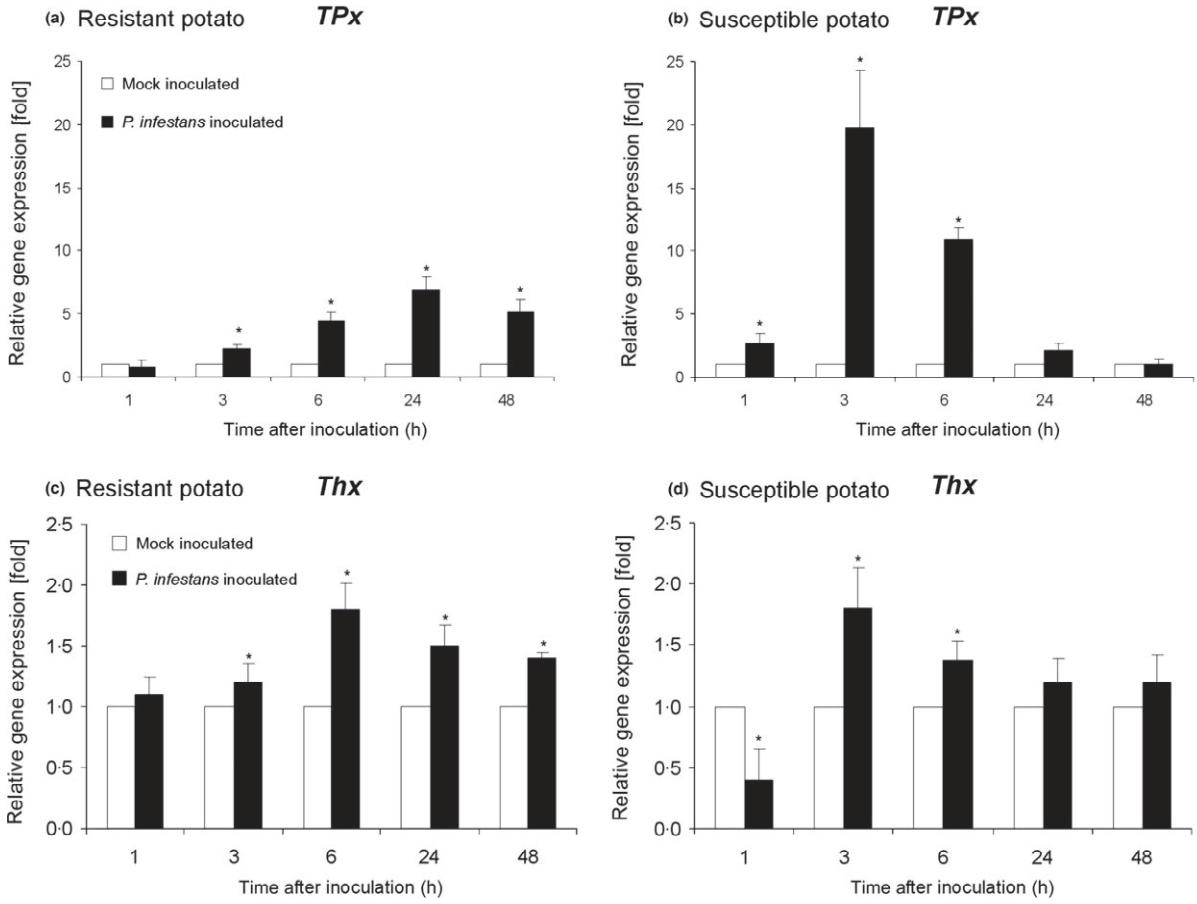


Figure 4 The qRT-PCR analysis of *TPx* and *Thx* gene expression in leaves of resistant (a, c) and susceptible (b, d) potato inoculated with *Phytophthora infestans*. The analyses were performed at 1, 3, 6, 24 and 48 h after challenge inoculation. Asterisks indicate values that differ significantly from the mock-inoculated (control) potato leaves at $P < 0{\cdot}05$ (*).

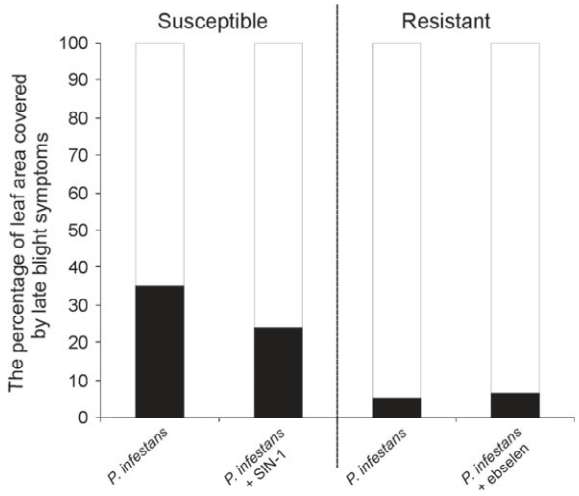


Figure 5 The effect of peroxynitrite donor (SIN-1) and scavenger (ebselen) pretreatment on late blight disease establishment in the susceptible and resistant potato cultivars. The percentage of leaf area infected by *Phytophthora infestans* was measured 3 days after pathogen challenge inoculation. Values are means from 30 leaves (10 leaves from each of three independent experiments).

cent dye, observed a significant accumulation of $ONOO^-$ at 3–4 h during *Arabidopsis* and an *avr Pseudomonas syringae* pv. *tomato* interaction, which visibly peaked at 7–8 h after pathogen treatment. Saito *et al.* (2006) found $ONOO^-$ generation in tobacco BY-2 cells treated with INF1, an elicitor that induces HR in tobacco. In this experimental model $ONOO^-$ formation occurred already within the first hour and reached a maximum level at 6–12 h after elicitation. This sequence of events seems to be quite different from the plant response during compatible interactions, in which $ONOO^-$ formation was detected at later stages of pathogen challenge. The potato *avr P. infestans* interaction was accompanied by the generation of considerable amounts of $ONOO^-$ starting from 24 hpi; however, an enhanced level of NO and superoxide was already recorded during the earlier phase of disease development. It should be stressed that $ONOO^-$ biosynthesis is not controlled in the narrower physiological sense, but equilibrated levels of both parent molecules could be precisely modified by various pathogen effectors and finally influence resistance.

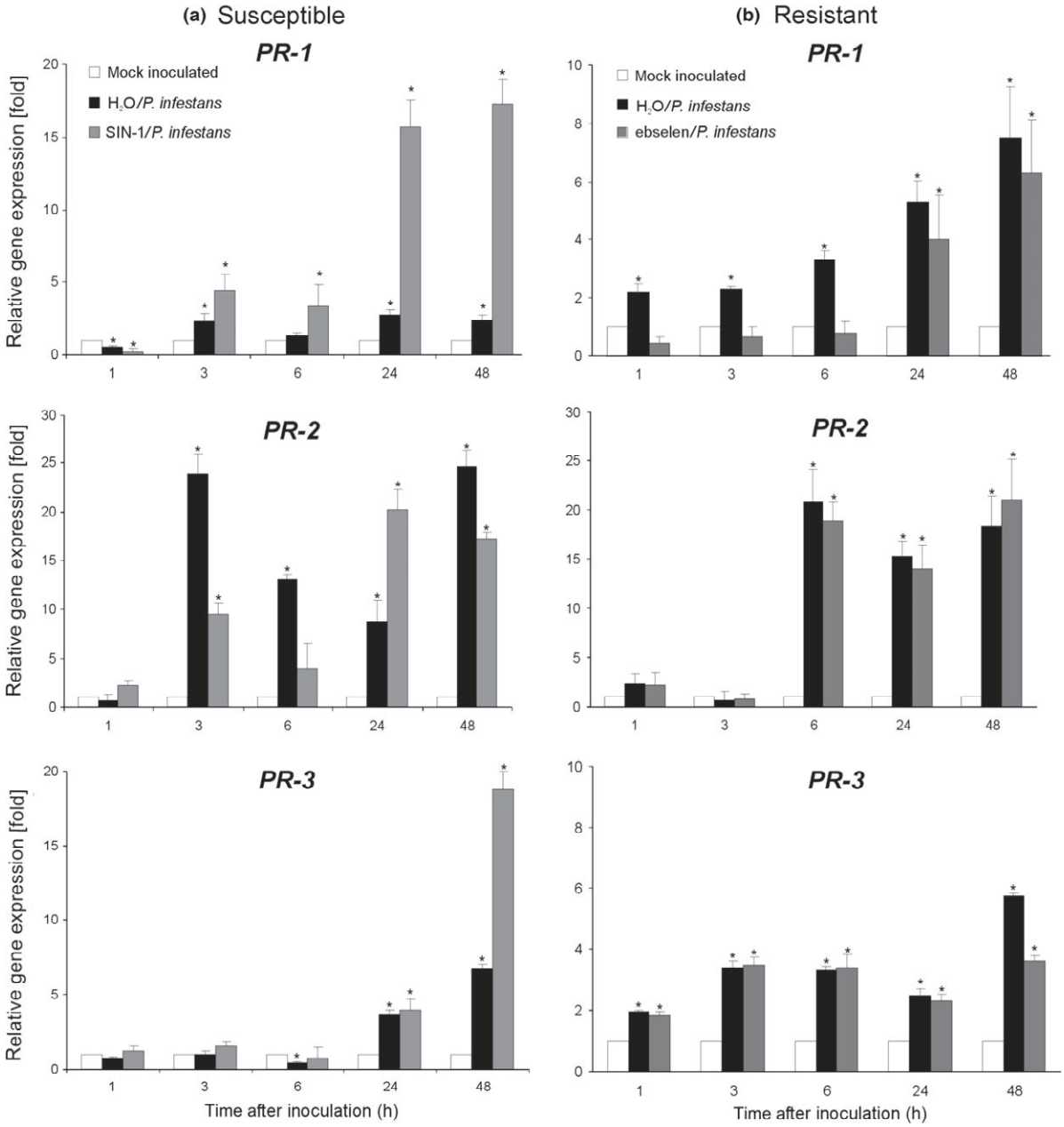


Figure 6 The effect of susceptible potato (a) pretreatment with peroxynitrite donor (50 μM SIN-1) and resistant potato (b) pretreatment with peroxynitrite scavenger (50 μM ebselen) followed by challenge inoculation with *Phytophthora infestans* on *PR-1*, *PR-2* and *PR-3* gene expression in potato leaves. The qRT-PCR analyses were performed at 1, 3, 6, 24 and 48 h after challenge inoculation. Asterisks indicate values that differ significantly from mock-inoculated leaves at $P < 0{\cdot}05$ (*).

It was earlier asserted by Alamillo & García-Olmedo (2001) that $ONOO^-$ is relevant to HR. However, when studying the mechanisms of RNS and ROS cooperation in triggering the hypersensitive cell death it was seen that $ONOO^-$ is not involved in NO-mediated cell death in plants (Delledonne *et al.*, 2001), although high external concentrations of $ONOO^-$ (1 mM) have been reported to induce tyrosine nitration in soybean and some necrotic lesions in *Arabidopsis* (Alamillo & García-Olmedo, 2001). More recently, Serrano *et al.* (2012) shed new light on $ONOO^-$ implication in plant cell death, with their results suggesting that $ONOO^-$ functions as a key signal molecule during developmental programmed cell death (PCD) of incompatible pollen in *Olea europaea*.

A potential signalling role of $ONOO^-$ during the induction of defence gene expression has also been proposed (Alamillo & García-Olmedo, 2001; Vandelle & Delledonne, 2011). As found in the present study, treat-

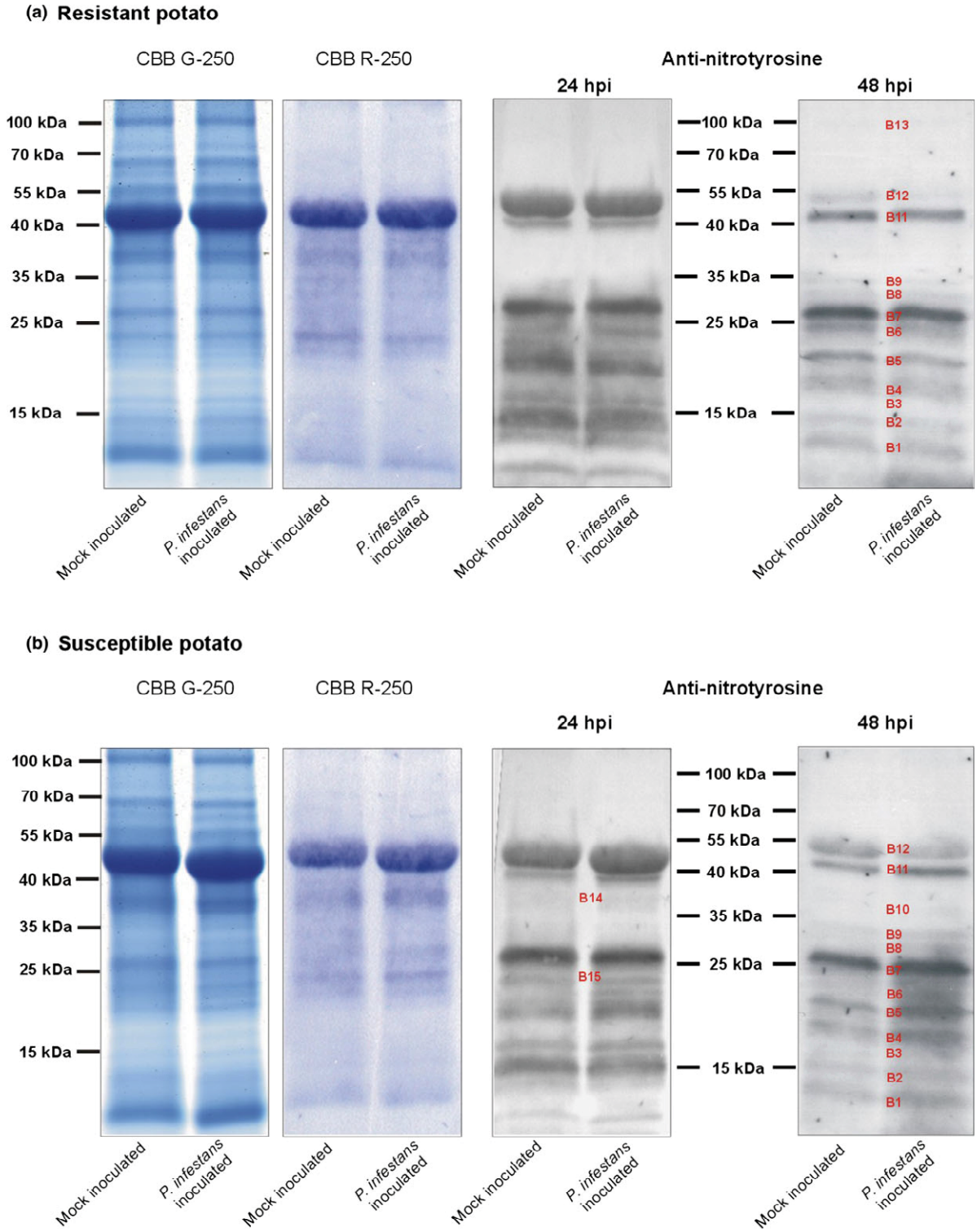


Figure 7 Tyrosine nitration pattern in potato leaves challenge-inoculated with *Phytophthora infestans* at 24 and 48 h post-inoculation (hpi) in resistant (a) and susceptible (b) genotypes. Coomassie Brilliant Blue (CBB) G-250-stained SDS PAGE (4–20%) gels, the loading control determined by staining the blot with CBB R-250, and western blot was probed with a rabbit anti-nitrotyrosine polyclonal antibody at a 1:1000 dilution. Numbers indicate the immunoreactive bands referring to the proteins listed in Table 2.

Table 2 Nitrated proteins in potato leaves inoculated with *Phytophthora infestans* isolated from PVDF membrane and identified by LC-MS-MS/MS

No.	Protein name	NCBI accession no.	BLAST score	Functional category
B1	40S ribosomal protein S14-2-like	gi|568215322	230	Translation
	PREDICTED: pathogenesis-related protein STH-2-like	gi|565347758	157	Defence response
	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	gi|162946541	110	Photosynthesis
B2	Putative kunitz-type proteinase inhibitor precursor P1D4	gi|20087001	132	Endopeptidase inhibitor activity
	Pathogenesis-related protein P2-like precursor	gi|568815624	102	Defence response
B3	PREDICTED: ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	gi|565383834	67	Photosynthesis
B4	Proteinase inhibitor	gi|994778	163	Endopeptidase inhibitor activity
	Ribosomal protein S7-like protein	gi|568214675	158	Translation
	Oxygen-evolving enhancer protein 2, chloroplastic precursor	gi|568214772	89	Photosynthesis
B5	PREDICTED: 2-Cys peroxiredoxin BAS1-like, chloroplastic-like	gi|565344108	216	Antioxidant activity
	60S ribosomal protein L15-like	gi|82621122	169	Translation
	PREDICTED: carbonic anhydrase, chloroplastic-like isoform X1	gi|565342863	94	Photosynthesis
	PREDICTED: carbonic anhydrase, chloroplastic-like isoform X2	gi|565342865	94	Photosynthesis
B6	60S ribosomal protein L19-like protein	gi|568214986	184	Translation
	Chitinase	gi|21495	99	Defence response
	PREDICTED: glutathione S-transferase L3-like	gi|565343884	84	Defence response
B7	Putative 40S ribosomal protein S8-like protein	gi|568215087	174	Translation
	PREDICTED: 60S ribosomal protein L6-like	gi|565371561	179	Translation
B8	Chloroplast manganese stabilizing protein	gi|313586398	355	Photosynthesis
	Glucan endo-1,3-β-glucosidase, basic isoform 2 precursor	gi|568214854	111	Defence response
B9	Pectin methyl esterase	gi|568214923	195	Hydrolase activity/cell wall modification
	Low temperature induced cysteine proteinase-like precursor	gi|568215000	65	Peptidase activity
	Class I chitinase precursor	gi|568214310	101	Hydrolase activity/chitinase activity/ carbohydrate metabolism
B10	PREDICTED: aldo-keto reductase-like	gi|565368099	256	Oxidoreductase activity
	PREDICTED: probable fructose-bisphosphate aldolase 2, chloroplastic-like	gi|565347367	94	Glycolysis
B11	Uncharacterized protein LOC102577718	gi|568215399	329	Unknown
	Anionic peroxidase, partial	gi|169555	138	Defence response
	60S ribosomal protein L3-like	gi|568214933	123	Translation
B12	Leucine aminopeptidase	gi|21487	1791	Stress response
	Serine hydroxymethyltransferase, mitochondrial	gi|568215114	1178	Glycine metabolic process
	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|108773138	933	Photosynthesis
	PREDICTED: ATP synthase subunit beta, mitochondrial-like	gi|565389366	632	ATP binding
	S-adenosyl-L-homocysteine hydrolase-like	gi|81075407	224	Carbon metabolic process/hydrolase activity
	Catalase	gi|587568	102	Defence response/response to oxidative stress
B13	PREDICTED: subtilisin-like protease-like	gi|565378238	1214	Proteolysis
	PREDICTED: subtilisin-like protease-like	gi|565378352	864	Proteolysis
B14	PREDICTED: quinone oxidoreductase-like protein At1g23740, chloroplastic-like	gi|565399387	186	Oxidoreductase activity
	PREDICTED: quinone oxidoreductase PIG3-like	gi|565360697	68	Oxidoreductase activity
B15	Unnamed protein product	gi|21465	130	Unknown
	Aspartic proteinase inhibitor	gi|21413	68	Protease inhibitor

ment of susceptible potato with SIN-1 before inoculation effectively activated key markers of plant defence against pathogens, including *PR-1*, *PR-2* and *PR-3*. Peroxynitrite implication in defence activation at the molecular level was even strengthened by the application of ebselen and subsequent inoculation with *P. infestans*, which provoked a significant down-regulation of *PR-1* expression in the resistant cultivar. This finding is in line with previous pharmacological reports showing the $ONOO^-$ association with *PR-1* gene expression in tobacco (Durner *et al.*, 1998). Moreover, the mRNA level of *PR-1* was reduced during *Arabidopsis* response to $ONOO^-$ scavenger treatment and subsequent inoculation with the virulent strain *P. syringae* pv. *phaseolicola* (*avr*RPM1$^-$). Also a transient peak of *PAL*, encoding a key enzyme of the phenylpropanoid pathway, observed at 4 h upon challenge with the avirulent (*avr*RPM1$^+$) strain of the bacterial pathogen, did not occur in the presence of urate, indicating that $ONOO^-$ is obligatory for the early induction of this defence gene (Alamillo & García-Olmedo, 2001). Most probably a specific redox imbalance created by $ONOO^-$ is crucial to unlock the sequence of events towards responses engaged in basal defence. Based on the potato incompatible response this

may include enhancement of the Thx pool, which may favour plant immunity via *NPR-1* regulation and subsequent *PR-1* gene expression (Tada *et al.*, 2008).

It is known that the production of $ONOO^-$ not only affects the redox balance, but also diminishes the concentration of bioavailable NO. In animal cells it means a switch of the redox environment from controlled by cytoprotective NO to controlled by the cytotoxic $ONOO^-$ (Burewicz *et al.*, 2013). In plants an early, transient and balanced/controlled burst of NO, occurring rapidly after pathogen recognition, is essential to immunity (Floryszak-Wieczorek *et al.*, 2007). In turn, an accurate resetting of the boosted NO signal prevents a homeostasis misbalance towards nitrosative stress. The accumulating data indicate that $ONOO^-$ is generated not only during plant pathological states, but might be continuously formed in healthy tissues as an inevitable event of cell metabolism, because plant cells are adapted to detoxify its excess by a broad range of decomposition mechanisms (Romero-Puertas *et al.*, 2008). Thus, the plant NO buffering system could also include an enhanced $ONOO^-$ formation, which provides an important regulatory loop for NO bioactivity, especially under stress conditions. An efficient mechanism for $ONOO^-$ detoxification could involve thiol-dependent peroxidases. In the present study, an early $ONOO^-$ over-production noted during the incompatible interaction was not accompanied by *TPx* gene expression. However, *TPx* mRNA gradually increased, reaching maximum expression at 24 hpi, which was negatively correlated with $ONOO^-$ accumulation. Thioredoxin peroxidases were proved to reduce $ONOO^-$, while they may also convert hydrogen peroxide or phospholipid hydroperoxides (Wood *et al.*, 2003). In this way *TPx* could control not only $ONOO^-$ concentration, but also indirectly mediate in hydrogen peroxide accumulation, which in orchestration with boosted NO synthesis, promoted HR observed in the plant–*avr* pathogen interaction.

Because the average distance of $ONOO^-$ diffusion is less than 100 μm, the molecule is able to travel short distances, reaching subcellular compartments and neighbouring cells (Szabó *et al.*, 2007). This characteristic may play a significant role in the amplification of the signal, especially during the incompatible interaction accompanied by a quick and transient $ONOO^-$ formation. Moreover, the identification of several proteins subjected to specific tyrosine nitration during HR supports the potential role of $ONOO^-$ as a mediator of NO-dependent signalling during defence responses (Cecconi *et al.*, 2009). Although it is generally accepted that $ONOO^-$ contributes to nitrosative stress in cells and tyrosine nitration has been associated with pathophysiological states during plant response to various abiotic stress stimuli (Chaki *et al.*, 2011; Tanou *et al.*, 2012; Signorelli *et al.*, 2013), nitration activity of $ONOO^-$ could be beneficial in terms of host defence against invading microorganisms. Increasing data point out that this post-translational modification may perform a signalling function in cellular behaviour through its positive or negative implication into existing tyrosine phosphorylation/dephosphorylation pathways, which could be crucial to plant defence responses (Vandelle & Delledonne, 2011).

A one-dimensional western blot analysis showed no intensification of nitrotyrosine immunoreactivity in the challenge-inoculated resistant potato, except for one protein band (85 kDa), which appeared only at 48 hpi. This indicates a subtilisin-like protease-like protein involved in proteolysis. Importantly, the tomato subtilase P69 has been identified as a pathogenesis-related protein and was shown to be one of several subtilases that are specifically induced following pathogen infection. Moreover, subtilisin-like protease promoters *P69B* and *P69C* may act as an early line of defence, because expression of both *P69B* and *P69C* occurs in cells distal from the necrotic lesion, being a consequence of HR during the incompatible interaction (Jordá & Vera, 2000). Some caspase-like activities are attributable to plant subtilisin-like proteases (Vartapetian *et al.*, 2011). Therefore, subtilisin-like proteases as potential sensors of $ONOO^-$ could be a functional link between $ONOO^-$ and active cell death under both physiological PCD (Serrano *et al.*, 2012) and plant immune responses. The nitroproteome profile did not show any visible differences in the resistant sunflower cultivar inoculated with the biotroph *Plasmopara halstedii* (Chaki *et al.*, 2009). In contrast, in susceptible sunflower plants the protein tyrosine nitration increased after infection with the pathogen. An enhanced expression of protein undergoing tyrosine nitration during the potato–*P. infestans* compatible interaction starting from 24 hpi was also found in the present study. Identified 3-nitrotyrosine targets from the susceptible potato genotype indicated proteins homologous to ones involved in gene transcription, translation, defence response, carbohydrate metabolism and photosynthesis. However, these proteins should be considered putatively nitrated until the nitrated sites have been identified by sequence analysis. Some of the identified proteins have been previously reported to be nitrated in other plant species and they included aldo-keto reductase, fructose-bisphosphate aldolase 2, the RuBisCo large and small subunit, ATP synthase subunit β, carbonic anhydrase 1 and 2, S-adenosyl-L-homocysteine hydrolase, endochitinase and peroxidase (Lozano-Juste *et al.*, 2011; Begara-Morales *et al.*, 2013; Chaki *et al.*, 2013). Importantly, the 38 kDa protein band (B10) appearing *de novo* at 48 hpi during the genetically compatible potato *P. infestans* interaction contained redox-regulated proteins, which modified by nitration could affect cellular redox balance contributing to plant susceptibility to pathogen attack. Moreover, anionic peroxidase located in the 45 kDa band (B11) was recently proved to be involved in potato resistance to *P. infestans* (Sorokan *et al.*, 2014). As nitration may result in reversible or irreversible down-regulation of the targeted protein, it is highly likely that these post-translational modifications of anionic peroxidase and the other up-regulated proteins identified in this study, could favour potato susceptibility to an oomycete pathogen.

In *Arabidopsis* challenged with an avirulent bacterial pathogen Cecconi *et al.* (2009) identified 11 proteins that undergo nitration during the progression of HR. The proteins were related to primary metabolism, such as nitrogen assimilation, ATP synthesis, the Calvin cycle and glycolysis. Moreover, in tobacco BY-2 suspension cells treated with a fungal elicitin, Saito *et al.* (2006) revealed the induction of tyrosine nitration in proteins with molecular masses in the range of 20–50 kDa.

The results presented here indicate that an early and transient $ONOO^-$ generation in cooperation with fine balanced *TPx* at the site of the *P. infestans* contact with the resistant potato most probably made it possible to generate sufficient amounts of $ONOO^-$, promoting defence responses. Moreover, selective nitration of tyrosine residues in a small number of proteins suggests that $ONOO^-$ might function as a key regulator of redox signalling in cells undergoing HR. In contrast, a time-delayed formation of $ONOO^-$, together with a prolonged and boosted NO accumulation in the compatible response, may strongly influence the redox milieu via elevated protein nitration, resulting in failed resistance. Tissue enrichment with $ONOO^-$ was able to reprogramme molecular mechanisms in susceptible potato cells, facilitating more potent defence responses to a subsequent pathogen attack, as shown by a significant induction of *PR* genes and reduced disease symptoms as a result of the sequential treatment with SIN-1 and *P. infestans*. Taken together, $ONOO^-$ is an important redox regulation player of defence responses engaged in basal resistance.

Acknowledgements

This work was supported by the grant of National Science Centre, project no. NCN 2011/01/B/NZ9/00243.

References

Alamillo JM, García-Olmedo F, 2001. Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. *The Plant Journal* **25**, 529–40.

Ali A, Alexandersson E, Sandin M *et al.*, 2014. Quantitative proteomics and transcriptomics of potato in response to *Phytophthora infestans* in compatible and incompatible interactions. *BMC Genomics* **15**, 497.

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, 2011. Understanding the fate of peroxynitrite in plant cells – from physiology to pathophysiology. *Phytochemistry* **72**, 681–8.

Begara-Morales JC, Chaki M, Sánchez-Calvo B *et al.*, 2013. Protein tyrosine nitration in pea roots during development and senescence. *Journal of Experimental Botany* **64**, 1121–34.

Burewicz A, Hazem D, Lu-Lin J, Malinski T, 2013. Nitric oxide/peroxynitrite redox imbalance in endothelial cells measured with amperometric nanosensors. *American Journal of Analytical Chemistry* **4**, 30–6.

Cecconi D, Orzetti S, Vandelle E, Rinalducci S, Zolla L, Delledonne M, 2009. Protein nitration during defense response in *Arabidopsis thaliana*. *Electrophoresis* **30**, 2460–8.

Chaki M, Valderrama R, Fernández-Ocaña AM *et al.*, 2009. Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. *Journal of Experimental Botany* **60**, 4221–34.

Chaki M, Valderrama R, Fernández-Ocaña AM *et al.*, 2011. High temperature triggers the metabolism of S-nitrosothiols in sunflower mediating a process of nitrosative stress which provokes the inhibition of ferredoxin-NADP reductase by tyrosine nitration. *Plant, Cell and Environment* **34**, 1803–18.

Chaki M, Carreras A, López-Jaramillo J *et al.*, 2013. Tyrosine nitration provokes inhibition of sunflower carbonic anhydrase (b-CA) activity under high temperature stress. *Nitric Oxide – Biology and Chemistry* **29**, 30–3.

Corpas FJ, Barroso JB, 2014. Peroxynitrite ($ONOO^-$) is endogenously produced in *Arabidopsis* peroxisomes and is overproduced under cadmium stress. *Annals of Botany* **113**, 87–96.

Delledonne M, Zeier J, Marocco A, Lamb C, 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences, USA* **98**, 13454–9.

Dietz KJ, 2011. Peroxiredoxins in plants and cyanobacteria. *Antioxidants and Redox Signaling* **15**, 1129–59.

Doke N, 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiological Plant Pathology* **23**, 345–57.

Durner J, Wendehenne D, Klessig DF, 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proceedings of the National Academy of Sciences, USA* **95**, 10328–33.

Ferrer-Sueta G, Radi R, 2009. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chemical Biology* **4**, 161–77.

Floryszak-Wieczorek J, Arasimowicz M, Milczarek G, Jelen H, Jackowiak H, 2007. Only an early nitric oxide burst and the following wave of secondary nitric oxide generation enhanced effective defence responses of pelargonium to a necrotrophic pathogen. *New Phytologist* **175**, 718–30.

Gaupels F, Spiazzi-Vandelle E, Yang D, Delledonne M, 2011. Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response. *Nitric Oxide – Biology and Chemistry* **25**, 222–8.

Gross F, Durner J, Gaupels F, 2013. Nitric oxide, antioxidants and prooxidants in plant defence responses. *Frontiers in Plant Science* **4**, 419.

Huang JC, Li DJ, Diao JC, Hou J, Yuan JL, Zou GL, 2007. A novel fluorescent method for determination of peroxynitrite using folic acid as a probe. *Talanta* **72**, 1283–7.

Jordá L, Vera P, 2000. Local and systemic induction of two defense-related subtilisin-like protease promoters in transgenic *Arabidopsis* plants. Luciferin induction of PR gene expression. *Plant Physiology* **124**, 1049–57.

Lozano-Juste J, Colom-Moreno R, León J, 2011. *In vivo* protein tyrosine nitration in *Arabidopsis thaliana*. *Journal of Experimental Botany* **62**, 3501–17.

Pfaffl MW, 2001. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* **29**, e45.

Romero-Puertas MC, Campostrini N, Mattè A *et al.*, 2008. Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Proteomics* **8**, 1459–69.

Saito S, Yamamoto-Katou A, Yoshioka H, Doke N, Kawakita K, 2006. Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. *Plant and Cell Physiology* **47**, 689–97.

Schlicht M, Kombrink E, 2013. The role of nitric oxide in the interaction of *Arabidopsis thaliana* with the biotrophic fungi, *Golovinomyces orontii* and *Erysiphe pisi*. *Frontiers in Plant Science* **4**, 351.

Serrano I, Romero-Puertas MC, Rodríguez-Serrano M, Sandalio LM, Olmedilla A, 2012. Peroxynitrite mediates programmed cell death both in papillar cells and in self-incompatible pollen in the olive (*Olea europaea* L.). *Journal of Experimental Botany* **63**, 1479–93.

Signorelli S, Corpas FJ, Borsani O, Barroso JB, Monza J, 2013. Water stress induces a differential and spatially distributed nitro-oxidative stress response in roots and leaves of *Lotus japonicus*. *Plant Science* **201–202**, 137–46.

Sorokan AV, Kuluev BR, Burkhanova GF, Maksimov IV, 2014. RNA silencing of the anionic peroxidase gene impairs potato plant resistance to *Phytophthora infestans* (Mont.) de Bary. *Molecular Biology* **48**, 709–17.

Szabó C, Ischiropoulos H, Radi R, 2007. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nature Reviews Drug Discovery* **6**, 662–80.

Tada Y, Spoel SH, Pajerowska-Mukhtar K *et al.*, 2008. Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science* **321**, 952–6.

Tanou G, Filippou P, Belghazi M *et al.*, 2012. Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress. *The Plant Journal* **72**, 585–99.

Vandelle E, Delledonne M, 2011. Peroxynitrite formation and function in plants. *Plant Science* **181**, 534–9.

Vartapetian AB, Tuzhikov AI, Chichkova NV, Taliansky M, Wolpert TJ, 2011. A plant alternative to animal caspases: subtilisin-like proteases. *Cell Death and Differentiation* **18**, 1289–97.

Wood ZA, Schröder E, Harris JR, Poole LB, 2003. Structure, mechanism and regulation of peroxiredoxins. *Trends in Biochemical Sciences* **28**, 32–40.

Zhao S, Fernald RD, 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *Journal of Computational Biology* **12**, 1047–64.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 The effect of peroxynitrite donor (50 μM SIN-1) on *TPx* gene expression in leaves of susceptible and resistant potato. The qRT-PCR analyses of *TPx* were performed at 1, 3, 6, 24 and 48 h after SIN-1 treatment. Asterisks indicate values that differ significantly from reference *EF1α* and *18S RNA* at $P < 0{\cdot}05$ (*).

Figure S2 The effect of peroxynitrite donor (50 μM SIN-1) on *PR-1*, *PR-2* and *PR-3* gene expression in leaves of susceptible potato. The qRT-PCR analyses of *PR* genes were performed at 1, 3, 6, 24 and 48 h after SIN-1 treatment. Asterisks indicate values that differ significantly from H_2O-pretreated leaves at $P < 0{\cdot}05$ (*).

Table S1 Amino acid sequence of the identified homologous proteins (matched peptides derived from *Solanum tuberosum* shown in bold red).

Supporting information

(https://onlinelibrary.wiley.com/doi/abs/10.1111/ppa.12471)

Figure S1. The effect of peroxynitrite donor (50 µm SIN-1) on *TPx* gene expression in leaves of susceptible and resistant potato. The qRT-PCR analyses of *TPx* were performed at 1, 3, 6, 24 and 48 h after SIN-1 treatment.

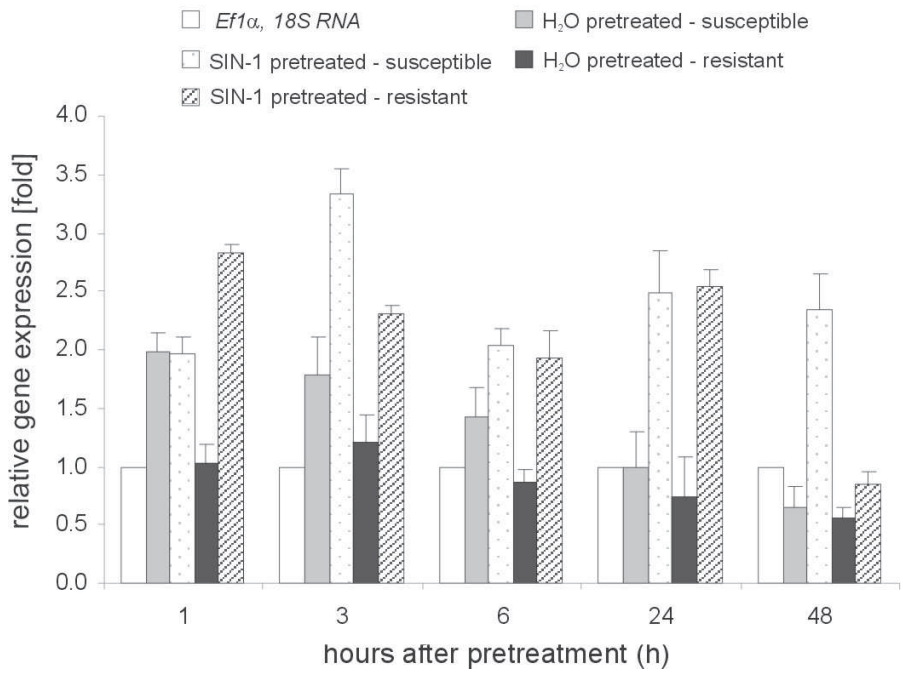


Figure S2. The effect of peroxynitrite donor (50 μm SIN-1) on *PR-1*, *PR-2* and *PR-3* gene expression in leaves of susceptible potato. The qRT-PCR analyses of *PR* genes were performed at 1, 3, 6, 24 and 48 h after SIN-1 treatment. Asterisks indicate values that differ significantly from H_2O-pretreated leaves at $P < 0.05$ (*).

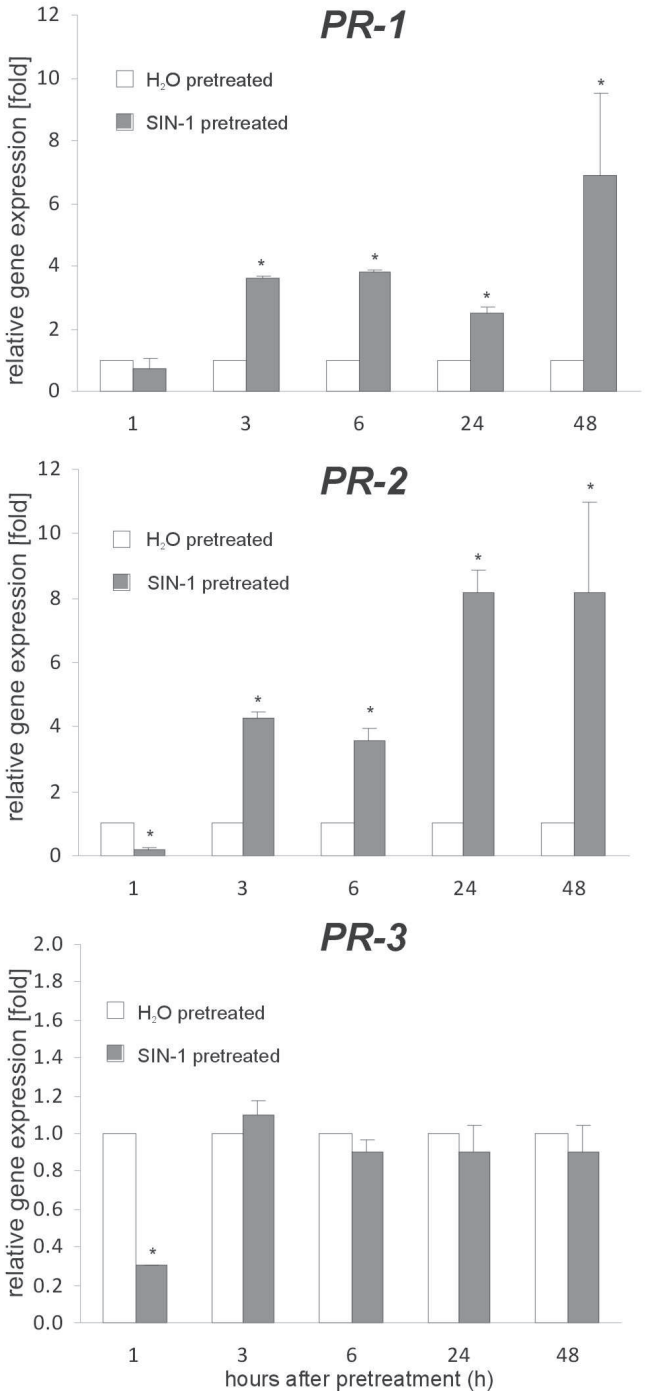


Table S1. Amino acid sequence of the identified homologous proteins (matched peptides derived from *Solanum tuberosum* shown in **Bold Red**)

No.	NCBI accession no.	Matched peptides (**BOLD RED**)
B1	**gil568215322**	1 MSKRRTREPK **EETVTLGPSV** REGELVFGVA HIFASFNDTF IHVTDLSGRE 51 TMVRITGGMK VK**ADRDESSP** **YAAMLAAQDV** **SQR**CKELGIN ALHIKLRATG 101 GNKTK**TPGPG** **AQSALR**ALAR SGMKIGR**IED** **VTPIPTDSTR** RKGGRRGRRL
	gil565347758	1 MGVNTF**THES** **TTTIAPTR**LF K**GLVLDFDSL** **VPK**LLSHDVK QFKPEEITAI 51 **AGSIK**QMNFV EGGPIKYLKH KIHAIDDKNL ETKYSLIEGD GVTVKKTGAA 101 YDVKFEAAGD GGCVCKTTTT YHTK**GDYVVS** **EEEHNVGK**GK
	gil162946541	1 MASSVISSAA VATRTNVTQA GSMIAPFTGL KSAATFPVSR KQNLDITSIA 51 SNGGRVRCMQ VWPPINTK**KY** **ETLSYLPDLT** **DEQLLK**EVEY LLKNGWVPCL 101 EFETEHGIVY REKHK**SPGYY** **DGR**YWNMWKL PMFGCTDATQ VLAEVQECKK 151 SYPQAWIR**II** **GFDNVR**QVQC ISFIAYKPEG Y
B2	**gil20087001**	1 MKCLFLLCLC LVPIVVFSST FTSQNPIN**LP** **SDATPVLDVT** **GK**ELDPRLSY 51 RIISIGR**GAL** **GGDVYLGK**SP NSDAPCANGV FR**YNSDVGPS** **GTPVR**FIGSS 101 SHFGPHIFED ELLNIQFAIS TSKLCVSYTI WKVGDYDASL GTMLLETGGT
	gil568815624	1 MERVNKLCVA FFVISMMMAM AAAQSATNVR ATYHLYNPQN INWDLRTASA 51 YCATWDADKP LAWRQRYGWT AFCGPAGPRG QASCGRCLR**V** **TNTGTGTQET** 101 **VR**IVDQCSNG GLDLDVNVFN RLDTNGLGYQ RGNLNVNYEF VNC
B3	**gil565383834**	1 MASSVISSAA VATRTNVTQA SSMVAPFTGL KSTPTFPVSR KQNLDITSIA 51 SNGGRVRCMQ VWPPINMKKY ETLSYLPDLT DEQLLKEVEY LLKNGWVPCL 101 EFETEHGFVY RENNKSPGYY DGRYWTMWKL PMFGCTDATQ VLAEVQECKN 151 AYPQAWIR**II** **GFDNVR**QVQC ISFIAYKPEG Y
B4	**gil994778**	1 MMKCLFFLCL CLFPILVFSS TFTSQNPINL PSESPVPK**PV** **LDTDGK**KLNP 51 NSSYRIISTF W**GALGGDVYL** **GK**SPNSDAPC PDGVFR**YNSD** **VGPSGTPVR**F 101 IPLSTNIFED QLLNIQFNIP TVKLCVSYTI WKVGNLNTHL WTMLLETGGT 151 IGKADSSYFK IVKSSKFGYN LLYCPITRPP IVCPFCRDDD FCAKVGVVIQ 201 NGKRR**LALVN** **ENPLDVLFQE** **V**
	gil568214675	1 MYTSKQKIHK DKDAEPSEFE VSVAQAFFDL ENTNQELKSE LKDLYINSAT 51 QIDVSGNRK**A** **VVIHVPYR**LR KAFRKVHVRL VRELEKKFSG KDVIFIATRR 101 IVRPPKRGSA AQRPRTR**TLT** **SVHDAILEDL** **VVPAEIVGK**R TRYRVDGSKI 151 MKVYLDPKER NNTEYK**LETF** **SAVYR**KLSGK DVVFEYPITE A
	gil568214772	1 MAASTQCFLH QYHALRSSPA RTSSVPSPKP NQLICRAQKQ DDANNTSNAV 51 SRRLALTLLI GTAAIGSKVS PADAAYGEAA NVFGKPKENT DFLPYNGDGF 101 KLQIPAKWNP SKEIEFPGQV LRYEDNFDST SNLMVAVTPT DKKSITDYGS 151 PEEFLSKVDY LLGKQAYFGK TDSEGGFESG AVATANLLEA SSATVGGKQY 201 YYLSVLTRTA DGDEGGK**HQL** **ITATVNDGK**L YICKAQAGDK RWFKGAKKFV
B5	**gil565344108**	1 MACSASSSTA LLSSTSRASI SPKSHISQSI SVPSAFNGLR NCKPFVSRVA 51 RSISTRVAQS ERRRFAVCAS SELPLVGNQA PDFEAEAVFD QEFIKVKLSE 101 YIGKKYVILF FYPLDFTFVC PTEITAFSDR YEEFEKVNTE VLGVSVDSVF 151 SHLAWVQTER KSGGLGDLNY PLISDVTKSI SK**SYNVLIPD** **QGIALR**GLFI 201 IDK**EGVIQHS** **TINNLGIGR**S VDETLRTLQA LQYVQENPDE VCPAGWKPGE
	gil82621122	1 MGAYTYVSEL WRKTSDVMSL AKVEVLEYRQ LPSMVRVTRP TRPDKARRLG 51 YKAKQGYVVY RVRVKRGGRK RPVSK**GIVYG** **KPTNQGVTQL** KFQRSKRSVA 101 EERAGRKLGG LR**VLNSYWIN** **EDSTYK**YF
	gil565342863	1 MSTASINNCL TISPAQASLK KPTRPVAFAR LGNSSSSSSI PSLIRNEPVF 51 AAPTPIINPI VREEMAKESY DQAIAALEKL LSEKAELGPV AAARVDQITA 101 ELKSADGSKA FDPVEHMKAG FIHFKTEKYD TNPALYGELA KGQSPKFMVF 151 ACSDSRVCPS HVLNFQPGEA FMVR**NIANMV** **PAYDK**VRYSG VGAAIEYAVL 201 HLKVENIVVI GHSACGGIKG LMSLPEDGSE STAFIEDWVK ICLPAKAKVL 251 ADHGDKEFGH QCTACEKEAV NVSLGNLLTY PFVREGLVKK TLALK**GGYYD** 301 **FVK**GGFELWG LEFGLSPPLS VKDVASILHW RLM
	gil565342865	1 MSTASINNCL TISPAQASLK KPTRPVAFAR LGNSSSSSSI PSLIRNEPVF

		51	AAPTPIINPI	VREEMAKESY	DQAIAALEKL	LSEKAELGPV	AAARVDQITA
		101	ELKSADGSKA	FDPVEHMKAG	FIHFKTEKYD	TNPALYGELA	KGQSPKFMVF
		151	ACSDSRVCPS	HVLNFQPGEA	FMVRNIANMV	PAYDKVRYSG	VGAAIEYAVL
		201	HLKVENIVVI	GHSACGGIKG	LMSLPEDGSE	STAFIEDWVK	ICLPAKAKVL
		251	ADHGDKEFGH	QCTACEKEAV	NVSLGNLLTY	PFVREGLVKK	TLALKGGYYD
		301	FVKGGFELWG	LEFGLSPPLS	V		
B6		1	MVSLKLQKRL	AASVLKCGRG	KVWLDPNEGN	EISMANSRQN	IRKLVKDGFI
		51	IRKPTKIHSR	SRARRMKEAK	RKGRHSGYGK	RKGTREARLP	TKVLWMRRLR
	gil568214986	101	VLRRLLRKYR	ESKKIDKHMY	HDMYMKVKGN	VFKNKRVLME	NIHKTKAEKA
		151	REKTLSDQFE	ARRAKNKASR	ERKFARREER	LAQGPGGEKP	VQQPAAPAAA
		201	APAQPAQGI				
		1	MRLLVLAWFS	VLCLKCVLAQ	DVGSLINKNL	FERIFLHRND	AACAAKGFYT
		51	YEAFITATKS	FAAFGTTGDT	NTRKKEIAAF	LAQTSHETTG	GWATAPDGPY
	gil21495	101	SWGYCFKQEQ	GSPGDYCVAS	QQWPCAPGKK	YFGRGPIQIS	YNYNYGPAGR
		151	AIGVNLLNNP	DLVANDAVVS	FKTALWFWMT	PQQPKPSAHD	VITGRWSPSA
		201	ADSAAGRVPG	FGVITNIING	GIECSKGSNA	QMDNRIGFYR	RYCQILGVDP
		1	MATPSVQQIR	PASLDSTSEP	PALFDGTTRL	YISYICPFAQ	RAWITRNFKG
		51	LQDKIELVPI	DLQNRPVWYK	EKVYPQNKVP	SLEHKNKVIG	ESLDLVKYID
	gil565343884	101	SNFEGPSLLP	DDPEKQKFAE	ELIAYSDTFL	KEIYGNFKGD	IEKHAGPQFD
		151	YLEKALDKFD	DGPFFLGQFS	QADIVYAPFV	ERFQIFLKEV	FDYDITSGRP
		201	KLAKWIEELN	KLDSYIQTKA	DPKEVVDLYK	KKYLA	
B7		1	MGISRDSMHK	RRATGGKKKS	WRKKRKYEMG	RQSANTKLVP	NAKTVRRIRV
		51	RGGNVKWRAL	RLDTGNYSWG	SEAVTRKTRL	LDVVYNASNN	ELVRTQTLVK
	gil568215087	101	SAIVQVDAAP	FKQWYLQHYG	VEIGRKKKSA	AKKEGEEAAE	GAAEEKKSNH
		151	VQRKLEKRQQ	DRKIDPHVEE	QFASGRLLAA	ISSRPGQCGR	ADGYILEGKE
		201	LEFYMKKLQK	KKGKGASGAT	A		
		1	MAAKKTARNP	ELIRGVGKFS	RSKMYHKKGL	WAIKKKNGGA	LPVHSKKPAA
		51	APAAEKSPKF	YPADDVKKPL	VNKHKPKPTK	LRSSITPGTV	LIILAGRFKG
	gil565371561	101	KRVVFLKQLV	SGLLLVTGPF	KFNGVPLRRV	NQAYVIGTST	KVDISGVSVD
		151	KIDDKYFAKQ	AEKKQKKGEG	EFFEEKKEEK	NVLPQEKKDD	QKAVDAALIK
		201	AIEAVPELKG	YLSARFSLKS	GMKPHELVF		
B8		1	AAATLMQPTK	VGARNNLQLR	SVQSVSKAFG	VEQGSARLTC	SLQTEIKELA
		51	QKCTDAAKIA	GFALATSALV	VSGANAEGVP	KRLTYDEIQS	KTYMEVKGTG
		101	TANQCPTIEG	GVGSFAFKPG	KYTAKKFCLE	PTSFTVKAEG	VSKNSAPDFQ
	gil313586398	151	KTKLMTRLTY	TLDEIEGPFE	VSPDGTVKFE	EKDGIDYAAV	TVQLPGGERV
		201	PFLFTIKQLV	ASGKPESFSG	EFLVPSYRGS	SFLDPKGRGG	STGYDNAVAL
		251	PAGGRGDEEE	LQKENVKNTA	SLTGKITLSV	TQSKPETGEV	IGVFESIQPS
		301	DTDLGAKVPK	DVKIQGIWYA	Q		
		1	MATSQIAVIV	LLGLLVATNI	HITEAQLGVC	YGMMGNNLPS	HSEVIQLYKS
		51	RNIGRLRLYD	PNQGALNALR	GSNIEVILGL	PNVDVKHIAS	GMEHARWWVQ
	gil568214854	101	KNVKDFWPDV	KIKYIAVGNE	ISPVTGTSSL	TSFQVPALVN	IYKAVGEAGL
		151	GNDIKVSTSV	DMTLIGNSYP	PSQGSFRNDV	RWFTDPIVGF	LRDTRAPLLV
		201	NIYPYFSYSG	NPGQISLPYA	LFTAPNVVVQ	DGSRQYRNLF	DAMLDSVYAA
		251	MERTGGGSVG	IVVSECGWPS	AGAFGATQDN	AATYLRNLIQ	HAKEGSPRKP
		301	GPIETYIFAM	FDENNKNPEL	EKHFGLFSPN	KQPKYNLNFG	VSERVWDISA
		351	ETNSTASSLI	SEM			
B9		1	MLDSGKQIDF	SKRKKKIYLA	IVASVLLVAA	VIGVVAGVKS	RSNNSDDDAD
		51	IMAISSSAHA	IVKSACSNTL	HPELCYSAIV	NVTDFSKKVT	SQKDVIELSL
		101	NITVKAVRRN	YYAVKELIKT	RKGLTPREKV	ALHDCLETMD	ETLDELHTAV
		151	ADLELYPNKK	SLKEHAEDLK	TLISSAITNQ	ETCLDGFSHD	EADKKVRKVL
	gil568214923	201	LKGQKHVEKM	CSNALAMICN	MTNTDIANEM	KLSGSRKLVE	DNGEWPEWLS
		251	AGDRRLLQSS	TVTPDVVVAA	DGSGDYKTVS	EAVAKAPEKS	SKRYVIRIKA
		301	GVYRENVDVP	KKKTNIMFMG	DGRSNTIITA	SRNVQDGSTT	FHSATVAAVG
		351	EKFLARDITF	QNTAGASKHQ	AVALRVGSDL	SAFYKCDILA	YQDTLYVHSN
		401	RQFFVQCLVA	GTVDFIFGNG	AAVLQDCDIH	ARRPGSGQKN	MVTAQGRTDP
		451	NQNTGIVIQK	CRIGATSDLR	PVQKSFPTYL	GRPWKEYSRT	VIMQSSITDV

	gil568215000	1 MAAHSSTLTI SLLLMLIFST LSSASDMSII SYDETHIHHR SDDEVSALYE 51 SWLIEHGKSY NALGEKDKRF QIFKDNLKYI DEQNSVPNQS YKLGLTKFAD 101 LTNEEYRSIY LGTKSSGDRR KLSKNKSDRY LPKVGDSLPE SVDWRDKGVL 151 VGVKDQGSCG SCWAFSAVAA MESINAIVTG NLISLSEQEL VDCDKSYNEG 201 CDGGLMDYAF EFVINNGGID TEEDYPYKER NDVCDQYRKN AKVVK**IDSYE** 251 **DVPVNNEK**AL QKAVAHQPVS IAIEAGGRDL QHYKSGIFTG KCGTAVDHGV 301 VAAGYGSENG MDYWIVRNSW GAKWGEKGYL RVQRNVASSS GLCGLATEPS
	gil568214310	1 MRLSACSLLF SLLLLTVSAE QCGRQAGGAP CAAGLCCSNV GWCGNTDDYC 51 GPGKCQSQCP SGPSPKPPTP GPGPSGGDIG DVISNSMFDQ LLMHRNENSC 101 EGKNNFYSYN AFINAAR**SFS** **GFGTTGDTTA** **R**KKEIAAFFA QTSHETTGGW 151 ASATDGPYAW GYCFIRERND HREHCTPSSQ WPCAPGRKYF GRGPIQISYN 201 YNYGPCGRAL EVDLLNNPDL VATDPVISFK SAIWFWMTPQ SPKPSCHDVI 251 IGSWQPSDAD RAANRLPGFG VITNIINGGK ECGHGNDTQV QDRIGFFRRY
B10	**gil565368099**	1 MASCSTFTPG IPSCRNFMRT LRPSSPSWRK SRVVTAKLAQ KNAMQYRKLG 51 DSDLNISEIT IGTMTFGEQN TEK**EAHEILS** **YAFDQGINII** **DTAEAYPVPM** 101 **RKETQGTTDL** **YISSWMK**SQP RDKVILATKV CGYSERSSYI RENAKVLRVD 151 AANIRESVEK SLKRLNTDYI DLLQIHWPDR **YVPLFGEFFY** **ETSK**WRPSVP 201 FVEQLRAFQE LIDEGKVRYI GVSNETSYGV MEFVHAAKVE GLPKIVSIQN 251 SYSLLVRCHF EVDLVEVCHP NNCNIGLLSY SPLAGGTLSG K**YLDNNSEAA** 301 **K**KGRLNLFPG YMERYNKSLA KEATKKYEEV AKKHGLTLVE LALGFAR**DRP** 351 **FMTSSIIGAT** **SVDQLK**EDID AFLTTERPLP PQVMDDIEDI FKRYRDPASR
	gil565347367	1 MASASLLKIS PVLDKTEFVK GQSLRLPSVS VRCHPASPSA LTVRASSYAD 51 ELVKTAKTIA SPGRGILAMD ESNATCGKR**L** **ASIGMENTEA** **NR**QAFRTLLV 101 SVPGLGEYIS GAILFEETLY QSTVEGKKMV DVLVEQNIVP GIKVDKGLVP 151 LAGSNNESWC QGLDGLASRS AAYYQQGARF AKWRTVVSIP NGPSALAVKE 201 AAWGLARYAA ISQDNGLVPI VEPEILLDGE HNIDRTFEVA KQVWAEVFFY 251 LAQNNVMFEG ILLKPSMVTP GAECKEKATP QQVADYTLSL LRQRIPPAVP 301 GIMFLSGGQS EVEATLNLNA MNQSPNPWHV SFSYARALQN TCLKTWSGRP 351 ENVKAAQDTL LVRAKANSLA QLGKYTGEGE SDEAKKGMFV KGYVY
	gil169555	1 SFVALALAGV AIYRNTYEAI IMNNGSLLQN ASPHFDSLES GVASILTLNN 51 KKRNSDMYLR QQLTPEACVF SAVR**GVVDSA** **IDAETR**MGAS LIRLHFHDCF 101 VDGCDGGILL DDINGTFTGE QNSPPNANSA RGYEVIAQAK QSVIDTCPNI 151 SVSCADILAI AARDSVAK**LG** **GQTYNVALGR** SDARTANFTG ALTQLPAPFD 201 NLTVQIQKFN DKNFTLREMV ALAGAHTVGF ARCSTVCTSG NVNPAAQLQC 251 NCSATLTDSD LQQLDTTPTM FDKVYYDNLN NNQGIMFSDQ VLTGDATTAG 301 FVTDYSNDVS VFLGDFAAAM IKMGDLPPSA GAQLEIRDVC SRVNPTSVAS 351 M
B11	**gil568215399**	1 MAPLRIPTTT VQSLENDMAT DSAAVPLPAV MKAPIRPDVV TYVHSNISKN 51 ARQPYAVSRK AGHQTSAESW GTGRAVSRIL GCSGGGTHRA GQGAFGNMCR 101 GGRMFAPTQI WRRWHRKIPV NQKRYAVASA IAASSVPSLV LARGHR**IESV** 151 **PELPLVISDS** **IEGIEK**TSNA IKALK**QIGAY** **PDAEK**AKDSH AIRQGKGKMR 201 NRRYISRKGP LIVYGTEGAK LVKAFRNIPG VEICHVDRLN LLK**LAPGGHL** 251 **GR**FIIWTKCA YEKLDGIYGT FDKPSLKKKG YLLPRPKMVN ADLAR**IINSD** 301 **EVQSVVRPIK** KDVNKRATLK KNPLKNLNVL LKLNPYAKTA RRMSLLAEAQ 351 RVKAKKEKLD KKRHQITKEE ASAIRGASHS WYK**TMISDSD** **YAEFDNFTK**W 401 LGVSQ
	gil568214933	1 MSHRKFEHPR HGSLGFLPRK RAARHRGKVK AFPKDDPSKP CKLTAFLGYK 51 AGMTHIVREV EKPGSKLHKK ETCEAVTIVE TPPMVIVGVV GYVKTPRGLR 101 CLNTVWAQHL SEDIKRRFYK NWCKSKKKAF LKYSKKYETD EGKKDIQAQL 151 EKLKKYACVI R**VLAHTQIR**K MKGLKQKKAH LMEIQVNGGS IAQKVDFAYG 201 FFEKQVPVDA VFQKDEMIDI IGVTKGKGYE GVVTRWGVTR LPRKTHRGLR 251 KVACIGAWHP ARVSFTVARA GQNGYHHRTE MNKKVYKLGK **VGQESHTAVT** 301 **EFDR**TEKEIT PIGGFAHYGV VKDDYLLIKG CCVGPKKRVV TLRQSLLNQT 351 SRVALEEIKL KFIDTSSKFG HGRFQTTQEK QKFYGRLKA
B12	**gil21487**	1 NPSVFTKCQS SPRWAFSFSV TPLCSRRSKR IVHC**IAGDTL** **GLTRPNESDA** 51 **PK**ISIGAK**DT** **DVVQWQGDLL** **AIGATENDLA** **R**DDNSKFKNP LLQRLDSKLN

	101	GLLSAASSEE	DFSGKSGQSI	NLRLPGGRIT	LVGLGSSASS	PTSYHSLGEA
	151	AAAAAKSAQA	RNIAVSLAST	DGLSAESKIN	SASAIATGVM	LGIFEDNRFR
	201	SESKTPALES	LDILGLGTGP	EIESKIKYAE	HVCAGVILGR	ELVNAPANIV
	251	TPGALAEEAK	KIASTYSDVI	TVNILDAEQC	KELKMGAYLG	VAAAATENPP
	301	YFIHLCFKTN	SRERKTKIAL	VGKGLTFDSG	GYNLKTGAGS	KIELMKNDMG
	351	GAAAVLGAAK	ALGEIKPRGV	EVHFIVAACE	NMISGAGMRP	GDIVTASNGK
	401	TIEVNNTDAE	GRLTLADALI	YACNQGVEKI	IDLATLTGAI	VTALGPSVAG
	451	AFTPSDGLAR	EVVVAAEASG	EKLWRMPMEE	SYWESMKSGV	ADMINTGPRD
	501	GGAITGALFL	KQFVDEKVQW	LHLDIAGPVW	SDEKKNATGY	GVSTLVEWVL
	551	RNSL				
gil568215114	1	MAMAIALRRL	SATVDKPVKS	LYNGGSLYYM	SSLPNEAVYD	KEKSGVAWPK
	51	QLNAPLEVVD	PEIADIIEHE	KARQWKGLEL	IPSENFTSVS	VMQAVGSVMT
	101	NKYSEGYPGA	RYYGGNEYID	MAETLCQKRA	LEAFRLDPAK	WGVNVQPLSG
	151	SPANFQVYTA	LLKPHERIMA	LDLPHGGHLS	HGYQTDTKKI	SAVSIFFETM
	201	PYRLDESTGY	IDYDQLEKSA	TLFRPKLIVA	GASAYARLYD	YDRIRKVCNK
	251	QKAILLADMA	HISGLVAAGV	IPSPFDYADV	VTTTTHKSLR	GPRGAMIFYR
	301	KGVKEVNKQG	KEVFYDYEDK	INQAVFPGLQ	GGPHNHTITG	LAVALKQATT
	351	PEYRAYQEQV	LSNSSKFAQA	LGEKGYELVS	GGTDNHLVLV	NMKNKGIDGS
	401	RVEKVLEAVH	IAANKNTVPG	DVSAMVPGGI	RMGTPALTSR	GFLEEDFVKV
	451	ADFFDAAVKI	AVKVKAETQG	TKLKDFVATL	ESSAPIKSEI	AKLRHDVEEY
	501	AKQFPTIGFE	KETMKYKN			
gil108773138	1	MSPQTETKAS	VGFKAGVKEY	KLTYYTPEYQ	TKDTDILAAF	RVTPQPGVPP
	51	EEAGAAVAAE	SSTGTWTTVW	TDGLTSLDRY	KGRCYRIERV	VGEKDQYIAY
	101	VAYPLDLFEE	GSVTNMLTSI	VGNVFGFKAL	RALRLEDLRI	PVAYVKTFQG
	151	PPHGIQVERD	KLNKYGRPLL	GCTIKPKLGL	SAKNYGRAVY	ECLRGGLDFT
	201	KDDENVNSQP	FMRWRDRFLF	CAEALFKAQA	ETGEIKGHYL	NATAGTCEEM
	251	MKRAVFAREL	GTPIVMHDYL	TGGFTANTTL	AHYCRDNGLL	LHIHRAMHAV
	301	IDRQKNHGMH	FRVLAKALRM	SGGDHIHSGT	VVGKLEGERD	ITLGFVDLLR
	351	DDFIEQDRSR	GIYFTQDWVS	LPGVLPVASG	GIHVWHMPAL	TEIFGDDSVL
	401	QFGGGTLGHP	WGNAPGAVAN	RVALEACVKA	RNEGRDLARE	GNEIIREAAK
	451	WSPELAAACE	VWKEIVFNFA	AMDVLDK		
gil565389366	1	MASRRLIASL	LRSSAQRGAG	SGGGPISRSS	IANSIARPAS	RASPKGFLLN
	51	RAVQYATSAA	APASKPSTPP	KSSDSEASGK	ITDEFTGAGA	IGKVCQVIGA
	101	VVDVRFDDGL	PPILTALEVL	DNQIRLVLEV	AQHLGENMVR	TIAMDGTEGL
	151	VRGQPVLNTG	SPITVPVGRS	TLGRIMNVIG	EAIDERGPIT	TDHFLPIHRE
	201	APAFVEQATE	QQILVTGIKV	VDLLAPYQRG	GKIGLFGGAG	VGKTVLIMEL
	251	INNVAKAHGG	FSVFAGVGER	TREGNDLYRE	MIESGVIKLG	EKQSESKCAL
	301	VYGQMNEPPG	ARARVGLTGL	TVAEHFRDAE	GQDVLLFIDN	IFRFTQANSE
	351	VSALLGRIPS	AVGYQPTLAT	DLGGLQERIT	TTKKGSITSV	QAIYVPADDL
	401	TDPAPATTFA	HLDATTVLSR	QISELGIYPA	VDPLDSTSRM	LSPHILGEDH
	451	YNTARGVQKV	LQNYKNLQDI	IAILGMDELS	EDDKMTVARA	RKIQRFLSQP
	501	FHVAEVFTGA	PGKYVDLKES	INSFQGVLDG	KYDDLSEQSF	YMVGGIDEVI
	551	AKAEKIAKES	AA			
gil81075407	1	MALLVEKTTS	GREYKVKDMS	QADFGRLEIE	LAEVEMPGLM	ASRTEFGPSQ
	51	PFKGAKITGS	LHMTIQTAVL	IETLTALGAE	VRWCSCNIFS	TQDHAAAAIA
	101	RDSAAVFAWK	GETLQEYWWC	TERALDWGPG	GGPDLIVDDG	GDATLLIHEG
	151	VKAEEEFAKN	GTIPDPTSTD	NVEFQLVLTI	IKESLKTDPL	RYTKMKERLV
	201	GVSEETTTGV	KRLYQMQANG	TLLFPAINVN	DSVTKSKFDN	LYGCRHSLPD
	251	GLMRATDVMI	AGKVALVAGY	GDVGKGCAAA	MKQAGARVIV	TEIDPICALQ
	301	ATMEGLQVLP	LEDVVSEVDI	FVTTTGNKDI	IMVDHMRKMK	NNAIVCNIGH
	351	FDNEIDMHGL	ETFPGVKRIT	ISSNDRWVFP	DTNSGIIVLA	EGRLMNLGCA
	401	TGHPSFVMSC	SFTNQVIAQL	ELWNERSSGK	YEKKVYVLPK	HLDEKVAALH
	451	LGKLGAKLTK	LTKDQADYIS	VPVEGPYKPA	HYRY	
gil587568	1	DPSKYRPSSA	YDTPFLTTNA	GGPVYNNVSS	LTVGPRGPVL	LEDYYLIEKL
	51	ATFDREKIPE	RVVHARGASA	KGFFEVTHDI	SHLTCADFLR	APGAQTPVIC
	101	RFSTVVHERG	SPESIRDIRG	FAVKFYTREG	NFDLVGNNVP	VFFNRDAKSF
	151	PDTIRALKPN	PKSHIQENWR	ILDFFSFLPE	SLHTFAFFYD	DVCLPTDYRH
	201	MEGFGVHAYQ	LINKEGKAHY	VKFHWKPTCG	VKSMSEEEAI	RVGGTNHSHA

		251	TKDLYDSIAA	GNYPEWKLFI	QTMDPEDVDK	FDFDPLDVTK	TWPEDLLPLI
		301	PVGRLVLNRN	IDNFFAENEQ	LAFNPGHIVP	GIYYSEDKLL	QTRIFAYADT
		351	QRHRIGPNYM	QLPVNAPKCG	HHNNHRDGAM	NMTHRDEEVD	YLPSRFDPCR
		401	PAEQYPIPAC	VLNGRRTNCV	IPKENNFKQA	GERYRSWESD	RQDRYITKWV
		451	ESLSDPRVTH	EIRSIWISYL	SQADKSCGQK	VASRLTVKPT	M
B13	gil565378238	1	MKNHHIIVFL	FSFFLIMSLR	GFVSCTDQET	KVYVVVYLGEH	NGEKTLKEIE
		51	DHHYSFLHSV	KGTTTSKEDV	RASLVHSYKN	VINGFSAVLT	PQEVDMISGM
		101	EGVVSVFHSD	PYEIRPHTTR	SWDFVSLLEG	TSLLNSREEL	LQNASYGKDI
		151	IVGVMDSGVW	PESLSFSDEG	MEPVPKSWNG	ICQEGVAFNA	SHCNRKLIGA
		201	RYYLKGYEAA	AGPLNETRDF	RSPRDVDGHG	THTAGTVGGR	RVANASAIGG
		251	FAKGTATGGA	PNVRLAIYKV	CWPAPDQSLA	EGNICATDDI	LAAFDDAIAD
		301	GVHVLSISLG	SLPKSTYYTE	NAIAIGSLHA	VKKNIVVACS	AGNDGPTPST
		351	VANVAPWVIT	VGASTIDRVF	SSPIMLGNGM	IVEGQTITQI	RRRRLHPLVY
		401	AGDVEIRGTT	ASNTTGACLP	GTLSRNLVRG	KVVLCLNSDI	QASMEVKRAG
		451	GVAAILGNPF	NEIQVIPFLN	PTTVTFLDGL	NTLLTYIRTE	KHPTATLVPG
		501	NTMIGTKTAP	VMAPFSSKGP	NVVDPNILKP	DITAPGFNIL	AAWSEASSPL
		551	NIPEDHRVVK	YNIDSGTSMS	CPHVSAVIAL	LKSIHPDWSS	AAVRSALMTT
		601	STINNVVGRP	IKNATGDDAN	PFEYGSGHFR	PSKAADPGLI	YDATYTDYLL
		651	YLCSQNIRPD	LSYNCPAKVP	AASNLNYPSL	AIANMRGSSK	TVTRVVTNVG
		701	KDNSTYVVAV	RSPPGYAVDI	VPKSLRFSKL	GEKHSFNITI	IRAQSSVDRR
		751	NEFSFGRYTW	SDGVHVVQSP	IAVSSSA		
	gil565378352	1	MRLGSFVSCT	EETKVYIVYL	GEHNGDKTLK	EIEDHHCSFL	HSVKGTTSKE
		51	DVRASLVHSY	KNVINGFSAV	LTPQEVDMIS	GMEGVVSVFH	SDPYEIRPHT
		101	TRSWDFVSLL	EGTSLLNSRE	ELLQNASYGK	DIIVGVMDSG	VWPESSSFND
		151	EGMEPVPKSW	NGICQEGVAF	NSSHCNRKLI	GARYYLKGYE	AAAGPLNETR
		201	DFRSPRDVDG	HGTHTAGTVG	GRRVANASAI	GGFAKGTATG	GAPNVRLAIY
		251	KVCWPVPDQS	LAEGNACATD	DILAAFDDAI	ADGVHVLSIS	LGSLPKSTYY
		301	TENAIAIGSL	HAVKKNIVVA	CSAGNDGPTP	STVGNVAPWI	ITVGASSIDR
		351	VFSSPIMLGN	GMIVEGQTVT	PIRRRRLHPL	VYAGDVEIRG	TTTNNTSGTC
		401	LPGTLSRNLV	RGKVVLCINN	LRAASMEVKR	AGGVAAILGN	RFNEIQVTPF
		451	LDTTTVVFSY	SLNTLLTYIR	TEKNPMATLV	PGNTLIGTKP	APVMASFTSK
		501	GPNIVDPNIL	KPDITAPGFN	ILAAWSEASS	PLKMPEDRRV	VKYNMQSGTS
		551	MSCPHVSAVI	ALLKSIHPDW	SSAAIRSALM	TTSTINNVVG	RPIKNATGDD
		601	ANPFEYGSGH	FRPSRAVDPG	LVYDATYTDY	LLYLCSQNIS	LDSSFSCPEK
		651	VPTASNLNYP	SLAIANMRGS	IRTVTRVVTN	VGKDNSTYVL	GVRSPPGYVV
		701	DIVPKSLHFS	KLGEKHSFNI	TIIRAQSSVE	RRNEFSFGWY	TWNDGVHVVR
		751	SPIAVSST				
B14	gil565399387	1	METLLSSTTL	QLKPLHPPSS	FSSLHSPFSS	TSALRVKGSR	KAETFIQRSQ
		51	FSTVLPVRVS	ASSQAAAAQT	STSISIPSEM	KAWSYTDYGS	VDVLKLESNV
		101	AVPDIKEDQV	LIKVVAAALN	PIDFKRRLGK	FKATDSPLPT	VPGYDVAGVV
		151	VKVGSQVKEL	KEGDEVYGDI	NEKAIDGPNQ	FGSLAEYTAV	EEKLVALKPK
		201	NLSFAEAAAL	PLVIETAYEG	LEKAGFSAGQ	SILVLGGAGG	VGSLVIQLAK
		251	HVFGASKVAA	TSSTGKLELL	KSLGADLAID	YTKDNFEDLP	DKFDVVYDSV
		301	GQGEKAVKAV	KEGGSVVVLT	GAVTPPGFRF	VVTSNGEMLK	KLNPYLESGK
		351	VKPVIDPKGP	FPFDKVVDAF	SHLETGRATG	KVVIYPIP	
	gil565360697	1	MKAVVITIPG	GPEVLKLQEV	EDPQIKDDEI	LIKIAATALN	RADTLQRQGK
		51	YPPPKGDSEY	PGLECSGTVE	AVGKDVTRWK	IGDQVCALIG	GGGYAEKVAV
		101	PTGQVLPIPS	GVSLQDAASF	PEVACTVWST	IFMTSKLSSG	ETFLIHGGSS
		151	GIGTFAIQMA	KCLGVKVFIT	AGSEEKLAAC	KELGADVCIN	YKTEDFVTRI
		201	KEETGGKGVD	VILDNIGGSY	FQRNLDSLNV	DGRLFIIGFM	GGTVTQVNLG
		251	CLLARRLTVQ	AAGLRSRSTK	NKAQIVREVE	KNVWPAIAAG	KVKPVVYKYF
		301	PLAEAAEAHQ	LMESSKHIGK	ILLTV		
B15	gil21465	1	TIFSLLFSLL	LLNASGSNVV	HRPDALCAPG	LCCSKFGWCG	NTNDYCGPGN
		51	CQSQCPGGPG	PSGDLGGVIS	NSMFDQMLNH	RNDNACQGKN	NFYSYNAFIS
		101	AAGSFPGFGT	TGDITARKRE	IAAFLAQTSH	ETTGGWPSAP	DGPYAWGYCF
		151	LREQGSPGDY	CTPSSQWPCA	PGRKYFGRGP	IQISHNYNYG	PCGRAIGVDL
		201	LNNPDLVATD	SVISFKSAIW	FWMTPQSPKP	SXHDVITGRW	QPSGADQAAN
		251	RVPGFGVITN	IINGGLECGH	GSDSRVQDRI	GFYRRYCGIL	GVSPGDNLDC

		301	GNQRSFGNGL	LVDTV			
	gil21413	**1**	MKCLFLLCLC	LVPIVVFSST	FTSKNPINLP	SDATPVLDVA	GKELDSRLSY
		51	RIISTFWGAL	GGDVYLGKSP	NSDAPCANGI	FR**YNSDVGPS**	**GTPVR**FSHFG
		101	QGIFENELLN	IQFAISTSKL	CVSYTIWKVG	DYDASLGTML	LETGGTIGQA
		151	DSSWFKIVKS	SQFGYNLLYC	PVTSTMSCPF	SSDDQFCLKV	GVVHQNGKRR
		201	LALVKDNPLD	VSFKQVQ			

Publikacja 3.

Izbiańska K., Floryszak-Wieczorek J., Gajewska J., Meller B., Kuźnicki D., Arasimowicz-Jelonek M. (2018) RNA and mRNA nitration as a novel metabolic link in potato immune response to *Phytophthora infestans*. *Frontiers in Plant Science*, 9: 672 (IF=3,678; 40 pkt MNiSW).

frontiers in Plant Science

ORIGINAL RESEARCH
published: 29 May 2018
doi: 10.3389/fpls.2018.00672

RNA and mRNA Nitration as a Novel Metabolic Link in Potato Immune Response to *Phytophthora infestans*

*Karolina Izbiańska[1], Jolanta Floryszak-Wieczorek[2], Joanna Gajewska[1], Barbara Meller[2], Daniel Kuźnicki[2] and Magdalena Arasimowicz-Jelonek[1]**

[1] Department of Plant Ecophysiology, Faculty of Biology, Adam Mickiewicz University in Poznań, Poznań, Poland,
[2] Department of Plant Physiology, Poznań University of Life Sciences, Poznań, Poland

Peroxynitrite ($ONOO^-$) exhibits a well-documented nitration activity in relation to proteins and lipids; however, the interaction of $ONOO^-$ with nucleic acids remains unknown in plants. The study uncovers RNA and mRNA nitration as an integral event in plant metabolism intensified during immune response. Using potato-*avr/vr Phytophthora infestans* systems and immunoassays we documented that potato immunity is accompanied by two waves of boosted $ONOO^-$ formation affecting guanine nucleotides embedded in RNA/mRNA and protein tyrosine residues. The early $ONOO^-$ generation was orchestrated with an elevated level of protein nitration and a huge accumulation of 8-nitroguanine (8-NO_2-G) in RNA and mRNA pools confirmed as a biomarker of nucleic acid nitration. Importantly, potato cells lacking $ONOO^-$ due to scavenger treatment and attacked by the *avr* pathogen exhibited a low level of 8-NO_2-G in the mRNA pool correlated with reduced symptoms of programmed cell death (PCD). The second burst of $ONOO^-$ coincided both with an enhanced level of tyrosine-nitrated proteins identified as subtilisine-like proteases and diminished protease activity in cells surrounding the PCD zone. Nitration of both RNA/mRNA and proteins *via* NO/$ONOO^-$ may constitute a new metabolic switch in redox regulation of PCD, potentially limiting its range in potato immunity to *avr P. infestans*.

Keywords: peroxynitrite, reactive nitrogen species, hypersensitive response, nucleic acid nitration, *Phytophthora infestans*, potato

OPEN ACCESS

Edited by:
Péter Poór,
University of Szeged, Hungary

Reviewed by:
Christian Lindermayr,
Helmholtz Zentrum München – Deutsches Forschungszentrum für Gesundheit und Umwelt, Germany
Sergey Morozov,
Lomonosov Moscow State University, Russia

****Correspondence:***
Magdalena Arasimowicz-Jelonek
marasimowicz@wp.pl;
arasim@amu.edu.pl

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *19 February 2018*
Accepted: *02 May 2018*
Published: *29 May 2018*

Citation:
Izbiańska K, Floryszak-Wieczorek J, Gajewska J, Meller B, Kuźnicki D and Arasimowicz-Jelonek M (2018) RNA and mRNA Nitration as a Novel Metabolic Link in Potato Immune Response to Phytophthora infestans. Front. Plant Sci. 9:672. doi: 10.3389/fpls.2018.00672

INTRODUCTION

Peroxynitrite ($ONOO^-$) is a product of the extremely rapid and diffusion-controlled reaction between the two radicals, nitric oxide (NO) and superoxide anion ($O_2^{\bullet-}$). The formation of the NO cognate has been detected *in vivo* in different cellular compartments in plants exposed to various stress factors (e.g., Saito et al., 2006; Corpas et al., 2009; Arasimowicz-Jelonek et al., 2011, 2012; Corpas and Barroso, 2014). However, unlike in animals, $ONOO^-$, does not appeared to be a such destructive to plant cell metabolism and is not an essential intermediate of plant cell death (Delledonne et al., 2001; Romero-Puertas et al., 2007). Moreover, it has also been revealed that incubation of *Arabidopsis* plants in a high concentration of $ONOO^-$ (3 mM) did not lead to the cell death, even in the Prx II E mutant line with the defective expression of *PrxII E* exhibiting peroxynitrite reductase activity (Romero-Puertas et al., 2007). Additionally, there are also several premises suggesting that ONOO production occurs in plants as an integral

event of cellular metabolism, which is exactly adopted to its accumulation (Romero-Puertas et al., 2004; Gzyl et al., 2016). Therefore $ONOO^-$ production could provide an important regulatory loop for NO bioactivity under both physiological and pathophysiological states, since $ONOO^-$ can provoke tyrosine nitration, recently considered to be a regulatory mechanism for protein activity (Gzyl et al., 2016).

It is well documented that $ONOO^-$ is one of the most important species engaged in nitration of various biomolecules. Apart from proteins it can modify lipids and oligonucleotides, significantly affecting their biochemistry (Jones, 2012). In plants, more than 100 protein involved in a wide range of biological processes in *Arabidopsis thaliana* and other model plants are known to be potential targets for tyrosine nitration, although the functional significance of this modification so far has been proven only for 13 proteins (Chaki et al., 2009, 2013; Álvarez et al., 2011; Galetskiy et al., 2011; Lozano-Juste et al., 2011; Melo et al., 2011; Begara-Morales et al., 2013, 2014, 2015; Corpas et al., 2013; Holzmeister et al., 2015; Sainz et al., 2015; Takahashi et al., 2015). The presence of endogenous nitro-linolenic acid in *Arabidopsis thaliana* was also evidenced, including data supporting the signaling role of these molecules in the tolerance mechanism against different abiotic stress factors such as wounding, salinity, cadmium, and low temperature (Mata-Pérez et al., 2016). The interaction of peroxynitrite with other important biomolecules, such as nucleic acid, to date has not been studied in plant systems. However, it is well established that various RNS, including $ONOO^-$ or nitrogen oxides, can nitrate guanine and related nucleosides and nucleotides either in the free form or embedded in DNA and/or RNA (Ihara et al., 2011). Notably, when peroxynitrite react with guanine, it forms several products, among which 8-oxoguanine (8-Oxy-G) and 8-nitroguanine (8-NO_2-G) are most abundant. Importantly, $ONOO^-$ reacts with the DNA and RNA bases at selected positions (Jena and Mishra, 2007). As indicated by Sodum and Fiala (2001), ONOO can mediate oxidation and nitration of guanine which occurs mainly at the C8 position.

Potato response to the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary is accompanied by changes in the NO metabolic status within the attacked cell (Kato et al., 2013; Abramowski et al., 2015; Arasimowicz-Jelonek et al., 2016; Floryszak-Wieczorek et al., 2016). These include $ONOO^-$ formation evidenced as an important redox regulator of defense responses involved in basal resistance (Arasimowicz-Jelonek et al., 2016). Furthermore, selective nitration of tyrosine residues in a small number of proteins recorded during the late phase of resistant response allowed us to form hypothesis that peroxynitrite might act as redox regulator also in cells undergoing hypersensitive cell death. Exploring the functional role of ONOO in the potato – *P. infestans* pathosystem, the present study demonstrates the first experimental evidence of nucleic acid nitration in plants, since the elevated formation of 8-NO_2-G within the RNA and mRNA pools was found in response to pathogen attack. What is more, the early $ONOO^-$-mediated modifications at mRNA and protein levels favor active cell death and limit its range in potato immunity to *avr P. infestans*.

MATERIALS AND METHODS

Plant Growth

The experiments were conducted on two sterile potato cultivars (*Solanum tuberosum* L.) – cv. Bintje (lacking R genes) which is highly susceptible to isolate 1.3.4.7.10.11. *P. infestans*, and cv. Bzura [carrying the R1 gene (Gebhardt et al., 2004) and the R2-like gene located in chromosome IV (Plich et al., 2015)] – highly resistant to 1.3.4.7.10.11. *P. infestans*. Plants of both cultivars derived from *in vitro* tissue culture were transferred to soil and grown for 4 weeks in a growth chamber with 16 h of light (180 μmol m^{-2} s^{-1}) at 18 ± 2°C and 60% humidity.

Pathogen Culture

Phytophthora infestans – isolate 1.3.4.7.10.11. was kindly obtained from the Plant Breeding and Acclimatization Institute, Research Division at Młochów, Poland. The oomycete was grown on a cereal-potato medium with an addition of dextrose.

Method of Inoculation

For *P. infestans* inoculation, the abaxial site of the detached leaves of both potato cultivars were sprayed with a zoospore suspension in water (conc. 2.0×10^5 per ml) and kept at 100% humidity in a growth chamber. The material for further analysis was taken until 96 h post inoculation (hpi). For the point inoculation experiment, 20 μl of the zoospore suspension were drop inoculated on the abaxial leaf surface and kept at 100% humidity in a growth chamber. The material for analysis was taken at the site of inoculation from an area of 0.5 cm in diameter (PCD zone, 1) and from the surrounding area within the radius 0.25 cm; (PCD distal zone, 2) until 96 hpi.

Peroxynitrite Donor and Scavenger Treatment

To estimate the effect of exogenous $ONOO^-$ detached potato leaves were sprayed with $ONOO^-$ donor – 50 μM SIN-1 (3-Morpholinosydnonimine, Calbiochem) which gradually decomposed to yield equimolar amounts of NO and $O_2^{\bullet-}$. Scavengers of $ONOO^-$ (50 μM ebselen or 1 mM epicatechin, Sigma) were used to evaluate the effect of endogenous on 8-NO_2-G formation. Control plants were treated with water. After 5 h of incubation leaves were gently dried and inoculated as described above.

Peroxynitrite Detection

Peroxynitrite formation was measured quantitatively using aminophenyl fluorescein (APF). Leaf disks (0.5 g) were incubated in darkness for 1 h in a mixture containing 5 μM APF in 100 mM phosphate buffer (pH 7.4). After the incubation, the probes were transferred into 24-well plates (1 ml per well) and the fluorescence was measured using spectrofluorometer at

485 nm excitation and 510 nm emission filters. Fluorescence was expressed as a relative fluorescence units.

TUNEL Assay

The TUNEL assay measures DNA fragmentation using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling method, which involves the TdT-mediated addition of fluorescein-12-dUTP to the 3′-OH ends of fragmented DNA. The samples were studied using TUNEL fluorescein kit (Roche; United States) according to Floryszak-Wieczorek and Arasimowicz-Jelonek (2016) and examined using a fluorescence microscope (Axiostar plus, Carl Zeiss, Germany) equipped with a digital camera, with excitation at 488 nm and emission at 515 nm. Experiments were repeated four times with ten slides per treatment. A region of 100 cells from at least 5 randomly selected slices in each treatment was counted and statistically analyzed.

RNA Extraction and Poly(A)-RNA Purification

Potato leaves were frozen in liquid nitrogen and stored at −80°C until use. For RNA extraction leaves (0.5 g) were ground to a fine powder in liquid nitrogen, and total RNA was extracted using TriReagent (Sigma) according to the manufacturer's instructions. The Poly(A)-RNA from previously prepared total RNA was prepared using the GenElute mRNA Miniprep Kit (Sigma Aldrich) according to the manufacturer's protocol. Briefly, the obtained total RNA was mixed with the binding buffer and oligo(dT) beads followed by 3-min incubation at 70°C and 10-min incubation at room temperature. After centrifugation the pellet of the oligo(dT) polystyrene beads: the mRNA complex was mixed with washing buffer and transferred to spin columns. After the second washing the mRNA was eluted at 70°C using an elution buffer (10 mM Tris-HCl, pH 7.5). The quantity and quality of obtained mRNA were measured by spectrophotometric methods.

8-NO_2-G Quantification

The level of 8-nitroguanine was determined using a competitive enzyme immunoassay OxiSelect^{TM} Nitrosative DNA/RNA Damage ELISA Kit (Cell Biolabs; STA-825), similar to Phookphan et al. (2017). For the analysis, 10 mg of the sample (total RNA or mRNA) was used. The unknown 8-NO_2-Gua samples or 8-NO_2-Gua standards were first added to an 8-NO_2-Gua-BSA conjugate preabsorbed microplate. After a brief incubation, an anti-8-NO_2-Gua monoclonal antibody was added, which bind to either the 8-nitroguanine of the samples or standards or to the 8-NO_2-Gua-BSA conjugate preabsorbed on the plate. The more 8-nitroguanine in the sample, the less free antibody available to bind to the conjugate on the plate. The absorbance of the samples was measured at a wavelength of 450 nm using an iMark microplate reader (BioRad). The 8-NO_2-G content was determined by comparing with the predetermined 8-NO_2-G standard curve. Each sample was analyzed in triplicate on ELISA microplates and values presented are means of three biological replicates ($n = 9$) ± SD.

Protein 3-Nitrotyrosine Assay

3-nitrotyrosine in a protein sample was measured using the OxiSelect^{TM} Nitrotyrosine ELISA Kit (Cell Biolabs; STA-305) according to the manufacturer's protocol. Briefly, nitrated BSA or samples were added to each well of 96-well plates and incubated with an anti-nitrotyrosine antibody at a 1:1000 dilution for 1 h on an orbital shaker. Wells were then washed three times, afterward the secondary antibody was added and the mixture incubated for 1 h at room temperature. Subsequently, the wells were washed three times and the substrate solution was added to each well. Finally after the color development the reaction was stopped and optical density was measured at 450 nm using an iMark microplate reader (BioRad). The 3-nitrotyrosine content in protein samples was determined by comparing with the predetermined 3-nitrotyrosine standard curve. Each sample was analyzed in triplicate on ELISA microplates and the values presented are means of three biological replicates ($n = 9$) ± SD.

Protease Activity Assay

Protease activity was determined according to Fernández et al. (2015) using azocasein as substrate in 50 mM sodium acetate buffer (pH 5.2) and 0.5 μg of isolated protease. Samples were incubated at 37°C for 2.5 h. Protease activity was measured as an increase in the absorbance at 335 nm of the supernatant.

Trypan Blue Staining

To visualize cell death the trypan blue dye was used according to Fernández-Bautista et al. (2016).

Statistical Analysis

All results are based on three biological replicates derived from three or four independent experiments. For each experiment, means of the obtained values were calculated along with standard deviations. The analysis of variance was performed (ANOVA) and the mean values were compared by Tukey's test ($\alpha = 0.05$).

RESULTS

P. infestans Provokes Two Waves of $ONOO^-$ Formation During Potato Immune Response

Based on the folic acid method, we previously reported that the *P. infestans* challenge provoked an early and transient program of boosted ONOO formation only in the resistant potato genotype (Arasimowicz-Jelonek et al., 2016). In the present study the $ONOO^-$ production in potato leaves was detected quantitatively using APF fluorochrome up to 96 hpi. Bio-monitoring confirmed that the potato–*avr P. infestans* interaction is accompanied by a significant ONOO accumulation within the first 6 hpi; however, monitoring within the successive hours revealed a second, lower burst of $ONOO^-$ at 72 hpi (**Figure 1A**). The potato–*vr P. infestans* pathosystem revealed $ONOO^-$ formation starting from 24 hpi, which reached the highest (*ca.* 2-fold increase) level at 72 hpi (**Figure 1B**).

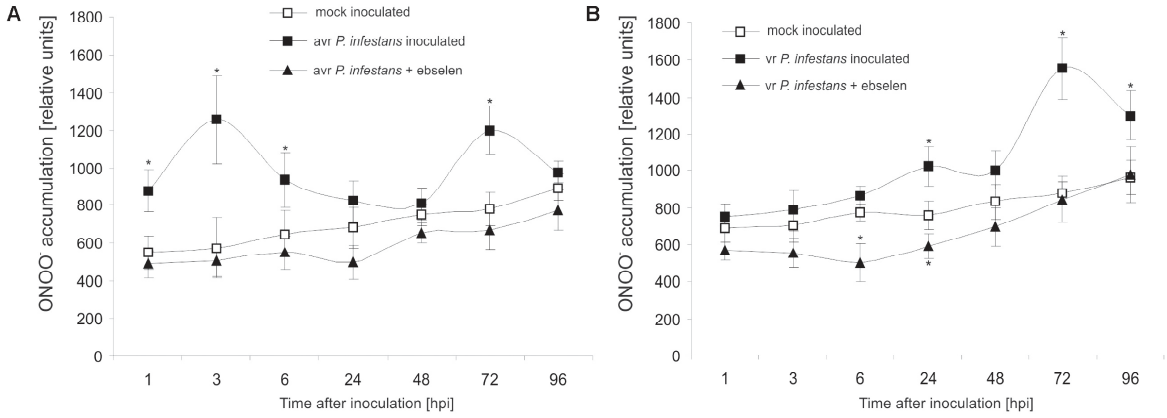


FIGURE 1 | Peroxynitrite formation measured as APF fluorescence in leaves of resistant **(A)** and susceptible **(B)** potato inoculated with *P. infestans*. Analyses were performed at 1, 3, 6, 24, 48, 72, and 96 h after challenge inoculation. Values represent the mean ± SD of at least three independent experiments (n = 9). Asterisks indicate values that differ significantly from mock inoculated (control) potato leaves at $P < 0.05$ (*).

Moreover, to verify that the detected pathogen-induced increase in APF fluorescence was caused by endogenous $ONOO^-$, its detection was performed in the presence of ebselen, an $ONOO^-$ scavenger. The used scavenger strongly suppressed fluorescence at 3rd and 24th hpi (**Figures 1A,B**), providing evidence that APF is a reliable tool to quantitatively investigate $ONOO^-$ formation in potato leaves.

The Presence of $ONOO^-$ Is Necessary for PCD During HR

The potato–*avr P. infestans* interaction resulted in HR-like cell death (Floryszak-Wieczorek and Arasimowicz-Jelonek, 2016). To gain insight into $ONOO^-$ participation in the death of pathogen attacked cells, the TUNEL assay illustrating the programmed DNA fragmentation in the presence of $ONOO^-$ scavengers was performed (**Figure 2**). Cells of potato leaves treated with *avr P. infestans* contained green-colored nuclei starting from 24 hpi (**Supplementary Figure S1**), while phenotype HR-like symptoms appeared 48 h after the *avr P. infestans* challenge (**Figure 2e**). The number of TUNEL–positive nuclei indicated that the effect was dependent on the $ONOO^-$ presence within potato leave cells, since the sequential treatment with epicatechin, a $ONOO^-$ scavenger and *P. infestans* effectively reduced the number of cells with symptoms of active death from 85 to 45% at 48 hpi (**Figures 2b,i**). Similarly, potato cells pretreated with ebselen and next elicited by the pathogen at 48 hpi showed 35% of nuclei with TUNEL–positive staining (**Figures 2c,i**). An independent application of both $ONOO^-$ scavengers resulted in reduced phenotype HR-like symptoms (**Figures 2f,g**).

Peroxynitrite Mediates 8-NO_2-G Formation Within RNA and mRNA Pools During Potato-*P. infestans* Interaction

To explore the functional role of $ONOO^-$ generation in potato leaves challenge-inoculated with *P. infestans* we examined whether, and to what extent, this RNS is able to target nucleic acids. Firstly, we confirmed that SIN-1 is effective in $ONOO^-$ formation within potato cells (**Supplementary Figure S2**). Secondly, to verify that $ONOO^-$ is able to provoke nitration at the RNA level in cells of potato, we monitored the accumulation of 8-NO_2-G, a marker of nucleic acid nitration, in healthy leaves treated with the $ONOO^-$ generator. As we expected, SIN-1 was effective in 8-NO_2-G RNA formation within potato cells of both genotypes (**Figures 3A,B**).

The potato–*avr P. infestans* interaction resulted in an impressive rise in the level of 8-NO_2-G within the RNA pool starting from the 1st hpi (**Figure 3C**). The highest increase of nitrated RNA in relation to mock inoculated leaves was noted at 3 hpi; however, a strong, *ca.* 5-fold enhancement of 8-NO_2-G RNA was observed also between 24 and 72 hpi. Then the level of 8-NO_2-G RNA declined. Surprisingly, RNA from cells of the susceptible genotype revealed a time-delayed and definitely lower level of 8-NO_2-G than did RNA of the resistant potato (**Figure 3D**). Only a 2-fold increase in RNA nitration was observed starting from 24 hpi and the enhancement was maintained during disease progress.

Since mRNA modifications have the potential to affect most post-transcriptional steps in gene expression (Gilbert et al., 2016), in the next set of experiments we verified if $ONOO^-$ targets mRNA in potato leaves as well. Interestingly, the use of poly(A)-RNA purified from total RNA showed a significant 2-fold increase in the level of 8-NO_2-G mRNA at 1 hpi, peaking at 3 and 48 hpi, respectively (**Figure 3E**). The amount of 8-NO_2-G mRNA in cells of the susceptible genotype was raised at 6 hpi, reaching the highest level at 72 hpi (**Figure 3F**). It should be highlighted that the steady-state level of 8-NO_2-G mRNA observed within the first 48 h of the experiment was significantly higher in healthy leaves of resistant potato than in the susceptible one (**Figures 3E,F**). What is more, cells of both potato genotypes lacking $ONOO^-$ due to the scavenger application and next attacked by the pathogen exhibited significantly lower levels of 8-NO_2-G both in RNA and mRNA (**Figures 3C–F**).

Protein Nitration as a Switch of the Redox Environment During HR Establishment

The nitrative modification of RNA and mRNA overlapped with the tyrosine residue nitration in proteins within the first 24 h after *avr P. infestans* challenge inoculation (**Figure 4A**). Based on the immunoassay (**Supplementary Figure S3**) we found the highest, *ca.* 30-fold increase in the total protein pool undergoing tyrosine nitration at 3 hpi (**Figure 4A**). Then, the level of nitrated proteins was gradually decreased and reached the amount recorded in mock inoculated leaves at 48 and 72 hpi. Interestingly, a *ca.* 3-fold increase of nitrated proteins at 96 hpi was followed by the second burst of $ONOO^-$ formation (**Figure 4A**). Additional experiment involving point inoculation revealed that only cells surrounding the PCD zone showed an elevated level of protein nitration at 96 hpi identified as subtilisin-like proteases (**Figures 5A–C** and **Supplementary Tables S1**,**S2**). Moreover, changes noted within the PCD distal zone coincided with the significantly reduced protease activity (**Figure 5D**). In contrast, disease progress observed during the potato-*vr P. infestans* interaction was accompanied by an increase of the nitrated protein pool starting from 24 hpi (**Figure 4B**).

DISCUSSION

The hypersensitive response (HR) considered as a form of programmed cell death (PCD) is a hallmark of effector-triggered immunity (ETI). According to current knowledge, properly balanced doses of NO and ROS co-operate to trigger PCD in plants (Delledonne et al., 2001; Zago et al., 2006). To date the role of $ONOO^-$ in HR has been ambiguous. Although application of 1 mM $ONOO^-$ has been reported to cause some necrotic lesions in *Arabidopsis* (Alamillo and García-Olmedo, 2001), high concentration of peroxynitrite does not seem to be essential to PCD induction (Delledonne et al., 2001; Romero-Puertas et al., 2007). That is probably due to the capacity of plants to detoxify $ONOO^-$ under normal physiological conditions.

Some evidence demonstrated that $ONOO^-$ in plants fulfills an important role in the creation of a cellular redox milieu promoting defense expression (Durner et al., 1998; Alamillo and García-Olmedo, 2001; Arasimowicz-Jelonek et al., 2016). Our earlier results showed that NO and O_2^- coexisted after potato leaf inoculation (Arasimowicz-Jelonek et al., 2016) and both molecules were needed to activate PCD in the potato-*avr P. infestans* system. Using the spectrofluorometric method for $ONOO^-$ detection we verified that the potato-*avr P. infestans* interaction is accompanied by $ONOO^-$ formation within the first 6 hpi and revealed an additional wave of $ONOO^-$ during the following hpi. The first burst of ONOO definitely preceded the TUNEL-positive reaction of cell nuclei. What is more, significantly reduced numbers of cells undergoing PCD were observed in potato leaves pretreated with epicatechin and ebselen, confirming that $ONOO^-$ next to NO and ROS participates in the active cell-death program.

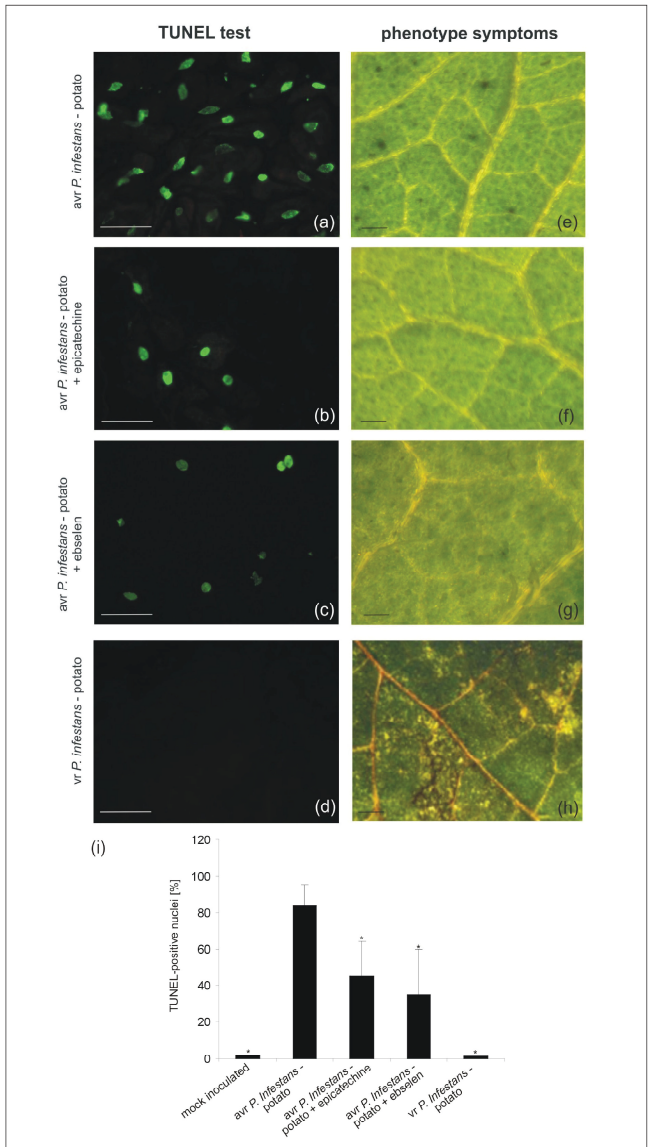


FIGURE 2 | TUNEL test illustrating programmed DNA fragmentation **(a–d)** and phenotype symptoms **(e–h)** on potato leaves inoculated with *P. infestans* at 48 hpi. Representative images of TUNEL-positive nuclei **(a)** and HR-like lesions **(e)** observed during the *avr P. infestans*-potato interaction; TUNEL-positive reaction in leaves of potato pretreated with epicatechin and next inoculated with *avr P. infestans* **(b)**; TUNEL-positive reaction in leaves of potato pretreated with ebselen and next inoculated with *avr P. infestans* **(c)**; TUNEL-negative reaction **(d)** and disease spots **(h)** observed during the *vr P. infestans*-potato interaction. Bars indicate 15 μm **(a–d)** and 500 μm **(e–h)**. The percentage of leaf cells exhibiting the TUNEL-positive reaction at 48 hpi **(i)**, 100 cells from at least 5 randomly selected slides were examined at each time point per treatment. Values represent the mean ± SD of four independent experiments ($n = 20$). Asterisks indicate values that differ significantly from *P. infestans* inoculated potato leaves at $P < 0.05$ (*).

Searching for a novel link connecting an early $ONOO^-$ formation with PCD during the potato-*avr P. infestans* interaction we found that not only protein tyrosine residues are affected by nitration activity of this RNS. In this study we

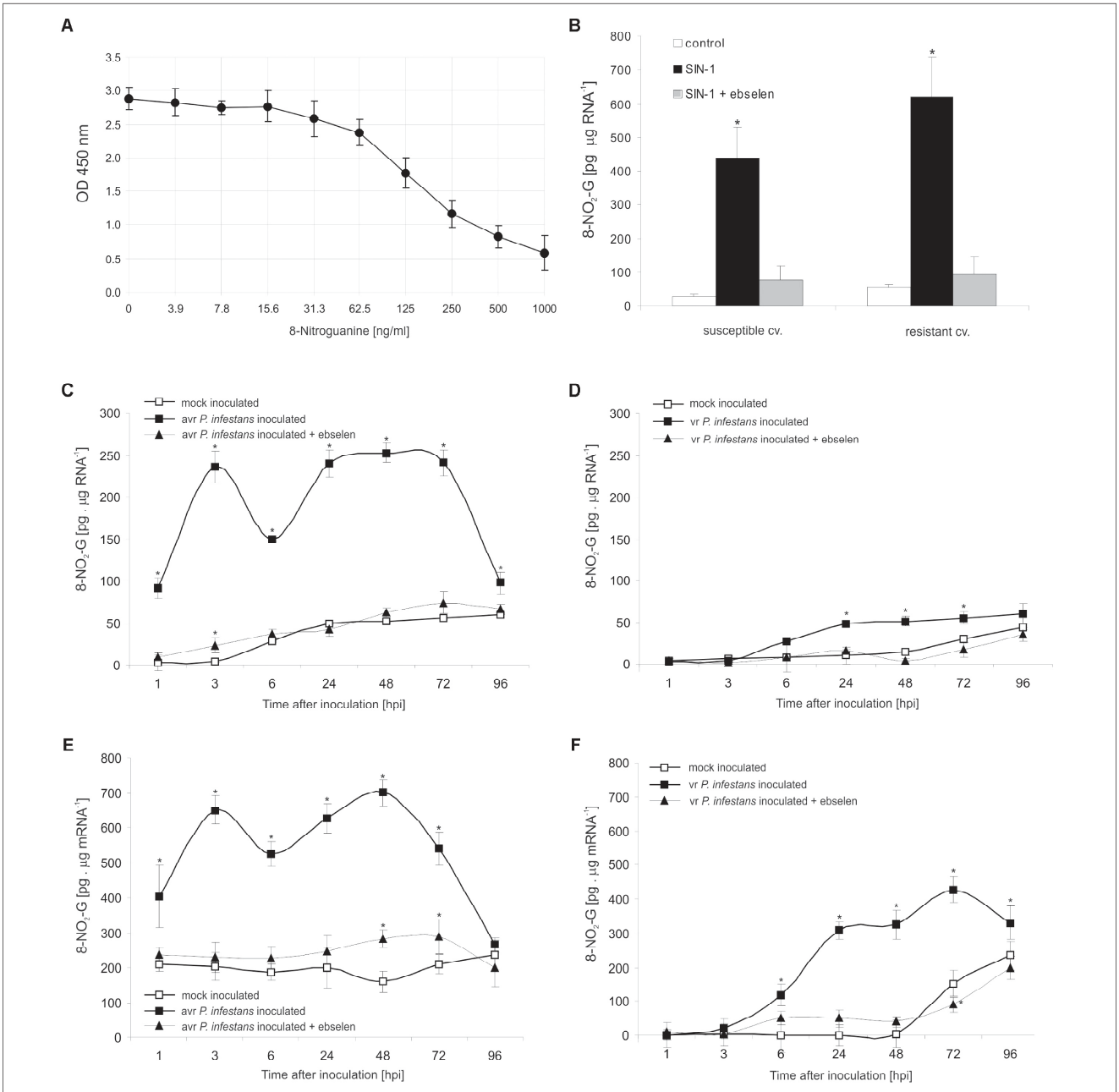


FIGURE 3 | RNA and mRNA nitration in healthy and challenge inoculated leaves of potato. 8-Nitroguanine ELISA standard curve **(A)** in the concentration range of 0 ng/mL to 1000 ng/mL; **(B)** quantification of nitrated RNA measured as 8-NO_2-G content in resistant and susceptible healthy potato leaves enriched with $ONOO^-$; 8-NO_2-G content was estimated 5 h after leaf pretreatment with 50 µM SIN-1. Quantification of nitrated RNA measured as 8-NO_2-G content in leaves of resistant **(C)** and susceptible **(D)** potato inoculated with *P. infestans*. Quantification of nitrated mRNA measured as 8-NO_2-G content in leaves of resistant **(E)** and susceptible **(F)** potato inoculated with *P. infestans*. Analyses were performed at 1, 3, 6, 24, 48, 72, and 96 h after challenge inoculation. Values represent the mean ± SD of at least three independent experiments ($n = 9$). Asterisks indicate values that differ significantly from mock inoculated (control) potato leaves at $^*P < 0.05$.

demonstrated for the first time that also the guanine nitration phenomenon occurs in plant cells. Nitration of nucleotides in DNA and RNA and the resulting 8-NO_2-G formed in response to RNS were first suggested to be activated in hamster livers infected with *Opisthorchis viverrini* (Pinlaor et al., 2003), and in the human gastric mucosa infected with *Helicobacter pylori* (Ma et al., 2004). In both cases the phenomenon was associated with infection- or inflammation-induced carcinogenesis (Terasaki et al., 2006). Although most of the studies regarding to nucleic acid nitration is focused on DNA, several reports indicate that RNA is more susceptible to this phenomenon (Shan et al., 2007; Liu et al., 2012; Hawkins et al., 2017).

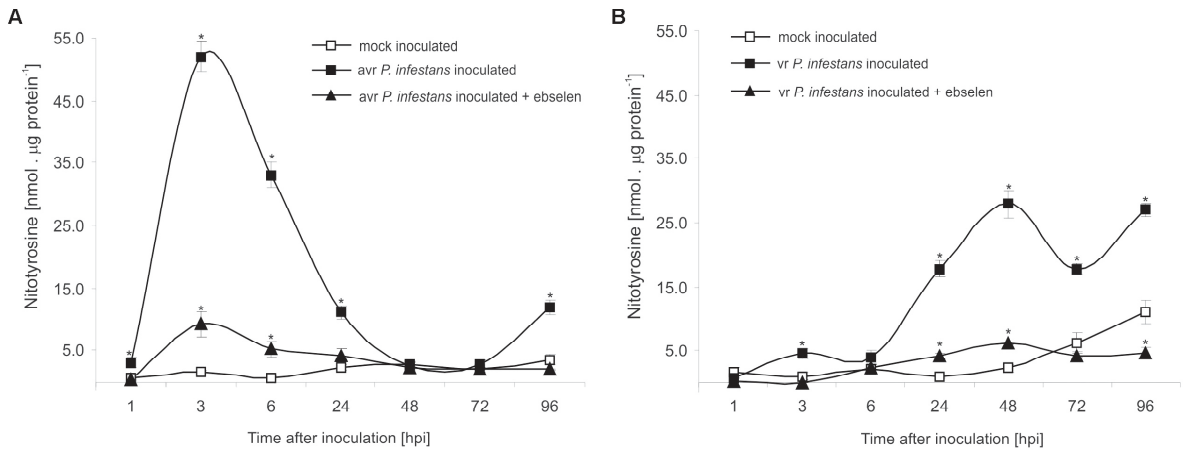


FIGURE 4 | Quantification of protein tyrosine nitration in leaves of resistant **(A)** and susceptible **(B)** potato inoculated with *P. infestans*. Analyses were performed at 1, 3, 6, 24, 48, 72, and 96 h after challenge inoculation. Values represent the mean ± SD of at least three independent experiments ($n = 9$). Asterisks indicate values that differ significantly from mock inoculated (control) potato leaves at $^*P < 0.05$.

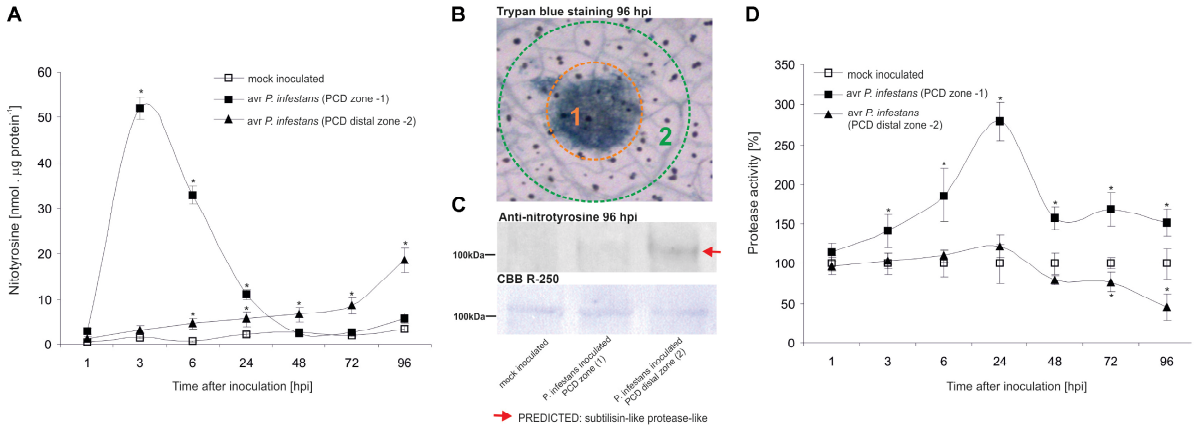


FIGURE 5 | Quantification of protein tyrosine nitration **(A)** and protease activity **(D)** in leaves of resistant potato at the site of *avr P. infestans* inoculation (PCD zone) and in the surrounding area (PCD distal zone). Analyses were performed at 1, 3, 6, 24, 48, 72, and 96 h after challenge inoculation. Values represent the mean ⊥ SD of at least three independent experiments ($n = 9$). Asterisks indicate values that differ significantly from mock inoculated (control) potato leaves at $^*P < 0.05$. **(B)** Trypan blue staining illustrating the PCD zone (1) and PCD distal zone (2) at 96 hpi. **(C)** Representative immunoblot showing immunoreactive bands containing subtilisin-like proteins undergoing tyrosine nitration in potato leaves challenge inoculated with *avr P. infestans* at 96 hpi. Western blot was probed with a rabbit anti-nitrotyrosine polyclonal antibody at a 1:1.000 dilution. The loading control was determined by staining the blot with CBB R-250. The indicated band was isolated from PVDF membrane and identified by LC-MS-MS/MS as described in **Supplementary Data Sheet S1**.

8-NO_2-G was detected in both potato genotypes challenge inoculated with the pathogen. However, only the resistant response was accompanied by a significant accumulation of the nitrated RNA pool starting from the first hpi. What is more, the modification dropped after 72 hpi. A similar trend was observed also within the mRNA pool. Thus, we conclude that mRNA nitration is an early event preceding or coincident with the first symptoms of PCD during HR and this process is not merely a consequence of dying cells. In confirmation, disease symptom development was accompanied by low levels of RNA/mRNA nitration starting from 24 hpi and correlated with necrotic cell dying. Formation of 8-NO_2-G in RNA may interfere with RNA functions and metabolism, similarly as RNA modification *via* ROS (Kong and Lin, 2011; Wang and He, 2014; Fimognari, 2015). It was earlier suggested that mRNA may be a major target of oxidative modification because of its relative abundance, widespread subcellular distribution, single-strand nature and, lack of protection from histone proteins (Shan et al., 2007). Messenger RNA modification *via* oxidation was found to be an early event prior to cell death in animals, rather than a simple consequence of an already dying cell, and it was shown to induce reduced protein expression (Shan et al., 2007). The effect of oxidized bases in mRNAs may cause ribosome stalling on the transcripts, leading to a decrease of protein expression or slowing the translation process. In a similar manner, an important consequence of RNA nitration could be

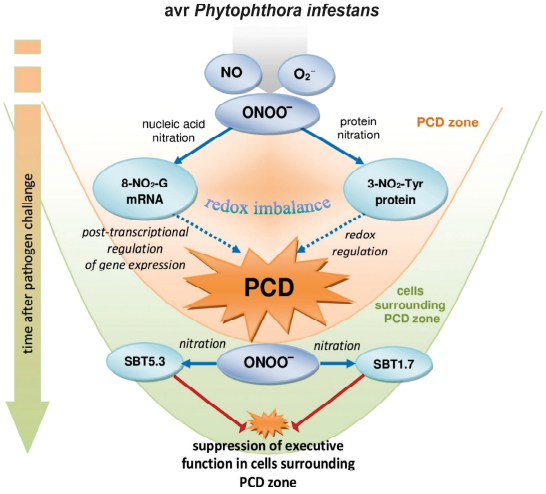


FIGURE 6 | A functional link between peroxynitrite and potato immunity in response to *avr P. infestans*: the early $ONOO^-$ generation facilitates nitrative modification of nucleic acids that could contribute to the post-transcriptional regulation of gene expression unlocking programmed cell death during HR; the second burst of $ONOO^-$ could favor protease inactivation *via* protein nitration suppressing the potential executer function of subtilisin-like proteases in cells distal from the zone of active dying cells.

an impairment of protein synthesis (Rivera-Mancía et al., 2017). Briefly, targeted RNA nitration might lead to the diminished expression of specified proteins and thus constitute a mechanism of post-transcriptional gene expression regulation. What is more, oxidized transcripts could be subjected to ribosome-based quality control and predestined for degradation through No-Go decay pathway (Simms et al., 2014). It should be noted that targeted mRNA oxidation has also been documented in plants. Namely, increased levels of 8-oxo-7,8-dihydroguanosine (8-OHG), which is a marker of RNA oxidation, were detected in sunflower seeds. Importantly, the observed modification was not a random process, but highly selective, directed toward a specific subset of 24 mRNAs, including mRNAs corresponding to genes associated with cell signaling (Bazin et al., 2011). So far, it is not clear what determine the susceptibility of mRNA to this type of modification. However, it has been proved that its sensitivity is not dependent on the abundance of specific mRNA species in the cell, the frequency of guanine in the sequence or the occurrence of any specific motif (Chang et al., 2008; Bazin et al., 2011). It should be noted that the observed increase in 8-NO_2-G mRNA in healthy leaves of susceptible potato might hinder protein synthesis or generate errors in the protein products related to senescence disorders.

Nitration of both guanine nucleotides embedded in RNA and mRNA and protein tyrosine residues can constitute an early switch of the redox environment facilitating HR establishment. Cecconi et al. (2009) observed *in vivo* an increase in nitrated proteins during the progression of hypersensitive response. In *A. thaliana* challenge inoculated with an avirulent bacterial pathogen defense responses were correlated with a modulation of nitrated proteins involved in regulation of a number of important cellular functions after 4 and 8 hpi confirming that Tyr-nitration could be a relevant physiological process in resistance (Gaupels et al., 2011). More recently, we found that *avr P. infestans* in potato leaves provoked nitration of subtilisin-like proteases, i.e., SBT1.7 and SBT5.3 belonging to serine-dependent enzymes at 48 hpi (Arasimowicz-Jelonek et al., 2016). Importantly, subtilisin-like protease (StSBTc-3) induced in potato leaves after *P. infestans* infection was found to exhibit caspase-3 like activity and display an executer function (Fernández et al., 2015). Since a physiological consequence of the $ONOO^-$ reaction with proteins often involves inactivation or impairment of its function, therefore nitration following the second burst of $ONOO^-$ may efficiently inhibit the activity of serine proteases and suppress a potential executer function in the distal zone from dying cells (**Figure 6**). In confirmation, an experiment applying point inoculation revealed nitration of SBT1.7 and SBT5.3 concomitant with reduced protease activity only in cells surrounding the PCD zone. It should be noted that the distal changes were not correlated with nitrative mRNA modification (data not presented).

CONCLUSION

Our study demonstrates that the nitration phenomenon presents a much more complex functionality in plant cells than it was assumed previously. The modification of RNA and mRNA *via* $ONOO^-$ is an integral part of plant cell metabolism and is intensified in response to pathogen attack. Although nitrative modification of bases in RNA and mRNA can be simply induced by an enhanced peroxynitrite level in the cellular milieu, the rate of $ONOO^-$ formation is dependent on the plant genetic makeup. An early and transient program of boosted $ONOO^-$ formation during the potato resistant response accelerated the time-dependent switch of the redox environment *via* the nitration phenomenon. The observed nitrative modification of RNA and mRNA could regulate the post-transcriptional gene expression and fine-tune cell signaling that contributes to PCD during HR (**Figure 6**). In confirmation, $ONOO^-$ elimination overlapped with a reduced pool of nitrated mRNA and the number of cells that undergo programmed cell death. In contrast, a time-delayed peroxynitrite over-accumulation in the potato-*vr P. infestans* interaction coincident with a relatively low level of 8-NO_2-G in the RNA/mRNA pools resulted in failed resistance. The challenge for the future is to understand the mechanisms of selective mRNA nitration and the physiological consequences of mRNA nitration. To this aim identification of mRNA nitration targets during plant responses to pathogen attack should be experimentally verified.

AUTHOR CONTRIBUTIONS

MA-J and JF-W planned and designed the research. KI, JG, BM, and DK performed the experiments. KI collected, analyzed the data and participated in writing the manuscript. MA-J wrote the manuscript.

FUNDING

This work was supported by the grant of National Science Center – project no. NCN 2014/13/B/NZ9/02177.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00672/full#supplementary-material

FIGURE S1 | Percentages of leaf cells exhibiting the TUNEL-positive reaction at 24, 48, and 72 hpi (i), 100 cells from at least 5 randomly selected slides were examined at each time point per treatment. Values represent the mean ± SD of at least four independent experiments ($n = 20$). Asterisks indicate values that differ significantly from *P. infestans* inoculated potato leaves at $^*P < 0.05$.

FIGURE S2 | $ONOO^-$ generation measured as APF fluorescence in healthy leaves of resistant and susceptible potato in response to increasing SIN-1 concentration. Values represent the mean ± SD of at least three independent experiments ($n = 9$). Asterisks indicate values that differ significantly from non-treated potato leaves at $^*P < 0.05$.

FIGURE S3 | (a) 3-Nitrotyrosine ELISA standard curve in the concentration range of 0–8000 nM; **(b)** quantification of nitrated proteins measured as 3-nitrotyrosine content in resistant and susceptible healthy potato leaves enriched with $ONOO^-$; 3-nitrotyrosine content was estimated 5 h after leaf pretreatment with 50 μM SIN-1.

TABLE S1 | Identification of nitrated proteins in potato leaves inoculated with *avr P. infestans* which appeared de novo at 96 hpi.

TABLE S2 | Amino acid sequence of the identified homologous proteins (matched peptides derived from *Solanum tuberosum* shown in Bold Red).

DATA SHEET S1 | Nitrotyrosine immunodetection and protein identification procedures.

REFERENCES

Abramowski, D., Arasimowicz-Jelonek, M., Izbiańska, K., Billert, H., and Floryszak-Wieczorek, J. (2015). Nitric oxide modulates redox-mediated defense in potato challenged with *Phytophthora infestans*. *Eur. J. Plant Pathol.* 143, 237–260.

Alamillo, J. M., and García-Olmedo, F. (2001). Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. *Plant J.* 25, 529–540.

Álvarez, C., Lozano-Juste, J., Romero, L. C., García, I., Gotor, C., and León, J. (2011). Inhibition of Arabidopsis O-acetylserine (thiol) lyase A1 by tyrosine nitration. *J. Biol. Chem.* 286, 578–586. doi: 10.1074/jbc.M110.147678

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., Deckert, J., Rucińska-Sobkowiak, R., Gzyl, J., Pawlak-Sprada, S., et al. (2012). Nitric oxide implication in cadmium-induced programmed cell death in roots and signaling response of yellow lupine plants. *Plant Physiol. Biochem.* 58, 124–134. doi: 10.1016/j.plaphy.2012.06.018

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., and Gwóźdź, E. A. (2011). The message of nitric oxide in cadmium challenged plants. *Plant Sci.* 181, 612–620. doi: 10.1016/j.plantsci.2011.03.019

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., Izbiańska, K., Gzyl, J., and Jelonek, T. (2016). Implication of peroxynitrite in defense responses of potato to *Phytophthora infestans*. *Plant Pathol.* 65, 754–766.

Bazin, J., Langlade, N., Vincourt, P., Arribat, S., Balzergue, S., and El-Maarouf-Bouteau, H. (2011). Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *Plant Cell* 23, 2196–2208. doi: 10.1105/tpc.111.086694

Begara-Morales, J. C., Chaki, M., Sánchez-Calvo, B., Mata-Pérez, C., Leterrier, M., Palma, J. M., et al. (2013). Protein tyrosine nitration in pea roots during development and senescence. *J. Exp. Bot.* 64, 1121–1134. doi: 10.1093/jxb/ert006

Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., Mata-Pérez, C., Valderrama, R., Padilla, M. N., et al. (2015). Differential molecular response of monodehydroascorbate reductase and glutathione reductase by nitration and S-nitrosylation. *J. Exp. Bot.* 66, 5983–5996. doi: 10.1093/jxb/erv306

Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., López-Jaramillo, J., et al. (2014). Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. *J. Exp. Bot.* 65, 527–538. doi: 10.1093/jxb/ert396

Cecconi, D., Orzetti, S., Vandelle, E., Rinalducci, S., Zolla, L., and Delledonne, M. (2009). Protein nitration during defense response in *Arabidopsis thaliana*. *Electrophoresis* 30, 2460–2468. doi: 10.1002/elps.200800826

Chaki, M., Carreras, A., López-Jaramillo, J., Begara-Morales, J. C., Sánchez-Calvo, B., Valderrama, R., et al. (2013). Tyrosine nitration provokes inhibition of sunflower carbonic anhydrase (β-CA) activity under high temperature stress. *Nitric Oxide* 29, 30–33. doi: 10.1016/j.niox.2012.12.003

Chaki, M., Valderrama, R., Fernández-Ocaña, A. M., Carreras, A., López-Jaramillo, J., Luque, F., et al. (2009). Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. *J. Exp. Bot.* 60, 4221–4234. doi: 10.1093/jxb/erp263

Chang, Y., Kong, Q., Shan, X., Tian, G., Llieva, H., and Cleveland, D. W. (2008). Messenger RNA oxidation occurs early in disease pathogenesis and promotes motor neuron degeneration in ALS. *PLoS One* 3:e2849. doi: 10.1371/journal.pone.0002849

Corpas, F. J., and Barroso, J. B. (2014). Peroxynitrite ($ONOO^-$) is endogenously produced in Arabidopsis peroxisomes and is overproduced under cadmium stress. *Ann. Bot.* 113, 87–96. doi: 10.1093/aob/mct260

Corpas, F. J., Hayashi, M., Mano, S., Nishimura, M., and Barroso, J. B. (2009). Peroxisomes are required for in vivo nitric oxide accumulation in the cytosol following salinity stress of Arabidopsis plants. *Plant Physiol.* 151, 2083–2094. doi: 10.1104/pp.109.146100

Corpas, F. J., Leterrier, M., Begara-Morales, J. C., Valderrama, R., Chaki, M., López-Jaramillo, J., et al. (2013). Inhibition of peroxisomal hydroxypyruvate reductase (HPR1) by tyrosine nitration. *Biochim. Biophys. Acta* 1830, 4981–4989. doi: 10.1016/j.bbagen.2013.07.002

Delledonne, M., Zeier, J., Marocco, A., and Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13454–13459.

Durner, J., Wendenhenne, D., and Klessig, D. F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10328–10333.

Fernández, M. B., Daleo, G. R., and Guevara, M. G. (2015). Isolation and characterization of a *Solanum tuberosum* subtilisin-like protein with caspase-3 activity (StSBTc-3). *Plant Physiol. Biochem.* 86, 137–146. doi: 10.1016/j.plaphy.2014.12.001

Fernández-Bautista, N., Domínguez-Núñez, J. A., Castellano Moreno, M. M., and Berrocal-Lobo, M. (2016). Plant tissue trypan blue staining during phytopathogen infection. *Bio Protocol* 6:E2078. doi: 10.21769/BioProtoc.2078

Fimognari, C. (2015). Role of oxidative RNA damage in chronic-degenerative diseases. *Oxid. Med. Cell Longev.* 2015:358713. doi: 10.1155/2015/358713

Floryszak-Wieczorek, J., and Arasimowicz-Jelonek, M. (2016). Contrasting regulation of NO and ROS in potato defense-associated metabolism in response to pathogens of different lifestyles. *PLoS One* 11:e0163546. doi: 10.1371/journal.pone.0163546

Floryszak-Wieczorek, J., Arasimowicz-Jelonek, M., and Izbiańska, K. (2016). The combined nitrate reductase and nitrite-dependent route of NO synthesis in potato immunity to *Phytophthora infestans*. *Plant Physiol. Biochem.* 108, 468–477. doi: 10.1016/j.plaphy.2016.08.009

Galetskiy, D., Lohscheider, J. N., Kononikhin, A. S., Popov, I. A., Nikolaev, E. N., and Adamska, I. (2011). Phosphorylation and nitration levels of photosynthetic

proteins are conversely regulated by light stress. *Plant Mol. Biol.* 77, 461–473. doi: 10.1007/s11103-011-9824-7

Gaupels, F., Spiazzi-Vandelle, E., Yang, D., and Delledonne, M. (2011). Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response. *Nitric Oxide* 25, 222–228. doi: 10.1016/j.niox.2011.01.009

Gebhardt, C., Ballvora, A., Walkemeier, B., Oberhagemann, P., and Schuler, K. (2004). Assessing genetic potential in germplasm collections of crop plants by marker-trait association: a case study for potatoes with quantitative variation of resistance to late blight and maturity type. *Mol. Breed.* 13, 93–102.

Gilbert, W. V., Bell, T. A., and Schaening, C. (2016). Messenger RNA modifications – Form, distribution, and function. *Science* 352, 1408–1412.

Gzyl, J., Izbiańska, K., Floryszak-Wieczorek, J., Jelonek, T., and Arasimowicz-Jelonek, M. (2016). Cadmium affects peroxynitrite generation and tyrosine nitration in seedling roots of soybean (*Glycine max* L.). *Environ. Exp. Bot.* 131, 155–163.

Hawkins, C. L., Pattison, D. I., Whiteman, M., and Davies, M. J. (2017). "Chlorination and nitration of DNA and nucleic acid components," in *Oxidative Damage to Nucleic Acids. Molecular Biology Intelligence Unit*, eds M. D. Evans, and M. S. Cook (New York, NY: Springer Science), 14–39.

Holzmeister, C., Gaupels, F., Geerlof, A., Sarioglu, H., Sattler, M., Durner, J., et al. (2015). Differential inhibition of Arabidopsis superoxide dismutases by peroxynitrite-mediated tyrosine nitration. *J. Exp. Bot.* 66, 989–999. doi: 10.1093/jxb/eru458

Ihara, H., Sawa, T., Nakabeppu, Y., and Akaike, T. (2011). Nucleotides function as endogenous chemical sensors for oxidative stress signaling. *J. Clin. Biochem. Nutr.* 48, 33–39. doi: 10.3164/jcbn.11-003FR

Jena, N. R., and Mishra, P. C. (2007). Formation of 8-nitroguanine and 8-oxoguanine due to reactions of peroxynitrite with guanine. *J. Comput. Chem.* 28, 1321–1335.

Jones, L. H. (2012). Chemistry and biology of biomolecule nitration. *Chem. Biol.* 19, 1086–1092. doi: 10.1016/j.chembiol.2012.07.019

Kato, H., Takemoto, D., and Kawakita, K. (2013). Proteomic analysis of S-nitrosylated proteins in potato plant. *Physiol. Plant.* 148, 371–386. doi: 10.1111/j.1399-3054.2012.01684.x

Kong, Q., and Lin, C. G. (2011). Oxidative damage to RNA: mechanisms, consequences, and diseases. *Cell Mol. Life Sci.* 67, 1817–1829. doi: 10.1007/s00018-010-0277-y

Liu, M., Gong, X., Alluri, R. K., Wu, J., Sablo, T., and Li, Z. (2012). Characterization of RNA damage under oxidative stress in *Escherichia coli*. *Biol. Chem.* 393, 123–132. doi: 10.1515/hsz-2011-0247

Lozano-Juste, J., Colom-Moreno, R., and León, J. (2011). In vivo protein tyrosine nitration in *Arabidopsis thaliana*. *J. Exp. Bot.* 62, 3501–3517. doi: 10.1093/jxb/err042

Ma, N., Adachi, Y., Hiraku, Y., Horiki, N., Horiike, S., Imoto, I., et al. (2004). Accumulation of 8-nitroguanine in human gastric epithelium induced by *Helicobacter pylori* infection. *Biochem. Biophys. Res. Commun.* 319, 506–510.

Mata-Pérez, C., Sánchez-Calvo, B., Padilla, M. N., Begara-Morales, J. C., Luque, F., Melguizo, M., et al. (2016). Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in Arabidopsis. *Plant Physiol.* 170, 686–701. doi: 10.1104/pp.15.01671

Melo, P. M., Silva, L. S., Ribeiro, I., Seabra, A. R., and Carvalho, H. G. (2011). Glutamine synthetase is a molecular target of nitric oxide in root nodules of *Medicago truncatula* and is regulated by tyrosine nitration. *Plant Physiol.* 157, 1505–1517. doi: 10.1104/pp.111.186056

Phookphan, P., Navasumrit, P., Waraprasit, S., Promvijit, J., Chaisatra, K., Ngaotepprutaram, T., et al. (2017). Hypomethylation of inflammatory genes (*COX2, EGR1,* and *SOCS3*) and increased urinary 8-nitroguanine in arsenic-exposed newborns and children. *Toxicol. Appl. Pharmacol.* 316, 36–47. doi: 10.1016/j.taap.2016.12.015

Pinlaor, S., Yongvanit, P., Hiraku, Y., Ma, N., Semba, R., Oikawa, S., et al. (2003). 8 Nitroguanine formation in the liver of hamsters infected with *Opisthorchis viverrini*. *Biochem. Biophys. Res. Commun.* 309, 567–571.

Plich, J., Tatarowska, B., Lebecka, R., Śliwka, J., Zimnoch-Guzowska, E., and Flis, B. (2015). *R2-like* gene contributes to resistance to *Phytophthora infestans* in polish potato cultivar Bzura. *Am. J. Potato Res.* 92, 350–358.

Rivera-Mancía, S., Tristán-López, L. A., and Montes, S. (2017). "Reactive nitrogen and oxygen species in hepatic encephalopathy," in *Liver Pathophysiology. Therapies and Antioxidants*, ed. P. Muriel (Cambridge, MA: Academic Press), 485–500.

Romero-Puertas, M. C., Laxa, M., Matte, A., Zaninotto, F., Finkemeier, I., Jones, A. M., et al. (2007). S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. *Plant Cell* 19, 4120–4130. doi: 10.1105/tpc.107.055061

Romero-Puertas, M. C., Perazzolli, M., Zago, E. D., and Delledonne, M. (2004). Nitric oxide signalling functions in plant–pathogen interactions. *Cell. Microbiol.* 6, 795–803.

Sainz, M., Calvo-Begueria, L., Pérez-Rontomé, C., Wienkoop, S., Abián, J., Staudinger, C., et al. (2015). Leghemoglobin is nitrated in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/peroxide – dependent mechanism. *Plant J.* 81, 723–735. doi: 10.1111/tpj.12762

Saito, S., Yamamoto-Katou, A., Yoshioka, H., Doke, N., and Kawakita, K. (2006). Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. *Plant Cell Physiol.* 47, 689–697.

Shan, X., Chang, Y., and Lin, C. L. (2007). Messenger RNA oxidation is an early event preceding cell death and causes reduced protein expression. *FASEB J.* 21, 2753–2764.

Simms, C. L., Hudson, B. H., Mosior, J. W., Rangwala, A. S., and Zaher, H. S. (2014). An active role for the ribosome in determining the fate of oxidized mRNA. *Cell Rep.* 9, 1256–1264. doi: 10.1016/j.celrep.2014.10.042

Sodum, R. S., and Fiala, E. S. (2001). Analysis of peroxynitrite reactions with guanine, xanthine, and adenine nucleosides by high-pressure liquid chromatography with electrochemical detection: C8-nitration and –oxidation. *Chem. Res. Toxicol.* 14, 438–450.

Takahashi, M., Shigeto, J., Sakamoto, A., Izumi, S., Asada, K., and Morikawa, H. (2015). Dual selective nitration in Arabidopsis: almost exclusive nitration of PsbO and PsbP, and highly susceptible nitration of four non- PSII proteins, including peroxiredoxin II E. *Electrophoresis* 36, 2569–2578.

Terasaki, Y., Akuta, T., Terasaki, M., Sawa, T., Mori, T., Okamoto, T., et al. (2006). Guanine nitration in idiopathic pulmonary fibrosis and its implication for carcinogenesis. *Am. J. Respir. Crit. Care Med.* 174, 665–673.

Wang, X., and He, C. (2014). Dynamic RNA modifications in posttranscriptional regulation. *Mol. Cell.* 56, 5–12. doi: 10.1016/j.molcel.2014.09.001

Zago, E., Morsa, S., Dat, J. F., Alard, P., Ferrarini, A., Inze, D., et al. (2006). Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. *Plant Physiol.* 141, 404–411.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

(https://www.frontiersin.org/articles/10.3389/fpls.2018.00672/full#supplementary-material)

FIGURE S1. Percentages of leaf cells exhibiting the TUNEL-positive reaction at 24, 48, and 72 hpi (i), 100 cells from at least 5 randomly selected slides were examined at each time point per treatment. Values represent the mean ± SD of at least four independent experiments (n = 20). Different letters indicate values that differ significantly from *P. infestans* inoculated potato leaves at $P < 0.05$.

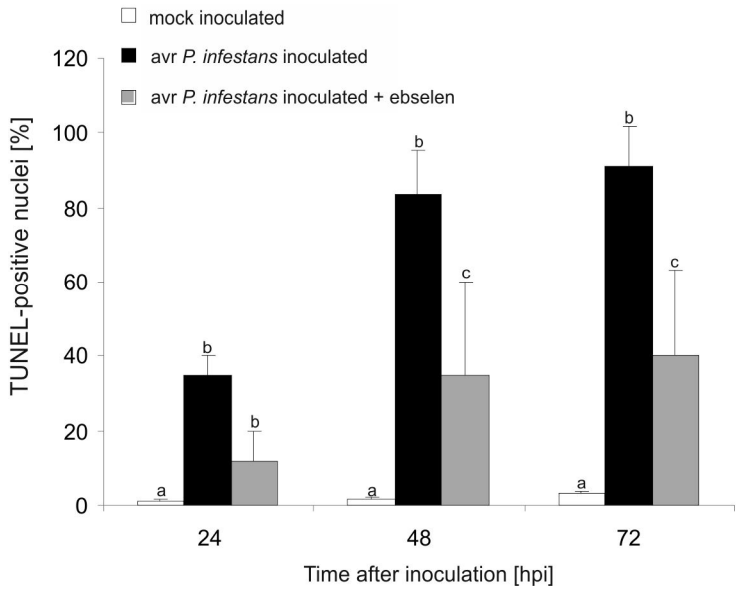


FIGURE S2. $ONOO^-$ generation measured as APF fluorescence in healthy leaves of resistant and susceptible potato in response to increasing SIN-1 concentration. Values represent the mean ± SD of at least three independent experiments (n = 9). Asterisks indicate values that differ significantly from non-treated potato leaves at $*P < 0.05$.

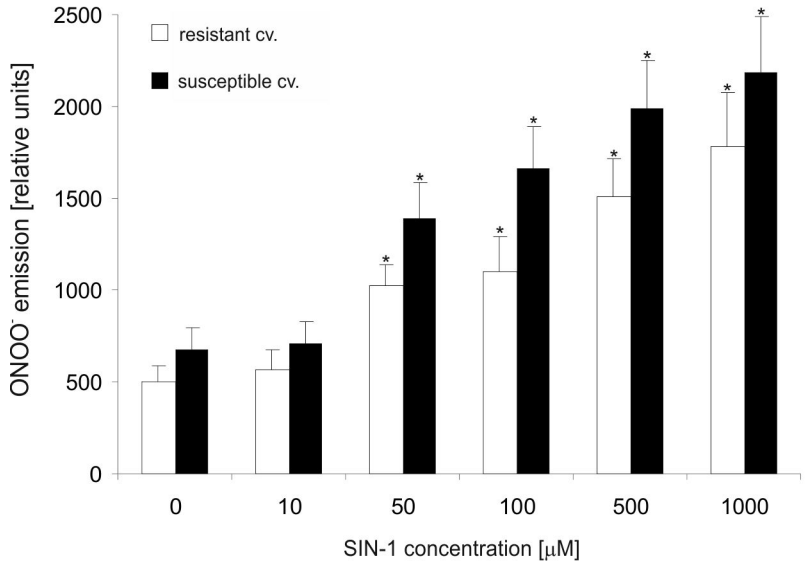


FIGURE S3. (a) 3-Nitrotyrosine ELISA standard curve in the concentration range of 0–8000 nM; (b) quantification of nitrated proteins measured as 3-nitrotyrosine content in resistant and susceptible healthy potato leaves enriched with $ONOO^-$; 3-nitrotyrosine content was estimated 5 h after leaf pretreatment with 50 μM SIN-1.

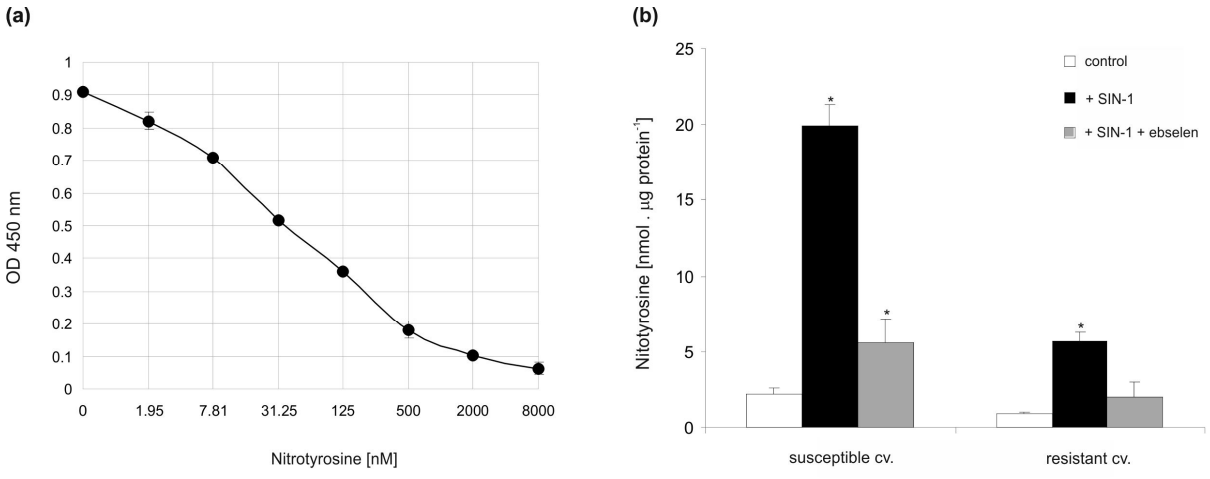


TABLE S1. Identification of nitrated proteins in potato leaves inoculated with avr *P. infestans* which appeared *de novo* at 96 hpi. Proteins were isolated from PVDF membrane and identified by LC-MS-MS/MS.

No.	Protein name	NCBI accession no.	Blast Score	Functional category
1	PREDICTED: subtilisin-like protease-like	gil565378238	885	Proteolysis
	PREDICTED: subtilisin-like protease-like	gil565378352	245	Proteolysis

TABLE S2. Amino acid sequence of the identified homologous proteins (matched peptides derived from *Solanum tuberosum* shown in Bold Red).

No.	NCBI accession no.						
1	gil565378238	1	MKNHHIIVFL	FSFFLIMSLR	GFVSCTDQET	KVYVVYLGEH	NGEKTLKEIE
		51	DHHYSFLHSV	KGTTTSKEDV	RASLVHSYKN	VINGFSAVLT	PQEVDMISGM
		101	EGVVSVFHSD	PYEIRPHTTR	SWDFVSLLEG	TSLLNSREEL	LQNASYGKDI
		151	IVGVMDSGVW	PESLSFSDEG	MEPVPKSWNG	ICQEGVAFNA	SHCNRKLIGA
		201	RYYLKGYEAA	AGPLNETRDF	RSPRDVDGHG	THTAGTVGGR	RVANASAIGG
		251	FAKGTATGGA	PNVRLAIYKV	CWPAPDQSLA	EGNICATDDI	LAAFDDAIAD
		301	GVIIVLSISLG	SLPK**STYYTE**	**NAIAIGSLIIA**	**VK**KNIVVACS	**AGNDGPTPST**
		351	**VANVAPWVIT**	**VGASTIDR**VF	SSPIMLGNGM	IVEGQTITQI	RRRR**LHPLVY**
		401	**AGDVEIR**GTT	ASNTTGACLP	GTLSRNLVRG	KVVLCLNSDI	QASMEVKRAG
		451	GVAAILGNPF	NEIQVIPFLN	PTTVTFLDGL	**NTLLTYIRTE**	**KHPTA**TLVPG
		501	**NTMIGTKTAP**	**VMAPFSSKGP**	**NVVDPNILKP**	**DITAPGFNIL**	AAWSEASSPL
		551	NIPEDHRVVK	YNIDSGTSMS	CPHVSAVIAL	LK**SIHPDWSS**	**AAVRSALMTT**
		601	**STINNVVGRP**	**IK**NATGDDAN	PFEYGSGHFR	PSKAADPGLI	YDATYTDYLL
		651	YLCSQNIRPD	LSYNCPAK**VP**	**AASNLNYPSL**	**AIANMR**GSSK	TVTRVVTNVG
		701	KDNSTYVVAV	**RSPPGYAVDI**	**VP**KSLRFSKL	GEKHSFNITI	IRAQSSVDRR
		751	NEFSFGRYTW	SDGVHVVQSP	IAVSSSA		
	gil565378352	1	MRLGSFVSCT	EETKVYIVYL	GEHNGDKTLK	EIEDHHCSFL	HSVKGTTSKE
		51	DVRASLVHSY	KNVINGFSAV	LTPQEVDMIS	GMEGVVSVFH	SDPYEIRPHT
		101	TRSWDFVSLL	EGTSLLNSRE	**ELLQNASYGK**	**DIIVGVMDSG**	VWPESSSFND
		151	EGMEPVPKSW	NGICQEGVAF	NSSHCNRKLI	GARYYLKGYE	AAAGPLNETR
		201	DFRSPRDVDG	HGTHTAGTVG	GRRVANASAI	GGFAKGTATG	GAPNVRLAIY
		251	KVCWPVPDQS	LAEGNACATD	DILAAFDDAI	ADGVHVLSIS	LGSLPKSTYY
		301	TENAIAIGSL	HAVKKNIVVA	CSAGNDGPTP	STVGNVAPWI	ITVGASSIDR
		351	VFSSPIMLGN	GMIVEGQTVT	**PIRRRRI.HPI.**	**VYAGDVEIR**G	TTTNNTSGTC
		401	LPGTLSRNLV	RGKVVLCINN	LRAASMEVKR	AGGVAAILGN	RFNEIQVTPF
		451	LDTTTVVFSY	SLNTLLTYIR	TEKNPMATLV	PGNTLIGTKP	APVMASFTSK
		501	GPNIVDPNIL	KPDITAPGFN	ILAAWSEASS	PLKMPEDRRV	**VKYNMQSGTS**
		551	**MSCP**HVSAVI	ALLKSIHPDW	SSAAIRSALM	TTSTINNVVG	RPIKNATGDD
		601	ANPFEYGSGH	FRPSRAVDPG	LVYDATYTDY	LLYLCSQNIS	LDSSFSCPEK
		651	VPTASNLNYP	SLAIANMRGS	IRTVTRVVTN	VGKDNSTYVL	GVRSPPGYVV
		701	DIVPKSLHFS	KLGEKHSFNI	TIIRAQSSVE	RRNEFSFGWY	TWNDGVHVVR

DATA SHEET S1. Nitrotyrosine immunodetection and protein identification procedures.

Protein extraction, SDS-PAGE and Western blot

Leaves (0.25 g) were ground in liquid N_2 to a fine powder and then suspended in a ratio of 1 to 3 (w/v) in 50 mM Tris-HCl buffer (pH 7.6) with 2 mM EDTA, 4 mM DTT, 0.6% PVPP, 1 mM PMSF and plant inhibitor cocktail (Sigma). The crude extracts were centrifuged at 10 000g for 15 min at 4ºC, then the concentration of supernatant proteins was determined with the Bradford assay, using BSA as a standard. The solution of proteins was mixed with the Laemmli sample buffer and boiled for 10 min at 70ºC. The sample of proteins (50 µg each) was separated on a gradient SDS-PAGE (4% - 20%, BioRad) and gels were stained with CBB-G250 or proteins were electroblotted to PVDF membranes. After transfer, membranes were blocked (5% BSA) and used for cross-reactivity assays with polyclonal antibodies against nitrotyrosine (Life Technologies) at a dilution of 1:1000. For immunodetection, the goat anti-rabbit antibody conjugated to horseradish peroxidase (Agrisera) and Lumi-Light Western Blotting Substrate (Roche) was used. The intensity of bands was quantified using a Gel Doc system (Bio-Rad) coupled with a highly sensitive camera.

MS and protein identification

Protein identification was performed using liquid chromatography coupled to the mass spectrometer at the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Raw data files were pre-processed with the Mascot Distiller software (version 2.4.2.0, MatrixScience). The obtained peptide masses and fragmentation spectra were matched to the National Center Biotechnology Information (NCBI) non-redundant database (37425594 sequences; 13257553858 residues), with a *Viridiplantae* filter (1760563 sequences) using the Mascot search engine (Mascot Daemon v. 2.4.0, Mascot Server v. 2.4.1, MatrixScience). The following search parameters were applied: enzyme specificity was set to semiTrypsin, peptide mass tolerance to ± 30 ppm and fragment mass tolerance to ± 0.1 Da. The protein mass was left as unrestricted, and mass values as monoisotopic with two missed cleavage being allowed. Alkylation of cysteine by carbamidomethylation as fixed, oxidation of methionine and carboxymethylation on lysine were set as a variable modification. Protein identification was performed using the Mascot search engine (MatrixScience), with the probability based algorithm. The expected value threshold of 0.05 was used for analysis, which means that all peptide identifications had less than 1 in 20 chance of being a random match.

WYKAZ POZOSTAŁYCH OSIĄGNIEĆ

PUBLIKACJE I MONOGRAFIE

1. Chmielowska-Bąk J., **Izbiańska K.**, Ekner-Grzyb A., Bayar M., Deckert J. (2018) Cadmium stress leads to rapid increase in RNA oxidative modifications in soybean seedlings. Frontiers in Plant Science 8: 2219 (IF=3,678; 40 pkt MNiSW)
2. Chmielowska-Bąk J., Arasimowicz-Jelonek M., **Izbiańska K**., Frontasyeva M., Zinicovscaia I., Guiance-Varela C., Deckert J. (2017) NADPH oxidase is involved in regulation of gene expression and ROS overproduction in soybean (Glycine max L.) seedlings exposed to cadmium. Acta Societatis Botanicorum Poloniae 86: 3551 (IF=1,321; 15 pkt MNiSW).
3. Gzyl J., **Izbiańska K**., Floryszak-Wieczorek J., Jelonek T., Arasimowicz-Jelonek M. (2016) Cadmium affects peroxynitrite generation and tyrosine nitration in seedling roots of soybean (*Glycine max* L.). Environmental and Experimental Botany 131: 155–163 (IF=3,712; 40 pkt MNiSW).
4. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K**. (2016) Chapter Six – Costs and Benefits of Nitric Oxide Generation in Plants Exposed to Cadmium. Advances in Botanical Research 77: 97–121 (IF=1,204; 30 pkt MNiSW).
5. Chmielowska-Bąk J., **Izbiańska K**., Deckert J. (2015) Products of lipid, protein and RNA oxidation as signals and regulators of gene expression in plants. Frontiers in Plant Science 6 (405) (IF=3,948; 40 pkt MNiSW).
6. Abramowski D., Arasimowicz-Jelonek M., **Izbiańska K**., Billert H., Floryszak-Wieczorek J. (2015) Nitric oxide modulates redox-mediated defense in potato challenged with *Phythophthora infestans*. European Journal of Plant Pathology 143: 237–260 (IF=1,494; 30 pkt MNiSW).
7. **Izbiańska K**., Arasimowicz-Jelonek M., Deckert J. (2014) Phenylpropanoid pathway metabolites promote tolerance response of lupine roots to lead stress. Ecotoxicology and Environmental Safety 110: 61–67 (IF=2,762; 30 pkt MNiSW).
8. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Abramowski D., **Izbiańska K**. (2014) Chapter 10 – Reactive nitrogen species and nitric oxide. Nitric Oxide in Plants: Metabolism and Role in Stress Physiology, Springer International Publishing Switzerland, 165–184.
9. Chmielowska-Bąk J., **Izbiańska K**., Deckert J. (2013) The toxic Doppelganger: on the ionic and molecular mimicry of cadmium. Acta Biochimica Polonica 60: 369–374 (IF=1,185; 15 pkt MNiSW).
10. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Drzewiecka K., Chmielowska-Bąk J., Abramowski D., **Izbiańska K**. (2013) Aluminum induces cross-resistance of potato to *Phytophthora infestans*. Planta 239: 679–694 (IF=3,347; 40 pkt MNiSW).

UDZIAŁ W KONFERENCJACH NAUKOWYCH

Konferencje Międzynarodowe

1. **Izbiańska K.**, Gajewska J., Gzyl J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Nitroproteome changes during the switch between *in vitro* and *in planta* growth of *Phytophthora infestans.* 7th Plant Nitric Oxide International Meeting, Nice (France) 24 – 26/10/2018

2. **Izbiańska K.**, Gajewska J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Peroxynitrite-mediated posttranslational regulation of β-1,3-glucanase activity in potato leaves inoculated with *Phytophthora infestans.* 7th Plant Nitric Oxide International Meeting, Nice (France) 24 – 26/10/2018

3. Gajewska J., **Izbiańska K.**, Bingöl O.A., Azzahra N.A., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Heavy metals affect RNS metabolism in *Phytophthora infestans* (Mont.) de Bary structures. 7th Plant Nitric Oxide International Meeting, Nice (France) 24 – 26/10/2018

4. Floryszak-Wieczorek J., **Izbiańska K.**, Gajewska J., Gzyl J., Arasimowicz-Jelonek M. New insight into the fate of peroxynitrite in plants. 8th Conference of the Polish Society of Experimental Plant Biology Białystok, (Poland) 12 – 15/09/2017

5. Gzyl J., **Izbiańska K.**, Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Nitric oxide status in roots of soybean seedlings exposed to cadmium. 8th Conference of the Polish Society of Experimental Plant Biology Białystok, (Poland) 12 – 15/09/2017

6. Gajewska J., **Izbiańska K.**, Floryszak-Wieczorek J., Arasimowicz-Jelonek M. The effect of reactive nitrogen species on *Phytophthora infestans*. 8th Conference of the Polish Society of Experimental Plant Biology Białystok, (Poland) 12 – 15/09/2017

7. **Izbiańska K.**, Gajewska J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Protein nitration and S-nitrosylation: what's new in *Phytophthora infestans*? 8th Conference of the Polish Society of Experimental Plant Biology. Białystok, (Poland) 12 – 15/09/2017

8. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K.**, Gajewska J. The role of nitric oxide in potato resistance to *Phytophthora infestans*. IV th International Conference on Research and Education Poznań (Poland) 6 – 8/04/2017

9. Gajewska J., **Izbiańska K.**, Arasimowicz-Jelonek M. Participation of nitrate reductase in response of potato leaves to *Phytophthora infestans*. IV th International Conference on Research and Education Poznań (Poland) 6 – 8/04/2017

10. **Izbiańska K.**, Gajewska J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Nitric oxide – mediated post-translational modifications in *Phytophthora infestans* structures. IV th International Conference on Research and Education Poznań (Poland) 6 – 8/04/2017

11. **Izbiańska K**., Gzyl J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Cadmium affects peroxynitrite generation and tyrosine nitration in seedling roots of soybean (*Glycine max* L.). 6th Plant Nitric Oxide International Meeting; Granada (Spain) 14-16/09/2016

12. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K**., Gajewska J. Understanding the fate of peroxynitrite in plant's and pathogen's struggle for existence. 6th Plant Nitric Oxide International Meeting; Granada (Spain) 14-16/09/2016

13. **Izbiańska K**., Gzyl J., Gajewska J., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. NO-mediated posttranslational modifications during potato response to *Phytophthora infestans.* Plant Biology Europe EPSO / FESPB 2016 Congress; Prague (Czech Republic) 26-30/06/2016

14. Chmielowska-Bąk J., **Izbiańska K**., Piasecki P., Deckert J. Short term cadmium stress leads to increased RNA and mRNA oxidation in soybean seedlings. Plant Biology Europe EPSO / FESPB 2016 Congress; Prague (Czech Republic) 26-30/06/2016

15. **Izbiańska K**., Abramowski D., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. The role of thioredoxin peroxidase in potato response to *Phytophthora infestans*. 7th Conference of the Polish Society for Experimental Plant Biology and the Intercollegiate Faculty of Biotechnology UG & MUG; Gdańsk (Poland) 08-11/09/2015

16. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Abramowski D., **Izbiańska K**. *Phytophthora infestans* generates nitric oxide to colonize potato leave. 7th Conference of the Polish Society for Experimental Plant Biology and the Intercollegiate Faculty of Biotechnology UG & MUG; Gdańsk (Poland) 08-11/09/2015

17. Abramowski D., Arasimowicz-Jelonek M., **Izbiańska K**., Billert H., Floryszak-Wieczorek J. Nitric oxide modulates redox-mediated defense in potato challenged with *Phytophthora infestans*. 7th Conference of the Polish Society for Experimental Plant Biology and the Intercollegiate Faculty of Biotechnology UG & MUG; Gdańsk (Poland) 08-11/09/2015

18. Gzyl J., **Izbiańska K**., Jelonek T., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Cadmium affects peroxynitrite generation and tyrosine nitration in soybean root seedlings7th Conference of the Polish Society for Experimental Plant Biology and the Intercollegiate Faculty of Biotechnology UG & MUG; Gdańsk (Poland) 08-11/09/2015

19. **Izbiańska K**., Floryszak-Wieczorek J., Gzyl J., Abramowski D., Arasimowicz-Jelonek M. Protein tyrosine nitration of potato leaves challenged with *Phytophthora infestans*. 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field; Verona (Italy) 24-26/06/2015

20. Abramowski D., Arasimowicz-Jelonek M., **Izbiańska K**., Floryszak-Wieczorek J. Acquisition of inherited systemic resistance of potato to *Phytophthora infestans*. 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field; Verona (Italy) 24-26/06/2015

21. Floryszak-Wieczorek J., Arasimowicz-Jelonek M., Abramowski D., **Izbiańska K**. Search for sources of synthesis and functioning of nitric oxide in resistance of potato leaves to *Phytophthora infestans.* 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field; Verona (Italy) 24-26/06/2015

22. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K**., Abramowski D. Peroxynitrite as an important player of potato defence to *Phytophthora infestans*. 12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field; Verona (Italy) 24-26/06/2015

23. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K.**, Abramowski D. (2014) Peroxynitrite mode of action during potato late blight development. 5th Plant NO Club Meeting; München (Germany) 24-25/07/2014

24. Floryszak-Wieczorek J., Arasimowicz-Jelonek M., Abramowski D., **Izbiańska K.** (2014) Synthesis and functioning of nitric oxide in resistance of potato leaves to *Phytophthora infestans*. 5th Plant NO Club Meeting; München (Germany) 24-25/07/2014

25. Arasimowicz-Jelonek M., Floryszak Wieczorek J., Abramowski D., **Izbiańska K**. The reactive nitrogen species affect potato immunity to *Phytophthora infestans*. 6th Conference of Polish Society of Experimental Plant Biology; Łódź (Poland) 16-19/09/2013

26. **Izbiańska K**., Arasimowicz-Jelonek M., Deckert J. The influence of phenylpropanoid pathway metabolites on *Lupinus luteus* seedlings exposed to Pb stress. 6th Conference of Polish Society of Experimental Plant Biology; Łódź (Poland) 16-19/09/2013

27. Floryszak-Wieczorek J., Arasimowicz-Jelonek M., Abramowski D., **Izbiańska K**. Redox-sensing responses in the potato-*Phytophthora infestans* system. Society for Experimental Biology Plant Symposium; Florence (Italy) 26-28/06/2013

28. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K**., Abramowski D. Peroxynitrite generation during potato-*Phytophthora infestans* interaction. Oxidative stress and cell death in plants: Mechanisms and implications. Society for Experimental Biology Plant Symposium; Florence (Italy) 26-28/06/2013

Konferencje krajowe

1. **Izbiańska K.**, Gajewska J., Arasimowicz-Jelonek M. Udział nadtlenoazotynu w regulacji aktywności β-1,3-glukanazy w liściach ziemniaka inokulowanych *Phytophthora infestans.* VI Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza w Poznaniu 15/06/2018

2. Gajewska J., Nur Azzahra A., Bingöl OA., **Izbiańska K.**, Arasimowicz-Jelonek M. Wpływ metali ciężkich na *Phytophthora infestans* – sprawcę zarazy ziemniaka. VI Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza w Poznaniu 15/06/2018

3. Katulska N., Misukiewicz A., **Izbiańska K**., Gajewska J., Arasimowicz-Jelonek M. Udział kwasu azelainowego i pipekolowego w immunizacji ziemniaka względem *Phytophthora infestans.* VI Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza w Poznaniu 15/06/2018

4. **Izbiańska K**., Gzyl J., Abramowski D., Floryszak-Wieczorek J., Arasimowicz-Jelonek M. Udział nadtlenoazotynu w odpowiedzi obronnej ziemniaka względem *Phytophthora infestans*. V Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza w Poznaniu; Ogród Botaniczny, Poznań 19/06/2015

5. Chmielowska-Bąk J., **Izbiańska K**., Deckert J. Obrazowanie poziomu reaktywnych form tlenu w siewkach soi traktowanych kadmem przy zastosowaniu fluorescencyjnego barwnika CM-H2DCFDA. V Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza w Poznaniu; Ogród Botaniczny, Poznań 19/06/2015

6. Arasimowicz-Jelonek M., Floryszak-Wieczorek J., **Izbiańska K**., Abramowski D.c Indukowana jonami glinu odporność krzyżowa ziemniaka na *Phytophthora infestans*. XII Ogólnopolska Konferencja Naukowa: Nauka dla Hodowli i Nasiennictwa Roślin Uprawnych; Zakopane 2-6/02/2015
7. Abramowski D., Arasimowicz-Jelonek M., **Izbiańska K**., Floryszak-Wieczorek J. Nabywanie dziedziczonej odporności systemicznej ziemniaka na *Phytophthora infestans* XII

Ogólnopolska Konferencja Naukowa: Nauka dla Hodowli i Nasiennictwa Roślin Uprawnych; Zakopane 2-6/02/2015

8. **Izbiańska K**., Gzyl J., Arasimowicz-Jelonek M. Cadmium stress affects reactive nitrogen species metabolism in roots of soybean seedlings. III Konferencja Naukowo-Dydaktyczna Wydziału Biologii Uniwersytetu im. Adama Mickiewicza w Poznaniu 10-12/04/2014
9. **Izbiańska K**., Deckert J. Rola metabolitów szlaku fenylopropanoidowego w tolerancji komórek korzeni łubinu żółtego na stres metali ciężkich. IV Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza. Ogród Botaniczny, Poznań 14/06/2013
10. Arasimowicz-Jelonek M., **Izbiańska K**., Abramowski D., Floryszak-Wieczorek J. Rola tlenku azotu w indukowanej glinem odporności krzyżowej ziemniaka względem *Phytophthora infestans*. IV Warsztaty Naukowe Instytutu Biologii Eksperymentalnej Uniwersytetu im. Adama Mickiewicza; Ogród Botaniczny, Poznań 14/06/2013

Udział w projektach naukowych

2017 – obecnie
Kierownik grantu „Analiza jakościowa i funkcjonalna nitrowanej beta-1,3-glukanazy w odporności liści ziemniaka na *Phythopthora infestans* (Mont.) de Bary" finansowanego przez Narodowe Centrum Nauki projekt nr 2016/23/N/NZ9/00181.

2016 – 2017
Kierownik Grantu Dziekana Wydziału Biologii „Wpływ nadtlenoazotynu na żywotność komórki roślinnej na przykładzie *Solanum tuberosum* L."

2015 – 2018
Wykonawca w grancie: „Identyfikacja i analiza funkcjonalna zdarzeń metabolicznych zależnych od tlenku azotu u *Phytophthora infestans* (Mont.) de Bary" finansowanego przez NCN 2014/13/B/NZ/02177. Kierownik projektu: dr hab. Magdalena Arasimowicz-Jelonek.

2015 – 2017
Wykonawca w grancie: „Udział oksydacyjnie modyfikowanego mRNA w odpowiedzi siewek soi na stres kadmowy" finansowanym przez NCN 2014/13/D/NZ9/04812. Kierownik dr Jagna Chmielowska-Bąk.

2014
Wykonawca w grancie: „Kontynuacja badań nad poszukiwaniem biomarkerów stanu patofizjologicznego w roślinie - w układzie modelowym ziemniak / *Phytophthora infestans*" finansowanym przez MNiSW „IUVENTUS PLUS" IP2011 000671. Kierownik projektu: dr hab. Magdalena Arasimowicz-Jelonek.

2013 – 2014
Wykonawca w grancie: „Poszukiwanie nowych źródeł syntezy i funkcjonowania tlenku azotu w odporności liści ziemniaka na *Phytophthora infestans*" finansowanym przez NCN 2011/01/B/NZ9/00243. Kierownik: prof. dr hab. Jolanta Floryszak-Wieczorek.