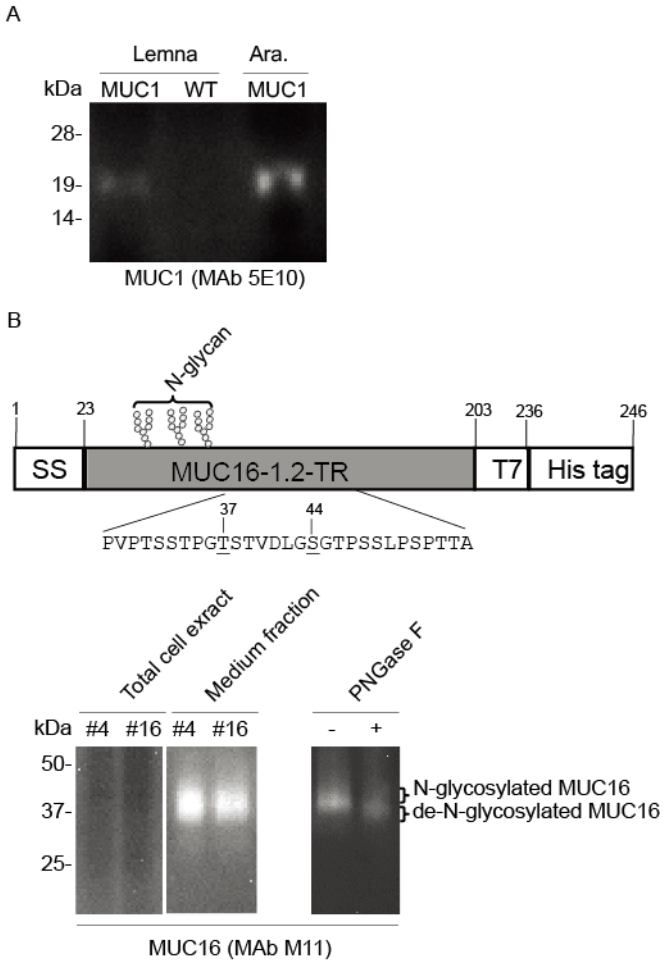
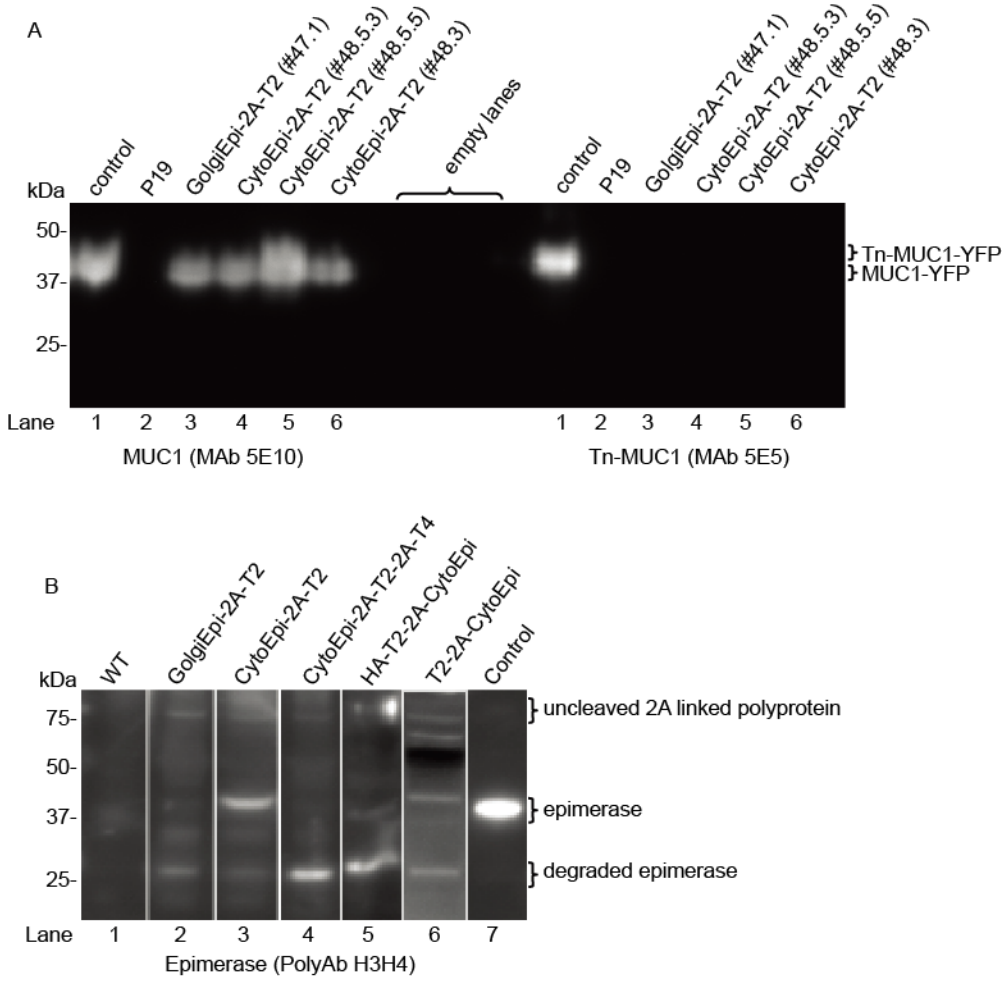
Yang et al. Supplemental Fig. S1



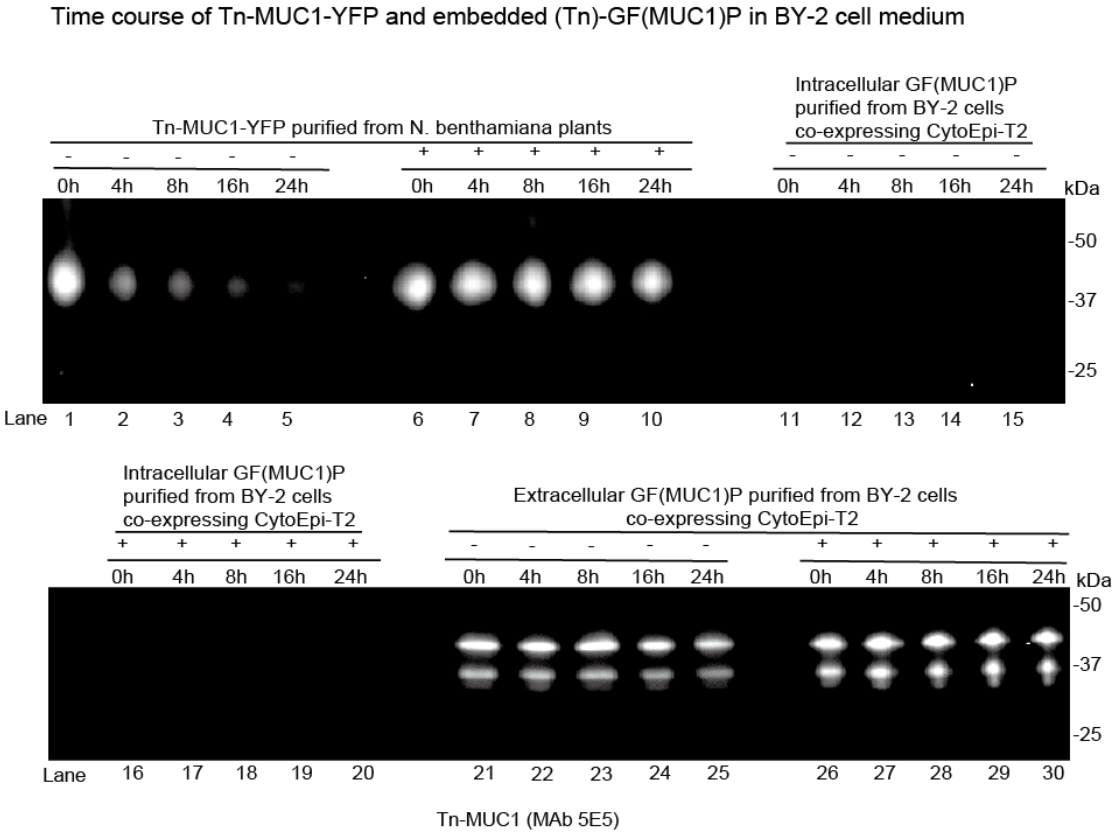

**Supplemental Figure S1.** Expression of the mucin type *O*-glycoproteins MUC1 and MUC16 in *Lemna minor* and BY-2 cells, respectively. (**A**) Western blot analysis, using MUC1 specific MAb 5E10, of total plants extracts of a *L. minor* line transgenic for MUC1-3.5TR (MUC1) with an *A. thaliana* line also transgenic for MUC1 serving as positive control. Approximately 30 µg total protein was loaded in each lane. (**B**) Western blot analysis of total cell extract and medium fractions of transgenic BY-2 cell lines #4 and #16 (left panel) transgenic for expression of MUC16-1.2TR using the MUC16 specific MAb M11. PNGase F mediated deglycosylation of *N*-linked glycans of MUC16 (line #4) in medium fractions indicates MUC16 is N-glycosylated in plant (right panel). Approximately 30 µg total protein of cell extract and 15 µl medium fraction were loaded in each lane.

Yang et al. Supplemental Fig. S2



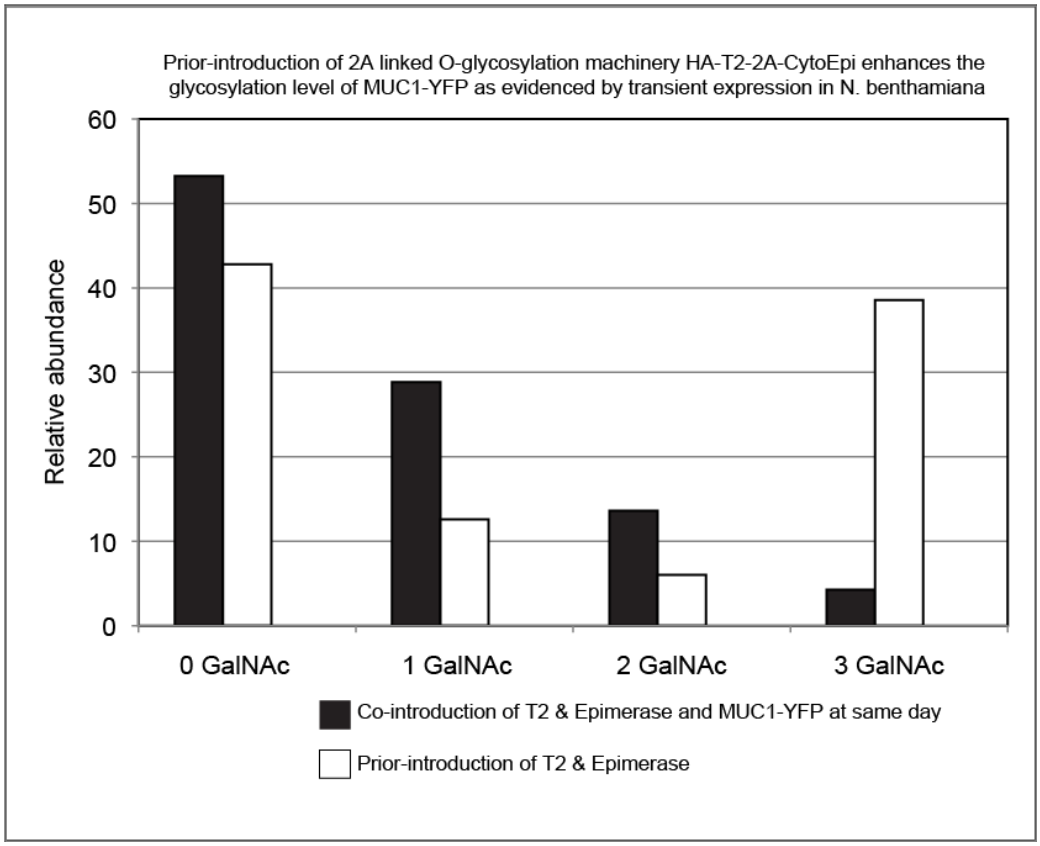

**Supplemental Figure S2.** (**A**) Western blot analysis of leaf extracts of transgenic *Arabidopsis thaliana* lines expressing either of the 2A linked glycosylation machineries GolgiEpi-2A-T2 (#47.1) or CytoEpi-2A-T2 (#48.5.3; #48.5.5; #48.3) in combination with MUC1-YFP. Co-expression of MUC1-YFP and HA-T2-2A-CytoEpi and expression of the gene silencing suppressor p19, transiently in leaves of *N. benthamiana*, served as positive and negative control, respectively. The blots were probed with the MUC1 and GalNAc-MUC1 (Tn-MUC1) specific MAbs, 5E10 and 5E5, respectively. (**B**) SDS-PAGE Western blot analysis of 2A linked *O*-glycosylation machinery reveals partial degradation and uncleaved polyproteins in transgenic *A. thaliana* and BY-2 cell lines when probed with mouse polyclonal Ab H3H4 raised against cytosolic epimerase. The cross-reacting bands of ca. 37, 75 and 25 kDa corresponds to the predicted MWs of intact 2A cleaved, uncleaved (2A linked epimerase and GalNAc-T2) and degraded epimerase, respectively. Lane 1: total proteinacious extracts of wt *N. benthamiana* leaves*;* lane 2-4: total proteinacious extracts of *A. thaliana* stably expressing 2A linked Golgi targeted epimerase and GalNAc-T2 (GolgiEpi-2A-T2), 2A linked cytosolic epimerase and GalNAc-T2 (CytoEpi-2A-T2); 2A linked cytosolic epimerase, GalNAc-T2 and -T4 (CytoEpi-2A-T2-2A-T4); lane 5-6: total proteinacious leaf extracts of *A. thaliana* and *Lemna minor* expressing 2A linked GalNAc-T2 (with or without an Hemagglutinin, HA tag) and cytoplasmic epimerase, *i.e.* HA-T2-2A-CytoEpi or T2-2A-CytoEpi. Approximately 30 µg total protein was loaded in each lane.

Yang et al. Supplemental Fig. S3




**Supplemental Figure S3.** MUC1 tandem repeats is stabilized by *O*-glycosylation and embedding into GFP in BY-2 cell culture. Time course, as visualized by GalNAc-MUC1 (Tn-MUC1) specific (MAb 5E5) Western blot analysis, of GalNAc glycosylated MUC1-YFP (Tn-MUC1-YFP) and GF(MUC1)P (Tn-GF(MUC1)P) incubated in 7 day old wt BY-2 cell culture medium fractions. Tn-MUC1-YFP transiently produced in leaves of *N. benthamiana* plants (lanes 1-10), intracellular (lanes 11-20) and extracellular (lanes 21-30) purified embedded GF(MUC1)P from a stably transformed BY-2 cell line co-expressing CytoEpi-T2. The isolated Tn-MUC1-YFP, GF(MUC1)P and Tn-GF(MUC1)P were added to 7 day old un-boiled (-) or boiled (+) wt BY-2 medium fractions, which were further incubated for up to 24 h under the same conditions. Approximately 5 µg Tn-MUC1-YFP, GF(MUC1)P or Tn-GF(MUC1)P were added to 1 ml BY-2 medium fractions, respectively. Ca. 15 µl were loaded and blots reacted with MUC1 specific MAb 5E10.

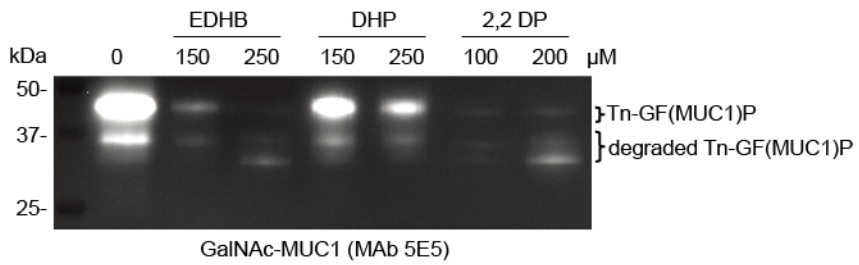
Yang et al. Supplemental Fig. S4




**Supplemental Figure S4.** Prior-introduction of *O*-glycosylation machinery enhanced the glycosylation level of MUC1-YFP in a transient expression system. Transient expression in *N. benthamiana* leaves: MUC1-YFP co-infiltrated with the *O*-glycosylation machinery (HA-T2-2A-CytoEpi) at same day (black) or with the *O*-glycosylation machinery additionally infiltrated two days before (prior-introduction of *O*-glycosylation machinery, white). The bar in the figure represents relative abundance of MUC1-1TR peaks in MALDI-TOF mass spectra.

Yang et al. Supplemental Fig. S5

A

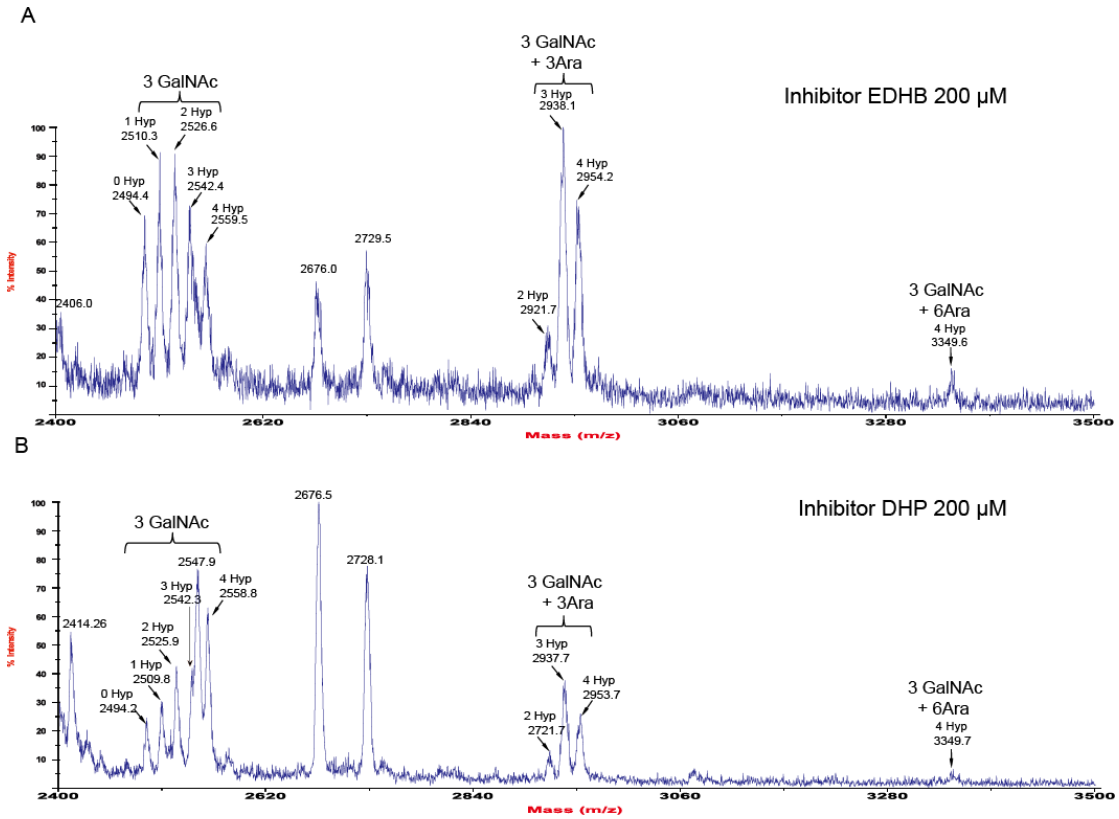



B

| Control | | EDHB | | DHP | | 2,2 DP | |
|---|---|---|---|---|---|---|---|
| µM | Cell volume | µM | Cell volume | µM | Cell volume | µM | Cell volume |
| 0 | 25 | 100 | 20 | 100 | 22,5 | 100 | 23 |
| | | 200 | 15 | 200 | 22 | 200 | 15 |
| | | 300 | 12,5 | 300 | 20 | 300 | 7,5 |
| | | 400 | 10 | 400 | 15 | 400 | 5 |
| | | 500 | 7,5 | 500 | 10 | 500 | 5 |

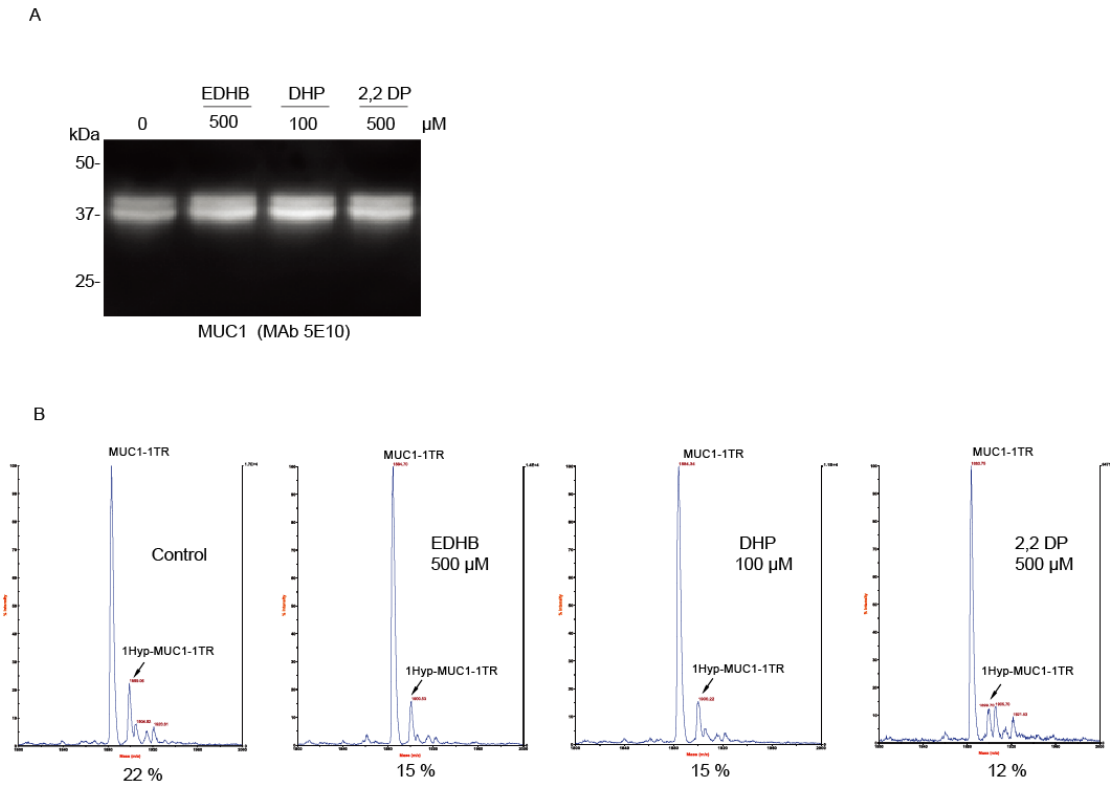
**Supplemental Figure S5.** Chemical inhibitors of prolyl 4-hydroxylases (P4Hs) reduce extracellular accumulation of GF(MUC1)P and inhibits growth of BY-2 cells. (**A**) Western blot analysis of a transgenic BY-2 cell line co-expressing embed GF(MUC1)P and the *O*-glycosylation machinery CytoEpi-T2 in the presence of 100-250 µM inhibitor, *i.e.* ethyl-3,4-dihydroxybenzoate (EDHB), 3,4-dehydroproline (DHP) or 2,2-dipyridyl (2,2 DP). Western blots were reacted with Tn-MUC1 specific MAb 5E5. Approximately 5 µg total protein was loaded in each lane. (**B**), BY-2 cell growth, measured as the total cell volume, was increasingly inhibited with increased concentrations of the three inhibitors.

Yang et al. Supplemental Fig. S6




**Supplemental Figure S6.** Chemical inhibition of proline hydroxylation of MUC1 expressed in BY-2 cells. MALDI-TOF-MS analysis of MUC1 tandem repeat (D**T**RPAPG**ST**APPAHGV**TS**AP, indicated by arrow) from His-tag purified Asp-N digested extracellular GF(MUC1)P of a BY-2 line co-expressing CytoEpi-T2, in the presence of 200 µM of the P4H inhibitors ethyl-3,4-dihydroxybenzoate (EDHB) or 3,4-dehydroproline (DHP). MALDI-TOF-MS analysis of purified GF(MUC1)P, expressed without inhibitors, is shown in Fig. 5 of the main text.

Yang et al. Supplemental Fig. S7




**Supplemental Figure S7.** Transient expression of MUC1-YFP with co-infiltration of different C4 proline hydroxylases inhibitors (P4Hs) in *N. benthamiana*. (**A**) Western blot analysis of total proteinacious extracts of *N. benthamiana* leaves transiently expressing MUC1-YFP in the presence of the three P4H inhibitors ethyl-3,4-dihydroxybenzoate (EDHB), 3,4-dehydroproline (DHP) and 2,2-dipyridyl (2,2 DP) at concentrations of 500 µM, 100 µM and 500 µM, respectively, in the infiltration buffer. Western blots were reacted with Tn-MUC1 specific MAb 5E5. Approximately 30 µg total protein was loaded in each lane. (**B**) MALDI-TOF-MS analysis of MUC1 tandem repeat from Asp-N digested MUC1-YFP purified from leaves in the presence of the different inhibitors. The degree of proline hydroxylation of MUC1 tandem repeats is indicated by the arrows.