Chapter 7
Stress Modulation in *Pinus* spp. Somatic Embryogenesis as Model for Climate Change Mitigation: Stress Is Not Always a Problem

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Abstract Climate change is leading to higher temperatures and lower precipitation; this fact can have a negative impact on plant performance and survival. Latest findings have revealed that the conditions in which zygotic embryogenesis takes place have an impact on the adaptive capacity of the resulting plants. Somatic embryogenesis provides us a potent biotechnological tool to manipulate the physical and chemical conditions (water availability) along the process and to study their effect in the final success of the process in terms of quantity and quality of somatic plants produced. The development of somatic cells to somatic plantlets comprises three stages: induction of embryonal masses, maturation of embryogenic tissues, and conversion into somatic plants. Our experience in somatic embryogenesis in *Pinus* spp. enables us to explore the possibility to modulate the quality in terms of abiotic stress tolerance of somatic plants modifying environmental conditions during the initial stages of the process. Our results have shown that the modification of environmental conditions affected not only the success of the process in some species of pines but also the water use efficiency of the somatic plants after several months in ex vitro conditions. In the chapter, we will show the different responses obtained in all the stages of the somatic embryogenesis process as well as the

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response obtained after drought periods in plants growing in the greenhouse under ex vitro conditions.

Keywords Stress modulation · Somatic embryogenesis · Climate change · Mitigation · *Pinus* spp

Introduction

As a result of being sessile organisms that are continuously exposed to a huge amount of external stimuli and changing environments, plants have evolved and managed to acquire different mechanisms to rapidly detect and respond to a great variety of stress factors, both biotic and abiotic (Xia et al. 2015). Among abiotic stresses, drought and high temperatures are among the most common environmental constraints for plants. In fact, due to climate change, extreme heat waves and long drought periods are becoming more and more frequent (Duliè et al. 2013), which cause a serious threat not only for natural ecosystems but also for all type of plantations and cultivars (Allen et al. 2010). These conditions are already challenging the survival and productivity of forests (Manzanera et al. 2017). Conifers, by far the largest and most diverse gymnosperm group that grow in a wide range of climate zones, are of great importance both ecologically and economically since they make up most of the biomass in many biogeographic zones of the Northern Hemisphere and provide wood and non-wood products (von Arnold et al. 2018). Nonetheless, the current situation driven by climate change foretells big difficulties to satisfy the future demand of forest products and services (Fenning et al. 2008).

At the molecular level, heat and drought are known to affect a great number of physiological traits. They trigger inhibition of photosynthesis, senescence, water, and osmotic imbalances and production of reactive oxygen species and modify the permeability and fluidity of cell membranes, among others (Larkindale and Knight 2002; Sangwan et al. 2002; Vacca et al. 2004; Feller and Vaseva 2014). However, the great adaptability and phenotypic plasticity of plants offer the opportunity to combine traditional breeding techniques with modern biotechnological tools to obtain plants pre-adapted to different environmental conditions (Conrath et al. 2015). This pre-adaptation strategy is mainly ruled by epigenetic variations, which endow plants with some kind of "memory" that results in modified gene expression patterns (Davies et al. 2011). In this context, somatic embryogenesis (SE) could be a useful tool to modulate future plant tolerance and therefore their behavior in ex vitro conditions. SE is a biotechnological tool in which embryos arise from somatic cells and commonly includes a set of stages such as induction, multiplication (proliferation), maturation, and conversion into plantlets (Heringer et al. 2018). Variation of the culture conditions during initial steps of the SE process is known to have

long-lasting effects in plants, even altering some developmental traits years later (Kvaalen and Johnsen 2008). Consequently, SE apart from being one of the most effective techniques to obtain large amounts of cloned material in conifers opens the door to a promising research field that could help to unravel the molecular mechanisms by which plants face stress.

To this purpose, in the last few years our research team has been applying different culture and environmental conditions during the different steps of SE, in order to assess if those changes in conditions can determine the success of the SE process itself in terms of plant production and plant quality, but also the behavior of somatic embryo-derived plants ex vitro. In parallel, numerous studies have been carried out to evaluate the effect of those conditions at morphological levels as well as to analyze different metabolites, aiming to detect early stress markers involved in the acquisition of tolerance. To enable a broader understanding of these mechanisms, two *Pinus* species from contrasting habitats have been employed in all experiments: *P. radiata* and *P. halepensis*.

Stress Modulation on Initial Stage of Somatic Embryogenesis

The Outcome of Different Water Availabilities and Temperatures at Initial Stages

As referred above, two *Pinus* species have been used in our experiments: *P. radiata* and *P. halepensis*. *R*adiata pine is a conifer native from California and México. It has a great economic relevance being suitable for a great range of uses due to its rapid and versatile growth and the desirable quality of its pulp and timber. It is the most widely planted pine in the world (Espinel et al. 1995; Dungey et al. 2009). *P. halepensis* Mill., commonly named Aleppo pine, is a species native to the Mediterranean region, widespread from Spain to Algeria (Botella et al. 2010). It is important for environmental conservation (Montalbán et al. 2013), due to its great ecological plasticity, since it can thrive under semi-arid climatic conditions in a wide variety of soils and can be used in large afforestations (Maestre and Cortina 2004).

With prior protocols established for SE in both species, two separate experiments *per* species that investigated the stress influence along the process by applying different environmental conditions, at induction and proliferation steps, were performed as indicated in Fig. 7.1.

In the initial set of experiments, different gellan gum concentrations (2, 3, and 4 gL^{-1}), to increase or reduce water availability, were added to the induction media, and the explants were then stored at three different temperatures (18, 23, and 28 °C). Immature megagametophytes from four seed families in *P. radiata* (García-Mendiguren et al. 2016a) and five in *P. halepensis* (Pereira et al. 2016) were used as explants. Different environmental conditions were applied only at the induction
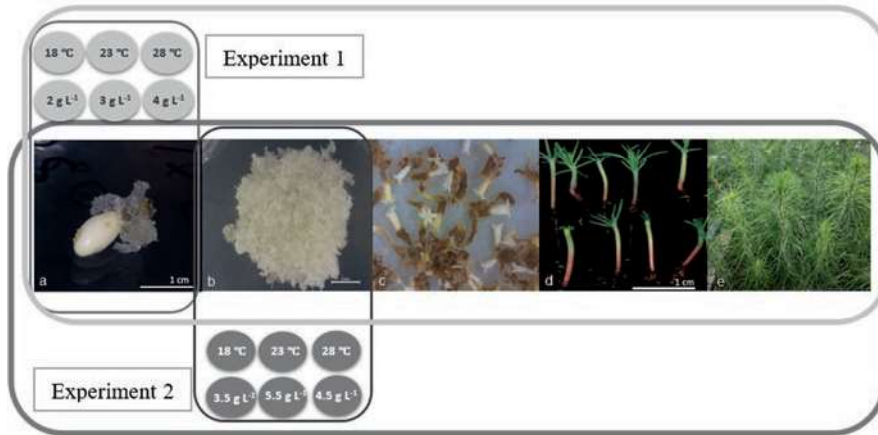
Fig. 7.1 Scheme of the experiments carried out in order to test the stress influence along the process by applying different environmental conditions, at induction and proliferation steps of somatic embryogenesis in *Pinus* spp. (**a**) Embryogenic mass extrusion from immature megagametophyte of *P. halepensis*. (**b**) Established embryogenic mass in proliferation of *P. halepensis*. (**c**) Somatic embryos obtained from embryogenic masses of *P. radiata*. (**d**) In vitro germinated somatic plantlets of *P. halepensis* and (**e**) acclimatized somatic plants of *P. radiata* growing in the greenhouse. Each experiment comprised nine different treatments and was individually developed for each species

stage, and then the cultures were maintained at standard conditions on the subsequent stages of the process. In both species, initiation rates of embryonal masses (EMs) were significantly different among seed families, temperatures, and gellan gum concentrations (García-Mendiguren et al. 2016a; Pereira et al. 2016). As expected, both showed significant differences on initiation rates between seed families. Since the effect of the mother plant cannot be equally reproduced unless the same families are used for a future experiment, a higher number of family seeds can reduce the variability of the results (Montalbán et al. 2013, 2015). Regarding the water availability, it is known that water availability in the culture media plays an important role in the phases of EM initiation and proliferation (Choudhury et al. 2008; García-Mendiguren et al. 2016a), and both species showed higher initiation rates in explants cultured with the lowest water availability (4 gL^{-1}). These results agree with reports in *P. monticola* (Percy et al. 2000) and *P. pinea* (Carneros et al. 2009) and are in contrast to some authors who have shown that when gellan gum increases, the initiation of embryogenesis is negatively affected (Harry and Thorpe 1991; Becwar et al. 1995; Pullman and Skryabina 2007). Nonetheless, the highest initiation rate of EMs in *P. halepensis* was obtained in cultures maintained at 23 °C (control temperature), while the lowest temperature, 18 °C, led to better initiation rates on *P. radiata*. Bonga et al. (2010) suggested that changes in temperature regimes might improve initiation and proliferation since temperature stress may promote cellular reprogramming. We relate this difference to the optimal temperature at which these species grow in the field, which is usually lower in the case of

radiata pine. Concerning the subsequent phases of SE in Aleppo pine, the initiation temperature only had a significant effect at proliferation stage, with initiation temperature 18 °C leading to lower rates. All embryogenic cell lines (ECLs) were able to produce somatic embryos (*se*), and there were no differences on the number of *se* produced or at the germination rates. García-Mendiguren et al. (2016a) showed that EMs of radiata initiated at 28 °C exhibited higher proliferation percentages and produced the highest number of *se*. A second experiment was developed with the same purpose, to evaluate the effect of different environmental conditions along the SE process, for both species at the proliferation stage. In this case, different gellan gum concentrations (3.5, 4.5, and 5.5 gL^{-1}) were added to the proliferation media, and again, the explants were stored at three different temperatures (18, 23, and 28 °C). Standard conditions were applied at all other stages of the process.

The results presented by García-Mendiguren et al. (2016a) on radiata pine showed that more water availability, 3.5 gL^{-1} gellan gum, led to lower proliferation rates. EMs incubated at the highest temperature, 28 °C, also showed lower proliferation rates. Nonetheless, no significant effects on maturation rates or the number of SE produced were observed. Pereira et al. (2017) gave results for Aleppo pine, reporting opposite findings in *P. radiata*, showed that the interaction between temperature and water availability significantly affected the number of *se* produced per gram, and germinations rates, in agreement with results obtained by Kvaalen and Johnsen (2008) in *Picea abies*. A significantly higher number of *se* were obtained when the cultures were proliferated at 28 °C with 3.5 gL^{-1} gellan gum, whereas the highest germination rate was achieved from EMs proliferated at 28 °C in a culture media supplemented with 5.5 gL^{-1} gellan gum. As a conclusion, it can be stated that regarding *P. radiata* the initiation stage is crucial to all further steps, from initiation to maturation of *se*, having long-term effects on embryogenesis. On the other hand, with *P. halepensis*, manipulation of environmental conditions during the initiation stage only influenced the success of initiation and proliferation, while during the proliferation of embryonal masses, it caused an effect only several months later, in the production of somatic plants at the germination stage.

Protein Profiles of Somatic Embryos Derived from Different Water Availabilities and Temperatures at Initials Stages

SE can occur in tissue and cell cultures of a wide range of species, from herbaceous to woody plants. Nonetheless, the mechanisms of initiation and *se* development are not fully understood, so in recent years SE has been studied using discovery proteomics, in order to get deeper insight into the process (Klubicová et al. 2017). Proteomics is a very useful tool for studying stress tolerance, allowing the identification and quantification of stress-tolerance-associated proteins (Pinheiro et al. 2014), with the possibility to use protein markers for improving selection. The utilization of the global transcript expression or protein profiling (Lippert et al. 2005;

Zhao et al. 2015) has been used as an approach to generate such markers and for a better understanding of SE regulation. In this sense, a 2D proteomic analysis was carried out, aiming to determine if the abovementioned environmental conditions during the induction phase of SE in *P. radiata* resulted in differences in the protein profiles of *se* (García-Mendiguren et al. 2016b). Protein patterns in *se* from ECLs from treatments producing high percentages of initiation (18/23 °C, 4 gL⁻¹) and low percentages of initiation (28 °C, 2/3 gL⁻¹) were evaluated and compared. In *se* from treatments that led to low percentages of initiation, a prominent group of proteins involved in defense responses, such as the osmotically inducible protein OsmC, chaperone protein, and vicilins, was identified. Chaperones are known as heat shock proteins (HSPs), whose biological role is to assist folding of unfolded or misfolded proteins under stress conditions (Efeoğlu 2009), and are also known for their roles in the maturation, as they act as oxidative stress regulators (Marsoni et al. 2008). Proteins associated with other functional groups, such as the response to ROS, important signaling molecules reported to act in plant responses to biotic and abiotic stresses (Delledone et al. 2001), and in the development of SE of conifers (Zhang et al. 2010), or proteins related to gene expression, were also found in lower proportions. The presence of proteins involved in stress and metabolic responses indicates that changing environmental conditions seem to influence all the SE process.

## Effect of Extreme Temperature Stress on the Induction Phase

Following on from the studies on the effects of different gelling agents and culture temperatures in the success of the SE process, further experiments were carried out in radiata pine with extreme temperature stress, in order to examine if those stresses can establish an epigenetic mark in the embryogenic cells, leading to plants with different ability to grow ex vitro under unfavorable conditions. For these studies, high temperatures (30, 40, 50, and 60 °C) for different incubation periods during initiation (Castander-Olarieta et al. 2019) were applied. Simultaneously, the success of the SE process was evaluated, and different morphological and molecular analyses were performed.

In line with previous studies (García-Mendiguren et al. 2016a; Pereira et al. 2016), the application of high temperatures for long exposure time periods during initiation had detrimental effects during the first steps of the SE process (initiation and proliferation), which reduced considerably the genetic diversity that could be induced by this technique. Nonetheless, the same treatments provoked an increase in the production of *se*, which agrees with the results reported by Fehér (2015). These authors postulated that stress acts like a selective pressure during initial phases of SE, improving the efficiency of the forthcoming steps. It is worth highlighting that the *se* obtained presented morphologies that usually led to low germination and rooting rates (Pullman et al. 2003). On the other hand, our results suggest that high temperatures for short periods did not provoke a decrease in initiation or

proliferation rates, but also promoted the production of *se*. Moreover, in this case, the embryos obtained were of better quality, bigger, and more elongated, suggesting that mild stresses enhance not only the production but also the quality of the embryos formed. For a better understanding of the effects of thermal stress during SE, an exhaustive micromorphological and ultrastructural analysis of the EMs was carried out. This study confirmed the presence of three cell types (embryogenic cells, tube-like cells, and suspensor cells) and three organizational structures (PEMI, PEMII, and PEMIII) in EMs, as already observed for other conifer species by Filonova et al. (2000) and Steiner et al. (2016). As a general trend, the application of high temperatures resulted in the loss of polarity and cellular disorganization of embryogenic structures (Fig. 7.2a, b, c, d), coupled with an increase in the number of vacuoles, plastolysome-like structures, whorls by cytoplasmic membranes, and starch grains around the nuclei (Fig. 7.3), all of them symptoms of programmed cell death (Smertenko and Bozhkov 2014).
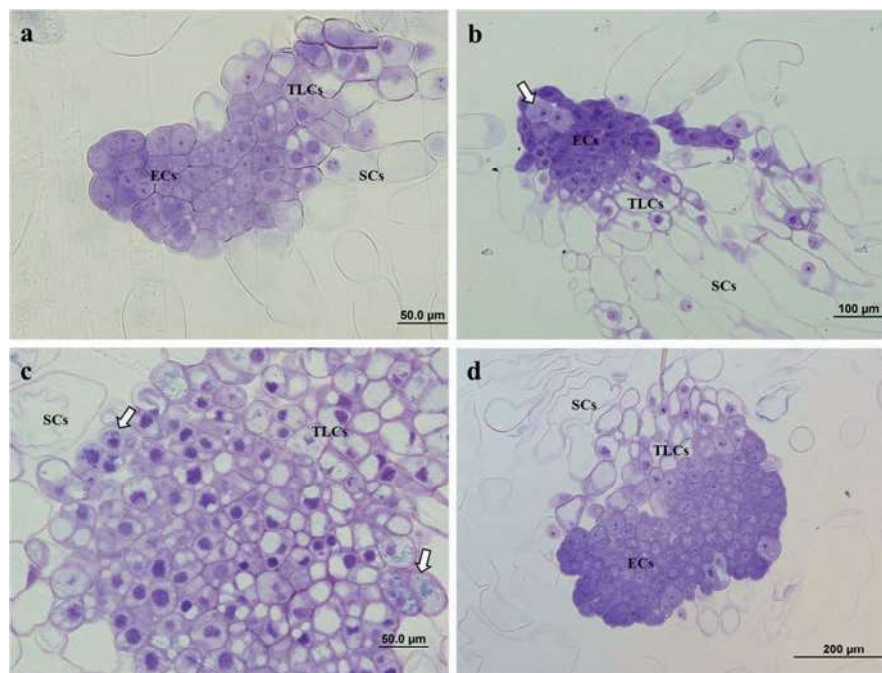
Fig. 7.2 Light microscopy and histochemical analyses of proembryogenic masses of *Pinus radiata* induced at different temperatures. The pictures show different cell types: embryogenic cells (ECs), tube-like cells (TLCs), and suspensor cells (SCs). (**a**) PEMIII from 23 °C presenting a clear polarization and well-organized structure. (**b**) PEMIII from 40 °C starting to lose polarization with enlarged TLCs arising in the middle of embryonal areas (arrow). (**c**) Embryonal area from 50 °C showing cells with advanced symptoms of programmed cell death (high vacuolation, compacted heterochromatin) and lost polarization. Small phenolic grains are also visible (arrows). (**d**) PEMIII from 60 °C forming a big cluster of compacted and well-organized ECs
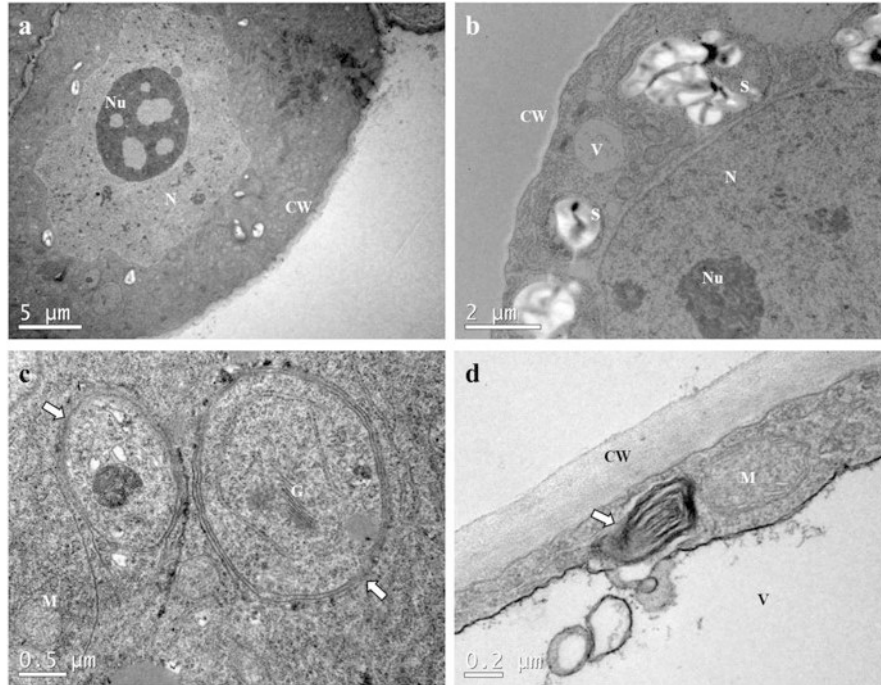
Fig. 7.3 Transmission electron microscopy images of ECs and TLCs from proembryogenic masses initiated at different temperatures. Cells present a great variety of organelles: nuclei (N), nucleoli (Nu), cell wall (CW), vacuole (V), mitochondria (M), Golgi bodies (G), and plastids with starch grains (S). (**a**) ECs from 23 °C with a dense cytoplasm and just a few S. (**b**) Details of ECs from 40 °C showing symptoms of programmed cell death such as a great number of V and S surrounding the N. Plastolysome-like structures engulfing different organelles (**c**) and whorls of cytoplasmatic membranes (**d**) from ECs and TLCs from 50 °C treatment, respectively (arrows)

Activation of programmed cell death by temperature was also reported by DaMatta and Cochicho Ramalho (2006). This phenomenon is known to increase the formation of suspensor cells (Abrahamsson et al. 2012), which together with the accumulation of starch grains around the nuclei are the principal causes of loss of embryogenic competence and formation of abnormal se (Morel et al. 2014; Merino et al. 2018). This was also observed with the application of high temperatures for long exposure times in *P. radiata* (Castander-Olarieta et al. 2019).

It is noticeable nevertheless that the same studies revealed an enhancement in the embryo formation when pulse-like treatments were applied. These conditions favored the presence of bigger and more developed embryogenic areas (Fig. 7.2d), which may explain the increase in embryo production and embryo quality previously observed.

Among the multiple fine-tuning strategies developed by plants against stress, readjustment of biochemical pathways and accumulation of elevated amounts of metabolites are two important examples. Heat and drought have overlapping roles

(Jia et al. 2017) and are known to activate similar molecular mechanisms driven by osmotic imbalances (Shinozaki et al. 2003; Gimeno et al. 2009). In forest species, these events lead to an enhanced synthesis and accumulation of soluble carbohydrates and amino acids (Patakas et al. 2002).

Sugars are described as taking an active part in numerous stress-mediated signal transduction pathways, and their activity as osmolytes, membrane stabilizers, and oxygen radical scavengers is well documented (Moradi et al. 2017; Woodrow et al. 2017). However, Castander-Olarieta et al. (2019) did not find pronounced differences in the carbohydrate profile of EMs exposed to different temperatures. However, the same authors detected significant differences in the amounts of certain amino acids, some of which are well known as playing important roles in plant stress tolerance acquisition. They are involved in osmotic adjustment, pH regulation, detoxification of reactive oxygen species, and synthesis of secondary metabolites (Corcuera et al. 2012; De Diego et al. 2015).

In opposition to numerous studies that highlight the importance of proline as one of the most active amino acids in stress responses (Wang et al. 2019). Castander-Olarieta et al. (2019) showed a clear participation of branched-chain amino acids in response to high temperatures. Isoleucine was the best example, whose levels increased with higher temperatures, suggesting its contribution to the synthesis of stress-induced proteins and to the regulation of gene expression (Joshi et al. 2010). Tyrosine also accumulated in all heat-shock treatments. This amino acid is a precursor of a wide range of secondary metabolites, among which phenolic compounds are of great importance because of their antioxidant role (Eliášová et al. 2017). Interestingly, light microscopy analysis carried out by the same authors revealed the presence of these compounds in the vacuoles of EMs subjected to the highest temperatures (Fig. 7.2c).

Future Perspectives

After having tested the feasibility of modulating the productivity and the quality of *se* by modifying different culture conditions during initial steps of SE in two pine species from contrasting habitats, our research team is also paying special attention to the effect of extreme temperatures (40, 50, and 60 °C) for different incubation periods, during the initiation phase, in SE of *P. halepensis*, for which information in this regard is still scarce. In that way we would be able to obtain more information about the different behaviors of this pine species and the mechanisms they employ to face stress.

Likewise, we consider it is of great interest to unravel the role that phytohormones are playing in these developmental and stress-response processes. Plant hormones, apart from modulating numerous physiological and developmental responses (Wani et al. 2016) and being essential during in vitro organogenesis and SE (Moncaleán et al. 2003, 2005, 2018; Montalbán et al. 2011), are active compounds during stress responses (De Diego et al. 2012, 2015).

Following the studies previously described (García-Mendiguren et al. 2016b), a deep study of the proteomic response of EMs and *se* to extreme temperatures will be performed. Proteomics is a very useful tool for studying stress response. The identification and quantification of proteins associated with stress-tolerance response accompanied with the possibility to use protein markers for improving selection can help the understanding of SE regulation, the mechanisms involved in the process, and the possible tolerance of the plants obtained (Klubicová et al. 2017).

Furthermore, we are also testing the drought resilience of somatic plants generated from EMs cultured under high temperatures, in order to assess if all the changes observed in previous studies at in vitro stages persist until reaching plant level and alter their capacity to face stress. This could prove a great advance toward the production of plants pre-adapted to unfavorable conditions. Finally, we are examining the expression patterns of stress-related genes and their methylation state on EMs, *se*, in vitro somatic plants and plants subjected to stress in the greenhouse.

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