UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE CIENCIAS QUÍMICAS

POSGRADO EN CIENCIAS EN BIOPROCESOS

Contribución del gen *PUT2* a la respuesta de defensa de *Arabidopsis* bajo interacción con *Pseudomonas*.

TESIS QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS EN BIOPROCESOS

PRESENTA:

FLORES HERNÁNDEZ EMMANUEL

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SAN LUIS POTOSÍ, S. L. P. NOVIEMBRE 2025

Este proyecto se realizó en el Laboratorio de Interacción Planta-Microorganismo adscrito a la Facultad de Ciencias de la Universidad Autónoma de San Luis Potosí en el periodo comprendido entre agosto del 2020 y septiembre de 2025, bajo la dirección de la Dra. Margarita Rodríguez Kessler y la Dra. María Elisa Gonzalez y fue apoyado por el Proyecto Ciencia de Frontera 2019 (CONAHCYT-1564453): "Metabolismo de poliaminas, especies reactivas de oxígeno y fitohormonas en la encrucijada de respuestas locales y sistémicas al estrés biótico en *Arabidopsis thaliana*: un enfoque fenotípico, funcional y metabolómico" de donde se obtuvieron recursos para la realización del trabajo.

El programa de Doctorado en Ciencias en Bioprocesos de la Universidad Autónoma de San Luis Potosí pertenece al Sistema Nacional de Posgrados de Calidad (SNP) del SECIHTI, registro 000590, en el Nivel Consolidado. Número de la beca otorgada por SECIHTI: 713109. Número de CVU: 935284.

Los datos del trabajo titulado "Contribución del gen *PUT2* a la respuesta de defensa de *Arabidopsis* bajo interacción con *Pseudomonas*" se encuentran bajo el resguardo de la Facultad de Ciencias y pertenecen a la Universidad Autónoma de San Luis Potosí.

FACULTAD DE CIENCIAS QUÍMICAS DE LA UASLP

POSGRADO EN CIENCIAS EN BIOPROCESOS

DOCTORADO

San Luis Potosí, 11 de noviembre del 2020

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Por este conducto solicito a Usted se lleve a cabo el registro de título de tesis de Doctorado en Ciencias en Bioprocesos de la siguiente manera:

Contribución del gen *AtPUT2* a las respuestas de defensa de *Arabidopsis* en interacción con *Pseudomonas.*

que desarrollará el (la) estudiante: **Emmanuel Flores Hernández**

Asimismo, le comunico que el proyecto en el cual trabajará el alumno involucrará **el manejo y/o generación de plantas de *Arabidopsis* genéticamente modificadas que no tienen valor comercial ni agroalimentario** y por lo tanto, si requiere de aval de Comité de Ética e investigación de la FCQ.

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Carta Cesión de Derechos

San Luis Potosí SLP a octubre/ día /2025

En la ciudad de San Luis Potosí el día 03 del mes de noviembre del año 2025. El que suscribe Emmanuel Flores Hernández alumno del programa de posgrado Ciencias en Bioprocesos adscrito a la Facultad de Ciencias Químicas manifiesta que es autor intelectual del presente trabajo terminal, realizado bajo la dirección de: Dra. Margarita Rodríguez y Domínguez Kessler y María Elisa Gonzalez y cede los derechos del trabajo titulado Contribución del gen *PUT2* a la respuesta de defensa de *Arabidopsis* bajo interacción con *Pseudomonas* a la **Universidad Autónoma de San Luis Potosí**, para su difusión con fines académicos y de investigación.

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Emmanuel Flores Hernández

UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ
Facultad de Ciencias Químicas
Centro de Investigación y Estudios de Posgrado
Posgrado en Ciencias en Bioprocesos
Programa de Doctorado
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Carta de Análisis de Similitud

San Luis Potosí SLP a 06/ 11 /2025

L.B. Reyna Nayeli Ortiz Quintero
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Asunto: Reporte de porcentaje de similitud de tesis de grado

Por este medio me permito informarle el porcentaje de similitud obtenido mediante Ithenticate para la tesis titulada "CONTRIBUCIÓN DEL GEN PUT2 A LA RESPUESTA DE DEFENSA DE ARABIDOPSIS BAJO INTERACCIÓN CON PSEUDOMONAS." presentada por el autor EMMANUEL FLORES HERNÁNDEZ. La tesis es requisito para obtener el grado de Doctorado en el Posgrado en Ciencias en Bioprocesos. El análisis reveló un porcentaje de similitud de 29% excluyendo referencias y metodología.

Agradezco sinceramente su valioso tiempo y dedicación para llevar a cabo una exhaustiva revisión de la tesis. Quedo a su disposición para cualquier consulta o inquietud que pueda surgir en el proceso.

Sin más por el momento, le envío un cordial saludo.

A T E N T A M E N T E

_____

Dr. Jaime David Pérez Martínez

Coordinador Académico del Posgrado en Ciencias en Bioprocesos

Agradecimientos Académicos

Agradecimientos Personales

Resumen

Las plantas interactúan constantemente con microorganismos, lo que ha impulsado la evolución de sistemas de defensa inducibles y finamente regulados. Entre los mediadores clave de estos sistemas se encuentran las poliaminas, cuyo metabolismo se regula en respuesta a patógenos con diferentes estilos de vida. A pesar de su importancia, el papel del transporte de poliaminas en la defensa e inmunidad, no se ha explorado. En este trabajo se estudió la familia génica *PUT/LAT* de *Arabidopsis thaliana*, que codifica transportadores de captación de poliaminas. La caracterización de líneas mutantes *put*, mostró que los genes *PUT2* y *PUT5* son necesarios para la resistencia a *Botrytis cinerea*. Mientras que, en interacción con *Pseudomonas syringae*, las líneas mutantes *put2-1*, *put4-1* y *put5-1*, mostraron un fenotipo de pérdida de la resistencia sistémica adquirida (SAR), manteniendo la resistencia basal. La sobreexpresión de *PUT2* generó resistencia contra ambos patógenos. Dada su importancia, se analizó el transcriptoma de la mutante *put2-1* bajo condiciones de SAR, encontrando que la expresión de genes implicados en la biosíntesis y señalización de los ácidos salicílico y pipecólico está alterada. Nuestro estudio determinó que PUT2 es necesario para transportar putrescina a tejidos distales bajo SAR, así como para modular los niveles de especies reactivas del oxígeno, ácido salicílico y otros compuestos fenólicos. Además, influye en la respuesta inmune a nivel de los estomas. Estos hallazgos demuestran que el transporte de poliaminas es un eje central en el proceso de defensa local y sistémica en *Arabidopsis*.

Palabras clave: Transportadores de poliaminas, *Pseudomonas*, *Arabidopsis,* Poliaminas, Resistencia sistémica adquirida, Putrescina.

Abstract

Plants constantly interact with a wide range of microorganisms, a relationship that has driven the evolution of inducible and finely regulated defense systems. Polyamines, which play a pivotal role in these systems, are subject to regulation in response to pathogens exhibiting different lifestyles. Despite their importance, the role of polyamine transport in defense and immunity remains to be elucidated. The present study investigated the PUT/LAT gene family in *Arabidopsis thaliana*, which encodes polyamine uptake transporters. Characterization of *put* mutant lines showed that the *PUT2* and *PUT5* genes are necessary for resistance to *Botrytis cinerea*. However, during interaction with *Pseudomonas syringae*, the *put2-1*, *put4-1* and *put5-1* mutant lines exhibited loss of systemic acquired resistance (SAR), while maintaining the basal resistance. Overexpression of *PUT2* generated resistance against both pathogens. Given the significance of the *put2-1* mutant phenotype, the transcriptome was analyzed under SAR conditions. The results obtained indicated that the expression of genes involved in the biosynthesis and signaling of salicylic and pipecolic acids is altered. Our study determined that PUT2 is essential for putrescine transport to distal tissues under SAR, as well as to modulate the levels of reactive oxygen species, salicylic acid, and other phenolic compounds. Additionally, we demonstrated that PUT2 influences the immune response at the stomatal level. These findings showed that polyamine transport is a central component in the local and systemic defense process in *Arabidopsis*.

Keywords: Polyamine Transporters, *Pseudomonas*, *Arabidopsis*, Polyamines, Systemic acquired resistance, Putrescine.

ÍNDICE GENERAL

INTRODUCCIÓN

Enfermedades de cultivos

Las enfermedades de las plantas representan una amenaza constante para la seguridad alimentaria mundial debido a su impacto directo en el rendimiento de los cultivos. El manejo eficaz de estas enfermedades sigue siendo un reto importante, principalmente por a la rápida evolución de los patógenos, la variabilidad genética entre los cultivos y las consecuencias del cambio climático (Purayannur et al., 2017). Si bien las estrategias actuales, como el uso de pesticidas y de agentes de control biológico, han contribuido al control de las enfermedades, su eficacia suele ser limitada y de corta duración. Además, el uso generalizado de productos agroquímicos ha suscitado preocupación debido a sus efectos negativos para el medio ambiente y la salud humana (Sharma et al., 2022).

Para poder diseñar estrategias eficientes de control de enfermedades en los cultivos, es importante comprender el proceso de defensa vegetal. La forma clásica de describir el sistema de defensa en las plantas es mediante el modelo de "zig-zag", que destaca la batalla evolutiva entre plantas y patógenos desde un punto de vista molecular. En este modelo, el proceso de defensa inicia tras el reconocimiento de patrones moleculares asociados a patógenos (PAMP), lo que genera una señalización que dar lugar a la inmunidad activada por patrones (PTI; Jones y Dangl, 2006). Sin embargo, algunos patógenos se han adaptado y son capaces de secretar factores de virulencia, conocidos como efectores, que suprimen la inmunidad del hospedero y permiten establecer la infección en un proceso conocido como susceptibilidad desencadenada por efectores. Algunas variedades de plantas han generado a través de la evolución proteínas de resistencia, que reconocen de manera directa o indirecta a los efectores, y activan la inmunidad desencadenada por efectores (ETI). En conjunto, la PTI y la ETI activan la respuesta de defensa, lo que evita el proceso infeccioso en la planta, al restringir la proliferación del patógeno (Jones y Dangl, 2006; Han, 2019; van der Burgh y Joosten, 2019).

La respuesta de defensa vegetal se caracteriza por una serie de eventos que incluyen influjos de Ca^{2+}, cierre estomático, deposición de callosa, producción de especies reactivas de oxígeno (ROS), óxido nítrico (NO), ácido fosfatídico, fitoalexinas y otros compuestos con actividad antimicrobiana. Estos procesos están acompañados por la activación de cascadas de proteínas quinasas dependientes de calcio (CDPKs) y proteínas quinasas

activadas por mitógenos (MAPKs). Como consecuencia, se induce la transcripción de genes de defensa y la acumulación de las fitohormonas especificas según el tipo de vida del patógeno: ácido jasmónico (JA) y etileno (Et) en el caso de patógenos necrotróficos y ácido salicílico (SA) frente a patógenos biotróficos y hemibiotróficos (Pieterse et al., 2012; Cui et al, 2015; Yu et al; 2017). Los patógenos biotróficos solo pueden prosperar en células vivas, mientras que los necrotróficos secretan toxinas y enzimas para matar las células del hospedador antes de consumir el tejido muerto. Por su parte, los hemibiotróficos presentan un estilo de vida intermedio: comienzan como biotróficos, viviendo en células vivas, y posteriormente transitan a una fase necrotrófica en la que se alimentan de los nutrientes de las células muertas (Liao et al., 2022). Finalmente, la acumulación de SA en el tejido infectado resulta fundamental para el establecimiento de la resistencia sistémica adquirida (SAR; Klessig, Choi y Dempsey, 2018; Lim, 2023).

Resistencia sistémica adquirida

La SAR es un mecanismo de defensa sistémico de las plantas que se activa en respuesta a una infección primaria (local). Esta respuesta confiere a la planta una mayor resistencia frente a infecciones secundarias (distales) causadas por un amplio espectro de patógenos (Vlot et al., 2021), por lo que se constituye una alternativa prometedora para el control de enfermedades en los cultivos (**Figura 1**).

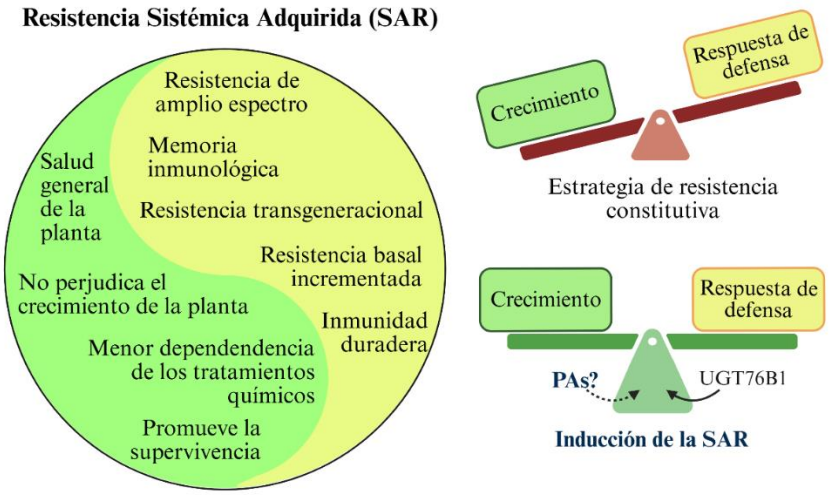


Figura 1. Resistencia sistémica adquirida en el control de enfermedades en plantas. A diferencia de estrategias experimentales que generan un estado de defensa constitutivo, donde la activación continua de rutas como la del ácido salicílico puede generar efectos negativos sobre el crecimiento vegetal (van Butselaar

y van den Ackerveken 2020), la SAR permite una activación rápida y regulada de la defensa, que se desencadena sólo tras la percepción de un patógeno. Esta respuesta optimiza el uso de recursos energéticos y metabólicos, reduciendo el compromiso fisiológico asociado a una defensa constante. Por ello, la SAR representa una estrategia más eficiente y sostenible, ya que equilibra la protección contra patógenos sin comprometer el desarrollo de la planta. Además, al ser una respuesta de amplio espectro, permite una protección eficaz contra múltiples patógenos, genera una memoria inmunitaria y puede ser heredable (Vlot et al., 2021). La glicosil transferasa UGT76B1 se ha propuesto como un regulador de este proceso (Cai et al., 2021); nosotros proponemos que las poliaminas podrían actuar de manera similar. La figura fue creada con BioRender (www.biorender.com).

El establecimiento de la SAR depende de una compleja red de señalización que viaja desde el sitio local de la infección hasta los tejidos distales, en la que intervienen ROS, Ca^{2+} y diversas "señales móviles" como el salicilato de metilo (MeSA), el ácido azelaico (AzA), el glicerol-3-fosfato (G3P) y el dehidroabietinal (DA) (Guerra et al., 2020; Kachroo y Kachroo, 2020; Vlot et al., 2021; Shine et al., 2022; Li et al., 2023). En última instancia, la activación de la SAR en los tejidos sistémicos requiere la acumulación del SA y del ácido pipecólico (Pip), ambos esenciales para la formación de una memoria inmunitaria hereditaria (Lim et al., 2023). Comprender los mecanismos que regulan esta respuesta ofrece una oportunidad valiosa para desarrollar estrategias innovadoras y sostenibles de protección de las plantas (**Figura 2**).

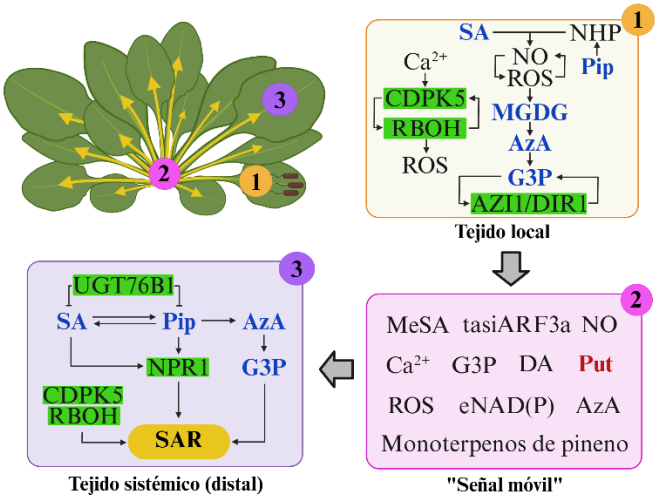


Figura 2. Esquema de la señalización y mecanismos moleculares de la resistencia sistémica adquirida (SAR). El contacto con el patógeno y su reconocimiento en el tejido local activan las cascadas de señalización (recuadro **1**). Se indican en color azul los metabolitos que constituyen la base para activar la SAR y en un recuadro verde las proteínas. En el recuadro **2** se enumeran los compuestos transportados entre el tejido local y el sistémico descritos hasta la fecha (septiembre de 2025) como parte de la conocida "señal móvil", también

se incluye la putrescina (Put) que se ha reportado que induce una respuesta similar a la SAR (Liu et al., 2021). Finalmente, el recuadro **3** muestra la señalización para el establecimiento de la SAR en tejido sistémico; en azul se muestran los principales metabolitos para esta acción y en un recuadro verde las proteínas. Abreviaturas AzA: Ácido azelaico; AZI1: Ácido azelaico inducido 1; Ca^{2+}: Calcio; CPK5: Proteína quinasa dependiente de calcio 5; DA, Dehidroabietinal; DIR1: Defectuoso en resistencia inducida 1; eNAD(P): fosfato de dinucleótido de nicotinamida y adenina extracelular; G3P: Glicerol-3-fosfato; MeSA: Salicilato de metilo; MGDG: Monogalactosildiacilglicerol; NHP: Ácido *N* -hidroxi-pipecólico; NO: Óxido nítrico; NPR1: No expresor de genes *PR1*; Pip: ácido pipecólico; Put: Putrescina; RBOHD: Proteína D homóloga de la oxidasa del estallido oxidativo; ROS: especies reactivas de oxígeno; SA: ácido salicílico; tasiARF3a: ARN3a pequeño de interferencia de acción en trans; UGT76B1: UDP-glicosiltransferasa 76B1. La figura fue creada con BioRender (www.biorender.com).

Poliaminas y estrés en plantas

Las poliaminas son compuestos alifáticos con dos o más grupos amino, presentes de forma ubicua en todas las células vivas, donde desempeñan funciones esenciales para la supervivencia (Kusano et al. 2008). En las plantas, las poliaminas intervienen en una amplia gama de procesos, que abarca desde la embriogénesis y la germinación de las semillas hasta la maduración y senescencia de los frutos, así como en las respuestas a estrés biótico y abiótico (**Figura 3**). Las poliaminas más estudiadas son la putrescina (Put), la espermidina (Spd), la espermina (Spm) y la termoespermina (tSpm) (Blázquez, 2024). La regulación del metabolismo de las poliaminas incluye su biosíntesis, catabolismo, transporte y conjugación durante la respuesta inmune es esencial para la defensa de las plantas contra los patógenos (González et al. 2021).

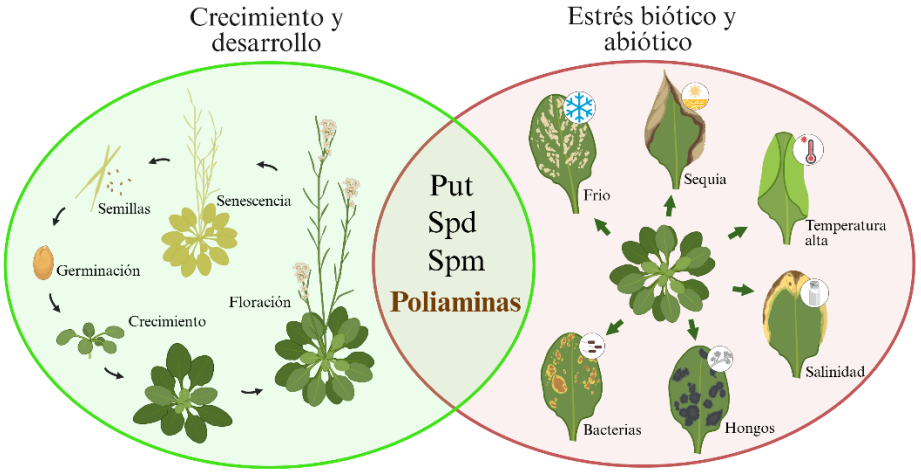


Figura 3: Importancia de las poliaminas en las plantas. Las poliaminas participan en el desarrollo normal en procesos como la embriogénesis, organogénesis, germinación de la semilla, desarrollo de hojas, floración,

generación y maduración del fruto y la senescencia (Imai et al., 2004; Kusano et al., 2008; Tavladoraki et al., 2016; Tsaniklidis et al., 2016; Ahmed et al., 2017; Sobieszczuk-Nowicka, 2017). Además, desempeñan un papel crucial en la respuesta de las plantas al estrés abiótico y biótico, contribuyendo a mitigar los efectos negativos del estrés inducido por salinidad, sequía y temperaturas extremas (Shen et al., 2016; Pál et al., 2018; Yin et al., 2019; Zhu et al., 2020). En cuanto al estrés biótico, se ha demostrado que las poliaminas actúan como mediadoras en la respuesta de defensa contra patógenos fúngicos y bacterianos, siendo su participación dependiente de la naturaleza del microorganismo involucrado (González et al., 2021; Blázquez, 2024). La figura fue creada con BioRender (www.biorender.com).

Transporte de poliaminas en *Arabidopsis*

En Arabidopsis, los transportadores de importe de poliaminas (PUT, Polyamine Uptake Transporters, por sus siglas en inglés) son los principales actores responsables de la movilización de poliaminas y algunos aminoácidos (**Figura 4**) (Mulangi et al., 2012). Se conocen cinco transportadores PUT localizados en diversos compartimentos celulares: PUT1 y PUT5 en el retículo endoplásmico, PUT2 en el aparato de Golgi, PUT2 y PUT3 en el cloroplasto, y PUT3 también en la membrana plasmática (Li et al., 2013; Fujita y Shinozaki, 2014; Ahmed et al., 2017). Además de poliaminas, algunos PUT pueden transportar aminoácidos como la leucina, vitamina B1 y el herbicida paraquat (Mulangi et al., 2012; Li et al., 2013; Martinis et al., 2016; Begam and Good, 2017). A pesar del avance en la investigación sobre el transporte de poliaminas, aun se sabe poco sobre su participación en la respuesta al estrés en plantas.

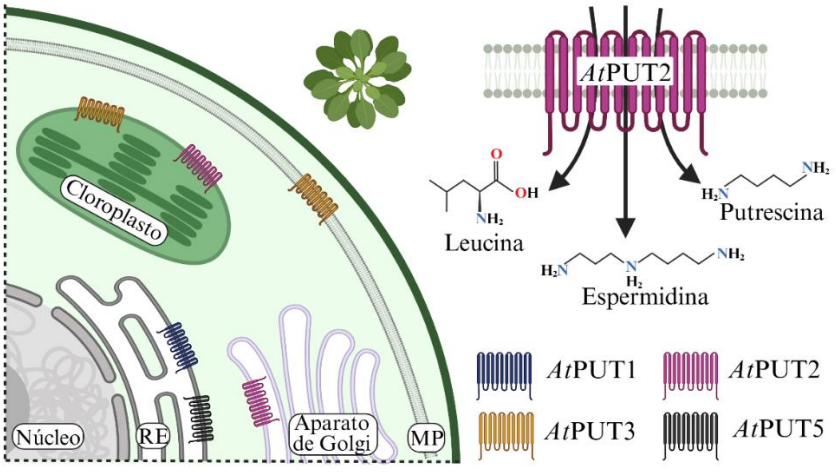


Figure 4: Transporte de poliaminas en *Arabidopsis thaliana*. Representación esquemática de la localización intracelular de los transportadores de poliaminas (exceptuando a *At*PUT4 cuya localización experimental no ha sido reportada) en *Arabidopsis*, lo que sugiere que cada transportador puede cumplir funciones específicas y diferenciadas en el transporte y regulación de poliaminas dentro de la célula vegetal. Además, se muestra un modelo de la topología predicha para el transportador *At*PUT2 y los metabolitos que han sido reportados como sus sustratos (Martinis et al., 2016). La figura fue creada con BioRender (www.biorender.com).

ANTECEDENTES

Las mutantes de los transportadores PUT participan en la respuesta de *Arabidopsis thaliana* a *Pseudomonas syringae*

En un estudio previo, utilizando líneas mutantes insercionales de T-DNA de los cinco genes *PUT* de *A. thaliana*, se observó que los transportadores de poliaminas son importantes para el desarrollo vegetal y la respuesta al estrés biótico inducido por *Pseudomonas syringae* (Flores-Hernandez, 2022; Tesis de Maestría).

El análisis del fenotipo de las líneas mutantes *put1-1*, *put2-1, put3-1*, *put4-1* y *put5-1* de cuatro semanas de edad, cultivadas bajo condiciones de día largo (16 h luz / 8 h oscuridad), mostró que el tamaño de la roseta de las mutantes fue menor que el del ecotipo silvestre, lo que sugiere que el transporte de poliaminas es necesario para el óptimo crecimiento de las plantas. Esta observación coincide con lo reportado para la mutante *put3-1,* que presenta raíces más cortas y un retraso de cinco días en la transición a la fase reproductiva, en comparación con el ecotipo silvestre (Martinis et al., 2016). Así como con lo descrito para la mutante *put5-1*, que es de menor tamaño que su parental a las cinco semanas de edad (Ahmed et al., 2017).

Estos resultados han sido complementados recientemente por un análisis genómico de la familia de genes *PUT* en tomate (*Solanum lycopersicum*), donde se identificaron ocho miembros de esta familia y se caracterizó particularmente el papel de *SlPUT2* en la respuesta al estrés salino. En este estudio, se observó que la sobreexpresión de *SlPUT2* confirió a las plantas una mayor tolerancia a la salinidad, evidenciada por un mejor crecimiento, una mayor relación K^+/Na^+, menor acumulación de ROS y una activación más eficiente del sistema antioxidante en comparación con plantas del ecotipo silvestre. Por el contrario, las líneas mutantes *Slput2* mostraron una mayor sensibilidad al estrés salino (Zhong et al., 2023). Este hallazgo refuerza la importancia de los transportadores de poliaminas en la respuesta al estrés abiótico en plantas de tomate, sugiriendo que los PUT desempeñan un papel importante para la respuesta al estrés.

Por otra parte, en ensayos de interacción entre plantas de *Arabidopsis* y la bacteria hemibiotrófica *Pseudomonas syringae* pv. *tomato* DC3000, se observó que las cinco líneas mutantes *put* mostraron un fenotipo de resistencia basal al patógeno, con una reducción en el número de unidades formadoras de colonias (UFC) en comparación con el ecotipo

silvestre (**Figura 5**). En respuesta a la infección, las líneas mutantes *put1-1*, *put3-1, put-5-1 y put5-2* presentaron un incremento en la producción del anión radical superóxido ($O_2^{\bullet-}$), mientras que las mutantes *put1-1*, *put2-1* y *put5-2* mostraron un nivel de expresión significativamente mayor en el gen *PR-1*, un marcador importante de la vía del SA. Estos resultados sugieren que alteraciones en el transporte de poliaminas, en las mutantes *put*, afectan vías de señalización hormonal y la homeostasis de ROS en la respuesta de defensa local. Considerando que tanto el SA como la acumulación de especies reactivas de oxígeno (como $O_2^{\bullet-}$) son esenciales para el establecimiento de la resistencia sistémica adquirida (SAR), este trabajo de tesis de doctorado, se determinó el papel de los transportadores de poliaminas y de las poliaminas en las distintas fases de la infección local y sistémica.

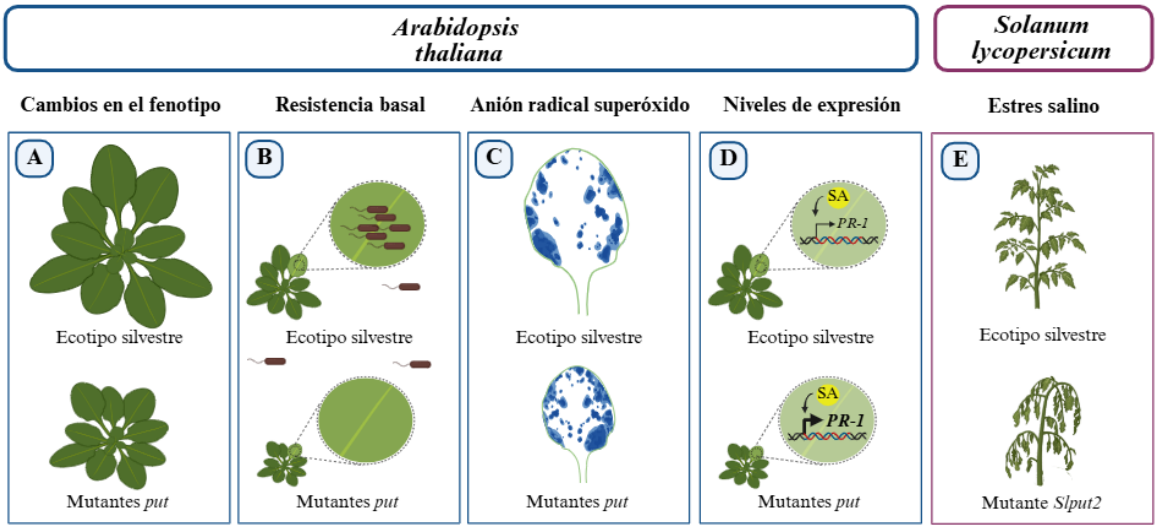


Figura 5. Alteraciones en el desarrollo y la respuesta inmune local en líneas mutantes *Atput* y *Slput.* En el panel **A** se muestra que las líneas mutantes *Atput* presentan un fenotipo de roseta de tamaño reducido en comparación con el ecotipo silvestre. En **B** se ilustra la resistencia local de las mutantes *Atput* frente a la bacteria *P. syringae*. En **C**, la tinción con NBT revela una mayor acumulación del anión radical superóxido ($O_2^{\bullet-}$) en hojas de las líneas mutantes *Atput1-1*, *Atput3-1*, *Atput5-1* y *Atput5-2* reflejada en una coloración azul más intensa. El panel **D** muestra la inducción significativa del gen marcador de defensa *PR1*, en las líneas mutantes *Atput1-1*, *Atput2-1* y *Atput5-2* en comparación con el ecotipo silvestre. Finalmente, el panel **E** muestra que la mutante *Slput2* es más susceptible al estrés salino respecto al ecotipo silvestre. La figura fue creada con BioRender (www.biorender.com).

JUSTIFICACIÓN

Las poliaminas son compuestos esenciales para la viabilidad celular. En las plantas, las poliaminas participan en la regulación y el establecimiento de las respuestas de defensa. Actualmente, se conoce que la biosíntesis, el catabolismo y la conjugación de estas aminas participan activamente en la respuesta de defensa. Sin embargo, aún se desconoce cómo el transporte de poliaminas, mediado por los transportadores PUT, contribuye a la respuesta inmunitaria.

Por lo anterior, este trabajo tiene como finalidad elucidar los mecanismos mediante los cuales el transporte de poliaminas, específicamente el mediado por PUT2, participa en el proceso de defensa basal, en el establecimiento de la resistencia sistémica adquirida, así como en la reprogramación transcripcional que presenta *Arabidopsis thaliana* durante el estrés biótico inducido por la bacteria *Pseudomonas syringae*.

Finalmente, conjuntar este conocimiento con el reportado previamente nos permitirá tener una visión más integral sobre la participación de las PAs en la defensa vegetal y, a partir de ello, proponer estrategias innovadoras para el control de fitopatógenos.

HIPÓTESIS

La alteración del transporte de poliaminas mediada por la pérdida de función del gen *PUT2* modifica la señalización molecular y la respuesta transcriptómica, favoreciendo la defensa basal de la planta frente a ataques de patógenos y alterando la respuesta de defensa sistémica.

OBJETIVOS

Objetivo general

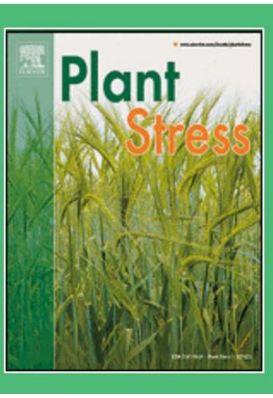
Evaluar la participación del transportador AtPUT2 en la respuesta molecular y fenotípica de *Arabidopsis thaliana* al estrés biótico inducido por *Pseudomonas syringae*.

Objetivos específicos

1. Caracterizar mediante cribado fenotípico de alto rendimiento las líneas mutantes insercionales de T-DNA de los genes *PUT* suplementadas con poliaminas.
2. Obtener líneas sobreexpresoras (*35S::AtPUT)* del gen que codifica el transportador PUT2.
3. Secuenciar masivamente el transcriptoma de la línea *put2-1* en condiciones SAR.
4. Determinar los niveles de poliaminas y aminoácidos en la línea mutante *put2-1* durante la SAR.
5. Evaluar los genes diferencialmente expresados implicados en defensa y SAR obtenidos del análisis transcriptómico de la mutante *put2-1*.
6. Suplementar con poliaminas las líneas mutantes y las sobreexpresoras del gen *PUT2* y evaluar su capacidad para establecer la SAR.

PRIMER ARTICULO

Polyamine uptake transporter 2 is essential for systemic acquired resistance establishment in *Arabidopsis*



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Title: Polyamine uptake transporter 2 is essential for systemic acquired resistance establishment in *Arabidopsis*

Running head: Polyamine transport in systemic acquired resistance

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ABSTRACT

The study of polyamine transport in plants has become increasingly important due to the central role of these amines in regulating growth, development, adaptation, and stress responses. This research focused on the *Arabidopsis thaliana Polyamine Uptake Transporters* gene family under conditions of systemic acquired resistance. We evaluated all single mutants of this gene family and found that the *put2-1* mutant abolished systemic acquired resistance while enhancing basal resistance to *Pseudomonas syringae* pv. *tomato* DC3000. In contrast, the *35S::PUT2* overexpression lines showed improved resistance and reduced bacterial titers compared to wild-type plants. RNA-seq analysis revealed that the *put2-1* mutant had deregulated expression of genes involved in the biosynthesis, signaling, and inactivation of salicylic acid and N-hydroxypipecolic acid. Most of these genes were transcriptionally upregulated by putrescine in wild-type plants, but not in the *put2-1* mutant. Putrescine supplementation increased endogenous putrescine and salicylic acid levels in wild-type plants but not in *put2-1*, highlighting the essential role of this transporter in facilitating putrescine mobilization and regulating salicylic acid in distal tissues. We found that the defective systemic acquired resistance phenotype in the *put2-1* mutant was linked to changes in the timing of polyamines, ROS, phenolic compound accumulation, and alterations in stomatal immunity. Our study emphasizes the key role of the Polyamine Uptake Transporter 2 (PUT2/LAT4) in establishing systemic acquired resistance in *Arabidopsis*, while also maintaining the plant's intrinsic basal resistance mechanisms. These findings offer valuable insights into the complex mechanisms of plant resistance, positioning polyamine transport as a central hub in systemic responses.

Keywords.

Putrescine, Polyamine transport, Reactive oxygen species, salicylic acid, systemic acquired resistance.

1. Introduction

Plants have evolved a sophisticated and highly regulated immune system capable of responding rapidly to pathogen threats through mechanisms activated by pathogen-associated molecular patterns (PAMPs), producing a cascade of signals that culminate in PAMP-triggered immunity (PTI). However, specific pathogens have adapted by deploying virulence factors known as effectors to suppress these innate immune responses. In response, plants have evolved the capacity to recognize these effectors through resistance proteins that activate effector-triggered immunity (ETI) (van der Burgh and Joosten, 2019).

Following the initial contact with a pathogen, plants fortify their local defenses and develop systemic acquired resistance (SAR), a state of heightened defensive readiness that confers long-lasting protection against a broad range of pathogens (Vlot et al., 2021). This systemic immunity is mediated by a complex network of signaling molecules like reactive oxygen species (ROS), Ca^{2+}, and "mobile signals" such as methyl salicylate (MeSA), azelaic acid (AzA), glycerol-3-phosphate (G3P), dehydroabietinal (DA), nitric oxide (NO), extracellular nicotinamide adenine dinucleotide phosphate [eNAD(P)] trans-acting siRNA-like RNAs (tasi-ARFs), and pinene monoterpenes (Guerra et al., 2020; Kachroo and Kachroo, 2020; Li et al., 2023; Vlot et al., 2021; Shine et al., 2022). SAR establishment leads to the production of salicylic acid (SA) and pipecolic acid (Pip), which facilitate communication between infected and distal tissues, effectively establishing an immune memory that primes the plant to respond more swiftly and robustly to subsequent infections (Lim, 2023).

Polyamines, aliphatic compounds with two or more amino groups, are usually protonated at physiological pH and play essential roles across a plant's life cycle, from embryogenesis and seed germination to fruit ripening and senescence (Blázquez, 2024). The most studied plant polyamines are the diamine putrescine (Put), the triamine spermidine (Spd), the tetraamines spermine (Spm), and thermospermine (tSpm) (Blázquez, 2024). However, their abundance depends on the plant species (Ćavar Zeljković et al., 2024). Polyamines are crucial mediators of the plant defense response (Jiménez-Bremont et al., 2014; Gonzalez et al., 2021). For instance, their catabolism, particularly through the action of polyamine oxidases (PAO), generates hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) compound that inhibits pathogen growth, strengthens cell walls, induces stomata closure, and acts as a second messenger in the signaling network regulating defense

gene expression (Wang et al., 2019). The *Atpao1-1 x Atpao2-1* double mutant, susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), has altered ROS levels (Jasso-Robles et al., 2020). Contrarily, overexpression of apoplastic *ZmPAO* in *Nicotiana tabacum* var. *Xanthi* increased tolerance to *P. syringae* pv. *tabaci* and the oomycete *Phytophthora parasitica* var. *nicotianae* (Moschou et al., 2009). Furthermore, endogenous Spm accumulation in *Arabidopsis thaliana* also confers resistance to *Pseudomonas viridiflava* (Gonzalez et al., 2011). However, polyamine catabolism is not always detrimental to pathogens, as the H_2O_2 produced by the *ZmPAO1* enzyme is essential for tumor development during the *Zea mays*-*Ustilago maydis* interaction, which favors pathogen survival (Jasso-Robles et al., 2016). The conjugation of polyamines with hydroxycinnamic acids produces compounds with antimicrobial activity. For instance, *p*-coumaroylputrescine bolsters the defense mechanisms of *A. thaliana* against the pathogen *Alternaria brassicicola* (Muroi et al., 2009).

While the intricate role of polyamine biosynthesis, conjugation, and catabolism in plant-pathogen interactions is partially known, our understanding of the implications of polyamine transport under stress conditions remains limited, especially regarding plant defense mechanisms. The *A. thaliana* genome encodes five genes for Polyamine Uptake Transporters (*PUT1-PUT5*), which belong to the L-type amino acid transporter (LAT) family of proteins. Structurally, these proteins have 10 to 12 transmembrane domains with cytoplasmic amino- and carboxy-terminal ends (Fujita and Shinozaki, 2014). In addition to polyamines, some PUT/LAT family members can transport amino acids (such as leucine), vitamin B1, and the herbicide paraquat (Mulangi et al., 2012; Li et al., 2013; Martinis et al., 2016; Begam and Good, 2017). The localization of PUTs in various cellular compartments, such as the endoplasmic reticulum (PUT1 and PUT5), Golgi apparatus (PUT2), chloroplast (PUT2 and PUT3), and plasma membrane (PUT3), suggests that these transporters play a significant role in the intracellular mobilization of polyamines in plants (Li et al., 2013; Fujita and Shinozaki, 2014; Ahmed et al., 2017).

Experimental evidence suggests that mobilizing plant polyamines under pathogen attack can promote plant resistance and, in some cases, pathogen virulence (Lowe-Power et al., 2018; Vilas et al., 2018). The recent observation that Put or its derivatives could serve as a signaling molecule to activate ETI and SAR in *Arabidopsis*, particularly in a process largely dependent on H_2O_2 (Liu et al., 2020), leads us to hypothesize that the transport of

polyamines or their derived metabolites via PUT/LAT transporters could significantly contribute to systemic defense responses. This study aimed to identify and functionally characterize PUT transporter(s) associated with SAR in *Arabidopsis*. Single mutants of the *PUT* gene family were assessed under SAR-inducing conditions to investigate how altered polyamine transport influences the plant's immune response, using the *Arabidopsis-Pseudomonas* pathosystem as a model. Using transcriptomic and metabolic approaches, we characterized the *put2-1* mutant to understand the basis of its SAR deficiency. Our findings provide key insights into the role of Put transport, highlighting its essential function in plant immunity.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (WT) and T-DNA mutant lines for members of the polyamine uptake transporter family in *Arabidopsis* [*put1-1* (GABI_890c10), *put2-1* (SALK_119707c), *put3-1* (SALK_206472c), *put4-1* (SAIL_1275_c06), and *put5-1* (SALK_007135)] were acquired from Salk Institute Genomic Analysis Laboratory (www.signal.salk.edu/cgi-bin/tdnaexpress). Homozygous lines were identified by PCR. The absence of the *PUT* gene expression in the corresponding T-DNA mutant line was confirmed by qPCR (**Fig. S1**)**.** Specific primers used for typing, cloning, and gene expression analysis are listed in **Table S1**.

For the experiments, seeds were stratified for 2 days in distilled water at 4 ºC before being germinated in pots containing a sterile commercial substrate composed of sunshine#3: vermiculite: perlite (3:1:1). Plants were grown in a controlled growth chamber at 22 ± 2 °C under short-day photoperiod (8 h light/16 h dark) for 6 weeks for assessing bacterial titers in SAR conditions, transcriptomic and metabolomic analysis as described below. Additionally, plants were grown under a long-day photoperiod (16/8 h light/dark regime) for 4 weeks to assess basal resistance.

2.2. Generation of the AtPUT2 overexpression lines

The *AtPUT2* ORF sequence (1485 bp) was PCR amplified and cloned into the pENTER-D-TOPO entry vector (Invitrogen, Carlsbad, CA, USA) and recombined into the

pEarley103 expression vector (Earley et al., 2006) using the Gateway LR clonase enzyme mix (Invitrogen). The expression clone (*35S::PUT2*) was transformed into *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) strain GV3101 by heat shock and then introduced into *A. thaliana* by floral dip. Transformed seeds were selected based on their resistance to glufosinate (Finale Ultra, BASF), following standard protocols (Harrison et al., 2006). Three independent overexpression lines were chosen for further analysis, *35S::PUT2-2, -18*, and *-27* (**Fig. S1**).

2.3. Bacterial strain and plant inoculation for SAR assays

Pseudomonas syringae pv. *tomato* DC3000 (*Pst*) and *Pseudomonas syringae avrRpt2* strains were used to induce SAR in *Arabidopsis* (Macho et al., 2010; Rufián et al., 2019). The strains were grown in King's B medium with rifampicin (50 μg/mL) at 28 ºC. The SAR assay was carried out as Rufián et al. (2019) described. Briefly, the lower rosette leaves (8, 9, and 10) of six-week-old plants were inoculated with bacteria (OD_{600}=0.005) to induce local priming (SAR) or with the Mock ($MgCl_2$ 10 mM, pH 7.0) as the control (native) condition. Then, 48 h after primary infection, upper leaves (13, 14, and 15) were systemically challenged with *Pst* (OD_{600}=0.0005). After a specific time, the upper leaves were collected for further analysis, assuming a native plant status or a SAR condition has been established.

2.4 Polyamine infiltration for studying SAR-like responses

Validation experiments were conducted to elucidate further the role of specific polyamines in regulating SAR-like responses. For this, the lower leaves of six-week-old plants were infiltrated with 30 μM of Put, Spd, Spm or Mock. Then, 6 h after polyamine infiltration, the upper leaves were collected to quantify the gene expression of specific genes related to the SA and Pip pathways. Another set of plants, 48 h after Put infiltration, the upper leaves were challenged with *Pst* and collected at 72 hours post-inoculation (hpi) for the estimation of bacterial titers. The upper leaves of Put-infiltrated plants were also collected at 6 and 24 h for further metabolic analysis.

2.5. Estimation of bacterial titers

Bacterial titers were determined in the upper leaves at 72 hpi. Six-leaf disks (∅ 0.5 cm) from each plant (n = 5) were homogenized in 0.2 mL of $MgCl_2$ (10 mM, pH 7.0) and

serially diluted (1:10 to 1:1x10^9). Then, 10 µL of each dilution was plated on solid King's B agar supplemented with rifampicin (50 µg/mL). Colony-forming units (CFU) were counted 24 h after incubation at 28 °C.

2.6. Fungal strain, spore collection, and plant inoculation

The *Botrytis cinerea* B05.10 strain was grown on Potato Dextrose Agar (PDA) for two weeks in the dark at 25 °C. The mycelium was scraped from the Petri dish, placed in a Tween 20 solution (0.02%, v/v), filtered through a mesh, and centrifuged to obtain spores. After washing with sterile distilled water, the spores were resuspended in Potato Dextrose Broth (PDB) medium supplemented with sucrose (10 mM) and KH_2PO_4 (10 mM) and adjusted to a final concentration of 1x10^5 spores/mL.

For broad-spectrum SAR assays, six-week-old WT or *put2-1* mutant plants were locally primed with *Pst* (OD_{600}=0.005) or Mock (10 mM $MgCl_2$) on the lower leaves and then systemically challenged with *B. cinerea* by inoculating a drop (5 µL) of spore suspension on the distal upper leaves. Inoculated leaves were collected at 24 and 48 hpi and stained with a 2.6 M Evans blue solution, following the protocol described by Vijayaraghavareddy et al. (2017). For tissue clarification, leaves were placed in an ethanol glycerol [90:10 (v/v)] solution and heated at 70 °C for 10 min or until no chlorophyll was visible. Clarified leaves were mounted on slides using a lactic acid:phenol:water [1:1:1 (v/v/v)] solution and photographed using a smartphone camera adapted to a stereoscope. Representative images were selected for each line and treatment, and lesion size was quantified using Image J Pro Plus 4.5 analysis software.

2.7. RNA isolation, Illumina sequencing, and data analysis

Total RNA was isolated using the TRI Reagent® method (Merck, Darmstadt, Germany) following SAR assays. The lower leaves were inoculated with *Pst* to induce local priming (SAR) or Mock treatment. At 24 hpi, the uninoculated upper leaves were collected for RNA extraction. In a second group of plants, 48 h after primary infection, upper leaves were systemically challenged with *Pst* and collected at 24 hpi for RNA extraction (see **Fig. 2a and b**).

Three biological replicates, each containing over 20 µg total RNA, were submitted to GENEWIZ® (https://www.genewiz.com/) for standard mRNA sequencing (by poly-A

selection) using the Illumina HiSeq platform with a 2×150 bp configuration. RNA-seq quality was analyzed using the FastQC v0.11.9 tool (Andrews, 2010), and the minimum read Phred score for the reads was 33. All reports from FastQC were collected using MultiQC v1.14. Neither adapter bias nor poor quality at the 3' end was evident in the RNA-seq data. There was no need to process the fragments. The Hisat v2.1.0 tool mapped the fastq files to the *Arabidopsis* genome. R internal scripts were used for the Hisat2 mapping statistics process (R core team, 2022). The featureCounts tool from the SubRead package was used to quantify the mapped fragments (Liao et al., 2019). An internal R script (R core team, 2022) produced a counts matrix using these quantifications.

Differential expression analysis was performed using the *edgeR* R package (Robinson et al., 2009); the calcNormFactors function was used to calculate the normalization factors, and the estimatedDisp function was used with default parameters to calculate the dispersion. The *glmFit* function fits the data to the Negative Binomial density distribution. The *glmLRT* function defined and performed the proposed contrast between conditions. Differentially expressed genes were defined as those with a fold change value of ≥ 2 and a *p*-value ≤ 0.05, and an FDR of ≤ 0.1. Genes of interest with significant differential expression were validated by qPCR.

Gene Ontology (GO) term enrichment was performed using a custom *R* script with a logistic regression model. The *glm* function with the Binomial density distribution family and the *logit* link was used to fit the logistic regression model $y=f(x)$, defining the classes y as class 1- transcripts in the GO term and class 0- transcripts not in the GO term. The score x was defined as $x=-log_{10}(FDR)sign(logFC)$.

The RNAseq data were deposited in the Gene Expression Omnibus database under the GEO accession GSE275478.

2.8. Gene expression analysis by RT-qPCR

Total isolated RNA was treated with DNAse I (Thermo Fisher Scientific, Massachusetts, USA) to eliminate gDNA. First-strand cDNA synthesis was performed using the RevertAid Reverse Transcriptase Kit (Thermo Fisher Scientific, Massachusetts, USA). Gene expression levels were quantified by qPCR using the Maxima SYBR green qPCR Master Mix (2x) (Thermo Scientific). Primers used are listed in **Table S1**. qPCR thermal cycling conditions consisted of 30 s at 95 ºC (initial denaturation), 40 PCR cycles of 10 s at

95 ºC (denaturation), and 30 s at 60 ºC (annealing/extension). Melting curves started at 65 ºC and gradually increased the temperature every 0.5 ºC up to 95 ºC. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the change in gene expression relative to the control samples, with *AtEF1α* (AT5G60390) as the reference gene.

2.9. Quantification of free polyamines

Six-week-old *Arabidopsis* plants were used to quantify the levels of free polyamines in the upper leaves after the different SAR treatments at 6 and 24 hpi. Briefly, lower leaves were inoculated with *Pst* to induce local priming (SAR) or Mock, and at 6 and 24 hpi the upper leaves were collected. In another set of plants, 48 h after the inoculation of the local leaves, the upper leaves were challenged with *Pst*, and samples were collected at 6 and 24 hpi. Leaves were snap-frozen in liquid nitrogen and stored at −80 °C. The samples were lyophilized, and the resulting dry weight (DW) was used for the targeted metabolic analysis.

Free polyamines were analyzed according to the method described by Ćavar Zeljković et al. (2024). 200 µL of 2 M NaOH was added to 200 µL of the supernatant of the extract described above, followed by 2.5 µL of benzoyl chloride in MeOH (50:50, v/v). The reaction mixture was vortexed for 5 s and then stirred for 40 min at 25 °C. After the reaction, 500 µL of saturated NaCl was added, and benzoylated polyamines were extracted with 2 × 500 µL of diethyl ether. The solvent was evaporated under a vacuum at 40 °C. Dry samples were dissolved in 200 µL of mobile phase and analyzed according to the protocol described by Ćavar Zeljković et al. (2024), with minor modifications to include additional target compounds. The analysis was performed in multiple reaction monitoring (MRM) mode, and transitions of the target compounds were as follows: Put (297.10>105.10, 297.10>77.05, 297.10>176.00), Dap (282.95>104.95, 282.95>77.00, 282.95>162.05), Dap-d_6 (288.95>112.95, 288.95>82.00, 288.95>170.05), Cad (311.10>104.95, 311.10>77.05, 311.10>190.20), nSpd (444.15>105.05, 444.15>162.00, 444.15>322.20), Spd (458.20>105.10, 458.20>162.15, 458.20>336.30), hSpd (472.20>247.20, 472.20>105.05, 472.20>176.05), nSpm (605.00>162.20, 605.00>105.20, 605.00>323.30), tSpm (619.25>105.00, 619.25>162.00, 619.25>337.20), Spm (619.25>497.30, 619.25>162.00, 619.25>105.00), and Agm (443.00>104.85, 443.00>375.00).

2.10. Quantification of free phenolic compounds

Analysis of phenolic compounds was performed according to the protocol described in Sedláková et al. (2023). The UHPLC-MS/MS measurements were carried out on an Ultra Performance LC-MS 8050 system (Shimadzu, Kyoto, Japan) with a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in negative mode. A 5 µL sample was injected into an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm, Waters, Milford, MA, USA), connected to the corresponding pre-column. The mobile phase consisted of a mixture of 15 mM formic acid (pH 3, adjusted with NH_4OH) (solvent A) and ACN (solvent B) at a flow rate of 0.4 mL/min. The linear gradient consisted of 10% B for 1 min, 10–13% B for 2 min, isocratic 13% B for 4 min, 13–25% B for 3 min, 25–70% B for 1.2 min, isocratic 75% B for 0.8 min, back to 10% B within 0.5 min, and equilibration for 3.5 min. The analysis was performed in multiple reaction monitoring (MRM) mode, and transitions of the target compounds were as follows: SAG (297.70>137.10, 297.70>93.10), pCA-d_6 (169.10>124.85, 169.10>97.15, 169.10>98.10), pCA (163.00>119.10, 163.00>93.00, 163.00>117.10), SiA (227.70>208.30, 222.70>192.20, 222.70>164.30), FA (193.10>134.25, 193.10>178.25), SA-d_4 (141.10>96.80, 141.10>69.10, 141.10>78.10), and SA (137.10>92.70, 137.10>65.00, 137.10>75.05). All standards and reagents were of the highest available purity and purchased from Sigma Aldrich Company (Prague, Czech Republic).

2.11. Stomatal aperture analysis

The stomatal aperture was assessed following Pantaleno et al. (2024). Epidermal peels were obtained from the abaxial side of leaves from six-week-old plants and placed in the opening buffer (5 mM MES pH 6.1, 50 mM KCl) for 3 h under light to encourage the optimal opening of most stomata. Then, the peels were treated with or without 1 µM flagellin 22 (flg22) for 0-, 15-, 30-, and 60-min periods. Stomata were observed under a microscope using a 40x objective and documented with a digital camera. The stomatal aperture was analyzed by Image J Pro Plus 4.5 analysis software.

2.12. Luminol assay

Luminol assays were conducted as outlined Bisceglia et al. (2015) with minor modifications. The lower leaves of six-week-old plants were infiltrated with Mock or *Pst* (as described above) to induce local priming, and the upper leaves were used for the luminol

assay. Discs were obtained from the midrib level using a sharp 4 mm biopsy punch from at least three leaves per plant and carefully transferred to a Petri dish containing ddH2O. Using a flat toothpick, one leaf disc was placed in each well of a 96-well luminometer plate, containing 150 µL of ddH_2O with the adaxial side facing upward. To minimize the impact of wounding, the plates were kept under the same growth conditions as the original plants for 20 h. Before measurement, water was removed from each well, and 100 µL of reaction solution (100 µM luminol, 10 µg/mL horseradish peroxidase, and 1 µM flg22) was added. Luminescence of the disc reaction mixture without the elicitor was monitored as the blank. Luminol luminescence was measured for one hour using the multimode reader Synergy H4 from BioTek, at 2-min intervals with an integration time of 1,000 ms.

2.13. Statistical analysis.

The results of each representative experiment are shown as the mean ± SE. Statistical differences ($p \leq 0.05$) were determined by Student's t-test using GraphPad Prism version 10.0.0; by Generalized Linear Mixed Models (GLMMs) with negative binomial distribution or by two-way, three-way, or four-way ANOVA with LSD Fisher comparison as appropriate using the InfoStat v2020e (https://www.infostat.com.ar/index.php?mod=page&id=15) statistical package. Graphs were obtained using GraphPad Prism version 10.0.0. Asterisks and different letters indicate statistically significant differences between the means.

The changes in the metabolic profile between lines and treatments were also analyzed using multivariate statistical analysis, specifically principal component analysis (PCA) and correlation matrices performed in RStudio (Version 1.1.463 – © 2009-2018 RStudio, Inc.) using the packages *factoextra, ggplot2, grid, ggrepel, ggnewscale* and *corrplot*.

3. Results

3.1. The AtPUT2 polyamine uptake transporter is essential for SAR establishment but not for basal resistance in Arabidopsis

SAR was induced in WT plants and single mutant lines of the *A. thaliana PUT* family (*put1-1*, *put2-1*, *put3-1*, *put4-1*, and *put5-1*). CFUs were estimated at 72 hpi following systemic challenge with *Pst* in six-week-old plants. In WT plants, bacterial titers were significantly lower in systemic leaves of locally challenged (primed) compared to the Mock-treated controls, indicating successful SAR establishment, as reported in previous studies (Rufián et al., 2019). We first analyzed bacterial titers in WT and mutant lines after Mock-infiltration. The *put1-1* behaved like WT, while the other mutants increased basal resistance **(Fig. 1a)**. Under SAR-induced conditions, *put1-1* and *put3-1* mutants reduced bacterial titers after priming as the WT (**Fig. 1a**). In contrast, the *put2-1*, *put4-1,* and *put5-1* mutant lines displayed a defective SAR. Notably, *put4-1* and *put5-1* mutants showed similar bacterial titers in primed plants as in the Mock-infiltrated ones (**Fig. 1a**).

The *put2-1* mutant exhibited the most pronounced SAR deficiency, with higher bacterial titers in primed plants than in the Mock-infiltrated ones, suggesting a key role for *At*PUT2 in SAR (**Fig. 1a**). To further study the role of *At*PUT2 in SAR, we generated three *Arabidopsis PUT2* overexpression lines (*35S::PUT2-2*, *-18,* and *-27*) and challenged them with *Pst*. All *35S::PUT2* overexpression lines were resistant to *Pst* inoculation, inducing SAR, and exhibited similar bacterial titers to the WT (**Fig. 1a**).

Following inoculation with the avirulent strain *P. syringae avrRpt2*, the *put2-1* mutant failed to establish SAR, as evidenced by increased bacterial titers compared to the native plant status (**Fig. 1b**). Interestingly, the *put2-1* mutant line also exhibited enhanced basal resistance (**Fig. 1a and b**). Specifically, *put2-1* plants inoculated with the Mock on the lower leaves and challenged on the upper leaves with *Pst* had lower bacterial titers than WT, a phenotype consistent even in four-week-old *put2-1* mutant plants (**Fig. S2**). This suggests a dual phenotype for the *put2-1* mutant, in which SAR is compromised, but basal resistance is enhanced.

3.2. Broad-spectrum SAR is lost in the Arabidopsis put2-1 mutant

SAR provides broad-spectrum protection against various pathogens. We examined whether *At*PUT2 influences this protection by testing SAR induced by *Pst* against an unrelated pathogen, *Botrytis cinerea*, in the WT and the *put2-1* mutant line (**Fig. 1c** and **d**). Plants in native or SAR status were systemically inoculated with *B. cinerea*, and the infected leaves were stained with Evans blue. In WT plants, local priming with *Pst* significantly reduced lesion sizes caused by *B. cinerea* at 48 hpi, demonstrating effective broad-spectrum SAR (**Fig. 1c**). However, the *put2-1* mutant showed larger fungal lesions in *Pst*-primed plants (**Fig. 1c** and **d**), indicating impaired broad-spectrum SAR. This result suggests that while the loss of *At*PUT2 function does not affect basal resistance to *Pst* in *Arabidopsis*, this transporter is crucial for managing SAR, even against unrelated pathogens.

3.3. Transcriptomic profiling reveals that the put2-1 mutant exhibits altered expression of genes associated with defense and SAR

To further investigate the role of the *AtPUT2* gene in plant defense, we conducted a transcriptome analysis of WT and *put2-1* mutant plants under native and SAR conditions at 24 hpi (**Fig. 2a** and **b;** complete gene list available at **File S1)**. For the native condition, the lower leaves of a set of plants were infiltrated with Mock solution, while the upper leaves were either kept in their native state or challenged with *Pst* (**Fig. 2a**). For the SAR condition, the lower leaves were infiltrated with *Pst,* while the upper leaves were either maintained in their native state or challenged with *Pst* (**Fig. 2a**). Samples were collected at 24 hpi as indicated (**Fig. 2b**). RNA-seq analysis revealed 28.8 ± 2.1 million fragments per library with high quality (minimum Phred score of 33) (**Fig. S3**). In the considered comparisons, 1,160 genes showed increased gene expression, and 3,857 showed decreased expression (**Fig. 2c** and **d**). In the *put2-1* mutant, the comparison of SAR $^{(1^{o}Pst\,/\,2^{o}Pst)}$ vs. native $^{(1^{o}\text{Mock}\,/\,2^{o}Pst)}$ had the highest number of differentially expressed genes (DE), with 1,107 increased and 3,811 decreased (**Fig. 2c** and **d**). Furthermore, there were only 9 genes with increased and 3 with reduced expression shared between *put2-1* [SAR $^{(1^{o}Pst\,/\,2^{o}Pst)}$ vs. native $^{(1^{o}\text{Mock}\,/\,2^{o}Pst)}$] and WT [SAR $^{(1^{o}Pst\,/\,2^{o}\text{Native})}$ vs. native $^{(1^{o}\text{Mock}\,/\,2^{o}\text{Native})}$] conditions (**Table S2**).

Gene Ontology (GO) enrichment analysis, based on the Differential Expression results, revealed deregulated terms associated with SAR (GO: 0009627), response to hydrogen peroxide (GO:0042542), regulation of defense response (GO:0031347), response

to fungus (GO:0009620), plant-type hypersensitive response (GO:0009626), fatty acid biosynthetic process (GO:0006633), thylakoid membrane organization (GO:0010027) and plant-type cell wall organization (GO:0009664) in the *put2-1* mutant compared to WT (**Fig. S4**). A heatmap summarizing the GO enrichment analysis of all contrasts is shown in **Fig. 2e**. Representative deregulated genes associated with SAR and defense in *put2-1* under SAR (^{1º}*Pst* / ^{2º}*Pst*) condition are listed in Table 1, with many linked to SA and Pip metabolism.

3.4. The put2-1 mutant displays deregulation in the SA and SAR marker genes

Expression of SAR and defense marker genes was evaluated in the upper (systemic) leaves of the WT and *put2-1* plants under native or SAR conditions (**Fig. 3**). The key examined genes included: SA synthesis and response (*PAD4*, *SARD1*, *ICS1*, and *PR1*), Pip and NHP biosynthesis (*ALD1, SARD4,* and *FMO1*), SAR signaling (*AZI1*), negative regulation of *NPR1* (*NIMIN1*), glycosylation of NHP and SA (*UGT76B1*), the ethylene- and jasmonate-responsive plant defensin (*PDF1.2*) and a Spm responsive gene (*NHL10*) (**Fig. 3**). The expression of most genes was influenced by the interaction among local priming, systemic challenge and genotype according to ANOVA (**File S2**). Under native (^{1º}Mock / ^{2º}Native) conditions, *put2-1* exhibited increased expression of most genes related to SA synthesis and response (*PAD4*, *SARD1*, *ICS1, PR1*), Pip, and NHP biosynthesis (*ALD1* and *FMO1*), as well as *AZI1* and *PDF1.2* compared to WT (**Fig. 3a-e**, **g**, **j** and **k**), consistent with its basal resistance phenotype (**Fig. 1a**). After local priming with *Pst* (^{1º}*Pst* / ^{2º}Native), expression of these marker genes showed slight changes in WT plants regarding the native uninoculated (^{1º}Mock / ^{2º}Native) condition (**Fig. 3**). At the same time, *put2-1* increased the expression of *PAD4, SARD4, NIMIN1, UGT76B1*, *AZI1*, and *NHL10* (**Fig. 3a**, **f**, **h-j**, and **l**), but decreased *PR1, ALD1,* and *FMO1*, as well as *PDF1.2* (**Fig. 3d, e, g** and **k**), compared to the native uninoculated condition. ß

In systemically challenged plants without priming (^{1º}Mock / ^{2º}*Pst*), both WT and *put2-1* upregulated most marker genes, particularly *SARD1* and *PR1* in the mutant (**Fig. 3b** and **d**). Interestingly, genes related to SA and NHP biosynthesis (*SARD1*, *ICS1*, *SARD4,* and *FMO1*), as well as the *PR1* gene, showed higher expression in the *put2-1* mutant compared to WT under the locally primed and systemically challenged (^{1º}*Pst* / ^{2º}*Pst*) condition (**Fig. 3b, c, f**, and **g**). In the same conditions, negative regulators (*NIMIN1* and *UGT76B1*) and *NHL10* were strongly induced in *put2-1* compared to WT (**Fig. 3h, i**, and **l**). Notably, *NIMIN1*, *UGT76B1,*

and *NHL10* displayed a consistent expression pattern in *put2-1* across all tested conditions, differing markedly from WT (**Fig. 3h, i**, and **l**). Overall, the simultaneous strong induction of both positive and negative SAR regulators in *put2-1* likely disrupts the balance between induction and attenuation of SAR, leading to an impaired systemic response.

The stronger expression of SA and SAR marker genes in *put2-1* under SAR ($^{1°Pst\,/\,2°Pst}$) condition compared to WT suggested a delayed response to secondary infection. To test this, we examined the expression of SAR-related genes (*ICS1, PR1, FMO1, SARD4, NHL10,* and *UGT76B1*) at an earlier time point (6 hpi) (**Fig. S5**). ANOVA revealed that, except for *ICS1*, the expression of all genes was influenced by the interaction among local priming, systemic challenge, and genotype (**File S2**). Our results confirmed that the WT responded more rapidly to systemic challenge without priming ($^{1°Mock\,/\,2°Pst}$), whereas *put2-1* exhibited a delayed response (**Fig. S5**). The exception was *SARD4*, which behaved similarly in both lines (**Fig. S5c**). Interestingly, in the SAR and systemically challenged condition ($^{1°Pst\,/\,2°Pst}$), most analyzed genes were less expressed in WT than in *put2-1*, except for *PR1* and *UGT76B1*. In WT, priming likely triggers a rapid response after the challenge of systemic leaves with *Pst*, and by 6 hpi, the expression levels of marker genes may have already decreased. This suggests that plant status differentially affects gene expression in the WT and the *put2-1* mutant.

3.5. AtPUT2 is crucial for the proper expression of SAR marker genes relying on putrescine.

To investigate the role of free polyamines and their transport in SAR, we locally infiltrated the lower leaves of six-week-old WT and *put2-1* mutant plants with Mock ($MgCl_2$) or 30 µM of selected polyamines (Put, Spd, Spm). Then, we measured the expression of SAR marker genes in the upper leaves at 24 hpi. ANOVA revealed that the interaction between polyamine type and genotype influenced gene expression (**File S2**). In WT, Put treatment significantly increased the expression of all marker genes, but the systemic effect of Put was impaired in the *put2-1* mutant (**Fig. 4**). Spd strongly induced *PR1*, *SARD4, FMO1,* and *NIMIN1* expression in WT, but had little effect on the other marker genes analyzed (**Fig. 4b-d**, and **h**). In *put2-1*, Spd treatment reduced marker gene expression (**Fig. 4**). Finally, Spm increased the expression of negative regulators of SAR (*UGT76B1* and *NIMIN1*) in *put2-1,* and to a lesser extent, *UGT76B1* expression in WT (**Fig. 4e** and **h**). These results align with previous findings that local Put treatment triggers a SAR-like response in *Arabidopsis* (Liu

et al., 2020), and demonstrate the crucial role of the *At*PUT2 transporter for relaying systemic signal(s) to distal leaves for proper SAR gene expression.

3.6. Local infiltration of Put results in a reduction of bacterial titers in systemic tissues

Since Put infiltration triggered the expression of SAR marker genes, we evaluated its impact on *Pst* systemic resistance. Six-week-old WT, *put2-1*, and *35S::PUT2* overexpression lines were locally infiltrated with 30 μM Put or Mock, followed by *Pst* inoculation of the upper leaves 48 h later. At 72 hpi, Put-treated WT plants showed significantly lower bacterial titers than the Mock-treated plants, indicating SAR activation (**Fig. 5**). Conversely, the *put2-1* consistently maintained low bacterial titers, with no significant differences between Mock and Put treatments (**Fig. 5**). Interestingly, the *35S::PUT2* overexpression lines exhibited low bacterial titers even in the Mock-treated plants, similar to the *put2-1* mutant, suggesting basal resistance. Furthermore, the *35S::PUT2* lines had enhanced systemic resistance under Put treatment (**Fig. 5**). These results indicate that Put or its derived metabolites trigger SAR induction in the WT plants (**Fig. 5**). Although Put is a less preferred substrate for PUT2 in yeast expression assays (Mulangi et al., 2012), our findings suggest that *in planta,* PUT2 play a role in Put transport that is crucial for conferring systemic resistance to *Pst*.

3.7. The put2-1 mutant line is affected in the Put mobilization.

As previously observed, Put infiltration upregulated the expression of SAR marker genes and a SAR-like phenotype in the WT, but not in *put2-1*, which aligns with the defective Put transport in this mutant line. Therefore, we assessed the effect of local infiltration with 30 μM Put on the content of free polyamines in distal leaves at 6- and 24-h post-infiltration (**Fig. 6**). A significant interaction was found among genotype, treatment, and time (**File S2**). In the WT, a notable increase in endogenous Put levels was detected in the distal leaves 6 h after Put infiltration of lower leaves, compared to the Mock treatment (**Fig. 6b**). This result demonstrates active mobilization of Put from lower to upper leaves. At this time, PCA also showed that Put and Spd were rapidly accumulated in the Put-infiltrated WT (**Fig. 6b, c,** and **e**). Contrary, following Put infiltration, *put2-1* exhibited either decreased (6 h) or unchanged (24 h) Put content in systemic leaves (**Fig. 6b**), indicating impaired Put mobilization from lower to upper leaves. Interestingly, the *35S::PUT2-27* overexpression line behaved

similarly to WT, increasing the endogenous Put content at 6 and 24 h after Put infiltration (**Fig. 6b**). Moreover, this line also rapidly accumulated other superior polyamines, such as Spm and tSpm, among others, but reduced their levels at 24 h, as happened in the WT (**Fig. 6e; Fig. S6**). These results may suggest that the Put fast transport is needed for the synthesis of the superior polyamines to regulate *Arabidopsis* SAR-induced response against *Pst*. This assumption was then further supported by the changes observed in the Put precursor Agm, which reduced in *put2-1* at 6 h and increased at 24 h, perhaps as a compensation response in the distal leaves for the Put reduced transport (**Fig. 6a**). In this regard, PC analysis indicated that in the *put2-1* mutant, Put infiltration induced Cad at 6 h, but didn't accumulate the higher polyamines until 24 h after the treatment (**Fig. 6e**).

3.8. Putrescine modulates SA levels in distal tissues

As described in previous sections, the *put2-1* mutant upregulated SA and SAR marker genes under native conditions (**Fig. 3, Fig. S5**), suggesting a deregulation of endogenous SA content. Therefore, a deeper analysis of phenolic compounds, including SA and SA-glucoside (SAG), was performed in the distal leaves of plants at 6 and 24 h after Put-infiltration (**Fig. 7**). The Mock-treated *put2-1* mutant exhibited higher SA content than the WT at 6 h (**Fig. 7a**). Following Put infiltration, the WT distal leaves showed a significant increase in SA levels at 6 h, which remained at 24 h (**Fig. 7a**). These results suggest that Put translocation to distal tissues may regulate SA levels in the WT. In contrast, *put2-1* displayed delayed SA accumulation, which only increased after 24 h, implying impaired Put-mediated systemic signaling (**Fig. 7a** and **7f**). The *35S::PUT2-27* overexpression line maintained elevated SA levels both with and without Put treatment (**Fig. 7a**), a feature also observed in the PCA analysis (**Fig. 7f**).

Put infiltration in the local leaves also influenced the SAG content of the distal leaves (**Fig. 7b**). For instance, the WT accumulated significantly higher SAG levels in Put-infiltrated plants at 24 h, most likely to regulate SA levels that were significantly increased at 6 h (**Fig. 7a, b,** and **f**). Similarly, the *put2-1* mutant enhanced SAG content at 24 h after Put infiltration (**Fig. 7b**). In contrast, the *35S::PUT2-27* overexpression line significantly raised SAG levels from 6 h after Put infiltration and kept them elevated at 24 h (**Fig. 7b** and **f**). Altogether, the data demonstrate that local Put infiltration and its transport via *At*PUT2 impact SA and SAG levels in systemic leaves. Furthermore, Put infiltration also modified

the endogenous content of other phenolic compounds, including hydroxycinnamic acids, such as sinapic (SiA), ferulic (FA), and *p*-coumaric acid (pCA) (**Fig. 7c-e**). Although no clear regulation of Put infiltration was observed regarding the changes of these last phenolic compounds, it was clear that *At*PUT2 modulation may contribute to regulating the levels of SiA, which significantly increased in the WT at 6 h after Put infiltration, but not in *put2-1* and *35S::PUT2-27* lines (**Fig. 7c**).

3.9. The put2-1 SAR deficiency is due to its disrupted polyamine homeostasis

We also measured the same aforementioned polyamines and phenolic compounds in the WT, *put2-1*, and *35S::PUT2-27* to understand the metabolic alterations due to *AtPUT2* disruption under SAR conditions (**Fig. 8; Fig. S7**). Significant differences were observed between the WT and the *put2-1* plants, while the *35S::PUT2-27* line behaved similarly to WT (**Fig. 8; File S2**). At 6 hpi under native conditions $^{(1ºMock\ /\ 2ºMock)}$, the *put2-1* mutant had increased content of several higher polyamines [Spd, hSpd, norspermidine (nSpd), Spm, tSpm, norspermine (nSpm)], as well as Cad and 1,3-diaminopropane (Dap), compared to the WT (**Fig. 8b; Fig. S7**). The *put2-1* mutant maintained increased levels of nSpd, Spm, nSpm, and tSpm at 24 hpi in native conditions, which align with the higher *ADC*, *SPMS*, and *ACL5* expression levels in this mutant (**Fig. 8g; Fig. S7 and S8**). Interestingly, there was a clear negatively correlation between Put and Spd and other polyamines like hSpd, Spm, tSpm, nSpd, and nSpm at 6 hpi (**Fig. 8f**). Maybe, the Put deficiency in *put2-1* is not only due to the limited Put transport but also rapid Spd conversion into the final product, such as Spm and tSpm, among others, which would condition the SAR. As a corroboration, *put2-1* reduced the Put content under SAR $^{(1ºPst\ /\ 2ºPst)}$ at 6 hpi (**Fig. 8a**). Contrarily, the WT and *35S::PUT2-27* accumulated higher polyamines and Dap under SAR $^{(1ºPst\ /\ 2ºPst)}$, whereas the *put2-1* mutant showed a correlation with phenolics, including SiA, SA, and SAG (**Fig. 8c, d, e; Fig. S7**). The elevated levels of SA and SAG in the *put2-1* mutant under SAR (**Fig. 8c** and **d**) were accompanied by the upregulated expression of *ICS1* and *UGT76B1* genes (**Fig. S5a** and **f**). Moreover, a clear correlation was observed between changes in concrete polyamine levels and phenolics (**Fig. 8f** and **h**). For instance, Spd showed a negative correlation with SAG at both 6 and 24 hpi, and SA and SAG content were also negatively correlated with Spd, Spm, and tSpm (**Fig. 8f** and **h**).

Altogether, our data suggest that higher polyamine accumulation precedes the accumulation of phenolic compounds (SA and hydroxycinnamic acid accumulation) under SAR conditions in the WT and the *35S::PUT2-27*, an aspect that was inverted in the *put2-1* mutant (**Fig. 8e** and **g**).

3.10. The SAR-deficient put2-1 is due to a ROS and stomatal immunity disruption.

To further explore systemic responses, we evaluated ROS and stomatal immunity in the WT, *put2-1,* and *35S::PUT2-27* lines (**Fig. 9**). First, superoxide radical ions were detected histochemically in response to local and systemic *Pst* inoculation (**Fig. 9a**). WT plants increased blue staining, indicating accumulation of superoxide radical ions in their leaves under SAR $^{(1^o Pst\ /\ 2^o Pst)}$. Contrarily, the *put2-1* mutant line only accumulated superoxide radical ions after local *Pst* inoculation $^{(1^o Mock\ /\ 2^o Pst)}$, but not under SAR $^{(1^o Pst\ /\ 2^o Pst)}$ (**Fig. 9a**), suggesting that the loss of *At*PUT2 function affects ROS accumulation during SAR. This pattern of ROS accumulation aligns with *RBOHD* gene expression, which is highly expressed after local *Pst* inoculation $^{(1^o Mock\ /\ 2^o Pst)}$ but significantly downregulated under SAR $^{(1^o Pst\ /\ 2^o Pst)}$ in the *put2-1* mutant (**Fig. S8e**).

To further explore the contribution of *AtPUT2* to the ROS response, ROS burst in discs taken from the upper (native leaves) was evaluated under flg22 elicitation 48 h after priming the lower leaves with Mock or *Pst* using the luminol assay (**Fig. 9b**). Again, different ROS responses were observed among lines. For instance, WT accelerated the ROS production in the *Pst*-primed (t_{max} ca. 10 min) plants compared to Mock-treated plants (t_{max} ca. 14 min), confirming that priming accelerates responsiveness to flg22 elicitation (**Fig. 9b** and **c**). A similar tendency was observed in the *35S::PUT2-27* line (**Fig. 9d**). However, the *put2-1* mutant showed similar ROS curves under both *Pst*-primed and Mock-treated plants (**Fig. 9b** and **c**). Moreover, the overall curves, calculated by integrating the area under the curve (AUC), were also compared for the three lines (**File S2**). Although the treatment was not significant, lines showed a clear difference in activating ROS signals, with the *put2-1* mutant exhibiting a substantial compromise in its capacity for ROS accumulation.

The ROS deficiency observed in the *Pst*-primed plants in *put2-1* under SAR and after flg22 elicitation (**Fig. 9a** and **c**) suggests that this line may have an alteration in the initial ROS-regulated immune responses occurring in the stomata (Song et al., 2014). To analyze this possibility, we observed stomatal changes in Mock or *Pst*-primed plants by eliciting the

distal leaves with flg22 (**Fig. 9e** and **f**). Under control conditions, WT and *put2-1* plants showed similar stomatal apertures, whereas the *35S::PUT2-27* line had reduced apertures. After flg22 elicitation, all lines induced stomata closure after 15 min (**Fig. 9d**). However, whereas the WT and *35S::PUT2-27* lines kept a closed status throughout the experiment, the *put2-1* mutant exhibited significantly larger stomatal apertures at 30 min of flg22 elicitation (**Fig. 9e** and **f**). Altogether, our results demonstrate that the *AtPUT2* gene regulates SAR by ROS signaling and stomatal closure in *Arabidopsis*.

4. Discussion

SAR enhances plant immunity by preparing defenses against future infections. Understanding the signaling molecules involved is crucial for deciphering systemic responses and improving sustainable disease resistance in crops. Our study investigated how restricting polyamine transport in *Arabidopsis* polyamine uptake transporter (*put*) mutant lines affects SAR activation. Our findings revealed differences in SAR efficacy among *Arabidopsis put* mutants, suggesting that specific polyamine transporters play a role in systemic immunity. While *put1-1* and *put3-1* mutants demonstrated SAR activation comparable to WT plants, the *put2-1*, *put4-1*, and *put5-1* lines exhibited noticeable SAR deficiencies. Notably, the *put2-1* mutant showed significant susceptibility to reinfection, with higher bacterial titers in SAR-induced plants compared to Mock-infiltrated controls. Further analysis of the *AtPUT2* gene revealed its involvement in broad-spectrum SAR, as the *put2-1* mutant could not limit *B. cinerea* infection following local priming. The dual phenotype observed in *put2-1*, compromised SAR but enhanced basal resistance to *Pst*, highlights a distinctive characteristic of this mutant, suggesting that polyamine transport is key for polyamine-mediated systemic immune responses. Furthermore, the enhanced basal resistance in the *put2-1* mutant remained unaffected by plant age or photoperiod, suggesting that this defense mechanism operates independently of the developmental stage and light-dependent signaling pathways.

Previous studies have shown that mutants defective in lipid biosynthesis and transport (i.e. *sfd1*, *sfd2*, *fad7*, *mgd1, dir1, mod1, lacs2*), the biosynthesis, transport and perception of the mobile signals (i. e. *azi1*, *earli1*, *che*), and epigenetic modifications (i. e. *rsi1*, *ref6*, *fve*, *gstt2*, *pwr*), as well as the lines overexpressing *RRTF1* and *PDLP*, lose SAR induction but maintain basal resistance against *P. syringae* pv. *maculicola* (*Psm*) or to *Pst*,

with bacterial titers comparable to those in WT (Maldonado et al., 2002; Nandi et al., 2004; Chaturvedi et al., 2008; Singh et al., 2013; 2023; Carella et al., 2015; Cecchini et al., 2015; Banday and Nandi, 2018; Chowdhury et al., 2020; Patil and Nandi, 2022; Cao et al., 2024). Conversely, mutants such as *npr1* and *ald1,* and overexpression of *UGT76B1*, which are involved in SA and Pip metabolism, lose both SAR and basal resistance (Cao et al., 1997; Bauer et al., 2021; Jiang et al., 2021). Interestingly, *put2-1* shares some features with these mutant lines but does not fully fit the described phenotypes. Unlike most SAR-deficient mutants, which retain basal resistance, *put2-1* shows enhanced local resistance, as evidenced by reduced bacterial titers.

Although local Put infiltration reduced bacterial titers in WT systemic leaves, mimicking SAR activation, the *put2-1* mutant showed no difference in bacterial titers between Mock and Put-treated plants, suggesting impaired perception or response to Put. Conversely, the *35S::PUT2* lines showed enhanced systemic resistance upon Put treatment. Quantitative analysis confirmed that both WT and *35S::PUT2* lines effectively translocated Put from local to distal tissues, whereas the *put2-1* mutant failed to do so (**Fig. 6**). These findings support the notion that *At*PUT2 is required for the long-distance transport of Put, which is essential for SAR activation and the regulation of SAR-associated gene expression. A recent study identified a tomato homolog of *PUT2* (phylogenetically clustered with the *Arabidopsis PUT1*, *PUT2,* and *PUT3* genes) as mediating shoot-to-root transport of polyamines (Spd and Put) during salt stress, a process regulated by the HB52 transcription factor (Yang et al., 2025) Additionally, the tomato PUT3 transporter is involved in polyamine uptake under salt stress (Yang et al., 2024), underscoring the significance of polyamine transport in mediating plant stress responses.

To further understand the SAR-deficient *put2-1* phenotype and the relevance of polyamine regulation, we conducted RNA-seq transcriptomic analysis. Several genes associated with systemic acquired resistance, regulation of defense response, plant-type hypersensitive response, response to hydrogen peroxide, photorespiration, and fatty acid biosynthetic process were deregulated in the *put2-1* mutant under SAR. Specifically, genes related to SA and NHP metabolism, which regulate local immunity and SAR, were altered in *put2-1*. Interestingly, the *ICS1* and *FMO1* genes, crucial for SA and NHP biosynthesis, were highly expressed in the *put2-1* mutant, especially at 24 hpi, when the WT had already reduced their expression. This result indicated that polyamine transport mediated by *At*PUT2

may be crucial for controlling SA and NHP biosynthesis under SAR. None of the known mobile SAR-inducing signals, such as MeSA, AzA, DA, G3P, NHP, and eNAD(P), have been reported to activate systemic SA biosynthesis directly (Kachroo and Kachroo, 2020; Vlot et al., 2021). Interestingly, our results showed a strong link between polyamines and SA biosynthesis because Put infiltration in lower leaves triggered SA accumulation in the upper (systemic) leaves, revealing a novel role for Put or a derived metabolite in regulating systemic SA levels. This extends previous observations that Put stimulates local SA accumulation and induces local and systemic transcriptional reprogramming of genes involved in SAR (Liu et al., 2020). Additional corroboration was that *At*PUT2 was essential for Put-mediated systemic induction of *ICS1*, *PR1*, *SARD4*, *FMO1*, *UGT76B1*, and *NHL10* genes, as this response was largely suppressed in the *put2-1* mutant. Based on our observations, polyamines may act as a key regulatory hub, contributing to the fine-tuning of the balance between activation and attenuation of SAR. While Put functions as an inducer of SAR and defense-related genes (Liu et al., 2020), we observed that higher polyamines, mainly Spm, promote the expression of genes encoding enzymes that inactivate SA and NHP, or their signaling pathways, such as *UGT76B1* and *NIMIN1* (**Fig. 4**).

UGT76B1, a glycosyltransferase that modifies SA, NHP, and isoleucine, plays a pivotal role in regulating the levels of the active forms that mediate plant immunity (Bauer et al., 2021; Mohnike et al., 2021). *UGT76B1* overexpression increases susceptibility to *Psm* and impairs SAR activation (Bauer et al., 2021). In contrast, the *ugt76b1* mutant exhibits constitutive SAR and accumulates elevated levels of free SA and NHP, indicating impaired inactivation and turnover of these signaling molecules (Bauer et al., 2021; Mohnike et al., 2021). Similarly, we observed decreased *UGT76B1* transcript levels in WT plants from 6 to 24 hpi under SAR $^{(1°Pst\,/\,2°Pst)}$, correlating with increased SA levels, which help bolster plant defense. Conversely, increased *UGT76B1* expression in the *put2-1* mutant under SAR conditions likely promotes the glycosylation and subsequent inactivation of SA, NHP, and ILA, compromising the systemic immune response. Interestingly, we also found that both Spm treatment and *put2-1* under SAR upregulated *UGT76B1* expression in *Arabidopsis*. Moreover, the high Spm levels in the mutant line indicate a clear connection between *UGT76B1* transcript levels and Spm. The *put2-1* mutant also exhibited elevated levels of other higher polyamines, including tSpm, nSpd, and nSpm, which aligned with increased *ADC2, SPMS,* and *ACL5* expression. Consistent with these findings, Kim et al., (2019)

reported Spm overaccumulation in seeds of the *put2-1* mutant. This accumulation suggests potential defects in polyamine distribution or metabolic regulation within the cell, reinforcing the idea that *At*PUT2 is critical for maintaining polyamine homeostasis and proper compartmentalization.

Polyamine supplementation experiments showed that Spd and Spm differently regulate gene expression. Spd treatment induced *NIMIN1* expression in WT plants but not in *put2-1*, while Spm increased *NIMIN1* and *UGT76B1* expression in *put2-1*. NIMIN1, a negative regulator of plant immune responses, acts by sequestering the SAR master regulator NPR1 (Mohan et al., 2016). *NIMIN1* was highly expressed in the *put2-1* mutant under native conditions and was further upregulated in response to local priming ($^{(1º\textit{Pst}\ /\ 2ºNative)}$), systemic challenge ($^{(1ºMock\ /\ 2º\textit{Pst})}$), and SAR ($^{(1º\textit{Pst}\ /\ 2º\textit{Pst})}$). Thus, affecting the defense responses in *put2-1*. Overexpression of *NIMIN1* leads to SA intolerance (Mohan et al., 2016), mimicking the *npr1* mutant phenotype characterized by reduced expression of SA-induced *PR* genes, including *PR1*, and impaired SAR. Interestingly, despite the loss of SAR in *put2-1*, *PR1* expression was not repressed, suggesting that a SA-independent pathway might regulate its expression.

ROS are key signaling molecules involved in regulating SAR and stomatal immunity. Previous studies have shown that Put induces *RBOH-D* and *RBOH-F*, which generate apoplastic ROS and contribute to SAR-like responses (Liu et al., 2020). In our study, the *put2-1* mutant line accumulated superoxide radical ions in response to local *Pst* inoculation ($^{(1ºMock\ /\ 2º\textit{Pst})}$) but not under SAR ($^{(1º\textit{Pst}\ /\ 2º\textit{Pst}),}$) suggesting a defect in systemic ROS signaling. Various experimental approaches in this study consistently demonstrated that the *put2-1* mutant displays impaired ROS accumulation and systemic signaling. For instance, luminol-based assays revealed that the *put2-1* mutant had weaker ROS signals than the WT and the *35S::PUT2-27* line. Following flg22 elicitation, the *put2-1* mutant showed no differences in ROS dynamics between Mock-treated and *Pst*-primed plants, confirming the compromised systemic response. Moreover, stomatal closure assays revealed that *put2-1* exhibited attenuated responsiveness to flg22 compared to WT, while the *35S::PUT2-27* showed an enhanced response. While stomatal closure mediated by the FLG22 receptor is known to be SA-dependent, polyamines (Put, Spd, and Spm) also significantly contribute to this process by enhancing nitric oxide and ROS production in guard cells. This polyamine response involves the activity of RBOH and amine oxidase enzymes, contributing to stomatal closure (Zheng et al., 2015; Agurla et al., 2018). Put stimulates NO production, thereby promoting

stomatal closure (Agurla et al., 2018), a process that may be impaired in *put2-1* due to its reduced Put content under SAR.

SA modulates polyamine metabolism in *Arabidopsis*, increasing Put accumulation via an NPR1-independent but partially MPK6-dependent mechanism, while *Pst* triggers Put accumulation in a SA-dependent manner (Rossi et al., 2021). Although the polyamines-ROS-SA crosstalk has primarily been studied at the biosynthesis level, our findings indicate that *At*PUT2-mediated polyamine transport also regulates SA and ROS levels, thereby modulating plant defense and stomatal immunity.

Another key finding of this study was that the *put2-1* mutant exhibited altered expression of genes encoding chloroplast enzymes, mainly related to galactolipid biosynthesis, suggesting a metabolic disorder in this organelle. In addition to its role in photosynthesis, the chloroplast is a crucial organelle involved in synthesizing Pip and precursors of SA, and coordinating plant defense responses (Kachroo et al., 2021). The *At*PUT2 transporter has been reported to localize to multiple intracellular compartments, including the chloroplast and the Golgi apparatus (Li et al., 2013; Fujita and Shinozaki, 2014; Ahmed et al., 2017), with additional localization predictions to the plasma membrane, vacuole, and mitochondria according to the SUBA4 database (Hooper et al., 2017). In chloroplasts, polyamines are key for stabilizing thylakoid structure, post-translational modifications of antenna proteins, and regulating photosynthesis, photoadaptation, and stress responses (Sobieszczuk-Nowicka and Legocka, 2014). Our findings suggest that *At*PUT2 may be involved in chloroplast-associated metabolic processes crucial for immune signaling and systemic resistance. However, other previously described roles for polyamines in the chloroplast may also be dependent on their transport.

Although further studies are needed to understand the chloroplast-associated regulation of *At*PUT2 in SAR, our assumptions are also supported by the fact that the *put2-1* mutant exhibits a similar phenotype to loss-of-function mutants of genes involved in galactolipid biosynthesis, with compromised SAR, while retaining basal resistance to *Pst* (Maldonado et al., 2002; Nandi et al., 2004; Chaturvedi et al., 2008; Lim et al., 2020). In these mutants, the loss of SAR has been linked to defects in plastid function (Chaturvedi et al., 2008). For example, the *MGDL* gene, which encodes a chloroplast A-type monogalactosyldiacylglycerol synthase required for galactolipid synthesis, plays a role in

generating precursors for mobile signals, such as Aza and G3P, which are essential for SAR (Kachroo et al., 2021).

Recently, it has been found that levels of various galactolipids, such as MGDG (18:3, 16:3), MGDG (18:3, 16:2), and MGDG (16:3), which are primary precursors of jasmonic acid (JA), increase in the Spm-deficient *spms* mutant that is *Pst* susceptible and *Bc* resistant (Zhang et al., 2023). Therefore, it is reasonable to suggest that the increased Spm levels in the *put2-1* mutant may also influence galactolipid and JA content as well as the defense responses to pathogens. Moreover, the increased susceptibility to *Bc* in systemic tissues of the *put2-1* mutant could also result from alterations in the ethylene-JA pathway, as recently shown by Zhang et al. (2023) and Peña-Lucio et al. (2025).

Our transcriptomic analysis also revealed that many genes associated with photosynthesis and chloroplast function (**Table S3**), including those involved in galactolipid synthesis in the chloroplast, such as *MGD1, FAD4,* and *FAD7,* were down-regulated in the *put2-1* mutant line (**Table 1, Fig. S9**). These findings reinforce the evidence that *At*PUT2 impacts chloroplast function, and that the SAR deficiency observed in the *put2-1* mutant may also result from disrupted production of galactolipid-derived mobile signals.

5. Conclusion

Our findings reveal the crucial role of the Polyamine Uptake Transporter 2 (*PUT2/LAT4)* in SAR and broad-spectrum SAR establishment in *Arabidopsis*. **Fig. 10** shows a model integrating the main findings related to the function of Put and *At*PUT2 in systemic responses. *At*PUT2 transporter is required for Put transport to systemic tissues, which regulates the expression of essential genes involved in SA and NHP biosynthesis and modulates SA levels. Under SAR ($^{(1°Pst\ /\ 2°Pst)}$), the *put2-1* mutant exhibits alterations in the expression of SAR-related genes, SA, ROS, and Put content, indicating that the proper timing of accumulation of each actor is crucial for SAR establishment. Particularly, ROS signaling is compromised in the *put2-1* mutant, affecting other key defense responses such as stomatal immunity. The downregulation of crucial genes involved in galactolipid synthesis in the *put2-1* mutant points to future research regarding the importance of polyamine transport in chloroplast-mediated SAR responses. Understanding how polyamine transport mechanisms contribute to plant defense could significantly enhance our understanding of plant stress resilience.

Figure captions.

Figure 1. Evaluation of SAR and broad-spectrum SAR in *Arabidopsis* polyamine uptake transporter mutants and overexpression lines. a) Bacterial titers in systemic leaves of WT and polyamine uptake transport mutants (*put1-1*, *put2-1*, *put3-1*, *put4-1*, and *put5-1*) and *AtPUT2* overexpression lines (*35S::PUT2-2*, *-18*, and *-27*) which were locally infiltrated with Mock, maintaining a native plant status, or challenged with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) to induce SAR. **b**) Bacterial titers in systemic leaves of WT and *put2-1*. Plants were locally infiltrated with Mock or challenged with the avirulent strain *P. syringae avrRpt2*. CFU, colony-forming units. Experiments were repeated twice with similar results. Values show the mean of five or six biological replicates ± SE. Different letters indicate significant differences ($p \leq 0.05$) between treatments according to two-way ANOVA and LSD Fisher's post-hoc test. **c**) Lesion size in WT and *put2-1* mutant plants, locally infiltrated with Mock or challenged with *Pst*, was assessed in systemic leaves after inoculation with *B. cinerea* (*Bc*). Values are the mean of four biological replicates (n=12 leaves) ± SE. Lesion size (cm^2) induced by *Bc* was quantified using ImageJ® software. Different letters denote significant differences ($p \leq 0.05$) between treatments via three-way ANOVA followed by LSD Fisher's post-hoc test. **d**) Evans blue staining at 24 and 48 hpi with *Bc*. Representative images of 12 leaves. Scale bar (0.5 cm). Each image displays a 2x digital zoom.

Figure 2. Transcriptomic analysis of *put2-1* mutant under native and SAR conditions. **a**) Experimental design and **b**) sampling. WT and *put2-1* plants were inoculated with *Pst* to establish the following conditions: native plant status and SAR. The lower leaves (8, 9, and 10) were inoculated with *Pst* at $OD_{600} = 0.005$ to induce local priming (SAR) or treated with a Mock solution ($MgCl_2$ 10 mM) to maintain a native plant status. At 24 hpi, the uninoculated upper leaves (13, 14, and 15) were collected for RNA extraction. In a second group of plants, 48 hours after primary infection, the upper leaves were systemically challenged with *Pst* at $OD_{600} = 0.0005$ and then collected 24 hpi for RNA extraction. For each condition, at least six plants were used. Scissors indicate the upper leaves that were harvested for the experiments. Venn diagrams representing the common and specific number of **c**) upregulated genes and **d**) down-regulated genes between the considered contrasts. **e**) Heatmap

summarizing the Gene Ontology (GO) enrichment for selected terms based on the contrasts. The scale color represents the enrichment score.

Figure 3. Gene expression analysis of SAR markers in the *put2-1* mutant line. Gene expression of **a**) *PAD4*, **b**) *SARD1*, **c**) *ICS1*, **d**) *PR1*, **e**) *ALD1*, **f**) *SARD4*, **g**) *FMO1*, **h**) *NIMIN1*, **i**) *UGT76B1*, **j**) *AZI1*, **k**) *PDF1.2*, and **l**) *NHL10* in the different plant status (native or SAR) at 24 hpi. Gene expression values were normalized to the *EF1α* reference gene and relative to WT plants in native status $^{(1ºMock\ /\ 2ºNative)}$ using the $2^{-\Delta\Delta Ct}$ method. Values are the mean of three replicates ± SE. Different letters denote significant differences ($p \leq 0.05$) between treatments according to three-way ANOVA and LSD Fisher's post-hoc test.

Figure 4. Regulation of SAR marker genes by polyamines. Gene expression of **a**) *ICS1*, **b**) *PR1*, **c**) *SARD4*, **d**) *FMO1*, **e**) *UGT76B1*, **f**) *AZI1*, **g**) *NHL10*, and **h**) *NIMIN1* in systemic leaves at 6 h after local infiltration with Mock or 30 μM Put, Spd, or Spm. Gene expression values were normalized to the *EF1α* reference gene and relative to WT Mock plants using the $2^{-\Delta\Delta Ct}$ method. Values are the mean of three replicates ± SE. Different letters indicate significant differences ($p \leq 0.05$) between treatments according to two-way ANOVA and LSD Fisher's post-hoc test.

Figure 5. Bacterial titers in distal leaves following the infiltration of Put in local leaves. Bacterial titers were assessed in distal leaves of the WT, *put2-1,* and *35S::PUT2* overexpression lines at 48 h after infiltrating lower leaves with either Mock, *Pst* priming, or 30 μM of Put. CFU, colony-forming units. The experiments were repeated twice, yielding consistent results. The values represent the mean of five biological replicates ± SE. Different letters indicate significant differences ($p \leq 0.05$) among Mock, *Pst* priming, or Put treatment in the WT, *put2-1* mutant, and *35S::PUT2* overexpression lines, according to a GLMM with negative binomial distribution followed by LSD Fisher's post-hoc test.

Figure 6. Estimation of free polyamine content in distal leaves following Put infiltration of local leaves. Content of **a**) Agm, **b**) Put, **c**) Spd, and **d**) Spm was assessed in distal leaves 6 and 24 h after infiltrating lower leaves with either 0 or 30 μM of Put. The values represent

the mean of six biological replicates ± SE. Different letters indicate significant differences ($p \leq 0.05$) among Mock or Put treatment in the WT, *put2-1* mutant, and the *35S::PUT2-27* overexpression line, according to three-way ANOVA followed by LSD Fisher's post-hoc test. **e**) PC analysis of common and uncommon polyamines in WT, *put2-1,* and *35S::PUT2-27* overexpression lines at 6 and 24 h after infiltrating lower leaves with either 0 or 30 μM of Put. The percentages of total variance represented by PC1 and PC2 are shown in parentheses.

Figure 7. Estimation of phenolic compounds content in distal leaves following Put infiltration of local leaves. The content of **a**) salycilic acid (SA), **b**) SA glycoside (SAG), **c**) *p*-coumaric acid (pCA), **d**) ferulic acid (FA), and **e**) sinapic acid (SiA) was assessed in distal leaves 6 and 24 h after infiltrating lower leaves with either 0 or 30 μM of Put. The values represent the mean of six biological replicates ± SE. Different letters indicate significant differences ($p \leq 0.05$) among Mock or Put treatment in the WT, *put2-1* mutant, and the *35S::PUT2-27* overexpression line, according to three-way ANOVA followed by LSD Fisher's post-hoc test. **f**) PC analysis of phenolic compounds in WT, *put2-1,* and *35S::PUT2-27* overexpression lines at 6 and 24 h after infiltrating lower leaves with either 0 or 30 μM of Put. The percentages of total variance represented by PC1 and PC2 are shown in parentheses.

Figure 8. Metabolic changes in WT, *put2-1*, and the *35S::PUT2-27* overexpression line in SAR. Content of **a**) Put, **b**) Spd, **c**) SA, and **d**) SAG in systemic leaves under native and SAR induction conditions at 6 and 24 hpi. Values are the mean of three biological replicates ±SE. Different letters indicate significant differences ($p \leq 0.05$) between treatments according to four-way ANOVA followed by LSD Fisher's post-hoc test.

Principal component analysis of free common and uncommon polyamines, and certain phenolic compounds in native (1ºMock / 2ºMock or 1ºMock / 2º*Pst*) and SAR (1º*Pst* / 2ºMock or 1º*Pst* / 2º*Pst*) conditions of the WT, *put2-1* mutant, and *35S::PUT2-27* overexpression line at **e**) 6 hpi and **g**) 24 hpi. The percentages of total variance presented by PC1 (Dim1) and PC2 (Dim2) are shown in parentheses. Pearson correlation matrix of free common and uncommon polyamines and certain phenolic compounds at **f**) 6 hpi and **h**) 24 h. Shades of blue indicate

increasing positive correlation coefficient; shades of brown indicate increasing negative correlation coefficient. The asterisks represent the significance of the correlation among variables. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 9. Histochemical detection of superoxide anion radical in WT and the *put2-1* mutant under SAR, ROS production after *flg22* treatment, and stomatal aperture. a) *In situ* production of $O_2^{\bullet-}$ was detected in rosette leaves of six-week-old plants by NBT staining 24 hpi in the following conditions: native plant status (1^{o}Mock / 2^{o}Native or 1^{o}Mock / 2^{o}*Pst*) and SAR (1^{o}*Pst* / 2^{o}Native or 1^{o}*Pst* / 2^{o}*Pst*). The blue color indicates the accumulation of $O_2^{\bullet-}$. Scale bar, 1 cm. **b)** Time of maximum relative luminescence units (t_{max} RLU) in WT, *put2-1*, and *35S::PUT2-27* after treatment with flg22 in discs of distal leaves derived from plants primed in local leaves with Mock or *Pst*. **c**) *put2-1* and **d**) *35S::PUT2-27* compared to WT: luminescence was measured over time after treatment with flg22 in discs of distal leaves derived from plants primed in local leaves with Mock or *Pst*. The data are presented as mean ± SE of n = 20. Statistical differences among the plant lines were determined using two-way ANOVA ($p < 0.05$), followed by a Tukey's HSD test. **e**) Estimation of stomatal aperture in response to flg22 treatment in epidermal peels of WT, *put2-1*, and *35S::PUT2-27*. Peels were preincubated for 3 h in opening buffer under light, then treated with 1 μM flg22. Box-and-whisker plots show n = 100 stomata. **f**) Representative images of stomata following flg22 treatment. Scale bar = 10 μm.

Figure 10. Proposed model of PUT2 involvement in SAR. PUT2 transports putrescine (or its derivatives) from local to distal tissues, promoting the accumulation of SA, Put, and the expression of SAR-related genes. In the *put2-1* mutant, the loss of SAR is associated with altered timing in the accumulation of SA, polyamines, and ROS, as well as in the expression of key SAR-related genes, compared to the wild-type. These changes suggest a disruption in the dynamics of the systemic defense response. Additionally, in SAR, the *put2-1* mutant exhibits increased expression of negative regulators of defense, especially *UGT76B1*.

Supplemental material.

Figure S1. Genomic organization and expression levels of the *Arabidopsis* Polyamine Uptake Transporter (*put*) mutant and overexpression (*35S::PUT*) lines characterized in this study.

Figure S2. Bacterial titers in WT and *put2-1* mutant line in response to local *Pst* inoculation.

Figure S3. Analysis of sequencing quality.

Figure S4. Complete gene ontology enrichment.

Figure S5. SAR marker gene expression in the *put2-1* mutant line at 6 hpi.

Figure S6. Estimation of free polyamine content in distal leaves following Put infiltration of local leaves.

Figure S7. Content of free common and uncommon polyamines and phenolic compounds in response to SAR at 6 and 24 hpi.

Figure S8. Expression of polyamine biosynthetic and catabolic genes, and *RBOH* genes in WT and *put2-1* mutant under SAR.

Figure S9. Expression profiles of genes associated with galactolipid metabolism.

Table S1. Specific primers used for typing, cloning, and gene expression analysis.

Table S2. Common increased and decreased genes between *put2-1* and WT under SAR-inducing conditions.

Table S3. Chloroplast-related up and down-regulated genes in the *put2-1* mutant in SAR $^{(1º\mathit{Pst}\,/\,2º\mathit{Pst})}$ vs. native $^{(1º\mathrm{Mock}\,/\,2º\mathit{Pst})}$ comparison.

File 1. List of differentially expressed genes.

File 2. Statistical data values

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Credit authorship contribution statement

Emmanuel Flores-Hernández: conceptualization, investigation, formal analysis, visualization, and writing-original draft. **María Elisa Gonzalez:** conceptualization, formal analysis, validation, writing-review, and editing. **Paulina Alvarado-Guitron:** investigation.

Francisco I. Jasso-Robles: investigation, formal analysis, and funding. **Cesaré M. Ovando-Vázquez:** data curation, software, and formal analysis. **Juan Francisco Jiménez-Bremont:** resources, funding, writing-review, and editing. **Sanja Ćavar-Zeljković**: investigation. **Markéta Ulbrichová:** investigation and formal analysis. **Nuria De Diego:** formal analysis, visualization, funding acquisition, resources, writing–review, and editing. **Margarita Rodríguez-Kessler:** conceptualization, formal analysis, visualization, resources, funding acquisition, supervision, writing–review, and editing.

Declaration of competing interest.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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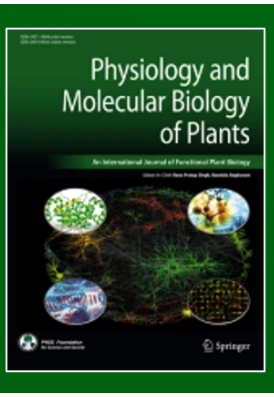
SEGUNDO ARTICULO

The polyamine uptake transporters PUT2/LAT4 and PUT5/LAT5 contribute to Arabidopsis defense response against *Botrytis cinerea*



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https://doi.org/10.1007/s12298-025-01630-1

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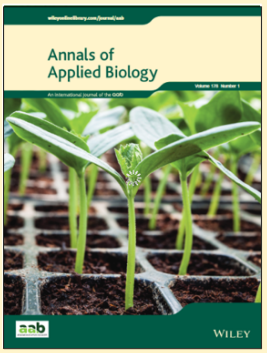
TERCER ARTICULO

Current status and perspectives on the role of polyamines in plant immunity



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https://doi.org/10.1111/AAB.12670

https://onlinelibrary.wiley.com/doi/abs/10.1111/aab.12670

CONCLUSIÓN

Los artículos presentados en esta tesis evidencian la participación central y diferenciada de los transportadores de poliaminas, específicamente PUT2/LAT4 y PUT5/LAT5 en la respuesta de defensa de *Arabidopsis thaliana* frente a dos patógenos con diferentes estilos de vida: el hongo necrotrófico *Botrytis cinerea* y la bacteria hemibiotrófica *Pseudomonas syringae* pv. *tomato* DC3000. La comparación entre estos patosistemas revela cómo la función de estos transportadores se ajusta según la naturaleza del patógeno y el tipo de respuesta requerida.

En el caso de *B. cinerea*, los resultados muestran que PUT2 y PUT5 son indispensables para una defensa local eficaz. En comparación con el ecotipo silvestre, las mutantes *put2-1* y *put5-1* mostraron una mayor susceptibilidad al hongo, efecto que se incrementó en la doble mutante (*put2-1xput5-1*). Esto sugiere una contribución no redundante de ambos transportadores en la resistencia contra este patógeno necrotrófico, posiblemente facilitando la movilización de poliaminas necesarias para la defensa local. En este contexto, la espermidina emerge como una poliamina clave, ya que la aplicación exógena de esta amina potencia la resistencia, siempre que el sistema de transporte sea funcional, lo que sugiere que su movilización es dependiente de los transportadores PUT2 y PUT5 y confirma que el transporte de espermidina es esencial para potenciar la defensa contra *B. cinerea*. Además, tras la infección, la actividad de las enzimas antioxidantes catalasa y ascorbato peroxidasas se inducen en el ecotipo silvestre pero no en los mutantes *put*, sugiriendo que el transporte de poliaminas es vital para la respuesta antioxidante, así como la regulación del catabolismo de las poliaminas mediado por las enzimas poliamina oxidasas.

Por su parte, el uso del patosistema *Arabidopsis thaliana-Pseudomonas syringae*, permitió identificar que la mutante *put2-1* abolió la resistencia sistémica adquirida, aunque paradójicamente mostró una resistencia basal incrementada al patógeno. Este fenotipo en la mutante *put2-1* parece estar asociado a una desregulación de las rutas del ácido salicílico y del ácido pipecólico, junto con la incapacidad para movilizar la putrescina. En este sentido, se encontró que la suplementación local con putrescina es capaz de establecer una respuesta similar a la resistencia sistémica adquirida en el ecotipo silvestre pero no en la mutante *put2-1* lo que evidencia el papel clave de PUT2 en la señalización de esta amina a tejidos distales para inducir la defensa sistémica. Además, se demostró que el tratamiento con putrescina,

induce la acumulación del ácido salicílico y de putrescina en tejidos sistémicos únicamente cuando PUT2 es funcional. La pérdida de la resistencia sistémica en *put2-1*, a pesar de su resistencia basal incrementada, muestra una interrupción entre la percepción local del patógeno y la propagación y/o percepción de señales sistémicas en ausencia de un transporte adecuado de poliaminas. Estos resultados muestran que la movilización de poliaminas, especialmente putrescina, y su impacto sobre metabolitos como el ácido salicílico requiere la acción específica de PUT2 y que no es compensada por otros transportadores de la familia PUT.

Aparte del tipo de patógeno, los estudios también revelan que la alteración en el transporte de poliaminas afecta procesos centrales como la inmunidad estomática, la acumulación y conjugación de poliaminas, y la producción de compuestos fenólicos. Estos elementos son esenciales tanto para la defensa local como para la respuesta sistémica, ya que condicionan la entrada del patógeno, la acumulación de compuestos antimicrobianos y la efectividad de la señalización hormonal. Además, estos procesos parecen estar regulados por la disponibilidad de poliaminas libres, la cual depende directamente del transporte activo facilitado por PUT2, particularmente en los tejidos distales involucrados en la defensa sistémica. Finalmente, los niveles de putrescina podrían funcionar como un indicador de infección, ya que sufre modificaciones de forma independiente del tipo de patógeno.

Es posible observar una relación entre PUT2 y PUT5 en función del contexto defensivo. El transportador PUT5 participa junto a PUT2 en la defensa local contra patógenos necrotróficos, y aunque no se exploró con detalle, también parece tener un rol destacado en la defensa sistémica. Por su parte, PUT2 resulta indispensable para la activación de respuestas sistémicas. Sin embargo, al igual que PUT5, afecta la resistencia basal contra patógenos hemibiotróficos. Estas diferencias funcionales reflejan la versatilidad del transporte de poliaminas como eje integrador de distintas señales de defensa, que se ajustan dependiendo de la naturaleza del patógeno, el tipo de respuesta requerida y si es un proceso local o sistémico (**Figura 6**).

En conjunto, esta evidencia sugiere que los transportadores PUT no simplemente movilizan poliaminas, sino que son actores clave en la regulación de la respuesta inmune de la planta. Su actividad permite modular la señalización y acumulación hormonal (particularmente de ácido salicílico), la movilización y/o acumulación de metabolitos clave y las respuestas celulares (homeostasis redox, inmunidad estomática) en la defensa local y

sistémica de las plantas. Comprender en profundidad cómo los mecanismos de transporte de poliaminas contribuyen a la defensa vegetal podría ampliar significativamente nuestro conocimiento sobre la resistencia de las plantas a los patógenos, lo que podría permitir diseñar estrategias de control de plagas en los cultivos.

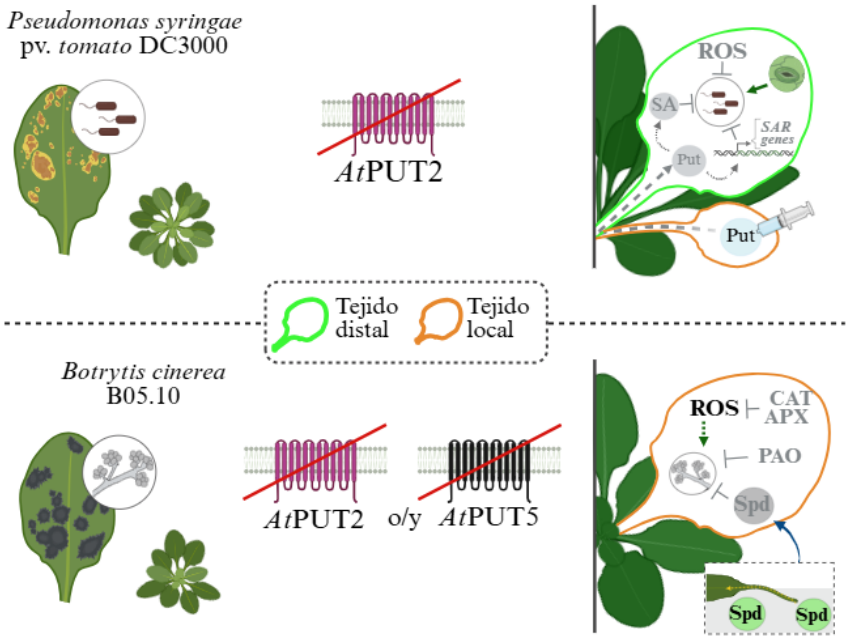

Figura 6. Funciones diferenciales de los transportadores PUT en la defensa local y sistémica de Arabidopsis frente a diferentes patógenos. Se ilustran los roles diferenciales de PUT2 y PUT5 en la defensa local contra el hongo necrotrófico *B. cinerea* y en la resistencia sistémica adquirida frente a la bacteria *Pseudomonas syringae* pv. *tomato* DC3000. Los transportadores PUT2 y PUT5 son necesarios para una respuesta local eficaz al hongo *B. cinerea* mediante el transporte de espermidina, producción controlada de ROS y activación de las enzimas antioxidantes: catalasa (CAT) y ascorbato peroxidasa (APX). Sin embargo, en las mutantes *put2-1* y *put5-1* se pierden estas respuestas (se indica en color gris) generando una mayor susceptibilidad al patógeno. Por otra parte, PUT2 es esencial para la movilización de putrescina desde el tejido local hacia tejidos distales, donde se acumula e induce la acumulación de SA y la activación de genes que participan en la activación de la resistencia sistémica. Además, regula la apertura estomática y los niveles de ROS. Por lo tanto, la ausencia de este transportador en la mutante *put2-1* compromete la defensa sistémica, la inmunidad estomática y la acumulación de metabolitos de defensa. En cada escenario se indica el método de suplementación de poliaminas, ya sea por infiltración con jeringa o suplementación del medio de incubación. Para el patosistema *Arabidopsis-B. cinerea* se analizaron plantas de 4 semanas de edad cultivadas en fotoperiodo de día largo, mientras que para el patosistema *Arabidopsis-P. syringae* se emplearon plantas de 6 semanas cultivadas bajo un fotoperiodo de día corto. La figura fue creada con BioRender (www.biorender.com).

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