**YUCCA8** and **YUCCA9** overexpression reveals a link between auxin signaling and lignification through the induction of ethylene biosynthesis

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Auxin is associated with the regulation of virtually every aspect of plant growth and development. Many previous genetic and biochemical studies revealed that, among the proposed routes for the production of auxin, the so-called indole-3-pyruvic acid (IPA) pathway is the main source for indole-3-acetic acid (IAA) in plants. The IPA pathway involves the action of 2 classes of enzymes, tryptophan-pyruvate aminotransferases (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAAIL)/TRYPTOPHAN AMINOTRANSFERASE RELATED (TAR)) and flavin monoxygenases (YUCCA). Both enzyme classes appear to be encoded by small gene families in *Arabidopsis* consisting of 5 and 11 members, respectively. We recently showed that it is possible to induce transcript accumulation of 2 YUCCA genes, YUC8 and YUC9, by methyl jasmonate treatment. Both gene products were demonstrated to contribute to auxin biosynthesis in planta.1 Here we report that the overexpression of YUC8 as well as YUC9 led to strong lignification of plant aerial tissues. Furthermore, new evidence indicates that this abnormally strong secondary growth is linked to increased levels of ethylene production.

IAA levels in the overexpressors when compared with wild-type plants and clearly auxin-related phenotypes. Consistent with other auxin overproducer lines, YUC8ox and YUC9ox are characterized by elongated hypocotyls and petioles, as well as epinastic growing cotyledons. In addition, the lines showed longer and narrower leave blades than wild-type *Arabidopsis*. However, not all of the observed phenotypes could be directly attributed to the detected significantly increased amount of endogenous IAA. Intriguingly, some of the strong overexpression lines showed aberrant secondary growth of the stem (Fig. 1). Indeed, in some cases the secondary growth was so pronounced that the stem was no longer able to follow the increased growth and the epidermis cracked open from the bottom to the top (Fig. 1A–D). As can be estimated from Figure 1E, the stem diameter of overexpressor lines reached about twice the size of wild-type stems. In fact, this can be attributed to a more pronounced cell expansion growth, particularly of the cortex, vascular bundle, and parenchyma cells, in the overexpressor lines, which confirms the previous finding that the transient overexpression both of YUC8 and YUC9 results in an induction of cell expansion by 2- to 2.5-fold.1 In addition, we observed that the overexpressors lost much less of their size when slowly dried at ambient temperatures (Fig. 1F). Comparing weight loss after drying, we did not observe significant differences.
between overexpressor lines and wild type, pointing out that the examined lines lost the same amount of water by vaporization. Remarkably, the fresh weight of the YUC8ox and YUC9ox lines (2.51 ± 0.05 g) was only slightly higher than that of the wild type (2.24 ± 0.03 g). Nevertheless, shrinkage of the YUC8ox and YUC9ox lines was apparently reduced relative to wild-type Arabidopsis.

Mechanical strength and plasticity of plants is to a great extent attributed to their cell walls. Cell walls are multilayered structures unique to plants that surround every cell providing sufficient rigidity to counteract the turgor pressure. The biosynthesis of this extracellular matrix that constitutes the boundary of the cell is a highly complex process that requires multiple coordinated enzymatic reaction steps. With respect to the described findings, we hypothesized that increased disposal of stabilizing biopolymers, e.g., lignin and embedded cellulosic compounds, in the secondary cell walls is likely to be the causal link for the abnormal phenotype of the overexpression lines relative to the wild type. Staining of various plant parts using phloroglucinol in the presence of alcohol and HCl, in fact, confirmed this hypothesis and revealed a substantially increased degree of lignification in YUC8ox and YUC9ox lines in comparison to the wild-type controls (Fig. 1G-I).

Both plant development and plant stress responses can be regulated by the mutual interop erability and congruence of plant hormones, a concept that is widely accepted. Various examples for the crosstalk between phytohormones and the underlying molecular bases can be found in the literature. For example, secondary plant growth can be stimulated by the interplay of auxin and strigolactone signaling, which seem to steer cambial activity. However, ethylene signaling is also assumed to contribute to radial (horizontal) growth by modulating vascular cell division.

The stimulation of ethylene emission from plant tissues by exogenously applied as well as endogenously produced auxin is a well-established phenomenon. Ethylene, for its part, also affects auxin biosynthesis and transport-dependent local auxin distribution. Remarkably, recent findings significantly augmented the current insight into this intimate relationship between auxin and ethylene biosynthesis. Auxin and ethylene production are metabolically linked by a pyridoxal-phosphate-dependent aminotransferase, REVERSAL OF sav3 PHENOTYPE (VAS1), that catalyzes the transamination of IPA to l-tryptophan and 2-oxo-4-methylthiobutyric acid, specifically using methionine as the amino donor. Given that vas1 accumulates more auxin and 1-aminocyclopropane-1-carboxylate (ACC) under normal growth conditions, VAS1 seemingly controls the amounts of these 2 plant hormones. Overall, it seems as if there is a circle of mutual activation and a tight metabolic link between the 2 plant hormonal pathways. Most relevant for our experiments, however, was the discovery that overproduction of IAA in transgenic plants induces the concomitant overproduction of ethylene. To examine whether YUC8- and YUC9-mediated IAA overproduction also affects ethylene biosynthesis, we tested the primary root growth inhibition by an ethylene biosynthesis inhibitor, 2-aminoisobutyric acid (AIB), in wild type and YUC8ox and YUC9ox lines (Fig. 2). Although higher concentrations of AIB ultimately suppressed primary root growth in wild-type Arabidopsis as well as YUC8ox and YUC9ox, the 2 overexpression lines clearly showed hyposensitivity toward AIB, pointing toward an increased resistance that is mediated by the stimulated formation of ethylene. This result was confirmed by quantitative transcript analyses, which highlighted transcript accumulation of a number of ethylene biosynthesis- and signal ing-related genes in YUC9ox (Table 1). Hence, we conclude that YUC8 and YUC9 overexpression-mediated overproduction of
IAA, in turn, triggers the induction of ethylene production and signaling, which in combination stimulates secondary growth and deposition of lignin into the cell walls.

Having in mind that jasmonates are capable of inducing the accumulation of YUC8 and YUC9 transcripts,1 thus being probably the initiator of a jasmonate/auxin/ethylene cascade, there is already evidence that coaction of ethylene and jasmonate is integrated through the ethylene-stabilized transcription factors EIN3 and EIL1, which physically interact with JASMONATE-ZIM-domain (JAZ) proteins to repress EIN3/EIL1.18 This leads to the emergence of a picture in which all 3 plant hormone signaling pathways may contribute to the stimulation of lignification in the overexpression lines.

There is mounting evidence that changes in lignification are linked to various plant hormone actions.19 For instances, the cellulose synthase mutant cev1 and the V-type ATPase mutant vha-a3 show ectopic lignification alongside with increased levels of JA-regulated genes and JA-precursors.20,21 In vha-a3, the AtMYB61 transcription factor22 appears to be misexpressed and suppression of AtMYB61 can restore the mutant phenotype. It is therefore possible that JA signaling is linked to lignin biosynthesis through the transcriptional regulation of AtMYB61. In addition, it has been shown that another MYB-type transcription factor, AtMYB32, is largely upregulated by IAA,23 while the KNOX gene family transcription factor BREVIPEDICELLUS (BP) that negatively regulates lignin biosynthesis is effectively repressed by IAA.24 Finally, also ethylene has been associated with the regulation of lignin biosynthesis. Characterization of a mutant in the chitinase-like protein AtCTL1, elp1, revealed that the phenotype of the mutant was due to the ectopic deposition of lignin and increased ethylene production.25 A similar lignin deposition phenotype has also been found in mutants of 2 leucine-rich-repeat receptor-like kinases, which seemingly link cell wall biosynthesis with ACC activity in Arabidopsis.26 Consistent with these circumstantial evidences, the transcription of AtMYB61 and AtMYB32 responds to both IAA and methyl jasmonate treatment, while BP additionally also responds to ACC treatment in a transient manner (http://jsp.weigelworld.org/expviz/expviz.jsp).

Although the underlying molecular mechanisms for the cross-regulation of these transcription factors is largely unknown, it may be reasonable to think that their coordinated expression, orchestrated by the 3 plant hormones, is a possible determinant to regulate lignin formation. How AtCTL1 and the 2 receptor-like kinases feed into this picture remains, however, uncertain. So far, there is only indication that their mutation translates into altered lignin deposition and the concomitant increase of ethylene and ACC levels, respectively. Changes in the transcript profiles of both YUC8ox and YUC9ox have not yet been assessed, and nothing is known about the differential regulation of lignin synthesis-related genes in these mutants. It will be interesting to determine the molecular bases for the increased strong secondary growth and aberrant lignin deposition in YUC8ox and YUC9ox.

**Table 1. Ethylene biosynthesis and signaling components significantly affected in their expression in the YUC9 overexpression line**

| Name  | Gene ID | p-value  | Fold |
|-------|---------|----------|------|
| **Ethylene biosynthesis-related genes** |
| ACS4  | At2g22810 | 6.18E-03 | +1.5 |
| ACS8  | At4g37770 | 3.37E-06 | +1.9 |
| ACS11 | At4g08040 | 4.47E-02 | +1.6 |
| ACO1  | At2g19590 | 4.10E-02 | +1.3 |
| ACO4  | At1g05010 | 5.89E-03 | +1.3 |
| ACO-like | At5g43440 | 1.01E-03 | +1.5 |
| **Ethylene signaling-related genes** |
| EIL1  | At2g27050 | 2.72E-03 | +1.4 |
| EIN3  | At3g20770 | 1.19E-02 | +1.3 |
| ETR2  | At3g23150 | 1.61E-03 | +1.5 |
| ESE3  | At5g25190 | 4.50E-04 | +2.1 |
| ERF-like | At5g61590 | 2.50E-07 | +2.8 |

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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