Plant innate immunity relies on a two-tiered defence response (Chisholm et al., 2006; Dangl et al., 2013; Jones & Dangl, 2006). In the first tier, cell-surface immune receptors recognize conserved molecular patterns from microbes to launch an induced defence response, called pattern-triggered immunity (PTI) (Bigeard et al., 2015; Dangl et al., 2013; Jones & Dangl, 2006; Wu & Zhou, 2013). However, pathogens continually compete for domination by secreting a series of effectors to the host plant to suppress

**SHORT COMMUNICATION**

**AvrRps4 effector family processing and recognition in lettuce**

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**Abstract**
During pathogenesis, effector proteins are secreted from the pathogen to the host plant to provide virulence activity for invasion of the host. However, once the host plant recognizes one of the delivered effectors, effector-triggered immunity activates a robust immune and hypersensitive response (HR). In planta, the effector AvrRps4 is processed into the N-terminus (AvrRps4N) and the C-terminus (AvrRps4C). AvrRps4C is sufficient to trigger HR in turnip and activate AtRRS1/AtRPS4-mediated immunity in Arabidopsis; on the other hand, AvrRps4N induces HR in lettuce. Furthermore, AvrRps4N-mediated HR requires a conserved arginine at position 112 (R112), which is also important for full-length AvrRps4 (AvrRps4F) processing. Here, we show that effector processing and effector recognition in lettuce are uncoupled for the AvrRps4 family. In addition, we compared effector recognition by lettuce of AvrRps4 and its homologues, HopK1 and XopO. Interestingly, unlike for AvrRps4 and HopK1, mutation of the conserved R111 in XopO by itself was insufficient to abolish recognition. The combination of amino acid substitutions arginine 111 to leucine with glutamate 114 to lysine abolished the XopO-mediated HR, suggesting that AvrRps4 family members have distinct structural requirements for perception by lettuce. Together, our results provide an insight into the processing and recognition of AvrRps4 and its homologues.

**KEYWORDS**
AvrRps4, effector-triggered immunity, hypersensitive response, processing, recognition, XopO
PTI (Jones & Dangl, 2006; Lapin & Van den Ackerveken, 2013; Su et al., 2018). As plants and pathogens have coevolved, a second tier of plant immunity has developed based on effector perception by resistance proteins (Andolfo & Ercolano, 2015; Cesari, 2018; Jones & Dangl, 2006). Specifically, resistance proteins directly or indirectly recognize effectors to induce a robust defence, the so-called effector-triggered immunity (ETI), that often manifests itself as rapid localized cell death known as the hypersensitive response (HR) (Nguyen et al., 2021; Saur et al., 2021).

An effector named AvrRps4 has been identified in the bacterial pathogen *Pseudomonas syringae* pv. *pisi* (Hinsch & Staskawicz, 1996). AvrRps4 is processed in planta, forming an N-terminal fragment (AvrRps4\textsuperscript{N}) of 133 amino acids and a C-terminal fragment (AvrRps4\textsuperscript{C}) of 88 amino acids (Sohn et al., 2009). The processing of AvrRps4 is dependent on an arginine at position 112 of AvrRps4\textsuperscript{N} (Sohn et al., 2009). AvrRps4 has been described as a bipartite effector (Halane et al., 2018), in which both processed fragments have effector functions. The locus of the resistance gene for AvrRps4, *RPS4*, has been identified, mapped, and characterized (Gassmann et al., 1999; Hinsch & Staskawicz, 1996). This gene encodes a toll/interleukin-1 receptor nucleotide-binding leucine-rich repeat receptor (TNL), which can complement the naturally susceptible phenotype of *Arabidopsis* RLD against bacteria expressing *avrRps4* (Gassmann et al., 1999). *RPS4* transcripts include not only full-length but also truncated open reading frames generated through alternative splicing activity. The combination of transcripts with full-length and truncated open reading frames of *RPS4* is required for the recognition of AvrRps4 (Zhang & Gassmann, 2003). *AvrRps4* is recognized by the linked gene pairs *RRS1-RPS4* and/or *RRS1B-RPS4B* in *Arabidopsis* (Guo et al., 2020; Sarris et al., 2015; Saucet et al., 2015). Conditional overexpression of *AvrRps4*\textsuperscript{N} in the *Arabidopsis* accession Columbia-0 (Col-0) triggers an HR similar to full-length AvrRps4 (AvrRps4\textsuperscript{N})-mediated ETI (Li et al., 2014), indicating that the C-terminus acts as a crucial effector domain of AvrRps4 in *Arabidopsis*. In addition, transient expression of AvrRps4\textsuperscript{C} is sufficient to trigger an HR in turnip (Sohn et al., 2009). While the C-terminus of AvrRps4 elicits ETI in *Arabidopsis* and turnip, its N-terminus can be recognized to induce HR in lettuce (Lactuca sativa 'Kordaat') (Halane et al., 2018; Su et al., 2021). In addition, not only AvrRps4\textsuperscript{N} but also AvrRps4\textsuperscript{C} interacts with EDS1, and both termini are required to trigger immunity in *Arabidopsis* when delivered by bacterial pathogens at natural protein levels (Bhattacharjee et al., 2011; Halane et al., 2018; Heidrich et al., 2011). These findings suggest the function of AvrRps4\textsuperscript{N}, which was formerly proposed to only contain a type III secretion system and a chloroplast targeting signal, to be a bona fide effector domain that acts beyond being a signal peptide (Halane et al., 2018; Su et al., 2021).

AvrRps4 has two close homologues, *P. syringae* pv. *tomato* HopK1 and *Xanthomonas campestris* pv. *vesicatoria* XopO (Halane et al., 2018; Li et al., 2014; Sohn et al., 2009; Su et al., 2021), which are together called the AvrRps4 effector family. These three effectors bear a striking similarity in amino acid sequences at the N-terminal domain, while their C-terminal domains are unrelated. Like AvrRps4, the N-terminus of HopK1 (HopK1\textsuperscript{N}), but not the C-terminus of HopK1, triggers an HR in lettuce (Halane et al., 2018), suggesting that the N-termini of the AvrRps4 family are evolutionarily and functionally identical. Moreover, the processing of AvrRps4 depends on arginine residue 112 (R112) (Sohn et al., 2009), which is conserved among the members of the AvrRps4 family (Su et al., 2021). Interestingly, we found that the R112 residue is important for effector processing and also effector recognition (Su et al., 2021). Indeed, mutation of R112 to leucine (R112L) abolishes AvrRps4-mediated HR in lettuce. While R112-mediated AvrRps4\textsuperscript{N} recognition is independent of R112-mediated AvrRps4\textsuperscript{C} processing, no other AvrRps4 mutation that blocked processing but not recognition, or vice versa, was described by Su et al. (2021). Therefore, due to the dual role of R112, it remained unclear whether AvrRps4\textsuperscript{F} processing is initially required for AvrRps4\textsuperscript{N} recognition. In this study, we found that AvrRps4/XopO processing was not necessary for effector recognition. Furthermore, mutation of the conserved R111 in XopO, if by itself, was insufficient for abolishing XopO recognition in lettuce.

First, we tested whether the N-terminus of XopO (XopO\textsuperscript{N}), which has not been studied previously, could trigger HR in lettuce similarly to AvrRps4\textsuperscript{N} and HopK1\textsuperscript{N}. As expected, like AvrRps4\textsuperscript{N} and HopK1\textsuperscript{N}, XopO\textsuperscript{N} induced an HR in lettuce cv. Kordaat (Figures 1a and 2a). Furthermore, quantitative ion leakage assays indicated that XopO\textsuperscript{N} elicited an even more robust HR than AvrRps4\textsuperscript{N} and HopK1\textsuperscript{N} at 6 and 11 h (Figure 1b). Then, we compared lettuce responses to AvrRps4, HopK1, XopO, and their mutants to gain an understanding of amino acid features required for effector processing and recognition in lettuce. Through site-directed mutagenesis, point mutation constructs of the conserved R112 were generated and cloned into the dexamethasone (Dex)-inducible vector pTA7002. Consistent with the previous study (Su et al., 2021), in our Dex-inducible system the R112L mutation abolished AvrRps4\textsuperscript{N}/AvrRps4\textsuperscript{C}-mediated HR and suppressed electrolyte conductivity in lettuce (Figure S1). Similar to AvrRps4, transient expression of HopK1 constructs showed an identical pattern. HopK1\textsuperscript{F} (R112L) and HopK1\textsuperscript{N} (R112L) successfully suppressed cell death (Figure S2a). Wild-type HopK1\textsuperscript{N} and HopK1\textsuperscript{F} induced increased electrolyte conductivity, representing stronger HR, compared to their R112 mutations (Figure S2b). Ion leakage from the R112L mutants was statistically slightly higher than that from the negative control. As we expected, the conserved R112 in HopK1 was also important for HopK1\textsuperscript{F} processing (Figure S2c).

Surprisingly, a distinct pattern was observed in XopO: as shown in Figure 2a, the R111L mutation in XopO failed to suppress the HR in lettuce. To quantify the amount of cell death, we conducted ion leakage assays. The amount of leakage caused by XopO\textsuperscript{N} (R111L) and XopO\textsuperscript{F} (R111L) was not significantly different compared to that caused by wild-type XopO\textsuperscript{N} and XopO\textsuperscript{F} (Figure 2b). However, R111L mutation in XopO blocked its processing, like R112 in AvrRps4 and HopK1 effector processing. To further confirm that the XopO\textsuperscript{R111L} mediated HR is specific, visual cell death assays using mutant sets of N-terminal or full-length effectors were performed at the same time. As shown in Figure S4, HR was exclusively observed with XopO\textsuperscript{R111L}...
mutants. These data indicate that XopO-mediated HR in lettuce is R111-independent, and that the HR and effector processing in XopO are uncoupled.

Because XopO<sup>(R111L)</sup> failed to suppress induction of cell death in lettuce, we expected that other or additional residue(s) would play a key role in effector recognition. From the protein alignment (Figure S3), we suspected and highlighted several candidate residues. To test whether another amino acid(s) were crucial for XopO- and AvrRps4-mediated HR in lettuce, we focused on another conserved arginine. Besides R112 in AvrRps4<sup>N</sup>, arginines at position 62 (R62) and position 88 (R88) were conserved among the three effectors (Figure S3). We chose to examine R88 (R87 in XopO) first due to the finding that truncated AvrRps4<sup>N</sup> (84–120) is sufficient to induce HR (Su et al., 2021). XopO<sup>R87L</sup> and AvrRps4<sup>R88L</sup> were generated in a similar way to the construction of the R112 mutants. Using XopO mutants, we found that the R87L single mutant and the R87L/R111L double mutant still induced an HR in lettuce (Figure S5), suggesting that R87 is not required for XopO recognition. In addition, XopO<sup>F</sup> (R87L) was processed in planta, indicating R87 is not necessary for XopO<sup>F</sup> processing (Figure 3c). However, it surprisingly behaved like R112L to block AvrRps4<sup>F</sup> processing (Figure 3c). In addition, the level of HR caused by AvrRps4<sup>F</sup> (R88L) was less than that caused by AvrRps4<sup>F</sup> (Figure 3b). In the case of AvrRps4<sup>N</sup> variants, AvrRps4<sup>N</sup> (R88L) triggered a similar HR level compared to wild-type AvrRps4<sup>N</sup> (Figure S6a,b). Equal protein expression levels of AvrRps4<sup>N</sup> (R88L) and AvrRps4<sup>N</sup> in protein blot assays confirmed the similarity of HR (Figure S6c).
These findings provided strong evidence that effector processing of the AvrRps4 family is not necessary for effector recognition. Effector processing seems to contribute to faster effector recognition because the cell death determinant is located at the N-terminus, which is processed from the full-length effector, and because AvrRps4\textsubscript{N} induces a stronger HR than AvrRps4\textsubscript{F} (Halane et al., 2018; Su et al., 2021).

Next, we investigated the function of the conserved R62 in AvrRps4. Unlike the conserved R112 and R88 residues, the substitution of R62 to leucine in AvrRps4 did not alter the AvrRps4 processing activity (Figure S7c). In addition, AvrRps4\textsubscript{R62L} induced an HR in lettuce (Figure S7a,b), indicating that R62 does not function in AvrRps4 recognition. This result is consistent with our previous data that the cell death determinant of AvrRps4 is from residues 84 to 120 (Su et al., 2021). Taken together, our data indicate that effector processing and effector recognition in the AvrRps4 family are uncoupled in lettuce, and different key residues for these are required for these activities.

Next, we investigated whether residues in addition to the conserved R111 are critical for XopO-triggered cell death in lettuce. For this, we focused on the AvrRps4 fragment from residues 84 to 120, as this region is necessary and sufficient for the recognition (Su et al., 2021) and is the most conserved region in the alignment of three effector sequences (Figure S3). We aimed to find positions for polymorphic amino acids among the three effector sequences. Because HopK1\textsubscript{R112L} and not XopO\textsubscript{R111L} behaved similarly to AvrRps4\textsubscript{R112L} (Figure S4), we hypothesized that the determinant residue(s) for XopO-triggered cell death should be polymorphic to those that are conserved at the same position between AvrRps4 and HopK1. Moreover, we also hypothesized that the determinant
residue(s) in XopO might have a different charge to the conserved residue(s) in AvrRps4 and HopK1. Different charged amino acids can cause different functional characteristics of proteins, which might result in the differential recognition of AvrRps4/HopK1 and XopO by lettuce. Four candidate amino acid residues between residues 84 to 120, were identified: D85_{AvrRps4/HopK1} (K84_{XopO}), N107_{AvrRps4/HopK1} (D106_{XopO}), Q111_{AvrRps4/HopK1} (E110_{XopO}), and K115_{AvrRps4/HopK1} (E114_{XopO}) (Figure S3). Among these four, we focused on E110 and E114 in XopO due to their positions being closer to the conserved R111. AvrRps4/HopK1 contain a positively charged K115, whereas XopO carries a negatively charged E114. XopO harbours negatively charged glutamic acid (E) at position 111, while AvrRps4/HopK1 have a corresponding neutral charged glutamine (Q). On the basis of this, we generated a XopO double mutant E110Q/R111L and triple mutant E110Q/R111L/E114K through site-directed mutagenesis. As shown in Figure S8, the double mutant XopO^{E110Q/R111L} failed to suppress the HR in lettuce. However, the triple mutant XopO^{E110Q/R111L/E114K} successfully abolished the HR, suggesting that E114 is involved in XopO recognition in lettuce. To further test whether E114 is sufficient for the XopO recognition, cell death assays were performed, using the single mutant XopO^{E114K} and double mutants E110Q/E114K and R111L/E114K (Figure 4a,b), with the triple mutant XopO^{E110Q/R111L/E114K} as a positive control of HR abolishment. Like, XopO^{R111L}, the single mutant XopO^{E114K} induced an HR in lettuce, indicating that E114 alone is insufficient for XopO recognition. Surprisingly, the double mutant XopO^{R111L/E114K}, but not XopO^{E110Q/E114K}, suppressed the HR in lettuce (Figure 4a,b). Because XopO^{E114K} mirrors AvrRps4 and XopO^{R111L/E114K} mirrors AvrRps4^{R112L} in cell death induction, we wondered whether the K115E mutation in AvrRps4 could compromise HR suppression by
R112L. To determine whether or not this hypothesis was correct, we tested the K115E single and R112L/K115E double mutant of AvrRps4 in cell death assays. The K115E single mutant did not affect AvrRps4-mediated HR (Figure S9). Like the R112L mutant, the R112L/K115E double mutant failed to induce an HR (Figure S9). Therefore, AvrRps4 R112L/K115E does not mirror XopO R111L in HR activation in lettuce, showing that R111 and E114 residues in XopO are specific for XopO recognition in lettuce. Our data indicate that R111 and E114, together, are essential for XopO recognition, suggesting key residues for effector recognition are different between XopO and AvrRps4.

To date, Arabidopsis immunity triggered by the C terminus of AvrRps4 has been well studied (Guo et al., 2020; Sarris et al., 2015; Saucet et al., 2015; Sohn et al., 2009). Our previous research raised a new model of AvrRps4-triggered immunity in lettuce by the N terminus, depending on R112 (Su et al., 2021). Interestingly,
HopK1 and XopO, whose conserved R112-dependent perceptions were not documented before, retain a conserved N-terminal domain with AvrRps4. This study compared the effector processing and recognition of AvrRps4, HopK1, and XopO. Due to the highly conserved amino acid sequence of the N-terminal fragment, HopK1 and XopO can induce HR in lettuce, like AvrRps4 (Figures 2, S2, and S3). We presume that a common corresponding resistance protein recognizes the N-termini of these three effectors in lettuce. AvrRps4/HopK1/XopO-mediated immunity in lettuce could be a direct recognition or an indirect recognition (with a guardee/decoy) (Figure 4d). The conserved arginine R112 in AvrRps4 plays a critical role in effector processing (Sohn et al., 2009) as well as effector recognition (Su et al., 2021). This binary function of R112 raised the question of whether effector processing is not required for AvrRps4 recognition, and proved that effector processing is not required for effector recognition. The putative resistance protein can recognize both full-length and N-terminal effectors. Blue arrows indicate the process of effector processing. Black arrows indicate the process of effector recognition. In AvrRps4, R88 or R112 is important for AvrRps4C-processing. However, only R112 is necessary for AvrRps4N/F-recognition (top). In XopO, the conserved R111, not the conserved R87, is important for XopO-processing. However, R111 and E114, together, are necessary for XopO N/F-recognition. Compared to AvrRps4, XopO is more easily recognized by lettuce due to the two key residues (bottom).

The electric charge of key residues plays an important role in protein function. For instance, negatively charged E175 and E187 in AvrRps4C are critical for AvrRps4-triggered immunity (Ma et al., 2018; Sohn et al., 2012); electropositive R493 of EDS1 is crucial for TNL-mediated resistance (Bhandari et al., 2019). Therefore, we suggest that R112 and its positive charge are probably vital for the interaction of AvrRps4 with the resistance protein (or guardee/decoy). Previously we proposed that the conserved R112 may also be functional in AvrRps4 homologues (Su et al., 2021). In this study, we proved that the hypothesis is correct for HopK1 (Figure 52) but not for XopO (Figure 2). Furthermore, we showed that the conserved R111 and nonconserved E114 work together for XopO recognition (Figure 4d). Compared to the positively charged K115 in AvrRps4 and HopK1, we propose that the negative charge of E114 makes a structural difference to XopO, which might lead to a stronger binding to its corresponding immune protein and a more robust HR in lettuce. If R112 is important for an electrostatic interaction of AvrRps4 with another protein, XopO might be able to function without R111 because other unique negatively charged residues are present in the vicinity that may still provide electrostatic interactions with adjacent amino acids in the partner protein. We also suppose that the three effectors (AvrRps4, HopK1, and XopO) were originally recognized identically by lettuce and other unknown plants. Over time, AvrRps4 and HopK1 could have evolutionarily escaped the effector recognition of the unknown plant species by changing the residue 115 to lysine, while XopO did not. However, the single mutation at residue 115 is insufficient for avoiding lettuce perception because R112 is still recognized. In the future, to identify why R111/E114 and only R112 are important for XopO and AvrRps4 recognition, respectively, it is important to perform structural analyses or protein crystallization of AvrRps4N and XopOβ. This study updates what we know about AvrRps4...
homologue effectors regarding their processing and recognition. For further studies, identifying a lettuce bacterial pathogen system for the natural delivery of effectors would be an important tool to characterize structural determinants of immune elicitation by AvrRps4 family members. Beyond the functions of key residues in effector processing and recognition, it would be interesting to discover their effects on the virulence functions of the AvrRps4 family in lettuce and whether effector processing is important for virulence functions. In addition, relative transcript expression of discover their effects on the virulence functions of the AvrRps4 in effector processing and recognition, it would be interesting to characterize structural determinants of immune elicitation by the AvrRps4 family could help describe the mechanism of AvrRps4/HopK1/XopO-mediated immunity in lettuce.

AUTHOR CONTRIBUTIONS
S.H.K. conceived the project. Q.M.N. and S.H.K. designed the experiments. Q.M.N., A.B.B.I., G.H.S., U.T.V., and J.L. performed experiments. Q.M.N. and S.H.K. analysed data and wrote the manuscript. W.G., J.-H.K., and S.H.K. edited the manuscript. S.H.K. supervised the project.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

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