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ORIGINAL ARTICLE

Heat shock transcription factors involved in seed desiccation tolerance and longevity retard vegetative senescence in transgenic tobacco

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Abstract

Main conclusion **Transcription factors normally expressed in sunflower seeds delayed vegetative senescence induced by severe stress in transgenic tobacco. This revealed a novel connection between seed heat shock factors, desiccation tolerance and vegetative longevity.**

HaHSFA9 and HaHSFA4a coactivate a genetic program that, in sunflower (*Helianthus annuus* L.), contributes to seed longevity and desiccation tolerance. We have shown that overexpression of HaHSFA9 in transgenic tobacco seedlings resulted in tolerance to drastic dehydration and oxidative stress. Overexpression of HaHSFA9 alone was linked to a remarkable protection of the photosynthetic apparatus. In addition, the combined overexpression of HaHSFA9 and HaHSFA4a enhanced all these stress-resistance phenotypes. Here, we find that HaHSFA9 confers protection against damage induced by different stress conditions that accelerate vegetative senescence during different stages of development. Seedlings and plants that overexpress HaHSFA9 survived lethal treatments of dark-induced senescence. HaHSFA9 overexpression induced resistance to effects of culture under darkness for several weeks. Only some homoiochlorophyllous resurrection plants are able to withstand this experimental severe stress condition. The combined overexpression of HaHSFA9 and HaHSFA4a did not result in further slowing of dark-induced seedling senescence. However, combined expression of the two transcription factors caused improved recovery of the photosynthetic organs of seedlings after lethal dark treatments. At later stages of vegetative development, HaHSFA9 delayed the appearance of senescence symptoms in leaves of plants grown under normal illumination. This delay was observed under either control or stress treatments. Thus, HaHSFA9 delayed both natural and stress-induced leaf senesce. These novel observations connect transcription factors involved in desiccation tolerance with leaf longevity.

Keywords Drastic stress tolerance · Heat shock factors · Leaf senescence · Seed longevity · Transgenic tobacco

Abbreviations

HSF	Heat shock factor
HSP	Heat shock proteins
sHSP	Small heat shock proteins
sHSP-P	Small heat shock protein (plastidial)
sHSP-CI	Small heat shock protein (cytosolic, Class I)
NT	Non-transgenic

Introduction

Drought stress is the single environmental factor that, worldwide, probably has the strongest negative influence on crop plant productivity (Chaves et al. 2002). Severe

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drought and heat wave events are increasing in frequency and intensity as a result of global climate change, and intensifying stress conditions are predicted to impair crop productivity in sustainable agriculture (Ahuja et al. 2010; Lobell and Gourdji 2012). Engineering transgenic plants with enhanced tolerance to stress factors such as elevated temperature, solar irradiation and drought, will be thus crucial in the immediate future. To achieve such goals, the analysis and manipulation of relevant genetic programs involved in stress tolerance is required. Knowledge obtained from seeds and resurrection plants could be particularly useful in understanding how the intensifying stress (that limits crop productivity) affects one of its major targets: the photosynthetic apparatus and, within it, photosystem II (PSII) (see Chaves et al. 2002; Munné-Bosch and Alegre 2002; Flexas et al. 2006; Takahashi and Badger 2011).

In plants the heat-shock response and some developmental processes are mainly controlled by a gene family of transcription factors known as the heat-shock transcription factors (HSFs). Plants contain the highest number of HSFs in eukaryotes. The potentially higher specialization of plant HSFs, compared with animal systems, is largely unexplored (reviewed by Scharf et al. 2012). There has been little functional analysis of HSFs from plants other than Arabidopsis. Our group has characterized HSFs that, in embryos of sunflower (*Helianthus annuus* L.), activate a genetic program that contributes to longevity, thermotolerance, and desiccation tolerance of seeds. HSFA9 (A9; *Helianthus annuus* Heat Shock Factor A9) is a unique HSF that, in sunflower (Almoguera et al. 2002) and Arabidopsis (Kotak et al. 2007), has been found to be expressed only in seeds. Monocot plants do not encode HSFA9 factors (Scharf et al. 2012). Gain of function as a consequence of overexpression of HaHSFA9 in seeds only, demonstrated the involvement of this transcription factor in seed longevity. Thus, the DS10:A9 seeds resisted accelerated aging (Prieto-Dapena et al. 2006). We have also shown that ectopic overexpression of A9 (in transgenic 35S:A9 tobacco plants) conferred resistance of vegetative tissues in young seedlings to severe dehydration (water potentials of ≈ -40 MPa; Prieto-Dapena et al. 2008). The tolerated stress conditions exceeded what "normal" or even "engineered" plants are able to withstand and resembled (though without fully reaching) the stress levels tolerated by resurrection plants (Prieto-Dapena et al. 2008). In DS10:A9 seeds and 35S:A9 plants, A9 activated a genetic program that includes subsets of genes encoding Heat Shock Proteins (HSP) normally expressed during zygotic embryogenesis in seeds. The HSP in this program—the "A9-program"—include HSP101 and multiple small HSP (sHSP), mostly a subset of cytosolic proteins of classes CI and CII (sHSP-CI, sHSP-CII). Plastidial sHSP (sHSP-P), but not late embryogenesis abundant (LEA) seed proteins, are also included in the same program (Almoguera et al. 2012). In other plants different from sunflower, developmental accumulation of seed HSP also occurs (for example in Arabidopsis, see Wehmeyer et al. 1996). We note that demonstration by gain-of-function in transgenic plants of enhanced desiccation tolerance and seed longevity is not available for HSFA9 different from HaHSFA9 (sunflower HSFA9). However, in Arabidopsis HSFA9 is activated by ABI3 (a key regulator of desiccation tolerance, see Kotak et al. 2007). Furthermore, in *Medicago truncatula* a regulatory network that includes ABI3 and HSFA9 has been linked to seed desiccation tolerance and longevity (Verdier et al. 2013). HSFA9 connects the desiccation tolerance and longevity modules in this network (Verdier et al. 2013).

Loss-of-function of the "A9-program" has also been reported from studies that used different modified forms of A9 specifically overexpressed in tobacco seeds. Transcription-inactive forms were found to be inefficient when compared to an active repressor form (A9-SRDX); using A9-SRDX only, specific, negative effects on HSP accumulation and a significant reduction of seed longevity were observed (Tejedor-Cano et al. 2010). The A9-SRDX seeds tolerated desiccation; this indicated redundancy of the A9-program with other embryonic programs of desiccation tolerance, for example with the programs that, in seeds and resurrection plants, include genes that encode LEA proteins (Illing et al. 2005; Boudet et al. 2006; Farrant and Moore 2011). The diminished longevity of A9-SRDX seeds indicated that HSFA9 is not the sole Class A HSF involved in transcriptional activation of the "A9-program" in developing seeds (Tejedor-Cano et al. 2010). Subsequently, HaHSFA4a—sunflower Heat Shock Factor (A4a)—was identified as one such accessory HSF (Tejedor-Cano et al. 2014). A9 and A4a show an unusual repression by the auxin/Indole-3-acetic acid (Aux/IAA) protein HaIAA27 (Carranco et al. 2010; Tejedor-Cano et al. 2014). A9 and A4a activate the same genetic program involving specific sHSP target genes. This has been confirmed by observing enhanced seed longevity and associated gene expression changes in DS10:A4a and DS10:A9/A4a transgenic tobacco lines, as compared to their respective sibling control lines (syngenic, single-transgenic). Similar analysis with 35S:A4a and 35S:A9/A4a lines revealed enhanced tolerance to vegetative severe dehydration and oxidative stress in young transgenic seedlings. Furthermore this also showed that A4a strictly requires A9 to cause the enhanced stress resistance (Personat et al. 2014).

The photosynthetic apparatus in young 35S:A9 seedlings showed an unusual resistance to very harsh conditions of dehydration and oxidative stress. Based in these results, we proposed that HaHSFA9 might protect seed non-

photosynthetic plastids and pro-plastids from developmental desiccation (Almoguera et al. 2012). The observed effects were quite complex, as they involved protection of diverse components of the two photosystems (PSII and PSI), the cycling of the labile D1 protein of PSII, and additional effects on the photosynthetic machinery that are not yet fully explained (Almoguera et al. 2012). The observed resistance to drastic stress of PSII, PSI, and of specific components of the two photosystems was comparable only to that described for resurrection plants (Almoguera et al. 2012; Dinakar et al. 2012). Resurrection plants tolerate desiccation in a way that is unlike normal plants but similar to most seeds (the orthodox seeds) of normal plants (Cushman and Oliver 2011; Gechev et al. 2012). It has been proposed that tolerance to vegetative desiccation in vascular resurrection plants has evolved at different times by changing the regulation of genes that in normal plants are active only in seeds (Oliver et al. 2000; Farrant and Moore 2011; Gaff and Oliver 2013).

It has been suggested that senescence might reduce crop yield when it is induced prematurely under adverse environmental conditions (Gregersen et al. 2013). Leaf senescence is a complex degenerative process that involves programed cell death regulation. Senescence is affected by multiple developmental signals that lead to the degradation of chlorophyll, protein, lipids, nucleic acids, and to the dismantling of chloroplasts and other cellular organelles; chloroplasts are the first organelles to be disorganized, while the nucleus and mitochondria remain active for longer (Lim et al. 2007). Leaf yellowing and death occur as the final stages of age-dependent senescence (Buchanan-Wollaston et al. 2003). Multiple environmental signals, including the signals induced by drought and darkness stress, also affect leaf senescence (Lim et al. 2007). The different stress signals appear to accelerate the same genetic program of developmental senescence. However, stress-induced senescence has been found to involve similar (but not exactly the same) molecular and cellular changes as for age-related senescence (Buchanan-Wollaston et al. 2005; Guo and Gan 2012). Delayed leaf senescence as a consequence of stay-green genotypes has been reported in many plants, including several crop species (Hörtensteiner 2009). Stay-green mutant genotypes can be assigned to two principal categories: functional and cosmetic. Functional stay-green plants retain chlorophyll and photosynthetic activity during leaf senescence, while cosmetic stay-green plants maintain only their green color due to a decrease in chlorophyll degradation (Hörtensteiner 2009). Functional stay-green genotypes have been shown to induce complex effects, for example resulting in protection of the PSII and causing regeneration of chloroplast ultrastructure (Luo et al. 2013).

Resurrection plants include species that either maintain (homoiochlorophyllous) or degrade (poikilochlorophyllous) chlorophyll and the photosynthetic apparatus during dehydration. Interestingly, only homoiochlorophyllous resurrection plants are able to avoid leaf senescence. The majority of their leaves do not senesce when the relative water content has declined, suggesting that stress-induced leaf senescence is inhibited in these resurrection plants (Griffiths et al. 2014). Furthermore, homoiochlorophyllous resurrection plants from the Gesneriaceae family can withstand dark treatments for very long periods that, in normal plants, would induce leaf senescence and death (Denev et al. 2012). Indeed, genes from homoiochlorophyllous resurrection plants were found to delay leaf senescence when overexpressed in normal plants (Islam et al. 2013). Furthermore, even in normal plants, resistance to drought and other stress conditions is linked to the delay of vegetative senescence; this is consistent with observations in engineered plants (Rivero et al. 2007) and stay green genotypes (Gregersen et al. 2013; Borrell et al. 2014). In particular, resistance of the photosynthetic apparatus to oxidative stress seems to be crucial to delay leaf senescence (Abbasi et al. 2009; Wu et al. 2012). These precedents led us to investigate whether 35S:A9 tobacco resists lethal conditions of dark-induced senescence. In addition, resistance to more moderate stress conditions that cause vegetative senescence was also investigated. We found that the overexpression of A9 caused persistent effects in plants beyond the young seedling stage. A9 induced resistance to prolonged dark stress and a delay in natural and stress-induced vegetative senescence; this newly observed stress-resistance was well beyond what can be withstood by normal plants. These effects occurred in parallel with ectopic accumulation of seed HSPs, including sHSP-P. Our results have revealed a novel connection between seed HSF and leaf longevity, which would further support the suggested roles of the A9-program in connection with the developing photosynthetic apparatus. We discuss these results further in the context of seed HSP accumulation and the evolution of vegetative desiccation tolerance.

Materials and methods

Plant material

We used three different pairs of single-transgenic (35S:A9) and sibling non-transgenic (NT, syngenic) lines. The previously described tobacco (*Nicotiana tabacum* L.) T_1/NT_1, T_2/NT_2, and T_3/NT_3 pairs (Almoguera et al. 2012) are named here as $A9_1/NT_1$, $A9_2/NT_2$, and $A9_3/NT_3$ to distinguish them from other lines used in the experiments of Fig. 4. We recently described (Personat et al. 2014) the

double-transgenic 35S:A9/A4a and, sibling, 35S:A9 lines used in Fig. 4: 35S:$A9_1$/$A4a_1$, 35S:$A9_1$; 35S:$A9_1$/$A4a_2$, 35S:$A9_1$; 35S:$A9_2$/A4a, 35S:$A9_2$, and 35S:$A9_3$/A4a, 35S:$A9_3$. In these double-transgenic lines $A9_1$, $A9_2$ and $A9_3$ denote the same A9-integration events present in the single-transgenic (35S:A9) homozygous lines (Almoguera et al. 2012; Personat et al. 2014).

Culture conditions and senescence assays

For the dark-induced senescence assays (Figs. 1, 3), seedlings were first grown for 3–4 weeks under photoperiod on filter paper (50 g/m^2) placed on MS medium in Petri dishes. Culture conditions consisted of a 16-h photoperiod, 25 °C day temperature, 20 °C night temperature and light intensity of 100 µmol m^{-2} s^{-1} within SANYO MLR-350H (Panasonic, Osaka, Japan) growth cabinets (Prieto-Dapena et al. 2008; Almoguera et al. 2012). Filter paper disks (with the 3 to 4-week old seedlings) were then transferred to new Petri dishes, each containing 4 filter paper disks (73 g/m^2) wetted with 4 mL MilliQ (Merck-Millipore, Billerica, MA, USA) water. Two additional filter paper disks (73 g/m^2) wetted with 2 mL MilliQ water were placed on top of the seedlings. The Petri dishes were sealed with Parafilm"M"® (Bemis Inc., Sheboygan Falls, WI, USA), wrapped in aluminum foil, and placed in the dark at 24 °C within SANYO MLR-350H growth cabinets for 1–5 weeks. Pigment content and the value of variable to maximal fluorescence (F_v/F_m, see below) was determined immediately before dark treatment and at the indicated dark treatment times using the seedlings of one Petri dish per experimental case and time: F_v/F_m values were obtained

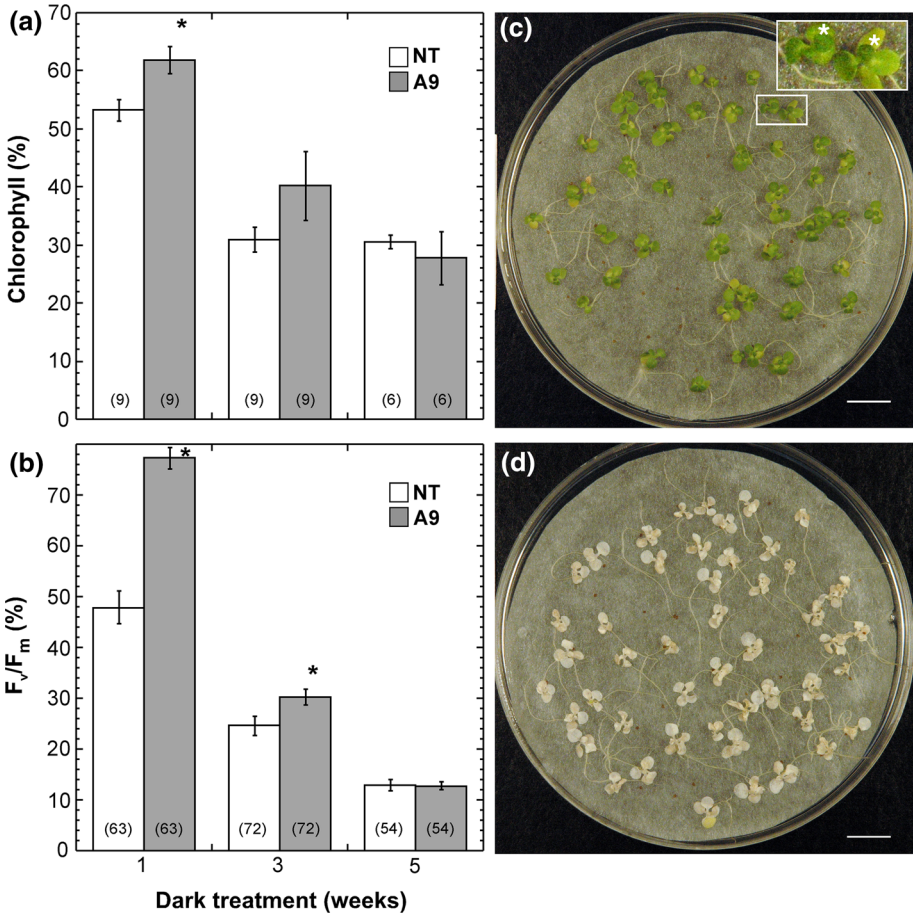

Fig. 1 Resistance to lethal conditions of dark-induced senescence in the 35S:A9 seedlings. The transgenic (*A9*) and sibling non-transgenic (*NT*) tobacco seedlings (3–4 weeks old) were cultured under darkness for the indicated time in Petri dishes. Total chlorophyll content (**a**) and the maximum yield of PSII (F_v/F_m, **b**) was quantified as the % of the initial values determined for each seedling type just before the beginning of the dark treatments. These values were: NT chlorophyll, 630.9 ± 20.9 µg/g FW; A9 chlorophyll, 773.9 ± 23.8 µg/g FW; NT F_v/F_m, 0.681 ± 0.005; A9 F_v/F_m, 0.777 ± 0.003. The 35S:A9 seedlings (**c**), but not the sibling NT seedlings (**d**) survived 3 weeks under darkness. The *inset* in **c** shows new leaves (marked with *asterisks*) that developed after seedling re-illumination. The photographs were taken 10 days after recovery under illumination. Other *asterisks* denote statistically significant difference (see Suppl. Table S1). *Numbers in brackets* denote sample size. *Scale bars* 1 cm. Represented data are for four independent experiments performed with seedlings from two to three different syngenic NT–A9 line pairs

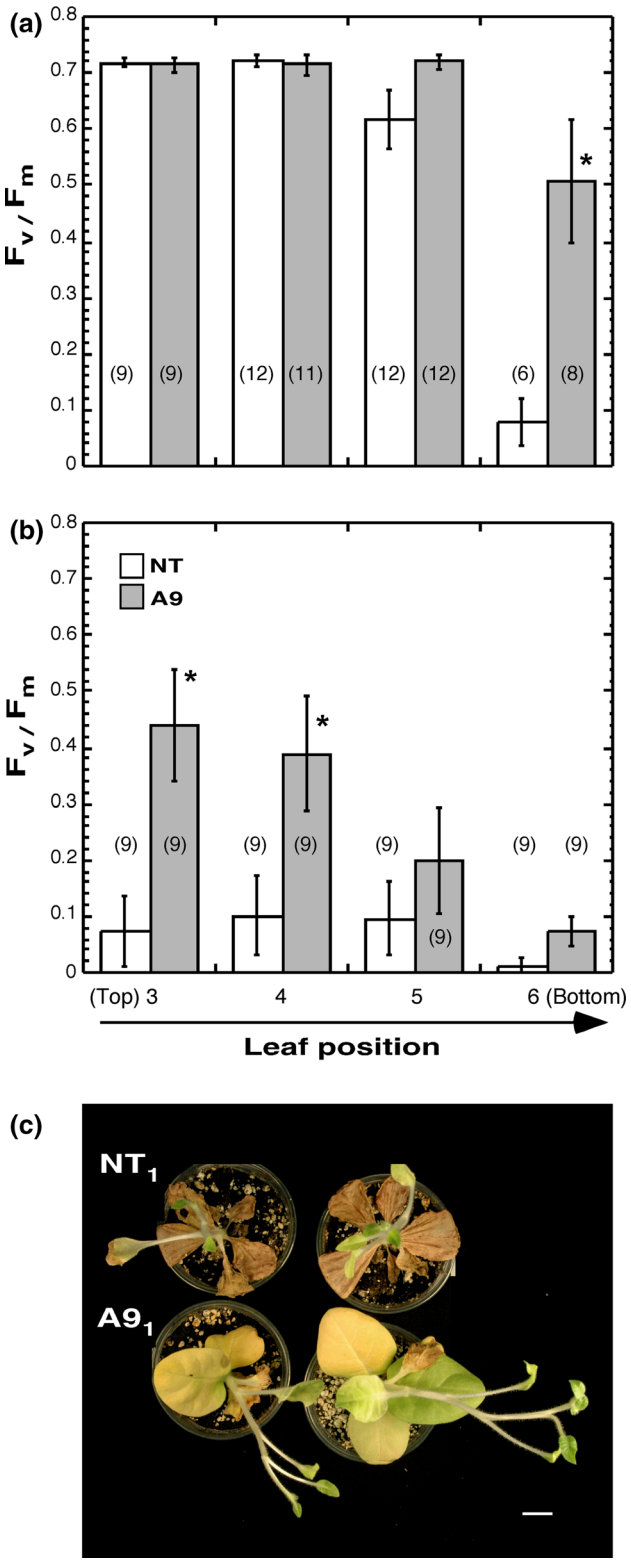

Fig. 2 The enhanced resistance to dark-induced senescence persists in 35S:A9 plants. Transgenic 35S:A9 (*A9*) and sibling non-transgenic (*NT*) plants grown on soil under illumination were transferred to complete darkness for 22 days (**a**) or 27 days (**b**). The F_v/F_m was determined in different leaves numbered according their position from the *top* to the *bottom* of the plant (the leaves closer to the top are younger). Data shown is for two independent experiments performed with plants representing three different syngenic NT–A9 lines pairs, using at least four plants per class and condition. Representative photographs illustrating whole plant survival after 27 days under darkness are presented for the $A9_1$–NT_1 line pair (**c**)

from 9 seedlings and the pigments (chlorophyll and carotenoid) extracted from 5 seedlings in 1 mL 80 % (v/v) acetone. The remaining seedlings (approximately 50 per dish) were used for the survival assays performed after the dark treatments. The filter paper disk with these seedlings was placed on MS medium in Petri dishes under photoperiod and photographed ten days after. To assess senescence under illumination in the experiments of Fig. 4, no filter paper disks were placed on top after transferring the seedlings to new dishes, and a photoperiod light intensity of 170 µmol m^{-2} s^{-1} was used for 6 extra days. In the experiments of Figs. 1, 3 and 4, percent survival reflects seedlings that regreened and/or resumed growth after the senescence-inducing treatments.

The plants used for the experiments of Figs. 2, 5, 6 and 7 were first grown for 3–4 weeks on Florabella® (Klasmann-Deilmann, Geeste, Germany) potting soil mixed with perlite (4:1). Thereafter, individual plants were transplanted to 200 mL pots filled with the same potting soil (Figs. 2, 7) or with vermiculite (Figs. 5, 6). Depending on the treatment, and as indicated in the text elsewhere, the pots were kept soaked at full capacity with either MilliQ water, or with the nutrient solution described by Arteca and Arteca (2000), used at 1/4 dilution. Senescence was induced under complete darkness using plants cultured for up to 8 weeks within a growth chamber at 24 °C. Alternatively, senescence was induced under illumination by prolonged culture with and without nutrient deprivation. The illumination and temperature conditions consisted of light intensity of 170 µmol m^{-2} s^{-1} (with 16-h photoperiod), and 25 °C day/20 °C night temperatures.

Photosynthetic pigment quantification and chlorophyll fluorescence determination

Total chlorophyll and carotenoid content was determined by UV–Vis spectroscopy; 80 % acetone-extracts of plant material were used for pigment quantification. Measurements at 470, 647 and 663 nm were performed and quantified as in Lichtenthaler and Buschmann (2001). Chlorophyll fluorescence was measured using a mini-PAM (Photosynthesis Yield Analyzer; Heinz Walz, Effeltrich, Germany). The procedures for F_v/F_m determination were described in detail by Almoguera et al. (2012).

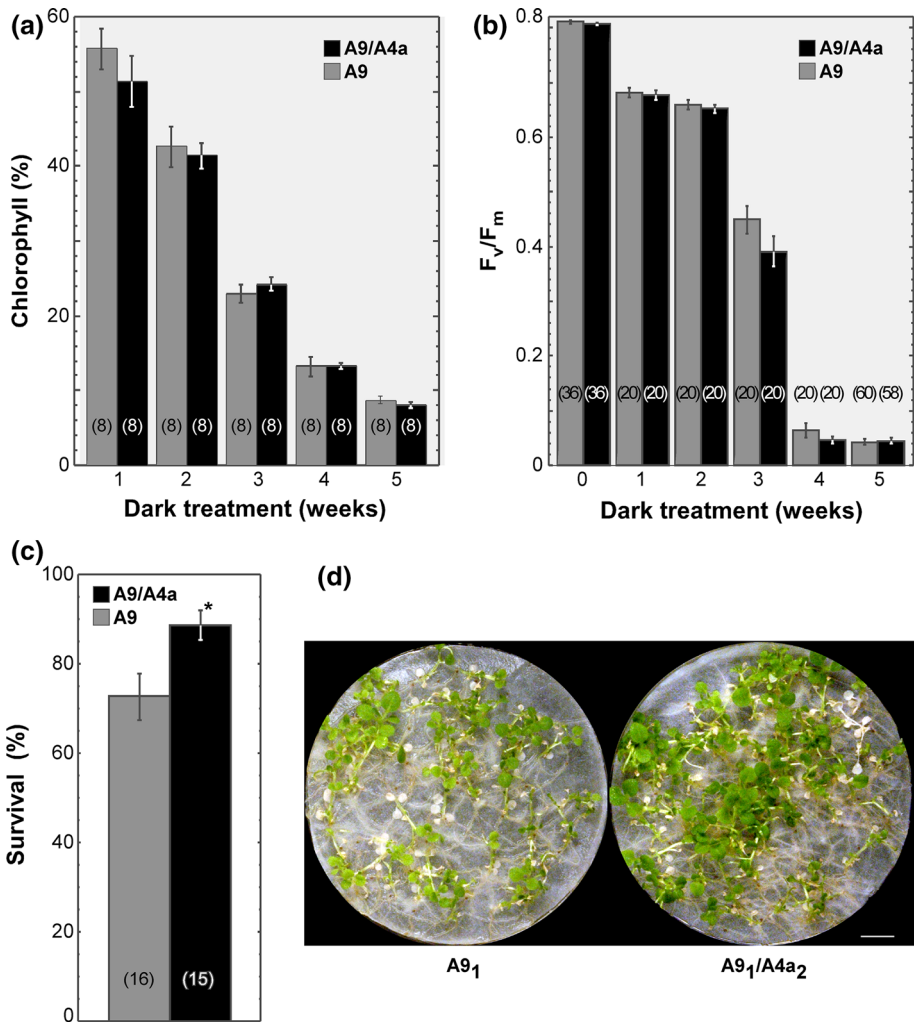

Fig. 3 Co-overexpression of HaHSFA9 and HaHSFA4a in the 35S:A9/A4a seedlings improves recovery from damage inflicted by prolonged darkness. Double transgenic (*A9/A4a*) and sibling single-transgenic (*A9*) seedlings were transferred to darkness for the indicated times. After the treatments, the % of total chlorophyll maintained by the seedlings (**a**) and the F_v/F_m (**b**) was quantified. Whole seedling survival was determined following the 5-week dark treatment and a subsequent transfer to photoperiod illumination for 10 additional days (**c**). Data shown is for three independent experiments performed with seedlings from four different A9–A9/A4a line pairs. Representative photographs of seedling damage recovery and survival are presented for the $A9_1$–$A9_1/A4a_2$ sibling seedlings (**d**). *Scale bar* 1 cm

Protein assays

The equivalent fresh weight (FW) protein extracts used in Fig. 6 were analyzed using Coomassie-blue stained 12.5 % (w/v) polyacrylamide SDS gels essentially as described by Zavaleta-Mancera et al. (1999). Extracts were prepared from 100 mg FW plant material using 1 mL 2× Laemmli buffer per sample. Gels were loaded with 20 µL (Fig. 6a) or 5 µL (Fig. 6b) extract per lane. In Fig. 6b, D1 accumulation was detected using the PsbA anti-C-terminal AS05 084 antibody (Agrisera AB, Vännäs, Sweden) at 1/6000 dilution (Almoguera et al. 2012). The western assays in Fig. 7 were performed using the same gel system as in Fig. 6, and 15 µg of total protein per lane (also in 2× Laemmli buffer). Detection with specific antibodies against sHSP-CI was as described by Prieto-Dapena et al. (2006). The accumulation of sHSP-P was detected with anti-AtHSP21 (AS08 285) from Agrisera, used at 1/3000 dilution. HSP101 was detected with the anti-ClpB (HSP101) N-terminal antibody (AS07 253) from Agrisera, used at 1/20,000 dilution.

Statistical analyses

ANOVA was used in experiments where data showed normal distribution or could be normalized by logarithmic transformation (Fig. 4, Suppl. Fig. S2). Alternatively, *t* Student tests were used when data could not be normalized (Figs. 1, 2, 3, 5 and Suppl. Figs. S1, S3 and S4); see the summarized data in Suppl. Table S1. We averaged the data for the different pairs of analogous sibling, NT and A9 lines. The statistical analysis of differences between the averaged data was consistent with results obtained when the differences were separately analyzed for each sibling line pair. Detailed procedures for these statistical analyses have been described in our former publications (Prieto-Dapena et al. 2006; Personat et al. 2014).

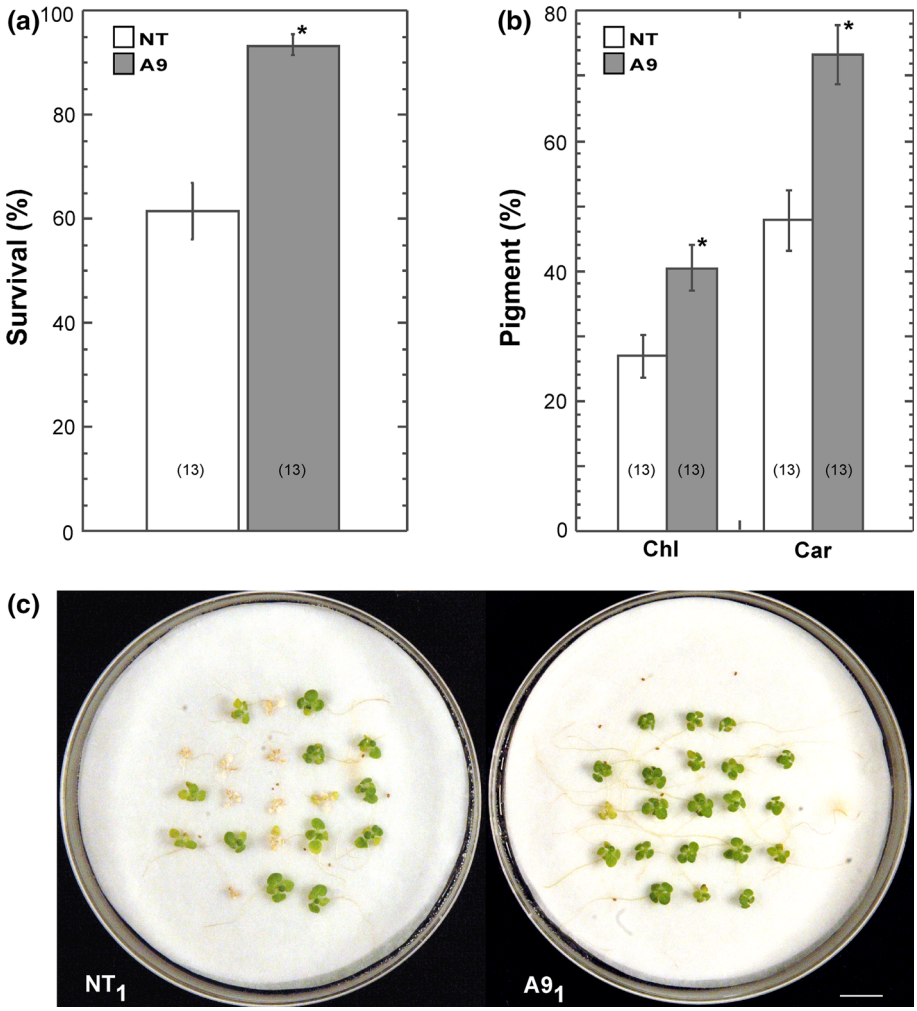

Fig. 4 The 35S:A9 seedlings also show delayed senescence symptoms under illumination. Young seedlings (3–4 weeks old) cultured under illumination on MS medium on filter paper were transferred to moist filter paper without MS medium. After six additional days under illumination, whole seedling survival (**a**) and total chlorophyll (*Chl*) and carotenoid (*Car*) was quantified (**b**). The % pigment content with respect to their initial values was determined (**b**). Data for two independent experiments performed with seedlings representing two different syngenic NT-A9 lines pairs are included. **c** Photographs with representative seedling survival for the NT_1–$A9_1$ sibling line pair are shown. *Scale bar* 1 cm

Results

The 35S:A9 seedlings and plants show resistance to lethal conditions of dark-induced senescence

We first analyzed the extent to which 35S:A9 seedlings could withstand prolonged dark treatments that induce senescence of the photosynthetic organs. Senescence induced under such conditions does not represent a physiologically relevant situation for normal plants. However, these dark treatments quickly induce senescence symptoms that are similar to those observed in natural leaf senescence or for senescence induced after other stress treatments (Lim et al. 2007; see additional results below). Figure 1 summarizes results of experiments performed with 4-week-old seedlings. Three different pairs of sibling transgenic (35S:A9) and non-transgenic (NT) lines were analyzed in separate experiments. It was observed that after 1 week of dark treatment the total chlorophyll content of the seedlings decreased, although to a lower level in the NT than in the 35S:A9 seedlings: to 53.2 ± 1.8 and 61.9 ± 2.3 % of the initial content, respectively (Fig. 1a). Chlorophyll degradation is the first visible symptom of leaf senescence (Buchanan-Wollaston et al. 2003). Thus, the lower decrease of chlorophyll content in the 35S:A9 seedlings indicates that A9 alleviates vegetative senescence. After 3 weeks of dark treatment the A9 effect on senescence was still evident as the chlorophyll content of the 35S:A9 seedlings was reduced to 40.2 ± 5.9 % compared with a reduction to 30.9 ± 2.1 % in the NT seedlings. However, the A9 effect on senescence was not observed after 5 weeks of dark treatment. At this time a similar drastic reduction of the chlorophyll content (to less than 30 % of the initial content) was observed for both the NT and the 35S:A9 seedlings (Fig. 1a). Supplementary Fig. S1 includes additional information on how the dark treatment differentially affected the chlorophyll a, chlorophyll b and total carotenoid content in the A9 and sibling NT seedlings. As for all figures in the manuscript, statistically significant differences are indicated by asterisks. Supplementary Table

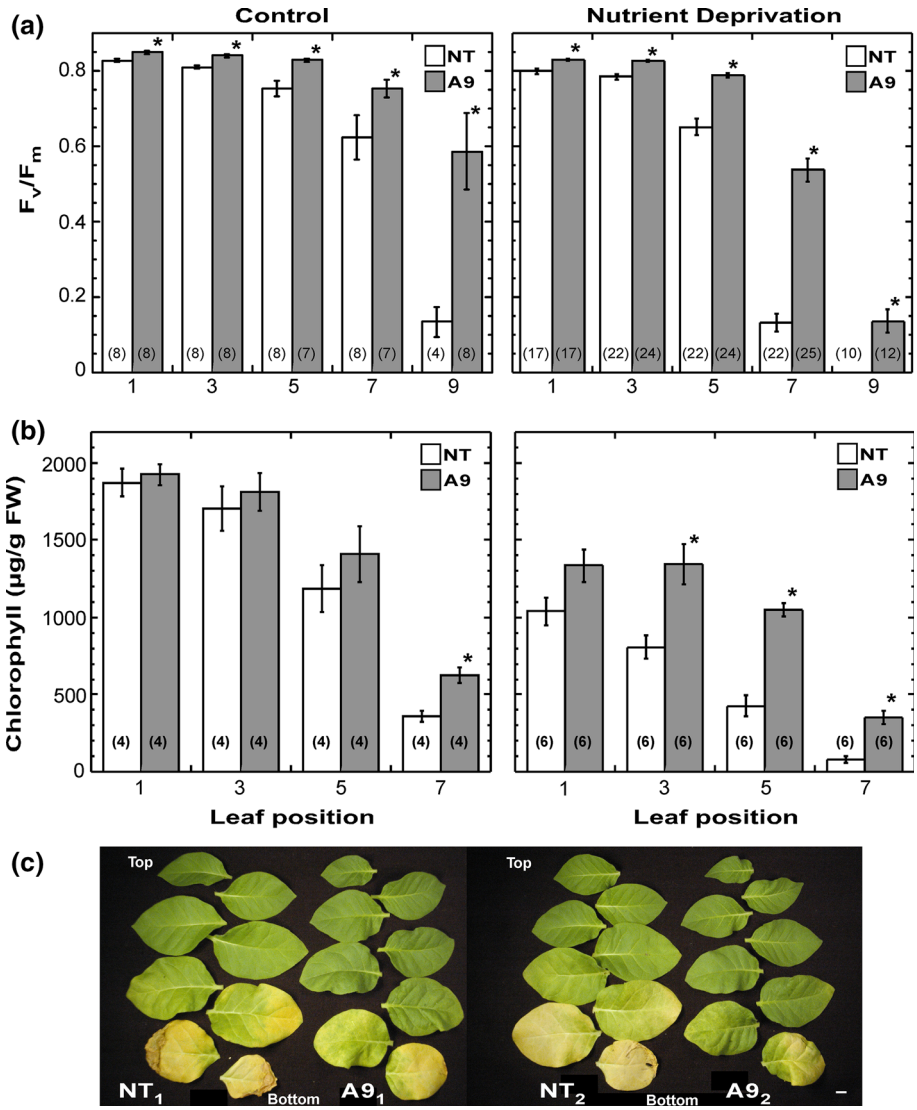

Fig. 5 Delayed natural and stress-induced leaf senescence in the 35S:A9 plants. Sibling 35S:A9 (*A9*) and non-transgenic (*NT*) plants were grown on vermiculite (see "Materials and methods"). Leaf senescence symptoms were evaluated 20 days after the nutrient solution soaking the vermiculite substrate was either maintained (Control), or replaced by water (Nutrient deprivation). The F_v/F_m (**a**) and total chlorophyll content (**b**) was determined in different leaves numbered according their position from the *top* to the *bottom* of the plant. Data shown is for three independent experiments performed with plants representing two different syngenic line pairs (NT_1, NT_2, $A9_1$, and $A9_2$), and using at least three plants per each *A9* or *NT* line. Chlorophyll content was determined from pooled leaf disks (diameter = 0.5 cm) taken from the same positioned leaves (four disks per leaf and plant). Immediately following F_v/F_m determination, whole leaves were detached and photographed; representative leaf pictures are shown for the two line pairs used (**c**)

S1 summarizes the statistical data that support these differences.

Protection of the maximum quantum yield of PSII (F_v/F_m) in the dark-treated 35S:A9 seedlings, compared to NT seedlings (Fig. 1b) was also observed. There was a notable decrease of F_v/F_m observed in the NT seedlings after only 1 week of dark treatment (to 47.9 ± 3.2 % of the initial value, compared to only 77.3 ± 2.1 % for the 35S:A9 seedlings). This suggests an A9-mediated protection of the PSII from dark-induced damage which, judging from the F_v/F_m values, appears to be similar to that caused by oxidative damage after drastic treatments of seedlings with 200 mM H_2O_2 for 24 h in the dark (Almoguera et al. 2012). PSII F_v/F_m protection was still evident after 3 weeks under dark conditions (24.6 ± 1.8 % for NT versus 30.2 ± 1.5 % for T), but disappeared after 5 weeks of dark treatment as observed for chlorophyll reduction (compare Fig. 1b, a).

The dark-induced senescence was not lethal after the 1-week treatment, as recovery of both the NT and 35S:A9 seedlings was similar following transfer to normal growth conditions with photoperiod illumination. In contrast, after 3 weeks of treatment, dark-induced senescence was lethal for the NT seedlings (0–5 % survival observed); however, the 35S:A9 seedlings recovered (80–100 %), as illustrated by the representative results shown in Fig. 1c, d. We conclude that A9 enhances vegetative longevity by protecting photosynthetic organs from dark-induced senescence. The protection was observed under very harsh stress conditions that are lethal for non-transgenic seedlings.

We next analyzed whether 35S:A9 plants resist prolonged dark treatments. We reasoned that the A9 effects on vegetative senescence determined in young seedlings (Fig. 1) might persist to further developmental stages after seedling establishment. Constitutive expression from the 35S sequences used in our experiments would allow

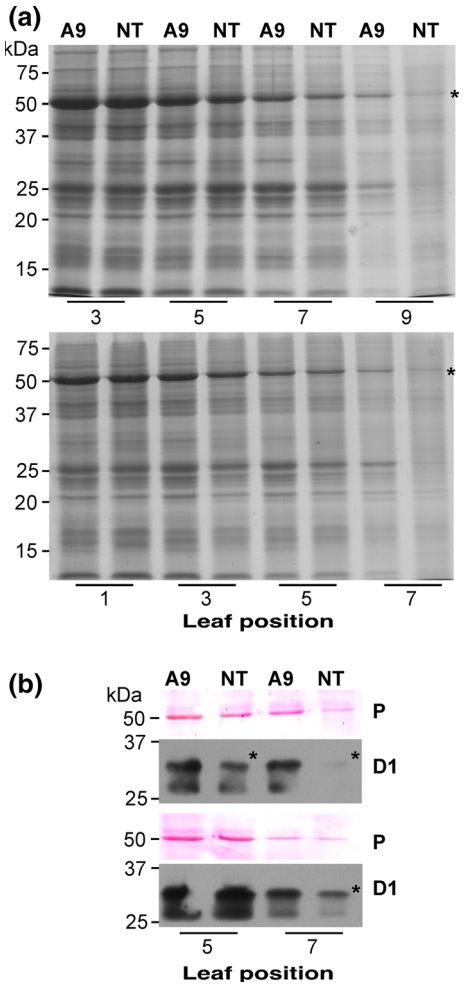

Fig. 6 Additional indications of delayed senescence in the 35S:A9 plants after nutrient deprivation. Total soluble leaf protein (**a**) and PSII D1 protein accumulation (**b**) was analyzed in the 35S:A9 (A9) and sibling non-transgenic (NT) plants used for the nutrient deprivation experiments of Fig. 5. Samples from different leaves numbered according their position from the top to the bottom of the plant were analyzed after SDS-PAGE. Coomassie blue-stained gels (**a**) showing the accumulation of abundant proteins in leaves 10 days (*top*) and 20 days (*bottom*) after the nutrient deprivation treatment started. The *asterisk* marks the Rubisco large subunit (RbcL) band mentioned in the text. Western-blot detection of the D1 protein was compared to Ponceau S-stained (P) RbcL band (**b**); the *asterisk* marks the major D1 band. Representative results for the two sibling line pairs used in Fig. 5 are depicted: NT_1–$A9_1$ (*top*) and NT_2–$A9_2$ (*bottom*). Gel lanes were loaded with protein extracts prepared on an equivalent FW basis (see "Materials and methods"). Left side, size markers (molecular mass, kDa)

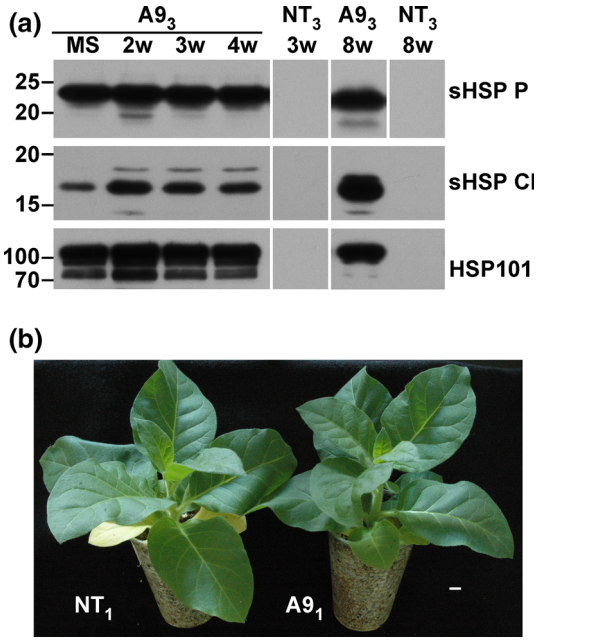

Fig. 7 The ectopic accumulation at normal growth temperatures of HSP (including plastidial sHSP-P) persists in 35S:A9 plants. Western-blot detection of the accumulation of the HSP indicated at the right side was compared using protein extracts from 35S:A9 (A9) young seedlings that were grown on MS medium in Petri-dishes (MS), and other extracts prepared from transgenic plants grown on potting substrate for 2, 3, 4, or 8 weeks (2–8 weeks). Representative results obtained with samples of the $A9_3$ line are shown; as a negative control samples from the sibling NT_3 line after 3 and 8 weeks' culture are included (**a**). Left side, size markers (molecular mass, kDa). In **b**, representative pictures of the size and development attained by the plants (NT_1 and $A9_1$ plants) after 8 weeks' culture are presented. *Scale bar* 1 cm

persistence of the A9 effects, unless additional limiting conditions were absolutely required (i.e., additional factors such as A4a, limiting post-translational modification of A9 later in development, etc.). Therefore, we investigated whether the A9-induced resistance to dark induced senescence observed with small seedlings (≈0.8 cm broad, with 2 pairs of true leaves, see Fig. 1) persisted in much larger and more developed plants. After 4 weeks of growth directly into potting soil under photoperiod illumination, the plants used for these experiments (≈10 cm broad) had 8 true leaves. F_v/F_m measurements were performed after 22 days (Fig. 2a) or 27 days (Fig. 2b) of subsequent exposure to dark culture conditions. The absolute F_v/F_m values in 35S:A9 leaves were higher than those of sibling NT plants after dark-induced senescence. The tobacco plants were quite resistant to 22 days under dark culture conditions (Fig. 2a); only in the older 5th and 6th leaves was senescence damage of PSII (i.e., significantly lower F_v/F_m values) evident in the NT lines, as compared to the sibling 35S:A9 lines. After dark culture for 27 days a much higher damage was evident for the NT plants. In this case, protection of PSII in the 35S:A9 leaves was more evident in the younger 3rd and 4th leaves than in the older leaves (Fig. 2b). After dark culture for 27 days the plants were transferred to normal photoperiod illumination conditions for 2 days. Leaf recovery from symptoms of induced senescence and leaf survival was clearly better in the 35S:A9

plants compared to the sibling NT plants. The 35S:A9 plants elongated under the prolonged dark culture conditions and developed more additional leaves than the NT plants when re-illuminated; the majority of the NT plants did not survive the 27-day dark treatment (Fig. 2c). These results indicate that protection from dark-induced senescence persists in 35S:A9 plants grown on soil, through to vegetative stages well beyond germination. Furthermore, other conditions beyond A9 overexpression (including the possible contribution of factors such as A4a) do not appear to drastically limit the A9 effect on vegetative senescence.

Improved recovery after prolonged dark treatments of the, double-transgenic, 35S:A9/A4a seedlings

Combined overexpression of A9 and A4a in tobacco seedlings has been shown to potentiate the previously described beneficial effects of A9 overexpression. Therefore, we used young seedlings to explore the possible, non-limiting, contribution of A4a to the A9 effect on vegetative senescence. We investigated whether A9 in combination with A4a also enhances the protection from dark-induced senescence observed upon overexpression of A9 alone (Fig. 1). Experiments were conducted exactly as for Fig. 1, except that a 5-week dark treatment was maintained for assessing seedling survival. This was necessary as the 35S:A9 seedlings showed quite good resistance to a 3-week dark treatment (see Fig. 1). We used seedlings from 4 different sibling pairs of single-transgenic (35S:A9) and double-transgenic (35S:A9/A4a) lines. At the end of the treatment, F_v/F_m and total chlorophyll content were measured; treated seedlings were then returned to normal growth conditions under photoperiod illumination for 2 weeks and photographed at the end of the experiments. Figure 3 summarizes the results of these experiments. The measurements of total chlorophyll content (Fig. 3a) and the F_v/F_m (Fig. 3b) did not reveal significant differences between the single and double-transgenic seedlings for any of the time points analyzed (1–5 weeks of dark treatment). Therefore, even after 5 weeks under dark the photosynthetic organs of both types of seedlings appear to be damaged to the same extent. However, the double-transgenic seedlings showed significantly better recovery than the single transgenic seedlings. This was quantified as percent survival rate after 5 weeks of dark treatment (Fig. 3c). Figure 3d illustrates a representative result of seedling survival for one of the pairs of single and double transgenic lines used in these experiments. The combined effect of A9 and A4a on senescence damage repair, rather than on senescence damage protection, may explain the enhanced recovery of the double-transgenic seedlings.

The 35S:A9 seedlings show delayed senescence when grown under photoperiod illumination

We next investigated whether the 35S:A9 seedlings would show delayed senescence symptoms under experimental conditions not involving prolonged dark treatments. The same three pairs of sibling NT and 35S:A9 lines utilized for the experiments of Fig. 1 were thus further analyzed. 3–4 week old seedlings that been grown on filter paper in MS medium plates under photoperiod illumination were transferred on water soaked paper circles to fresh plates without MS (see "Materials and methods"). Following 6 days of additional culture under the same illumination conditions, seedling senescence symptoms were evaluated (Fig. 4). This treatment might impose some nutrient stress (see additional experiments described below), but was not lethal for either the NT or the 35S:A9 seedlings, as most of them subsequently survived. However, survival of the 35S:A9 seedlings was significantly higher than of the sibling NT seedlings (respectively 93.41 ± 2.06 and 61.54 ± 5.51 %, Fig. 4a). Furthermore, immediately after treatment the 35S:A9 seedlings retained a significantly higher fraction of the initial total chlorophyll content than that observed for the sibling NT seedlings (respectively 40.38 ± 3.74 and 26.83 ± 3.36 %, Fig. 4b). Similarly, 35S:A9 seedlings also retained a higher fraction of the initial total carotenoid content (Fig. 4b). Figure 4c illustrates representative seedling survival for one of the pairs of NT and 35S:A9 lines employed in these experiments. Thus, the 35S:A9 seedlings also showed alleviated symptoms of vegetative senescence under conditions not involving prolonged dark culture.

The 35S:A9 plants show delayed senescence when grown under photoperiod illumination

We performed pilot experiments using NT and sibling 35S:A9 plants grown on soil in small pots (see Suppl. Fig. S2). These plants were kept well irrigated under normal photoperiod illumination culture conditions for a total of 7 weeks. The F_v/F_m, and total chlorophyll content of individual leaves located at different positions within the plants was then determined. The leaves of NT plants showed more senescence symptoms (yellowish color, dead tissue; decrease of F_v/F_m and chlorophyll) than the leaves of A9 plants. Moreover these senescence symptoms appeared earlier in "younger" leaves (placed near the top) in the NT lines than in the A9 lines. These results confirmed protection from senescence damage in the leaves of A9 plants; thus, the damage would affect PSII more in the NT plant leaves than in the sibling A9 plant leaves. However, we could not exclude that the observed senescence

symptoms were caused by micronutrient and/or macronutrient deprivation after prolonged culture in the small pots that were used (see for example, Thomas and De Villiers 1996; Wingler et al. 2005).

To try to differentiate between natural senescence (senescence symptoms caused by aging independently of exogenous stress) and nutrient-stress-induced senescence, additional experiments were performed. 3–4 week seedlings germinated on potting soil were individually transplanted to 200 mL pots. However, in this case the pots contained vermiculite (as the solid culture substrate). After 3 weeks culture on vermiculite substrate, well irrigated with a complete nutrient solution, the solution was substituted by deionized water in half of the pots (nutrient deprivation treatment), and in the other half (control treatment) irrigation with the nutrient solution was maintained. Both the control and the nutrient deprivation treatments induced leaf senescence symptoms, which were more evident and occurred earlier in the NT than in sibling 35S:A9 plants. Ten days after initiation of the control treatment, F_v/F_m measurements did not reveal a significant decrease except for the bottom NT leaves. At the same timing under the nutrient deprivation treatment, F_v/F_m started to decrease in the bottom leaves of the 35S:A9 plants; however such a decrease was significantly higher in the leaves of the NT sibling plants (Suppl. Fig. S3). By 20 days after starting the treatments, the differences between the effects of the two treatments on the F_v/F_m of NT and 35S:A9 leaves were more evident (Fig. 5a). For example, under the control treatment a significant decrease of F_v/F_m was observed in all NT leaves compared to leaves at the same position in the 35S:A9 sibling plants. After 20 days of nutrient deprivation treatment, there was a greater decrease in F_v/F_m for leaves placed at positions 5, 7, and 9 (bottom leaves); a significantly higher value of F_v/F_m was measured in 35S:A9 leaves as opposed to corresponding NT leaves. In fact, the bottom NT leaves (at position 9) were dead by this time and the F_v/F_m could be measured at this leaf position only in the 35S:A9 sibling plants (Fig. 5a). Total chlorophyll determination in individual leaves, 20 days after starting the two treatments, confirmed the senescence symptoms and the differences observed between treatments for the NT and 35S:A9 leaves (Fig. 5b). For example, after 20 days of control treatment the initial chlorophyll content of leaves decreased only in leaves positioned near the bottom of the plant (positions 5 and 7), but only leaf 7 of the 35S:A9 plants maintained a significantly higher content of chlorophyll than the same leaf in the NT sibling plants. By 20 days under the nutrient deprivation treatment a sharp decrease of total chlorophyll content was observed in all leaves (from top to bottom positions: 1–7); the chlorophyll content was significantly higher in the 35S:A9 leaves than in sibling NT leaves for all positions (Fig. 5b). At the same timing and for the same leaves, total carotenoid content was analyzed. The results included in Suppl. Fig. S4 show that nutrient deprivation accelerated the decrease of carotenoid content observed in the older leaves. This decrease was significantly lower in the A9 leaves compared to NT leaves. The leaves from plants that have been nutrient-deprived for 20 days showed macroscopic senescence symptoms (yellowish color, necrotic lesions, etc.). These senescence symptoms appeared in "younger" leaves (placed near the top) in the NT lines than in the sibling 35S:A9 lines. This is shown with photographs of the leaves from 35S:A9 and NT plants that represent two of the sibling line pairs used in these experiments (Fig. 5c).

Additional senescence symptoms were analyzed: the decrease of total soluble protein in leaves and the reduction of the abundance of some major polypeptide bands that can be easily visualized in Coomassie blue-stained gels (Zavaleta-Mancera et al. 1999). After only 10 days under nutrient deprivation treatment the protein content clearly decreased, but only in leaves placed closer to the bottom of the plant (positions 5, 7 and 9) and more distinctively in the NT plants than in the sibling 35S:A9 plants. The accumulation level of the major polypeptide band (of $\approx$50 kDa), that corresponds to ribulose-1,5-bisphosphate carboxylase (Rubisco) large subunit, and that of other abundant polypeptides decreased in these leaves, but to a lesser extent in the 35S:A9 plants compared to the NT sibling plants (Fig. 6a, top). By 20 days of nutrient deprivation the decrease of protein content also affected the top leaves (see for example position 3), and the differences in protein content and in polypeptide abundance between the NT and 35S:A9 plants occurred in younger leaves (placed closer to the top), and these differences were increased (Fig. 6a, compare top and bottom panels). The accumulation level of the PSII D1 protein was also analyzed in leaves at two intermediate positions (5 and 7) after 14 days of nutrient deprivation (Fig. 6b). After this time, the abundance of the Rubisco large subunit polypeptide was still similar between the NT and 35S:A9 leaves. This was shown with Ponceau-S staining of the same membranes use to detect the D1 protein by western-blot. However, the accumulation of D1 protein was distinctly lower in NT leaves than in 35S:A9 leaves (Fig. 6b). The reduction in the accumulation level of D1 in these leaves would indicate an early senescence symptom that is delayed in the leaves of the 35S:A9 plants (see "Discussion"). To recapitulate, the results of the experiments summarized in Figs. 5 and 6 indicate that nutrient deprivation treatment accelerates leaf aging. Under our experimental conditions, different leaf senescence symptoms were delayed in the 35S:A9 plants compared to the NT siblings after the control and nutrient deprivation treatments. The observed delay would thus affect natural and stress-induced senescence.

The ectopic accumulation of HSPs caused by A9 overexpression persists in transgenic plants well after seedling establishment

Ectopic HSP accumulation at normal growth temperature (under unstressed conditions), which is a consequence of A9 overexpression (Prieto-Dapena et al. 2008; Almoguera et al. 2012), was determined in plants directly grown on soil for different times (as for the senescence experiments in this report: see Figs. 2 and 5). The observed HSP accumulation was compared with that of seedlings grown on Petri dishes (the original conditions used in all our previous reports). The accumulation of sHSP-P, sHSP-CI and HSP101 persisted at similarly high levels in plants grown on potting soil for 4 weeks. When ectopic HSP accumulation was analyzed in 35S:A9 plants later in vegetative development, a similar persistence of sHSP-P, sHSP-CI and HSP101 was observed, for at least four additional weeks (Fig. 7a). Plant culture in these conditions allowed a substantial vegetative development compared to that of seedling grown in Petri dishes (conditions used in the experiments of Figs. 1, 3 and 4, and in all our previous reports). Figure 7b illustrates the size and development of the plants after 8 weeks culture (the same scale bar as in Fig. 1 is used).

Discussion

Previous research by our group has shown that, in sunflower and closely related dicot plant species, seed longevity and desiccation tolerance is in part determined by a genetic program coactivated by A9 and A4a: the A9-program. When A9 was overexpressed in young tobacco seedlings, we found stress-tolerance for severe dehydration and oxidative stress; this included a remarkable protection of the photosynthetic apparatus. The described functional effects of A9 coincided with ectopic seed-HSP accumulation at normal growth temperatures. We note that our previous characterization of 35S:A9 plants did not reveal any evident negative impacts on the growth of the transgenic plants (see the "Discussion" by Prieto-Dapena et al. 2008 and Fig. S2 therein). The constitutive activation of the A9-program did not have obvious metabolic costs or other trade-offs, at least for plants cultured under controlled laboratory conditions. Here, we describe new effects of overexpressed A9 and A4a in seedlings and plants cultivated well beyond the seedling stages. The ectopic seed-HSP accumulation persisted in these plants. The present results support and further extend the suggested roles of the A9-program in connection with the developing photosynthetic apparatus (Almoguera et al. 2012) and with the evolution of vegetative desiccation tolerance (Gaff and Oliver 2013).

The new ectopic effects of A9 observed in 35S:A9 seedlings and plants include marked resistance to treatments of prolonged darkness. The dark stress conditions endured by the 35S:A9 plants are not fully comparable with what homoiochlorophyllous resurrection plants withstand (Denev et al. 2012). However, as previously shown for dehydration tolerance and oxidative stress (Prieto-Dapena et al. 2008; Almoguera et al. 2012), the new A9-effects described here conferred resistance to stress levels well beyond those withstood by normal plants. The results in Figs. 2 and 7 represent the first evidence showing that the ectopic effects of A9 persist beyond the seedling stage. We have previously postulated the involvement of the A9-program in the evolution of vegetative desiccation tolerance in some resurrection plants (Prieto-Dapena et al. 2008). The persistent A9-effect that we report here would strengthen and extend our initial proposal. Our results indicate that some plant species could have evolved resistance to drastic stress just by changing the seed-specific expression of a single transcription factor (HSFA9) to constitutive expression; that (or similar) regulatory modification would suffice to extend the drastic stress resistance to stages well after germination. We note that our proposal fits the constitutive accumulation of HSPs in *Craterostigma plantagineum* (Alamillo et al. 1995); our hypothesis is also consistent with protection of the photosynthetic apparatus from drastic stress conditions (including darkness) that is exhibited by *Craterostigma plantagineum* and similar homoiochlorophyllous resurrection plants (Denev et al. 2012; Dinakar et al. 2012; Griffiths et al. 2014). The orthologous HSFA9 in these plants would have lost the initial seed-specific expression that still prevails in most dicot plants. The required mutations might involve (multiple) changes in HSFA9 cis-regulatory elements and (or) in protein interaction interphases; the target genes of the mutated regulators would be maintained (Tsong et al. 2006). The proposed change in regulation would extend protection of PSII by the A9-program from seeds to adult organs, where it would contribute to drastic stress tolerance and enhance vegetative longevity (see below). To gain full vegetative desiccation tolerance, additional evolutionary changes would be necessary. These changes might, for example, affect transcriptional regulation of different, seed-specific, genetic programs of desiccation tolerance. Regarding the A9-program, the regulation of HSFs such as A4a could have co-evolved with that of A9. The A9-program would be one of the seed-specific genetic programs assumed to have evolved to originate vegetative desiccation tolerance in some dicot land plants (Oliver et al. 2000; Farrant and Moore 2011; Gaff and Oliver 2013). Note that in monocot plants a seed-specific HSFA9 is not conserved (Scharf et al. 2012), implying different origins of vegetative desiccation tolerance not

involving the A9-program. Indeed, different types of regulatory switches involved in the implementation of desiccation tolerance may vary from one taxon to another as the happenstances of discrete evolutionary events separated in geological time (Gaff and Oliver 2013).

A9 retarded vegetative senescence in the 35S:A9 seedlings and plants. This was indicated by the results in Figs. 1 and 2, and it was confirmed by experiments using different senescence-inducing conditions not involving prolonged darkness (Figs. 4, 5). The lesser reduction of F_v/F_m values and pigment content observed in 35S:A9 seedlings and plants compared to NT siblings are consistent with retarded senescence. We also observed additional visual (leaf color and integrity changes) and molecular hints of retarded senescence that included a slower decrease of the accumulation level of the PSII D1 protein (Fig. 6b). We note that D1 protein degradation is an early symptom of nutrient stress-induced leaf senescence in Arabidopsis (Thomas and De Villiers 1996). Furthermore, D1 protein accumulation following dark-induced senescence has been shown to remain at higher levels in stay-green genotypes, compared to wild type (WT) plants (Yamatani et al. 2013). In all, the 35S:A9 seedlings and plants display a phenotype consistent with "functional stay-green" characteristics (Hörtensteiner 2009). The delayed leaf senescence in resurrection plants has been also compared to that of stay-green genotypes (Griffiths et al. 2014). Our results for the 35S:A9 plants are thus similar to both stay-green genotypes and resurrection plants, offering a new system to analyze leaf longevity by gain-of-function. We could only find one precedent linking a plant HSF with leaf senescence: a study where mutation of Arabidopsis HSFB1 accelerated leaf senescence and reduced tolerance to drought stress (Breeze et al. 2008). In Arabidopsis, HSFB1 has been shown to enhance acquired thermotolerance of vegetative organs (Ikeda et al. 2011); however, HSFB1 would not have other functions in seeds.

Ectopic HSP accumulation was analyzed in the seedlings and plants used to demonstrate the persistent, 35S:A9, phenotypes. The results in Fig. 7 show that high accumulation levels of HSP101, sHSP-CI and sHSP-P persisted in all the analyzed developmental stages, and even in older 35S:A9 plants (Fig. 7, 8 weeks). We conclude that the sHSP-P and sHSP-CI assumed to contribute to protection and recovery of the photosynthetic apparatus from drastic dehydration and oxidative stress in the 35S:A9 seedlings (Almoguera et al. 2012) are available during later vegetative development in the 35S:A9 plants. However, the photosynthesis-related phenotypes reported here and elsewhere (Almoguera et al. 2012) are too complex to be explained only by the ectopic accumulation of seed HSPs. For example, A9 together with A4a appear to enhance the recovery (rather than protection) of photosynthetic organs (Fig. 3). A4a might enhance previously described A9 effects that are relevant for the recovery of PSII, such as perhaps the repair synthesis of the D1 protein (Almoguera et al. 2012). Interestingly, the recovery of the photosynthetic machinery appears also to be enhanced in some stay-green genotypes of normal plants (Luo et al. 2013). Similarly, a resurrection plant showed enhanced expression of a protein involved in PSII assembly/stability (Oliver et al. 2011). The A9 effect on vegetative senescence also includes results (Fig. 6) consistent with decreased proteolysis of total soluble and thylakoidal (D1) or stromal Rubisco large subunit proteins. This might also occur in some resurrection plants, where the expression of a cysteine protease inhibitor gene is enhanced (Blomstedt et al. 2010). This would reduce senescence-relevant proteolysis (Roberts et al. 2012), and thus contribute to delaying senescence. Considering the A9 effects on vegetative senescence, we note that similar effects caused in Arabidopsis by JUB1 (a NAC transcription factor) involved activation of a response network that comprised 14 HSP genes (including CI, CII and CIII sHSPs), and also that of many non-HSP genes (Wu et al. 2012). Thus, A9 (with A4a) might cause broad effects on non-HSP genes potentially involved in the protection and assembly of photosynthetic membranes in seed embryos. Future work by our group will involve identifying these non-HSP genes activated by A9 and A4a.

We conclude that ectopic overexpression of A9 and A4a caused complex effects in vegetative organs of the transgenic plants and induced phenotypes analogous to those of resurrection plants. A9 affected the photosynthetic organs by inducing delayed vegetative senescence and resistance to prolonged darkness, which is lethal for normal plants. This has revealed a novel connection between seed HSF and vegetative longevity.

Author contribution statement CA conceived and designed research and conducted most of the experiments. PPD helped CA and performed the statistical analyses. JMP performed the experiments with the 35SA9/A4a seedlings, some experiments with the 35S:A9 seedlings, and analyzed the persistent accumulation of seed HSPs in the 35S:A9 plants. JJ supervised the work and wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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