**Results**

**WOX5 homeostasis is essential for the maintenance of QC identity**

To investigate the role of WOX5 in mediating auxin signaling for root QC maintenance, we conducted experiments involving the overexpression of *35S::WOX5-GFP* plants and assessed their impact on root QC identity. In 5-day-old wild-type (WT) seedlings, fewer than 10% exhibited root QC division, where the QC cell underwent horizontal division to form two cells. In contrast, approximately 80% of *wox5-1* mutant seedlings displayed QC division in their roots (Fig. 1A, B), suggesting that the loss of WOX5 function was responsible for this phenotype. Interestingly, *35S::WOX5-GFP* seedlings also exhibited approximately 30% QC division (Fig. 1A, B), while *pWOX5:WOX5-GFP* seedlings showed 15% QC division (Fig. 1A, B). These findings collectively demonstrate that both overexpression and functional loss of WOX5 can result in an increased rate of QC division. Thus, maintaining WOX5 homeostasis is essential for the maintenance of root QC identity.

**MPK3/MPK6 interact with and phosphorylate WOX5**

MPK3/MPK6 have been demonstrated to play a role in regulating root development. To investigate whether WOX5 homeostasis is influenced by MPK3 and MPK6, we initiated our study by examining the protein interaction between WOX5 and MPK3/MPK6 in *Arabidopsis* protoplasts through a bimolecular fluorescence complementation (BiFC) assay. Our findings revealed robust fluorescence signals when CYFP-WOX5 and NYFP-MPK3 or NYFP-MPK6 were co-expressed in protoplasts, contrasting with the absence of YFP signal in negative controls (Fig. 2A). This result clearly indicates a physical interaction between WOX5 and MPK3/MPK6. Subsequently, we confirmed this protein interaction using a yeast two-hybrid experiment system (Fig. 2B). Furthermore, we validated the interaction between WOX5 and MPK3/MPK6 through co-immunoprecipitation (Co-IP) assays in *Arabidopsis* mesophyll protoplasts, where YFP-MPK3 or YFP-MPK6 proteins were immunoprecipitated by anti-MYC antibodies co-expressing WOX5-MYC (Fig. 2C and D). Taken together, these results strongly support the conclusion that WOX5 indeed interacts with MPK3/MPK6.

Given the interaction between MPK3/MPK6 and WOX5, we next examined whether MPK3/MPK6 phosphorylate WOX5. We conducted in vitro phosphorylation experiments involving recombinant GST-labeled WOX5, MPK3, MPK6, and MKK5DD proteins. Activation of MPK3 and MPK6 was achieved in vitro through MKK5DD, which is the constitutively active form of MKK5. The results clearly demonstrated robust phosphorylation of WOX5 when GST-MPK3 or GST-MPK6 was activated by MKK5DD (Fig 3A).

To pinpoint the exact phosphorylation sites on WOX5 targeted by MPK3/MPK6, we carried out in vitro phosphorylation mass spectrometry assays. These assays detected phosphorylation sites at T45 and S107 when MKK5DD-MPK3-WOX5 was analyzed (Fig S1A), while S107 was identified as a phosphorylation site in MKK5DD-MPK6-WOX5 (Fig S1B). Notably, when WOX5 protein was assessed alone, no phosphorylation sites were detected. Subsequently, we conducted in vitro phosphorylation assays after replacing the serine or threonine residue at the corresponding site of WOX5 with alanine, thus creating non-phosphorylatable forms (WOX5T45A, WOX5S107A, and WOX5T45A/S107A) (Fig S2). These experiments revealed that when both serine/threonine residues were mutated to alanine (WOX5T45A/S107A), the phosphorylation of WOX5 by MPK3/MPK6 was significantly reduced (Fig 3B). These findings collectively indicate that MPK3 and MPK6 phosphorylate WOX5, with the main phosphorylation sites being Thr 45 and Ser 107. The significance of these two specific amino acid sites for WOX5's function was underscored by the successful complementation of the *wox5*-1 mutant phenotype using both a phosphor-inactive variant, denoted as *pWOX5:WOX5T45A/S107A*, and a phosphor-mimic variant, designated as *pWOX5:WOX5T45D/S107D* (Fig S3-Fig S4).

**MPK3/MPK6 homeostasis is necessary for QC maintenance**

To investigate the role of MPK3/MPK6 in regulating root QC maintenance, we conducted a comprehensive analysis of QC phenotypes in various genetic backgrounds, including *mpk3-1*, *mpk6-3*, the *mpk3 mpk6* double mutant, *MPK3pro:MPK3-GFP*, and *MPK6pro:MPK6-GFP* seedlings. In comparison to the WT, which displayed a QC division rate of less than 10%, *mpk3-1* and *mpk6-3* single mutant seedlings exhibited approximately 30% and 65% QC division, respectively (Fig 4A and B). Notably, to circumvent the embryo lethality associated with double mutants of *MPK3* and *MPK6*, we employed the *MPK3SR* line, which carries the *MPK3pro:MPK3TG* transgene. By treating *MPK3SR* seedlings with 4-amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine (NA-PP1), an inhibitor of *MPK3pro:MPK3TG*, we observed that 89% of mutant seedlings displayed QC division (Fig 4A and B). This observation indicates that MPK3 and MPK6 function redundantly in regulating QC division.

Furthermore, in *MPK3pro:MPK3-GFP* and *MPK6pro:MPK6-GFP* seedlings, approximately 50% and 42% of seedlings, respectively, exhibited root QC division (Fig 4A and B). These results underscore the importance of maintaining optimal homeostasis of MPK3/MPK6 for QC maintenance. The endogenous expression levels of MPK3 and MPK6 in plant root tips are evidently critical for maintaining the low division state of the QC, emphasizing the intricate balance required for this crucial process.

**MPK3/MPK6 increase the accumulation of WOX5 protein**

To investigate the impact of MPK3/MPK6 on WOX5 homeostasis, we explored their effects on WOX5 at both transcriptional and protein levels. Starting with transcriptional regulation, we compared the expression of *pWOX5-GFP* in *MPK6SR* and WT. Notably, *MPK6SR* carries a transgene of *MPK6pro:MPK6TG* in the background of the *mpk3 mpk6* double mutant, and this transgene can also be inhibited by NA-PP1, effectively simulating the *mpk3 mpk6* double mutant condition. In line with prior observations, our results demonstrated a substantial increase in GFP signals in the QC of root tips in *pWOX5-GFP*/*MPK6SR* seedlings compared to those in *pWOX5-GFP*/WT, indicating that MPK3/MPK6 suppress *WOX5* expression at the transcriptional level (Fig 5A, B).

Then we assessed the effect of MPK3/MPK6 on WOX5 protein levels through comparing the fluorescence expression of *pWOX5:WOX5-GFP* between WT and the *mpk3 mpk6* double mutant. Our findings revealed a significant reduction in the fluorescence signal of WOX5-GFP in the QC of seven different translation seedlings carrying *pWOX5:WOX5-GFP*/*MPK3SR* compared to those in *pWOX5:WOX5-GFP*/WT, signifying that MPK3/MPK6 play a positive role in regulating the accumulation of WOX5 protein (Fig 5A, B).

**Auxin regulates WOX5 homeostasis in QC through MPK3/MPK6**

MPK3/MPK6 play a pivotal role in maintaining WOX5 homeostasis within the QC by regulating both its transcriptional and protein levels. The precise auxin concentration within the QC is critical for the preservation of the root tip stem cell niche (SCN). To investigate whether MPK3/MPK6 mediate WOX5 regulation via auxin signaling, we meticulously examined the effects of altering auxin levels on WOX5 transcription and protein expression in both WT and *mpk3 mpk6* mutants. In this study, we employed a variety of seedlings, namely *pWOX5-GFP*/WT, *pWOX5-GFP*/*MPK6SR*, *pWOX5:WOX5-GFP*/WT, and *pWOX5:WOX5-GFP*/*MPK3SR*, and subjected them to treatments with Kynurenine (Kyn) or auxin (NAA) with the aim of manipulating internal auxin levels of examined seedlings. Our objective was to assess the influence of auxin on WOX5 transcription and protein expression levels in both WT and *mpk3 mpk6* mutant plants.

In line with previous findings, the fluorescence signals of WOX5-GFP within the QC of *pWOX5-GFP*/WT plants were significantly reduced after NAA treatment, while they were enhanced following Kyn treatment, suggesting that auxin inhibits *WOX5* transcription (Fig 6A, B). Interestingly, under the same conditions, the WOX5-GFP signal in *pWOX5:WOX5-GFP*/WT root QC slightly increased compared to that in *pWOX5-GFP*/WT after Kyn treatment. Conversely, the WOX5-GFP signal at the root tip QC moderately decreased in *pWOX5:WOX5-GFP*/WT compared to *pWOX5-GFP*/WT following NAA treatment (Fig 6A, B). These results collectively indicate that auxin has a negative regulatory effect on *WOX5* transcription but exerts a positive influence on WOX5 protein accumulation.

To investigate whether auxin-mediated *WOX5* transcriptional inhibition involves MPK3/MPK6, we compared the fluorescence signals of *pWOX5-GFP* in WT and *MPK6SR* seedlings after altering auxin levels. The results revealed that the changes in WOX5 fluorescence signals in response to NAA and Kyn treatment were less pronounced in *MPK6SR* seedlings compared to WT (Fig 6A, C), indicating that auxin indeed inhibits *WOX5* transcription through the mediation of MPK3/MPK6. Similarly, to examine whether auxin positively regulates WOX5 protein accumulation via MPK3/MPK6, we compared the fluorescence signals of *pWOX5:WOX5-GFP* in WT and *mpk3 mpk6* mutant seedlings subjected to varying auxin levels. The results demonstrated that the changes in fluorescence signals following NAA and Kyn treatment were less sensitive in *MPK3SR* seedlings compared to WT (Fig 6A, D), suggesting that auxin promotes WOX5 protein accumulation through the mediation of MPK3/MPK6.

In summary, these findings underscore the critical role of MPK3/MPK6 in mediating auxin signaling to maintain WOX5 homeostasis in the QC through a dual regulatory mechanism at both the transcriptional and post-translational levels.

**MPK3/MPK6 regulate root stem cell niche identity partially dependent on WOX5**

To further elucidate whether the maintenance of root QC homeostasis by MPK3/MPK6 is dependent on WOX5, we examined the root QC cell division phenotype in *pWOX5:WOX5-GFP*/*MPK3SR*. The result showed that root QC division phenotype in *MPK3SR* was only partially rescued in the *pWOX5:WOX5-GFP*/*MPK3SR* seedlings (Fig 7A, B), indicating that MPK3/MPK6 regulated QC identity is partially dependent on WOX5.

Similar to WOX5, which regulates both QC identity and root distal stem cell (DSC) differentiation (Fig S5A, Fig 1A), MPK3/MPK6 also have been demonstrated to not only regulate QC identity but also play a role in shaping DSC identity within the root. Consistent with earlier findings, in *mpk3-1*, *mpk6-3*, and *MPK3SR* seedlings, approximately 30%, 75%, and 100% of the roots lacked DSC, respectively. Furthermore, in *MPK3pro:MPK3-GFP* and *MPK6pro:MPK6-GFP* seedlings, about 50% and 11% of seedlings displayed two layers of DSC, respectively (Fig S5B, Fig 3A), implying a critical positive role of MPK3/MPK6 in maintaining root DSC identity. Moreover, the substantially increased differentiation of root distal stem cells (DSCs) in *MPK3SR* was partially rescued in *pWOX5:WOX5-GFP*/*MPK3SR* seedlings, in which only 92% of seedling roots exhibited altered DSC differentiation, as compare to the 100% observed in the *MPK3SR* alone (Fig S5C, Fig 7A). Collectively, these results suggest that MPK3/MPK6 partially regulate the maintenance of DSC in the root through their interaction with WOX5.