miR172 Regulates WUS during Somatic Embryogenesis in Arabidopsis via AP2

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Abstract: In plants, the embryogenic transition of somatic cells requires the reprogramming of the cell transcriptome, which is under the control of genetic and epigenetic factors. Correspondingly, the extensive modulation of genes encoding transcription factors and miRNAs has been indicated as controlling the induction of somatic embryogenesis in Arabidopsis and other plants. Among the miRNAs that have a differential expression during somatic embryogenesis, members of the MIRNA172 family have been identified, which implies a role of miR172 in controlling the embryogenic transition in Arabidopsis. In the present study, we found a disturbed expression of both MIRNA172 and candidate miR172-target genes, including AP2, TOE1, TOE2, TOE3, SMZ and SNZ, that negatively affected the embryogenic response of transgenic explants. Next, we examined the role of AP2 in the miR172-mediated mechanism that controls the embryogenic response. We found some evidence that by controlling AP2, miR172 might repress the WUS that has an important function in embryogenic induction. We showed that the mechanism of the miR172-AP2-controlled repression of WUS involves histone acetylation. We observed the upregulation of the WUS transcripts in an embryogenic culture that was overexpressing AP2 and treated with trichostatin A (TSA), which is an inhibitor of HDAC histone deacetylases. The increased expression of the WUS gene in the embryogenic culture of the hdac mutants further confirmed the role of histone acetylation in WUS control during somatic embryogenesis. A chromatin-immunoprecipitation analysis provided evidence about the contribution of HDA6/19-mediated histone deacetylation to AP2-controlled WUS repression during embryogenic induction. The upstream regulatory elements of the miR172-AP2-WUS pathway might involve the miR156-controlled SPL9/SPL10, which control the level of mature miR172 in an embryogenic culture.

Keywords: somatic embryogenesis; miR172; AP2; WUS; miR156; SPLs

1. Introduction

Somatic embryogenesis (SE), a plant-specific developmental process, results in the formation of embryos from in vitro-cultured somatic cells. For years, SE has been widely explored in plant biotechnology for the efficient regeneration, micropropagation and genetic modifications of plants [1]. Moreover, studies on SE have also provided an attractive research model for understanding the regulatory processes that control the embryogenic transition of somatic cells and, in a broader sense, the developmental plasticity and toti/pluripotency of plants [2].

The research of a model plant of Arabidopsis has contributed the most to revealing the molecular mechanisms that control SE induction [3]. The studies provided evidence that complex interactions between the genetic and epigenetic factors, including the transcription
factors, miRNAs (microRNAs), DNA methylation and chromatin modifications, control the embryogenic reprogramming of somatic cells [4,5]. Consistent with a decisive function of the transcription factors (TFs) in the genetic reprogramming of somatic cells in plants and animals [6,7], hundreds of TF genes have shown an extensively modulated expression in the SE of different plants, including Arabidopsis [8–13]. Moreover, several TFs have been found to have an essential function in embryogenic transition, including BABY BOOM—BBM [14], LEAFY COTYLEDON1 and 2—LEC1 and LEC2 [15,16], WUSCHEL—WUS [17], AGAMOUS-like15—AGL15 [18], MYELOBLASTOSISI18—MYB118 [19] and EMBRYOMAKER—EMK [20]. Most SE-essential TFs control embryogenic induction by regulating the phytohormone-related pathways, mainly metabolism, transport and the signaling of auxin [3,5,21].

It is worth noting that the SE-essential TFs of Arabidopsis have been used to improve the regeneration efficiency of in vitro-recalcitrant crops [22–25]. This finding suggests that the SE-induction mechanism in different plants might have common regulators.

In concert with TFs, microRNAs control SE induction and a differential expression of numerous MIRNAS has been found in embryogenic cultures of Arabidopsis [26] and other plants [27–29]. Only a few miRNAs, including miR160 and miR166/165 [30], miR167 [31], miR393 [32], miR156 [33], miR396 [34] and miR528 [35], have been functionally analyzed during SE. The functions of miRNA in SE induction involve the regulation of the SE-involved genes. During SE, the miRNA-controlled TF-encoding genes regulate the genes that are involved in metabolism and the signaling of different phytohormones, mainly auxin [30,32–35], and the stress response [33,35].

The accumulation of the primary transcripts of MIRNA172 genes in an embryogenic culture of Arabidopsis suggests SE-related functions of miR172 [26]. In vivo, miR172 controls the juvenile-to-adult phase transition and flowering in Arabidopsis [36,37]. Furthermore, the involvement of miR172 in regulating stem-cell fate [38], sex determination [36,39], fruit growth [40,41] and spike architecture [42] has been postulated. The function of miR172 in integrating various endogenous and exogenous cues during the developmental transitions that are associated with plant flowering has also been reported [43–45]. The regulatory mechanisms that control the development of the generative organs and the embryogenic transition of somatic cells seem to be partially convergent [8,11]. Thus, the role of miR172 in the genetic network controlling embryogenic induction in plant somatic cells might be hypothesized.

In Arabidopsis, five members (MIR172a–e) of a conserved MIRNA172 gene family are spatially and temporally regulated and play redundant and specific roles in plant development [44–46]. Recently, the function of individual MIRNA172 genes in regulating the flowering time in the leaves and the shoot apical meristem (SAM) was revealed [43]. The targets of miR172 include members of the AP2-like (APETALA-like) subfamily from the AP2/ERF (APETALA2/ETHYLENE RESPONSE FACTOR) family of TFs [47]. The AP2 protein family contains numerous members that are critical for SE in Arabidopsis and other plants, including BBM [14,48], the PLETHORA [34,49], EMK [20] and Mt SOMATIC EMBRYO RELATED FACTOR1—MtSERF1 [50].

The miR172-controlled AP2-like genes include AP2 (APETALA2), TOE1 (TARGET OF EAT1), TOE2, TOE3, SMZ (SCHLAFMÜTZE) and SNZ (SCHNARCHZAPFEN), which regulate flowering and the development of the floral organs [37,51,52]. AP2-like TFs regulate the genes by the AP2-DNA-binding domain [47] and the targeted genes have been found to control the flower [53] and embryo development [14], spikelet meristem determinacy [54] and leaf epidermal-cell identity [55]. The AP2 targets during floral development include the floral repressor AGL15 [56] and the stem-cell-niche regulator of the floral meristem, WUS [57]. Because AGL15 and WUS have a regulatory function in embryogenic induction in vitro [17,18], the role of AP2 in the regulatory circuit that controls SE could be assumed.

Besides the TFs and miRNAs, chromatin modifications, including histone acetylation (Hac), also control embryogenic development [58–60]. In Hac-mediated gene regulation,
two types of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), affect the accessibility of the chromatin-binding proteins to DNA via changes in the acetylation status of the histones [61]. The antagonist activity of HAT and HDAC promotes an open vs. closed chromatin state, which results in the activation vs. repression of gene transcription [62].

The central role of the epigenetic processes, including Hac in the SE-regulatory network, has been postulated [4]. However, the evidence on the Hac function during SE is still quite limited and mostly indirect. A substantial deregulation of the HAT and HDAC genes have been documented in SE induction in Arabidopsis [13] and other plants [63–65]. Moreover, the inhibition of HDAC by trichostatin A (TSA) treatment promoted the development of embryogenic structures in the seedlings and explants of Arabidopsis [66,67] and improved the embryogenic response in conifers [68,69]. The SE-promoting activity of TSA has been associated with the upregulation of LEC1, LEC2, BBM and PHB (PHABOULOSA), which implies a role of Hac in the regulation of the SE-involved TF genes [67]. However, the mechanisms and regulatory elements, including HDAC in the Hac-mediated regulation of specific genes in SE induction, remain mostly unexplored.

Of the HDACs, members of the RPD3/HDA1 (REDUCED POTASSIUM DEPENDENCE 3/HISTONE DEACETYLASE 1) family, including HDA6 and HDA19, have a regulatory function during various processes in plant development [70]. HDA6 and HDA19 redundantly control the repression of an embryo-specific gene function, and the repression of the HDA6/19 function results in a spontaneous somatic-embryo development in germinating seedlings [66], thereby suggesting the role of these HDACs during SE control. Recently, the cooperation of HDA6/HDA19 with the AGL15 and the TOPLESS co-repressors of the SIN3/HDAC (SWI-INDEPENDENT 3/HISTONE DEACETYLASE) silencing complex was revealed to control the miRNA biogenesis genes in SE induction [71].

The differential expression of the MIRNA172 in the embryogenic culture of Arabidopsis [26], together with the key role of miR172 in the developmental transition of plants [44–46] motivated us to explore the function of miR172 in the embryogenic transition that is induced in in vitro-cultured explants of Arabidopsis. The goal of the study was to reveal the role of miR172 in the genetic network that controls SE induction by identifying the down- and upstream targets of miR172 in an embryogenic culture. The present study demonstrated that miR172 controls SE by targeting AP2, which represses the WUS expression. It also revealed that the miR172-AP2-imposed control of WUS in SE involves histone acetylation via the AP2-mediated recruitment of the histone deacetylases HDA6/HDA19 to the target gene. Moreover, it indicated upstream regulatory elements of the miR172-AP2-WUS module that involve miR156-controlled SPLs regulating the level of mature miR172 during SE. The results provided new components of the miRNA- and Hac-mediated pathways of the SE-regulatory network.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The Columbia (Col-0) seeds of Arabidopsis thaliana (L.) Heynh were supplied by NASC (The Nottingham Arabidopsis Stock Centre, Nottingham University, Nottingham, UK). The transgenic lines with a mutation in the MIRNA172 (miR172b—N670873; miR172c—N673321; miR172d—N866316), miR172 target genes (toe1—N668454; toe2—N655709, toe3—CS825725, smz—N664087, snz—N668027, ap2—N571140) were purchased from the SALK Institute Genomic Analysis Laboratory database and the Syngenta Arabidopsis Insertion Library (SAIL). The 35S::MIR172 line was kindly provided by Dr. J. Palatnik (Research Council, Institute of Molecular and Cell Biology in Rosario, Argentina), 35S::MIM172 from Dr. Detlef Weigel (Max Planck Institute for Developmental Biology, Tübingen, Germany) and hda6 and hda6 hda19 by Kim Boutillier (Wageningen University & Research, Wageningen, The Netherlands). The 35S::MIR172D line had a higher level of mature miR172 molecules (Supplementary Figure S1). In the MIM lines (35S::MIM172, 35S::MIM156), the overexpressed transcripts with multiple miRNA-binding sites competed with the endoge-
nous targets, thereby abolishing the function of all of the miRNA family members [72]. The 35S::AP2-ER and 35S::SPL9-ER lines with a β-estradiol-induced overexpression were purchased from the TRANSPLANTA collection [73]. In addition, the insertional mutants in TOPLESS (tp1—N68599, tp1—N529936, tp4—N502209), SPLs (6mSPL10—N66332, 6mSPL11—N66336) and 35S::MIM156 (N9953) were studied. In the 6mSPL10 and 6mSPL11 lines, the mutation disrupted the miR156-binding site [74]. The plants that were used for the explants for the in vitro cultures were grown in Jiffy pots (Jiffy, Zwijndrecht, The Netherlands) in a “walk-in” type Phytotron under controlled conditions (20–22 °C, 16 h/8 h L/D photoperiod, light intensity of 100 µE m⁻² s⁻¹). The cultures that were grown in vitro were maintained in a controlled-growth chamber at 22 °C, 16 h/8 h (light/dark) and a light intensity of 40 µM m⁻² s⁻¹.

2.2. Somatic Embryogenesis Induction

Somatic embryogenesis was induced in vitro according to Gaj [75] and immature zygotic embryos (IZE) in the bent cotyledonary stage (10–12 DAP) were used as the explants. A solid B5-based [76] medium (E5) that had been supplemented with 20 g/L sucrose, 8 g/L Oxoid agar (Oxoid, Hampshire, UK) and 5 µM 2,4-D (2,4-dichlorophenoxyacetic acid, Sigma, St. Louis, MO, USA) was used to induce SE. In some of the experiments, the E5 medium was supplemented with a chemical inhibitor of the HDAC activity, trichostatin A (TSA; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 µM or β-estradiol (5 µM; Sigma-Aldrich, St. Louis, MO, USA) to induce an overexpression in the transgenic lines.

The morphogenic potential of the transgenic lines for SE was evaluated using two parameters: SE efficiency, which was calculated as the frequency of the explants that produced somatic embryos, and SE productivity, which was calculated as the average number of somatic embryos per explant. Thirty explants in three replicates were evaluated for each genotype.

2.3. Analysis of Mature miRNA and Target Genes Expression

Total RNA was isolated from the explants that had been induced on the E5, E5 + E, and E5 + TSA media for 0, 5 and 10 days. To isolate the RNA from the 0 d culture, IZEs that had been dissected from siliques in a drop of water were immediately transferred to 5 mL of RNAlater (Life Technologies, Carlsbad, CA, USA) and then treated the same way as the explants that had been cultured for 5 and 10 d. Total RNA with miRNA were isolated using an miRVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). Depending on the age of the culture, 250 (0 d) to 50 (10 d) explant-derived cultures were used for RNA isolation in one biological replicate. RNA concentrations were measured using a Nano-Drop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The DNA was removed from the RNA samples by digesting them with RQ1 RNase-free DNase (Promega, Medison, WI, USA). The miRNA-specific and oligo-dT primers and a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) were used to produce the cDNA. The mature miRNAs were identified according to the Speth and Laubinger [77] method. The cDNA was diluted 1:4 and used for the Real-Time qPCR analysis. The qPCR was performed using a LightCycler® 480 SYBR Green I Master kit (Roche, Basel, Switzerland) and the primers that were relevant to the genes being studied were used to determine the Real-Time RT qPCR reactions (Supplementary Table S1). The control gene had a constant expression level (C_T = 18 ± 1) in all of the tissue samples. The Ct values were calculated using LinRegPCR software (version 11, Academic Medical Centre, Amsterdam, The Netherlands). The plant tissues for the Real-Time qPCR analysis were produced in three biological repetitions and two technical replicates of each repetition were analyzed. The relative expression level was calculated using 2^{ΔΔCT}, where ΔΔCT represents ΔC_{T_{reference}} - ΔC_{T_{compared condition}}.
2.4. Chromatin Immunoprecipitation (ChIP)

The ChIP method of Yelagandula et al. [78] with some modifications that were described by Nowak et al. [71] was used. Chromatin was extracted from the cultured explants (~50 mg of tissue) that had been treated with 1% formaldehyde for 20 min on ice under a vacuum. The chromatin was sheared using sonication (Bioruptor® Plus, Diagenode, Denville, NJ, USA). The complex of proteins and DNA fragments was then immunoprecipitated using the polyclonal antibodies against the acetylated forms of histone H3 (2 μg; Merck, St. Louis, MO, USA, Cat. no. 06-599). The DNA that was cross-linked to the immunoprecipitated proteins was reversed and analyzed using qPCR and the gene-specific primers. A LightCycler 480 (Roche, Basel, Switzerland) real-time detection system was used to analyze the relative acetylation level of the WUS-gene-associated chromatin. The qPCR reaction was conducted according to Nowak et al. [71]. The primers that were used in the qPCR were designed using Primer3Plus software (version 3, Molbi, Michelstand, Germany). The genomic sequence that was analyzed in the WUS gene was localized in the TSS + 300 bp region and 3′UTR (Supplementary Table S1). The Ct values were calculated using LinRegPCR software (version 11, Academic Medical Centre, Amsterdam, The Netherlands). The ChIP–qPCR data were normalized using the percent-input method. The H3ac level is presented as 2(adjusted input − Ct (xx gene) * 100%. Three biological replicates and two technical replicates were analyzed for each combination.

2.5. Statistical Analysis

The Student’s t-test and a two-way ANOVA (p < 0.05) followed by Tukey’s honestly-significant-difference test (Tukey HSD-test) (p < 0.05) were used to calculate any significant differences between the experimental combinations. The graphs show the average values with the standard deviation (SD); the statistical analysis was performed using the medians.

3. Results

3.1. Functional Analysis of the MIRNA172 Genes during SE

In a global analysis of pri-miRNA during SE, we found that five members of the MIRNA172 gene family (MIR172a-e) were differentially expressed in the SE-induced explants of Arabidopsis [26].

To verify the hypothesis on the SE-related function of miR172, we analyzed the embryonic potential of different lines with a disturbed expression/function of miR172, including T-DNA insertion mutants (miR172b, miR172c, miR172d), MIM line (35S::MIM172) and the overexpression line (35S::MIR172D). We found that all of the miR172-defective lines significantly impaired the SE response and both SE parameters, and that SE efficiency and SE productivity were reduced in the mutant cultures compared to the WT (wild type), Col-0 (Figure 1A,B).

The results indicate that a disturbed transcription of MIR172 genes results in an impaired embryonic response in the explants. The results also suggest that different MIRNA genes, including MIR172b, c, and d contribute to SE induction and that the tightly regulated miR172 level determines an efficient embryonic response. Therefore, the contribution of miR172 to SE regulation might be postulated in Arabidopsis.
Figure 1. miR172 contributes to the embryogenic potential of a culture. There was a significant decrease in the SE efficiency and SE productivity of the miR172-affected genotypes, including the miR172 mutants (miR172b, miR172c, miR172d), the MIR172D-overexpressing line (35S::MIR172D) and the MIM line (35S::MIM172) vs. the WT control (Col-0) culture (A). There was also a decrease in the embryogenic responses of the miR172b, miR172c, miR172d mutants compared to the Col-0 culture (B). The explants were induced on an auxin (E5) medium and the embryogenic response was evaluated in 21-day-old cultures. * values significantly different from the WT, Col-0 (p < 0.05; n = 3 ± SD).

3.2. Targets of miR172 in SE Induction

The candidate miR172 targets include the transcription factors (TFs) that control plant flowering in vivo [79]. We used quantitative PCR to verify whether the expression of the miR172 targets is modulated during SE induction in the Col-0 genotype. An analysis of the candidates revealed a significant modulation of the TOE1, 2, 3, SMZ, SNZ and AP2 transcripts and an increased TOE1 and TOE2 expression and decreased SMZ, SNZ, AP2 and TOE3 transcription in the SE-induced explants of Col-0 (Figure 2).

To validate the involvement of the candidate miR172 targets in SE induction, we evaluated the embryogenic response of the toe1, 2, 3, smz, snz and ap2 mutants and the 35S::AP2-ER-overexpression line. The results showed that all of the analyzed genotypes had a significantly defective embryogenic potential, which was manifested by a reduced SE efficiency and/or productivity (Figure 3A,B). The results support the assumption that the TOE1, 2, 3, SMZ, SNZ and AP2 genes contribute to SE induction in Arabidopsis.
Figure 2. The expression level of the candidate miR172 targets, including TOE1, TOE2, TOE3, SNZ, SMZ and AP2 in the embryogenic culture of Col-0. The relative transcript level was normalized to an internal control (At4g27090) and calibrated to 0 d of the culture. * values significantly different from the freshly isolated 0 d explants ($p < 0.05; n = 3 \pm SD$).

To further verify the regulatory relationship between miR172 and the candidate targets in SE induction, the transcription levels of TOE1, 2, 3, SMZ, SNZ and AP2 were evaluated in the 35S::MIM172 and 35S::MIR172D cultures, which had a disrupted miR172 function and an increased miR172 level, respectively (Figure 4). The analysis showed that two candidate targets of miR172, TOE1 and AP2, had contrasting expression levels in the 35S::MIM172 vs. the 35S::MIR172D cultures. Accordingly, the TOE1 and AP2 transcripts were substantially increased in the MIM172 (Figure 4A) and decreased in the 35S::MIR172D culture (Figure 4B). The results infer a role of miR172 in the control of AP2 and TOE1 in SE induction. In contrast to TOE1 and AP2, the expression profiles of TOE2, 3, SNZ and SMZ in the 35S::MIM172 and 35S::MIR172D cultures were not indicative of their regulatory dependence on miR172 in SE. In plant development in vivo, the WUS TFs that have a regulatory function in SE was indicated among AP2 targets [17,57,80]. Hence, we focused our further analysis on the role of the miR172-AP2-WUS pathway in controlling the embryogenic response.
Figure 3. The impaired embryogenic response in the cultures affected the candidate miR172 target genes, including the smz, snz, toe1, toe2, toe3 and ap2 mutants and the AP2-induced overexpression line, 35S::AP2-ER, compared to the WT culture, Col-0. The significantly impaired SE efficiency and productivity (A) of the analyzed genotypes were exemplified by the reduced embryogenic response of the representative mutants snz, toe3, ap2 (B). The explants were induced on an auxin (E5) medium and the embryogenic response was evaluated in 21-day-old cultures. The AP2 overexpression was induced with β-estradiol (+E). * values significantly different from the WT, Col-0 (p < 0.05; n = 3 ± SD); #—values significantly different from the 35S::AP2-ER -E culture (p < 0.05; n = 3 ± SD).
Figure 4. Expression analysis of the candidate miR172 target genes (TOE1, TOE2, TOE3, SNZ, SMZ, AP2) in the embryogenic cultures of the 35S::MIM172 (A) and 35S::MIR172D (B) lines with a disrupted miR172 function and an increased miR172 level, respectively. The relative transcript level was normalized to an internal control (At4g27090) and calibrated to the WT (Col-0) culture of the same age (0d, 5d and 10d). * values significantly different from the Col-0 culture of the same age (p < 0.05; n = 3 ± SD).

3.3. miR172 Regulates WUS TF of Critical Function during SE via AP2

To gain insight into the regulatory relationship between AP2 and WUS in SE induction, the expression of WUS was analyzed in the SE-induced explants with an increased (35S::AP2-ER) or defective (ap2) AP2 activity. We found that the level of the WUS transcript was decreased and increased in the SE-explants of increased (35S::AP2-ER) and an impaired (ap2) AP2 expression, respectively, which suggests that AP2 negatively regulates WUS in SE induction (Figure 5A). Because we had assumed that AP2 would be under the control of miR172 during SE (Figure 4), we examined whether disrupting the miR172 activity would affect the expression level of the candidate AP2 target, WUS. The results showed a decrease in the WUS transcripts in the 35S::MIM172 culture, which suggests a regulatory relationship between miR172 and WUS during SE (Figure 5B).

To summarize, the results provided evidence that miR172 might indirectly regulate the expression of the WUS TFs that play a critical role in SE induction by controlling AP2.
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Figure 5. Expression analysis of WUS in the SE culture in AP2-induced overexpression line and ap2 mutant (A) and 35S::MM172 line with a disrupted miR172 function (B). AP2 overexpression was induced with β-estradiol (+E). The relative transcript level was normalized to an internal control (At4g27090) and calibrated to the WT (Col-0) culture of the same age (0d, 5d and 10d). * values significantly different from the Col-0 culture of the same age (p < 0.05; n = 3 ± SD); #—values significantly different from the 35S::AP2-ER +E culture of the same age (p < 0.05; n = 3 ± SD).

3.4. AP2 Represses the WUS Expression via a HDAC-Mediated Histone Acetylation

We assumed that the AP2-mediated repression of WUS in SE induction might involve a HDAC-controlled histone deacetylation. It is known that histone acetylation is involved in the AP2-mediated mechanism of target-gene repression during plant development [81,82]. To verify whether Hac contributes to the AP2-controlled WUS expression during SE, we analyzed the WUS transcription level in the explants that overexpressed AP2 and those that had been treated with an inhibitor of HDAC, trichostatin A (TSA), to block the HDAC function. The TSA-induced explants had an accumulation of the WUS transcript in response to an AP2 overexpression (Figure 6A). A similar tendency was observed for the Col-0 culture that had been treated with TSA. The results suggest that a repressive activity of AP2 on WUS expression might be exerted via the HDAC-related pathway. The candidate HDAC with an assumed role in controlling the SE-involved genes include the HDA6 and HDA19 histone deacetylases [66,71]. Our analysis of the WUS expression relative to the HDA6/HDA19 expression revealed an elevated WUS transcript in the SE-induced explants of the hda6 and hda6 hda19 mutants (Figure 6B). The results imply that AP2 might repress the expression of the WUS gene during SE via a HDA6/HDA19-mediated histone deacetylation. To find further evidence for the assumption that Hac is involved in the AP2-mediated regulation of the WUS expression, we examined changes in the H3 histone acetylation marks (H3K9ac and H3K14ac) in the chromatin that are associated with the TSS + 300 bp fragment of the WUS relative to the AP2 expression. The results of the ChIP analysis indicated that the level of H3 acetylation in the WUS-bound chromatin region (TSS + 300 bp) was increased in the ap2 mutant and decreased in the AP2 overexpressing cultures, respectively (Figure 6C). In contrast to the TSS + 300 bp, the chromatin fragment that was bound to the 3′UTR of
WUS had a similar H3ac level in the cultures of Col-0, the AP2-overexpression line and the ap2 mutant (Supplementary Figure S2). To summarize, we documented that AP2 might negatively control the WUS gene in SE induction via a Hac-related mechanism in which HDA6 and HDA19 seem to be involved.

**Figure 6.** A Hac-related mechanism is involved in the AP2-mediated repression of WUS during SE. Expression analysis of WUS in the SE culture in the AP2-overexpression line untreated (-TSA) and the one that had been treated (+TSA) with TSA (trichostatin A) (A) and the hda6 and hda6 hda19 mutants (B). Hac enrichment in the chromatin fragment that was bound to the TSS + 300 bp region of the WUS gene promoter in the explants of Col-0 (WT), 35S::AP2-ER and ap2 (C). AP2 overexpression was induced with β-estradiol (+E). The HDAC activity was inhibited by the TSA treatment in the Col-0 control (Col-0 + TSA) and in the AP2-overexpressing (35S::AP2-ER + TSA) culture. The relative transcript level was normalized to an internal control (At4g27090) and calibrated to the WT (Col-0) culture of the same age (0 d, 5 d, and 10 d). * values significantly different from the Col-0 culture of the same age (p < 0.05; n = 3 ± SD); #—values significantly different from the 35S::AP2-ER + E culture of the same age (p < 0.05; n = 3 ± SD).

3.5. TPL Co-Repressors Might Contribute to the AP2-Mediated Repression of WUS during Embryogenic Induction

Next, we hypothesized that the TOPLESS (TPL) co-repressors that directly interact with AP2 during plant development [81] would also be involved in the AP2-mediated repression of the WUS gene during SE. To verify this assumption, we evaluated the expression level of WUS in the tpi/tpr mutant cultures and compared it to the Col-0 control of the same age. The analysis showed that the WUS gene was significantly up-regulated in the SE-induced explants of the tpi, tpr1 and tpr4 mutants (Figure 7). The results suggest a contribution of the TPL (TPL, TPR1 and TPR4) co-repressors to the AP2-mediated negative control of WUS in SE induction.
3.6. miR156 Might Control the miR172-AP Regulatory Module during SE by Targeting SPL9/10

Our results provide some evidence that miR172 might control the WUS gene in SE induction by repressing AP2. Next, we attempted to identify the upstream regulators of the miR172-AP2-WUS regulatory module. The candidate regulators included the miR156-repressed SPL (SQUAMOSA-PROMOTER BINDING PROTEIN-like) TFs that control the miR172-AP2 module during the floral transition [45,51,83]. Therefore, to verify the regulatory relationship between the miR156- and miR172-controlled modules in SE induction, we evaluated the accumulation of miR172 in the SE-induced explants with an abolished miR156 function (35S::MIM156) (Figure 8A) and an overexpression of three of the SPL genes, SPL9/SPL10/SPL11 in the culture of 35S::SPL9-ER, 6mSPL10 and 6mSPL11 lines (Figure 8B). The results showed a decrease in the mature miR172 levels in 35S::MIM156 and SPL9/10-overexpressing lines (Figure 8). Thus, the results imply that miR156 might negatively control miR172 in embryogenic induction in Arabidopsis by targeting SPL9 and 10.

To summarize, the study provides several pieces of evidence on new, miR172-related regulatory components of the genetic network that underlies SE induction. In this mechanism, miR172-controlled AP2 in cooperation with the TPL co-repressors and HDA6/HDA19 negatively controls WUS during SE. The upstream-acting elements that control the miR172-AP2 module during SE might also involve the miR156-SPL regulatory module (Figure 9).
Figure 8. Level of mature miR172 in the embryogenic culture of 35S::MIM156 with a disrupted miR156 function (A) and an SPL9-overexpression line (35S::SPL9-ER) and the 6mSPL10 and 6mSPL11 mutants with a disrupted miR156-binding site (B). SPL9 overexpression was induced with β-estradiol (+E). The relative miRNA level was normalized to an internal control (At4g27090) and calibrated to the WT (Col-0) culture of the same age (0 d, 5 d, and 10 d). *—value significantly different from the Col-0 culture of the same age (p < 0.05; n = 3 ± SD).

Figure 9. A putative model of the miR172-mediated regulatory interactions that control SE induction by repressing AP2 controls the WUS TF with a regulatory function in embryogenic induction. The AP2-controlled repression of WUS involves a Hac-related mechanism in which AP2 might recruit TOPESS co-repressors (TPL, TPR1, TPR4) and histone deacetylases, HDA6 and HDA19, which are the central components of the SIN3/HDAC gene-silencing complex. Other miR172-related regulatory elements that might regulate SE induction include the miR156-SPL9/10 module, which has a negative impact on miR172.
4. Discussion

4.1. miR172 Controls SE Induction by Targeting AP2

The genetic network that controls the SE induction involves complex regulatory interactions in which miRNA-mediated gene repression plays a substantial role [4]. Consistent with this notion, the differential expression of numerous pri-miRNAs of different MIRNA gene families, including MIRNA172, was found in SE-induced explants of Arabidopsis [26]. Like Arabidopsis, a differential transcription and accumulation of MIRNA172 and mature miR172, respectively, have also been associated with SE induction in other plants, thus further ensuring the role of miR172 in SE regulation [27,28,84–86]. However, the miR172-mediated regulatory mechanism, including the up- and downstream targets of miR172 in the embryogenic induction, is as yet unknown.

Therefore, we conducted a functional analysis of miR172 in the SE of Arabidopsis. The results showed that both an overexpression (35S::MIR172D) and a disturbed function (35S::MIM172) of miR172 resulted in an impaired embryogenic response of the explants (Figure 1). Congruently, the genotypes that were affected in AP2, which is the miR172 target, including the ap2 mutant and AP2-overexpression line, also had a reduced SE induction (Figure 3). The impaired SE response was also characteristic of the genotypes with an increased or decreased expression/activity of other SE-regulators, including LEC2, ARF5 (AUXIN RESPONSE FACTORS), and miR393 [16,32,87,88]. Thus, we assume that a strictly controlled level of miR172 and AP2 seems to be required for efficient SE induction.

Two alternative modes of miR172-mediated gene regulation, cleavage and a translation inhibition of the target mRNA, have been suggested [89–92]. The opposite level of miR172 vs. the AP2 and TOE1 transcripts (Figure 4) suggests that miR172 might control these genes in SE at the transcriptional level via mRNA cleavage. The regulatory role of the miR172-AP2 module in the developmental transition that is associated with plant flowering [80] and a postulated similarity of the flower regulators to that controlling the embryogenic transition of somatic cells [12,48] motivated us to investigate the downstream target of the AP2 TF during SE. The results of the gene-expression profiling in the miR172-affected cultures (Figure 4) suggests that unlike AP2 and TOE1, other potential targets, including TOE2, 3, SNZ and SMZ, seem not to be regulatory dependent on miR172 in SE. In contrast to SE, miR172 controls these genes during plant flowering and the development of the floral organs [37,51,52]. The report and the present results imply that the targets of miR172 differ between the developmental processes. In line with the distinctly different expression profiles of the members of the MIRNA172 gene family in plant developmental processes, including SE [26,44,45,93,94], the regulatory relations between miR172 and the targets are highly specific to the developmental context. In support of this, miR172 controls the AP2 gene in flower development [93], whereas it targets TOE1 and TOE2 to promote juvenile epidermal identity in the production of the trichomes [51]. It is worth noting that the two closest AP2 homologues, TOE3 and TOE1, do not act redundantly to AP2 in stem-cell maintenance [57]. In the control of flowering time, SMZ, SNZ, TOE1 and TOE2 are involved but not TOE3 [46].

4.2. The AP2 Module Controls WUS in Embryogenic Induction via a Hac-Related Repression

In the shoot apical meristem, AP2 regulates WUS in order to control the WUS-CLV3 (CLAVATTA3) feedback loop that has an essential function in stem-cell maintenance [57]. WUS, a member of the plant-specific homeobox superfamily of WOX TFs, has also been implicated in controlling auxin-induced SE [17]. The early induction of WUS expression is critical for embryo development in the embryogenic callus of Arabidopsis [95]. The interaction of WUS with the LEC-controlled pathway of SE induction has also been postulated [96]. However, the upstream regulators that control the auxin-gradient-induced WUS expression in SE have not yet been identified.

We provide some evidence that AP2 might repress WUS expression in controlling the embryogenic transition in Arabidopsis. In support of this, we found that there was an opposite effect of the ap2 mutation vs. AP2 overexpression of WUS expression during SE
The presence of the AP2-recognized cis-element in the WUS promotor suggests that AP2 might directly control WUS transcription. Further analysis is required to verify the role of the AP2-WUS interactions in regulating WUS expression during SE.

We found histone-acetylation-related mechanisms of the AP2-mediated repression of the WUS gene in SE induction. In support of this, the repressive effect of AP2 on the WUS transcripts was abolished in cultures that had been treated with TSA, which is an HDAC inhibitor (Figure 6). In addition, the AP2 expression negatively affected the H3ac level in the WUS TSS + 300 region in the SE-induced explants.

Both activating and repressive functions of AP2 in controlling gene expression have been documented [80]. AP2 interacts with the SIN3/HDAC-silencing-complex components, including the transcriptional co-repressors and HDAC, in order to repress the target genes [81,82]. The histone deacetylases that cooperate with AP2 include HDA6 and HDA19 [82]. Our findings of an increased WUS expression in the SE-induced hda6 and hda6 had19 explants (Figure 6) support the assumption of the role of HDA6 and HDA19 in the AP2-mediated repression of the WUS gene during embryogenic transition.

In the gene-repression mechanism, TFs cooperate with the transcriptional co-repressors, and in order to repress its targets, AP2 recruits the TOPOLESS co-repressors into the SIN3/HDAC-silencing complex [81,82]. The results imply a role of TPL, TPR1 and TPR4 in the AP2-mediated repression of the WUS gene in an embryogenic culture (Figure 7). Accordingly, the tpl/tpr mutant cultures had a higher WUS expression. Two of these co-repressors, TPL and TPR1, can physically interact with AP2 via the EAR (Ethylene-responsive element-binding factor that is associated with amphiphilic repression) domain [81], but the character of the interactions between AP2-TPL/TPR1 needs to be verified during SE.

The importance of gene repression in plant development, including SE induction, is increasingly being recognized [97–99]. However, identifying the regulatory components of the gene-silencing mechanisms during SE has just begun. Recently, the SIN3/HDAC-silencing complex was reported to transcriptionally control the genes that are involved in the miRNA biogenesis during SE [71]. In this mechanism, AGL15 recruits the TPL/TPR co-repressors and cooperates with HDA6/HDA19 in order to repress the targets, DCL1 (DICER-like3), SERRATE and HEN1 (HUA-ENHACER1) [71]. We postulated that a similar silencing complex might repress WUS transcription in SE induction. To silence the WUS in SE, AP2 TF might recruit the TPL, TPR1 and TPR4 co-repressors and HDA6/HDA19 into the SIN3/HDAC complex.

TPL/TPRs might also physically interact with numerous other TFs, including members of the AUX/IAA, MYB, NAC and MADS gene families that have been indicated/suggested as being involved in SE induction [8,12,13,17,100,101]. Thus, we assumed that some of these TFs might have a regulatory function in SE induction as a part of the SIN3/HDAC complex. Identifying the TFs that contribute to the SIN3/HDAC-complex-mediated regulation seems to be interesting in order to further reveal the SE-regulatory pathways.

### 4.3. miR156-Targeted SPL9 and SPL10 Negatively Regulate the miR172 Level during SE

The study revealed the role of the miR172-AP2 module in regulating WUS in SE induction. During plant development, the upstream regulators of miR172-AP2 and other miR172-AP2-like modules, include the miR156-SPLs [46]. The regulatory functions of the miR156-SPLs in the fundamental developmental processes have been documented [102], including the involvement of the miR156 and SPL10 and 11 genes in zygotic-embryo development [74]. We assumed that the miR156-SPL module might also control SE. In support of this hypothesis was the opposite expression levels of the mature miR156 vs. the SPL9, 10 and 11 transcripts that have been reported in an embryogenic culture of Arabidopsis [26]. Moreover, an analysis of transgenic lines in a callus culture of citrus provided some evidence that miR156 enhanced the SE induction by silencing SPL3 and SPL14 [33].

During the floral transition, the miR156-regulated SPLs, including SPL9/10 and 15, positively affect the expression of the MIRNA172 genes [45,51,103]. Our analysis of the
Arabidopsis explants that overexpressed components of the miR156-SPL module indicated that the SPL9 and SPL10 negatively control the amount of miR172 in SE induction (Figure 8).

The SPL-mediated regulatory mechanisms involve the positive control of targets by direct binding of SPLs to target promoters and competitive interactions with other regulatory proteins, including TCP4 (TEOSINTE BRANCHED 1 CYCLOIDEA, PCF1 FAMILY TRANSCRIPTION FACTOR 4) and the CUC (CUP-SHAPED) TFs, in the transcriptional complexes [104,105]. On the other hand, the SPL-mediated repression of gene transcription has also been documented. In support of this, SPL9 negatively controlled the LAS (LATERAL SUPPRESSOR), which is a regulator in the formation of the axillary bud in Arabidopsis [106].

The presence of the EAR repression domain whose role is to recruit HDAC into the silencing complex [107,108] in some of the SPLs (PlantEAR database- [109]) provides a hint that Hac might be involved in SPL-controlled repression, including the negative control of miR172 by SPL10 during SE.

The assumed mechanism by which SPL9, which lacks the EAR domain, represses miR172 during SE might involve SPL9 interactions with the components of the phytohormone-signaling pathways such as gibberellin (GA) and strigolactone (SL) [110,111]. Given the central function of the phytohormones, including GA, in controlling the SE-involved genes, the contribution of the DELLA and D53-like regulators of GA and SL signaling pathways, respectively, to the SPL9-mediated repression of miR172 might also be of interest in future studies on SE [112].

To summarize, we provide some evidence that miR156-regulated SPL9/SPL10 [26] might control the miR172-AP2 node in order to repress WUS expression in SE induction in Arabidopsis. Similarly, the direct and indirect impact of the miR156-SPLs on WUS in controlling the SAM size has also been reported [113]. Moreover, the physical interactions of the SPL9 and WUS proteins that have been found for some soybean orthologues suggest that the SPL proteins might also post-translationally regulate WUS activity [114]. Further analyses are required in order to verify the regulatory interactions between the components of the miR156-SPLs-miR172-AP2-WUS during SE.

4.4. Other Candidate Components of the miR172-Mediated Pathways in SE, AGL15 and TOE1

The versatile regulatory interactions of the miR172-AP2 module during plant development [46] suggest that besides WUS, other genes might be targeted by AP2 during SE. Insight into the genes that are controlled by miR172-AP2 during the floral transition [115] that have a postulated regulatory similarity to SE induction [8,11,12] revealed that AGL15 plays a role during SE [18]. AGL15 contributes to SE induction by controlling the genes involved in the hormone and stress responses and miRNA biogenesis [71,116–118]. Regulatory interactions between AGL15 and other SE-involved TFs, including the LEC genes, have also been documented [3]. However, little is known about the upstream elements that control the AGL15 expression during the embryogenic response. We assume that AP2 might activate the AGL15 expression during SE as we found a positive impact of AP2 on AGL15 expression (Supplementary Figure S3). The role of AP2-AGL15 regulatory interactions and the relevance of the AGL15 expression to miR172 during SE remain to be explored in future research.

Our results suggest that, besides AP2, miR172 might repress another AP2-like TF, TOE1, during SE. The hypothesis on the TOE1 function in SE induction is of particular interest given the interactions of TOE1 with KANADI1 (KAN1), which is the regulator of the development of the abaxial trichome of leaves [119]. Like leaves, the cotyledons have an adaxial–abaxial polarity [120] and the adaxial side of cotyledon exclusively contributes to somatic-embryo development in Arabidopsis [121,122]. In support of the role of the cotyledon polarity in the SE-induction mechanism, miR165/miR166 and PHB had adaxial-specific expression patterns in the SE-induced explants [30,123]. Importantly, during SE, to control the abaxial leaf identity, KAN1 targets the genes that are engaged in auxin biosynthesis, auxin transport and auxin response [124], which play central roles in the
SE-induction mechanism [3]. Therefore, the TOE1 function relative to KAN1 and the adaxial-specific SE induction needs to be addressed in future research on the miR172-mediated control during SE.

5. Conclusions

The coordinated actions between the miRNAs and epigenetic modifications are believed to significantly contribute to regulating gene expression during developmental processes [35,125,126]. The present study suggests a contribution of miRNAs and Hac to the regulation of WUS during the embryogenic response of Arabidopsis explants. We provide some evidence that the miR172-AP2 regulatory node under the control of the miR156-SPL module might repress WUS expression in SE induction in Arabidopsis via a Hac-related mechanism. In this mechanism, the cooperation of the AP2 TF with the TPL/TPR/TPR4 co-repressors results in the recruitment of HDACs such as HDA6/HDA19 into the SIN3/HDAC-silencing complex.

Other candidates in the HDAC-mediated regulation include hundreds of TFs that have the EAR repression domain [109]. Within these, the transcriptional repressors VAL1 and VAL2, which have been indicated as playing a role in SE induction [127], seem to be of particular interest in studies on epigenetics, including a Hac-mediated gene regulation during SE. VAL1/VAL2 recruit the histone-methylation-related Polycomb Repressive Complex 2 (PRC2) into gene silencing in Arabidopsis [128]. Interestingly, VAL1 has recently been found to couple the PRC2-controlled histone methylation with HDAC in the mechanism of gene repression [129]. Thus, we assume that the VAL genes would be of particular interest in studies on the embryogenic reprogramming of the plant somatic cells and, in particular, on the interplay of Hac with other epigenetic modifications such as histone methylation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells11040718/s1, Figure S1. Level of mature miR172 in the embryogenic culture and 14-day-old seedlings of the 35S::MIR172D line. The relative miRNA level was normalized to an internal control (At4g27090) and calibrated to the WT (Col-0) culture of the same age (0 d, 5 d, and 10 d) and 14-day-old seedlings. *—value significantly different from the Col-0 culture of the same age (p < 0.05; n = 3 ± SD) and 14-day-old seedlings. Table S1: List of the primers that were used for the Real Time qPCR analysis. Figure S2. H3ac enrichment in the chromatin fragment that was bound to the 3′UTR of the WUS gene in the explants of Col-0 (WT), 35S::AP2-ER and ap2. AP2 overexpression was induced with β-estradiol (+E). A statistical analysis indicated no differences between the compared combinations (p < 0.05; n = 3 ± SD). Figure S3: Expression analysis of AGL15 in the SE culture in AP2-induced overexpressor line and the ap2 mutant. The relative transcript level was normalized to an internal control (At4g27090) and calibrated to the WT (Col-0) culture of the same age. * values significantly different from the Col-0 culture of the same age (p < 0.05; n = 3 ± SD); #—values significantly different from the 35S::AP2-ER-E culture of the same age (p < 0.05; n = 3 ± SD).

Author Contributions: Conceptualisation, K.N. and M.D.G.; methodology, K.N., J.M., M.G. and A.S.-S.; formal analysis, K.N., J.M., M.G. and A.S.-S.; investigation, K.N., J.M., M.G. and A.S.-S.; data curation, K.N., J.M. and A.S.-S.; writing—original draft preparation, K.N.; writing—review and editing, M.D.G.; visualisation, K.N., J.M. and M.G.; supervision, M.D.G.; project administration, M.D.G.; funding acquisition, M.D.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a research grant from the National Science Centre in Poland (OPUS13 2017/25/B/NZ1/01615) and co-financed by funds granted under the Research Excellence Initiative of the University of Silesia in Katowice.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.
Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

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