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Brief Communication

# Depletion of the *Nb*CORE receptor drastically improves agroinfiltration productivity in older *Nicotiana benthamiana* plants

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## Summary

*Nicotiana benthamiana* is increasingly used for transient gene expression to produce antibodies, vaccines, and other pharmaceutical proteins but transient gene expression is low in fully developed, 6–8-week old plants. This low gene expression is thought to be caused by the perception of the cold shock protein (CSP) of *Agrobacterium tumefaciens*. The CSP receptor is contested because both *Nb*CSPR and *Nb*CORE have been claimed to perceive CSP. Here, we demonstrate that CSP perception is abolished in 6-week-old plants silenced for *Nb*CORE but not *Nb*CSPR. Importantly, older *Nb*CORE-silenced plants support a highly increased level of GFP fluorescence and protein upon agroinfiltration. The drastic increase in transient protein production in *Nb*CORE-depleted plants offers new opportunities for molecular farming, where older plants with larger biomass can now be used for efficient protein expression.

*Nicotiana benthamiana* is frequently used for transient gene expression (Bally *et al*., 2018). In addition to studies on subcellular localization, protein–protein interaction and enzymatic activities, transient gene expression is commercially used to produce antibodies, vaccines, and other pharmaceutical proteins (Sainsbury, 2020; Schillberg and Spiegel, 2022). Transient expression is achieved by infiltrating leaves or whole plants with disarmed *Agrobacterium tumefaciens* harbouring a binary vector that carries genes-of-interest on the transfer DNA (T-DNA). *A. tumefaciens* transfers this T-DNA into the plant cell, where it is expressed.

The success of transient gene expression decreases with the age of the *N. benthamiana* plants, despite having more biomass and large leaves that are easy to infiltrate (Lai and Chen, 2012; Saur *et al*., 2016). Best expression is achieved in 3–5 week-old plants and poor expression in older, 6–8-week-old plants that start flowering. The poor gene expression is thought to be caused by the perception of cold shock protein (CSP) of *A. tumefaciens* (Saur *et al*., 2016). A 22 amino acid fragment of CSP called csp22 is sufficient to trigger immune responses including a burst of reactive oxygen species (ROS) (Felix and Boller, 2003; Saur *et al*., 2016). The csp22-induced ROS burst is observed from leaf discs from old plants, but not from young plants (Saur *et al*., 2016), implicating that CSP recognition might indeed underpin the success of transient gene expression in older plants.

Two distinct receptors, the receptor-like protein *Nb*CSPR (Saur *et al*., 2016) and the receptor-like kinase *Nb*CORE (Wang *et al*., 2016), respectively, have been proposed to act as CSP receptors. Transcripts of both receptors, encoding proteins with only 29.9% amino acid identity, are detectable only in older *N. benthamiana* plants. *Nb*CSPR was reported to interact with csp22 and to be required for its perception because depletion of *Nb*CSPR by virus-induced gene silencing (VIGS) suppressed the csp22-induced ROS response. The silencing of *Nb*CSPR also resulted in higher transient expression in older plants after Agroinfiltration of a reporter gene. In disagreement with this report, Wang *et al*. (2016) could not confirm the role of *Nb*CSPR in csp22 perception. Rather, they report that CORE tomato (*Sl*CORE) forms the specific, high-affinity receptor binding site that is required and sufficient for csp22 perception (Wang *et al*., 2016). They also found that its ortholog in *N. benthamiana*, *Nb*CORE, but not *Nb*CSPR, confers csp22 responsiveness when transformed into *Arabidopsis thaliana*, which is otherwise insensitive to csp22 (Wang *et al*., 2016).

Here, we depleted *Nb*CSPR and *Nb*CORE by VIGS to investigate if CSP perception hampers recombinant protein production in older plants. To silence *Nb*CSPR, we used the same 299 bp gene fragment of *Nb*CSPR used earlier (Saur *et al*., 2016; Tables S1 and S2), and cloned this into a vector expressing *RNA2* of tobacco rattle virus (TRV2gg). Similarly, a 300 bp fragment specific to *Nb*CORE was cloned into TRV2gg. Alignments of the used silencing fragments with the coding sequences of *Nb*CORE and *Nb*CSPR show that cross-silencing is unlikely (Figure S1). TRV carrying a fragment of beta-glucuronidase (*TRV::GUS*) was included as a negative control. 2-week-old *N. benthamiana* plants were infected with TRV carrying the silencing fragments. The *TRV::NbCSPR* and *TRV::NbCORE* plants have no developmental phenotypes compared to *TRV::GUS* plants (Figure 1a).

Leaf discs from 4-week and 6-week-old VIGS plants were tested for a csp22-induced oxidative burst. Importantly, the csp22-induced ROS burst is absent from 6-week-old *TRV::NbCORE* plants and is present in *TRV::NbCSPR* plants, comparable to *TRV::GUS* control plants (Figure 1b and Figure S2). As reported before, younger, 4-week-old plants, only have very

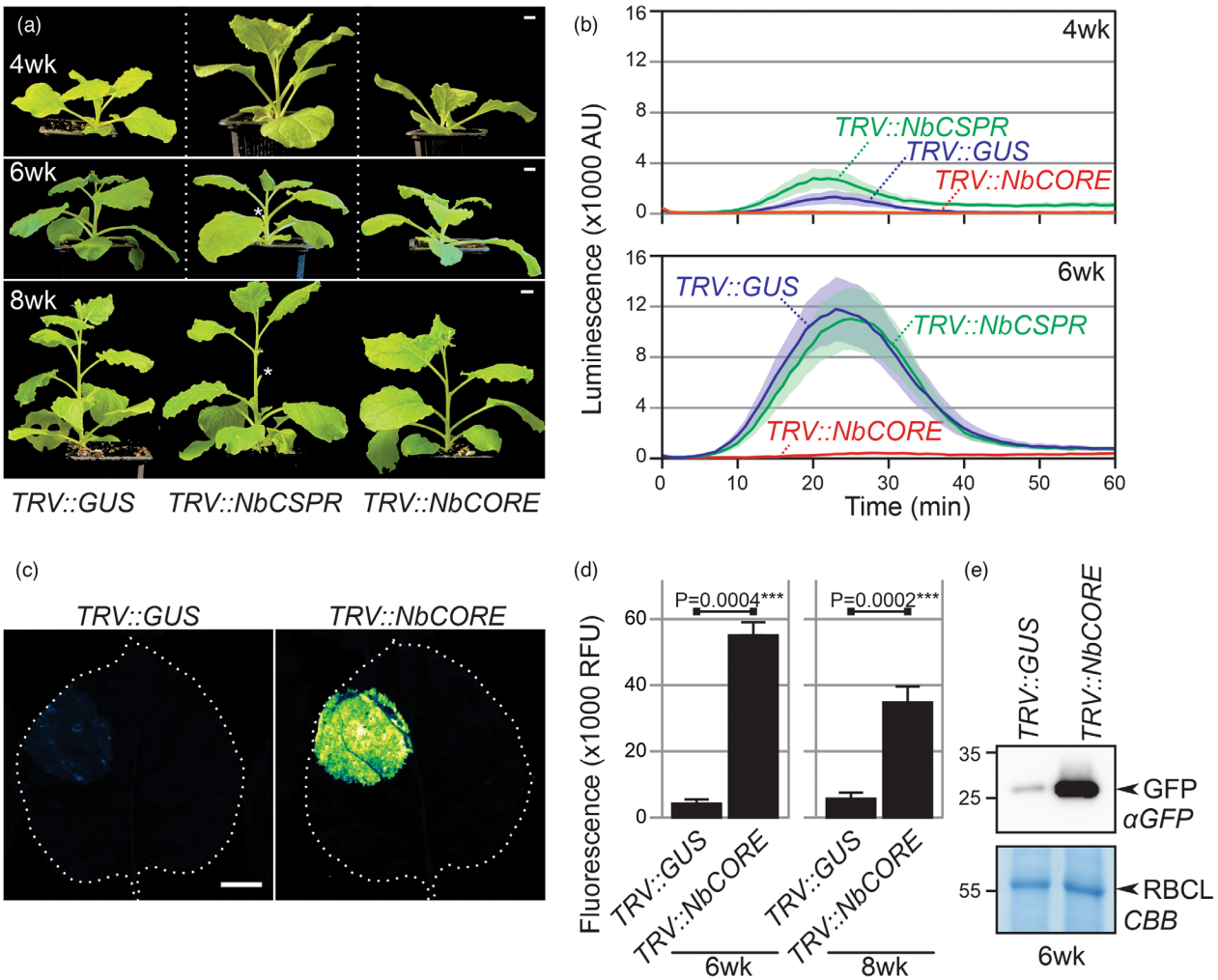


**Figure 1** *Nb*CORE silencing removes csp22 responsiveness and increases transient protein production in older *N. benthamiana* plants. (a) *TRV::NbCSPR* and *TRV::NbCORE* plants have no additional developmental phenotype compared to *TRV::GUS* plants. Scale bars, 1 cm. *, removed sample leaves. (b) The csp22-induced oxidative burst is absent from 6-week-old *TRV::NbCORE* plants but present in *TRV::GUS* and *TRV::NbCSPR* plants. Error shades represent the standard error of $n = 6$ leaf discs. (c) *Nb*CORE depletion causes bright GFP fluorescence upon agroinfiltration of 6-week-old plants. The image was taken 5 days agroinfiltration with 35S:eGFP. Scale bar, 1 cm. (d) Significant increase in GFP fluorescence upon *Nb*CORE depletion. GFP fluorescence was quantified from images of $n = 4$ biological replicates of 6-week and 8-week-old VIGS plants agroinfiltrated with 35S:eGFP 5 days before fluorescence scanning. Fluorescence was quantified using ImageJ and normalized by leaf area. ****, *P* value = 0.0000084 (*t*-test). (e) *TRV::NbCORE* plants accumulate much more GFP protein upon agroinfiltration than *TRV::GUS* plants. Total leaf proteins were extracted from VIGS plants, 5 days after agroinfiltration with *35S:eGFP*, and analysed by anti-GFP western blot. CBB, Coomassie brilliant blue.

weak csp22-induced responses that can vary per batch of plants (Figure 1b and Figure S2). These data demonstrate that *Nb*CORE is essential for the csp22-induced oxidative burst in older *N. benthamiana* plants. Our data indicate that *Nb*CSPR is not necessary for csp22 perception, consistent with the findings of Wang *et al*. (2016) that *Nb*CSPR is not sufficient for csp22 perception.

To investigate to what level the depletion of *Nb*CORE promotes transient gene expression, we agroinfiltrated 6w-old *TRV::GUS* and *TRV::NbCORE* plants with Agrobacterium delivering enhanced GFP driven by a strong 35S promoter (35S:eGFP, Kourelis *et al*., 2021), and scanned the agroinfiltrated leaves for fluorescence five days later. Bright GFP fluorescence was detected in *TRV::NbCORE* plants, whereas hardly any fluorescence was detected in *TRV::GUS* plants (Figure 1c), corresponding to a nearly eight-fold increased GFP fluorescence (Figure 1d). A similar increased fluorescence was observed upon infiltrating 8-week-old plants (Figure 1d). Western blot analysis confirmed a drastically increased GFP protein level in *TRV::NbCORE* plants compared to the *TRV::GUS* control plants (Figure 1e).

Our data showing that *Nb*CORE is required for csp22-induced oxidative burst is consistent with reports that *Nb*CORE binds csp22 with high affinity (Kd = 6 nM, Wang *et al*., 2016) and that transient expression of *Nb*CORE confers csp22-responsiveness to leaves of young plants (Wei *et al*., 2018). The csp22-induced ROS burst in *TRV::NbCSPR* plants was similar to the *TRV::GUS* control, which contradicts earlier work (Saur *et al*., 2016). Our data is, however, consistent with experiments that *Nb*CSPR is unable to confer csp22-responsiveness (Wang *et al*., 2016). A more recent report also shows that *Nb*CSPR is identical to RE02, the receptor for VmE02, a conserved Cys-rich protein secreted by diverse microbes (Nie *et al*., 2021).

 

Our results offer new opportunities in molecular farming, where older plants with larger biomass can now be used for efficient transient gene expression. A more durable depletion of *Nb*CORE can be achieved by genome editing, or by engineering *A. tumefaciens* strains to contain a CSP that is no longer recognized by *Nb*CORE. Both approaches will drastically improve transient protein production in older *N. benthamiana* plants, without the need for a licence to work with TRV to deplete *Nb*CORE by VIGS.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

ID and CC performed experiments and analysed the data; ID, PB and RH designed experiments; ID and RH wrote the paper with help from all authors. All the authors read and approved the final manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1.** Supplemental Methods, Figures and Tables.

 