Signalling in gene silencing

The mechanisms involved in post-transcriptional gene silencing (PTGS) are being unravelled. Firm evidence exists that PTGS is a mechanism by which targeted RNAs are removed from the plant cytoplasm. Recent studies using localized DNA introduction techniques have demonstrated that PTGS involves three steps: initiation, propagation and maintenance[1,2]. Initiation is manifested by processes that program single cells for degradation of targeted RNAs and is associated with the production of a sequence-specific silencing signal. The existence of such a signal has been previously shown using an elegant approach[3]. Using a grafting procedure, it was demonstrated that transgene-specific post-transcriptional silencing can be efficiently transmitted from silenced stocks to non-silenced scions. The localized DNA introduction experiments confirmed that the silencing signal spreads via cell-to-cell and long-distance vascular movement (propagation). Systemic acquired silencing (SAS) requires stable transmission of the silencing signal throughout the plant and is probably connected to the capacity of cells receiving the primary signal to amplify this signal (maintenance).

Similarities between gene silencing and natural viral defence mechanisms

Non-transgenic plants sometimes recover from viral infection by a PTGS-like mechanism[4]. Although primary infection is normal in recovered plants and the virus spreads systemically, newly developing leaves lack symptoms and viral RNAs fail to accumulate because of viral RNA degradation.

Infection with viruses containing homology to endogenous or transgene sequences is associated with viral resistance and PTGS in recovered tissue, indicating that these processes are based on a similar mechanism. Interestingly, it has been shown that infection of *Nicotiana benthamiana* with a chimeric potato virus X-phytoene desaturase (PVX-PDS) led to inactivation of endogenous PDS, whereas PVX-PDS accumulated to high levels[5]. By contrast, both the viral RNA and the mRNA of the highly expressed transgene encoding green fluorescent protein (GFP) were degraded when PVX-GFP was used for infection. The presence of a highly transcribed gene appears to enhance recovery whenever the infecting virus shares homology to this gene. This also shows that PVX-induced PTGS might be too weak to block viral replication but it is sufficiently strong to inactivate genes expressed at low levels.

Localized induction of silencing

The initiation, maintenance and propagation of PTGS were investigated by biolistic treatment of transgenic tobacco plants[2]. Localized introduction of a sense nitrate reductase (Nia) transgene into leaves initiated cosuppression of host Nia genes and previously introduced Nia transgenes (Fig. 1a). When wild-type tobacco (class 0) was used, PTGS initiation was occasionally observed in the bombarded area as chlorotic spots. This localized acquired silencing (LAS) was seen 12–15 days post-bombardment in transgenic plants from homozygous lines expressing Nia (trans)genes during their lifetime (class I) and the chlorotic spots were larger than those observed in wild-type plants. In addition to class 0 and class I plants, transgenic class II plants were used. In each homozygous class II line, spontaneous triggering of Nia cosuppression occurs at each generation, affecting only a limited but constant fraction of the population. Upon bombardment, non-silenced homozygous class II plants also displayed LAS but in these plants, LAS was often followed by SAS. These results suggest that only class II cells are able to amplify a silencing signal delivered from the bombarded cells, allowing its propagation through the whole plant.

These observations are in agreement with results obtained from grafting experiments using identical tobacco lines[6] (Fig. 1b). Silenced class II plant stocks initiated systemic Nia gene silencing in class I and non-silenced class II scions but not in wild-type scions. Removal of the silenced scions and regrafting onto wild-type rootstocks revealed that only homozygous class II scions maintained grafting-induced silencing. Newly developing leaves of class I and hemizygous class II scions successively lost chlorosis.

As found in the virus recovery phenomenon, grafting experiments revealed that PTGS was influenced by the transcription rate. The non-transgenic tobacco line NIA30 accumulates host Nia mRNA above the level of wild-type plants, because of metabolic derepression. After grafting onto silenced class II stocks, silencing of the Nia genes appeared in the scions. In the reverse experiment, class II plant scions lost their susceptibility to grafting-induced silencing when Nia transgene transcription was blocked by genetic crosses with a silencer locus before grafting.

Initiation of silencing

Consistent results have been obtained for initiation of PTGS (Refs 1,2). Whether PTGS is initiated by DNA–DNA, RNA–DNA or RNA–RNA interactions was investigated using *Agrobacterium* infiltration or biolistic transformation of *N. benthamiana* plants expressing a GFP transgene[1]. Although definitive evidence was not obtained, the data suggest a direct interaction between the transferred DNA and the transgene. According to previous models, transgene transcription would be perturbed by this interaction, leading to the production of aberrant RNAs. These aberrant RNAs could then serve as templates for a cellular RNA-directed RNA polymerase[7] that produces antisense RNA. The antisense RNA could then target mRNA for double-strand-specific RNase degradation and is proposed to be a component of a moveable silencing signal[1]. The DNA pairing model is supported by the fact that ssDNA, promoterless, sense and antisense DNA constructs can initiate PTGS (Refs 1,2).

Nevertheless, several observations point to an initiation process that is based on RNA–DNA or RNA–RNA interactions. The latter possibility is supported by the observation that the PDS host gene was not inactivated when the PDS-specific insert of a chimeric PVX was targeted to an intron[5]. A delayed occurrence of LAS and a lower efficiency of SAS using promoterless constructs, as compared with promoter-regulated Nia constructs, has been reported[2]. This suggests that transient transcription of the introduced DNA has an impact on the initiation process. Moreover, PTGS is efficiently triggered in flies[8], worms[9] and trypanosomes[10] by localized introduction of dsRNA indicating that DNA–DNA interactions are not required for initiation, at least in these organisms.

Double stranded RNA can also initiate gene silencing in tobacco plants[11]. In this study, separate expression of sense and antisense RNA was a less efficient trigger of silencing than simultaneous expression of both RNAs. The observation that promoter-driven antisense Nia constructs mediated the fastest LAS response and the highest efficiency of SAS induction[2], might reflect the importance of dsRNAs in PTGS. Although triggering of silencing by localized introduction of promoterless constructs argues for the DNA pairing hypothesis[1,2], it should be noted that mechanically inoculated promoterless dsDNA and ssDNA are transcribed in tomato leaf cells[12].

Propagation of silencing

In class II (Ref. 2) or GFP (Ref. 1) plants, systemic silencing can be observed following local initiation of PTGS. Therefore, propagation appears as a simple mechanism that is dependent on the production of a sequence-specific signal that moves intercellularly via plasmodesmata and over long distances via the phloem. The nature of this propagatable signal is unclear. To account for its sequence specificity, it is probable that it contains a nucleic

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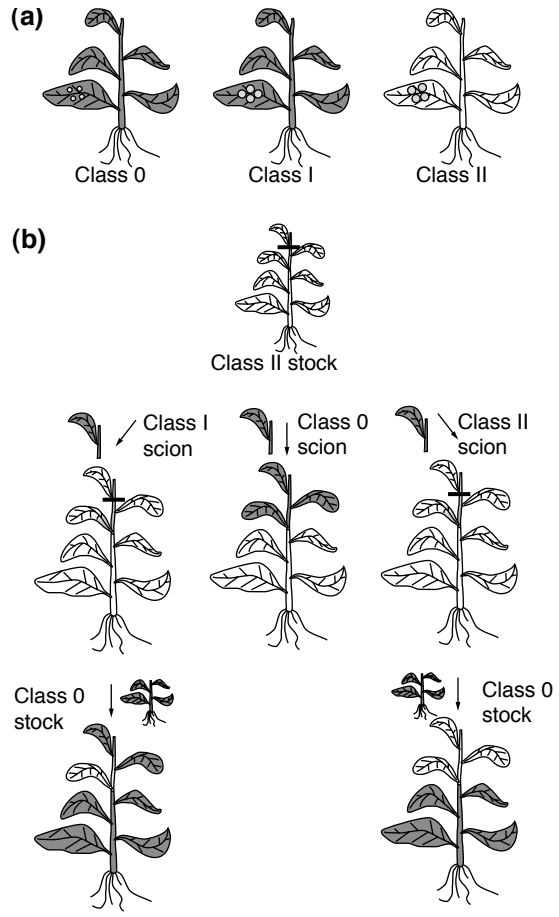

Fig. 1. Schematic of biolistic- and grafting-induced Nia (trans)gene cosuppression. Nia-expressing tissue is represented in dark-gray and the non-expressing (silenced) tissue in white. (a) Localized introduction of Nia DNA fragments leads to localized acquired silencing (LAS) in bombarded wild-type tobacco (class 0) leaves (small light-gray spots). LAS is more pronounced (large light-gray spots) in bombarded homozygous class I and class II plant leaves but LAS is followed by systemic acquired silencing (SAS) in class II plants. (b) Scions of class 0, Nia transgene-expressing homozygous class I, and Nia transgene-expressing homozygous class II plants have been grafted onto silenced class II stocks. Developing leaves of class I and class II scions display SAS whereas Nia cosuppression is not observed in class 0 scions. Regrafting of the SAS-displaying class I and class II scions onto class 0 stocks reveals that only class II scions maintain cosuppression. Previously silenced class I scions recover from cosuppression and newly developed

acid component, probably RNA. Similar to non-coding viroid RNA, this RNA could move as a ribonucleoprotein complex to systemically invade the plant (for a review see Ref. 13).

Maintenance of silencing

Maintenance of silencing is dependent on the synthesis of a signal that specifically targets mRNA for degradation. Wild-type plant scions receiving the silencing signal from class II plant stocks are not able to undergo PTGS. On the contrary, bombarded wild-type plant cells show LAS (Fig. 1). Assuming that more signal molecules are provided by biolistic transformation than by class II plant stocks, the inability to undergo silencing indicates that, on the one hand, the level of signal molecules was below a critical threshold in grafted class 0 plants. On the other hand, the lower stock-provided signal concentration led to PTGS in class I and non-transgenic NIA30 plant scions. Unlike wild-type plants, both of these plant lines displayed high Nia transcription. If a low signal concentration is compensated by high transcription, it seems reasonable that the signal can interact with the transcribed loci, the transcript itself, or with both.

The concept of such a signal–transcription interaction is in agreement with observations of pronounced LAS in bombarded class I plants. Biolistically transformed cells can be thought of as silenced class II stocks. In such a scenario, class 0 plant cells that surround a bombarded cell are not silenced, which is similar to wild-type scions. Class I plant cells, immediately adjacent to the initiated 'stock', receive sufficient signal for initiation of RNA degradation. With increasing distance from the initiated 'stock', the signal is diluted out, reflecting the situation when class I plant scions are removed from the silenced class II stock. Finally, only homozygous class II plants can maintain silencing, irrespective of whether they have been initiated by grafting or by biolistic transformation (Fig. 1).

Interestingly, it has been shown that in homozygous class II plants, a reduction in the amount of bombarded DNA was associated with LAS, a pattern typical of class I plants[2]. This suggests that in class II plant cells a certain signal threshold is required to initiate SAS and to maintain PTGS. From these results, we conclude that a defined signal threshold is required to initiate degradation in a single cell. To maintain silencing and to induce SAS, the signal concentration has to reach a second threshold.

Conclusion

At present, the available data only allow us to speculate on the molecules and processes that are involved in PTGS. In a simplified model, PTGS can be considered as a mechanism in which a mobile signal exceeds a threshold. Although the nature of such a signal is unknown, it is thought that it contains dsRNA. However, degradation of dsRNA is assumed to be responsible for the removal of mRNA during PTGS, so it appears unlikely that the signal is an unprotected dsRNA molecule. It is worth investigating whether the signal molecules are protected by proteins or whether they can escape degradation, for example, by heteroduplex formation. To investigate the role of dsRNA in PTGS further, biolistic techniques for the transfer of dsRNAs into plant cells could be very helpful.

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Stephen Hales and the cohesion theory

In science, theories can be born more than once, as the mendelian laws have shown. In this context, the cohesion theory of water movement in plants has been variously ascribed to Josef Böhm[1], Henry H. Dixon and John Joly[2], and Eugen Askenasy[3]. However, all of the elements of this theory were first described in 1727 by the English clergyman Stephen Hales in his book *Vegetable Statics*[4]. Unfortunately, Hales' ideas were not understood at the time, so his findings failed to influence the debate on water transport in plants in the 19th century.

Hales' insight appears to have been influenced by his relations with Isaac Newton. Although Newton left Cambridge in 1696 when Hales entered, they met in 1718 when Hales was elected a fellow of the Royal Society. As chairman of the society, Newton gave Hales' book his imprimatur.

The most relevant section of *Vegetable Statics* is exp.33. After citing Newton's *Optics* (2nd edn, 1717; query 31, in which mercury is lifted 60–70 inches in a barometer tube by cohesion compared with a water pillar of over 60 feet) Hales wrote, 'And by the same principle it is, that we see, in the preceding experiments, plants imbibe moisture so vigorously up their fine capillary vessels; which moisture, as it is carried off in perspiration, (by the action of warmth) thereby gives the sap-vessels liberty to be almost continually attracting of fresh supplies; which they could not do, if they were full saturate with moisture: for without perspiration the sap must necessary stagnate, notwithstanding the sap-vessels are so curiously adapted by their exceeding fineness, to raise a sap to great heights, in a reciprocal proportion to their very minute diameters.' Hales' discussion of water conduction in plants is based on sound experiments, such as his measurements of tensions in transpiring branches. He noted that tensions of up to 12 inches of mercury are not the full tension as air is sucked out of a branch simultaneously. He also produced an early dendrometer, and measured imbibition forces in peas.

As with so many original arguments, Hales' sounds superficial on first reading, although there can be no doubt of the mechanism involved. Hales also failed to name his theory. Thus, four editions and translations of his book were insufficient to connect the name of this versatile clergyman with the cohesion theory.

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Retraction by Jeff Schell

In a recent article in *Science* (1999) 238, 1987–1989 it is said that I had no plans to publish retractions of the papers in the journals in which they originally appeared. In fact, I wanted to stress the point that the first responsibility the collaborating colleagues in- and outside the Max-Planck Institute and I had felt was to publish our results showing that the previously published data could not be reproduced by another, more objective, method. Therefore, the members of the investigating team decided to publish all further data re-evaluating this fraud as a regular scientific paper in *The Plant Journal*. After peer-review and acceptance of the paper it was agreed with the Editor-in-Chief, Prof. Dianna Bowles, that after publication short correction statements should be sent to individual journals, which could refer to the paper for full details of the new experiments, confirming the irreproducibility of the protoplast assays in question. As the paper has now appeared, we hereby retract officially the results regarding phytohormone effects on division of tobacco protoplast-derived cells in our papers:

Hayashi *et al.* (1992) *Science* 258, 1350–1353
Walden *et al.* (1994) *EMBO J.* 13, 4729–4736
Röhrig *et al.* (1995) *Science* 269, 841–843
Miklashevichs *et al.* (1996) *Trends Plant Sci.* 1, 411
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Harling *et al.* (1997) *EMBO J.* 16, 5855–5866
Miklashevichs *et al.* (1997) *Plant J.* 12, 489–498

Please refer to our new results published in Schell *et al.* (1999) *Plant J.* 17, 461–466.

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