Optimization of Immune Receptor-Related Hypersensitive Cell Death Response Assay Using Agrobacterium-Mediated Transient Expression in Tobacco Plants

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Research Article

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Abstract

Background

The study of the regulatory mechanisms of evolutionarily conserved Nucleotide-binding leucine-rich repeat (NLR) resistance (R) proteins in animals and plants is of increasing importance due to the understanding of basic immunity and the value of various crop engineering applications of NLR immune receptors. The importance of temperature is also emerging when applying NLR to crops responding to global climate change. In particular, studies of pathogen effector recognition and autoimmune activity of NLRs in plants can quickly and easily determine their function in tobacco using agro-mediated transient assay. However, there are conditions that should not be overlooked in these cell death-related assays in tobacco.

Results

Environmental conditions play an important role in the immune response of plants. The system used in this study was to establish conditions for optimal hypertensive response (HR) cell death analysis by utilizing the paired NLR RPS/RRS1 autoimmune and AvrRps4 effector recognition system. The most suitable greenhouse temperature for growing plants was fixed at 22°C. In this study, RPS4/RRS1-mediated autoimmune activity, RPS4 TIR domain dependent cell death, and RPS4/RRS1-mediated HR cell death upon AvrRps4 perception significantly inhibited under conditions of 65% humidity. The HR is strongly activated when the humidity is below 10%. In addition, the leaf position of tobacco is important for the HR cell death. Position #4 of the leaf from the top in 4-5 weeks old tobacco plants showed the most effective HR cell death.

Conclusions

As genome sequencing of various crops continues, different types of NLRs and their functions will be studied. At this time, if we optimize the conditions for evaluating NLR-mediated HR cell death, it will help to more accurately identify the function of NLRs. In addition, it will be possible to contribute to crop development in response to global climate change through NLR engineering.

Introduction

Recent climate change is changing the map of crop production. An increase in temperature can aggravate crop damage caused by pathogens [1, 2]. In order to actively respond to these rapid changes, rapid response through genetic engineering is required.

Plant pathogens inject effector proteins by manipulating cellular processes via type three secreted system (T3SEs) into host cells [3]. Nucleotide-binding leucine-rich repeat receptor (NLR) proteins recognize specific effectors and trigger effector-triggered immunity (ETI), which is often associated with localized programmed cell death, known as the hypersensitive response (HR) and limits pathogen proliferation [4].

Plant NLRs share many similar structural features to animals [5]. How plant NLRs signal immune responses remains largely unknown when compared to similar animal systems.

Like animals, paired NLRs exist in plants, and their functions are divided into sensor NLR and helper NLR. In particular, sensor NLRs have a role in recognizing effectors via integrated domain and inhibit the autoimmune activity of helper NLRs [6]. For example, this is the case with RPS4/RRS1. RRS1 is known to recognize effectors secreted by three different pathogens [7, 8]. Interestingly, artificially extending the N- or C-terminus of RRS1 activates RPS4 autoimmunity without effector recognition [9]. That is, an autoimmune system and an effector recognition system using RPS4/RRS1 paired NLR can be studied together.

Agrobacterium-mediated transient analysis is widely used to evaluate the function of NLRs. This is because the clear phenotype of HR cell death can be assessed with various combinations of NLRs and effectors in a relatively short time without obtaining the transgenic plants. It is known that NLR-mediated cell death is influenced by environmental factors such as temperature and humidity [10, 11]. In particular, the immunity of plants decreases with increasing temperature conditions and affects effector recognition of NLRs [12]. If so, it is important to clarify the conditions for an effective HR cell death assay using *agrobacterium*-mediated transient system in tobacco plant.

In this study, we confirmed that humidity and plant leaf position, excluding temperature conditions, were important for effectively confirming HR cell death assay in tobacco plant. The RPS4/RRS1-mediated cell death was activated by AvrRps4 perception in tobacco plants, these HR cell death was suppressed at 65% humidity. However, in 10% humidity, the HR cell death was enhanced. In addition, the infiltrated-leaf position of tobacco is important. Position #4 of recent leaves from the tops in 4-5 weeks old plants showed the most effective HR cell death. Under these distinct conditions, performing NLR-mediated HR upon effector recognition or autoimmune activation may yield more accurate results.

Results

The NLR RPS4/RRS1 pair can be used in the cell death assay system, which is divided into autoimmune activity and effector-triggerred hypersensitive cell death response

Two NLR immune receptor proteins encoded by genetically linked genes function together as paired NLRs. They are divided into sensor NLR and helper NLR according to their function. Sensor NLR frequently has a non-canonical domain called the integrated domain (ID) which can recognize cognate effector protein [13, 14]. Overexpression of helper NLR caused HR-like cell death in absence of sensor NLR. If coexpressed sensor/helper NLRs, the HR cell death is inhibited (Figure 1A) [13, 15]. When a protein such as fluorescent protein was linked to the N-terminus or C-terminus of the sensor NLR RRS1, RPS4-dependent autoimmunity appeared without effector perception (Figure 1A) [9]. In the engineering of sensor NLRs, it must be careful when engineering to utilize specific proteins. TIR (Toll-like, Interleukin-1 receptor, Resistance protein) or CC (coiled-coil) of the helper NLR act as a signaling domain. TIR^{RPS4} domain alone can activate TIR-dependent autoimmune and TIR^{RRS1} can suppress this HR cell death [16]. TIR domain

mutant RPS4(SH/AA) fail to activate HR, suggesting that TIR^{RPS4} oligomerization is required for generating the HR signal (Figure 1B). RRS1-R from accessions Ws-2 forms an immune receptor complex with RPS4 that recognizes *Pseudomonas syringae* AvrRps4, *Ralstonia solanacearum* PopP2, and unknown effector [7, 8, 17, 18]. Thus the paired RPS4/RRS1-R can prevent infection by three distinct pathogens and it can be used as a very useful tool for crop engineering (Figure 1C). We summarize the following experimental procedures based on the RPS4/RRS1 system capable of performing these three types of HR cell death assays using the *Agrobacterium*-mediated transient expression in tobacco plant (Figure 1D).

Both RRS1-S and RRS1-R fused with fluorescent protein under low humidity conditions exhibited RPS4-dependent HR cell death

Sensor NLR is a good material for developing crops that recognize various pathogens by introducing new IDs. The positioning may alter the overall sensor NLR protein size or fuse new IDs into the N- or C-terminus [19]. Bimolecular fluorescence complementation (BiFC) methods to confirm intra-/intercellular interactions should evaluate their function when applied to sensor NLRs [9, 20]. We found that overexpression of *cCFP-RRS1-S-nCerulean* activated RPS4-depenent HR cell death in low humidity condition but this cell death was suppressed in the high humidity condition (Figure 2A). At 4 dpi, we could not detect any difference between *cCFP-RRS1-S-nCerulean/RPS4-HA* mediated autoimmune activity and *cCFP-RRS1-S-nCerulean/RPS4-HA* mediated HR cell death upon AvrRps4 perception (Figure 2A). As the result, it was confirmed that the humidity condition at a temperature of 22°C affected HR cell death.

In the case of RRS1-R, unlike RRS1-S, 83 amino acids are extended at the C-terminus, which is known to play a decisive role in recognizing PopP2 [21]. We tested changes in autoimmunity under difference humidity conditions using RRS1R-cCFP and RRS1-R-nCerulean. Consistent with *cCFP-RRS1-S-nCerulean*, both *RRS1-R-cCFP* and *RRS1-R-nCerulean* activated RPS4-dependent HR cell death under low humidity. High humidity can suppress these cell death in *N. tabaccum* (Figure 2B). Finally, humidity is an important determinant of HR cell death activation by paired NLR autoimmune.

Agro-infiltrated leaf location in the tobacco plant is also a critical factor in HR cell death assays

HR cell death assays using tobacco plants often show differences in the intensities of HR cell death. In particular, if agro-infiltration is performed using various leaves in one plant, the same cell death cannot be obtained. To confirm that these differences occurred, leaf positions of 4-5 week old *N. benthamiana* were numbered from top to bottom. In the leaf position #4, coexpression of *RRS1-R-cCFP/RPS4-Myc or RRS1-R-nCerulean/ RPS4-Myc* exhibited strong HR cell death but not leaf position #5 (Figure 3A). Consistently, this autoimmune activity was suppressed by high humidity in *N. benthamiana* (Figure 3B). These results suggested that HR cell death is also affected by infiltrated leaf position and can be more easily detected under a relatively low humidity condition.

Agro-infiltrating leaf position is an important factor to evaluate HR cell death in both TIR ^{RPS4} -mediated cell death and RPS4/RRS1-mediated effector triggered cell death

NLRs are divided into two groups depending on the type of N-terminal domain. The N-terminal coiled-coil (CC) domain is called CNLs (CC-NLRs), and those with the N-terminal Toll/interleukin-1 receptor (TIR) domain is called TNLs (TIR-NLRs) [22]. Evolutionally, bacterial TIR domain proteins have NADase enzymatic activity that generates a non-canonical variant cyclic ADPR (cADPR) molecule and cleave NAD^+ (nicotinamide adenine dinucleotide) [23]. The TIR domain of plant NLR also has NADase activity and is required downstream signaling [24, 25]. The overexpression of TIR^{RPS4} domain is sufficient to activate autoimmune and oligomerization of TIR^{RPS4} domain is required for plant immune signaling [16]. In the TIR-mediated immunity studies, the importance of TIR function evaluation is increasing.

The *N. benthamiana* is an excellent system for studying cell death induced by the TIR/CC domains. To test whether TIR^{RPS4} can induce HR cell death in *N. benthamiana* and which leaf positions are most suitable for HR cell death assays. When co-delivered *35S::TIR^{RPS4}-GFP/ 35S::GFP* control in leaf positions #4-#7, the HR cell death is detected in leaf position #4 and #5 (Figure 4A). In the leaf position #4, TIR^{RPS4}-mediated autoimmune cell death is more potent than leaf position #5 (Figure 4A). In leaf positions #6 and #7, HR cell death is not detected (Figure 4A). If co-expressed *35S::TIR$^{RPS4(SH/AA)}$-GFP/ 35S::GFP* control, It cannot induce HR cell death because of inhibition of TIR^{RPS4} oligomerization (Figure 4A).

We also checked whether RPS4/RRS1 is functional in *N. benthamiana* system. When co-delivered *35S::RRS1-R-HF/ 35S::RPS4-Myc/ 35S::AvrRps4-GFP*, clear HR cell death was observed in the leaf positions #4, but weak HR cell death was exhibited in the leaf positions #5 (Figure 4B). However, RPS4/RRS1-mediated HR cell death upon AvrRps4 perception is not observed in leaf positions #6-#7 (Figure 4B). We attempted to determine whether HR intensity was determined by leaf position in *N. tabacum* as in the *N. benthamiana*. Similarly, combination of *35S::RRS1-R-HF/ 35S::RPS4-Myc/ 35S::AvrRps4-GFP* was co-expressed at leaf positions #4-#5 in *N. tabacum*. HR cell death phenotype was checked at 4 dpi. As expected, HR cell death was stronger in leaf position #4 than #5 (Figure 4C). In the effector triggered HR cell death, the position of the leaf used for Agro-infiltration affects the intensity of HR cell death both *N. benthamiana* and *N. tabacum*.

Based on the results, we have summarized the important factors in the tobacco-based Agro-infiltration cell death assay (Figure 5). In most reported cases, high temperature suppressed HR as well as disease resistance. The high temperature suppressed plant resistance under various experimental conditions [10, 11]. In the HR cell death analysis, humidity and leaf position used for infiltration were found to be the most important factors. Ultimately, it is expected to be of great help in experiments to confirm the cell death phenotype that exhibits various NLRs under these optimal conditions. In addition, when preparing a sample for co-immunoprecipitation (co-IP) or western blot analysis rather than a cell death assay, if the humidity is high and the sample is collected from a location other than location #4, sufficient experiments are possible.

Discussion

In this study, we developed and optimized an Agro-mediated transient cell death assay in tobacco plant. Tobacco plants are the most effective functional research system as various types of plant NLRs have been discovered through whole genome sequencing (WGS) [26, 27, 28]. In particular, it is possible to quickly and easily identify which effector NLR recognizes or which domain regulates NLR autoimmune activity through phenotypes such as HR cell death. On the other hand, it is difficult to confirm the intracellular localization of the NLR fused with a fluorescent protein due to autoimmune activity. Some NLRs do not produce distinct HR responses in tobacco plant. That is, updated low-humidity conditions and infiltrated leaf positions can be utilized to identify optimal HR cell death phenotypes of NLR studies.

Regulation mechanism of NLR-mediated humidity-sensitive HR is not fully understood. However, this is probably similar to the temperature-sensitive HR cell death. Although there are exceptions and not much research has been done, high temperature and humidity suppressed HR and affected plant immunity [10, 11, 29, 30]. For example, dwarf phenotype of autoimmune *snc1-1* is suppressed at 28°C [31]. *Arabidopsis* U-box ubiquitin ligase SAUL1 regulated senescence and cell death. The *saul1-1* mutant showed the autoimmune activity. An autoimmune phenotype of *saul1-1* was rescued by higher relative humidity and higher temperature. The *saul1-1* phenotype regulated by EDS1/PAD4 dependent signaling pathway [29]. This implied that NLR-mediated cell death also might be connected with EDS1/PAD4 pathway. Salicylic acid (SA) may play an important role in the thermoregulation of plant NLR-mediated cell death [30].

Leaf senescence is one of the programmed cell death (PCD) and is regulated by ethylene (ET) hormone [32]. Although SA regulates key pathways of plant cell death and immunity, this regulation is inhibited by ET [30]. Interestingly, ET did not affect leaf senescence in young seedlings [33]. There is a sufficient possibility that the newly generated upper leaves can escape the effects of ET when used for agro-infiltration. Arabidopsis onset of leaf death (*old*) mutants is allelic to *CONSTITUTIVE EXPRESSER OF PR GENE5* (*CPR5*), which showed senescence symptoms in young seedlings [34, 35]. Interestingly, *CPR5* maintains the steady state level of nicotinamide adenine dinucleotide (NAD) [36], suggesting that NAD homeostasis also may affect NLR-mediated cell death including autoimmune activity. CPR5 associated with a novel nucleoporin PLANT NUCLEAR ENVELOPE TRANSMEMBRANE 1 (PNET1) [37]. Function of human PNET1 homolog is important to the cell cycle regulation [38]. This suggests that CPR5-PNET1 may have a dual function between cell cycle and immune pathways in the nuclear pore. Thus, leaf position-dependent HR cell death might be associated with cell cycle and immunity.

Conclusions

We proposed optimized transient HR cell death assay conditions for NLR studies using tobacco plant. When temperature, humidity and leaf position conditions are optimally adjusted, various NLR-mediated effector triggered HR cell death and autoimmune phenotypes can be observed.

Methods

Plant Materials and Growth Conditions

Nicotiana tabacum cv. Petite Gerard and *Nicotiana benthamiana* plants were sown on soil and grown at 22°C under long day condition (16 h light/8 h dark) with 55% relative humidity.

Agrobacterium strains and vector constructions

Agrobacterium tumefaciens GV3101 was used in infiltration assays with tobacco leaves. The *Agrobacterium* strain GV3101 competent cells were thawed on ice and added 1 µL recombined plant expression vector, then kept in ice for 5 min. Then the mixture was fast frozen in liquid nitrogen for 5 min, followed by an incubation at 37 ℃ for 5 min. After that, the mixture was kept in ice for 5 min and added 1 mL fresh Luria Broth (LB) liquid medium. After a culture in shaker for 1 h at 28 ℃, 200 rpm, 100 µL cells were plated on a LB agar plate containing rifampicin (25 mg/L) and kanamycin (100 mg/L), and then cultured for 2 days at 28 ℃.

Briefly, genomic fragments of full-length RRS1-R and RRS1-S were PCR-amplified from *Arabidopsis thaliana* genomic DNA, accessions Ws-2 and Col-0. The genomic fragments of RRS1 were PCR-amplified with primers containing 4bp specific overhangs and *Bsa*I recognition sequence and cloned into the pCR8/GW/TOPO (ThermoFisher). The resulting pCR8 constructs were subsequently used for Golden Gate assembly in pICH86988 [39]. C-cCFP, C-nCerulean, and N-cCFP tags were introduced into the RRS1 [21]. *35S::RPS4-HA*, *35S::RPS4-Myc*, *35S::RRS1-R-HF*, *35S::AvrRps4-GFP*, *35S::AvrRps4^{E187A}-GFP*, *35S::AvrRps4$^{KRVY/AAAA}$-GFP*, *35S::TIR^{RPS4}-GFP*, *35S::TIR$^{RPS4(SH/AA)^-}$GFP*, and *35S::TIR$^{RRS1(SH/AA)^-}$GFP* used in this study were described previously [15, 16, 17, 21].

Agrobacterium tumefaciens infiltration in tobacco plant

A single positive colony of *Agrobacterium* was inoculated in 3 mL LB liquid medium (25 mg/L rifampicin and 100 mg/L kanamycin) and cultured for 1 day in a shaker at 28 ℃, 200 rpm. Then, 100 µL *Agrobacterium* cells were transferred into 5 mL fresh LB liquid medium supplemented with above-mentioned antibiotics and cultured overnight at 28 ℃, 200 rpm. The *agrobacterium* cells were pelleted by centrifugation (3000 rpm, 10 min) and resuspended with 1 mL infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6). The re-suspended agro-cells were then diluted to OD_{600} = 0.5 with infiltration buffer. Fully expanded leaves of 4-5 weeks old tobacco plant were syringe-infiltrated. The leaf-infiltrated plants were dried with paper, and the humidity in the greenhouse was maintained at 55%. After 1 day, the plants were transferred to growth chamber with a16-h light/8-h dark photoperiod at 22°C, 65% humidity or transferred to growth chamber at 22°C, 10% humidity. It is possible to test in a laboratory with a relative humidity of 10% and at 20-22°C. The HR phenotype was confirmed at 3-5 dpi.

Abbreviations

NLR
Nucleotide-binding leucine-rich repeat (NLR) resistance (R) proteins
HR
hypertensive response
T3SE
type three secreted system
ETI
effector-triggered immunity
ID
integrated domain
TIR
Toll-like, Interleukin-1 receptor, Resistance protein
CC
coiled-coil
BiFC
Bimolecular fluorescence complementation
CNL
CC-NLR
TNL
CC-NLR
NAD+
nicotinamide adenine dinucleotide
cADPR
non-canonical variant cyclic ADPR
WGS
whole genome sequencing
PCD
programmed cell death
CPR5
CONSTITUTIVE EXPRESSER OF PR GENE5
PNET1
PLANT NUCLEAR ENVELOPE TRANSMEMBRANE 1

Declarations

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Contributions

S.U.H designed the research, performed the experiments, analyzed the data, wrote the read and approved the final manuscript.

Availability of data and materials

All data and material generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

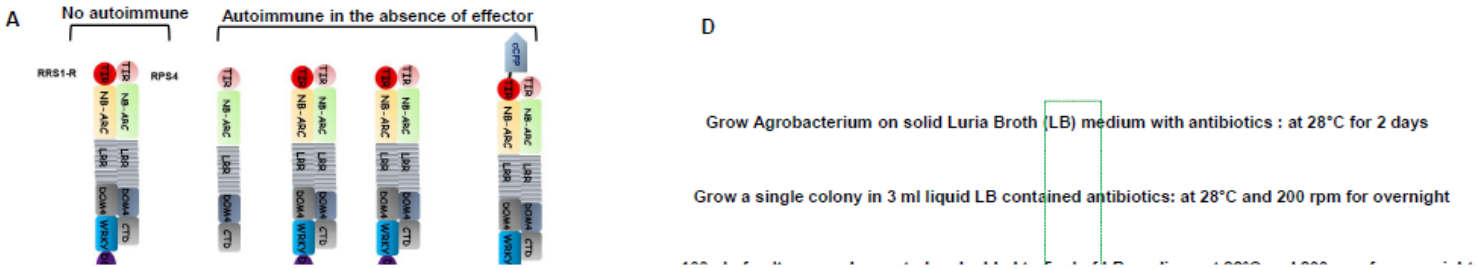

Figure 1

Three HR cell death systems utilizing the paired NLR RPS4/RRS1

A. The helper RPS4 autoimmune activity is suppressed by sensor RRS1. In the absence of RRS1, RPS4 exhibits autoimmune activity. When cCFP or nCerulean is fused to N- or C- of RRS1, it causes structural modification of RRS1. Thus, the ability of RRS1 to inhibit RPS4 autoimmune activity is lost. In the absence of effectors, the autoimmune activity of paired NLRs can be assessed. B. TIR domain of helper RPS4 is capable of generating an autoimmune signal by itself. Oligomerization of TIR^{RPS4} is important for generating an HR cell death signal. This TIR^{RPS4}-dependent HR can be repressed by coexpressing of TIR domain of RRS1. C. RPS4/RRS1 directly recognizes effectors and induces NLR-mediated HR cell death. Sensor RRS1 has an integration domain (ID) WRKY and is currently known to recognize three different pathogen effectors: AvrRps4 (*Pseudomonas syringae*), PopP2 (*Ralstonia solanacearum*), and an unknown effector (*Colletotrichum higginsianum*). D. Schematic of *Agrobacterium*-mediated transient expression for HR cell death assay.

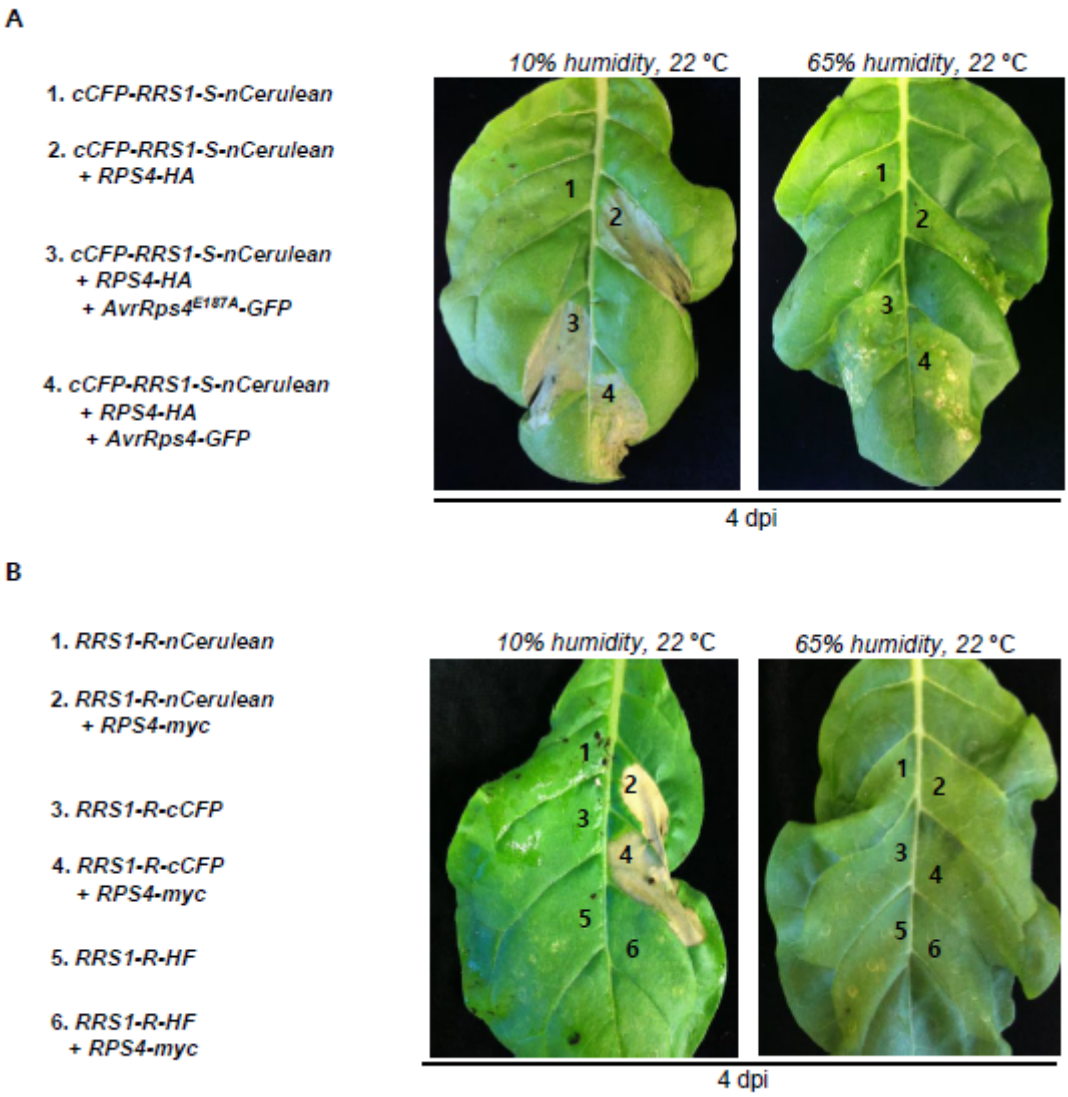

Figure 2

RPS4/RRS1 autoimmune activity is enhanced by low humidity

A. cCFP-RRS1S-nCerulean triggered RPS4-depentent autoimmune cell death in *N. tabacum*. *35S::cCFP-RRS1-S-nCerulean* or *35S::cCFP-RRS1-S-nCerulean/ 35S::RPS4-HA or 35S::cCFP-RRS1-S-nCerulean/ 35S::RPS4-HA/ 35S::AvrRps4-GFP* or *35S::cCFP-RRS1-S-nCerulean/ 35S::RPS4-HA/ 35S::AvrRps4^{E187A}-GFP* were transiently co-expressed in *N. tabacum* leaves. After agro-infiltration, the tobacco plants stayed at 55% humidity and 22 °C for 1 day. After 1 day, the tobacco plants were transferred to 10% and 65% humidity conditions, respectively. The HR cell death was confirmed on the 4 days. B. RRS1-R-nCerulean and RRS1-R-nCFP triggered RPS4-depentent autoimmune cell death in *N. tabacum*. *35S::RRS1-R-nCerulean* or *35S::RRS1-R-nCerulean/ 35S::RPS4-Myc* or *35S::RRS1-R-cCFP* or *35S::RRS1-R-cCFP/ 35S::RPS4-Myc* or *35S::RRS1-R-HF or 35S::RRS1-R-HF/35S::RPS4-Myc* were transiently co-expressed in *N. tabacum* leaves. After agro-infiltration, the tobacco plants stayed at 55% humidity and

22 °C for 1 day. After 1 day, the tobacco plants were transferred to 10% and 65% humidity conditions, respectively. The HR cell death was confirmed on the 4 days.

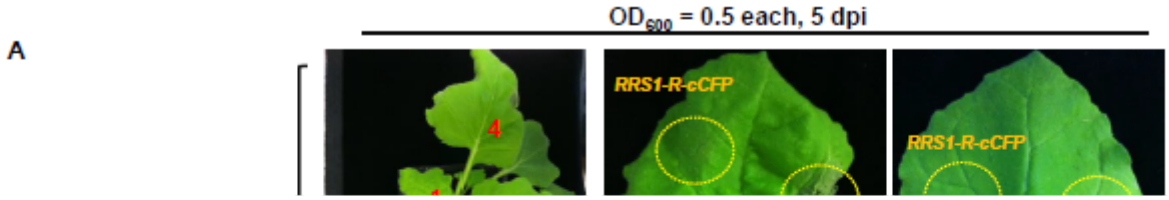

Figure 3

Effect of leaf position and humidity on RPS4/RRS1-mediated autoimmunity in *N. benthamiana*

A. The autoimmune activity of RPS4-mediated RRS1-cCFP or RRS1-nCerulean at leaf positions #4 and #5 was different at low humidity. The positions of the leaves are numbered from top to bottom. *35S::RRS1-R-cCFP* or *35S::RRS1-R-cCFP/ 35S::RPS4-Myc or 35S::RRS1-R-nCerulean* or *35S::RRS1-R-nCerulean/ 35S::RPS4-Myc* were transiently co-expressed in *N. benthamiana* leaf positions #4 and #5 at 55% humidity and 22 °C for 1 day. After 1 day, the tobacco plants were transferred to 10% humidity condition. The HR cell death was confirmed on the 5 days. B. The autoimmune activity of RPS4-mediated RRS1-cCFP or RRS1-nCerulean at leaf positions #4 and #5 was suppressed by high humidity. *35S::RRS1-R-cCFP/ 35S::RPS4-Myc* or *35S::RRS1-R-nCerulean/ 35S::RPS4-Myc* were transiently co-expressed in *N.*

benthamiana leaf positions #4 and #5 at 55% humidity and 22 °C for 1 day. After 1 day, the tobacco plants were transferred to 65% humidity condition. The HR cell death was confirmed on the 5 days.

Figure 4

Effect of leaf position on TIR^{RPS4}-mediated autoimmune and RPS4/RRS1-mediated effector triggerred HR cell death in tobacco plants

A. TIR^{RPS4}-dependent autoimmune activity is different for each leaf position. *N. benthamiana* leaf was transiently agro-infiltrated with *35S::TIR^{RPS4}-GFP/ 35S::GFP* or inactive mutant *35S::$TIR^{RPS4(SH/AA)}$-GFP/ 35S::GFP* or *35S::TIR^{RRS1}-GFP/ 35S::GFP* or *35S::$TIR^{RPS4(SH/AA)}$-GFP*/inactive mutant *35S::$TIR^{RRS1(SH/AA)}$-GFP* at leaf positions #4-#7. TIR^{RPS4}-mediated autoimmune activity could not be confirmed at leaf positions #6-#7, and the HR cell death for leaf positions #4-#5 was enlarged. The HR cell death was confirmed on the 3 days. B. RPS4/RRS1-mediated effector triggered HR cell death exhibited differently infiltrated leaf position in *N. benthamiana*. Agro-infiltration was performed with transiently coexpressed *35S::RRS1-R-HF/ 35S::RPS4-Myc with 35S::GFP or 35S::AvrRps4-GFP or* non-functional mutant *35S::$AvrRps4^{KRVY/AAAA}$-GFP* at leaf positions #4-#7 in *N. benthamiana*. RPS4/RRS1-mediated HR cell death upon AvrRps4 perception could not be confirmed at leaf positions #6-#7, and the HR cell death for leaf positions #4-#5 was enlarged. The HR cell death was confirmed on the 3 days. C. RPS4/RRS1-mediated effector triggered HR cell death exhibited differently infiltrated leaf position in *N. tabacum*. Agro-infiltration was performed with transiently coexpressed *35S::RRS1-R-HF/ 35S::RPS4-Myc with 35S::GFP or 35S::AvrRps4-GFP or* non-functional mutant *35S::$AvrRps4^{KRVY/AAAA}$-GFP* at leaf positions #4-#5 in *N. tabacum*. The HR cell death for leaf positions #4-#5 was enlarged. The HR cell death was confirmed on the 4 days.

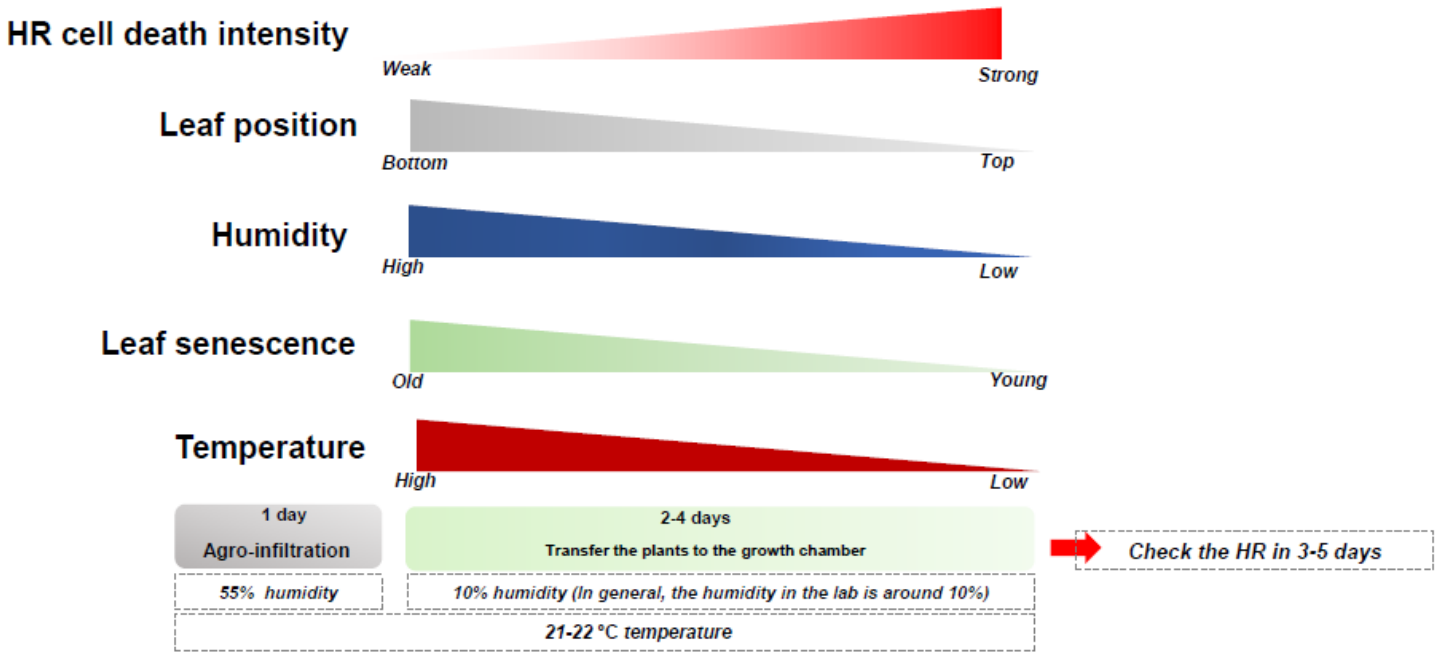

Figure 5

NLR-mediated HR cell death intensity depends on leaf position and humidity

The fastest and most accurate characterization of plant NLRs is the transient HR cell death assay using tobacco. Of course, NLRs that form very strong apoptosis signals can ignore various environmental conditions and confirm a phenotype of apoptosis. However, in HR cell death analysis using various NLR autoimmunity and effector recognition, cell death related to NLR function can be clearly identified when a low humidity of about 10% is used. Humidity conditions seem to behave very similarly to temperature conditions. High temperatures are often accompanied by high humidity. Therefore, it is known that the temperature condition is also a very important factor. Additionally, the leaf position of tobacco used for infiltration is considered to be another important factor. It is not yet known what kind of regulatory action leads to HR cell death, but it is thought to be somewhat related to leaf aging. As a result, it is believed that the assays to confirm HR cell death under normal plant culture conditions are affected by various environmental factors.