Is KIPI30 the main virulence target of the *Phytophthora infestans* effector protein Avr3a?

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Abstract

Plant parasitic oomycetes like *Phytophthora infestans* secrete diverse classes of effector proteins to modulate host innate immune reactions. Two different alleles of *Avr3a*, which belongs to the RxLR effector family, are known, but only the KI-form, in contrast to the EM-form, is recognized by the NBS-LRR resistance protein R3a (1). To date the main virulence target of any eukaryotic effector protein is unknown. By means of a Yeast-2-Hybrid-Screen using Avr3a as bait the strongest interactor of both forms, Avr3a^{EM}and Avr3a^{KI}, turned out to be an N-terminally truncated pyruvate kinase-like protein, named KIPI30. Silencing of KIPI30 in R3a-transgenic *Nicotiana benthamiana* plants via VIGS caused a significant decrease of the HR response after *Agrobacterium tumefaciens*-mediated expression of Avr3a^{KI}. Localization studies show that the truncated KIPI30 is localized in vesicle-like structures, while the full-length protein is chloroplast-associated. However, a co-localization with either form of Avr3a could not be observed. Moreover, *in vivo* interaction studies using BiFC in *N.benthamiana* or Yeast-2-Hybrid did not reveal any interaction between either form of Avr3a and the full-length KIPI30. Currently we focus our investigation on optimizing the VIGS-conditions and on interaction studies via pull-down assays to figure out if KIPI30 is a genuine virulence target of Avr3a, and to investigate if it is the mediator in R3a recognition.

Introduction

As shown below, R3a-mediated hypersensitive response occurs only in the presence of Avr3a^{KI}. The other form, Avr3a^{EM}, is not recognized by the cytoplasmic NBS-LRR resistance protein R3a and, in contrast to the KI-form, does not suppress cell death in *N. benthamiana* triggered by the *P.infestans* PAMP INF1 (2). It has recently been demonstrated that the deletion of the C-terminal tyrosine (Y147) of Avr3a^{KI} abolishes the suppression of INF-mediated cell death, but does not affect R3a-recognition (3). This feature separation supports the idea that this effector interacts with more than one host protein (4). Here we aim at investigating one particular protein from potato, named KIPI30, for its putative role as virulence target and mediator in R3a recognition.

Avr3a^{KI}, but not Avr3a^{EM} is recognized by R3a (1)

Avr3a^{KI}, but not Avr3a^{EM} suppresses INF-mediated cell death (2)

deletion of C-terminal tyrosin (C147) of Avr3a^{KI} abolishes INF1-mediated cell death, but...

...doesn't affect recognition by R3a (3)

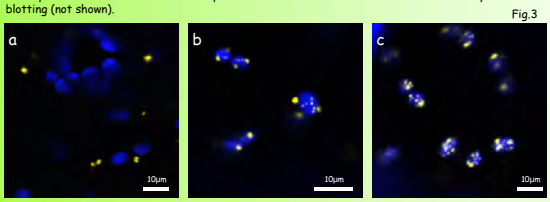
Results

We conducted a Yeast-2-Hybrid screen against a cDNA library from *P.infestans*-infected potato using the Invitrogen Gal4-based Y2H system with both forms of Avr3a as bait to identify potential host targets. The most interesting candidate turned out to be an N-terminally truncated pyruvate kinase, named KIPI30ΔN (Fig.1). By means of 5'-RACE we identified two different alleles of the full-length KIPI30 (a1 and a2). Interestingly, only the full-length alleles harbor a chloroplast-targeting signal.

Fig.1

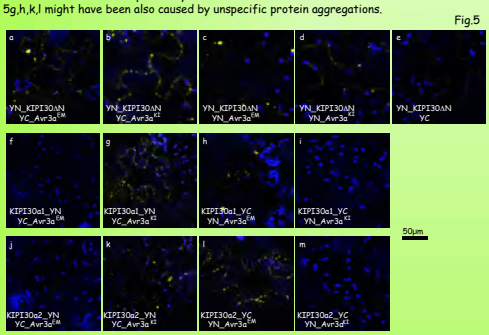
To investigate the subcellular localisation of KIPI30, we constructed Yellow-Fluorescent-Protein (YFP) fusions and transiently expressed them via *Agrobacterium tumefaciens*-infiltration in *N.benthamiana* plants. As shown in Fig.3, the fluorescence caused by YFP-KIPI30ΔN (a) was observed in small vesicle-like structures, which did not colocalize to peroxisome or Golgi marker (not shown). In contrast, KIPI30 full-length fusions exclusively localized to the chloroplasts (a1 in b and and a2 in c). It has to be determined, if the full-length KIPI30 localizes to chloroplasts or to chloroplast-associated vesicles. The expression of the fusion constructs was confirmed by Western blotting (not shown).

Fig.3



We attempted the direct visualization of KIPI30-Avr3a interaction in living plant cells by transiently co-expressing split-YFP (sYFP) fusions via *Agrobacterium tumefaciens*-infiltration in *N.benthamiana* leaves. For that purpose we fused both alleles, the truncated form of KIPI30 and both forms of Avr3a to both halfs of YFP (YN and YC). As shown in Fig.5, after co-expression of KIPI30ΔN- and Avr3a-sYFP fusions we obtained fluorescence signals, which 1. were considerably higher compared to co-expression of a sYFP-fusion with an untagged YN or YC, respectively (Fig.5e), and 2. resembled fluorescence signals from localization studies (Fig.3a) supporting the idea of an specific interaction (Fig.5a-d). However, we did not detect such a consistent specific interaction after co-expression of full-length KIPI30- and Avr3a-sYFP fusions (Fig.5f-m), particularly not as chloroplast-associated fluorescence, which might be due to an instability of the KIPI30 fusions (the amount of fusion proteins was below detection level in Western blot analyses) or, in case of the presence of transmembrane helices in KIPI30 (which have been predicted for the full-length KIPI30 by some, but not all programmes which are available online) the orientation of KIPI30-sYFP fusions across a membrane avoids a close proximity of both YN and YC to reconstitute. Fluorescence observed in Fig. 5g,h,k,l might have been also caused by unspecific protein aggregations.

Fig.5

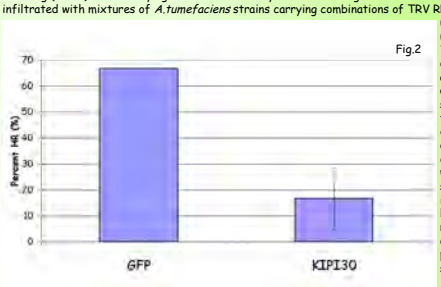


Conclusion and Outlook

- the strongest interactor of Avr3a in a Y2H screen turned out to be an N-terminally truncated pyruvate kinase, named KIPI30; this interaction was verified by independent Y2H- and sYFP experiments
- KIPI30-location is associated with chloroplasts
- in Y2H and sYFP full-length KIPI30 does not specifically interact with Avr3a
- KIPI30 silenced R3a transgenic *N.benthamiana* plants seem to be impaired in R3a-mediated recognition of Avr3a
- future optimization of VIGS experiments as well as pull-down approaches are necessary to gain insight into the putative role of KIPI30 as genuine virulence target and mediator of R3a-recognition

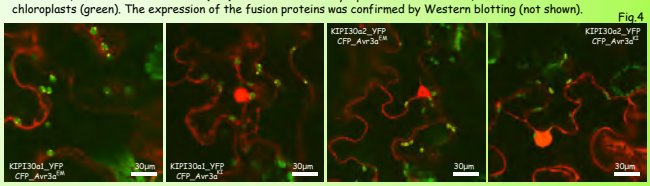
In order to assess if KIPI30 is involved in R3a-mediated recognition of Avr3a^{KI}, we employed Virus-induced gene silencing (VIGS) followed by agro-infiltration assays. Five-leaf stage *N.benthamiana* R3a-transgenic plants were infiltrated with mixtures of *A.tumefaciens* strains carrying combinations of TRV RNA1 and either pTV-GFP (vector control) or pTV-KIPI30 (TRV RNA2). Three weeks after TRV infection, we transiently expressed Avr3a^{KI} in leaf 3 and 4 above the VIGS-infiltrated leafs via agroinfiltration. Preliminary results show (Fig.2), that on GFP control treated plants, a HR cell death was clearly visible at 7 dpi. In contrast, on KIPI30-silenced plants, cell death was considerably reduced, which makes it tempting to speculate that KIPI30 is an important part in triggering the hypersensitive response after R3a mediated recognition of Avr3a^{KI}. Currently we are confirming the particular efficiency of the KIPI30 silencing by means of real-time PCR.

Fig.2



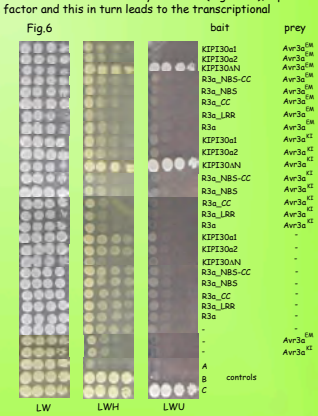
KIPI30 might be a genuine virulence target of Avr3a and as shown above (Fig.2), it appears to play an important role in R3a-mediated recognition of Avr3a^{KI}. Therefore an interaction between KIPI30 and Avr3a seems to be likely. To study this putative protein-protein interaction, we first performed co-localization assays in *N.benthamiana* leaves. We transiently expressed KIPI30-YFP fusions together with CFP-Avr3a fusions via agroinfiltration. As shown in Fig.4, KIPI30 and Avr3a do not colocalize, as fluorescence of both KIPI30-YFP fusions (yellow) is chloroplast-associated, and, in contrast, both CFP-Avr3a fusions (red) are located in the cytoplasm and the nucleus, but do not localize to the chloroplasts (green). The expression of the fusion proteins was confirmed by Western blotting (not shown).

Fig.4



The Yeast-2-Hybrid system offers another experimental approach to investigate protein-protein interactions *in vivo*. For that purpose we cloned both alleles and the N-terminally truncated form of KIPI30, the potato resistance protein R3a and subfragments of it and both alleles of Avr3a in both pGADT7 and pGBKT7 and assessed for putative protein-protein interactions via the Invitrogen ProQuest Gal4-based Y2H-system. After transformation of yeast cells (Fig.6 LW), a positive interaction of two proteins reconstitutes the Gal4-transcription factor and this in turn leads to the transcriptional activation of reporter genes, from which two of them enable transformed yeast cells to grow on particular dropout-media (Fig.6 LWH and LWU). As shown in Fig.6, only yeast cells expressing the N-terminally truncated KIPI30 and either form of Avr3a indicates by its growth on both dropout-media an interaction, which confirms the screening result (Fig.1). As no growth is observable by using negative control vector combinations ("-" in Fig.6), the interaction between KIPI30ΔN and either Avr3a seems to be specific. In contrast to that, transformation of yeast cells with full-length KIPI30 and either Avr3a did not lead to growth on dropout-media, similarly to yeast cells transformed with R3a or its subfragments and either form of Avr3a. After vector swapping we obtained the same results (not shown). This might have different reasons: If, as mentioned earlier (Fig.5), full-length KIPI30 contains a transmembrane domain, the Y2H-approach wouldn't be the appropriate one. Another imaginable reason could be that there isn't a specific interaction between Avr3a and full-length KIPI30. That would mean, that the observed interaction between Avr3a and KIPI30ΔN is false-positiv. As an interaction between R3a or one of its subfragments with either form of Avr3a cannot be detected, one can assume, that R3a recognizes Avr3a within the plant cytoplasm in an indirect manner. Although the Yeast-2-Hybrid system is a nice and easy method for investigating protein-protein interactions, the subcellular conditions do not reflect plant cytoplasmic conditions and its results should not be overinterpreted, unless other approaches tend to similar results.

Fig.6



References:
(1) Armstrong *et al.* 2005. PNAS 102 (21), pp. 7766-7771
(2) Bos *et al.* 2006. Plant J. 48, pp. 165-176
(3) Bos *et al.* 2009. MPMI 22 (3), pp. 269-281
(4) Birch *et al.* 2009. J.Exp.Bot. 60 (4), pp. 1133-1140