**Summary**

By screening a T-DNA population of Arabidopsis mutants for alterations in inflorescence stem vasculature, we have isolated a mutant with a dramatic increase in vascular tissue development, characterized by a continuous ring of xylem/phloem. This phenotype is the consequence of premature and numerous cambial cell divisions in both the fascicular and interfascicular regions that result in the loss of the alternate vascular bundle/fiber organization typically observed in Arabidopsis stems. The mutant was therefore designated high cambial activity (**hca**). The **hca** mutation also resulted in pleiotropic effects including stunting and a delay in developmental events such as flowering and senescence. The physiological characterization of **hca** seedlings in vitro revealed an altered auxin and cytokinin response and, most strikingly, an enhanced sensitivity to cytokinin. These results were substantiated by comparative microarray analysis between **hca** and wild-type plants. The genetic analysis of **hca** indicated that the mutant phenotype was not tagged by the T-DNA and that the **hca** mutation segregated as a single recessive locus, mapping to the long arm of chromosome 4. We propose that **hca** is involved in mechanisms controlling the arrangement of vascular bundles throughout the plant by regulating the auxin–cytokinin sensitivity of vascular cambial cells. Thus, the **hca** mutant is a useful model for examining the genetic and hormonal control of cambial growth and differentiation.

**Keywords:** Arabidopsis thaliana, cambium, secondary xylem, cytokinin hypersensitivity, vascular patterning, Complete Arabidopsis Transcriptome MicroArray (CATMA).

**Introduction**

Vascular tissues in plants are either primary or secondary. Primary xylem and phloem differentiate within the vascular cylinder during primary growth of stems and roots. Later in development, when elongation ceases and plants undergo secondary growth, a vascular cambium develops that gives rise to secondary xylem and secondary phloem (Esau, 1965). The dividing cambial initials produce phloem or xylem mother cells which subsequently undergo one or several rounds of division before differentiation. The ‘cambial zone’ includes the cambial initials and phloem and xylem mother cells (Lachaud et al., 1999; Larson, 1994). During both primary and secondary growth, positional information is necessary to coordinate the spatio-temporal formation of vascular strands not only throughout the plant organ but also within vascular bundles. Our current knowledge concerning the molecular mechanisms underlying many aspects of vascular development, such as control of cambial cell division, primary and secondary xylem/phloem differentiation and patterning, is still fragmented.
It is well known that plant hormones, and in particular auxin, play a crucial role in the developmental control of primary and secondary vascular tissues and cambial activity (Little and Pharis, 1995; Ye, 2002) by regulating both radial (Sundberg et al., 2001; Uggla et al., 1996) and longitudinal vascular pattern formation in plants (Berleth et al., 2000; Sieburth, 1999). Developing buds and young shoots are major sources of auxin, but indole acetic acid (IAA) is also synthesized in young root tissues (Ljung et al., 2002). Auxin is transported through the plant in a basipetal polar fashion (for reviews see Lomax et al., 1995; Swarup and Bennett, 2003). In the canalization hypothesis proposed by Sachs (1981) this polar auxin transport has been related to the differentiation of procambial strands. In recent years, genetic studies of Arabidopsis mutants altered in auxin metabolism have increased our understanding of auxin transport and signal transduction in relation to vascular differentiation and patterning (for reviews see Berleth and Mattsson, 2000; Berleth et al., 2000; Hobbie, 1998). It is known that polar auxin transport is mediated to a great extent by the efflux carrier PIN1. Based on the pin1 mutant phenotype, it was also demonstrated that auxin transport has a central role in the formation of vascular strands (Gälweiler et al., 1998). Many proteins involved in responses to auxin have been characterized, and at least two of them, PINOID, a putative protein kinase, and MP, an auxin response factor, have been shown to be important for vascular differentiation and patterning (Christensen et al., 2000; Hardtke and Berleth, 1998).

Although there is some evidence pointing to a role for cytokinin in procambial cell division and xylem differentiation, its mode of action is not well understood (Saks et al., 1984; for review see Aloni, 1995). In vitro studies of xylogenesis in Zinnia elegans indicate that auxin alone is not sufficient to induce the differentiation of leaf mesophyll cells into tracheary elements (TE), and that cytokinin is a strict requirement for differentiation of TE (Fukuda and Komamine, 1980). More recently, the characterization of the wooden leg (woI) mutant and the subsequent cloning of the corresponding gene, CYTOKININ RESPONSE (CRE1), demonstrated that cytokinin regulates vascular development by controlling procambial cell division (Inoue et al., 2001; Mähonen et al., 2000). As a result of defective procambial cell divisions, all procambial cells in roots differentiate uniquely into protoxylem, with neither protophloem nor metaxylem forming in the stele. The WOL/CRE1 gene encodes a cytokinin receptor which belongs to the two-component signal transducer family. It is expressed in procambial cells of primary roots.

Although we are still a long way from establishing an integrated model that thoroughly explains the differentiation of cambial cells into functional vascular bundles, important headway has been made in dissecting certain steps of vascular differentiation and patterning per se by carrying out mutant screening for vascular defects. Many mutants with a variety of phenotypes have been characterized (for reviews see Fukuda, 2004; Scarpella and Meijer, 2004; Turner and Sieburth, 2002). In some case, mutant analysis has resulted in a more precise function for some genes already known to be involved in secondary cell deposition. This is the case for irx3 encoding cellulose synthase (Taylor et al., 1999) and irx4 encoding cinnamoyl Co-A reductase (Jones et al., 2001). In others cases, genetic analysis has revealed the role of important regulatory genes in vascular formation. Recently, a MYB transcription factor, ALTERED PHLOEM DEVELOPMENT (APL), has been identified as a key player in phloem development (Bonke et al., 2003). Another regulatory gene INTERFASCICULAR FIBERLESS1 (IFL1), belonging to the homeodomain-leucine zipper protein (HD-ZIP) is essential for proper fiber differentiation in Arabidopsis stems (Ratcliffe et al., 2000; Zhong and Ye, 1999; Zhong et al., 1997). Although numerous vascular mutants have been described, to our knowledge mutants with altered cambial activity and/or secondary growth are extremely rare (Oyama et al., 1997).

Contrary to preconceived notions, Arabidopsis is considered to be an excellent model for the study of secondary growth (Chaffey et al., 2002). Under normal growth conditions, hypocotyls of mature plants possess a vascular cambium and undergo extensive secondary growth (Busse and Evert, 1999; Ye et al., 2002). Secondary growth has also been described in the inflorescence stem (Altamura et al., 2001), and this phenomenon is enhanced when plants are grown at a low population density and/or with repeated removal of all newly emerging inflorescences stems. Taking advantage of this characteristic, Zhao et al. (2000) constructed a cDNA library from root-hypocotyl sections enriched in developing xylem cells. Moreover, genes related to secondary growth were identified by hybridizing the Arabidopsis Genome GeneChip arrays with cDNA extracted from stems with enhanced secondary xylem formation (Ko et al., 2004; Oh et al., 2003).

Since Arabidopsis possesses a vascular cambium and undergoes secondary growth it should be possible to identify molecular and physiological factors that control cambial activity by identifying mutants altered in amounts or patterns of secondary vascular tissues. An assumption may be made that these alterations would be the direct consequence of altered cambial activity. To address this issue, we have screened a T-DNA collection of Arabidopsis (for 'high cambial activity'), the extensive secondary growth altered the organization of the stem vasculature leading to a continuous ring of vascular tissues; the mutant is impaired in cambial activity and secondary growth throughout the plant body. We have demonstrated by multiple, independent assays that...
responses to both auxin and cytokinin were affected in the hca mutant.

Results

Isolation of hca mutant with unusually high cambial activity

To dissect the molecular determinants that control vascular differentiation in plants, we screened an Arabidopsis T-DNA mutant collection (Beschtold et al., 1993) for altered vascular lature. The basal portion of inflorescence stems from 6-week-old plants were systematically sectioned freehand and observed using light and UV fluorescence microscopy. This screening procedure allowed us to isolate a mutant line with a dramatically altered organization of vascular tissues. The vascular system of wild-type Arabidopsis is classically organized in discrete collateral bundles with the xylem towards the inside and the phloem towards the outside of the bundle. Vascular bundles are separated from each other by lignified interfascicular fibers (Figure 1a–c). In contrast, the mutant line exhibited a continuous ring of vascular tissues. The altered growth pattern in hca resembles that of the stem of young seedlings in wild-type plants. More-over, the diameter of xylem cells was considerably reduced (Figure 2a,b). In 5-week-old hypocotyls, while the majority of the surface area is occupied by secondary xylem in wild-type plants, hca exhibited fewer files of xylem with indentations towards the outside of the section. There is no secondary growth apparent, and as a consequence the diameter of the xylem is smaller than in wild type (Figure 2c,d). This result suggests that the vascular meristems are differentially regulated in shoots and hypocotyls in hca.

To investigate the altered vasculature of hca in more detail, thin sections from the base of the stem were observed (Figure 1g–j). In the wild type (Figure 1i), the vascular bundles and the interfascicular fibers are separated from the cortex by a single layer of large parenchyma cells called in the literature either the starch sheath or the endodermis. Within each bundle, an intrafascicular cambium is visible between the xylem and the phloem (see large arrows in Figure 1i). Under our growth conditions, only primary vascular tissues were observed. In contrast, hca (Figure 1j) is characterized by a continuous vascular cambium undergoing periclinal divisions leading to radial files of xylem daughter cells that differentiate into secondary xylem (see large arrows in Figure 1j). The presence of primary xylem poles reminiscent of vascular organization typical of wild-type Arabidopsis is still visible immediately inside the ring of secondary xylem (see Xyl in Figure 1h). Surrounding the phloem, we observed the presence of two or three additional layers of parenchyma cells which form a ring inside the starch sheath (see open arrowheads on Figure 1j).

The altered growth pattern in hca resulted in a dramatic effect on the surface area of stem tissues. Tissue areas were calculated from digitized images of transverse sections of 20 greenhouse-grown plants at the flowering stage. The total surface area of hca stem sections is half that of wild type (0.59 ± 0.08 and 1.37 ± 0.21 mm² respectively). The relative proportions of tissue type were also compared in wild type and hca. The proportion of pith in hca is considerably lower (12% in hca versus 55% in wild type) with most of the surface area comprising vascular tissue (46% in hca versus 20% in wild type).

Abnormal vasculature was observed not only at the base of the stem of hca but also at the subapical and middle portions (Figure 1k–n). No interfascicular fibers were present in the subapical zone of hca. Parenchyma cells were found in the interfascicular fiber position between enlarged vascular bundles (Figure 1i). In the middle portion of the stem, a continuous ring of xylem/phloem could already be observed (Figure 1n). In contrast, in wild type, as is the case for the basal portion of the stem (Figure 1b,e), triangular vascular bundles alternate with interfascicular fibers forming an arch-shaped pattern around the stem both in the subapical and the middle portion (Figure 1k,m).

The effect of the hca mutation on vasculature was further examined throughout the plant. In roots, vascular tissues from hca did not exhibit the diarch organization of xylem and phloem typically observed in wild-type plants. Moreover, the diameter of xylem cells was considerably reduced (Figure 2a,b). In 5-week-old hypocotyls, while the majority of the surface area is occupied by secondary xylem in wild-type plants, hca exhibited fewer files of xylem with indentations towards the outside of the section. There is no secondary growth apparent, and as a consequence the diameter of the hca hypocotyl is smaller than the wild type (Figure 2c,d). This result suggests that the vascular meristems are differentially regulated in shoots and hypocotyls in hca.

To further understand the effects of the hca mutation on cambial activity, we monitored the expression pattern of pAttb8::GUS plants were performed and the F₂ mutant progeny were analyzed. In the basal portion of 6-week-old
Figure 1. Transverse sections of inflorescence stem of 6-week-old wild type and hca: (a–c, g, i, k, m) wild type, (d–f, h, j, l, n) hca.
(a–f) Hand-cut sections of the basal portion of the stem (a–f) observed under bright field (a, d) or UV light (c, f). Sections were stained with phloroglucinol in (b) and (e).
(g–j) Thin sections of the basal portion of the stem stained with toluidine blue. (i, j) Higher magnifications of wild-type and hca vascular tissues respectively. A continuous cambium with numerous cell divisions is visualized in hca whereas the cambium is restricted to the fascicular region in wild-type stems (see large open arrows in i and j). Open arrowheads in (j) indicate additional parenchyma cells in hca between the starch sheath and the phloem.
(k–n) Phloroglucinol stained hand-cut sections from the top (k and l) and middle (m and n) portions of the stem. (k) In the top of the wild-type stem, lignified xylem cells in the vascular bundles stained dark red and alternated with lignified interfascicular fibers. (l) In the top portion of hca there are no interfascicular fibers and the vascular bundles are wider than the wild type. C, cortex; If, interfascicular fibers; P, pith; Ph, phloem; S, starch sheath; VB, vascular bundle; Xyl, primary xylem; Xyll, secondary xylem; F, fiber cells. Scale bars: (a–h) 100 μm, (i–n) 50 μm.
wild-type stems (Figure 3a,b), \textit{ATHB-8} is expressed in parenchyma cells surrounding vessel elements, with only trace amounts of expression in the fascicular cambium (see large arrows in Figure 3b). On the contrary, in the basal portion of \textit{hca} stems (Figure 3c,d), \textit{ATHB-8} is highly expressed in parenchyma cells associated with primary xylem, but also in the continuous cambium (see large arrows in Figure 3d). These results demonstrate an up-regulation of \textit{ATHB-8} in \textit{hca} plants and suggest that the continuous cambium formed as a result of the \textit{hca} mutation is highly active. Interestingly, \textit{ATHB-8} is also expressed in a continuous ring in the subapical portion of the \textit{hca} stem, even prior to the formation of the continuous ring of xylem (Figure 3f). Since \textit{ATHB-8} is expressed during early stages of plant development (Baima \textit{et al.}, 1995), we examined the expression pattern of \textit{pAthb8::GUS} in mature embryos 24 h after imbibition in both \textit{hca} and wild-type backgrounds. A more intense staining is visible in the root apical meristem (RAM) of \textit{hca} as compared with the wild type (Figure 3g,h). The upregulation of \textit{ATHB-8} in the RAM in \textit{hca} suggests that the \textit{HCA} gene also acts as a regulatory factor in primary meristems. However, it is interesting to note that primary vascular organization in cotyledons and leaves is not altered in \textit{hca} (Figure 3i–l).

\textbf{The \textit{hca} mutation has pleiotropic effects on plant morphology}

Beyond aspects on vascular patterning, the \textit{hca} mutation caused pleiotropic developmental changes throughout the life cycle. At the flowering stage, \textit{hca} plants are eightfold smaller than wild type and the rosette is composed of twisted, dark-green leaves when grown under long-day conditions (Figure 4a). This difference in rosette morphology is even more pronounced under short-day conditions. \textit{hca} leaves are more twisted and smaller than those of wild-type plants (Figure 4b). Measurements of the morphometric characteristics of 6-week-old plants (Table 1) showed that \textit{hca} plants have very short primary stems (23.8 cm compared with 55.1 cm for wild type). This difference is due to a reduction in internode length. Together with reduced

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Vascular organization of hypocotyls and roots of wild type and \textit{hca}: (a, c, e) wild type, (b, d, f) \textit{hca}. (a, b) Transverse section of the stele of 7-day-old roots observed after staining with carminio green. Xylem cells are identified by green staining of their lignified walls whereas phloem cells are stained pink for cellulose. (c, d) Transverse hand-cut sections of hypocotyls from 5-week-old plants observed under UV light. (e, f) Transverse thin sections in the basal portion of the stele of 10-day-old hypocotyls. In (a) and (c) the arrows indicate the xylem and phloem poles respectively. In (f) large arrows indicate extensive division in the pericycle and cambium of \textit{hca}. Ph, phloem; Xy, xylem; XyII, secondary xylem. Scale bars: (a, b) 30 \textmu m, (c, d) 100 \textmu m, (e, f) 50 \textmu m.}
\end{figure}
internode length, hca produced more secondary stems which contribute to the bushy aspect of adult hca plants. hca flowers were morphologically normal and fertile and the number of siliques per plant was similar in hca and wild-type plants (Table 1). hca exhibited a continuous but slower growth rate, with a delay in developmental events such as flowering (8 days) and senescence (4 weeks).

The effect of the hca mutation was also determined in in vitro-grown seedlings. Seven-day-old hca seedlings had smaller cotyledons than wild-type seedlings with significantly shorter hypocotyls and roots (Figure 4c,d). A more in-depth examination of hypocotyls using scanning electron microscopy indicated a dramatic reduction in cell size in hca plants (Figure 4e,f), suggesting that the mutation caused a defect in cell elongation. The architecture and development of hca roots was also strongly altered: the root of hca formed a tripod whereas the wild type produced a long primary root (Figure 4c,d). The tripod is visible between days 4 and 6. The primary root emerges first, followed rapidly by the two other roots; one forms directly at the basal end of the hypocotyl and the second one on the upper portion of the primary root. At day 7, the three roots are of almost equal length. The emergence of lateral roots was delayed by 5 days in hca but there was little difference in the number produced (data not shown). When seedlings were grown in vitro in the dark, three major differences were observed between hca and wild-type plantlets (Figure 4g,h):

Wild-type seedlings were characterized by an inhibition of secondary root formation whereas secondary root formation in hca was stimulated (see white arrow in Figure 4h).

Short roots appeared on elongated hypocotyls on wild-type seedlings (see white arrows in Figure 4g). In contrast, the emergence of roots from hypocotyls was never observed on hca seedlings.

Compared with in vitro light-grown conditions, the average length of wild-type primary roots is significantly reduced in dark-grown conditions versus light-grown (light $33.6 \pm 9.7$ mm versus dark $2.6 \pm 7.7$ mm),

whereas the average length of hca mains roots is even longer in the dark (light 20.9 ± 7.9 mm versus dark 28.6 ± 8.6 mm).

The vascular organization of the double mutant hca/cov is similar to the hca mutant alone

As it has been previously described that the cov1 Arabidopsis mutant is characterized by a continuous ring of vascular tissue in the stem (Parker et al., 2003), we generated a double mutant, hca/cov. The hca/cov grown in the greenhouse under long-day conditions was shorter and had twisted leaves as compared with hca and cov1 alone (Figure 5a). When grown in vitro, roots of 3-week-old hca/cov were twice as short as both single mutants and three to four times shorter than wild-type plants (Figure 5b). Microscopic examination of the stem and hypocotyl vasculature of 6-week-old plants did not reveal any striking differences between hca/cov and hca alone, except that the diameter of hca/cov hypocotyls was smaller compared with each mutant individually and both wild types (Figure 5c–l).

Table 1 Morphometric characteristics of 6-week-old greenhouse-grown wild-type and hca

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>hca</th>
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<tbody>
<tr>
<td>Primary stem length (cm)</td>
<td>55.1 (2.4)</td>
<td>23.8 (1.8)</td>
</tr>
<tr>
<td>Number of side branches</td>
<td>3.3 (0.6)</td>
<td>3.7 (0.4)</td>
</tr>
<tr>
<td>Number of secondary stems</td>
<td>4.2 (0.7)</td>
<td>6.0 (2.0)</td>
</tr>
<tr>
<td>Number of internodes per stem</td>
<td>3.4 (0.9)</td>
<td>4.1 (0.9)</td>
</tr>
<tr>
<td>Internode length (cm)</td>
<td>4.3 (3.4)</td>
<td>1.6 (1.4)</td>
</tr>
<tr>
<td>Number of siliques per plant</td>
<td>477.8 (74)</td>
<td>437.1 (56)</td>
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</tbody>
</table>

Each measurement represents the average of 10 plants. The number in brackets represent the SEM. Number of internodes per stem and internode length were calculated on primary and secondary stems. The sub-apical portion was excluded. Side branches, primary and secondary stems are indicated in Figure 4(a).

Figure 5. hca/cov1 morphology and vasculature organization.
(a) Five week-old plants grown in the greenhouse under long-day conditions.
(b) Three-week-old in vitro-grown plants.
(c–l) Transverse stem (c–g) and hypocotyl (h–l) sections of 6-week-old plants. (c, h) WS; (d, i) hca; (e, j) hca/cov1 double mutant; (f, k) cov1; (g, l) Ler. Scale bars: (c–l) 100 μm.
The hca mutant displays altered sensitivity towards auxin and cytokinin

The phenotypic modifications caused by the hca mutation suggested an alteration in hormonal balance and/or sensitivity. To assess the role of hca in responses to auxin/cytokinin, we first examined root elongation of in vitro-grown seedlings in the presence of various concentrations of exogenous auxin (Figure 6a). In 9-day-old seedlings, measurements of root elongation indicated that, in the
presence of $10^{-8}$ M 2,4-dichlorophenoxyacetic acid (2,4-D), the root elongation of hca was proportionally less inhibited (23%) than in wild-type roots (37%) (Figure 6a). This result suggests a lower sensitivity of hca to synthetic auxin compared with the wild type. However, at a higher 2,4-D concentration ($10^{-6}$ M), both hca and wild-type seedlings were severely affected. Although the seeds germinated, the seedlings did not elongate and became agravitropic (Figure 6a). Interestingly, the difference in sensitivity to auxin between hca and wild-type seedlings was accentuated over time. Indeed, when plantlets were grown for 20 days in the presence of $10^{-8}$ M 2,4-D, hca roots continued to grow whereas there was virtually no wild-type root growth between days 9 and 20 (white marks indicate root length at 9 days on Figure 6a). In vitro root elongation assays were also performed with two auxin transport inhibitors: TIBA (2,3,5-triiodobenzoic-acid) and NPA (1-naphthalene acetic acid). Root growth of wild-type and hca seedlings was inhibited at identical concentrations of TIBA and NPA, and both lines became agravitropic (data not shown), suggesting that basipetal auxin transport was not affected in hca. These results were also confirmed by radioactive measurements of polar auxin transport assays (data not shown). In conclusion, the hca mutant displays a reduced sensitivity towards exogenous auxin thereby suggesting that the hca gene is in some way required for proper auxin perception and/or response. It is noteworthy that all attempts to rescue the hca phenotype by the addition of exogenous auxin were unsuccessful.

To elucidate the role of hca in cytokinin response, similar root elongation assays were performed in the presence of different concentrations of benzyladenine (BA) (Figure 6b). At day 9, $10^{-8}$ M BA provoked only very slight effects on the elongation of wild type roots (9% inhibition) whereas at $10^{-7}$ M, primary root elongation was dramatically inhibited (80%). On the contrary, hca root elongation was greatly inhibited even at $10^{-6}$ M (55% inhibition). The apparent increase in sensitivity of hca to BA was also evident when comparing the aerial portions of the plantlets (Figure 6b). Wild-type plantlets grown for 20 days on medium supplemented with $10^{-8}$ and $10^{-7}$ M BA were small with pale yellow rosette leaves. At the same BA concentrations, hca plantlets were smaller than the wild type and accumulated anthocyanins (Deikman and Hammer, 1995) in the petioles and the basal portion of the stem (see box in Figure 6b). Taken together, these results indicate an increased sensitivity of hca to cytokinin, suggesting a role for HCA in cytokinin perception and/or response.

_The hca mutation affects the response to cytokinin/auxin ratios in in vitro tissue culture assays_

In order to more precisely evaluate the role of hca in response to auxin/cytokinin, excised hypocotyls from wild-type and hca seedlings were cultured in the presence of various auxin/cytokinin concentrations. When hypocotyl explants were excised from 7-day-old plantlets grown under dim light and cultured for 3 weeks with kinetin and 2,4-D, three main differences could be observed between wild-type and hca hypocotyls (see red boxes in Figure 7). Firstly, in the absence of auxin/cytokinin, or at low auxin/cytokinin concentrations ($\leq 30$ ng ml$^{-1}$) wild-type hypocotyls only produced roots (red boxes with solid line). The first callus from wild-type plants was observed when the medium was supplemented with a minimum of $30$ ng ml$^{-1}$ of both hormones. At these hormone concentrations, hca hypocotyls never produced roots. Secondly, at elevated 2,4-D concentrations (1000 and 3000 ng ml$^{-1}$) and in the absence of cytokinin, or at low cytokinin concentrations ($\leq 30$ ng ml$^{-1}$), wild-type hypocotyls elongated and produced small yellow calli at the explant extremities (30 ng ml$^{-1}$ of kinetin). In contrast, hypocotyls of hca produced small yellow calli all along the hypocotyls, even in the absence of kinetin (see red boxes with dashed line). Thirdly, at elevated 2,4-D concentrations (1000 or 3000 ng ml$^{-1}$) and kinetin concentrations above 300 ng ml$^{-1}$, callogenesis from wild-type hypocotyls was inhibited at 10 000 ng ml$^{-1}$ of kinetin whereas callus formation from hca hypocotyls was inhibited at much lower kinetin concentrations ($\geq 300$ ng ml$^{-1}$, see red boxes with dotted line). However, the inhibition of callogenesis in hca by kinetin was not observed when hypocotyls were grown on media containing auxin concentrations below 1000 ng ml$^{-1}$.

Both the root elongation response and tissue culture experiments indicated that the hca mutant displayed a much greater sensitivity to cytokinin, and that this sensitivity is dependent on the concentration of exogenous auxin. Moreover, the capacity of hca to induce callus formation in the absence of kinetin suggested that levels of endogenous cytokinin and/or auxin may have been different between hca and wild type. Measurements of endogenous IAA and cytokinin content were therefore performed in both the aerial parts and roots of 15-day-old wild-type and hca plants (Figure 8).

As for cytokinins (Figure 8a), a reduced level of isopentenyladenosine-5'-monophosphate (iPMP) was observed in hca shoots as compared with wild type. The pool sizes of zeatin-type cytokinin were unchanged in the shoot of the mutant. In roots, (Figure 8b) zeatin ribotide (ZMP), zeatin riboside (ZR) and zeatin-3-O-glucoside (ZOG) were all significantly less abundant in hca. The levels of iP-type cytokinins were not altered in hca roots. For IAA, the content was slightly lower in both roots and shoots in the hca mutant in comparison with wild type (Figure 8c). A lower level of IAA in the hca mutant was also observed in hypocotyls of adult plants (hca 108 ± 13 ng g$^{-1}$ fresh weight; wild type 198 ± 59 ng g$^{-1}$ fresh weight). In contrast, there was no difference in IAA content in the upper part of the inflorescence
Figure 7. The Effect of auxin and cytokinin concentration on excised hypocotyls of wild-type and hca seedlings. Hypocotyls were excised from seedlings grown under reduced light for 7 days and transferred to media containing various concentrations of 2,4-D and kinetin for 3 weeks. Ten hypocotyls of each genotype were examined for each hormone combination.

and just below the lowermost leaf (data not shown). In conclusion, the hca mutation leads to a reduced levels of the major zeatin-type cytokinins and IAA levels in young hca plantlets compared with wild type.

Microarray analysis sheds light on certain aspects of the hca phenotype

To determine the influence of the hca mutation on global gene expression, transcriptome profiling was performed on 10-day-old WS versus hca mutant plantlets using the complete Arabidopsis transcriptome microarray (CATMA) (Crowe et al., 2003). The CATMA contained 24,576 GSTs corresponding to known and predicted genes. In 10-day-old hca plantlets, a total of 239 genes (about 1% of the genes on the array) had a significant differential expression, with 165 genes upregulated and 74 downregulated. These genes are listed in Supplementary data S1. Interestingly, among the 165 upregulated genes, a large proportion of cell wall-related genes were identified (expansins, extensins, chitinases, arabinogalactan proteins, hydroxyproline-rich proteins etc.). Another striking feature of hca gene expression is the upregulation of several members of the zinc finger transcription factor family, mainly the C3HC4 type and the Dof type. Some Dof transcription factors have been shown to be specifically expressed in the vascular system of plants (Papi et al., 2002). Interestingly, the most upregulated transcription factor belongs to the Scarecrow transcription factor family (At4g36710) which plays an essential role in the radial patterning of both roots and shoots (Wysocka-Diller et al., 2000). A second Scarecrow-like protein is also upregulated, but to lesser extent. An AP2 domain-containing transcription factor (At3g57600) which interacts with scarecrow, is also upregulated in hca (Aida et al., 2004).

Genetic analysis and hca mapping

hca was originally isolated as a T-DNA-tagged mutant from the INRA Versailles collection (Beschtold et al., 1993). The homozygous mutant line was backcrossed with wild-type WS and the resulting F1 progeny was selfed. PCR and kanamycin segregation tests on the F2 progeny revealed that the T-DNA and the hca phenotype were not linked. Segregation of different phenotypic traits of hca including twisted leaves and abnormal vasculature was performed by analyzing 174 F2 progeny. A 100% correlation was observed between twisted leaves and abnormal vasculature and the partition of these characters was very near to 25% of the total plant population (22.4%) ($\chi^2$, $\alpha < 5\%$). These results indicated that the overall hca phenotype is due to a monogenic, recessive mutation. The localization of the hca locus was undertaken by generating an F2-mapping population by crossing the hca line with Columbia Arabidopsis Col-0. Ninety-eight mutants were isolated and screened with SSLP

Figure 8. Auxin and cytokinin content in 15-day-old wild-type and hca seedlings grown in vitro. 
(a, b) cytokinin content in shoots and roots respectively. 
(c) IAA content in shoots and roots. Error bars represent standard deviations.
Abbreviations: Z, zeatin; ZMP, zeatin riboside-5¢-monophosphate; iPMP, isopentenyladenosine-5¢-monophosphate; ZOG, zeatin-O-glucoside; iPA, isopentenyl adenosine; ZR, zeatin riboside; ZROG, zeatin riboside-O-glucoside.
(simple sequence length polymorphism) markers spanning the entire Arabidopsis genome (Bell and Ecker, 1994; Ponce et al., 1999). The hca mutation was located on the lower arm of chromosome 4 between nga1107 (10.5 cM) and nga1139 (4.9 cM). For more detailed mapping, additional SSLP markers were generated within the region containing the hca mutation. These SSLP markers were used to screen a test cross population to identify critical recombination near the mutation. The test cross population was generated by two successive crosses. The first cross was made between hca and Col-0 wild-type plants and the second between the F1 progeny of the first cross and hca. The phenotype of plants from this population was 50% mutant and 50% wild type (heterozygous). More than 1500 plants were screened with these additional SSLP markers. This allowed us to reduce the mutated region to 240 kb. This region contains 68 predicted open reading frames (ORFs) from At4g35295 to At4g35940. As no supplementary recombination was observed in this region, another recombinant population is currently being constructed using a line containing a T-DNA in one of these ORFs. The presence of the T-DNA will enable us to use kanamycin resistance as a phenotypical marker to screen the population.

Discussion

hca is characterized by abnormal cambial activity leading to an altered pattern of vascular tissues. In inflorescence stems, an interfascicular cambium is formed very early in development, resulting in the production of large amounts of vascular tissue (46% in hca versus 20% in wild type). As a consequence, hca does not exhibit the alternate vascular bundle/sclerenchyma fiber organization typically observed in wild-type plants. Instead, a continuous ring of vascular tissue with some sclerenchyma fibers dispersed throughout the ring was observed. This vascular organization is reminiscent of the vasculature of herbaceous species with secondary growth, i.e. tobacco and woody species. However, in contrast to woody species, this extensive secondary growth is not accompanied by an enlargement of the stem diameter. Interestingly, despite the fact that hca acts early during plant development as observed by upregulation of ATHB-8 in root meristem and increased cell division in 10-day-old hypocotyls, the organization of primary xylem in stems, sensu stricto, was not modified. Indeed, the xylem poles were correctly positioned under the ring of secondary xylem. We therefore hypothesize that hca exhibits premature secondary growth. A continuous ring of vascular tissue has been described in the stem of the Arabidopsis mutant, cov1 (Parker et al., 2003). The COV1 gene encodes a membrane protein of unknown function. In keeping with the cov1 phenotype, the fact that auxin response was not altered, and that auxin content was two times lower in the hypocotyl, the authors suggested that the COV1 gene could be involved in a mechanism that negatively regulates the differentiation of vascular tissues. Based on the map position, HCA is not allelic to COV1. Moreover hca exhibited significant differences in the organization of the vasculature compared with cov. In stems hca vasculature forms a continuous ring of secondary tissues, whereas the cov vasculature still exhibits arch-shaped vascular bundles and no secondary growth. In mature hypocotyls there is no difference in secondary xylem formation between cov and wild type, whereas hca produced less xylem compared with wild type. The vasculature of the double mutant hca/cov is more similar to hca than to cov in both stems and hypocotyls. However, the alteration of vasculature of hca/cov was no greater than either mutant alone. Based on this observation we cannot conclude to whether HCA and COV act in the same pathway or not.

Previous studies on aspen have clearly underlined the role of auxin on cambial activity and subsequent secondary growth (Sundberg et al., 2001; Uggla et al., 1996). Furthermore, several Arabidopsis mutants impaired in auxin response or transport that exhibit alterations in vascular differentiation or patterning have been described (for reviews see Berleth and Mattsson, 2000; Berleth et al., 2000; Hobbie, 1998). In this context, we performed an in-depth examination of the auxin status in the hca mutant. Different experimental assays pointed to an altered response to auxin in hca. Firstly, in root elongation assays, hca is less sensitive to exogenous auxin than the wild type. Secondly, in tissue culture experiments, although callus formation could be induced at the same auxin concentration in wild-type and hca hypocotyls, the inhibition of callogenesis in hca was observed at lower auxin concentrations than in the wild type. These results indicate that the response to auxin in hca is altered in an organ-specific manner. This alteration in auxin response is not directly related to auxin transport, since auxin transport is not affected in hca. Moreover, both the vascular alteration and overall phenotype of hca are different from that in mutants affected in auxin transport, such as pin (Gälweiler et al., 1998) and lop (Carland and McHale, 1996), auxin-inducible genes, such as iaaaxr (Hobbie et al., 2000), or transcription factors that regulate these genes, such as monopteros (Berleth and Jürgens, 1993). At present, a direct link between altered vascular patterning and auxin response and content in hca cannot be established.

Many of the phenotypic characteristics of hca, including delay of leaf senescence, loss of apical dominance and inhibition of hypocotyl elongation, suggest an altered cytokinin metabolism and/or response to cytokinin (for review see Mok and Mok, 2001). We have therefore performed a detailed investigation of the effect of exogenous cytokinin in various in vitro assays. Normally, the addition of exogenous cytokinin to plants grown in vitro induces callogenesis and inhibition of root primordia and root hair formation. Interestingly, all of these cytokinin responses were observed in
hca in the absence of cytokinin. Moreover, callus formation was severely inhibited at a cytokinin concentration 30 times lower in hca than in the wild type. The increased sensitivity of hca to exogenous cytokinin was also observed in in vitro plantlets. An inhibition of root growth and a pronounced accumulation of anthocyanin in leaves were observed at a lower BA concentration compared with wild type.

Taken together, these results suggested that hca was either an overproducer of cytokinin or had an increased sensitivity to cytokinin. Interestingly, our analysis of cytokinin pool sizes revealed a clear downregulation of the pathway involving zeatin-type cytokinins while the iP-related pathway was more or less unaltered. This effect on cytokinin homeostasis suggests that the suppression of cytokinin biosynthesis in hca could be explained by potential cross-talk between homeostasis and signal transduction; basically, a hypersensitivity to cytokinin could induce feedback inhibition of the synthesis. The fact that the cytokinin content in hca plantlets was even lower than in the wild type thereby favors the increased response hypothesis. We postulate that hca could interfere directly or indirectly with mechanisms regulating cytokinin response and may act as a switch in maintaining the downstream cytokinin signaling cascade in the ‘on’ position (i.e. in a constitutive fashion). In support of this hypothesis, microarray analysis of hca indicated that the gene ARR16 involved in cytokinin response (D’Agostino et al., 2000; Kiba et al., 2002; To et al., 2004) was upregulated in hca plantlets. This gene was also shown to be upregulated in wild-type plantlets treated with exogenous BA (Rashotte et al., 2003). In this way, constitutive activation of the cytokinin pathway could conceivably lead to a hypersensitivity to exogenous cytokinin. This hypersensitivity might induce a feedback regulation that in turn decreases the cytokinin levels observed in the hca mutant.

An increase in cytokinin sensitivity has already been described in two types of mutants affected in their cytokinin signaling pathways. Kubo and Kakimoto (2000) reported that callus produced by ckh1 and ckh2 exhibited typical cytokinin responses at lower levels of kinetin than in wild type. The authors speculate that CKH1 and CKH2 gene products negatively regulate the signaling pathway of cytokinin perception that leads to cell proliferation and chloroplast development. That said, beyond the cytokinin response, ckh mutants do not exhibit an altered vascular system (C. Pineau, unpublished data). Recently, the family of ARR genes has also been characterized as negative regulators of the cytokinin signaling pathway since the inhibition of root elongation in response to cytokinin is more sensitive in a multiple arr mutant than in wild type (To et al., 2004). However, with the exception of delayed leaf senescence, both the multiple arr mutants and the ckh mutant are phenotypically very different from hca. To date, we cannot conclude if an altered cytokinin response is a primary or secondary effect of the hca mutation. Keeping in mind that auxin promotes and is required for cell division in the cambium, it is also tempting to speculate that the activation of cambial cell division in the interfascicular region of hca stems is a result of an enhanced sensitivity of these cells to cytokinin. Cytokinin is well known for its role in the cell cycle (D’Agostino and Kieber, 1999) and Shibaoka (1994) reported that cytokinin influences the plane of cell division with an increased number of periclinal rather than anticlinal divisions.

Taken together, the physiological data presented here suggest that the hca mutation leads directly or indirectly to an enhanced sensitivity toward cytokinin, and as a result a modification of the response to auxin and cytokinin. In this way, we hypothesize that auxin response of hca could be a secondary effect of the mutation and should be viewed in an integrative response scheme that may include others hormones and environmental factors. For example, interaction between light and cytokinin signaling pathway has been described in the det1 mutant (Chory et al., 1994), and more recently Cluis et al. (2004) reported that the transcription factor HY5 integrates light and auxin signaling pathways. In keeping with these observations, it is interesting to note that light had a significant effect on the root phenotype of hca. When grown under light conditions, the emergence of secondary roots is delayed in hca when compared with wild type. In contrast, under dark-grown conditions, hca seedlings exhibit enhanced root formation. Synergistic, antagonistic and additive interactions among plant hormones have been demonstrated in many aspects of plant development (Coenen and Lomax, 1997; Nordström et al., 2004). Mutants that respond abnormally to one phytohormone often respond abnormally to others, reflecting the complex interacting network of hormone response pathways. Alterations in auxin and cytokinin responses associated with abnormal vascular organization have been described in the rice mutant ral1 (Scarpella et al., 2003) and the pls mutant in Arabidopsis (Casson et al., 2002). The ral1 mutant is impaired in procambial development and exhibits altered leaf venation patterning. To date the RADICLELESS1 (RAL1) gene has not yet been cloned, but physiological data indicated that the gene may play an important role in the establishment of vascular patterns and proper response to auxin and cytokinin. In the same way, the POLARIS (PLS) gene which encodes a small peptide (Casson et al., 2002) is required for correct auxin–cytokinin homeostasis to mediate leaf vascular patterning. Based on the map position of HCA, it is unlikely that HCA is allelic to POLARIS.

To date, the molecular nature of the HCA gene product is not known. Thus, it is possible that the hca mutation directly affects a step in the cytokinin transduction pathway. Alternatively, as hca exhibited a pleiotropic phenotype it is also possible that the hca gene is involved in a very early step in controlling plant development by acting on the commitment of cambial cells via
modification of hormone sensitivity. As a consequence, extensive cell division could occur in both the fascicular and interfascicular regions leading to early and increased secondary growth in stems. One attractive model would be that hca gene interacts with INTERFASCULAR FIBERLESS1 (Ratcliffe et al., 2000; Zhong and Ye, 1999; Zhong et al., 1997) to control the status of cell division and differentiation in the interfascicular region in the stem. The fact that hca is characterized by few fibers and that these are dispersed throughout the xylem ring supports this hypothesis. The future cloning of the hca gene will allow us to establish the link between proper response to auxin and cytokinin and vasculature development.

Experimental procedures

Plant material and mutant isolation

Microscopic screening was performed on 4-week-old plants from T-DNA-mutagenized T₂ lines of Arabidopsis thaliana (Beschtold et al., 1993). Hand sections of 12 different plant stems per line were observed under bright field and UV light, or stained with phloroglucinol according to standard protocols (Nakano and Meshitsuka, 1992).

The pATHb-8::GUS seeds were provided by the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA).

The double mutant, hca/cov1, was obtained by crossing homozygous hca and cov1 lines. The first generation (double heterozygotes) was selfed and the double homozygous mutant hca/cov1 was identified using SSLP genetic markers designed for mapping the two mutants. This screening method was possible since hca is in a WS genetic background and cov1 in a Ler (Landsberg erecta) background.

Growth conditions for seedlings and adult plants

Seeds were surface-sterilized for 25 min in sterile water containing 25% bleach and 0.1% Triton X-100, and washed three times in sterile water. The seeds were then imbibed in 0.2% agarose for 2 days at 4°C in the dark and subsequently sown on the basal growth medium composed of 4.32 g l⁻¹ Murashige and Skoog medium (Sigma Lyon, France, M5519), 1% sucrose, 10 ml l⁻¹ of 5% 2-(N-morpholino)ethane sulfonic acid pH 7.5, 100 mg l⁻¹ inositol, 10 mg l⁻¹ thiamine-HCl, 1 mg l⁻¹ pyridoxine-HCl, 1 mg l⁻¹ nicotinic acid and 0.6% phytagel (Sigma). For phenotypic observations and root elongation assays, seedlings were excised and placed on basal medium as described above, supplemented with 1 mg l⁻¹ of biotin and various concentrations of the kinetin and 2,4-D. Hypocotyls were cultivated for 3–4 weeks at 23°C under light conditions (16 h day length at 100 μmol m⁻² sec⁻¹). Ten hypocotyls were tested for each combination of hormone concentrations. A representative callus for each hormonal concentration was photographed.

In vitro root elongation assays

Root elongation assays were performed on seedlings germinated on MS medium described above in the presence of various concentrations of 2,4-D or BA. Root length was measured on Petri plates and growth was measured between 5 and 9 days. Plates were photographed at 9 and 20 days. For hca, root length was considered as the mean length of the three roots comprising the tripod root. Hormonal stimulation or inhibition of root elongation for wild type and hca was calculated as a percentage of root elongation on hormone-free MS medium. These per cent values were used to compare the hormone response of wild type and hca. Lateral root emergence was quantified at 12 days.

In vitro callogenesis assays

Hypocotyls from 7-day-old wild-type and hca seedlings were excised and placed on basal medium as described above, supplemented with 1 mg l⁻¹ of 5% 2-(N-morpholino)ethane sulfonic acid prepared in 0.1 M sodium phosphate (pH 7.3) and a secondary fixation procedure was performed with 1% osmium tetroxide prepared in 0.1 M sodium phosphate (pH 7.3) solution. Tissues were rinsed and dehydrated in a successive ethanol series (10, 30, 50, 70, 95, 100%), and infiltrated step-wise with Spurr resin (25, 50, 70, 100% in ethanol). Sections 1 μm thick were stained with 1% toluidine blue w/v.

Hand-cut sections from fresh Arabidopsis stems or roots were observed using an inverted Leica microscope (Leitz, Rueil-Malmaison, France, DMIRBE), equipped with epifluorescence illumination (excitation filter BP 340–380 nm, suppression filter LP 430 nm). Images were registered using a CDD camera (Colour Coolview, Photonic Science, Robertsbridge, UK) and treated by image analysis (Image Pro-plus, Media Cybernetics, Silver Spring, MD, USA).

Measurements of endogenous cytokinin and IAA content

Endogenous IAA measurements on 15-day-old in vitro-grown plantlets were performed using the isotope dilution MS method according to Edlund et al. (1995). Frozen plant material was ground with a mortar and pestle in liquid nitrogen. For cytokinin analysis, tissue was extracted in the extraction buffer MeOH:H₂O:HOCH₂ (15:4:1 v/v) for 2 h (−20°C) in the presence of heavy labeled internal standards ₁H₂-Z, ₁H₂-ZR, ₁H₂-ZTG, ₁H₂-ZTG, ₁H₂-ZOG, ₁H₂-ZROG, ₁H₂-ZMP, ₁H₂-ip, ₁H₂-ipA, ₁H₂-HPMP (Z, zeatin; ZMP, ZR-5’-monophosphate; iMP, isopentenyladenosine-5’-monophosphate; ZOG, zeatin-O-glucoside; iP, isopentenyl adenosine; ZR, zeatin riboside; ZROG, zeatin riboside-O-glucoside). Cytokinins were purified as described by Nordström et al. (2004) and derivatized as described in Astot et al. (1998). Cytokinin analysis by mass spectrometry was performed in selective reaction monitoring mode. Chromatographic separation was performed using a drop-in guard cartridge 10 × 1 mm BetaMax Neutral, with 5 μm particle size (Thermo Hypersil-Keystone, Cambridge, UK) as analytical column. Before loading samples in the autosampler the propionylated samples were dissolved in 1.3 μl acetonitrile containing 3% formic acid followed by 11.7 μl H₂O also containing 3% formic acid. At a fixation, plant material was rinsed in 0.1 M sodium phosphate (pH 7.3) and a secondary fixation procedure was performed with 1% osmium tetroxide prepared in 0.1 M sodium phosphate (pH 7.3) solution. Tissues were rinsed and dehydrated in a successive ethanol series (10, 30, 50, 70, 95, 100%), and infiltrated step-wise with Spurr resin (25, 50, 70, 100% in ethanol). Sections 1 μm thick were stained with 1% toluidine blue w/v.

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flow rate of 10 µl min⁻¹ the following binary gradient was used: 0 to 2 min, isotropic elution of 5% solvent B; 2 to 10, a linear gradient to 55% B; followed by isotropic elution of 85% solvent B. After analysis, the column was washed by 100% solvent B at a flow rate of 80 µl min⁻¹ and equilibrated for initial conditions. Solvent A consisted of 3% (v/v) formic acid in water, and solvent B consisted of 3% (v/v) formic acid in acetonitrile. Eluents from the chromatographic column were introduced to a Micromass Quattro Ultima mass spectrometer (Micromass, Manchester, UK) via an electrospray ion source. The following mass spectrometric set-up was used: capillary voltage -3 kV, source temperature 90°C, desolvation temperature 250°C, cone gas flow 12 l h⁻¹, desolvation gas flow 800 l h⁻¹. The collision cell pressure was kept between 2 and 2.5 mbar with argon gas and the cone voltage and collision energies were as follows [cone voltage (V)/collision cell energy (eV)]: Z (60/14), iP (60/15), PRO-ZOG (80/25), PRO-iPMP (80/18), PRO-ZMP (80/20), PRO-DHZR (60/19), PRO-Z7G,Z9G (100/22), PRO-ZROG (85/27). Data were processed using Masslynx software (Micromass, Manchester, UK).

**Location of GUS activity**

GUS activity analysis was performed according to Jefferson et al. (1987) in a homozygous hca line containing a pATHb-8::GUS construct. Briefly, sections were hand-cut or mature embryos were pulled out from their tegument after 1 nights’ imbibition and incubated for several hours to overnight in 2 mM X-Gluc (5-bromo-4-iodo-3-indolyl-α-D-glucuronidase), 0.1% Triton X-100, 50 mM sodium phosphate pH 7.0, and 1 mM each of K⁺ ferri/ferrocyanide. Depending of tissue toughness (old leaves, floral stems), an additional vacuum infiltration step was performed with the same reaction medium for 10 min before incubation. After the reaction, the GUS staining was fixed for 1 h in 5% formaldehyde, 5% acetic acid and 45% ethanol. Green tissues were depigmented in 70% ethanol.

**Microarray analysis**

The microarray analysis was performed with the CATMA array containing 24576 gene-specific tags (GSTs) from Arabidopsis thaliana (Crowe et al., 2003; Hilson et al., 2004). The GST amplicons were purified on Multiscreen plates (Millipore, Bedford, MA, USA) and resuspended in Tris EDTA-DMSO at 100 ng µl⁻¹. The purified probes were transferred to 1536-well plates with a Genesis workstation (TECAN, Männedorf, Switzerland) and spotted on UltraGAPS slides (Corning, New York, NY, USA) using a Microgrid II (Genomic Solution, Huntingdon, UK). The current CATMA version printed at the Unité de Recherche en Génomique Végétale (URGV) consists of 3 metablocks, each composed of 64 blocks of 144 spots. A block is a set of spots printed with the same print-tip. In these arrays, a print-tip is used three times to print a block in each metablock.

For the transcriptome studies, whole seedlings were harvested at 10 days after germination, at the stage where cotyledons opened fully according to Boyes et al. (2001) from 3 x 300 plants of A. thaliana WS and hca. One dye swap was performed for each biological replicate. Plants were grown in vitro on Petri plates under light conditions (16 h at 100 µmol m⁻² sec⁻¹) at 20°C. RNA was extracted from these samples using TRIzol® extraction (Invitrogen, Carlsbad, CA, USA) followed by two ethanol precipitations, then checked for RNA integrity with the Bioanalyzer from Agilent (Waldbroon, Germany). cRNAs were produced from 2 μg of total RNA from each pool with the ‘Message Amp aRNA’ kit (Ambion, Austin, TX, USA). Then 5 μg of cRNAs were reverse transcribed in the presence of 200 U of SuperScript II (Invitrogen), cy3-dUTP and cy5-dUTP (NEN, Boston, MA, USA) according to Puskas et al. (2002) for each slide. Samples were combined, purified and concentrated with YM30 Microcon columns (Millipore). Slides were pre-hybridized for 1 h and hybridized overnight at 42°C in 25% formamide. Slides were washed in 2x SSC + 0.1% SDS (4 min), 1x SSC (4 min), 0.2x SSC (4 min), 0.05x SSC (1 min) and dried by centrifugation. Six hybridizations (three dye swaps) were carried out. The arrays were scanned on a GenePix 4000A scanner (Axon Instruments, Foster City, CA, USA) and images were analyzed by GenePix Pro 3.0 (Axon Instruments).

**Statistical analysis of microarray data**

The statistical analysis was performed as described in Lurin et al. (2004), based on two dye swaps, i.e. four arrays each containing the 24576 GSTs and 384 controls. The controls were used for assessing the quality of the hybridizations but were not included in the statistical tests. For each array, the raw data comprised the logarithm of the median feature pixel intensity at wavelength 635 nm (red) and 552 nm (green). No background was subtracted. In the following description, log-ratio refers to the differential expression between the mutant and the control. An array-by-array normalization was performed to remove systematic biases. Then we performed a global intensity-dependent normalization using the loess procedure (see Yang et al., 2002) to correct the dye bias. Finally, on each block, the log-ratio median is subtracted from each value of the log-ratio of the block to correct a print-tip effect on each metablock. To determine differentially expressed genes, we performed a paired t-test on the log-ratios. The number of observations per spot varied between two and six and is inadequate for calculating a gene-specific variance. For this reason we assume that the variance of the log-ratios is the same for all genes. The raw P-values were adjusted by the Bonferroni method, which controls the family wise error rate (FWER).

**Gene mapping**

The F₂ mapping population was screened with SSLP PCR-based markers designed by Bell and Ecker (1994). The primer sequences of the markers nga1139 and nga1107 were TTTTTT-CCTTGTGTTGCATTCC – TAGCCCGATGAGTTGCTACG and CG-ACGAACTGCAGAATTAGG – GCGAAAAAACAAAAAAATCCA, respectively.

DNA was extracted from cotyledons in a 96-well PCR plate as described for covf1 mapping (Parker et al., 2003). Cotyledons were ground in ice in 0.4 M NaOH. Seven microliters of extract were neutralized in 150 µl of 0.3 mol cold Tris-HCl buffer (pH 8.0). The PCR was performed with Life Technology TAQ polymerase using a classic PCR program. The PCR products were run on 3% agarose gel to detect polymorphisms.

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Supplementary Material

The following supplementary material is available for this article online:

Supplementary data S1. List of all differentially expressed genes in 10-day-old plantlets of hca as compared with wild type. Microarray analysis was performed using the CATMA (complete Arabidopsis transcriptome microarray). Genes are listed according to their log2 ratio from the most upregulated to the most downregulated. For each gene, the locus name (At number), the CATMA number (Crowe et al., 2003) and the putative ID are indicated. Note that the complete results of this experiment will soon be available on the Array Express website (http://www.ebi.ac.uk/arrayexpress/).

This material is available as part of the online article from http://www.blackwell-synergy.com

References


