Mechanisms of the light-dependent induction of cell death in tobacco plants with delayed senescence

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Received 10 May 2005; Accepted 5 August 2005

Abstract

The relationship between leaf senescence and cell death was investigated using tobacco with delayed senescence due to auto-regulated production of cytokinin (SAG12-IPT). Although leaf senescence ultimately results in cell death, the results show that senescence and cell death can be uncoupled: in nutrient-deficient, but not in fertilized SAG12-IPT plants, necrotic lesions were detected in old, but otherwise green leaves. By contrast, wild-type leaves of the same age were yellow, but not necrotic. Chlorophyll fluorescence analysis revealed an over-reduction of the electron transport chain in old SAG12-IPT leaves, in combination with characteristic spatial patterns of minimum fluorescence ($F_0$), quantum efficiency of open photosystem II centres ($F_v/F_m$) and non-photochemical quenching (NPQ), as determined by fluorescence imaging. The same patterns of $F_0$, $F_v/F_m$ and NPQ were induced by incubation of leaf discs from nutrient-deficient SAG12-IPT plants under illumination, but not in the dark, indicating that light-dependent reactions were responsible for the cell death. RT-PCR analysis showed that the pathogenesis-related (PR) genes PR-1b and PR-Q were strongly induced in old SAG12-IPT tobacco leaves with necrotic lesions. In addition, the ethylene-synthesis gene ACO was induced before lesions became visible in SAG12-IPT. It is proposed that over-reduction of the electron transport chain in combination with decreased electron consumption due to nutrient-deficiency led to oxidative stress, which, mediated by ethylene formation, can induce PR gene expression and hypersensitive cell death. Probably as a consequence of inefficient nutrient mobilization, flower development was prematurely aborted and reproduction thereby impaired in nutrient-deficient SAG12-IPT plants.

Key words: Apoptosis, cytokinin, defence, hypersensitive response, oxidative stress, programmed cell death, senescence, tobacco.

Introduction

The main function of leaf senescence is the recycling of nutrients, such as nitrogen, potassium, and phosphorus from the old leaves (Himelblau and Amasino, 2001). Leaf senescence can therefore be defined as a nutrient remobilization process that accompanies the decline in photosynthetic activity in ageing leaves. To allow efficient nutrient recycling, cellular integrity has to be maintained until late in the senescence process. Lipid peroxidation, for example, only occurs during the late stages of senescence (Berger et al., 2001). There has been some debate whether or not leaf senescence is a form of apoptosis or programmed cell death (Noodén et al., 1997; Buchanan-Wollaston et al., 2003; Thomas et al., 2003; Yoshida, 2003; van Doorn and Woltering, 2004). Whereas van Doorn and Woltering (2004) argue that senescence is part of the programme leading to cell death, Thomas et al. (2003) conclude that senescence is distinct from death-related processes. Experimental evidence supporting both views has been published. Although hallmarks of apoptosis, such as condensation of chromatin (Simeonova et al., 2000) and DNA laddering (Yen and Yang, 1998; Coupe et al., 2004) have been found in senescing leaves of some species, Lee and Chen (2002) found no indication of DNA laddering, condensation of...
nuclear material, fragmentation of nuclei, or formation of apoptotic bodies in senescing rice leaves. Furthermore, membrane integrity and cellular compartmentalization were maintained until late into the senescence programme, suggesting that leaf senescence is non-apoptotic (Lee and Chen, 2002). The capacity of leaves to re-green (Zavala-Manterra et al., 1999a, b) also demonstrates that senescing mesophyll cells remain functional and that cell death is not an inevitable consequence of senescence.

Instead of promoting cell death, senescence-dependent processes may play an important role in preventing premature death. Often leaves are still green when photosynthetic activity starts to decline (Stessman et al., 2002). During this stage, continued capture of light energy by chlorophyll, especially free chlorophyll released from pigment complexes, could result in premature cell death through the formation of reactive oxygen species (ROS). To prevent photo-oxidative stress, the photosynthetic apparatus has to be dismantled in an ordered manner. Remobilization of the nitrogen contained in photosynthetic proteins requires the co-ordination of several pathways for the breakdown of soluble proteins, thylakoid proteins, and pigments (Hörtensteiner and Feller, 2002; Thomas et al., 2002). Although the nitrogen contained in chlorophyll cannot be retrieved from the senescing leaf, chlorophyll degradation is required to prevent the accumulation of free chlorophyll and of toxic chlorophyll catabolites (Hörtensteiner, 2004). In addition, photoprotective mechanisms, including increased non-photochemical quenching (Wingler et al., 2004) and the adjustment of minor light-harvesting complexes (Humbeck and Krupinska, 2003), may play an important role in protecting senescing leaves against oxidative processes.

Transgenic plants with delayed senescence have been used to investigate the role of leaf senescence in photoprotection and nutrient remobilization. Delayed senescence can be achieved by over-expression of a gene for isopentenyl transferase (IPT) under the control of a senescence-specific promoter, resulting in the auto-regulated production of cytokinin (Gan and Amasino, 1995). Although the leaves of transgenic tobacco plants with delayed senescence stay green, photosynthetic activity does eventually decline (Wingler et al., 1998; Jordi et al., 2000). Interestingly, some transgenic Lolium multiflorum plants with delayed senescence developed spontaneous lesions (Li et al., 2004), while leaves of maize plants expressing the same construct progressed directly from fully green to bleached and desiccated without an intervening yellowing phase (Robson et al., 2004). These observations demonstrate that age-dependent cell death is not necessarily a consequence of senescence, but occurs independently of the senescence process. Furthermore, effects of delayed senescence on nutrient remobilization have been described. In transgenic tobacco with delayed senescence, altered patterns of nitrogen allocation from the old leaves led to reduced contents of protein and chlorophyll in the young leaves (Jordi et al., 2000). Similarly, the young, upper leaves of transgenic lettuce and maize plants with delayed senescence were paler than those of wild-type plants (McCabe et al., 2001; Robson et al., 2004).

Transgenic tobacco plants with delayed senescence (Gan and Amasino, 1995) were used here to analyse the mechanisms that lead to premature cell death. Chlorophyll fluorescence patterns and gene expression in plants grown under nutrient-limiting conditions suggest that increased excitation pressure and induction of defence pathways trigger cell death without prior senescence.

Materials and methods

Plant material and growth conditions

Wild-type tobacco (Nicotiana tabacum cv. Wisconsin) and P_{SAG12-IPT} plants (SAG12-IPT) with delayed senescence due to auto-regulated production of cytokinin (Gan and Amasino, 1995) were germinated on compost (Murphy’s Multi Purpose Compost, Murphy Garden Products, Ipswich, UK). After 4 weeks, the plants were transferred into 3.0 l pots containing a 1:1 v:v mixture of compost and sand (Horticultural Silver Sand, CEM-PAK, Dewsbury, UK) for low nutrient treatments (LN) or compost only for high nutrient treatments (HN). HN plants were fertilized weekly with a fertilizer solution containing N:P:K in a ratio of 14:10:27 (Plant Food, Photostrogen, Corwen, UK). The plants were kept in a glasshouse at a temperature of 20–24 °C under natural daylight. Additional illumination was provided for 14 h d^{-1} with 400 W metal-halide lamps (HQI-BT, Osram, München, Germany).

Incubation of leaf discs

Twelve weeks after transfer to the LN or HN conditions, leaf discs were cut from the 5th leaf (from the bottom). At this stage, the bottom leaves of wild-type plants grown under low nutrient supply had started to senesce, but senescence was not yet visible in the 5th leaf. The discs were floated on water and incubated in the dark or under illumination for 16 h d^{-1} at a photon flux density of 50 μmol m^{-2} s^{-1}. The temperature was 22 °C during the photoperiod and 18 °C at night.

Chlorophyll fluorescence analysis

Chlorophyll a fluorescence was analysed using a modulated fluorometer (FMS-2, Hansatech, King’s Lynn, UK). Minimum fluorescence (F_{0}) was measured by exposing dark-adapted leaves to modulated red light, before a saturating flash of white light was applied to record maximum fluorescence (F_{m}). Leaves were then illuminated with actinic light (290 μmol m^{-2} s^{-1}) and saturating flashes of 0.7 s duration were applied every 1.5 min. After 15 min of illumination, maximum fluorescence of light-adapted leaves (F_{m}), steady-state fluorescence (F_{s}) and minimum fluorescence (F_{0}) were recorded. The following equations were used for calculating photosynthetic parameters: Quantum efficiency of open photosystem II centres, F_{v}/F_{m}=(F_{m}-F_{0})/F_{m}; photochemical quenching, qP=(F_{m}-F_{s})/(F_{m}-F_{0}); non-photochemical quenching, NPQ=(F_{m}-F_{0})/F_{m}.

Chlorophyll fluorescence images were captured with a FluorCam 700MF imaging fluorometer (Photon Systems Instruments, Brno, Czech Republic) as described by Wingler et al. (2004). After determination of F_{s}/F_{m} in dark-adapted plants, the leaves were illuminated with actinic light (200 μmol m^{-2} s^{-1}) and saturating flashes of 0.8 s duration were applied every 2 min to determine NPQ.
Seventeen weeks after transfer to the LN conditions, total RNA was extracted following the protocol of Logemann et al. (1987) from leaves 11 (old) and 20 (young) from the bottom. As whole leaves were used, the material for the old SAG12-IPT leaves was a mixture of necrotic and green tissue. First-strand cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, Crawley, UK). Reverse transcription was initiated in the presence of oligo (dT) and random p(dN)₆ (Roche Diagnostics, Lewes, UK) primers (42°C, 1 h). The PCR was performed after heat inactivation of the reverse transcriptase at 95°C for 5 min. The PCR cycle profile consisted of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C. The following primer pairs were used: Chlorophyll a/b binding protein (CAP, accession number M29868) forward: 5'-GTTTGGAACTACTGGTAGATGAT-3', reverse: 5'-ATGTTGAACTAAGCTAAGGTCAGT-3'; ACC oxidase (ACO, accession number AB012857) forward: 5'-GAGCTAAGGTTAGCAACTATCCA-3', reverse: 5'-CTTTCTCAACTAAAGTTGG-3'; acidic chitinase (PR-Q, accession number M29868) forward: 5'-GTTTGGAACTACTGGTAGATGAT-3', reverse: 5'-ATGTTGAACTAAGCTAAGGTCAGT-3'; pathogenesis-related protein 1b (PR-1b, accession number X05453) forward: 5'-TGGATGCCCTATACACGGCT-3', reverse: 5'-CCCCCCCTTAATTAAGACCACT-3'; elongation factor 1-a (EF1α, accession number AF120093) forward: 5'-TCACTACAACTTGTGCTATGGTGG-3', reverse: 5'-TGATCTGTCAGGCTCAGAAG-3'. The PCR cycle number was 25 for CAP, PR-1b, PR-Q, and EF1α genes, and 30 for the ACO gene. No PCR products were detected in negative controls without reverse transcriptase.

Results

Cell death in plants with delayed senescence

In nutrient-deficient, but not in fertilized (not shown) SAG12-IPT plants, necrotic lesions became visible in the interveinal areas of the old leaves from about week 15 onwards (Fig. 1B). The necrosis was observed in all SAG12-IPT plants from two independent experiments, but only in the lower leaves. In some leaves, the necrotic lesions occurred without any visible yellowing. By contrast, wild-type leaves of the same position and age showed extensive yellowing before cell death occurred (Fig. 1A). Necrosis in the SAG12-IPT plants was accompanied by changes in chlorophyll fluorescence characteristics. Compared with the wild type (Fig. 1C, E, G), F₀ was increased in the necrotic regions (Fig. 1D), whereas Fₐ/Fₘ was reduced (Fig. 1F), indicating chronic photoinhibition. NPQ was higher than in wild-type leaves, especially in the green regions (Fig. 1H), suggesting increased dissipation of energy as heat.

To investigate what triggered premature cell death in the SAG12-IPT plants, chlorophyll fluorescence characteristics were analysed more quantitatively over the development of leaf 10 in plants grown at low nutrient supply (Fig. 2). Only the non-necrotic areas were analysed in order to record effects before the occurrence of cell death. The parameter 1-qP, correlating with a reduction of the photosystem II electron acceptor QA, increased with leaf age, both in wild-type and in SAG12-IPT plants (Fig. 2A). From week 13 onwards, values were higher in the plants with delayed senescence. This suggests that the electron transport chain was over-reduced, probably due to an increase in light capture relative to electron utilization. Furthermore, as already indicated by the fluorescence images (Fig. 1), NPQ was significantly higher in the SAG12-IPT leaves in weeks 15 and 17 (Fig. 2B).

Light-dependent induction of cell death

Fluorescence parameters indicate that cell death in the SAG12-IPT plants was triggered by an imbalance of light capture and energy utilization. To confirm that cell death was light-dependent, leaf discs taken before senescence or cell death became apparent were incubated in the dark or under illumination (Fig. 3). While no clear differences were found between wild-type and transgenic leaf discs from plants originally grown at high nutrient supply, differences became apparent in discs from plants grown at low nutrient supply. After 11 d in the dark, discs from wild-type plants showed clear senescence, as indicated by reduced Fₐ/Fₘ values (Fig. 3B) in combination with visible yellowing,
but no necrotic lesions (not shown). In the dark, discs of SAG12-IPT plants had higher $F_v/F_m$ values than wild-type discs and there was no indication of cell death. However, illuminated discs of nutrient-deficient SAG12-IPT plants showed the same symptoms of cell death that also occurred in planta, i.e. necrotic lesions (not shown) with increased $F_0$ (Fig. 3A) and decreased $F_v/F_m$ (Fig. 3B). In addition, NPQ was increased in the non-necrotic areas (Fig. 3C). These results show that cell death in the SAG12-IPT plants grown at low nutrient supply was light-dependent.

**Changes in gene expression**

Since the necrotic lesions found in nutrient-deficient SAG12-IPT tobacco resemble hypersensitive cell death, the expression of genes involved in pathogen response was studied (Fig. 4). The pathogenesis-related genes $PR-1b$ and $PR-Q$ were strongly induced in old leaves of the SAG12-IPT plants, but not in the old leaves of wild-type tobacco. Expression of photosynthetic genes, such as $CAB$, is typically down-regulated in senescing leaves. The finding that $CAB$ gene expression was higher in young than in old leaves was therefore expected. Surprisingly, expression of $CAB$ was lower in old leaves of the SAG12-IPT plants than in old leaves of the wild type. The reason for this could be that, in the case of SAG12-IPT, whole leaves containing necrotic and green tissue were analysed. $ACO$, an important gene in ethylene synthesis, was up-regulated in old compared with young leaves of wild-type tobacco, and also more strongly expressed in young SAG12-IPT leaves than in young wild-type leaves. Expression of the senescence-enhanced cysteine protease gene NTCP-23 (Ueda et al., 2000) was also determined to monitor the extent of senescence (results not shown). However, it was found that NTCP-23 was also strongly expressed in young leaf tissue and the extent of senescence could therefore not be assessed.

**Effect on fecundity**

Premature cell death could result in inefficient nutrient remobilization. To assess the effect on resource allocation to reproductive organs, flowering and fruit formation were determined. At high nutrient supply, delayed senescence did not affect flowering or fruit formation (Fig. 5). Nutrient
deficiency reduced flowering and fruit formation in wild-type plants and, more severely, in the transgenic plants with the delayed senescence. At low nutrient supply, production of buds was delayed in SAG12-IPT plants compared with the wild type (Fig. 5A), and fewer buds developed into flowers and fruits (Fig. 5B, C). Although the total number of buds, prematurely aborted buds, open flowers, and fruits was not reduced compared with the wild type at the final time point (Fig. 5D), this was mainly due to the presence of prematurely aborted buds (Fig. 6A) that did not develop into seed-containing fruits. As a consequence, fruit dry weight was significantly reduced in SAG12-IPT plants grown at low nutrient supply, but not at high nutrient supply (Fig. 6B). In addition to effects on fruit formation, SAG12-IPT plants grown at low nutrient supply were shorter than wild-type plants and had a lower photosynthetic activity and chlorophyll content in the young leaves (data not shown).

Discussion

The results presented here show that leaf senescence and cell death can be uncoupled in ageing leaves (Fig. 1). Changes in photosynthetic parameters (Figs 1, 2, 3) and in gene expression (Fig. 4) indicate that cell death in the
SAG12-IPT plants was due to light-dependent effects causing photo-oxidative stress and induction of defence pathways. Cellular integrity in wild-type leaves, on the other hand, was maintained until late in the senescence process, allowing efficient nutrient recycling for fruit formation under nutrient-limiting conditions (Figs 5, 6).

Changes in photosynthetic parameters underlying the light-dependent induction of cell death

Similar to the necrotic lesions described here, cell death has also been observed in other transgenic plants with autoregulated production of cytokinin (Li et al., 2004; Robson et al., 2004), but it does not occur under all growth conditions in the SAG12-IPT tobacco. Even under nutrient-limiting conditions, the extent of cell death varied between different experiments and it is not clear what the exact environmental factors are that determine this effect.

Nevertheless, this phenotype shows that senescence and cell death are independent processes.

Cell death in the SAG12-IPT plants is light-dependent (Fig. 3). The fact that this effect was only observed in nutrient-starved plants suggests that an imbalance between energy capture and consumption may be responsible. For example, cytokinin could alter the source–sink balance in the SAG12-IPT plants by inducing extracellular invertase (Balibrea Lara et al., 2004). Nitrogen deficiency under these conditions could lead to a decrease in Calvin cycle enzymes or reduced energy consumption for nitrogen assimilation. This hypothesis is supported by the analysis of photosynthetic parameters. Values for F0 values increased with leaf age in the SAG12-IPT plants, despite the continued presence of chlorophyll. This could result in over-reduction of the electron transport chain, as indicated by increased $1-qP$ values (Fig. 2), and in the production of ROS, which could trigger lipid peroxidation and cell death. Although $NPQ$ values increased with leaf age in the SAG12-IPT plants, energy dissipation may not have been sufficient to prevent cell death. Increased $F_0$ values (Figs 1, 3) indicate that free chlorophyll that is not bound in protein–pigment complexes may be present in the necrotic lesions.

The changes in fluorescence characteristics observed here resemble the effects found during a mastoparan-induced hypersensitive response (Allen et al., 1999). Mastoparan treatment results in an oxidative burst, an increase in non-photochemical quenching and a decrease in photochemical quenching. Allen et al. (1999) conclude that light-dependent electron transport can stimulate cell death by enhancing the oxidative burst during the hypersensitive response. This is in agreement with the light-dependent cell death in the SAG12-IPT tobacco (Fig. 3).

Despite the light-dependence of the cell death phenotype, it cannot be completely excluded that it may have been caused by an interaction of cytokinin accumulation with illumination. Recently, it has been shown that cytokinins can trigger apoptosis in cell cultures (Mlejnek and Procházka, 2002; Mlejnek et al., 2003; Carimi et al., 2003, 2004). However, in contrast to the effect observed in the SAG12-IPT plants, treatment of Arabidopsis plants with the synthetic cytokinin benzylaminopurine resulted in leaf yellowing and not in a stay-green phenotype (Carimi et al., 2003).

Interaction of delayed senescence with oxidative stress and defence pathways

The necrotic lesions in the SAG12-IPT tobacco resemble symptoms of a pathogen-induced hypersensitive response or ozone-induced cell death. However, in contrast to the
large necrotic lesions in the interveinal regions described here, ozone stress initially becomes visible as small necrotic spots or lesions near the mid vein (Dutilleul et al., 2003; Pourtau et al., 2003). Rather than being caused by the uptake of external ozone, oxidative stress in the SAG12-IPT tobacco was, therefore, probably caused by photo-oxidative processes within the mesophyll cells. Overall, the symptoms observed in the SAG12-IPT tobacco resemble the phenotype described for the acd1 mutant of Arabidopsis (Greenberg and Ausubel, 1993), which lacks an important enzyme in chlorophyll catabolism, pheophorbide a oxygenase (Hörtensteiner, 2004). Similar to the symptoms described here, senescence or pathogen treatment results in necrotic lesions without visible chlorosis in acd1 (Greenberg and Ausubel, 1993).

Interactions of delayed chlorophyll degradation with activation of defence pathways are supported by induction of PR-1b and PR-Q gene expression in old leaves from the SAG12-IPT tobacco (Fig. 4). Increased expression of PR genes and proteins has also been reported for plants expressing IPT under the control of the Rubisco small subunit promoter (Synková et al., 2004) or in the fruits (Martineau et al., 1994). Enhanced age-dependent sugar accumulation in the transgenic tobacco (Wingler et al., 1998) could be responsible for the induction of PR-1b and PR-Q (Herbers et al., 1995). In addition, the induction of PR-1b could result from the formation of ROS (Ernst et al., 1992; Green and Fluur, 1995). As PR-1b is ethylene-inducible (Xu et al., 1994) and ethylene is involved in the ozone-dependent induction of PR-1b (Grimmig et al., 2003), there could also be a link between oxidative stress, increased expression of the ethylene synthesis gene ACO in the young leaves of the SAG12-IPT tobacco and induction of PR-1b. Ethylene has also been shown to induce lipoxigenase (Griffiths et al., 1999), which, in turn, could lead to hypersensitive cell death. The observation that neither PR-1b or PR-Q were induced in senescing wild-type tobacco was surprising, since Arabidopsis PR genes are induced during leaf senescence (Hanfrey et al., 1996; Robatzek and Somssich, 2001).

It has been reported previously that the pathways involved in senescence, defense, and oxidative stress overlap. However, it is still not clear whether production of ROS is required to trigger leaf senescence. Dertinger et al. (2003) have shown that the normal senescence-dependent decline in glutathione reductase, superoxide dismutase, and ascorbate peroxidase is delayed in SAG12-IPT tobacco. They conclude that the decline in anti-oxidative activities is a consequence and not the cause of senescence. While some SAGs are induced by oxidative stress (Navabpour et al., 2003) others, such as SAG12, are not stress-inducible (Miller et al., 1999). Nevertheless, salicylic acid, a signaling molecule in oxidative stress and pathogen defence, is required for the induction of SAG12 (Morris et al., 2000): reduced necrosis and reduced expression of SAG12 in a

**Impact on resource allocation**

The importance of senescence-dependent nutrient recycling to young leaves has been described before (Jordi et al., 2000; McCabe et al., 2001; Robson et al., 2004). In the experiments described here, delayed senescence under nutrient-limiting conditions did not only result in reduced photosynthetic activity in the young leaves (data not shown), but also led to premature abortion of bud development without fruit production (Figs 5, 6). Similarly, it has been shown that leaf senescence is related to fruit production in different Arabidopsis ecotypes (Levey and Wingler, 2005). However, the results also show that the importance of leaf senescence for fecundity is largely dependent on nutrient availability. If nutrient supply is not limiting, extended photosynthetic activity due to delayed senescence increases carbon availability and could thereby even result in higher seed production as, for example, shown for stay-green mutants of durum wheat (Spano et al., 2003) and also in the SAG12-IPT tobacco (Gan and Asamino, 1995).

Using a SAG12-GUS construct, Grbić (2002) has shown that the SAG12 promoter is active in senescing flowers. Therefore it cannot be excluded that, in addition to delayed leaf senescence, cytokinin production in the flowers affects fruit development in the SAG12-IPT tobacco. However, production of cytokinin in the flowers of petunia transformed with the SAG12-IPT construct resulted in delayed floral senescence rather than abortion of bud development (Chang et al., 2003). The phenotype observed here is also different from the effects of auto-regulated production of cytokinin described for Nicotiana alata (Schroeder et al., 2001). In N. alata, expression of the SAG12-IPT construct resulted in a reduced number of flowers per branch, but also in increased branching of the inflorescence. As a consequence, the total number of flowers was slightly increased and not reduced (Schroeder et al., 2001). It is therefore likely that the phenotype observed here is due to limited nutrient availability for flower and fruit development and not to the accumulation of cytokinin.

In summary, the results suggest that, under nutrient-limiting conditions, delayed leaf senescence in SAG12-IPT plants can lead to premature cell death in a process resembling the hypersensitive response during pathogen infection. Changes in photosynthetic parameters in the SAG12-IPT plants indicate that over-reduction of the electron transport chain, probably resulting in oxidative stress, was primarily responsible for the light-dependent induction of cell death (Fig. 7). Premature cell death could impair nutrient recycling and thereby growth and fecundity. By contrast, ordered degradation of the photosynthetic apparatus in wild-type plants ensures that cell death only occurs after nutrients have been recycled.
Fig. 7. Scheme showing senescence-dependent and senescence-independent pathways leading to cell death.

**Acknowledgements**

We would like to thank Richard Amasino (University of Wisconsin) for providing the plants. Work on this project was supported by a BBSRC grant (31/P16341).

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